Yi-Wei Tang
Charles W. Stratton *Editors*

Advanced Techniques in Diagnostic Microbiology

Second Edition

Springer

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Editors
Yi-Wei Tang
Department of Laboratory Medicine
Memorial Sloan-Kettering Cancer Center
New York, NY, USA

Charles W. Stratton
Departments of Pathology and Medicine
Vanderbilt University Medical Center
Nashville, TN, USA

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Preface

In the United States, hospitals annually report over five million cases of infectious-disease-related illnesses: clinical microbiology laboratories in these hospitals are engaged in detecting and identifying the pathogenic microorganisms in clinical specimens collected from patients with suspected infections. Clearly, the timely and accurate detection/identification of these microbial pathogens is critical for patient treatment decisions and outcomes for millions of patients each year. Despite an appreciation that the outcome of an infectious-disease-related illness is directly related to the time required to detect and identify a microbial pathogen, clinical microbiology laboratories in the United States as well as worldwide have long been hampered by traditional culture-based assays, which may require prolonged incubation time for slowly growing microorganisms such as *Mycobacterium tuberculosis*. Moreover, traditional culture-based assays often require multiple steps with additional time needed for discernment of species and/or detection of antimicrobial resistance. Finally, these traditional, slow multistep culture-based assays are labor-intensive and require skilled clinical microbiologists at the bench.

Over the past several decades, advanced molecular techniques in diagnostic microbiology quietly have been revolutionizing the practice of clinical microbiology in the hospital setting. Indeed, molecular diagnostic testing in general and nucleic acid-based amplification methods in particular have been heralded as diagnostic tools for the new millennium. There is no question that the development of rapid molecular techniques for nucleic acid amplification/characterization combined with automation and user-friendly software has greatly broadened the diagnostic capabilities of the clinical microbiology laboratory. These technical advances in molecular microbiology over the first decade of the twenty-first century have profoundly influenced the physical structure of clinical microbiology laboratories as well as their staffing patterns, workflow, and turnaround time. These molecular microbiology advances have also resulted in the need for a revised and updated second edition of *Advanced Techniques in Diagnostic Microbiology*. This second edition again provides an updated and comprehensive description of the ongoing

evolution of molecular methods for the diagnosis of infectious diseases. In addition, many new chapters have been added, including a chapter on the clinical interpretation and relevance of advanced technique results. The second edition, like the first edition, includes both a "techniques" section describing the latest molecular techniques and an "applications" section describing how these advanced molecular techniques are being used in the clinical setting. Finally, the second edition, like the first edition, utilizes a diverse team of authors who have compiled chapters that provide the reader with comprehensive and useable information on advanced molecular microbiology techniques.

New York, NY, USA — Yi-Wei Tang
Nashville, TN, USA — Charles W. Stratton

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Contributors

Jaber Aslanzadeh Department of Pathology, Hartford Hospital and Clinical Laboratory Partners, Hartford, CT, USA

Ellen Jo Baron Department of Pathology, Stanford University School of Medicine, Palo Alto, CA, USACepheid, Sunnyvale, CA, USA

Sándor Belák The National Veterinary Institute The Swedish University of Agricultural Sciences, Uppsala, Sweden

Lawrence B. Blyn Ibis Biosciences, Inc., an Abbott Company, Carlsbad, CA, USA

Nyasha O. Bullock Department of Pathology, Hartford Hospital and Clinical Laboratory Partners, Hartford, CT, USA

Sheldon Campbell Pathology and Laboratory Medicine Service VA Connecticut Department of Laboratory Medicine, Yale University School of Medicine, New Haven, CT, USA

Karen C. Carroll Division of Medical Microbiology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

Tsung Chain Chang Department of Medical Laboratory Science and Biotechnology, School of Medicine National Cheng Kung University, Taiwan, Republic of China

Vishnu Chaturvedi Wadsworth Center New York State Department of Health Department of Biomedical Sciences, University at Albany School of Public Health, Albany, NY, USA

Lunbiao Cui Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China

Miao Cui Department of Pathology, Mount Sinai School of Medicine New York University, New York, NY, USA

Phyllis Della-Latta Department of Pathology, Columbia University Medical Center New York-Presbyterian Hospital, New York, NY, USA

Jianli Dong Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA

Sherry A. Dunbar Luminex Corporation, Austin, TX, USA

David J. Ecker Ibis Biosciences, Inc., an Abbott Company, Carlsbad, CA, USA

Jiang Fan Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, USA

Tao Feng Department of Pathology, Mount Sinai School of Medicine, New York University, New York, NY, USA

Patrice Francois Department of Specialties in Medicine, University of Geneva Hospital, Geneva, Switzerland

Terry J. Gentry Department of Soil and Crop Sciences, Texas A&M University, College Station, TX, USA

Richard V. Goering Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha, NE, USA

Jian Han HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA

Xiang Y. Han Department of Laboratory Medicine, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

Heather Harbottle Office of New Animal Drug Evaluation Center for Veterinary Medicine, U.S. Food and Drug Administration, Derwood, MD, USA

Jie He Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, USA

Tao Hong Department of Pathology, Hackensack University Medical Center, Hackensack, NJ, USA

Yuan Hu Northeast Regional Laboratory, U.S. Food and Drug Administration, Jamaica, NY, USA

Shihai Huang Abbott Molecular Inc., Des Plaines, IL, USA

Kai Man Kam Public Health Laboratory Services Branch, Department of Health, Kowloon, Hong Kong

Abdullah Kilic Department of Microbiology, Gulhane Military Medical Academy, Ankara, Turkey

Marie L. Landry Department of Laboratory Medicine, Yale University School of Medicine, New Haven, CT, USA

Susanna K.P. Lau Department of Microbiology, The University of Hong Kong, Pokfulam, Hong Kong

Ivy Lee Department of Pathology, Mount Sinai School of Medicine, New York University, New York, NY, USA

Haijing Li Department of Pathology, Vanderbilt University School of Medicine, Nashville, TN, USA

Dongyou Liu Biosecurity Quality Assurance Program, Royal College of Pathologists of Australia, Surry Hills, NSW, Australia

Lihong Liu The National Veterinary Institute, The Swedish University of Agricultural Sciences, Uppsala, Sweden

Angus C.T. Lo Public Health Laboratory Services Branch, Department of Health, Kowloon, Hong Kong

Michael J. Loeffelholz Department of Pathology, University of Texas Medical Branch, Galveston, TX, USA

Vladimir Majerciak HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Elizabeth M. Marlowe SCPMG Regional Reference Laboratories, Kaiser Permanente, North Hollywood, CA, USA

Christian Massire Ibis Biosciences, Inc., an Abbott Company, Carlsbad, CA, USA

Alexander J. McAdam Department of Laboratory Medicine and Pathology, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA

Alexander Mellmann Institute of Hygiene, University Hospital Münster, Münster, Germany

David Metzgar Ibis Biosciences, Inc., an Abbott Company, Carlsbad, CA, USA

Kobayashi Miwako Department of Pathology and Laboratory Medicine, Emory University and Grady Memorial Hospital, Atlanta, GA, USA

Nima Mosammaparast Department of Laboratory Medicine and Pathology, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA

Johannes Müthing Institute of Hygiene, University Hospital Münster, Münster, Germany

Nicole Parrish Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD, USA

Bruce K. Patterson IncellDx, Inc., Menlo Park, CA, USA

Michael Pendrak Laboratory of Pathology, National Cancer Institute, NIH, Bethesda, MD, USA

Criziel Quinn Department of Pathology, Vanderbilt University Medical Center, Nashville, TN, USA

Edmunds Reineks Department of Clinical Pathology, Cleveland Clinic, Cleveland, OH, USA

Stefan Riedel Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD, USA

Rangarajan Sampath Ibis Biosciences, Inc., an Abbott Company, Carlsbad, CA, USA

Jacques Schrenzel Department of Specialties in Medicine, University of Geneva Hospital, Geneva, Switzerland

Audrey N. Schuetz Departments of Pathology & Laboratory Medicine, and Medicine, Weill Cornell Medical College, The New York-Presbyterian Hospital, New York, NY, USA

Ted E. Schutzbank Covance Central Laboratory Services Inc., Indianapolis, IN, USA

Susan Sefers Department of Pathology, Vanderbilt University Medical Center, Nashville, TN, USA

Rosemary C. She SCPMG Regional Reference Labs, Kaiser Permanente, North Hollywood, CA, USA

Wun-Ju Shieh Division of High-Consequence Pathogens & Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA

Jeong Hwan Shin Department of Laboratory Medicine, Busan Paik Hospital, Inje University College of Medicine, Busan, South Korea

Charles W. Stratton Departments of Pathology and Medicine, Vanderbilt University Medical Center, Nashville, TN, USA

Yi-Wei Tang Departments of Pathology and Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA

Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Jade L.L. Teng Department of Microbiology, The University of Hong Kong, Pokfulam, Hong Kong

Joy D. Van Nostrand Institute for Environmental Genomics Department of Botany and Microbiology, University of Oklahoma, Norman, OK, USA

Chunlin Wang Stanford Genome Technology Center, Department of Biochemistry, Stanford University, Palo Alto, CA, USA

Sihe Wang Department of Clinical Pathology, Cleveland Clinic, Cleveland, OH, USA

Yun F. (Wayne) Wang Emory University and Grady Memorial Hospital, Atlanta, GA, USA

Donna M. Wolk Department of Pathology, University of Arizona College of Medicine, Tucson, AZ, USA

Patrick C.Y. Woo Department of Microbiology, The University of Hong Kong, Pokfulam, Hong Kong

Fann Wu Department of Pathology, Columbia University Medical Center New York-Presbyterian Hospital, New York, NY, USA

Josephine Wu Department of Pathology, Mount Sinai School of Medicine New York University, New York, NY, USA

Xiao Xu ACEA Biosciences, Inc., San Diego, CA, USA

Fei Ye Department of Pathology, Mount Sinai School of Medicine New York University, New York, NY, USA

Bingjiao Yin Department of Pathology, Mount Sinai School of Medicine New York University, New York, NY, USA

Sherif R. Zaki Division of High-Consequence Pathogens & Pathology, National Center for Emerging and Zoonotic Infectious Diseases Centers for Disease Control and Prevention, Atlanta, GA, USA

David Zhang Department of Pathology, Mount Sinai School of Medicine New York University, New York, NY, USA

Wan-Ming Zhang Department of Clinical Pathology, Cleveland Clinic, Cleveland, OH, USA

Min Zheng ACEA Biosciences, Inc., San Diego, CA, USA

State Key Laboratory of Diagnostic and Treatment for Infectious Diseases, First Affiliated Hospital Zhejiang University School of Medicine, Hangzhou, China

Zhi-Ming Zheng HIV and AIDS Malignancy Branch, National Cancer Institute National Institutes of Health, Bethesda, MD, USA

Jizhong Zhou Institute for Environmental Genomics, Department of Botany and Microbiology, University of Oklahoma, Norman, OK, USA

Part I
Methods

Chapter 1
Automated Blood Cultures

Xiang Y. Han

Introduction

A clinically suspected infection is ultimately confirmed by isolation or detection of the infectious agent. Timely identification of the microorganism and antibiotic susceptibility tests further guide effective antimicrobial therapy. Bloodstream infection is systemic and is the most severe form of infection. It is frequently life threatening, and blood culture to detect circulating microorganisms has been the diagnostic standard. Much of the scientific and technologic advances of blood culture have been made through the 1970s to 1990s; this chapter briefly reviews various aspects of it with emphasis on automated culturing systems.

Principles

The principles and scientific basis to optimize the diagnostic yield of blood cultures have been reviewed and summarized [1, 2]. Most parameters were initially established for manual blood culture systems that used basal culture media. A recent study addressed some of these parameters for newer culture systems and media and found them to be mostly valid nowadays [3]. Major features are summarized as follows.

Host and microbial factors. Invasion of the bloodstream by microorganisms reflects the failure of initial host defense, such as the loss of integrity of skin and mucosa and weakening of the innate and acquired immunity, to prevent such invasion or spill from a localized infection site. Among those patients bearing

X.Y. Han (✉)
Department of Laboratory Medicine, Unit 84, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA
e-mail: xhan@mdanderson.org

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intravascular device or using recreational drugs intravenously, direct blood seeding of microorganism is also possible. Once in the bloodstream, microbes are constantly attacked by host defenses, such as complements, phagocytic leukocytes, antibodies, and other factors. The ability of invading microorganisms to evade or shield off host defense or antimicrobics favors their survival and dissemination in the bloodstream. On the other hand, if the host defense is paralyzed, such as leucopenia and immune suppression, even the least pathogenic organisms can cause fatal infections. Therefore, both the host and microbial factors determine the occurrence, severity, and duration of septic episodes, which may also affect the yield of culture recovery. The presence of antimicrobial agents in the circulation may also reduce culture recovery.

Timing, volume, and frequency of cultures. Timing the blood-draw has bearings on culture recovery. Most bacteremia or fungemia are not constant except in case of endocarditis; thus, the host responses, such as rising fever, likely herald the best time to draw blood culture. Blood should also be drawn, if at all possible, before initiation of empiric antimicrobial therapy.

For each septic episode, two to three sets of cultures over a 24-h period provide maximum recovery for the offending microorganism(s). A set of blood cultures usually means an aerobic bottle and an anaerobic bottle each inoculated with 10 ml blood for an adult patient. This practice requires draws of a total of 40–60 ml blood from two to three venipunctures from different arms. In a culture bottle, the blood sample is diluted by the culture broth to reach a blood–broth ratio of 1:2.5 to 1:10, which may dilute inhibitory blood components to favor microbial growth. It is generally accepted and hence practiced that 40 ml for two culture sets offer good culture recovery as well as maintain cost-effective microbiology. A couple of laboratories showed recently that 60 ml blood draw consistently yield higher culture recoveries than lower volume draws [4, 5]. Lower volume reduces culture sensitivity whereas higher volume adds to the cost and iatrogenic anemia to the patient.

The need to draw repeat culture hinges on the patient's response to initial treatment, culture findings, and antimicrobial susceptibility test results. It may take a few days for a patient under adequate therapy to show obvious clinical response. The patient may still spike a fever for 2–3 days while clearing the killed and dying microorganism in the circulation. Persistence of fever during therapy is a common reason to repeat culture.

Atmosphere and length of incubation. Traditionally, both aerobic and anaerobic cultures have been used and thus recommended. However, the declining proportion of bacteremias due to obligate anaerobes has led to suggestion that routine anaerobic cultures are not needed and can be tailored to the needs of individual institution and patient population. Anaerobic cultures are valuable for patients with surgery or gynecologic/obstetric procedures because of the high number of anaerobes in the lower gastrointestinal and urogenitary tracts.

How long to incubate blood culture? Several studies on different culturing systems have shown that a 5-day culturing and testing is sufficient to recover nearly all significant microorganisms (~99%) [3, 6–9]. The vast majority of organisms become

culture positive in the first 3 days, and most fastidious bacteria can be recovered during the extra 2 days, such as the HACEK organisms (*Haemophilus aphrophilus*, *Aggregatibacter* (*Actinobacillus) actinomycetemcomitans*, *Cardiobacterium* species, *Eikenella corrodens*, and *Kingella kingae*), *Brucella* spp., and nutritionally variant streptococci (currently known as *Abiotrophia* species and *Granulicatella* species) [10]. A new species, *Cardiobacterium valvarum*, proposed by us as well as a cause of endocarditis, can be cultured within 3 days [11]. The length of culture for *Brucella* spp. had been controversial until studies done in the past decade or so by Bannatyne et al. [12] showed that 90 of 97 such bacteremic patients became culture positive within 5 days and by Baysallar et al. [13] that 30 of 30 were positive within 4 days. Blood cultures for *Francisella tularensis*, fewer than a dozen such culture-positive cases in the United States nowadays, mostly become positive after incubation for 3–8 days [10, 14]. Yeasts, such as *Candida* species that have been within the list of the most common ten blood culture organisms [3, 6, 8], can also be cultured within the 5 days.

Interpretation of significance. Interpretation of a positive blood culture is not always straightforward. Both the isolated microorganism and the host factors need to be considered on a case-by-case basis. The pathogenic potential of an organism should be considered and can be roughly divided into three categories: strict pathogens irrespective of host factors, such as *F. tularensis*, *Mycobacterium tuberculosis*, *Brucella* species, *Yersinia pestis*, *Bacillus anthracis*, and others; usual pathogens, such as *Staphylococcus aureus*, almost all members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Candida albicans*, and most other gram-negative bacilli; and occasional or conditional pathogens with low pathogenic potential, such as *Bacillus* species (excluding *B. anthracis*) that are soil dwellers, coagulase-negative staphylococci (CoNS), coryneform bacilli, alpha-hemolytic or viridans group streptococci, and *Propionibacterium acne* that are common skin and/or gut flora. Those usual pathogens are common blood isolates and are almost always clinically significant once isolated. On the other hand, those occasional pathogens are common blood culture contaminants and usually become positive late during the 5-day incubation period. In most hospitals, isolation of an occasional pathogen from a single bottle means contaminant whereas from two or more bottles likely denotes true infection. Therefore, with more and more patients having immune defects in one way or another or carrying intravascular devices that are prone to colonization and infection, each positive culture entails clinical correlation with other findings and sound judgment to make final assessment [15, 16]. For instance, we found that, in patients with cancer who have mucositis and severe neutropenia or immune suppression, isolation of viridans group streptococci means true infection most of the time instead of contaminants [17].

Rapid microbial identification after culture. After positive culture signal, it is necessary to subculture the microorganism for purity and quantity on agar plates for further identification and susceptibility test. Depending on the growth rate of the organism, these steps may take one to three days or even longer, which may cause delay in obtaining accurate information for optimal management of the patient.

Thus, in recent years, efforts have been made to rapidly identify the microorganism in a positive culture. Because CoNS and *S. aureus* are gram-positive cocci and common blood isolates and yet have different pathogenic potential and thus therapeutic and prognostic significance, rapid differentiation of these staphylococci, particularly methicillin-resistant *S. aureus*, upon culture recovery is important. Studies found that differentiation of *S. aureus* and CoNS in a few hours can be achieved with tube coagulase test, peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) (AdvanDx, Woburn, Mass.), or API RAPIDEC staph (API) (bioMerieux, Durham, NC) [18–20]. The tube coagulase test is cheap and easy to perform and can be used in small hospital settings. Recently, Qian et al. [21] also found that a latex agglutination test based on penicillin binding protein 2a could detect methicillin-resistant *S. aureus* from positive blood broth with sensitivity, specificity, and predictive values all >90%. Larger scale confirmatory studies on this method may be needed. Other rapid microbial identification methods from blood culture bottles are also being evaluated, including direct inoculation into commercial systems for microbial identification and susceptibility (such as VITEK instruments, BD PHOENIX system, and MicroScan system), various polymerase chain reaction-based methods, and others. These methods have inherent costs and also potential benefits. The many literature articles are not cited here in view of the scope of this article.

Other trends. Some noticeable trends in the past decades are the following: increasing number and life span of immuno-compromized or -suppressed patients, and thus emergence of more opportunistic pathogens; more frequent use of antibiotics and associated resistance, in fact, up to 29% of blood cultures came from patients with active antimicrobic therapy; more use of indwelling devices, such as intravascular catheters and others; and emergence of more *Candida* and other fungal infections [3, 22].

Automated Culturing Systems

Blood culture has evolved over four decades from manual methods, now hardly being used, to automated culturing systems. The major advantage of an automated system, such as BACTEC NR660, is the obviation of manual inspection or examination to detect microbial growth because each system automatically does so by monitoring microbial CO_2 production. Agitation of culture bottles also improves mixing and aeration to promote the growth of aerobes and facultative anaerobes. These features make blind subcultures of negative bottles unnecessary, as shown in a few studies reviewed by Reimer et al. [2]. Automation has improved the practice of blood culture enormously in terms of timely report of positive culture and more laboratory efficiency, and consequently better patient care.

Continuously monitoring blood culturing systems (CMBCSs) are most commonly used nowadays. Introduced in the early 1990s, CMBCSs have added nearly continuous (every 10–12 min) monitoring of microbial growth to the automated

Table 1.1 Commercial continuously monitoring blood culturing systems (CMBCSs)

Manufacturer	Current system, year	Test interval (min)	Microbial detection mechanism	Initial system since early 1990s
BioMerieux	BacT/Alert 3D, 2001	10	Colorimetric for CO_2 production	BacT/Alert series for varying holding capacity
Becton-Dickinson	BACTEC FX, 2009	10	Fluorescent for CO_2 production	BACTEC series for varying holding capacity
Trek	VersaTrek, 2004	12	Manometric for gas (CO_2 and other gases)	ESP series for varying holding capacity

Table 1.2 Performance of culture media with or without lytic agents or additives

Compared systems and/or media (bottle)	Findings	Reference
BacT/Alert FAN vs. BacT/Alert standard	BacT/Alert FAN improved recovery of *S. aureus*, CoNS, and enterics	[24]
BacT/Alert FAN vs. BACTEC Plus/F	Comparable	[25]
BacT/Alert FAN vs. Trek ESP 80A, in pediatric patients	Overall comparable. BacT/Alert FAN better for *S. aureus* and antibiotic-treated samples; ESP 80A better for streptococci and enterococci	[27]
BacT/Alert FAN vs. Trek ESP 80A	BacT/Alert FAN improved recovery of *S. aureus*, enterics, and non-*Pseudomonas aeruginosa* gram-negative rods	[26]
BacT/Alert FAN vs. BACTEC fungal medium	Comparable to detect fungemia	[28]
BACTEC Plus Anaerobic/F bottles vs. Standard Anaerobic/F bottles	BACTEC Plus Anaerobic/F bottles detected more microorganisms	[29]
BacT/Alert standard vs. BACTEC 9240 standard	BacT/Alert standard improved recovery of *S. aureus*, CoNS, and yeasts	[30]
BacT/Alert FA Medium in plastic vs. glass bottles	Comparable	[31, 32]
BacT/Alert 3D SA and SN vs. VersaTrek Redox media	Overall comparable for bacteria and fungi. VersaTrek better in detecting streptococci and enterococci	[33]

systems. Currently, three CMBCSs are available in the United States and they are briefly shown in Table 1.1. More detailed description can be found elsewhere [23]. New versions of CMBCS, available for the past several years, have kept the key elements from earlier versions while refining the hardware, computer system, data processing, and report. The trend is to increase user-friendly features for space, operation, and flexibility.

Numerous studies have been published to compare the performance of the CMBCSs and associated media with or without various lytic agents or additives to remove blood antimicrobics, and several recent ones are summarized as follows (Table 1.2). McDonald et al. [24] compared the BacT/Alert standard bottle with

BacT/Alert FAN bottle that contains Ecosorb™, an antimicrobic-absorbing substance, and they found that FAN bottle recovered significantly more microbes from all septic episodes, especially *S. aureus*, CoNS, and members of *Enterobacteriaceae*. Along with this, however, recovery of all contaminants, including CoNS, was also higher. The performance of the BacT/Alert FAN bottle and BACTEC Plus aerobic/F bottle (with resins to absorb antimicrobics) was also compared and the two systems were found to be comparable [25]. In a study comparing BacT/Alert FAN vs. Trek ESP 80A, Doern et al. [26] found that BacT/Alert FAN recovered more *S. aureus*, enterics, and non-*Pseudomonas aeruginosa* gram-negative rods, along with more contaminants too. In a similar study in pediatric patients [27], the two systems were found to be overall comparable with BacT/Alert FAN recovering more *S. aureus* and better for antibiotic-containing samples and ESP 80A recovering more streptococci and enterococci. Since yeasts are an increasing cause of nosocomial bloodstream infections, McDonald et al. [28] compared BacT/Alert FAN with BACTEC fungal medium for their recovery, and the two systems were found comparable. The anaerobic culture media have also been compared; a recent study by Wilson et al. [29] found that the BACTEC Plus Anaerobic/F bottles detected more microorganisms and episodes of bacteremia and fungemia than the BACTEC Standard Anaerobic/F bottles. Mirrett et al. [30] compared BacT/Alert standard bottle and BACTEC standard bottle and found that the former significantly improved the recovery of *S. aureus*, CoNS, and yeasts. Two recent studies found that, in the BacT/Alert system, the plastic culture bottles were comparable to the glass bottles [31, 32].

In a recent study of the newer version CMBCS, Mirrett et al. [33] compared the BacT/Alert 3D standard media and VersaTrek Redox media and found that they were overall comparable. Together, these data show that CMBCSs, each with cost, strength, and weakness, perform well overall in delivering timely and accurate diagnosis of bloodstream infections. Incorporation of lytic or antimicrobic-absorbing substances in these systems has consistently improved the recovery of *S. aureus* and members of *Enterobacteriaceae*, particularly from treated patients.

Blood Culture and CMBCS for Mycobacteria

Bacteremia due to rapidly growing mycobacteria (RGM) can be detected by blood cultures, similar to other fastidious organisms. In a recent study, we summarized our experience with 115 consecutive clinical RGM strains [34]. Of them, 46 (40%) were isolated from blood cultures using the BACTEC 9240 and/or the Isolator 10 system (Wampole Laboratories, Princeton, NJ). These RGM typically grow in 3–5 days and the bacteremias are usually associated with the use of intravascular catheter. Among the 46 blood RGM isolates, *M. mucogenicum* was most common (24 of 46, or 52%), followed by *M. abscessus* and *M. fortuitum*.

In contrast to RGM, *M. avium* and *M. tuberculosis* are two species of many slowly growing mycobacteria. Blood culture has been useful to detect and monitor *M. avium* bacteremia in patients with AIDS. *M. avium* bacteremia usually occurs when

the CD4+ cell count falls below 50/mm^3 [35]. Circulating *M. avium*, exclusively within monocytes, usually ranges in 10–10^3 colony forming units (CFUs) per ml blood, but can be as high as 10^6 CFU/ml [35]. A number of blood culture systems have been used: the earlier BACTEC 13A radiometric system and Isolator 10 system and the more recent CMBCS, such as BACTEC 9240 with MYCO/F LYTIC medium and BacT/Alert MB. Several studies have evaluated these systems; for example, in a controlled comparison of the performance of these systems, Crump et al. [36] found that these systems perform comparably well in detecting *M. avium* bacteremia and other mycobacterial and fungal sepsis. In addition, the two CMBCSs detect *M. avium* bacteremia 2–3 days sooner than the earlier systems. On average, an incubation of 14 days is required.

Blood culture also detects *M. tuberculosis* bacteremia [36]. *M. tuberculosis* bacteremia seems particularly common in AIDS patients in developing countries. For examples, in Tanzania, it is the most common organism of all sepsis-causing microorganisms, accounting for 48% (57 of 118 patients) [37]. In Thailand, it ranks the second (27 of 114 patients), following *Cryptococcus neoformans* (30 of 114) and surpassing *M. avium* (24 of 114) [38]. In Brazil, it is also the most common cause of mycobacterial sepsis [39]. Culture of blood is as sensitive as culture of bone marrow to detect *M. tuberculosis* and its role seems expanding [40]. *M. tuberculosis* bacteremia in patients with AIDS carries a high and quick mortality [41]. Recent efforts in developing countries have evaluated the performance of a few systems in culturing *M. tuberculosis* bacteremia, such as the usual Isolator 10 system and BACTEC with MYCO/F LYTIC medium, conventional BacT/Alert FA, and BacT/Alert MB [41–44]. Crump et al. [44] found that BacT/Alert MB detected *M. tuberculosis* bacteremia more rapidly than the manual methods BACTEC with MYCO/F LYTIC medium and Isolator 10 system, but the mean time to positivity exceeded 3 weeks. The mean CFUs per milliliter blood were found to be 30 CFU/ml in one study [44] and 8 CFU/ml in another study [41]. Together, these studies may assist timely empiric antibiotic coverage against tuberculosis in patients with AIDS in these countries to reduce immediate mortality once the patient is seen at the hospital. These data may also impact public health policies and health care priorities in their respective countries.

Concluding Remarks

In conclusion, automatic blood cultures have become the diagnostic mainstay for bloodstream infections. The systems are refined and able to cultivate various bacteria, fungi, and mycobacteria. The laboratories have seen improved efficiency through automation and a 5-day culturing cycle (except mycobacteria). With the vast majority of significant isolates being detected within the first 72 h of culture, the timely care of patients is facilitated. The future challenge remains that the sooner the identification of cultured organism is rendered the better the patient care will be.

References

1. Weinstein MP (1996) Current blood culture methods and systems: clinical concepts, technology, and interpretation of results. Clin Infect Dis 23:40–46
2. Reimer LG, Wilson ML, Weinstein MP (1997) Update on detection of bacteremia and fungemia. Clin Microbiol Rev 10:444–465
3. Cockerill FR 3, Wilson JW, Vetter EA, Goodman KM, Torgerson CA, Harmsen WS, Schleck CD, Ilstrup DM, Washington JA 2, Wilson WR (2004) Optimal testing parameters for blood cultures. Clin Infect Dis 38:1724–1730
4. Lee A, Mirrett S, Reller LB, Weinstein MP (2007) Detection of bloodstream infections in adults: how many blood cultures are needed? J Clin Microbiol 45:3546–3548
5. Patel R, Vetter EA, Harmsen WS, Schleck CD, Fadel HJ, Cockerill FR, 3 (2011) Optimized pathogen detection with 30- compared to 20-milliliter blood culture draws. J Clin Microbiol 49(12):4047–51
6. Wilson ML (1997) Clinically relevant, cost-effective clinical microbiology. Strategies to decrease unnecessary testing. Am J Clin Pathol 107:154–167
7. Doern GV, Brueggemann AB, Dunne WM, Jenkins SG, Halstead DC, McLaughlin JC (1997) Four-day incubation period for blood culture bottles processed with the Difco ESP blood culture system. J Clin Microbiol 35:1290–1292
8. Han XY, Truant AL (1999) The detection of positive cultures by the Accumed ESP-384 system: the clinical significance of three day testing. Diagn Microbiol Infect Dis 33:1–6
9. Bourbeau PP, Foltzer M (2005) Routine incubation of BacT/ALERT FA and FN blood culture bottles for more than 3 days may not be necessary. J Clin Microbiol 43(5):2506–2509
10. Doern GV, Davaro R, George M, Campognone P (1996) Lack of requirement for prolonged incubation of Septi-Chek blood culture bottles in patients with bacteremia due to fastidious bacteria. Diagn Microbiol Infect Dis 24:141–143
11. Han XY, Meltzer MC, Woods JT, Fainstein V (2004) Endocarditis with ruptured cerebral aneurysm caused by *Cardiobacterium valvarum* sp. nov. J Clin Microbiol 42:1590–1595
12. Bannatyne RM, Jackson MC, Memish Z (1997) Rapid diagnosis of *Brucella* bacteremia by using the BACTEC 9240 system. J Clin Microbiol 35:2673–2674
13. Baysallar M, Aydogan H, Kilic A, Kucukkaraaslan A, Senses Z, Doganci L (2006) Evaluation of the BacT/ALERT and BACTEC 9240 automated blood culture systems for growth time of *Brucella* species in a Turkish tertiary hospital. Med Sci Monit 12(7):BR235–BR238
14. Han XY, Ho LX, Safdar A (2004) *Francisella tularensis* peritonitis in stomach cancer patient. Emerg Infect Dis 10(12):2238–2240
15. Mirrett S, Weinstein MP, Reimer LG, Wilson ML, Reller LB (2001) Relevance of the number of positive bottles in determining clinical significance of coagulase-negative staphylococci in blood cultures. J Clin Microbiol 39:3279–3281
16. Weinstein MP (2003) Blood culture contamination: persisting problems and partial progress. J Clin Microbiol 41:2275–2278
17. Han XY, Kamana M, Rolston KV (2006) Viridans streptococci isolated by cultures from blood of cancer patients: clinical and microbiologic analysis of 50 cases. J Clin Microbiol 44: 160–165
18. Chapin K, Musgnug M (2003) Evaluation of three rapid methods for the direct identification of *Staphylococcus aureus* from positive blood cultures. J Clin Microbiol 41:4324–4327
19. Oliveira K, Brecher SM, Durbin A et al (2003) Direct identification of Staphylococcus aureus from positive blood culture bottles. J Clin Microbiol 41(2):889–891
20. Qian Q, Eichelberger K, Kirby JE (2007) Rapid identification of *Staphylococcus aureus* in blood cultures by use of the direct tube coagulase test. J Clin Microbiol 45(7):2267–2269
21. Qian Q, Venkataraman L, Kirby JE, Gold HS, Yamazumi T (2001) Direct detection of methicillin resistance in *Staphylococcus aureus* in blood culture broth by use of a penicillin binding protein 2a latex agglutination test. J Clin Microbiol 48(4):1420–1421

22. Weinstein MP, Towns ML, Quartey SM, Mirrett S, Reimer LG, Parmigiani G, Reller LB (1997) The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. Clin Infect Dis 24:584–602
23. Weinstein MP, Reller LB (2002) Commercial blood culture systems and methods. In: Truant AL (ed) Manual of commercial methods in clinical microbiology. ASM, Washington, DC, pp 12–21
24. McDonald LC, Fune J, Gaido LB, Weinstein MP, Reimer LG, Flynn TM, Wilson ML, Mirrett S, Reller LB (1996) Clinical importance of increased sensitivity of BacT/Alert FAN aerobic and anaerobic blood culture bottles. J Clin Microbiol 34:2180–2184
25. Jorgensen JH, Mirrett S, McDonald LC, Murray PR, Weinstein MP, Fune J, Trippy CW, Masterson M, Reller LB (1997) Controlled clinical laboratory comparison of BACTEC plus aerobic/F resin medium with BacT/Alert aerobic FAN medium for detection of bacteremia and fungemia. J Clin Microbiol 35:53–58
26. Doern GV, Barton A, Rao S (1998) Controlled comparative evaluation of BacT/Alert FAN and ESP 80A aerobic media as means for detecting bacteremia and fungemia. J Clin Microbiol 36:2686–2689
27. Welby-Sellenriek PL, Keller DS, Ferrett RJ, Storch GA (1997) Comparison of the BacT/Alert FAN aerobic and the Difco ESP 80A aerobic bottles for pediatric blood cultures. J Clin Microbiol 35:1166–1171
28. McDonald LC, Weinstein MP, Fune J, Mirrett S, Reimer LG, Reller LB (2001) Controlled comparison of BacT/Alert FAN aerobic medium and BACTEC fungal blood culture medium for detection of fungemia. J Clin Microbiol 39:622–624
29. Wilson ML, Mirrett S, Meredith FT, Weinstein MP, Scotto V, Reller LB (2001) Controlled clinical comparison of BACTEC plus anaerobic/F to standard anaerobic/F as the anaerobic companion bottle to plus aerobic/F medium for culturing blood from adults. J Clin Microbiol 39:983–989
30. Mirrett S, Reller LB, Petti CA, Woods CW, Vazirani B, Sivadas R, Weinstein MP (2003) Controlled clinical comparison of BacT/Alert standard aerobic medium with BACTEC standard aerobic medium for culturing blood. J Clin Microbiol 41:2391–2394
31. Petti CA, Mirrett S, Woods CW, Reller LB (2005) Controlled clinical comparison of plastic and glass bottles of BacT/ALERT FA medium for culturing organisms from blood of adult patients. J Clin Microbiol 43(4):1960–1962
32. Riley JA, Heiter BJ, Bourbeau PP (2005) Comparative recovery of microorganisms from BacT/ALERT plastic and glass FA and FN blood culture bottles. J Clin Microbiol 43(7):3244–3246
33. Mirrett S, Hanson KE, Reller LB (2007) Controlled clinical comparison of VersaTREK and BacT/ALERT blood culture systems. J Clin Microbiol 45(2):299–302
34. Han XY, De I, Jacobson KL (2007) Rapidly growing mycobacteria: clinical and microbiologic analyses of 115 cases. Am J Clin Pathol 128:612–621
35. Inderlied CB, Kemper CA, Bermudez LM (1993) The *Mycobacterium avium* complex. Clin Microbiol Rev 6:266–310
36. Crump JA, Reller LB (2003) Two decades of disseminated tuberculosis at a university medical center: the expanding role of mycobacterial blood culture. Clin Infect Dis 37:1037–1043
37. Archibald LK, den Dulk MO, Pallangyo KJ, Reller LB (1998) Fatal *Mycobacterium tuberculosis* bloodstream infections in febrile hospitalized adults in Dar es Salaam, Tanzania. Clin Infect Dis 26:290–296
38. Archibald LK, McDonald LC, Rheanpumikankit S, Tansuphaswadikul S, Chaovanich A, Eampokalap B, Banerjee SN, Reller LB, Jarvis WR (1999) Fever and human immunodeficiency virus infection as sentinels for emerging mycobacterial and fungal bloodstream infections in hospitalized patients >/=15 years old, Bangkok. J Infect Dis 180:87–92
39. Grinsztejn B, Fandinho FC, Veloso VG, Joao EC, Lourenco MC, Nogueira SA, Fonseca LS, Werneck-Barroso E (1997) Mycobacteremia in patients with the acquired immunodeficiency syndrome. Arch Intern Med 157:2359–2363

40. Crump JA, Tanner DC, Mirrett S, McKnight CM, Reller LB (2003) Controlled comparison of BACTEC 13A, MYCO/F LYTIC, BacT/Alert MB, and Isolator 10 systems for detection of mycobacteremia. J Clin Microbiol 41:1987–1990
41. Munseri PJ, Talbot EA, Bakari M, Matee M, Teixeira JP, von Reyn CF (2011) The bacteraemia of disseminated tuberculosis among HIV-infected patients with prolonged fever in Tanzania. Scand J Infect Dis 43:696–701
42. Hanscheid T, Monteiro C, Cristino JM, Lito LM, Salgado MJ (2005) Growth of *Mycobacterium tuberculosis* in conventional BacT/ALERT FA blood culture bottles allows reliable diagnosis of Mycobacteremia. J Clin Microbiol 43(2):890–891
43. Gopinath K, Kumar S, Singh S (2008) Prevalence of mycobacteremia in Indian HIV-infected patients detected by the MB/BacT automated culture system. Eur J Clin Microbiol Infect Dis 27(6):423–431
44. Crump JA, Morrissey AB, Ramadhani HO, Njau BN, Maro VP, Reller LB (2011) Controlled comparison of BacT/Alert MB system, manual Myco/F lytic procedure, and isolator 10 system for diagnosis of *Mycobacterium tuberculosis* bacteremia. J Clin Microbiol 49(8):3054–3057

Chapter 2
Breath Tests for Detection of *Helicobacter pylori* and *Aspergillus fumigatus*

Sihe Wang, Wan-Ming Zhang, and Edmunds Reineks

Introduction

Helicobacter pylori

The association of *Helicobacter pylori* with peptic ulcer disease and gastric cancer was first proposed by Warren and Marshall in 1983 [1]. In February 1994, the National Institutes of Health Consensus Development Conference concluded that *H. pylori* infection is the major cause of peptic ulcer disease, and all patients with confirmed peptic ulcer disease associated with *H. pylori* infection should receive treatment with antimicrobial agents [2]. The International Agency for Research on Cancer Working Group of the World Health Organization categorized *H. pylori* as a group I, or definite, human carcinogen [3]. Based on the data retrieved during the National Health Interview Survey of 1989, 10% of adult US residents reported physician-diagnosed ulcer disease, among whom one-third had an ulcer in the past year [4]. In developing countries, the prevalence of *H. pylori* carriers can be as high as 70–90%. The prevalence of the infection in developed countries is lower, ranging from 25 to 50% with most patients acquiring the infection at childhood [5]. Seroprevalence studies in adults demonstrated an increasing rate of 3–4% per decade [3, 6–8].

H. pylori-infected patients may develop chronic gastric inflammation that can be asymptomatic. *H. pylori* infection is associated with peptic ulcer disease and eradication of the infection leads to cure of the ulcers [5]. *H. pylori* infection is also associated with gastric adenocarcinoma [9] and mucosa-associated lymphoid tissue (MALT) lymphoma [10]. The American Medical Association published guidelines in 2000 for testing and treatment of *H. pylori*-related disease [11]. More recently,

S. Wang (✉) • W.-M. Zhang • E. Reineks
Department of Clinical Pathology, Cleveland Clinic,
9500 Euclid Avenue, Cleveland, OH 44195, USA
e-mail: wangs2@ccf.org

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two updated guidelines for testing-and-treating strategies were published by American College of Gastroenterology and the European Helicobacter Study Group [12, 13]. The American College of Gastroenterology guidelines recommend testing for *H. pylori* in patients with active ulcers, a history of ulcers, gastric MALT lymphomas, a history of endoscopic resection of early gastric cancer, and uninvestigated dyspepsia [12].

In the USA, the recommended primary treatment for the infection includes a proton pump inhibitor (PPI), clarithromycin, and amoxicillin, or metronidazole for 14 days or a PPI or histamine-2 receptor antagoinist (H2RA), bismuth, metronidazole, and tetracycline for 10–14 days [12]. The "MOC" therapy, consisting of metronidazole, omeprazole, and clarithromycin administered for 7–14 days, may offer greater than 90% eradication rate [3]. Because of resistance developed by the organism, quadruple therapy (PPI, tetracycline, metronidazole, and a bismuth salt) has been used to improve the efficacy and is associated with fewer side effects [5]. The quadruple therapy should be considered as first-line treatment for *H. pylori* infection in areas where the prevalence of resistance to clarithromycin or metronidazole is high [14, 15]. An *H. pylori* vaccination strategy was investigated and showed promising results, but unfortunately the sponsorship for the research was later withdrawn [16, 17].

Aspergillus spp.

Aspergillus is a spore-forming fungus consisting of ~200 species among which less than 20 have been found to cause infection in human, known as aspergillosis. *A. fumigatus* is the most frequently identified pathogen in human aspergillosis [18]. The primary route of infection by *Aspergilli* is via inhalation of the airborne conidia followed by deposition of these conidia in the bronchioles or alveolar spaces [19]. In general the alveolar macrophages are the first line of defense against the inhaled *Aspergillus* via oxidation and phagolysosomal acidification [20, 21]. *Aspergillus* is an opportunistic pathogen and disease development depends on the host's immune function. Aspergillosis is frequently found in immunocompromised patient populations, such as those with chronic granulomatous disease, AIDS, acute leukemia, and neutropenia and those treated with immunosuppressants [22].

Human aspergillosis can be clinically divided into three types: saprophytic, allergic, or invasive. Saprophytic aspergillosis is defined as a colonization of *Aspergillus* in the respiratory tract, usually does not invade or damage tissues, and may present as aspergilloma [23]. Management of the saprophytic forms of aspergillosis depends on the condition. For example, single pulmonary aspergillomas may be treated by surgical resection, while chronic cavitary and chronic necrotizing pulmonary aspergillosis may require long-term medical treatment [24]. Allergic aspergillosis is often referred to as allergic bronchopulmonary aspergillosis (ABPA) which is caused by a hypersensitivity reaction to *A. fumigatus* [25]. The treatments for ABPA are focused on controlling the immunologic activity and attenuating the fungal burden. Steroids and antifungal agents are the two major means to achieve

these goals [24, 25]. The invasive aspergillosis (IA) is the most severe form of *Aspergillus* infection and causes high mortality and morbidity [24, 26]. The majority of the IA cases were found to be restricted to lungs (56%), followed by disseminated infection (19%), central nervous system (CNS) infection (6%), and skin and sinus infections (5% each) [27]. Of the numerous *Aspergillus* spp., *A. fumigatus* accounts for about 90% of the IA cases [28]. The primary treatments for IA infections include voriconazole, amphotericin B, and liposomal amphotericin B, while caspofungin, amphotericin B lipid complex, or posaconazole can be used as alternatives [24, 26]. Early diagnosis and prompt initiation of treatment are the key factors to patient outcome [24, 26].

Conventional Diagnostic Tests

Detection of H. pylori

Detection of the organism in biopsy tissue specimens. Patients infected with *H. pylori* can be diagnosed by examining biopsy tissue specimens obtained through endoscopy [29, 30]. The organism can be directly detected in hematoxylin and eosin (H/E)-stained histology tissue samples, or in imprint cytology specimens with the same staining [31]. The organisms in tissue can also be stained with silver, Giemsa, or Ganta staining methods [3, 29, 32]. *H. pylori* can also be indirectly detected in the gastric biopsy tissue by testing its urease activity (rapid urease test). This enzyme (organism), present in the specimen, converts urea in the testing medium into ammonia. The elevated pH, as a result of the reaction, can be observed with a color pH indicator in the testing medium [3]. These methods are reasonably sensitive, specific, and easy to perform. However, invasive procedures are required.

Antibody detection. *H. pylori*-specific antibodies, IgG or IgA, can be detected in infected patient serum samples by immunological tests, most commonly ELISA assays [33]. These assays are commercially available in both laboratory-based and point of care-based formats. The disadvantage is that these antibodies may persist for months or years after infection and results need careful interpretation [34]. Because the sensitivity and specificity for detection of antibodies against *H. pylori* vary widely in children, it is not to be solely relied on in this age group in either diagnosis of infection or confirmation of eradication [34].

Detection of antigen in stool. *H. pylori*-specific antigen can be detected in stool samples from infected patients by monoclonal or polyclonal antibody-based assays [35–38]. The tests using monoclonal antibodies have better diagnostic accuracy compared to those with polyclonal antibodies [39]. In a meta-analysis of 13 pre-treatment studies, Gisbert et al. found that sensitivity and specificity of the tests using monoclonal antibodies were 0.95 (ranged 0.93–0.96) and 0.96 (0.94–0.98), respectively. When polyclonal antibody-based tests were used in the same studies, specificity (0.96, ranged 0.94–0.97) was similar, but sensitivity (0.83; 0.80–0.85)

was significantly lower [40]. The stool antigen tests are also useful for monitoring the therapeutic response and confirming *H. pylori* eradication with a sensitivity of 0.93 and specificity of 0.96 [40].

Molecular detection. *H. pylori* can be detected by molecular methods, such as polymerase chain reaction (PCR) targeting *H. pylori* species-specific genes [41–43]. The specimens that these methods may work on include blood, saliva, feces, and biopsy tissues. The PCR methods are also used for genotyping of *H. pylori* and detecting antibiotic resistance genes and gene mutations [44–47]. Therefore, the PCR methods are useful not only for diagnosis but also for selection of therapeutic drugs. However, the PCR methods cannot distinguish dead and living organisms and should not be used to confirm eradication [43]. Also, the PCR methods may produce false-positive results partially due to the detection of cDNA from non-*H. pylori* organisms [48].

Detection of Aspergillus spp.

Histological detection and culture. Direct detection of *Aspergillus* in tissue or fluid specimens is used to confirm the diagnosis of IA. *Aspergillus* hyphae may be stained with a fungal-specific stain, for instance, Gomori's methenamine silver stain [49]. Under direct microscopic observation, *Aspergillus* spp. in tissue sections typically appear as slender septate hyphae that exhibit angular dichotomous branching [50]. The *Aspergillus* hyphae may also be stained with *Aspergillus* spp.-specific or species-specific antibodies or probes [51–53]. A culture from a biopsy or sputum specimen yielding *Aspergillus* not only provides evidence of *Aspergillus* infection, but also allows for susceptibility testing. A positive culture indicates IA disease in immunocompromised patients [54, 55].

Antibody detection. The antibodies against *Aspergillus* spp. are detected in sera from patients with chronic pulmonary aspergillosis [56, 57]. Because it takes time for a patient with aspergillosis to generate sufficient antibodies against *Aspergillus*, antibody detection may not be effective for patients with compromised immune systems [58]. Anti-*Aspergillus* antibody level is very low at the onset of IA, but specific antibodies in some patients may increase significantly by day 14 or later [59].

Antigen detection. Several surface antigens of *Aspergillus* may be detected in the blood samples from patients with aspergillosis [60]. Immunoassays for galactomannan and beta-glucan were most commonly used for diagnosis and therapeutic monitoring of IA [61–63].

Galactomannan is a polysaccharide that presents in the cell wall of most *Aspergillus* species [64]. It may be present in tissues and blood, as well as body fluids including CSF, urine, and bronchoalveolar lavage fluid [65]. The level of galactomannan in blood is associated with fungal load in the tissues [66, 67]. Sulahian et al. reported that detection of galactomannan by an ELISA assay preceded the onset of radiologic signs by 1 week in 65% of the patients with IA [68].

(1,3)-Beta-D-glucan (BG) is a component of the cell wall of a wide variety of fungi and is a sensitive biomarker for diagnosis of Aspergillosis. For example, the detection of serum BG preceded clinical diagnosis of IA by 18 days in four probable IA cases [63]. Combination of BG and galactomannan tests was proven to significantly improve the specificity and diagnostic accuracy for IA [69].

Molecular detection. Nucleic acid probes (AccuProbe, Gen-Probe, San Diego, CA) are commercially available for identification of Aspergillus species on purified isolates. PCR detection of *Aspergillus* in either blood or Bronchoalveolar lavage is highly sensitive and specific for diagnosis of IA [70, 71]. *Aspergillus* DNA may also be detected in other samples, such as CSF, tissue biopsies, or paraffin-embedded tissues [72, 73].

Breath Tests

Introduction

Testing of a patient's breath was found useful to detect substances associated with specific diseases. For instance, distinct changes in breath components were noted in the breath of patients with liver diseases or chronic renal failure [74]. The exhaled air specimen contains aqueous and gaseous phases. The aqueous component consists mainly of water vapor and aerosol compounds formed in the lower respiratory tract, and the gaseous component contains inorganic gases, such as NO, CO_2, and various volatile organic compounds (VOCs). The exhaled VOCs can be divided into endogenous and exogenous VOCs. The endogenous VOCs are produced from various metabolic processes and are distributed through the alveolar-interface or are produced in the airway or oral cavity. VOCs can also be derived from an exogenous source, such as alcohol use, environmental pollutants and microbial infection, and colonization [75].

Sample Collection

Exhaled air samples are collected into various containers that can be directly or indirectly connected to analytical instruments. The exhaled air usually contains a mixture of alveolar air and ambient air retained in the respiratory dead space. The sampling approaches can be divided into alveolar air or mixed air collection. The latter is most often used due to its simplicity. However, the VOCs are usually diluted and contaminated in the mixed air, which may result in large variations. The collection apparatus for alveolar air is designed to optimally sample alveolar air while diverting dead space air to a reservoir. For each breath of the tested

subject, only alveolar air stored in the sample port is removed and analyzed [76]. The concentrations of VOCs in inspiratory air or the ambient air are measured as background [77]. Because the concentration of VOCs in the exhaled specimen is usually very low (at the level of parts per billion or trillion), a number of concentration techniques are utilized prior to analysis. Enrichment of VOCs in the exhaled air specimen can be achieved by using solid-phase microextraction (SPME), in which VOCs are adsorbed onto coated fibers, or sorbent traps. The adsorbed analytes are released from traps or fibers by thermodesorption [78]. Enrichment can also be achieved by cryofocusing [79, 80]. The enriched VOCs are measured by gas chromatography-mass spectrometry (GC-MS).

Urea Breath Test for H. pylori

Urea breath tests can detect current *H. pylori* infection. This test is based on the knowledge that *H. pylori* produces abundant active urease, an enzyme that converts urea to ammonium and carbon dioxide (CO_2) [81, 82]. When infected with *H. pylori*, high urease activity is present in the patient's stomach. A dose of urea labeled with either ^{13}C or ^{14}C is ingested by the subject. The urease-catalyzed reaction then takes place in the mucus layer where *H. pylori* is present, the labeled CO_2 diffuses to the epithelial cells, and then is carried in the bloodstream. Ultimately it is released in exhaled air. The labeled CO_2 in the subject's breath can be measured. The amount of the labeled CO_2 relates to the urease activity, which indicates the presence or absence of *H. pylori* infection [81–83]. The amounts of isotopic CO_2 can be measured by various techniques and the results are expressed relative to endogenous CO_2 production. The sensitivity and specificity of breath tests range from 95 to 97%, although this method has been reported to be less reliable for patients with gastric surgery or in patients who take PPI or ranitidine [82]. In a study involving 20 volunteers, Cutler et al. found that ranitidine at a standard (150 mg b.i.d.) or high dose (300 mg b.i.d.) does not significantly alter breath test results, and suggested that ranitidine does not need to be discontinued before a urea breath test [84]. However, this observation has been challenged by a later study showing that false negative results in urea breath test were obtained in patients taking pantoprazole and ranitidine even if these patients were pretreated with citric acid [85].

^{14}C-Urea breath test. Conventionally, patient preparation for this test requires fasting for at least 4 h and oral ingestion of 5 μCi ^{14}C-urea in 20 mL water. Breath is collected 20 min post dosing in a CO_2-absorbing solution (examples are hyamine–methanol solution or benzethonium hydroxide–methanol with a pH indicator) [86–88]. Radioactivity in the sample is measured by a scintillation counter and the result is expressed as counts per minute (cpm) or as a specific activity at a specific post-dosing time (AS_{time}) [86–88]:

$$AS_{time} = (\%^{14}CO_2 \text{ dose excreted} / \text{mmol of } CO_2) \times \text{weight (kg)},$$

where the ^{14}C-urea dose is calculated from measurements of standard solutions with known concentrations of ^{14}C-urea.

$$^{14}CO_2 \text{ dose excreted} = \text{counts at the specific time} - \text{counts at baseline.}$$

This parameter is corrected for the patient's weight in the first equation [86]. The conventional ^{14}C-urea test using β-scintillation is suitable for diagnosis of *H. pylori* as well as confirmation of eradication following antibiotic treatment [86–88].

The two parameters that have been subject to modification are the ^{14}C-urea dose and breath-collection times [89, 90]. A reduced dose of ^{14}C-urea of 1 μCi has been shown to be highly sensitive and specific (equivalent to the conventional test) for both diagnosis and posttreatment confirmation of eradication [91, 92]. Further reduction of the collection time to 10 min post ^{14}C-urea dosing has been shown to be appropriate for the clinical diagnosis of *H. pylori* [93, 94]. Though the dose of radioactive ^{14}C-urea is minimal, strict regulations must be followed to ensure patient safety. The test has not been approved for use in pregnant women or children in the USA. However, with a reduced dose of ^{14}C-urea (1 μCi), the amount of radiation that a patient receives from the ^{14}C-urea breath test is actually less than that acquired from the natural environment in 1 day [95]. It is now suggested by some clinicians that the ^{14}C-urea breath test can be safely used in pediatric patients [95, 96], especially in developing countries where the ^{13}C-urea breath test is not usually available and the infection rate of *H. pylori* in children is very high [95].

False positive results can be obtained in a ^{14}C-urea breath test with non-capsulated ^{14}C-urea due to urease-producing bacteria present in the oropharynx. The measured values due to oral micro flora declined by 91 and 96% at 10 min, and 94% and 98% at 15 min in patients without and with mouth cleansing, respectively. These results indicate that the $^{14}CO_2$ excretion by oral micro flora becomes negligible in 10 min with and 15 min without mouth cleansing. The authors suggested that two breath samples should be taken at 15 and 20 min for without and at 10 and 15 min with mouth cleansing protocols to ensure the exclusion of $^{14}CO_2$ produced by oral micro flora [97].

^{13}C-Urea breath test. ^{13}C-urea breath test is considered a standard noninvasive test for both initial diagnosis and eradication confirmation. Compared to the ^{14}C-urea breath test, the ^{13}C element is a nonradioactive isotope of ^{12}C with a natural relative abundance of 1.1% and no special handling is necessary. The general procedure is to take a simple test meal to delay gastric emptying and maximize the distribution of ^{13}C-urea after fasting followed by ingesting the ^{13}C-urea dose in water or tablet form. If the ^{13}C-urea dose is taken in a water solution, immediate mouth rinsing with water is recommended to prevent false-positive results caused by oral bacteria with urease activity [98–102]. This mouth-rinsing step can be eliminated by taking a film-coated tablet-formulated ^{13}C-urea dose that is not soluble in the oral cavity but readily soluble in the stomach [100]. A breath sample is then taken at both baseline and the specified post-dose time points, usually at 20 or 30 min. The conventional detection is by isotope ratio mass spectrometry (IRMS), which differentiates $^{13}CO_2$

and $^{12}CO_2$. Less expensive GC/MS has also been used to measure the specimens [103]. The delta over the baseline of $^{13}CO_2$ excess is used as the diagnostic parameter. The formula is expressed as the following [101]:

$$\delta = (R_{\text{sample}} - R_{\text{ref}}) / R_{\text{ref}} \times 1{,}000‰$$

where R is the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the sample and in a reference gas. The reference gas is traceable to an international primary standard, Pee Dee belemnite calcium carbonate [83, 104]. The test results are expressed as the difference in relative enrichment between pre-dose and post-dose breath samples (delta over baseline or DOB) [101]. Cutoff values vary based on ^{13}C-urea doses, different administration methods, formulation of ^{13}C-urea and test meals, sample collection time, and detection techniques.

Other detection techniques have been developed to eliminate the cost of a mass spectrometer. Based on the slightly different absorption spectra between $^{13}CO_2$ and $^{12}CO_2$, the ratio of $^{13}CO_2/^{12}CO_2$ can be accurately determined by a nondispersive isotope-selective infrared spectrometer (NISIR). The sensitivity and specificity of the ^{13}C-urea breath test using NISIR are comparable to those measured by mass spectrometry [105–108]. This detection technique is less expensive compared to mass spectrometry. It can also be placed in a regular laboratory, clinic, or even in a doctor's office. Another technique to detect the ratio of $^{13}CO_2/^{12}CO_2$ is using a laser-assisted ratio analyzer (LARA). The detection principle is based on the optogalvanic effect, which is an electrical signal in response to optical stimulation of a resonance transition in an electrical discharge species. The optogalvanic effect is due to changes in the effective electrical impedance of the gas discharge, which results from an optically induced change in the electron energy distribution function in the molecules. The laser-induced stimulation modifies the ionization rate in the discharge cell, which enables measurement of electron energy to determine the gas concentration in the specimen [109, 110]. The LARA is based on two unique light sources, $^{13}CO_2$ and $^{12}CO_2$ charging lamps. The use of the two charging lamps ensures that light absorption is due to the existence of $^{13}CO_2$ and $^{12}CO_2$ only, in the gas mixture. It also reduces the background radiation leading to a highly sensitive and specific technique [111]. The application of this technique has been proven to be an effective alternative to the traditional IRMS [109, 111–114].

Since its initial description using 350 mg of ^{13}C-urea [115], the test has been modified extensively on two major areas to reduce the cost and increase patient comfort level: ^{13}C-urea dose and duration of the test. Reduction of the ^{13}C-urea dose to 100 mg for a test duration of 30 min without a test meal has been shown to be highly sensitive and specific [101]. Tests employing a dose of 100 or 75 mg ^{13}C-urea for a duration of 30 min have been proven to be as accurate and less expensive compared to larger doses [98, 99, 101, 116]. The test meal can be milk, orange juice, or a citric acid solution [98, 99, 116, 117]. Reduction of dose to 50 mg ^{13}C-urea and test duration to 15 min has also proven to be sufficient [99]. Further modification using a tablet containing 50 mg ^{13}C-urea and 456 mg citric acid without a test meal for a duration as short as 10 min provides sufficient sensitivity and specificity when

endoscopy was used as a "gold standard" diagnosing *H. pylori* infection [118, 119]. In the case of ingestion of 100 mg ^{13}C-urea in 50 mL water with no test meal after a 6-h fasting, the earliest optimal time for discriminating *H. pylori*-positive and -negative patients is 2 min with endoscopic administration and 6 min with a conventional method of administration [102]. Another study involving 202 patients showed no significant difference between the conventional tests (75 mg ^{13}C-urea in 50 mL water) with or without a test meal (200 mL 0.1N citric acid) [120]. However, most studies showed that pretreatment with citric acid in the urea breath test displayed higher DOB values compared to other test meals or no test meals [121–124]. Citric acid slows gastric emptying and thus increases the contact between the bacterial urease and the substrate [124, 125]. In addition, *H. pylori* urease activity is pH dependent with higher activity at lower pH [126]. Therefore it is expected that citric acid should increase DOB values mostly in infected patients and with little or no change of DOB in uninfected patients.

A further modification incorporating endoscopy showed highly accurate diagnosis of *H. pylori* and confirmation of eradication [127]. The most important feature of the technique (endoscopic ^{13}C-urea breath test, EUBT) is the direct spray of ^{13}C-urea over the entire gastric mucosa under endoscopic observation. However, this technique requires a lot of patient preparation, including an oral intake of 80 mg dimethylpolysiloxane to remove adherent gastric mucus 10 min before the endoscopy, along with an oral intake of 200 mg lidocaine to anesthetize the pharyngeal areas, and intramuscular injection of 20 mg scopolamine butylbromide 5 min before the endoscopy [127]. The ^{13}C-urea breath test is not affected by bleeding peptic ulcers while the rapid urease test shows significantly decreased sensitivity in this condition [128]. One drawback with the ^{13}C-urea breath test is that dubious or false-negative results often occur in patients on antisecretory medications, for example, H2-blockers. This problem can be resolved by taking the ^{13}C-urea in a tablet formulation supplemented with citric acid [117]. The ^{13}C-urea breath test results fluctuate in different age groups and genders. DOB results decrease as age increases in both genders. At age 30, the DOB reaches a nadir and moderately increases after age 60 in both genders. In general, females have significantly higher DOB than males even in early childhood and throughout old ages [129].

The diagnosis of *H. pylori* using a ^{13}C-urea breath test has been explored in infants and adolescents. The commonly accepted method is using 75 mg ^{13}C-urea with breath samples taken at baseline, 20 and 30 min. This approach was shown to be highly sensitive (100%). The specificity is lower in children less than 6 years of age (88.1% vs. 97.8%) compared to the older group [130]. This could be due to the lower production of endogenous CO_2 in younger children compared to adults, resulting in relatively higher isotopic ratio of $^{13}CO_2/^{12}CO_2$ with the same dose of ^{13}C-urea [131]. Because of some overlap, defining a gray zone seems to be appropriate in this age group [130]. The breath test has been shown to have excellent sensitivity and specificity for confirmation of eradication of *H. pylori* (100%) in 72 children aged 3–18 years. The diagnostic specificity (95%) and sensitivity (100%) have also been shown to be comparable to histology, rapid urease test, and

serology [132]. Reduction of ^{13}C-urea dose to 50 mg in children is sufficient for diagnosis of *H. pylori* [133–135]. A fatty test meal and 50 mg ^{13}C-urea with breath sampled at 30 min have been shown to give the best sensitivity (98%) and specificity (98%) in a multicenter study [133].

Current available tests. Urea breath tests from two companies have been approved by the FDA for *H. pylori* diagnosis. EZ-HBT Helicobacter Blood Test using a Finnigan BreathMAT Plus Isotope Ratio Mass Spectrometer is offered by Metabolic Solutions, Inc (Nashua, NH). The company provides test kits and offers lab test service. A granulated powder containing 75 mg ^{13}C-urea, citric acid, aspartate, and mannitol is provided within the kit for preparing the oral dose. Baseline and 15-min post-dose breath samples are collected. Patients are instructed to fast for at least 1 h and avoid PPI, antibiotics, bismuth, or any treatment for *H. pylori* within 2 weeks prior to the test. The claimed sensitivity and specificity are 95% (Metabolic Solution Web site, 2011). BreathTek (Meretek Diagnostics, Inc., Rockville, MD) is the other FDA-cleared test (Package insert, 2010). The test can be administered in the doctor's office, clinic, or patient service center. The patient should abstain from antibiotics, PPIs, and bismuth 14 days before the initial testing or 4 weeks after treatment prior to testing for confirmation of eradication. The patient is also required not to have anything in his/her mouth 1 h prior to the testing. Immediately after a baseline breath sample is collected by blowing into a collection bag to determine the initial ratio of $^{13}CO_2$ and $^{12}CO_2$, the patient is given a lemon-flavored Pranactin-Citric solution by mouth. Each 3 g dose of the Pranactin-Citric powder is supplied in a pouch containing 75 mg ^{13}C-urea, citric acid, aspartame, and mannitol. The second breath sample is then collected 15 min after the dose by blowing into the second collection bag. Urease produced by *H. pylori* hydrolyzes ^{13}C-Pranactin-Citric to form $^{13}CO_2$, which is expelled and detectable in the second breath sample. The samples are analyzed on a UBiT-IR300 Infrared Spectrometer or a POCone® Infrared Spectrophotometer for the measurement of $^{13}CO_2$ and $^{12}CO_2$ in breath samples. Analysis by the UBiT-IR300 spectrometer can be set up and operated by each individual laboratory or test facility. A cutoff of 2.4 is used for both initial diagnosis and posttreatment monitoring of *H. pylori*. However, the test's performance for patients under 18 has not been established. There is also lack of an established correlation between the number of *H. pylori* organisms in the stomach and the breath test results (Package insert, 2010).

Helikit (Isodiagnostika, Edmonton, Alberta, Canada) also incorporates ^{13}C-urea formulation with the possibilities of both IRMS and infrared point-of-care (ISOMAX) detections. The post-dose breath collection is set at 30 min, and the sensitivity and specificity are claimed to be 98 and 95%, respectively (Package insert, 2011). BreathID system is offered by Exalenz Bioscience Inc (Wilmington, DE). The detection of $^{13}CO_2/^{12}CO_2$ is achieved by Mass Correlation Spectrometry via continuous breath sampling at a point-of-care environment. The BreathID technology enables healthcare providers to perform the breath test by pushing a single button and results are printed within 10 min in most cases.

Breath Test for Aspergillosis

Unique VOCs were found to be produced by five *Aspergillus* species (*A. fumigatus*, *A. versicolor*, *A. sydowi*, *A. flavus*, and *A. niger*) cultivated on malt extract agar and gypsum board [136]. In another study, 2-Pentylfuran (2PF) was consistently detected in the media of *A. fumigatus*, *Fusarium* spp., *A. terreus*, *A. flavus*, and to a lesser extent by *A. niger*. 2PF was not detected from most of the other bacterial strains tested except *Streptococcus pneumoniae*. In addition, 2PF was detected in breath samples from 4/4 patients with CF and *A. fumigatus* colonization, 3/7 patients with CF but no microbiological evidence of *A. fumigatus*, and none of the 10 healthy controls [137]. In a later study involving more study subjects, 2PF was not detected in the breath samples collected from the 14 healthy controls but was detected in 1 of 10 neutropenic patients and in 17 of 32 subjects with respiratory disease. Using culture results of sputum specimens collected within 2 months as gold standard, the sensitivity and specificity of the 2PF detection for *Aspergillus* infection were determined to be 77 and 78%, respectively [138]. In both studies, breath samples were collected in 4- or 5-L tedlar bags. A single breath sample was collected by forced expiration, and pre-concentrated by using SPME. VOCs in the breath samples were analyzed by GC/MS [137, 138]. Further prospective clinical studies are needed to validate the clinical usefulness of this biomarker in diagnosis and monitoring of *Aspergillus* infection [139]. Furthermore, other VOCs have been found to be promising biomarkers for other infections with examples of 2-aminoacetophenone for *Pseudomonas aeruginosa* [140] and nicotinic acid for *Mycobacterium tuberculosis* [141].

Conclusions

In summary, urea breath tests are intended to detect active infections. They are non-invasive and can be highly accurate. Newer assay formats and instruments are much simpler, more cost effective, more user friendly, and thus may provide suitable alternative choices for clinical diagnosis of microbial infections.

References

1. Marshall BJ, Warren JR (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1:1311–1315
2. Yamada N (1994) *Helicobacter pylori* in peptic ulcer disease. NIH Consens Statement 12:1–23
3. Versalovic J (2003) Helicobacter pylori. Pathology and diagnostic strategies. Am J Clin Pathol 119:403–412
4. Sonnenberg A, Everhart JE (1996) The prevalence of self-reported peptic ulcer in the United States. Am J Public Health 86:200–205

5. Dunn BE, Cohen H, Blaser MJ (1997) *Helicobacter pylori*. Clin Microbiol Rev 10:720–741
6. Kosunen TU, Aromaa A, Knekt P et al (1997) *Helicobacter* antibodies in 1973 and 1994 in the adult population of Vammala, Finland. Epidemiol Infect 119:29–34
7. Cullen DJ, Collins BJ, Christiansen KJ et al (1993) When is *Helicobacter pylori* infection acquired? Gut 34:1681–1682
8. Sipponen P, Kosunen TU, Samloff IM, Heinonen OP, Siurala M (1996) Rate of *Helicobacter pylori* acquisition among Finnish adults: a fifteen year follow-up. Scand J Gastroenterol 31:229–232
9. O'Connor F, Buckley M, O'Morain C (1996) *Helicobacter pylori*: the cancer link. J R Soc Med 89:674–678
10. Isaacson PG (1994) Gastric lymphoma and *Helicobacter pylori*. N Engl J Med 330:1310–1311
11. Peterson WL, Fendrick AM, Cave DR, Peura DA, Garabedian-Ruffalo SM, Laine L (2000) *Helicobacter pylori*-related disease: guidelines for testing and treatment. Arch Intern Med 160:1285–1291
12. Chey WD, Wong BC (2007) American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. Am J Gastroenterol 102:1808–1825
13. Malfertheiner P, Megraud F, O'Morain C et al (2007) Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. Gut 56:772–781
14. McColl KE (2010) Clinical practice. *Helicobacter pylori* infection. N Engl J Med 362:1597–1604
15. Fischbach L, Evans EL (2007) Meta-analysis: the effect of antibiotic resistance status on the efficacy of triple and quadruple first-line therapies for *Helicobacter pylori*. Aliment Pharmacol Ther 26:343–357
16. Malfertheiner P, Schultze V, Rosenkranz B et al (2008) Safety and immunogenicity of an intramuscular *Helicobacter pylori* vaccine in noninfected volunteers: a phase I study. Gastroenterology 135:787–795
17. Czinn SJ, Blanchard T (2011) Vaccinating against *Helicobacter pylori* infection. Nat Rev Gastroenterol Hepatol 8:133–140
18. Hohl TM, Feldmesser M (2007) *Aspergillus fumigatus*: principles of pathogenesis and host defense. Eukaryot Cell 6:1953–1963
19. Dagenais TR, Keller NP (2009) Pathogenesis of *Aspergillus fumigatus* in invasive Aspergillosis. Clin Microbiol Rev 22:447–465
20. Philippe B, Ibrahim-Granet O, Prevost MC et al (2003) Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. Infect Immun 71:3034–3042
21. Ibrahim-Granet O, Philippe B, Boleti H et al (2003) Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. Infect Immun 71:891–903
22. Segal BH (2009) Aspergillosis. N Engl J Med 360:1870–1884
23. Daly P, Kavanagh K (2001) Pulmonary aspergillosis: clinical presentation, diagnosis and therapy. Br J Biomed Sci 58:197–205
24. Walsh TJ, Anaissie EJ, Denning DW et al (2008) Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327–360
25. Agarwal R (2009) Allergic bronchopulmonary aspergillosis. Chest 135:805–826
26. Maschmeyer G, Haas A, Cornely OA (2007) Invasive aspergillosis: epidemiology, diagnosis and management in immunocompromised patients. Drugs 67:1567–1601
27. Patterson TF, Kirkpatrick WR, White M et al (2000) Invasive aspergillosis. Disease spectrum, treatment practices, and outcomes. I3 Aspergillus Study Group. Medicine (Baltimore) 79:250–260
28. Denning DW (1998) Invasive aspergillosis. Clin Infect Dis 26:781–805
29. Loffeld RJ, Stobberingh E, Flendrig JA, Arends JW (1991) *Helicobacter pylori* in gastric biopsy specimens. Comparison of culture, modified giemsa stain, and immunohistochemistry. A retrospective study. J Pathol 165:69–73

30. Faraker CA (1996) Diagnosis of *Helicobacter pylori* in gastric brush and biopsy specimens stained by Romanowsky and immunocytochemical methods: comparison with the CLOtest. Cytopathology 7:108–119
31. Mostaghni AA, Afarid M, Eghbali S, Kumar P (2008) Evaluation of brushing cytology in the diagnosis of *Helicobacter pylori* gastritis. Acta Cytol 52:597–601
32. Anim JT, Al-Sobkie N, Prasad A, John B, Sharma PN, Al-Hamar I (2000) Assessment of different methods for staining *Helicobacter pylori* in endoscopic gastric biopsies. Acta Histochem 102:129–137
33. Vaira D, Holton J, Menegatti M et al (1999) New immunological assays for the diagnosis of *Helicobacter pylori* infection. Gut 45(Suppl 1):I23–I27
34. Guarner J, Kalach N, Elitsur Y, Koletzko S (2010) *Helicobacter pylori* diagnostic tests in children: review of the literature from 1999 to 2009. Eur J Pediatr 169:15–25
35. Agha-Amiri K, Mainz D, Peitz U, Kahl S, Leodolter A, Malfertheiner P (1999) Evaluation of an enzyme immunoassay for detecting *Helicobacter pylori* antigens in human stool samples. Z Gastroenterol 37:1145–1149
36. Agha-Amiri K, Peitz U, Mainz D, Kahl S, Leodolter A, Malfertheiner P (2001) A novel immunoassay based on monoclonal antibodies for the detection of *Helicobacter pylori* antigens in human stool. Z Gastroenterol 39:555–560
37. Suzuki N, Wakasugi M, Nakaya S et al (2002) Catalase, a specific antigen in the feces of human subjects infected with *Helicobacter pylori*. Clin Diagn Lab Immunol 9:784–788
38. Suzuki N, Wakasugi M, Nakaya S et al (2002) Production and application of new monoclonal antibodies specific for a fecal *Helicobacter pylori* antigen. Clin Diagn Lab Immunol 9:75–78
39. Gisbert JP, Pajares JM (2004) Stool antigen test for the diagnosis of *Helicobacter pylori* infection: a systematic review. Helicobacter 9:347–368
40. Gisbert JP, de la Morena F, Abraira V (2006) Accuracy of monoclonal stool antigen test for the diagnosis of *H. pylori* infection: a systematic review and meta-analysis. Am J Gastroenterol 101:1921–1930
41. Gramley WA, Asghar A, Frierson HF Jr, Powell SM (1999) Detection of *Helicobacter pylori* DNA in fecal samples from infected individuals. J Clin Microbiol 37:2236–2240
42. Nagasawa S, Azuma T, Motani H et al (2009) Detection of *Helicobacter pylori* (*H. pylori*) DNA in digestive systems from cadavers by real-time PCR. Leg Med (Tokyo) 11(Suppl 1):S458–459
43. Kabir S (2004) Detection of *Helicobacter pylori* DNA in feces and saliva by polymerase chain reaction: a review. Helicobacter 9:115–123
44. Glocker E, Kist M (2004) Rapid detection of point mutations in the gyrA gene of *Helicobacter pylori* conferring resistance to ciprofloxacin by a fluorescence resonance energy transfer-based real-time PCR approach. J Clin Microbiol 42:2241–2246
45. Oleastro M, Gerhard M, Lopes AI et al (2003) *Helicobacter pylori* virulence genotypes in Portuguese children and adults with gastroduodenal pathology. Eur J Clin Microbiol Infect Dis 22:85–91
46. Oleastro M, Menard A, Santos A et al (2003) Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in *Helicobacter pylori*. J Clin Microbiol 41:397–402
47. Ribeiro ML, Gerrits MM, Benvengo YH et al (2004) Detection of high-level tetracycline resistance in clinical isolates of *Helicobacter pylori* using PCR-RFLP. FEMS Immunol Med Microbiol 40:57–61
48. Sugimoto M, Wu JY, Abudayyeh S et al (2009) Unreliability of results of PCR detection of *Helicobacter pylori* in clinical or environmental samples. J Clin Microbiol 47:738–742
49. Grocott RG (1955) A stain for fungi in tissue sections and smears using Gomori's methenamine-silver nitrate technic. Am J Clin Pathol 25:975–979
50. Hope WW, Walsh TJ, Denning DW (2005) Laboratory diagnosis of invasive aspergillosis. Lancet Infect Dis 5:609–622

51. De Vos MM, Nelis HJ (2003) Detection of *Aspergillus fumigatus* hyphae by solid phase cytometry. J Microbiol Methods 55:557–564
52. Kaufman L, Standard PG, Jalbert M, Kraft DE (1997) Immunohistologic identification of Aspergillus spp. and other hyaline fungi by using polyclonal fluorescent antibodies. J Clin Microbiol 35:2206–2209
53. Zimmerman RL, Montone KT, Fogt F, Norris AH (2000) Ultra fast identification of *Aspergillus* species in pulmonary cytology specimens by in situ hybridization. Int J Mol Med 5:427–429
54. Horvath JA, Dummer S (1996) The use of respiratory-tract cultures in the diagnosis of invasive pulmonary aspergillosis. Am J Med 100:171–178
55. Yu VL, Muder RR, Poorsattar A (1986) Significance of isolation of *Aspergillus* from the respiratory tract in diagnosis of invasive pulmonary aspergillosis. Results from a three-year prospective study. Am J Med 81:249–254
56. Huizinga M, Stevens E, Berrens L (1985) Detection of class-specific antibodies against *Aspergillus fumigatus* antigens in various pulmonary diseases. Ann Inst Pasteur Immunol 136C:343–352
57. Tomee JF, van der Werf TS, Latge JP, Koeter GH, Dubois AE, Kauffman HF (1995) Serologic monitoring of disease and treatment in a patient with pulmonary aspergilloma. Am J Respir Crit Care Med 151:199–204
58. Chan CM, Woo PC, Leung AS et al (2002) Detection of antibodies specific to an antigenic cell wall galactomannoprotein for serodiagnosis of *Aspergillus fumigatus* aspergillosis. J Clin Microbiol 40:2041–2045
59. Marier R, Smith W, Jansen M, Andriole VT (1979) A solid-phase radioimmunoassay for the measurement of antibody to *Aspergillus* in invasive aspergillosis. J Infect Dis 140:771–779
60. Yeo SF, Wong B (2002) Current status of nonculture methods for diagnosis of invasive fungal infections. Clin Microbiol Rev 15:465–484
61. Mennink-Kersten MA, Donnelly JP, Verweij PE (2004) Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. Lancet Infect Dis 4:349–357
62. Mennink-Kersten MA, Ruegebrink D, Wasei N, Melchers WJ, Verweij PE (2006) In vitro release by *Aspergillus fumigatus* of galactofuranose antigens, 1,3-beta-D-glucan, and DNA, surrogate markers used for diagnosis of invasive aspergillosis. J Clin Microbiol 44:1711–1718
63. Odabasi Z, Mattiuzzi G, Estey E et al (2004) Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. Clin Infect Dis 39:199–205
64. Latge JP, Kobayashi H, Debeaupuis JP et al (1994) Chemical and immunological characterization of the extracellular galactomannan of Aspergillus fumigatus. Infect Immun 62:5424–5433
65. Klont RR, Mennink-Kersten MA, Verweij PE (2004) Utility of *Aspergillus* antigen detection in specimens other than serum specimens. Clin Infect Dis 39:1467–1474
66. Francis P, Lee JW, Hoffman A et al (1994) Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: the potential role of bronchoalveolar D-mannitol and serum galactomannan as markers of infection. J Infect Dis 169:356–368
67. Marr KA, Balajee SA, McLaughlin L, Tabouret M, Bentsen C, Walsh TJ (2004) Detection of galactomannan antigenemia by enzyme immunoassay for the diagnosis of invasive aspergillosis: variables that affect performance. J Infect Dis 190:641–649
68. Sulahian A, Boutboul F, Ribaud P, Leblanc T, Lacroix C, Derouin F (2001) Value of antigen detection using an enzyme immunoassay in the diagnosis and prediction of invasive aspergillosis in two adult and pediatric hematology units during a 4-year prospective study. Cancer 91:311–318
69. Pazos C, Ponton J, Del Palacio A (2005) Contribution of (1->3)-beta-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. J Clin Microbiol 43:299–305

70. Jones ME, Fox AJ, Barnes AJ et al (1998) PCR-ELISA for the early diagnosis of invasive pulmonary aspergillus infection in neutropenic patients. J Clin Pathol 51:652–656
71. Kami M, Fukui T, Ogawa S et al (2001) Use of real-time PCR on blood samples for diagnosis of invasive aspergillosis. Clin Infect Dis 33:1504–1512
72. Hummel M, Spiess B, Kentouche K et al (2006) Detection of *Aspergillus* DNA in cerebrospinal fluid from patients with cerebral aspergillosis by a nested PCR assay. J Clin Microbiol 44:3989–3993
73. Rickerts V, Mousset S, Lambrecht E et al (2007) Comparison of histopathological analysis, culture, and polymerase chain reaction assays to detect invasive mold infections from biopsy specimens. Clin Infect Dis 44:1078–1083
74. Manolis A (1983) The diagnostic potential of breath analysis. Clin Chem 29:5–15
75. Cao W, Duan Y (2006) Breath analysis: potential for clinical diagnosis and exposure assessment. Clin Chem 52:800–811
76. Phillips M (1997) Method for the collection and assay of volatile organic compounds in breath. Anal Biochem 247:272–278
77. Miekisch W, Schubert JK, Noeldge-Schomburg GF (2004) Diagnostic potential of breath analysis—focus on volatile organic compounds. Clin Chim Acta 347:25–39
78. Hyspler R, Crhova S, Gasparic J, Zadak Z, Cizkova M, Balasova V (2000) Determination of isoprene in human expired breath using solid-phase microextraction and gas chromatography-mass spectrometry. J Chromatogr B Biomed Sci Appl 739:183–190
79. Knutson MD, Viteri FE (1996) Concentrating breath samples using liquid nitrogen: a reliable method for the simultaneous determination of ethane and pentane. Anal Biochem 242:129–135
80. Phillips M, Greenberg J (1992) Ion-trap detection of volatile organic compounds in alveolar breath. Clin Chem 38:60–65
81. Bazzoli F, Zagari M, Fossi S et al (1997) Urea breath tests for the detection of *Helicobacter pylori* infection. Helicobacter 2(Suppl 1):S34–S37
82. Vakil N, Vaira D (2004) Non-invasive tests for the diagnosis of *H. pylori* infection. Rev Gastroenterol Disord 4:1–6
83. Logan RP (1993) Urea breath tests for the detection of *Helicobacter-pylori* infection. Eur J Gastroenterol Hepatol 5:S46–S49
84. Cutler AF, Elnaggar M, Brooks E, O'Mara K (1998) Effect of standard and high dose ranitidine on [13C]urea breath test results. Am J Gastroenterol 93:1297–1299
85. Ozturk E, Yesilova Z, Ilgan S, Ozguven M, Dagalp K (2009) Performance of acidified 14C-urea capsule breath test during pantoprazole and ranitidine treatment. J Gastroenterol Hepatol 24:1248–1251
86. Desroches JJ, Lahaie RG, Picard M et al (1997) Methodological validation and clinical usefulness of carbon-14-urea breath test for documentation of presence and eradication of *Helicobacter pylori* infection. J Nucl Med 38:1141–1145
87. Marshall BJ, Plankey MW, Hoffman SR et al (1991) A 20-minute breath test for *Helicobacter* pylori. Am J Gastroenterol 86:438–445
88. Rollan A, Giancaspero R, Arrese M et al (1997) Accuracy of invasive and noninvasive tests to diagnose *Helicobacter pylori* infection after antibiotic treatment. Am J Gastroenterol 92:1268–1274
89. Abukhadir BA, Heneghan MA, Kearns M, Little CL, McCarthy CF (1998) Evaluation of a 20 minute 14C urea breath test for the diagnosis of *Helicobacter pylori* infection. Ir Med J 91:23–25
90. Kao CH, Huang CK, Wang SJ, Hsu CY, Lin WY, Chen GH (1993) Accuracy of a rapid 10-minute carbon-14 urea breath test for the diagnosis of *Helicobacter pylori*-associated peptic ulcer disease. Eur J Nucl Med 20:708–711
91. Hegedus O, Ryden J, Rehnberg AS, Nilsson S, Hellstrom PM (2002) Validated accuracy of a novel urea breath test for rapid *Helicobacter pylori* detection and in-office analysis. Eur J Gastroenterol Hepatol 14:513–520

92. Raju GS, Smith MJ, Morton D, Bardhan KD (1994) Mini-dose (1-microCi) 14C-urea breath test for the detection of *Helicobacter pylori*. Am J Gastroenterol 89:1027–1031
93. Ozturk E, Yesilova Z, Ilgan S et al (2003) A new, practical, low-dose 14C-urea breath test for the diagnosis of *Helicobacter pylori* infection: clinical validation and comparison with the standard method. Eur J Nucl Med Mol Imaging 30:1457–1462
94. Peura DA, Pambianco DJ, Dye KR et al (1996) Microdose 14C-urea breath test offers diagnosis of *Helicobacter pylori* in 10 minutes. Am J Gastroenterol 91:233–238
95. Pathak CM, Kaur B, Khanduja KL (2010) 14C-urea breath test is safe for pediatric patients. Nucl Med Commun 31:830–835
96. Gunnarsson M, Leide-Svegborn S, Stenstrom K et al (2002) No radiation protection reasons for restrictions on 14C urea breath tests in children. Br J Radiol 75:982–986
97. Pathak CM, Avti PK, Bunger D, Khanduja KL (2008) Kinetics of 14carbon dioxide excretion from 14C-urea by oral commensal flora. J Gastroenterol Hepatol 23:1603–1607
98. Epple HJ, Kirstein FW, Bojarski C et al (1997) 13C-urea breath test in *Helicobacter pylori* diagnosis and eradication. Correlation to histology, origin of 'false' results, and influence of food intake. Scand J Gastroenterol 32:308–314
99. Liao CC, Lee CL, Chiang TC et al (2002) The 13C-urea breath test to detect *Helicobacter pylori* infection: a validated simple methodology with 50 mg 13C-urea. Aliment Pharmacol Ther 16:787–792
100. Ohara S, Kato M, Saito M et al (2004) Comparison between a new 13C-urea breath test, using a film-coated tablet, and the conventional 13C-urea breath test for the detection of *Helicobacter pylori* infection. J Gastroenterol 39:621–628
101. Oksanen A, Bergstrom M, Sjostedt S, Gad A, Hammarlund B, Seensalu R (1997) Accurate detection of *Helicobacter pylori* infection with a simplified 13C urea breath test. Scand J Clin Lab Invest 57:689–694
102. Peng NJ, Lai KH, Liu RS et al (2001) Clinical significance of oral urease in diagnosis of *Helicobacter pylori* infection by [13C]urea breath test. Dig Dis Sci 46:1772–1778
103. Lee HS, Gwee KA, Teng LY et al (1998) Validation of [13C]urea breath test for *Helicobacter pylori* using a simple gas chromatograph-mass selective detector. Eur J Gastroenterol Hepatol 10:569–572
104. Thomas JE, Dale A, Harding M et al (1999) Interpreting the 13C-urea breath test among a large population of young children from a developing country. Pediatr Res 46:147–151
105. Braden B, Caspary WF, Lembcke B (1999) Nondispersive infrared spectrometry for 13CO2/12CO2-measurements: a clinically feasible analyzer for stable isotope breath tests in gastroenterology. Z Gastroenterol 37:477–481
106. Isomoto H, Inoue K, Mizuta Y et al (2003) Validation of endoscopic 13C-urea breath test with nondispersive infrared spectrometric analysis in the management of *Helicobacter pylori* infection. Hepatogastroenterology 50:422–425
107. Kato M, Saito M, Fukuda S et al (2004) 13C-Urea breath test, using a new compact nondispersive isotope-selective infrared spectrophotometer: comparison with mass spectrometry. J Gastroenterol 39:629–634
108. Savarino V, Mela GS, Zentilin P et al (1999) Comparison of isotope ratio mass spectrometry and nondispersive isotope-selective infrared spectroscopy for 13C-urea breath test. Am J Gastroenterol 94:1203–1208
109. Braden B, Gelbmann C, Dietrich CF, Caspary WF, Scholmerich J, Lock G (2001) Qualitative and quantitative clinical evaluation of the laser-assisted ratio analyser for detection of *Helicobacter pylori* infection by (13)C-urea breath tests. Eur J Gastroenterol Hepatol 13:807–810
110. Murnick DE, Peer BJ (1994) Laser-based analysis of carbon isotope ratios. Science 263:945–947
111. Shirin H, Kenet G, Shevah O et al (2001) Evaluation of a novel continuous real time (13)C urea breath analyser for *Helicobacter pylori*. Aliment Pharmacol Ther 15:389–394
112. Cave DR, Zanten SV, Carter E et al (1999) A multicentre evaluation of the laser assisted ratio analyser (LARA): a novel device for measurement of $13CO_2$ in the 13C-urea breath test for the detection of *Helicobacter pylori* infection. Aliment Pharmacol Ther 13:747–752

113. Minoli G, Prada A, Schuman R, Murnick D, Rigas B (1998) A simplified urea breath test for the diagnosis of *Helicobacter pylori* infection using the LARA System. Laser assisted ratio analyzer. J Clin Gastroenterol 26:264–266
114. Savarino V, Landi F, Dulbecco P et al (2000) Isotope ratio mass spectrometry (IRMS) versus laser-assisted ratio analyzer (LARA): a comparative study using two doses of. Dig Dis Sci 45:2168–2174
115. Graham DY, Klein PD, Evans DJ Jr et al (1987) *Campylobacter pylori* detected noninvasively by the 13C-urea breath test. Lancet 1:1174–1177
116. Labenz J, Barsch G, Peitz U et al (1996) Validity of a novel biopsy urease test (HUT) and a simplified 13C-urea breath test for diagnosis of *Helicobacter pylori* infection and estimation of the severity of gastritis. Digestion 57:391–397
117. Hamlet A, Stage L, Lonroth H, Cahlin C, Nystrom C, Pettersson A (1999) A novel tablet-based 13C urea breath test for *Helicobacter pylori* with enhanced performance during acid suppression therapy. Scand J Gastroenterol 34:367–374
118. Gatta L, Vakil N, Ricci C et al (2003) A rapid, low-dose, 13C-urea tablet for the detection of *Helicobacter pylori* infection before and after treatment. Aliment Pharmacol Ther 17: 793–798
119. Wong WM, Lam SK, Lai KC et al (2003) A rapid-release 50-mg tablet-based 13C-urea breath test for the diagnosis of *Helicobacter pylori* infection. Aliment Pharmacol Ther 17: 253–257
120. Wong WM, Wong BC, Wong KW et al (2000) (13)C-urea breath test without a test meal is highly accurate for the detection of *Helicobacter pylori* infection in Chinese. Aliment Pharmacol Ther 14:1353–1358
121. Dominguez-Munoz JE, Leodolter A, Sauerbruch T, Malfertheiner P (1997) A citric acid solution is an optimal test drink in the 13C-urea breath test for the diagnosis of Helicobacter pylori infection. Gut 40:459–462
122. Gisbert JP, Vazquez MA, Jimenez I et al (2000) 13C-urea breath test for the diagnosis of *Helicobacter pylori* infection before treatment: is citric acid necessary? Dig Liver Dis 32:20–24
123. Kopacova M, Bures J, Vorisek V et al (2005) Comparison of different protocols for 13C-urea breath test for the diagnosis of *Helicobacter pylori* infection in healthy volunteers. Scand J Clin Lab Invest 65:491–498
124. Leodolter A, Dominguez-Munoz JE, Von Arnim U, Malfertheiner P (1999) Citric acid or orange juice for the 13C-urea breath test: the impact of pH and gastric emptying. Aliment Pharmacol Ther 13:1057–1062
125. Graham DY (1998) Gastrin sensitivity and acid in *H. pylori*: revisited. Gastroenterology 115:509–510
126. Miederer SE, Grubel P (1996) Profound increase of *Helicobacter pylori* urease activity in gastric antral mucosa at low pH. Dig Dis Sci 41:944–949
127. Suto H, Azuma T, Ito S et al (1999) Evaluation of endoscopic 13C-urea breath test for assessment of *Helicobacter pylori* eradication. J Gastroenterol 34(Suppl 11):67–71
128. Wildner-Christensen M, Touborg Lassen A, Lindebjerg J, Schaffalitzky de Muckadell OB (2002) Diagnosis of *Helicobacter pylori* in bleeding peptic ulcer patients, evaluation of urea-based tests. Digestion 66:9–13
129. Zevit N, Niv Y, Shirin H, Shamir R (2011) Age and gender differences in urea breath test results. Eur J Clin Invest 41(7):767–772
130. Kindermann A, Demmelmair H, Koletzko B, Krauss-Etschmann S, Wiebecke B, Koletzko S (2000) Influence of age on 13C-urea breath test results in children. J Pediatr Gastroenterol Nutr 30:85–91
131. Yang HR, Ko JS, Seo JK (2008) Does the diagnostic accuracy of the 13C-urea breath test vary with age even after the application of urea hydrolysis rate? Helicobacter 13:239–244
132. Yoshimura N, Tajiri H, Sawada A et al (2001) A 13C-urea breath test in children with *Helicobacter pylori* infection: assessment of eradication therapy and follow-up after treatment. J Gastroenterol 36:606–611

133. Bazzoli F, Cecchini L, Corvaglia L et al (2000) Validation of the 13C-urea breath test for the diagnosis of *Helicobacter pylori* infection in children: a multicenter study. Am J Gastroenterol 95:646–650
134. Canete A, Abunaji Y, Alvarez-Calatayud G et al (2003) Breath test using a single 50-mg dose of 13C-urea to detect *Helicobacter pylori* infection in children. J Pediatr Gastroenterol Nutr 36:105–111
135. Kawakami E, Machado RS, Reber M, Patricio FR (2002) 13 C-urea breath test with infrared spectroscopy for diagnosing *Helicobacter pylori* infection in children and adolescents. J Pediatr Gastroenterol Nutr 35:39–43
136. Gao P, Korley F, Martin J, Chen BT (2002) Determination of unique microbial volatile organic compounds produced by five *Aspergillus* species commonly found in problem buildings. AIHA J (Fairfax, VA) 63:135–140
137. Syhre M, Scotter JM, Chambers ST (2008) Investigation into the production of 2-Pentylfuran by *Aspergillus fumigatus* and other respiratory pathogens in vitro and human breath samples. Med Mycol 46:209–215
138. Chambers ST, Syhre M, Murdoch DR, McCartin F, Epton MJ (2009) Detection of 2-pentylfuran in the breath of patients with *Aspergillus fumigatus*. Med Mycol 47:468–476
139. Chambers ST, Bhandari S, Scott-Thomas A, Syhre M (2011) Novel diagnostics: progress toward a breath test for invasive *Aspergillus fumigatus*. Med Mycol 49:S54–S61
140. Scott-Thomas AJ, Syhre M, Pattemore PK et al (2010) 2-Aminoacetophenone as a potential breath biomarker for *Pseudomonas aeruginosa* in the cystic fibrosis lung. BMC Pulm Med 10:56
141. Syhre M, Manning L, Phuanukoonnon S, Harino P, Chambers ST (2009) The scent of *Mycobacterium tuberculosis* - Part II breath. Tuberculosis 89:263–266

Chapter 3
Rapid Antigen Tests

Sheldon Campbell and Marie L. Landry

Introduction

Immunoassays for the detection of the antigens of microorganisms remain important tools for the diagnosis and management of infectious diseases. Great strides have been made since the introduction of the early precipitation and agglutination assays in increasing the sensitivity, specificity, standardization, and automation of antigen tests [1–4]. Antigen tests have long been used to detect infectious agents that are difficult, slow, or hazardous to culture. However, antigen detection methods are especially useful for rapid diagnosis, whether in the clinic, emergency department, doctor's office, or central laboratory. Simple one-step assays can provide results in 15 min with dramatic benefits to physician decision-making.

The basis for antigen detection assays is the specific binding of an antigen (protein or glycoprotein or polysaccharide) to an antibody. Antigen assays are generally more economical than either culture or molecular techniques. However, they do not amplify their target, as culture amplifies infectious organisms, or as polymerase chain reaction amplifies nucleic acid. Thus, they are generally less sensitive than these other methods. Since antigen immunoassays traditionally detect only the antigen originally present in the sample, optimal sample collection and handling are key to good results.

S. Campbell
Pathology and Laboratory Medicine Service, VA Connecticut,
West Haven, CT, USA

Department of Laboratory Medicine, Yale University School of Medicine,
New Haven, CT 06520-8035, USA
e-mail: sheldon.campbell@yale.edu

M.L. Landry (✉)
Department of Laboratory Medicine, Yale University School of Medicine,
New Haven, CT 06520-8035, USA
e-mail: marie.landry@yale.edu

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Antigen detection methods are also very valuable for the specific identification of infectious agents after amplification in culture. However, since culture requires at least an overnight incubation, these methods are not discussed here. In this chapter, we consider only those tests that detect antigens directly in clinical samples, with results available within minutes to several hours after sample receipt. First, we briefly review the principles and characteristics of major techniques and then we discuss their application to detection of microorganisms and viruses in clinical specimens.

Principles of the Techniques

Agglutination

Agglutination methods utilize the antibody–antigen bond to create clumping (agglutination) of particles. Agglutination tests to detect antigens employ fixed red cells (hemagglutination), latex beads, gelatin, or synthetic microbeads coated with specific antibody as carrier or indicator particles. In a typical agglutination assay for detection of microbial antigen, a drop of liquid suspension of antibody-coated particles is placed on a card, and the specimen is added and mixed. The card is then incubated, often on an oscillating mixer, and read by visually observing the clumping reaction (Fig. 3.1). No washing is required. Agglutination assays can be made semiquantitative by performing serial dilutions of the specimen and reporting the greatest dilution which results in a positive reaction.

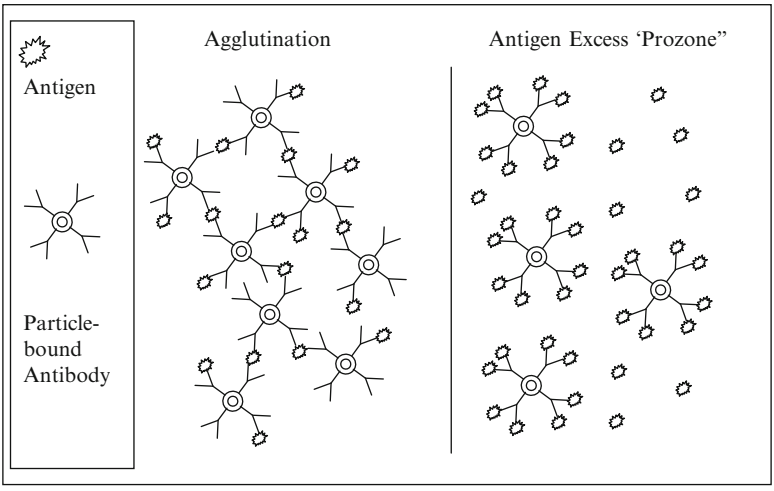

Fig. 3.1 Particle agglutination and prozoning

A major source of error in agglutination tests is the prozone reaction, which occurs when antigen is present in excess. “Prozoning” is observed at high antigen concentrations where excess antigen occupies most antibody binding sites with unique antigen molecules, thus preventing the multiple antibody binding of each antigen that causes the particles to clump (Fig. 3.1). These false-negative reactions can be detected by repeating the test at a higher dilution of sample, which reduces the antigen concentration into the range that produces agglutination.

Compared with other methods, agglutination tests tend to be very rapid, and require minimal training and equipment. However, test sensitivity is usually less than that for other techniques, as a greater quantity of antigen is required to produce visible agglutination. Factors which limit the specificity of agglutination methods include heterophile and rheumatoid factor antibodies which may cause agglutination in the absence of specific antigen; mucus and other substances which may agglutinate particles nonspecifically; and lipemia and other opaque materials which interfere with interpretation.

Immunofluorescence

Immunofluorescence (IF) is a microscopic technique that uses specific antibodies labeled with fluorochromes to detect, localize, or quantify microorganisms, or proteins expressed in virus-infected cells, in samples applied to slides. A variety of fluorochromes are available, but the most commonly used are fluorescein and rhodamine. Several fluorochromes can be used simultaneously to detect more than one organism. Fluorochromes are excited by UV light and, in returning to their resting state, emit photons at a specific wavelength. Visualization requires a microscope with a dark-field condenser and filters for each fluorochrome that allow only the emitted fluorescent light to be seen. In the direct method, the primary antibody is labeled with the fluorochrome (Fig. 3.2). In the indirect method, the specific antibody is unlabeled, but a second anti-species antibody that reacts with the antigen–antibody complex is labeled, and allows detection. The direct technique is shorter and simpler, whereas the indirect method is cheaper and in theory more sensitive.

Prior to IF, clinical specimens may be washed to remove material that can itself fluoresce or trap stain. After application to a glass slide, the sample is fixed by heat, cold acetone, or occasionally formalin. The sample affixed to the slide is allowed to react with specific antibodies, and then washed to remove non-reacting materials. Mounting oil and a coverslip are applied. Time to result after fixation is less than 1 h for direct and about 2 h for the indirect method.

IF requires an expensive fluorescence microscope, which must be well maintained, kept in a dark room, and the bulb life monitored as intensity declines with use. IF allows microscopic visualization of sample quality, and thus the opportunity to recollect inadequate samples. IF also allows the detection of only one or two infected cells or few microorganisms, making it potentially more sensitive than

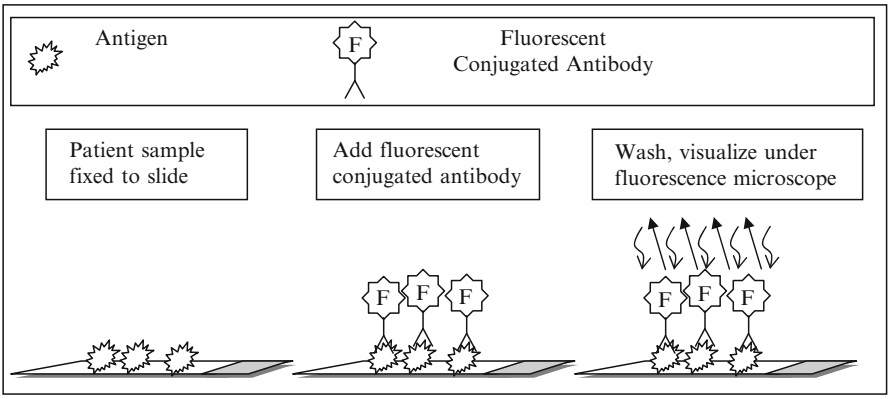

Fig. 3.2 Direct immunofluorescence

other immunoassays. However, significant training and judgment are required to ensure good quality preparations and accurate interpretation. Performance characteristics for IF must be established by each laboratory, for each reader, and for each analyte. Slides can be saved at 4°C for weeks for quality control purposes and correlation with culture results.

Enzyme Immunoassay

Enzyme immunoassay (EIA) is the generic term for a large number of methods that link an antigen–antibody reaction to an enzymatic reaction to produce a colorimetric, fluorimetric, or chemiluminescent readout. A variety of enzymes may be used, but the most common are alkaline phosphatase and horseradish peroxidase. EIA methods are used in formats that range from self-contained kits sold for home use to methods that run on high-throughput, random-access laboratory instruments. Typical assay times are 2–3 h, though self-contained membrane EIAs and competitive EIAs can be significantly faster. EIAs thus allow manufacturers to offer tests in a wide variety of formats to suit different clinical applications.

More automated EIA methods such as fluorescent particle immunoassay (FPIA) and chemiluminescent immunoassays tend to be utilized for higher-volume testing such as drug and hormone assays. Testing for some microbial antigens, such as hepatitis B surface antigen (HBsAg), is sufficiently high volume to merit these automated formats.

Enzyme-linked immunosorbent assay (ELISA) is a specific category of EIA in which one of the antibodies is "adsorbed" or bound to a solid phase (immuno*sorbent*). ELISAs typically are implemented in a microwell, tube, or bead format

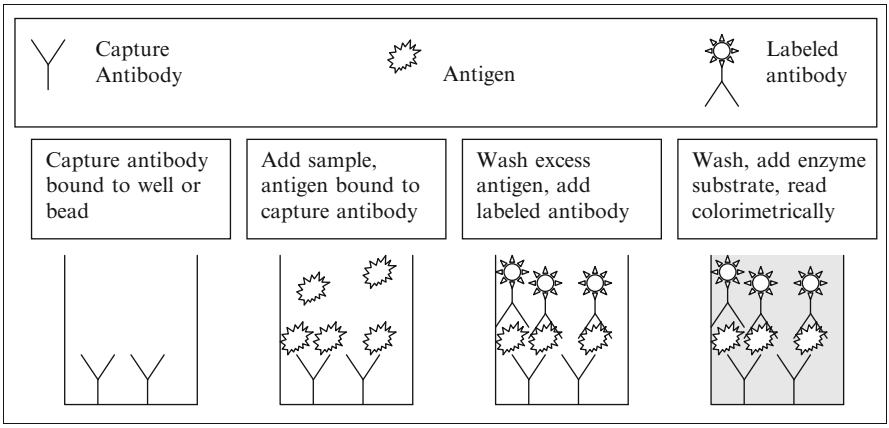

Fig. 3.3 Antigen capture ELISA

(Fig. 3.3). The label can be carried on a single labeled antibody, or a sandwich of an antigen-specific antibody and a label. In the latter case, the label is borne either on a second anti-species antibody that reacts with the antigen–antibody complex or on an antibody-binding protein such as staphylococcal protein A. Another strategy uses biotin-labeled antibody and streptavidin–horseradish peroxidase conjugate. The sandwich-type methods increase sensitivity, but may increase time and cost. Various steps of the process can be automated by plate washers and readers, and by more comprehensive automated ELISA systems.

Competitive ELISAs may be set up with either antibody or antigen on the solid phase. Labeled antigen is added either simultaneously with or after the patient specimen is reacted with the first antibody. The signal generated is inversely proportional to the amount of antigen in the specimen. In comparison with direct or non-competitive formats, competitive ELISAs tend to be more rapid and specific, but less sensitive.

In qualitative antigen detection, a quantitative cutoff divides positive from negative results. The precise value of the cutoff, which is usually expressed as a signal relative to that generated by a negative control sample, depends on the method and the desired mix of sensitivity and specificity needed for clinical purposes; lower cutoffs provide more sensitivity but less specificity. Receiver–operator curve (ROC) analysis may be used to optimize the cutoff; ROC curves demonstrate the relationship between sensitivity and specificity as cutoff values vary, and allow assessment of the effect of changing cutoff values on test performance.

Significant interferences in EIA testing arise from “hook effects,” heterophile antibodies in blood, and nonspecific binding of specimen constituents producing high backgrounds. “Hook effects” arise when extremely high quantities of antigen are present; however, the mechanism of interference with EIAs is not as clearly

defined as with agglutination. Heterophile antibodies can produce either negative or positive interference, depending on the details of the assay construction. Nonspecific binding of specimen constituents is particularly troublesome in respiratory specimens, where mucoid specimens may be associated with false-positives.

Advantages of EIAs include ability to run large numbers of samples with minimal hands-on time, modest personnel training requirements, ability to automate, and objective end-points. Disadvantages include inability to assess specimen quality, need to set sometimes arbitrary cutoffs, hook effects, interfering substances, including rheumatoid factors and heterophile antibodies, and need for careful and thorough washing to avoid false-positive results.

Chemiluminescent Methods

Chemiluminescence (ChL) is the emission of light that occurs when a substrate decays to a ground state from an excited state produced by a chemical reaction, most often an oxidation. The emission is read with a luminometer or may be captured on photographic film. Chemiluminescence is the most sensitive reporter system for immunoassays, since light emission can be detected at very low levels, and there are few naturally occurring molecules which emit light under the conditions used for chemiluminescence, leading to very low backgrounds. Chemiluminescent readouts can employ either a chemiluminescent readout from an enzyme assay or a directly chemiluminescent labeled antibody. The most common ChL compounds are acridinium esters and derivatives of isoluminol, both of which are excited by sodium hydroxide and hydrogen peroxide. In addition, 1,2-dioxetane molecules are used as substrates for alkaline phosphatase in many commercial immunoassays. Finally, electrochemiluminescent detection of ruthenium-labeled antibodies has been employed in systems for the detection of biological weapon agents in environmental samples and in general immunochemical platforms.

Lateral Flow Immunochromatography and Fluoroimmunoassay

Membrane EIAs usually involve a series of steps: addition of sample, wash step, addition of conjugate, wash step, and addition of substrate, and then stop reagent. The result is read as a colored spot or a triangle on a solid surface. By substituting an IgG binding dye (e.g., staphylococcal protein A-gold reagent) for the anti-immunoglobulin conjugate, the procedure can be shortened by one step. However, immunochromatographic or lateral flow assays usually require no reagent additions, and thus are extremely simple to perform. Like membrane EIAs, most of

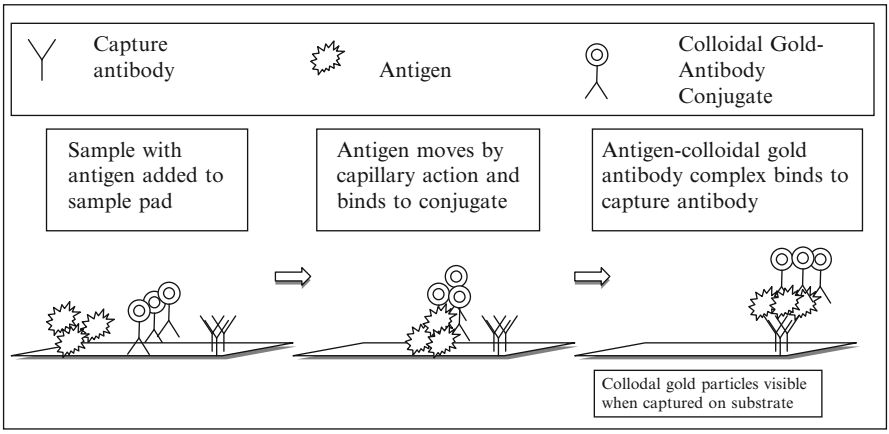

Fig. 3.4 Lateral flow immunochromatography

these tests include a built-in control; if the test differentiates two different agents (e.g., influenza A and B), two controls are included. These tests utilize antibodies spotted onto nitrocellulose membranes with lateral or vertical flow of sample or reagents to interact with immobilized antibody (Fig. 3.4). Use of an antibody sandwich increases sensitivity. Specific antibody is adsorbed onto a nitrocellulose membrane in the sample line, and a control antibody is adsorbed onto same membrane as second line. Both antibodies are conjugated to visualizing particles that are dried onto an inert fibrous support. Conjugate pad and striped membrane are combined to construct the test strip. An extracted sample is added at one end and moves along membrane by capillary action to reach the immobilized antibody stripes. Alternatively, a test strip can be inserted vertically into a tube containing the extracted sample.

Disadvantages of rapid membrane assays in general include subjective interpretation, lack of automation, and possible errors if the reader is color-blind. Although simple to perform, inexperience and lack of attention to technique can lead to errors. Samples must disperse within specified time limits and pipettes must be held vertically for correct delivery of reagent volumes. Accurate timing can be adversely affected when multiple samples are tested. Results must be visually read within a narrow time window, which can be difficult in a busy clinic or laboratory.

Recent improvements to lateral flow assays applied to respiratory virus detection include use of a fluorescent label to enhance sensitivity of detection and insertion of sample cassettes into a fluorescent reader for objective readout, accurate timing, and printed results. Use of bar-coded samples and an interface with the laboratory information system reduce transcription errors and save labor.

Characteristics of the Techniques

The characteristics of the techniques are presented in Table 3.1, stratified by training requirements. IF requires the most intensive training and quality control. EIA methods are widely used for many analytes, high-quality commercial kits are available, and automation is common. Rapid membrane and agglutination assays, while generally simple, vary in number of steps. Lateral flow methods usually require no wash steps or reagent additions. Each laboratory needs to evaluate these methods and establish performance characteristics in its own settings and patient populations. Decisions on which tests to employ should take into account clinical needs, test volumes, time to result, cost of materials and labor, equipment required, and staff expertise.

Applications of the Techniques

A summary of the applications of antigen techniques to specific pathogens is given in Table 3.2, and common uses are discussed below.

Bacteria

Rapid antigen testing is routine for diagnosis of group A streptococcal pharyngitis. Although rapid antigen tests offer less than 100% sensitivity, their wide availability at the point of care (POC) allows practitioners to diagnose and treat this common childhood illness in a single office visit in most cases, reserving culture for antigen-negative patients [5].

The value of detection of *Streptococcus pneumoniae* antigen in urine for the diagnosis of pneumonia is limited by the positive results obtained in patients with mere oropharyngeal colonization, occurring especially in children, and by sensitivities of only 50–85%. The role of this test in management of patients with community-acquired pneumonia is still evolving, but current guidelines for the management of community-acquired pneumonia suggest the use of this antigen test in patients with severe disease [6–8].

Antigen detection in urine is a major diagnostic procedure for *Legionella* infections. Although available tests detect only 80–90% of the serotypes associated with human disease, the method is sensitive and specific for those serotypes, and is much more rapid than culture. Urinary antigen can remain positive for days to weeks after therapy is begun, and thus can be performed on treated patients. Because non-serogroup I is more common in health care-associated infections than in community-acquired disease, the urinary antigen test is most sensitive in detecting community-acquired legionellosis. The antigen test is also more sensitive in severe than in mild disease. DFA testing of respiratory specimens for *Legionella* is

Table 3.1 Characteristics of the techniques

Method	Time to result	Equipment	Training	Advantages	Limitations
Immuno-fluorescence	1–2 h	Cytospin, centrifuge, fume hood, fluorescence microscope	Extensive	Can assess sample quality; can detect 1–2 infected cells; can multiplex detection of multiple viruses; can quantitate infected cells	Need adequate target cells, expert slide preparation, and interpretation; subjective; performance must be established in each laboratory
Microwell, tube, or bead ELISA	1 h 15 min to 2 h	Spectrophotometer Pipettors	Moderate	Most suited to high-volume testing; can be automated	Interference due to Hook effects, heterophile antibodies, and nonspecific binding
Agglutination	15 min	Vortex (optional) Oscillating mixer (optional)	Minimal	Very rapid, simple, no wash steps	Prozone reaction, subjective; may be less sensitive
Membrane and other rapid EIA	15–30 min	Pipettors or none	Minimal	Rapid, simple, can be used at POC	Subjective interpretation, lack of automation, possible errors if the reader is color-blind; thick or mucoid samples may not enter strip within specified time limits; inaccurate timing of steps when testing multiple samples; must read promptly for valid results
Lateral flow immuno-chromatography	10–20 min	None	Minimal	Rapid, very simple, some have no reagent additions or wash steps; can be used at POC	Similar to rapid EIA
Lateral flow immuno-fluorescence	20 min	IF reader	Minimal	No reagent additions; can read bar-coded samples and interface with LIS; sample cassette inserted into reader; test read objectively at the end of incubation and report printed; more sensitive than LFIC	Reader may have limited throughput

EIA enzyme immunoassay, *ELISA* enzyme-linked immunosorbent assay, *LFIC* lateral flow immunochromatography, *LIS* laboratory information system, *POC* point of care

Table 3.2 Application of techniques to detection of specific pathogens

Pathogen	Methods	Specimen	Sensitivity[a]	Specificity	Comments
Bacteria					
Streptococcus Group A	Agglutination, rapid EIA, OIA, LFIC	Throat swab	70–90+%	>95%	Often performed at POC. Negatives usually must be evaluated by culture.
Streptococcus pneumoniae	Rapid EIA, LFIC	Urine	50–85%	94%	Clinical role still evolving. Provides adjunct, but not definitive, diagnostic information in patients at risk for *S. pneumoniae* disease.
Legionella spp.	IF	Respiratory	25–75%	90%+	Requires FA microscope. Cross-reactions with some other bacteria, especially with polyclonal reagents. No gold standard for comparison.
	EIA or LFIC	Urine	80–99%	99%	Test characteristics well established only for *L. pneumophila* group 1.
Clostridium difficile	Agglutination, rapid EIA, ELISA, LFIC	Stool	65–100%	88–100%	Toxin tests are significantly insensitive relative to more laborious and expensive methods such as PCR and cytotoxicity. Combination algorithms for GDH and toxin antigen testing are more sensitive, but still may not approach PCR.
Helicobacter pylori	ELISA, LFIC	Stool	89%	90–94%	Used as an alternative to serology and urea breath testing; usable as test of cure.
Chlamydia trachomatis	ELISA	Genital	60–70%	97%	Being phased out, but POC versions might be valuable if sensitivity improves. No single-test format available. Not useful for screening low-prevalence populations due to poor specificity.
Meningitis panel (*H. influenzae*, *N. meningitidis*, *S. pneumoniae*, group B *Streptococcus*)	Agglutination	CSF, urine			Inadequate sensitivity/specificity for routine clinical use. Empiric therapy given for CSF neutrophilia covers these pathogens, until culture results available. Positive predictive value of antigen tests is very low in patients without CSF leukocytosis.

Fungi					
Aspergillus	ELISA	Serum	Varies	Varies	Used for surveillance in neutropenic patients, allows for early initiation of antifungal therapy. Roughly 2/3 of patients have positive antigenemia before diagnosis.
Cryptococcus	Agglutination, ELISA	CSF, serum	99%+	Very high if heat or pronase pretreatment used	Sensitivity may exceed culture. Cross-reactivity with (very rare) systemic *Trichosporon* infections. Prozone is a problem in high-level infections.
Pneumocystis jiroveci (formerly *P. carinii*)	IF	Respiratory	Variable	High	Requires fluorescence microscope. Sensitivity is highest for antibodies that detect antigens present in trophozoites and cysts. No significant sensitivity or specificity advantages over conventional and Calcofluor white stains.
Parasites					
Giardia	IF, LFIC, ELISA, rapid EIA	Stool	Higher than microscopy	100%	No "gold standard" available for comparison. Specimen treatment (e.g., fixed, unfixed, or frozen) varies with different tests.
Cryptosporidium	IF, LFIC, ELISA, rapid EIA	Stool	Higher than microscopy	93–100%	No "gold standard" available for comparison. Some kits detect both *Giardia* and *Cryptosporidium.*
Entamoeba histolytica/dispar group	ELISA	Stool	Higher than microscopy	>95%	Reagents are available to distinguish between *E. histolytica* and *E. dispar.*
Trichomonas vaginalis	IF, LA, LFIC	Genital	85%	High	Alternatives include wet prep (60% sensitivity relative to culture), culture, molecular detection. Wet prep is limited by specimen stability.

(continued)

Table 3.2 (continued)

Pathogen	Methods	Specimen	Sensitivity[a]	Specificity	Comments
Plasmodium species	LFIC and other rapid formats	Blood	Similar to microscopy	High	One FDA-approved test available. Cost limits use in endemic areas, but development is a high priority.
Lymphatic filariases	ELISA, LFIC	Blood	Equivalent to microscopy; similar to or higher than concentration methods	>95%	No "gold standard." Cost limits use in endemic areas.
Viruses					
Respiratory syncytial	ELISA, LFIC	NP swab or aspirate, BAL, sputum	80–95%	97–99%	Very sensitive in young infants who shed high titers of virus. Mucoid samples may not disperse properly and may give rise to erroneous results.
	IF	NP swab or aspirate, BAL, sputum	90–100%	>99%	More sensitive than culture or other antigen tests. Can be multiplexed with other antibodies. IF allows assessment of sample quality.
Influenza A and B	EIA, LFIC	NP swab or aspirate, BAL, sputum, throat swab	50–90%[a] 10–79%[b]	95–99% 48–98%[b]	Sensitivity higher in children, and with NP aspirates and washes. Some kits require the use of special swab. Simpler rapid tests suitable for POC. Mucoid samples may not disperse properly and may give rise to erroneous results.
	IF	NP swab, NP aspirate, nasal wash, BAL	85–98%[a] 46–93%[b]	95–99%	Performance must be established in each laboratory. Can be more sensitive than other rapid tests. Cytospin preparation of slides improves results. Use of pooled antibodies can be used to screen a single cell spot for multiple respiratory viruses. IF allows assessment of sample quality.

Parainfluenza	IF	NP swab or aspirate, BAL, sputum	80–95%	95–99%	Only rapid method available. Cytospin preparation of slides improves results. Antibodies to types 1, 2, 3, but not type 4, are included in commercial antibody pool.
Human metapneumovirus	IF	NP swab or aspirate, BAL, sputum	85%[b]	99%	Available as a separate reagent or in a multiplex reagent with seven other respiratory viruses.
	LFIC	NP swab or aspirate	63%[b]	97%	Since HMPV grows poorly in culture, RT-PCR is the reference method.
Adenovirus	IF	NP swab or aspirate, BAL, sputum	50–70%	99%	IF for adenovirus not as sensitive as for other respiratory viruses. Cytospin preparation of slides improves results.
	EIA	Stool	90%	99%	Test available for detection of all adenovirus types in culture fluids or stools; does not differentiate among types.
Adenovirus, enteric types 40, 41	EIA Agglutination	Stool	98%	99%	Test available to detect only enteric types 40 and 41 which do not grow in routine cell cultures.
Rotavirus	EIA, agglutination, LFIC	Stool	90–98%	90–98%	Rotavirus does not grow in routine cell cultures, so rapid tests are compared to EM. Rotavirus sheds in high titers in stools of infants and young children.
Astrovius	EIA	Stool	97%	99%	Rapid tests compared to EM.
Norovirus	EIA	Stool	30–40%	94–99%	Sensitivity compared to RT-PCR. Limited by antigenic variation and rapid onset and resolution of illness.

(continued)

Table 3.2 (continued)

Pathogen	Methods	Specimen	Sensitivity[a]	Specificity	Comments
Herpes simplex	IF	Skin lesions, genital lesions, oral lesions, BAL, brain tissue	80–95%	>99%	Sensitivity enhanced by cytospin preparation of slides. Sensitivity is higher for skin lesions than for mucosal lesions. Pooled HSV and VZV antibodies labeled with different fluorochromes can be used to test for both viruses in a single cell spot.
Varicella zoster	IF	Skin lesions, BAL	>99%	>99%	IF for VZV in skin lesions is more sensitive than culture. VZV and HSV antibodies can be pooled for dual detection.
Cytomegalo virus	IF IP	Blood leukocytes	90–97%	>99%	Quantitative detection of CMV pp65 antigenemia is very useful in rapid diagnosis and in monitoring therapy. More sensitive than culture and equivalent to Amplicor PCR in plasma. Not suitable for severely neutropenic patients.
Human immuno-deficiency virus p24 antigen	ChLIA	Blood	80% of antibody-negative, RNA-positive infections	95–99%	4th generation HIV screening tests combine p24 antigen with antibody detection to detect early HIV-1 infection before anti-HIV antibody appears. Molecular test needed to confirm specificity of result.
Hepatitis B surface antigen (HBsAg) and e antigen (HBeAg)	EIA, ChLIA	Blood	99%	>99%	Free HBsAg is produced in 100- to 1,000-fold excess over complete virus particles. Thus HBsAg can be more sensitive than DNA techniques. HBeAg is a marker for high levels of viral replication. Viral mutations can lead to falsely negative IA results.

[a]Test performance compared to culture unless otherwise stated
[b]Test performance for 2009 pandemic influenza compared to RT-PCR

insensitive, even relative to culture, and requires a skilled reader to limit false-positives. Monoclonal reagents are significantly more specific than polyclonal reagents, but both have been described to cross-react with non-*Legionella* species, and contamination of water, buffers, and the environment with environmental *Legionella* also may produce false-positives. The true sensitivity and specificity of antigen detection methods in *Legionella* infections are difficult to determine, since culture itself is insensitive, and molecular methods are only available in a limited number of places [9, 10].

For diagnosis of enterocolitis due to *Clostridium difficile* toxins, there is no "gold standard." Rather, a variety of diagnostic techniques are employed, including toxigenic culture, tissue culture cytotoxicity with antibody neutralization, rapid and conventional toxin EIAs, and molecular diagnostics. The various EIA methods are the most widely employed because of their modest technical requirements and rapid time to result; tests which detect both toxin A and toxin B are more sensitive than methods which detect only toxin A. As simpler molecular methods have become available, the role of antigen and other methods has become increasingly controversial. Toxin-only antigen testing is quite insensitive relative to toxigenic culture or PCR; on the other hand, PCR testing may detect patients with asymptomatic colonization with *C. difficile*. Optimal antigen-based algorithms involve a screening *C. difficile* glutamate dehydrogenase (GDH) antigen test, which is more sensitive than a toxin assay but detects non-toxigenic strains, followed by a toxin antigen test for confirmation. PCR or repeat testing may be used to address discrepant results [11, 12]. A cartridge-type test incorporating both GDH and toxin antigen detection is available.

Antigen testing of stool for *Helicobacter pylori* is available both in a laboratory-based EIA and a rapid format. It serves as a diagnostic option to the urea breath test, serology, and endoscopy. It may be particularly useful in children, where the urea breath test may be difficult to perform, and in patients in whom serologic testing is likely to be problematic, such as steroid-treated or HIV-infected patients [13]. False-negative results are common in patients on proton-pump inhibitor therapy, bismuth, or antibiotics. The stool antigen test can also be used as a test-of-cure, though the time required after treatment is still unclear [14].

Antigen testing for genital *Chlamydia* infections has been almost entirely replaced by nucleic acid testing, which is substantially more sensitive and specific. Rapid tests have the potential for POC use, but none is yet FDA approved, and sensitivity remains an issue [15]. Due to the lack of infrastructure for nucleic acid tests, and the high prevalence and disease burden of *Chlamydia* in the less developed parts of the world, *Chlamydia* antigen tests may have a role in those settings despite disappointing sensitivity [16, 17].

Bacterial antigen testing for meningitis is rapid, but has fallen out of use in recent years due to inadequate sensitivity and specificity and the use of empiric antibiotic therapy. The presence of neutrophils in CSF generally leads to therapy in patients with compatible syndromes, while the positive predictive value of antigen testing performed on patients with acellular CSF is dismal. Empiric antibiotic choices cover the organisms detected by the antigen tests [18, 19].

Current guidelines for detection of Shiga-toxin-producing *E. coli* (STEC) recommend the use of a Shiga-toxin antigen test. Antigen testing may be performed directly on stool, but improved sensitivity is available if an overnight enrichment in selective broth is performed. The sensitivity of antigen tests, even after broth enrichment, falls short of 100%, so selective culture on sorbitol-MacConkey agar is still recommended. Sorbitol-negative colonies of *E. coli* may be tested with a rapid antigen test for the O:157 antigen. For cases of STEC detected either by culture or antigen testing, the stool sample or the isolate should be forwarded to public health laboratory for analysis, confirmation, and strain typing for outbreak investigation [20].

Fungi

Detection of invasive Aspergillus infections using the galactomannan ELISA has become an important aspect of management of bone-marrow transplant and other profoundly neutropenic patient populations. Aspergillus galactomannan antigen is used for surveillance in at-risk patients, and may also be of value in monitoring therapy. The combination of radiologic and antigen testing allows early initiation of antifungal therapy and improves outcome in neutropenic patients. The test is less sensitive in non-neutropenic patients, due likely to lower organism loads. False-positives are a problem, occurring frequently in patients who receive piperacillin–tazobactam or amoxicillin–clavulanate, and in patients with other fungal infections [21].

For *Cryptococcus*, antigen testing is the mainstay of diagnosis. The sensitivity in cryptococcal meningitis approaches that of culture while providing more rapid diagnosis [22]. Cryptococcal antigen testing of both serum and CSF is important in diagnosis, staging, and monitoring of cryptococcal disease in at-risk patients [23].

By contrast, IF staining of respiratory specimens for *Pneumocystis jiroveci* is one of several techniques of similar sensitivity for detection of *Pneumocystis* pneumonia. The choice of IF, conventional stain, or Calcofluor white depends on the laboratory. IF and Calcofluor require fluorescent microscopes, and IF reagents are expensive. Calcofluor and conventional staining methods require the reader to discriminate between *Pneumocystis*, yeasts, other pathogens, and cellular structures morphologically, which requires more interpretive skill than IF staining [24, 25]. IF appears to be insensitive compared with PCR for *Pneumocystis*, especially in non-AIDS patients who frequently have lower organism burdens, but PCR is positive in persons who are most likely asymptomatically colonized [26].

Parasites

For infections by *Giardia* and *Cryptosporidium*, antigen testing has become the method of choice, with sensitivities that exceed those of routine microscopic exam [27]. Cost-saving strategies using pooled specimens screened with antigen detection

have been described. Many different formats are available, and laboratories select a method based on technical (e.g., availability of fluorescence microscope, test format) and operational (e.g., specimen requirements, test volume) differences [28, 29].

EIA methods are also available for *Entamoeba histolytica*. *E. histolytica* is morphologically indistinguishable from a nonpathogenic relative, *E. dispar*. Several tests are available, but the EIA from Techlab (*E. histolytica* II) is comparatively specific for pathogenic *E. histolytica* and is useful for distinguishing it from *E. dispar*. Because *E. histolytica* is relatively rare in the USA, antigen tests are not as widely used as for *Giardia* and *Cryptosporidium*. Different publications [30–32] differ markedly in their assessment of the sensitivity of antigen testing versus PCR, but PCR is not yet widely available.

Since *Trichomonas* rapidly loses motility below body temperature, the wet prep has always been an insensitive approach to diagnosis, particularly if specimens needed to be transported prior to viewing. Commercially available IF and latex agglutination methods provide better sensitivity; nucleic acid amplification, direct nucleic acid hybridization, and culture are more sensitive but more complex [33].

Rapid diagnosis of malaria by antigen detection, primarily using lateral-flow immunochromatography, is a promising approach to field diagnosis. The proliferation of chloroquine-resistant strains and the expense of newer antimalarial drugs may make these tests economical in endemic regions. An FDA-approved test has separate channels for a specific *Plasmodium falciparum* antigen and for an interspecies common antigen. It does not replace thick and thin smears but provides a simple, rapid diagnostic test usable in virtually any laboratory, with a sensitivity of roughly 95% for *P. falciparum* and 69% for *P. vivax*. Worldwide, rapid malarial antigen tests vary significantly in sensitivity, ease of use, and quality control, and dissemination of high-quality tests is a priority for worldwide malaria control [34].

Rapid antigen tests have also been evaluated for *Wuchereria bancrofti* infections. They appear to be more sensitive than direct microscopy, and approach or exceed the sensitivity of concentration techniques in some studies [35].

Viruses

Antigen tests have long been the diagnostic mainstay for viruses that do not grow in routine cell culture, such as hepatitis B and rotavirus. Hospitals that serve infants and children have provided rapid antigen testing for RSV since the 1980s to aid in infection control and patient management [36, 37]. With the advent of therapy with neuraminidase inhibitors, rapid testing for influenza increased dramatically in clinics, emergency departments, and hospitals, for patients of all ages. In pediatric patients, having a test result available within 10–30 min has been shown to reduce the use of antibiotics and ordering of diagnostic tests, allow earlier discharge, and permit early administration of antiviral agents [38–40]. Rapid antigen tests are relatively inexpensive, require minimal training, and allow on-demand testing, including at the POC. While less sensitive than other methods, these tests can perform sufficiently

well if samples contain high titers of virus. Current tests require approximately 100,000 viral copies for a positive result, a titer most commonly found in samples from very young children. The 2009 H1N1 influenza pandemic focused attention on the insensitivity of rapid flu tests, with some hospitals reporting abysmal specificity as well [41, 42]. Nevertheless, many hospitals still consider rapid antigen tests their best option [43] and efforts are underway to improve accuracy. Preliminary studies indicate that incorporating fluorescent immunoassay technology into the lateral flow format, together with a small instrument to analyze the data, can increase sensitivity significantly while retaining a 20-min time to result [44, 45].

IF can detect a broader array of viruses, and has the ability to multiplex reagents and to quantitate infected cells. Use of cytocentrifugation to prepare slides enhances slide quality and test sensitivity. Since IF is commonly done in virology laboratories for identification of culture isolates, the equipment and some expertise are usually available. Nevertheless, the accuracy of IF directly on clinical samples is highly variable between laboratories. A significant commitment to training, monitoring, and quality control is critical. Without careful attention to detail, nonspecific staining can be misinterpreted as positive, or small numbers of positive cells can be overlooked.

Respiratory virus screening by IF was initially most widely applied to RSV, but since 2000 the use of pooled antibodies has allowed detection up to eight viruses in a single cell spot with results in 1–2 h [46, 47]. When done well, IF is significantly more sensitive than other rapid influenza virus antigen tests. For the 2009 influenza pandemic, IF sensitivities in adults of 82–93% compared to RT-PCR were achieved by some laboratories [48, 49]. Recent IF innovations include cell fixation and staining in suspension rather than affixed to slides to shorten time to result [47]. Antibodies labeled with different fluorochromes can also be pooled to screen skin lesion samples for HSV and VZV [50]. The use of IF to rapidly detect and quantitate CMV in peripheral blood revolutionized the diagnosis and monitoring of CMV infections [51]. Although largely replaced by real-time PCR in high-volume laboratories, CMV antigenemia retains advantages for on-site testing in smaller laboratories. IF is less expensive than PCR, takes 1–2 h to complete, can be done repeatedly during laboratory hours, and is more suitable to low-volume testing, including a single urgent sample when needed [52]. In addition, virology laboratories that use rapid antigen testing to provide results within 15 min to 2 h can save resources by canceling cultures or molecular tests on many samples that have a positive rapid antigen result.

In an important advance in the diagnosis of acute HIV infections, fourth-generation HIV antibody tests incorporate HIV-1 p24 antigen detection in a combination antigen/antibody assay. Used in Europe for over a decade, the first HIV antigen–antibody combo assay became available in the USA in 2010. The antigen–antibody test format greatly facilitates the diagnosis of early infections before antibody has developed, detecting 80% of RNA-positive, antibody-negative infections [53]. Furthermore, when performed on random access instruments using chemiluminescence, results are available within an hour.

References

1. Carpenter AB (2002) Antibody-based methods. In: Rose NR, Hamilton RG, Detrick B (eds) Manual of clinical laboratory immunology. ASM, Washington, DC, p 2002
2. Constantine NT, Lana DP (2003) Immunoassays for the diagnosis of infectious diseases. In: Murray PR, Baron EJ, Jorgenesen JH, Pfaller MA, Yolken RH (eds) Manual of clinical microbiology. ASM, Washington, DC
3. Hage DS (1999) Immunoassays. Anal Chem 71:294R–304R
4. Peruski AH, Peruski LF Jr (2003) Immunological methods for detection and identification of infectious disease and biological warfare agents. Clin Diagn Lab Immunol 10:506–513
5. Bisno AL, Gerber MA, Gwaltney JM Jr, Kaplan EL, Schwartz RH, Infectious Diseases Society of America (2002) Practice guidelines for the diagnosis and management of group A streptococcal pharyngitis. Clin Infect Dis 35:113–125
6. Roson B, Fernandez-Sabe N, Carratala J, Verdaguer R, Dorca J, Manresa F, Gudiol F (2004) Contribution of a urinary antigen assay (Binax NOW) to the early diagnosis of pneumococcal pneumonia. Clin Infect Dis 38:222–226
7. Smith MD, Derrington P, Evans R, Creek M, Morris R, Dance DA, Cartwright K (2003) Rapid diagnosis of bacteremic pneumococcal infections in adults by using the Binax NOW *Streptococcus pneumoniae* urinary antigen test, a prospective, controlled clinical evaluation. J Clin Microbiol 41:2810–2813
8. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, Dowell SF, File TM Jr, Musher DM, Niederman MS, Torres A, Whitney CG (2007) Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis 44(Suppl 2):S27–S72
9. Waterer GW, Baselski VS, Wunderink RG (2001) *Legionella* and community-acquired pneumonia, a review of current diagnostic tests from a clinician's viewpoint. Am J Med 110:41–48
10. Edelstein PH (2007) Legionella. In: Murray P, Barron EJ, Jorgensen JH, Landry ML, Pfaller MA (eds) Manual of clinical microbiology, 9th edn. ASM, Washington, DC
11. Wilcox MH, Planche T, Fang FC (2010) What is the current role of algorithmic approaches for diagnosis of *Clostridium difficile* infection? J Clin Microbiol 48:4347–4353
12. Eastwood K, Else P, Charlett A, Wilcox M (2009) Comparison of nine commercially available *Clostridium difficile* toxin detection assays, a real-time PCR assay for *C. difficile* tcdB, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods. J Clin Microbiol 47:3211–3217
13. Versalovic J (2003) *Helicobacter pylori*. Pathology and diagnostic strategies. Am J Clin Pathol 119:403–412
14. Chey WD, Wong BC, The Practice Parameters Committee of the American College of Gastroenterology (2007) American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. Am J Gastroenterol 102:1808–1825
15. Mahoney JB, Coombes BK, Chernesky MA (2003) *Chlamydia* and *Chlamydophilia*. In: Murray P, Barron EJ, Jorgensen JH, Landry ML, Pfaller MA (eds) Manual of clinical microbiology, 9th edn. ASM, Washington, DC
16. Sabido M, Hernandez G, Gonzalez V, Valles X, Montoliu A, Figuerola J, Isern V, Vinado B, Figueroa L, Casabona J (2009) Clinic-based evaluation of a rapid POC test for *Chlamydia trachomatis* among sex workers in Escuintla, Guatemala. J Clin Microbiol 47:475–476
17. Peeling RW, Holmes KK, Mabey D, Ronald A (2006) Rapid tests for sexually-transmitted infections (STIs): the way forward. Sex Transm Infect 83(Suppl 5):S1–S6
18. Kiska DL, Jones MC, Mangum ME, Orkiszewski D, Gilligan PH (1995) Quality assurance study of bacterial antigen testing of cerebrospinal fluid. J Clin Microbiol 33:1141–1144
19. Thompson RB, Miller JM (2003) Specimen collection, transport, and processing, bacteriology. In: Murray P, Barron EJ, Jorgensen JH, Landry ML, Pfaller MA (eds) Manual of clinical microbiology, 9th edn. ASM, Washington, DC

20. CDC (Centers for Disease Control and Prevention) (2009) Recommendations for diagnosis of shiga toxin-producing *Escherichia coli* infections by clinical laboratories. MMWR 58:RR-12
21. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens DA, van Burik J, Wingard JR, Patterson TF (2008) Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327–360
22. Perfect JR, Casadevall A (2002) Cryptococcosis. Infect Dis Clin North Am 16:837–874
23. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrell TC (2010) Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. Clin Infect Dis 50:291–322
24. Cruciani M, Marcati P, Malena M, Bosco O, Serpelloni G, Mengoli C (2002) Meta-analysis of diagnostic procedures for *Pneumocystis carinii* pneumonia in HIV-1-infected patients. Eur Respir J 20:982–989
25. Thomas CF Jr, Limper AH (2004) Pneumocystis pneumonia. N Engl J Med 350:2487–2498
26. Hauser PM, Bille J, Lass-Florl C, Geltner C, Feldmesser M, Levi M, Patel H, Muggia V, Alexander B, Hughes M, Follett SA, Cui X, Leung F, Morgan G, Moody A, Perlin DS, Denning DW (2011) Multicenter, prospective clinical evaluation of respiratory samples from subjects at risk for *Pneumocystis jirovecii* infection by use of a commercial real-time PCR assay. J Clin Microbiol 49:1872–1878
27. Garcia LS (2001) Antibody and antigen detection in parasitic infections. In: Garcia LS (ed) Diagnostic medical parasitology, 4th edn. ASM, Washington, DC
28. CDC (Centers for Disease Control and Prevention), National Center for Infectious Diseases, Division of Parasitic Diseases (2004) Diagnostic procedures for stool specimens: detection of parasite antigens. http://wwwdpdcdcgov/dpdx/HTML/DiagnosticProcedures.htm
29. Johnston SP, Ballard MM, Beach MJ, Causer L, Wilkins PP (2003) Evaluation of three commercial assays for detection of *Giardia* and *Cryptosporidium* organisms in fecal specimens. J Clin Microbiol 41:623–626
30. Roy S, Kabir M, Mondal D, Ali IKM, Petri WA, Haque R (2005) Real-Time-PCR assay for diagnosis of *Entamoeba histolytica* infection. J Clin Microbiol 43:2168–2172
31. Solaymani-Mohammadi S, Rezaian M, Babaei Z, Rajabpour A, Meamar AR, Pourbabai AA, Petri WA (2006) Comparison of a stool antigen detection kit and PCR for diagnosis of *Entamoeba histolytica* and *Entamoeba dispar* infections in asymptomatic cyst passers in Iran. J Clin Microbiol 44:2258–2261
32. Stark D, van Hal S, Fotedar R, Butcher A, Marriott D, Ellis J, Harkness J (2008) Comparison of stool antigen detection kits to PCR for diagnosis of amebiasis. J Clin Microbiol 46:1678–1681
33. Leber AL, Novak-Weekley S (2007) Intestinal and urogenital amebae, flagellates, and ciliates. In: Murray P, Barron EJ, Jorgensen JH, Landry ML, Pfaller MA (eds) Manual of clinical microbiology, 9th edn. ASM, Washington, DC
34. Murray CK, Gasser RA, Magill AJ, Miller RS (2008) Update on rapid diagnostic testing for malaria. Clin Microbiol Rev 21:97–110
35. Chandrasena TG, Premaratna R, Abeyewickrema W, de Silva NR (2002) Evaluation of the ICT whole-blood antigen card test to detect infection due to *Wuchereria bancrofti* in Sri Lanka. Trans R Soc Trop Med Hyg 96:60–63
36. Slinger R, Milk R, Gaboury I, Diaz-Mitoma F (2004) Evaluation of the QuickLab RSV test, a new rapid lateral-flow immunoassay for detection of respiratory syncytial virus antigen. J Clin Microbiol 42:3731–3733
37. Wilhelmi I, Colomina J, Martin-Rodrigo D, Roman E, Sanchez-Fauquier A (2001) New immunochromatographic method for rapid detection of rotaviruses in stool samples compared with standard enzyme immunoassay and latex agglutination techniques. Eur J Clin Microbiol Infect Dis 20:741–743
38. Barenfanger J, Drake C, Leon N, Mueller T, Troutt T (2000) Clinical and financial benefits of rapid detection of respiratory viruses: an outcomes study. J Clin Microbiol 38:2824–2828

39. Bonner AB, Monroe KW, Talley LI, Klasner AE, Kimberlin DW (2003) Impact of the rapid diagnosis of influenza on physician decision-making and patient management in the pediatric emergency department: results of a randomized, prospective, controlled trail. Pediatrics 112:363–367
40. Jennings LC, Skopnik H, Burckhardt I, Hribar I, Del Piero L, Deichmann KA (2009) Effect of rapid influenza testing on the clinical management of paediatric influenza. Influenza Other Respi Viruses 3:91–98
41. Landry ML (2011) Diagnostic tests for influenza infection. Curr Opin Pediatr 23:91–97
42. Sambol AR, Abdalhamid B, Lyden ER, Aden TA, Noel RK, Hinrichs SH (2009) Use of rapid influenza diagnostic tests under field conditions as a screening tool during an outbreak of the 2009 novel influenza virus, practical considerations. J Clin Virol 47:229–233
43. Welch DF, Ginocchio CC (2010) Role of rapid immunochromatographic antigen testing in diagnosis of influenza A virus 2009 H1N1 infection. J Clin Microbiol 48:22–25
44. Dale SE, Mayer C, Mayer MC, Menegus MA (2008) Analytical and clinical sensitivity of the 3M rapid detection influenza A+B assay. J Clin Microbiol 46:3804–3807
45. Egan R, Hale M, Nasser N, et al (2011) High sensitivity of immunofluorescence influenza A+B assay with analyzer. Poster M2. 27th annual clinical virology symposium, Daytona Beach, FL, 8–11 May 2011
46. Landry ML, Ferguson D (2000) SimulFluor Respiratory Screen for rapid detection of multiple respiratory viruses in clinical specimens by immunofluorescence staining. J Clin Microbiol 38:708–711
47. Landry ML (2009) Developments in immunologic assays for respiratory viruses. Clin Lab Med 294:635–647
48. Pollock NR, Duong S, Cheng A, Han LL, Smole S, Kirby JE (2009) Ruling out novel H1N1 influenza virus infection with direct fluorescent antigen testing. Clin Infect Dis 49:e66–e68
49. Landry ML, Ferguson D (2010) Cytospin-enhanced immunofluorescence and impact of sample quality on detection of novel swine origin (H1N1). influenza virus. J Clin Microbiol 48:957–959
50. Scicchitano LM, Shetterly B, Bourbeau PP (1999) Evaluation of light diagnostics SimulFluor HSV/VZV immunofluorescence assay. Diagn Microbiol Infect Dis 35:205–208
51. Gerna G, Revello MG, Percivalle E, Morini F (1992) Comparison of different immunostaining techniques and monoclonal antibodies to the lower matrix phosphoprotein (pp 65) for optimal quantitation of human cytomegalovirus antigenemia. J Clin Microbiol 30:1232–1237
52. Landry ML, Ferguson D (2000) 2-hour cytomegalovirus pp 65 antigenemia assay for rapid quantitation of cytomegalovirus in blood samples. J Clin Microbiol 38:427–428
53. Branson BM (2010) The future of HIV testing. J Acquir Immune Defic Syndr 55(Suppl 2):S102–S105

Chapter 4
Antibody Detection: Principles and Applications

Yun F. (Wayne) Wang and Miwako Kobayashi

Introduction

Antibody detection methods by immunoassay have been developed, commonly used, and will be expanded further for rapid and accurate diagnosis of the common or newly emerging infection-causing agents such as viruses in clinical as well as public health laboratories. Since the first competitive radioimmunoassay was developed over 50 years ago for human insulin detection [1], immunoassays have been developed with emphasis on fast and sensitive detection technologies and automation. Due to the demand of large screening for epidemiology, blood bank, prenatal care, and diagnosis of HIV and hepatitis, more immunodiagnostic procedures are performed using instruments and reagents similar to immunochemistry platforms used in clinical chemistry or core laboratory. Immunoassay for detection of host-produced antibodies directed against microorganisms, particularly viruses, has been one of the most widely used analytical techniques in laboratory medicine [2, 3]. Automation and random access application have been implemented for rapid diagnosis of infectious diseases.

This chapter reviews the antibody detection assays and limitations for detection and identification of infectious agents and looks into the application of those technologies with emphasis on detection methods such as chemiluminescent and multi-analyte profile (xMAP), automation, and their clinical application in the areas of diagnosing HIV, viral, and bacterial infections.

Y.F. (Wayne) Wang (✉) • M. Kobayashi
Emory University and Grady Memorial Hospital,
P.O. Box 26248, 80 Jesse Hill Dr. SE, Atlanta, GA 30303, USA
e-mail: yfwang@emory.edu; miwako.kobayashi@emory.edu

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Principles and Characteristics of Techniques

Successful immunoassays for antibody (Ab) detection rely upon three important factors: (1) the specific antigen used to capture target antibody; (2) the target antibody if present, and the detector or secondary antibody used for indirect detection of antibody; and (3) the detection method. First two factors are important for the efficiency of antigen–antibody complex formation and the third one is about the ability to detect these complexes and is a critical factor for sensitivity and automation.

Immunoassays shall have high sensitivity to detect low concentrations of antibodies against infectious agents, as well as high specificity so as to have no cross-recognition of antigenically related antigens and produce no false positive results. In reality, a highly sensitive assay has a low chance to produce false negative findings and is suitable for screening large numbers of samples. The specific antigens such as the killed or neutralized virus lysate, synthetic peptides, or recombinant proteins are usually developed in research and development phase for specificity.

The principles of antibody detection assays can be grouped according to the method of analysis, such as direct or indirect assays, or competitive inhibition assays. Since most direct immunoassays are used for antigen detection, and most indirect immunoassays can be used as competitive inhibition assays, we only cover the indirect immunoassays in this chapter. The indirect immunoassay, the most commonly used type of immunoassay, is illustrated in Fig. 4.1. In brief, the capture antigen used can be either bound on solid phase (1A) or microparticle in liquid phase (1B). The target antibody that needs to be detected shall bind to specific antigen. The detector or the so-called secondary antibody, is conjugated for signal detection.

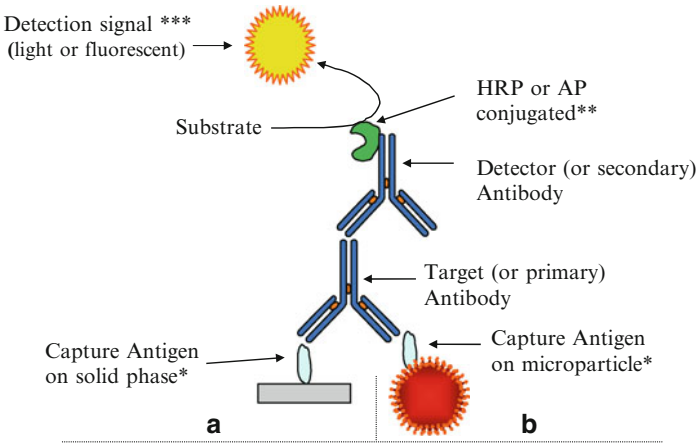

Fig. 4.1 Model for EIA detection method. Model for target antibody detection in two typical indirect immunoassays. *Capture antigen is bound to solid phase such as microwell plate (**a**), or antigen can be labeled or bound to microparticle or microsphere in liquid phase (**b**). **Horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated secondary antibody (usually anti-human IgG). ***Detection signal can be generated by colorimetric, chemiluminescent, or fluorescent methods

The signal detection system such as conjugate, substrate, and detection methods such as color or fluorescent, are critical in the immunoassay.

The immunoassays can be grouped into several categories according to the type of detection systems used (Table 4.1): (1) colorimetric, (2) radiometric, (3) chemiluminescent, or (4) fluorescent [2, 4–6].

Enzymes are effective labels because they catalyze chemical reactions, which can produce a signal. Because a single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction, these labels are effective at amplifying assay signals. As shown in Table 4.1, most enzyme–substrate reactions used for immunoassays utilize chromogenic, chemiluminescent, or fluorescent substrates that produce a signal detectable with the naked eye, a spectrophotometer, luminometer, or fluorometer.

Colorimetric or Chromogenic Substrate

Colorimetric method is the substrate color change that can be detected by naked eye or optic density using a specific wavelength of light detected by spectrophotometer. Latex agglutination is a photometric immunoassay that is used more in antigen detection than antibody detection and thus is not covered in this chapter.

Enzyme immunoassay (*EIA*) *or enzyme-linked immunosorbant assay* (*ELISA*) is indirect or "sandwich"-type colorimetrical assay that is the backbone of the immunology technique. Solid-phase enzyme-coupled reagent assays were developed 40 years ago [6]. In indirect ELISA (Fig. 4.1), antibody, if present in test sample, forms immune complex first with the capture antigen affixed to the solid phase (plastic microwell plate or tube). The primary or target antibodies in serum sample can bind to the target or capture antigens immobilized on plate wells by using enzyme-linked detector (or secondary, conjugate) Abs, such as goat, mouse, or rabbit anti-human immunoglobulin G (IgG) Abs. Secondary Ab labeled by chemical conjugation of an enzyme bounds the immune complex. The enzyme "fixed" on the solid phase through immune complex interacts with the substrate, catalyzes a chemical reaction with a substrate, and yields a colored product that can be visualized and measured by optical density measured by spectrophotometer. The intensity of substrate color change is proportional to the amount of enzyme-linked secondary antibodies, which is proportional to the amount of primary antibodies in the sample.

Indirect EIA or ELISA shall reduce or eliminate the nonspecific Ab binding and interfering serum factors (e.g., rheumatoid factors), thus providing low background and high sensitivity and specificity. Some assays use avidin–biotin complexes between Abs and antigens to increase assay sensitivities. The most commonly used enzymes for EIA are Alkaline Phosphatase (AP) and Horseradish Peroxidase (HRP) as the detection enzyme because of their stability, turnover number, and lack of interferences. HRP is a relatively small enzyme with a high turnover and is derived from nonmammalian sources. When used with a variety of substrates such as ABTS, HRP generates large signals from the production of the colored products (a deep

Table 4.1 Types of antibody detection methods

Method	Capture antigen (Ag)	Sample target antibody	Label conjugated on detector (secondary) antibody	Detection method
Colorimetric or chromogenic			Usually goat, mouse, or rabbit anti-human IgG	
Latex agglutination	Ag on bead suspension	Serum		Visible agglutination
ELISA	Ag on solid phase or microparticle	Serum	Enzyme (HRP or AP[a])	Visible color, optic density (OD) by spectrophotometer
Immunoblotting	Ag on nitrocellulose membrane	Serum	Enzyme (HRP or AP[a])	Visible band
Lateral flow diffusion (handheld)	Ag colloidal gold-labeled on nitrocellulose or nylon membrane	Serum, blood, oral fluid	Colloidal gold (chromatographic lateral flow)	Visible line
Radioimmunoassay (RIA)	Ag on bead (or radiolabeled)	Serum	Radiolabeled (^{125}I, ^{14}C, ^{3}H)	Radioactivity by gamma counter
Chemiluminescence (CLIA) Enhanced CLIA	Ag on solid phase or microparticle	Serum	Luminol (dioxetane through HRP or AP*) or acridinum ester	Photon output or light by Luminometer
Electro-chemiluminescence (ECL)	Ag on magnetic beads	Serum	Chelate ruthenium (Ru) as electron carrier (TPA as substrate)	Photon output by flow cell with photon detector
Fluorescence				
Indirect fluorescent (IFA)	Ag bound on slide or microparticle	Serum	Fluorescein isothiocyanate (FITC) conjugated	Fluorescence by microscope under UV light or fluorometer
Time-resolved fluoroimmunoassay (TRF)	Ag bound on microparticle	Serum	Fluorescein biotinylated with Lanthanide chelate (europium) in low pH	Fluorescence by fluorometer
Flow cytometry (FC) Multi-analyte profile (xMAP)	Ag-coated dyed microsphere	Serum	Fluorescein (through biotin-Ab to streptavidin)	Fluorescence cell scanner (flow cytometer) Fluorescence with FlowMetrix (Luminex)

Most EIA assays are indirect or "sandwich" assays

[a]Horseradish peroxidase (HRP) or alkaline phosphatase (AP)

green color) in the presence of hydrogen peroxide, which can be seen without a spectrophotometer. The amount of color generated is then measured after a fixed incubation time at a specific wavelength. The optical density obtained is then related back to the concentration of the antigen in the sample. Due to the need of random access without batching for quick clinical turnaround time, certain EIA or ELISA Ab methods have been gradually replaced by other rapid methods that are discussed in this chapter.

Conformational antibody test such as immunoblotting method is another technique for antibody detection. In the so-called Western Blot or immunoblot method, the capture antigens such as proteins, peptides, or viral lysates are electrotransferred to a nitrocellulose membrane. If target antibodies are present in the specimen, they will bind to the antigens present on the nitrocellulose strips. Visualization of the antibodies bound to antigen is accomplished using a series of reactions with goat anti-human IgG conjugated with biotin, avidin conjugated with HRP, and the HRP substrate. Multiple bands corresponding to the antigens seen on the nitrocellulose strip can be used as confirmation or supplementary antibody in cases of HIV [7] or hepatitis C virus (HCV).

Rapid antibody detection has been achieved by using lateral flow diffusion (handheld, portable device) method. Initially designed more for the antigen-specific immunoassay, rapid assays have been used more in antibody detection largely due to the use of HIV rapid antibody testing [7]. Assay uses colloidal gold, carbon, paramagnetic, or color latex beads for visible line in capture zone on the nitrocellulose or nylon membrane. Labeled capture antigen–antibody complex migrates by capillary action.

Known as "handheld" assays, lateral flow assays were initially developed for drugs and pregnancy testing [8], they are simple to use require minimal training, and require no special storage conditions. In most cases, the manufacturer provides simple instructions that include pictures of positive and negative results. The assays are typically designed on nitrocellulose or nylon membranes contained within a plastic or cardboard housing. In the antibody detection format, a capture antigen is bound to the membrane, and a secondary labeled antibody is placed on a sample application pad. As the sample migrates down the membrane by capillary action, antibody present in the sample binds to the labeled antigen and is captured as the complex passes. Colloidal gold, carbon, paramagnetic, or colored latex beads are commonly used particles that create a visible line in the capture zone of the assay membrane for a positive result. This is useful particularly when the control line is built in so that general quality control can be performed only once daily.

Radioimmunoassay

Due to strict regulation on radiation safety, radioimmunoassay (RIA) that uses radiolabels for measurement of antigen-binding antibody has been used much less in the clinical laboratories and thus is not discussed in detail. In general, antibody in a test serum binds radiolabeled antigen to form antigen–antibody complex in liquid phase. Subsequent protein A-Sepharose or protein G-Sepharose beads bind

Ag–Ab complexes. The RIA uses ^{125}I-, ^{14}C-, or ^{3}H-labeled antigens as the so-called tracers, and detector (secondary) antibody is radiolabeled. Radioactivity can be measured by collecting beads after centrifugation and by gamma counter.

Chemiluminescence-Immunoassay

Due to the ease of process that can fit into automation, chemiluminescence-immunoassay (CLIA) and enhanced chemiluminescence have been developed and implemented more in the clinical setting. CLIA and enhanced CLIAs are the chemical generation of visible light by a reaction and as such do not use any light source and can be measured by luminometer [4, 9]. Thus the need for optical wavelength filtering systems is eliminated. Chemiluminescent systems fall into three classes: (1) indirect CLIA, (2) direct CLIA, and (3) enhanced CLIA.

Indirect chemiluminescence (*CLIA*) uses an enzyme as the label. The enzymes are used to produce the chemiluminescent signal. Typically either HRP or AP is used and the amount present is determined by the addition of substrates that under the influence of the enzyme system give rise to visible emission. One example is CSPD or dioxetane converts to metastable intermediate by alkaline phosphatase and emits "glow" light. The chemiluminescent substrate, a phosphate ester of adamantyl dioxetane, undergoes hydrolysis in the presence of AP to yield an unstable intermediate. The continuous production of the intermediate results in the sustained emission of light for photon output signal measured by the luminometer. Using this type of signal enhancement has allowed immunoassays to be developed that are faster and more sensitive than any traditional colorimetric assay. Light intensity is a linear function of the amount of label enzyme, and the luminescence intensity at any time point is a direct measure of the concentration of the enzyme. The low background signal of the system allows a high degree of discrimination between negative and (true) positive serum samples [4]. Luminol is the substrate to the HRP. Amplify by turnover of the chosen substrate, a single enzyme label can convert $>10^7$ molecules per minute, a millionfold increase.

Direct chemiluminescence (*CLIA*) is a nonenzymatic system. Substrate linked to antibody–antigen is the label. One oxidation event liberates one molecule of label with release of set number of photons. A nonenzymatic system uses direct chemiluminescent labels which have lower background signals than the enzyme systems, and will typically give rise to very fast times to elicit signals. Luminol reaction is widely used as a chemiluminescent fast or "flash" reaction, but unlike the peroxyoxalate systems does not require an organic/mixed solvent system. The chemiluminescent emitter is a "direct descendent" of the oxidation of luminol by an oxidant in basic aqueous solution. Probably the most useful oxidant is hydrogen peroxide (H_2O_2). With the acridinium ester system, after the immunological binding and subsequent wash step, the signal takes only 2 seconds to develop, compared with 30 min or longer for an enzyme-generated system. This molecule can turn to chemiluminescence that will speed up most assays by an assay of tenfold or more sensitive and can be easily detected [10].

One reason for the growing popularity of chemiluminescent assays is their exquisite detection sensitivity. Unlike absorbance (colorimetric) or fluorescent measurements, assay samples typically contribute little or no native background chemiluminescence. The lack of inherent background and the ability to easily measure very low and very high light intensities with simple instrumentation provide a large potential dynamic range of measurement. Linear measurement over a dynamic range of 10^6 or 10^7 using purified compounds and standards is routine.

Enhanced chemiluminescence. Like indirect or enzymatic CLIA, HRP enzyme is the label, luminol is the substrate, and the so-called enhancers act as catalysts. Enhancers speed the oxidation of the luminol by HRP by as much as 1,000 times. Thus, HRP oxidation of luminol as enhancement leads to eventual light by luminol.

Electrochemiluminescent

Another antibody detection method is electrochemiluminescence (ECL), a promising technology similar to ELISA except that the secondary antibody is labeled with a chemiluminescent label ruthenium (Ru) and magnetic beads provide greater surface for soluble and target capture and separation [2]. Electron transfer between Ru atom and substrate tripropylamine (TPA) results in photon production. Excitation results in light emission can be detected by photon detector that detects electrochemiluminescent signal in electrochemical flow cell for magnetic-bead-Ru-tagged immune complex. The magnetic beads are usually small spherical and range from a few nanometers to micrometers in sizes. The advantage of magnetic beads that contain paramagnetic magnetite (Fe_3O_4) is the capability of rapid separation of captured antigen–antibody complex when placed in a magnetic field.

ECL assay, for one example, can use immunomagnetic separation (IMS by ORIGEN system, IGEN) and magnetic ECL detection system [11–13]. Detection of ECL is accompanied by heavy metal chelate ruthenium conjugated to a detector antibody. Initially Ru and TPA in the buffer are oxidized at the surface of an anode when an electric field is applied to the electrode. TPA loses a proton and becomes a reducer, which causes Ru to enter a high-energy state by a high-energy electron transfer from the electron carrier TPA. A rapid electron transfer reaction between the substrate TPA and the Ru atom occurs, resulting in the production of photons in light transmission, which in turn is sensed by the photon detector at 620 nm. A linear dynamic range spans six orders of magnitude [14] and thus makes ECL assay another method of choice for antibody detection.

Fluorescent Immunoassays

Immunoassays using fluorescent detection methods can be categorized into four groups: (1) direct fluorescent assay (DFA), (2) indirect immunofluorescence (IFA), (3) time-resolved fluorescence (TRF), and (4) flow cytometry (FC). DFA is commonly

used for antigen test and thus is not covered here. IFA such as the slide method for microscopic examination under the UV light is used much less in antibody detection than antigen detection and is not covered as well. However, IFA techniques such as used in the TRF and FC (Table 4.1) are discussed briefly.

TRF assays use lanthanide chelate such as europium (Eu^{3+}) labels with unique properties such as long fluorescence decay time so as to lower background. TRF is similar to ELISA, except that the capture antigen affixed to the solid phase is mixed with the specimen and the complex if any is mixed to diluted detector antibody that is labeled with lanthanide chelate such as europium or samarium. A low-pH enhancement solution can cause lanthanide to dissociate from the labeled compound and is highly fluorescent [15, 16]. TRF exploits the differential fluorescence life span of lanthanide chelate labels compared to background fluorescence. The labels have an intense long-lived fluorescence signal and a large stokes shift, resulting in assays with a very high signal-to-noise ratio and excellent sensitivity [17]. TRF produces its signal through the excitation of the lanthanide chelate by a specific wavelength of light. Fluorescence with a pulse of excitation energy, repeatedly and reproducibly, is initiated in TRF.

Flow cytometry is the commonly used IFA. The first use of flow cytometry for analysis of microsphere-based immunoassays was published in 1977 [18, 19]. Initially different-sized microspheres were used for simultaneous analysis of different analytes [19]. A fluorescent probe is added to a liquid suspension with sample, which is then streamed past a laser beam where the probe is excited. A detector analyzes the fluorescent properties of the sample as it passes through the laser beam. Using the same laser excitation source, the fluorescence may be split into different color components so that several different fluorophores can be measured simultaneously and analyzed by specialized software. A flow cytometer has the ability to discriminate different particles on the basis of size or color, thus making multiplexed analysis possible with different microsphere populations in a single tube and in the same sample at the same time. The FC technology is the basis of the xMAP technology.

Multi-Analyte Profile Technology

xMAP technology has been called differently, namely, multiplexed particle-based flow cytometric assays technology, fluorescent microsphere immunoassay (MIA), fluorescence covalent microbead immunosorbent assay (FCMIA), multiplexed indirect immunofluorescence assay, or multiplex flow cytometry. This two-step suspension method is based on fluorescent detection using the FlowMetrix analysis system [20]. Systems using xMAP technology perform assays on the surface of color-coded beads (microspheres) that are covered with capture antigens that react with the target antibodies. The microbeads with surface binding characteristics and a dying process to create up to 100 unique dye ratios can be used. Specific dyes permeate the polystyrene microspheres that are 5.5 μm (5.5 micron) in diameter and are composed of polystyrene and methacrylate, to provide surface carboxylate functional

groups on the surface. Each antigen was covalently linked by a carbodiimide conjugation method [21] such as EDC coupling method to beads of uniform size, which were colored with different amounts of red and orange fluorescent dyes (in a unique ratio) to allow for discrimination based upon the relative emission intensities at the wavelengths of the two fluorescent dyes.

There are 64 different ratios of red and orange fluorescence, which identify 64 distinctly colored sets of microspheres. Differently colored microsphere sets can be individually coupled via the surface carboxylate moiety to a specific probe for a unique target. The flow cytometer analyzes individual microspheres by size and fluorescence, distinguishing three fluorescent colors: green (530 nm), orange (585 nm), and red (>650 nm) simultaneously. Microsphere size, determined by 90° light scatter, is used to eliminate microsphere aggregates from the analysis. All fluorescent molecules are labeled with a green-emitting fluorophore. Each fluorochrome has a characteristic emission spectrum, requiring a unique compensation setting for spillover into the orange fluorescence channel. Green-emitting fluorochrome can be used as a reporter.

Microspheres conjugated with antigen can be added in to the well, in the sample, as well as the Fluorescein-conjugate [red-phycoerythrin (R-PE) through biotin and streptavidin] antispecies detector or secondary antibody [22]. The red laser excites specific dyes to identify the analyte [red and orange fluorescent dyes (detected by FL2/FL3)]; the green laser excites a different dye to quantify the result (a green fluorescent reporter dye FL1) [23, 24]. The fluorescence emission of each bead of the specific antigen was determined with a fluorescence-activated cell scanner (FACScan, Becton-Dickinson), a benchtop flow cytometer (multiparameter flow cytometer that is based on a single 488-nm excitation laser), with FlowMetrix hardware for data acquisition and analysis (Luminex Corp., Austin, Texas).

The software allows rapid classification of microsphere sets on the basis of the simultaneous gating on orange and red fluorescence. The Luminex instrument is a dual-laser flow analyzer. The first laser excites the fluorochrome mixture intrinsic to the microspheres, enabling the bead identity to be determined as the beads pass single file through the laser path in the flow cell. The second laser excites the extrinsic fluorochrome (R-PE) that is covalently attached to the secondary antibodies. The dual lasers allow the operator to mix beads with different antigens together in a well of a filter plate, thus enabling multiplex analysis of different antibody specificities at one time. Orange and red fluorescence are used for microsphere classification, and green fluorescence is used for analyte measurement [20]. The xMAP technology used by Luminex has been useful for detection of multiple antibodies in a single assay.

Contrast of These Techniques

The contrast to immunoassay techniques is shown in Table 4.1.

- *EIA or ELISA* assays are relatively inexpensive, can be adapted for high-throughput use, and thus are commonly used in research and clinical laboratories. The enzymes and substrates used for ELISA might be unstable and require specialized

storage to maintain activity. Most commercial products have been validated and have had overcome this issue. Manual format of ELISA has more hands-on time and can only measure one analyte at a time. It cannot meet the demand of random access and stat test requirements. Automated system using the batched samples shall be useful for large screening purpose and can be used as open platform for different tests.

- *Rapid test or handheld system*. Signal-to-noise ratio and the limit of detection of antibody have been improved for handheld system to overcome the concern of lower sensitivity as compared to regular EIA. Many handheld systems have been approved and used for rapid diagnosis of HIV. The better the avidity and affinity of the antibody, the more sensitive and specific the assay. In general, only one or two agents that can be detected per assay strip with certain sensitivity levels. Another limitation of handheld systems is that assessment of a result is qualitative and subject to interpretation. The handheld system has made point-of-care antibody detection available for certain clinical and resource-limited settings.
- *RIA*. RIA is relatively slow and difficult to automate, and is susceptible to interfering immunoglobulin M (IgM) rheumatoid factor or high backgrounds with some sera. The labile nature of some radioactivity molecules (some might decay quicker) and the regulatory constraints in their use (particularly exposure potential and disposal regulation) in clinical laboratory make radioactivity no longer the test of choice.
- *Chemiluminescent* (*CLIA*) *detection*. The use of a more sensitive detection method such as chemiluminescence allows for a faster assay system, as well as a lower limit of detection. Assays are often more sensitive than enzyme-based immunoassays. CLIA techniques have been widely accepted and implemented for automation because assay samples typically contribute little or no native background chemiluminescence and because detection procedure is simple. It requires no excitation source (as does fluorescence and phosphorescence), and only a single light (photon) detector such as a photomultiplier tube. Most samples have no "background" signal, i.e., they do not themselves emit light. No interfering signal limits sensitivity [25–27]. Most commercially developed chemiluminescent reactions use labeling either with a chemiluminescent compound or with an enzyme and use a chemiluminescent substrate, [4, 9] as shown in Table 4.2. CLIA-based method can be used for automation, random access, and stat detection of antibody in the clinical setting.
- *ECL*. ECL background signal is constant with time, and steady-state ECL signal is proportional to the rate of substrate turnover, which is different with the colorimetric background signal that accumulates with time. Light is the consequence of chemical reaction, luminol undergoes oxidative bond cleavage to yield an excited state species that decays by a radioactive pathway, and HRP (in the conjugate reagent) catalyzes the one-electron oxidation of luminol and expends hydrogen peroxide. The reaction does not suffer from the same surface steric and diffusion limitations as compared to conventional EIA or ELISA as the magnetic beads provide a greater surface area.

Fluorescence immunoassay and indirect fluorescence assay (*IFA*). Fluorescent assay will allow more sensitive or faster detection than colorimetric methods. However, it could suffer from possible high background contamination due to the intrinsic fluorescence of some proteins and light-scattering effects. While simple to perform and requiring minimal equipment and reagents, significant expertise is necessary to interpret the results of IFA by slide microscopic method [28]. IFA can be performed if batching is not required.

TRF and *flow cytometry*. The limitation for TRF is similar as ELISA. In addition, dedicated measuring instrument and rigorous washing techniques are important to avoid lanthanide contamination since lanthanide label is highly fluorescent [16]. A major strength of FC technology is its ability to be multiplexed with little or no loss of sensitivity [23, 29]. FC by BD Biosciences (San Jose, CA, USA) has many applications in biomedical research and is commonplace in most large clinical laboratories. However, FC has several disadvantages. Assays typically lack the sensitivity of those based on ECL or TRF. The system itself is relatively complicated, requiring training and expertise to operate.

xMAP. Unlike traditional ELISA and other immunoassays that allow one test for each specific antibody at one time, many antibodies can be measured at the same time, in a single well or tube by using xMAP multiplexed technology (Luminex). The xMAP technology was originally developed using the principles of flow cytometry (FACScan) that has the multiparametric resolving power. Unlike general flow cytometry on different sizes of beads, the xMAP technology detects identically sized microspheres with two different dyes, emitting in two different wavelengths, allows aggregates to be distinguished, and permits discrimination of at least 64 different sets of microspheres. Due to multiplexing, xMAP technology delivers more data with comparable results to ELISA within the same sample. Automation of xMAP technology has been developed and will be further expanded in the clinical setting.

Application of the Techniques in the Diagnostic Microbiology

Clinical Applications

Clinical application of immunodiagnostics can be best demonstrated in availed immunoassays for HIV [7, 30] and hepatitis. Immunoassays have been developed to detect anti-HIV antibodies or viral antigens present in serum, plasma, dried blood spots, urine, and saliva. Assay formats range from EIAs, ELISA-based Western blot assays, IFA assays, and even rapid handheld immunoassays. In general, the EIA remains the most widely used serologic test for detecting antibodies to HIV. HIV-1, 2, and O or HIV antigen–antibody combo immunoassays represent the advances in antibody detection technologies to detect and identify infectious agents. Study comparing ELISA methods with Western blotting, Microagglutination, IFA, and flow cytometry for detection of antibodies to *Francisella tularensis* and diagnosis of tularemia is another source to demonstrate the utilization of antibody detection

techniques [31]. In this study, the combined usage of ELISA and confirmatory blotting seems to be the most suitable approach for serodiagnosis of tularemia.

Immunoanalyzers for broad application range will help meet the challenges of immunodiagnosis of infectious diseases such as automation, random access, multiplexing, and high throughput. The main focus of this section of clinical application will be general utilization of technologies and automation in terms of methods for antibody detection.

EIA detection. *EIA or* ELISA assays are still the methods of choice for large-scale investigations during outbreak or epidemiological surveillance studies. Because of its relative simplicity and good sensitivity, ELISA has been used for screening large numbers of small-volume samples and has had great impact in epidemiology and in the diagnosis of infection, particularly in the diagnosis of the bacteria and viruses such as West Nile (WN) virus [32, 33] that is difficult to culture, not to mention that these assays have been used extensively in HIV and hepatitis testing [7].

There are continuous developments in EIAs for screening and diagnosis of HIV. Third-generation EIAs include recombinant or synthetic peptide antigens derived from both HIV-1 and HIV-2 as capture antigens, and allow detection of both IgG and IgM, which enable improved sensitivity to early detection of HIV antibody [34]. FDA approved the first fourth-generation assay for use in the USA in June 2010, which detects both HIV antibodies and HIV p24 antigen. The fourth-generation HIV antigen–antibody combo assay is designed to detect acute HIV infection even in those who have not yet begun to produce HIV-specific antibodies [35].

Other than HIV, IgM and or IgG capture ELISAs have been developed and used as the most useful and widely used tests for diagnosis of arboviral encephalitis [36]. Serum or CSF IgM testing has been recommended in the diagnosis of West Nile Virus (WNV) infection [36]; however, issue of cross-reactivity especially with the flaviviruses has been reported [37, 38]. The Plaque Reduction Neutralization Test is considered as the gold standard test and can be used to confirm a positive ELISA test [37, 39]. This combination of assays is highly sensitive and specific, but performing a complete panel of ELISAs requires 2–3 working days to complete, as overnight incubations are deemed necessary to enhance sensitivity. IFAs may also be used for diagnosis, but they are not suitable for a high throughput of specimens and they are less sensitive than ELISA. Alternative methods that allow a more rapid detection of anti-WNV virus antibodies now exist, which include an anti-WNV IgG optical fiber immunoassay that uses biosensors and chemiluminescence [37, 40].

Immunoblotting method. Cross-reactivity could result from an antibody that binds to structurally distinct but similar epitopes present on different antigens or from an antibody that binds to structurally identical epitopes on different antigens. This is why confirmatory tests are needed in certain tests such as HIV using more specific assays such as the Western blot [41]. The separated HIV-1 proteins are electotransferred to a nitrocellulose membrane. If antibodies to any of the major HIV-1 antigens are present in the specimen, bands corresponding to the HIV-1 proteins (p) or glycoproteins (gp) such as gp24, gp41, or gp120 will be seen on the nitrocellulose strip. Antibodies can thus be detected by using enzyme-conjugated secondary antibody (to human IgG) and demonstrated by darkly colored lines on the membrane

Table 4.2 Type of commercially available automated antibody detection systems

Method	Detection method	Automated system (company)	High throughput	Full automation
Colorimetric	Enzyme colorimetric	Evolis (Bio-Rad) ETI-Max (DiaSorin) Triturus (Grifols) DSX (Alere) DS2 (INOVA) QUANTA-Lyser (INOVA)	Yes	Yes
Chemiluminescence (CL) immunoassay	CLIA	ACCESS (Beckman) UniCel DxI (Beckman) ADVIA Centaur (Siemens) Architect (Abbott) Immulite (Siemens) Liaison (DiaSorin)	Yes	Yes
	Enhanced CLIA	VITROS (Ortho)	Yes	Yes
	Electro-CLIA (ECL)	Cobas (Roche) ORIGEN (IGEN, Roche)	Yes	Yes
Fluorescence	Fluorescence	AxSYM (Abbott) VIDAS (bioMerieux) Nexgen Four (Adaltis)	Yes	Yes
	Flow cytometry (FC)	FACScan (Becton-Dickinson)		
	Multiple-analyte profile (xMAP)	AtheNA Multi-Lyte (Zeus, Alere) Bio-Plex (Bio-Rad)		
Dual technology	EIA and IFA	Margo 45 (Diamedix)		

Note: Many can handle antibody detection assays such as anti-HIV, anti-HAV, anti-HCV, anti-HBs, anti-HBc, CMV, and Rubella. Throughput is generally high from 80 to 400 tests per hour

under the substrate. However, newer-generation EIAs often detect HIV infection earlier than Western blotting, and with the improvement in the sensitivity and the specificity of EIAs and rapid tests, the role of Western blotting in HIV diagnosis may change in the near future [42].

The recombinant immunoblot assay (RIBA) or strip immunoblot assay (SIA) for detecting NS5 and c33c recombinant proteins and c100p, 5-1-1p, and c22p synthetic peptides of HCV is intended as a supplemental test that was originally developed to confirm the results of a positive EIA test. However, the specificity of the third-generation EIA results that exceed particular signal/cutoff ratios is extremely high, and given the widespread availability of nucleic acid testing, the role of RIBA testing in HCV diagnosis is limited [43]. Semi-automated or automated processing instrumentation is available for immunoblotting.

Rapid or handheld assay. *Rapid immunoassay or* handheld immunoassays have evolved significantly in the past decade. Development of self-contained miniaturized devices allows an immunoassay to be performed in a field or in the point-of-care setting. Currently available handheld immunoassays include those used for detection of antibodies against Epstein–Barr virus (EBV), *Helicobacter pylori*, and not to mention HIV. Since 2002, the FDA has approved six rapid HIV tests for use in the USA, which utilize either immunochromatography (lateral flow) or immunoconcentration (flow-through) techniques [44]. A recent study evaluating six rapid HIV tests showed that all tests present high sensitivity and specificity compared to the third-generation EIAs [44]. In 2010, The OraQuick® HCV Rapid Antibody Test was approved by FDA for HCV diagnosis. This assay utilizes antigens from the core, NS3 and NS4 regions of the HCV genome, and has shown to have sensitivity and specificity that are comparable to laboratory EIA [45].

RIA application. Even though unpopular in the clinical setting, RIA is still available for research settings. One example is the Human Papilloma Virus (HPV) type-specific competitive RIA (cRIA) used to evaluate HPV type-specific antibody titers in HPV vaccine trials [46, 47]. Briefly, HPV L1 virus-like particle (VLP) antigens (HPV-6 and HPV-11) are coated onto solid-phase polystyrene beads and incubated with equal volumes of sera and diluted monoclonal Ab, as well as the ^{125}I-labeled secondary antibody [48]. However, a competitive, multiplexed, Luminex-based immunoassay could increase assay throughput and specificity and thus replace cRIA.

Chemiluminescence. Chemiluminescence is discussed in the "Automation" section.

Fluorescence immunoassay. Though TRF has been used to detect various viral antigens recent studies have utilized this method to measure immunity or antibody response after vaccination against Varicella Zoster Virus [49–51]. Another type of fluorescent technology is fluorescent polarization (FP), which is a phenomenon seen when polarized light excites a fluorescent dye causing photons to be emitted in the same plane as the exciting light. FP assays can be used for detecting antibodies [52]. Because of the limited need for sample processing, FP antibody detection assays are particularly useful for high-throughput screening such as AxSYM (Abbott Laboratories, Abbot Park, IL, USA).

Flow cytometry. McHugh described a duplex immunoassay for antibodies to cytomegalovirus and herpes simplex virus using two distinct sizes of microspheres [53, 54]. Scillian further described its use for simultaneous detection of four different HIV recombinant DNA proteins. Size discrimination of microspheres allows simultaneous detection of small numbers of analytes, but the inability to distinguish aggregates of smaller microspheres from larger microspheres limits the extent of multiplexing that can be achieved [55].

Multiplexed bead (*xMAP*) *assay*. Diagnosis of infection often requires testing for multiple antibodies or multiple markers. Bead-based immunoassays allow a quantitative and qualitative analysis of multiple targets rapidly with excellent sensitivity and specificity [56]. The xMAP technique applications include detection of antibodies against infectious agents, disease surveillance, and screening of donated blood [57]. It has been used to test a panel of respiratory viruses, including influenza A and B viruses; adenovirus; parainfluenza viruses 1, 2, and 3; and respiratory syncytial virus [58], and for *Bacillus anthracis* anti-protective antigen (PA)-specific immunoglobulin G (anti-PA IgG) [59]. When compared with the ELISA method [60], this technology usually has good positive correlation, better sensitivity and speed, and enhanced dynamic range. It uses smaller sample volume and can be multiplexed, that is, measure more than one analyte simultaneously [61]. For example, a Luminex xMAP-based technology was compared with ELISA for quantitation of antibodies to the toxoids of *Clostridium tetani* (Tet) for tetanus, *Corynebacterium diphtheriae* (Dip) for diphtheria, and *Haemophilus influenzae* type b (Hib) polysaccharide. The correlations (R^2) between ELISA and Luminex of the 81 samples were 0.96, 0.96, and 0.91 for Tet, Dip, and Hib, respectively [62]; the Luminex technology has been successful in detecting targets from dried blood-spot specimens: antibodies to HIV-1 p24, gp160, and gp120 eluted from dried blood-spot specimens from newborns were detected simultaneously [63, 64], as well as the HCV antibody and Hepatitis B Virus surface (HBs) antigen with HIV antibodies [65].

This technology is also applied to vaccine development by testing antibody response. Simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides (PnPs) has been developed [61], which showed results similar to another xMAP assay developed for antibodies to PnPs [66]. The assay simultaneously determines serum IgG concentrations to 14 PnPs serotypes. Recently, a multiplexed bead-based immunoassay using xMAP to quantify 17 pneumococcal proteins was developed in pursuit of a pneumococcal vaccine that would provide protection regardless of serotypes, as opposed to the currently available vaccines [67].

The xMAP assay may also be useful in areas where cross-reaction is an issue. A WNV recombinant antigen microsphere (suspended-microsphere) diagnostic immunoassay has been developed for detection of human anti-flavivirus antibodies [68], which can be performed rapidly (in less than 3 h) and with small amount of specimen (≤30 μL). A duplex microsphere method that allows detection of anti-WNV and anti-St. Louis encephalitis was developed that has the advantages of being faster to perform and providing a more definitive answer regarding the infecting virus, as opposed to simply yielding two results [69, 70].

An advantage of the 96-well plate Luminex assay format is that it avails itself to automation, such as the Tecan Genesis liquid handler to automate the assay. The automation such as Bio-Plex system (Bio-Rad Laboratories, Hercules, CA, USA) employing the Luminex xMAP allows individual and multiplex analysis of up to 100 different analytes in a single microtiter well [23] and is used for detecting 15 human cytokines [71]. A multiplexed bead assay was evaluated for assessment of EBV immunologic status using FDA-cleared IgM and IgG EBV assays on a fully automated BioPlex 2200 system (Bio-Rad). Concordance between results generated by the BioPlex system and conventional assays showed 97.9, 91.4, and 96.9% agreement for viral capsid antigen (VCA) IgM, VCA IgG, and EBNA-1 IgG assays, respectively [72].

Automation

Commercially available immunoanalyzers have been widely used to facilitate the analysis of large numbers of samples by improving the throughput and automation (Table 4.2). The first generation of immunoassay systems was developed more than a decade ago to automate what had been labor-intensive manual laboratory tests. Advances in clinical immunology, and the demand for faster turnaround times and reduced costs, have helped technology developments in immunoassay, as well as the integrated immunochemistry analyzers. The high-volume immunoassay will have a significant impact on laboratory performance by reducing errors, turnaround times, and labor requirements for those tests. Capability of interfacing with laboratory information system (LIS) further enables the utilization of commercial systems.

The ideal immunoassay system will have the following capabilities to provide optimal productivity and a comprehensive disease-focused menu: no-pause loading of all reagents, samples, and supplies; continuous sample loading for fast turnaround time; high-throughput process efficiency; random access; reduced operator intervention; minimal hands-on time with large onboard capacity for reagents; and ability to interface with the LIS for increased efficiency with easy-to-use software. The above features are critical to those assays for HIV and complete hepatitis antibody detections.

Any technology and system, as sophisticated as it may appear, needs to be validated. The reliability of instrument and immunoassays and their clinical utility used under real-time clinical conditions need to be well studied. The decision to switch will be made on the basis of adequate quality through validation of assays and analysis of the cost. As methods change, the new automated assays must be validated against the existing ones for better sensitivity, specificity, and predictive values, and clinical utility.

Most chemiluminescent reactions can be adapted to this assay format by labeling either with a chemiluminescent compound or with an enzyme and using a chemiluminescent substrate. Most commercially developed immunoassays are of this type (Table 4.2). For example, Lumi-Phos 530 of Luminol CLIA is used as the detection reagent in the Access immunoassay analyzer (Beckman Coulter Inc., Fullerton, CA, USA). Lumigen PPD and enhancer are incorporated in the chemiluminescent detection

reagent used in the Immulite Immunoassay Analyzer from Siemens. The AxSYM immunoassay system (Abbott) is based on the microparticle enzyme immunoassay technology [73–75]. Immulite (Siemens, Deerfield, IL, USA) is a benchtop immunoassay analyzer with continuous random-access capabilities that uses enzyme-amplified chemiluminescence chemistry for antibody or antigen detection [76]. Multiple high-throughput systems that can provide streamlined operations to reduce total processing time are available in the market, and some are capable in running different types of immunoassays. Many types of immunoassays can be developed on the automated systems for hepatitis virus A, B, and C, cytomegalovirus, and HIV assays.

With the availability of EIA, CLIA, and multiplex flow immunoassay that allow automated high-throughput testing for syphilis, some laboratories have adopted reverse sequence screening in which a treponemal EIA or CLIA is followed by a nontreponemal testing for positive specimens in order to reduce the time and labor required for syphilis screening [77]. This has created new problems, especially when the treponemal specific screening test is positive but the nontreponemal tests that follow are negative [78]. CDC's recent recommendation on such discordant results is to use the *Treponema pallidum* particle agglutination (TP-PA) assay, and if the TP-PA is nonreactive, syphilis is considered to be unlikely [77]. The Fluorescent treponemal antibody (FTA) has been considered the gold standard; however, it is now considered to have a lower specificity and sensitivity compared to other nontreponemal tests [77, 79].

Summary

Immunodiagnostic technologies have been developed to identify the infectious agents for better sensitivity and specificity to ensure that every true positive case is diagnosed over the past 20 years. Antibody-based methods used to be the tool for the detection and epidemiological analysis of slow-growing, difficult-to-culture, uncultivatable, or emerging infectious agents.

Antibody detection methods such as CLIA, ECL, and TRF detection formats become predominant technologies compared to conventional EIA or ELISA for diagnosis of infectious agents. Emerging antibody detection methods such as rapid or handheld assay and multiplexed flow cytometry have been proved to be the promising technologies in the clinical setting.

References

1. Yalow RS, Berson SA (1960) Immunoassay of endogenous plasma insulin in man. J Clin Invest 39:1157–1175
2. Peruski AH, Peruski LF Jr (2003) Immunological methods for detection and identification of infectious disease and biological warfare agents. Clin Diagn Lab Immunol 10:506–513
3. Andreotti PE, Ludwig GV, Peruski AH, Tuite JJ, Morse SS, Peruski LF Jr (2003) Immunoassay of infectious agents. BioTechniques 35:850–859

4. Kricka LJ (1991) Chemiluminescent and bioluminescent techniques. Clin Chem 37: 1472–1481
5. Engvall E, Perlmann P (1972) Enzyme-linked immunosorbent assay, ELISA: quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J Immunol Methods 109:129–135
6. Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871–874
7. Wang YF, Eaton ME, Schuetz AN, Nesheim SR (2009) Human immunodeficiency virus. ASM, Washington, DC
8. Zuk RF, Ginsberg VK, Gouts T et al (1985) Enzyme immunochromatography: a quantitative immunoassay requiring no instrumentation. Clin Chem 31:1144–50
9. Kricka LJ (1996) The clinical and research potential of bioluminescence and chemiluminescence in medicine. Wiley, Chichester
10. Weeks I, Wodhead JS (1991) Chemiluminescent assays based on acridinium labels. Wiley, Chichester
11. Yu H (1998) Comparative studies of magnetic particle-based solid phase fluorogenic and electrochemiluminescent immunoassay. J Immunol Methods 218:1–8
12. Haukanes BL, Kyam B (1993) Application of magnetic beads in bioassays. Biotechnology (N Y) 11:60–63
13. Blackburn GF, Shah HP, Kenten JH et al (1991) Electrochemiluminescence detection for development of immunoassays and DNA probe assays for clinical diagnosis. Clin Chem 37:1534–1539
14. Yang H, Leland JK, Massey RJ (1994) Electrochemiluminenscence: a new diagnostic and research tool. ECL detection technology promises scientist new "yardsticks" for quantification. Biotechnology (N Y) 12:193–194
15. Peruski AH, Johnson LH, Peruski LF Jr (2002) Rapid and sensitive detection of biological warefare agents using time-resolved fluorescence assays. J Immunol Methods 263:35–41
16. Aggerbeck H, Norgaard-Pedersen B, Heron I (1996) Simultaneous quantitation of diphtheria and tetanus antibodies by double antigen, time-resolved fluorescence immunoassay. J Immunol Methods 190:171–183
17. Hemmila I, Dakubu S, Mukkala VM, Siitari H, Lovgren T (1984) Europium as a label in time-resolved immuofluorometric assays. Anal Biochem 137:335–43
18. McHugh TM (1994) Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. Methods Cell Biol 42:575–595
19. Horan PK, Wheeless LL (1977) Quantitative single cell analysis and sorting. Science 198:149–57
20. Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR (1997) Advanced multiplexed analysis with the FlowMetrix system. Clin Chem 43:1749–1756
21. Staros JV, Wright RW, Swingle DM (1986) Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. Anal Biochem 156:220–222
22. Jones LP, Zheng HQ, Karron RA, Peret TCT, Tsou C, Anderson LA (2002) Multiplex assay for detection of strain-specific antibodies agains the two variable regions of the G protein of Respiratory Syncytial Virus. Clin Diagn Lab Immunol 9:633–638
23. Vignali DA (2000) Multiplexed particle-based flow cytometric assays. J Immunol Methods 243:243–255
24. Mandy FF, Nakamura T, Bergeron M, Sekiguchi K (2001) Overview and application suspension array technology. Clin Lab Med 21:713–729
25. Nieman T (1995) Chemiluminescence: theory and instrumentation, overview. In: Encyclopedia of analytical science. Academic, Orlando. pp 608–613
26. Campbell AK (1988) Detection and quantification of chemiluminescence. Ellis Horwood, Chichester
27. Berthrold F (1990) Instrumentation for chemiluminescence immunoassays. CRC, Boca Raton, FL
28. Nuwayhid NF (1995) Laboratory tests for detection of human immunodeficiency virus type 1 infection. Clin Diag Lab Immunol 2:637–645

29. Carson RT, Vignali DA (1999) Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric asssay. J Immunol Methods 227:41–52
30. Nielsen K, Bryson YJ (2000) Diagnosis of HIV infection in children. Pediatr Clin North Am 47:39–63
31. Porsch-Ozcurumez M, Kischel N, Priebe H, Splettstosser W, Finke E-J, Grunow R (2004) Comparison of enzyme-linked immunosorbent assay, western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. Clin Diag Lab Immunol 11:1008–15
32. MMWR (2000) Guidelines for surveillance, prevention, and control of West Nile virus infection—United States. MMWR Morb Mortal Wkly Rep 49:25–28
33. Nash D, Mostashari F, Fine A et al (2001) The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med 344:1807–1814
34. Louie B, Pandori MW, Wong E, Klausner JD, Liska S (2006) Use of an acute seroconversion panel to evaluate a third-generation enzyme-linked immunoassay for detection of human immunodeficiency virus-specific antibodies relative to multiple other assays. J Clin Microbiol 44:1856–1858
35. Pandori MW, Hackett J Jr, Louie B et al (2009) Assessment of the ability of a fourth-generation immunoassay for human immunodeficiency virus (HIV) antibody and p24 antigen to detect both acute and recent HIV infections in a high-risk setting. J Clin Microbiol 47:2639–2642
36. Tunkel AR, Glaser CA, Bloch KC et al (2008) The management of encephalitis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis 47:303–327
37. Dauphin G, Zientara S (2007) West Nile virus: recent trends in diagnosis and vaccine development. Vaccine 25:5563–5576
38. Martin DA, Biggerstaff BJ, Allen B, Johnson AJ, Lanciotti RS, Roehrig JT (2002) Use of immunoglobulin m cross-reactions in differential diagnosis of human flaviviral encephalitis infections in the United States. Clin Diagn Lab Immunol 9:544–549
39. Lindsey HS, Calisher CH, Mathews JH (1976) Serum dilution neutralization test for California group virus identification and serology. J Clin Microbiol 4:503–510
40. Herrmann S, Leshem B, Landes S, Rager-Zisman B, Marks RS (2005) Chemiluminescent optical fiber immunosensor for the detection of anti-West Nile virus IgG. Talanta 66:6–14
41. Jackson JB, Parsons JS, Nichols LS, Knoble N, Kennedy S, Piwowar EM (1997) Detection of human immunodeficiency virus type 1 (HIV-1) antibody by western blotting and HIV-1 DNA by PCR in patients with AIDS. J Clin Microbiol 35:1118–1121
42. Owen SM, Yang C, Spira T et al (2008) Alternative algorithms for human immunodeficiency virus infection diagnosis using tests that are licensed in the United States. J Clin Microbiol 46:1588–1595
43. Ghany MG, Strader DB, Thomas DL, Seeff LB (2009) Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 49:1335–1374
44. Delaney KP, Branson BM, Uniyal A et al (2011) Evaluation of the performance characteristics of 6 rapid HIV antibody tests. Clin Infect Dis 52:257–263
45. Lee SR, Yearwood GD, Guillon GB et al (2010) Evaluation of a rapid, point-of-care test device for the diagnosis of hepatitis C infection. J Clin Virol 48:15–17
46. Frazer IH (2010) Measuring serum antibody to human papillomavirus following infection or vaccination. Gynecol Oncol 118:S8–S11
47. Smith JF, Kowalski R, Esser MT, Brown MJ, Bryan JT (2008) Evolution of type-specific immunoassays to evaluate the functional immune response to Gardasil: a vaccine for human papillomavirus types 16, 18, 6 and 11. Hum Vaccin 4:134–142
48. Opalka D, Lachman CE, MacMullen SA et al (2003) Simultaneous quantitation of antibodies to neutralizing epitopes on virus-like particles for human papillomavirus types 6, 11, 16, and 18 by a multiplexed luminex assay. Clin Diagn Lab Immunol 10:108–115
49. Maple PA, Gray J, Breuer J, Kafatos G, Parker S, Brown D (2006) Performance of a time-resolved fluorescence immunoassay for measuring varicella-zoster virus immunoglobulin G levels in adults and comparison with commercial enzyme immunoassays and Merck glycoprotein enzyme immunoassay. Clin Vaccine Immunol 13:214–218

50. ChrisMaple PA, Gray J, Brown K, Brown D (2009) Performance characteristics of a quantitative, standardised varicella zoster IgG time resolved fluorescence immunoassay (VZV TRFIA) for measuring antibody following natural infection. J Virol Methods 157:90–92
51. McDonald SL, Maple PA, Andrews N et al (2011) Evaluation of the time resolved fluorescence immunoassay (TRFIA) for the detection of varicella zoster virus (VZV) antibodies following vaccination of healthcare workers. J Virol Methods 172:60–65
52. Nielsen K, Gall D, Jolley M et al (1996) A homogeneous fluorescence polarization assay for detection of antibody to Brucella abortus. J Immunol Methods 195:161–168
53. McHugh TM, Miner RC, Logan LH, Stites DP (1988) Simultaneous detection of antibodies to cytomegalovirus and herpes simplex virus by using flow cytometry and a microsphere-based fluorescence immunoassay. J Clin Microbiol 26:1957–1961
54. Jani IV, Janossy G, Brown DW, Mandy F (2002) Multiplexed immunoassays by flow cytometry for diagnosis and surveillance of infectious diseases in resource-poor settings. Lancet Infect Dis 2:243–250
55. Scillian JJ, McHugh TM, Busch MP et al (1989) Early detection of antibodies against rDNA-produced HIV proteins with a flow cytometric assay. Blood 73:2041–2048
56. Fonseca BP, Marques CF, Nascimento LD et al (2011) Development of a multiplex bead-based assay for detection of hepatitis C virus. Clin Vaccine Immunol 18:802–806
57. Kellar KL, Iannone MA (2002) Multiplexed microsphere-based flow cytometric assays. Exp Hematol 30:1227–1237
58. Martins TB (2002) Development of internal controls for the Luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile. Clin Diagn Lab Immunol 9:41–45
59. Biagini RE, Sammons DL, Smith JP et al (2004) Comparison of a multiplexed fluorescent covalent microsphere immunoassay and an enzyme-linked immunosorbent assay for measurement of human immunoglobulin G antibodies to anthrax toxins. Clin Diagn Lab Immunol 11:50–55
60. Quinn CP, Semenova VA, Elie CM et al (2002) Specific, sensitive, and quantitative enzyme-linked immunosorbent assay for human immunoglobulin G antibodies to anthrax toxin protective antigen. Emerg Infect Dis 8:1103–1110
61. Biagini RE, Schlottmann SA, Sammons DL et al (2003) Method for simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides. Clin Diagn Lab Immunol 10:744–750
62. Pickering JW, Martins TB, Schroder MC, Hill HR (2002) Comparison of a multiplex flow cytometric assay with enzyme-linked immunosorbent assay for auantitation of antibodies to tetanus, diphtheria, and Haemophilus influenzae Type b. Clin Diagn Lab Immunol 9:872–876
63. Bellisario R, Colinas RJ, Pass KA (2001) Simultaneous measurement of antibodies to three HIV-1 antigens in newborn dried blood-spot specimens using a multiplexed microsphere-based immunoassay. Early Hum Dev 64:21–25
64. Faucher S, Martel A, Sherring A et al (2004) Protein bead array for the detection of HIV-1 antibodies from fresh plasma and dried-blood-spot specimens. Clin Chem 50:1250–1253
65. Lukacs Z, Dietrich A, Ganschow R, Kohlschutter A, Kruithof R (2005) Simultaneous determination of HIV antibodies, hepatitis C antibodies, and hepatitis B antigens in dried blood spots—a feasibility study using a multi-analyte immunoassay. Clin Chem Lab Med 43: 141–145
66. Pickering JW, Martins TB, Greer RW et al (2002) A multiplexed fluorescent microsphere immunoassay for antibodies to pneumococcal capsular polysaccharides. Am J Clin Pathol 117:589–596
67. Shoma S, Verkaik NJ, de Vogel CP et al (2011) Development of a multiplexed bead-based immunoassay for the simultaneous detection of antibodies to 17 pneumococcal proteins. Eur J Clin Microbiol Infect Dis 30:521–526
68. Wong SJ, Demarest VL, Boyle RH et al (2004) Detection of human anti-flavivirus antibodies with a west nile virus recombinant antigen microsphere immunoassay. J Clin Microbiol 42:65–72

69. Johnson AJ, Cheshier RC, Cosentino G et al (2007) Validation of a microsphere-based immunoassay for detection of anti-West Nile virus and anti-St. Louis encephalitis virus immunoglobulin m antibodies. Clin Vaccine Immunol 14:1084–1093
70. Johnson AJ, Noga AJ, Kosoy O, Lanciotti RS, Johnson AA, Biggerstaff BJ (2005) Duplex microsphere-based immunoassay for detection of anti-West Nile virus and anti-St. Louis encephalitis virus immunoglobulin m antibodies. Clin Diagn Lab Immunol 12:566–574
71. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT (2003) Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 10:133–139
72. Binnicker MJ, Jespersen DJ, Harring JA, Rollins LO, Beito EM (2008) Evaluation of a multiplex flow immunoassay for detection of Epstein-Barr virus-specific antibodies. Clin Vaccine Immunol 15:1410–1413
73. Fiore M, Mitchell J, Doan T et al (1988) The Abbott IMx automated benchtop immunochemistry analyzer system. Clin Chem 34:1726–1732
74. Hennig H, Schlenke P, Kirchner H, Bauer I, Schulte-Kellinghaus B, Bludau H (2000) Evaluation of newly developed microparticle enzyme immunoassays for the detection of HCV antibodies. J Virol Methods 84:181–190
75. Lazzarotto T, Galli C, Pulvirenti R et al (2001) Evaluation of the Abbott AxSYM cytomegalovirus (CMV) immunoglobulin M (IgM) assay in conjunction with other CMV IgM tests and a CMV IgG avidity assay. Clin Diagn Lab Immunol 8:196–198
76. Li TM, Chuang T, Tse S, Hovanec-Burns D (2004) El Shami AS. Development and validation of a third generation allergen-specific IgE assay on the continuous random access IMMULITE 2000 analyzer. Ann Clin Lab Sci 34:67–74
77. MMWR (2011) Discordant results from reverse sequence syphilis screening–five laboratories, United States, 2006–2010. MMWR Morb Mortal Wkly Rep 60:133–137
78. Binnicker MJ, Jespersen DJ, Rollins LO (2011) Treponema-specific tests for serodiagnosis of syphilis: comparative evaluation of seven assays. J Clin Microbiol 49:1313–1317
79. Marangoni A, Sambri V, Storni E, D'Antuono A, Negosanti M, Cevenini R (2000) Treponema pallidum surface immunofluorescence assay for serologic diagnosis of syphilis. Clin Diagn Lab Immunol 7:417–421

Chapter 5
Cytometry-Based Antimicrobial Resistance Techniques

Vishnu Chaturvedi

Introduction

Flow cytometry (FC) has been regarded as one of the most promising technologies for the clinical microbiology laboratories for nearly three decades. The accuracy, versatility, and quantitative nature of the FC measurements are both complementary and superior to many microscopic and other techniques used in the clinical microbiology laboratories. Since early 1990s, numerous proof-of-concept studies have appeared in the literature for the FC identification of pathogenic bacteria, fungi, parasites, and viruses, and for the rapid testing of susceptibility of these pathogens to antimicrobials and other compounds. The reader is referred to a number of comprehensive review articles that describe the principles of FC, reagents, protocols, and a variety of applications for specific pathogens [1–6]. There are also excellent studies describing the use of FC and bead arrays, using antigen, antibody, or nucleic acid probes, for the identification and genotyping of microbes [7–9].

The emergence and spread of antimicrobial resistance have led to a vigorous search for the methods for rapid detection. Many nucleic acid-based approaches have quickly become the methods of choice and some have been adapted in ready-to-use commercial devices. The pace of innovation of FC methods for antimicrobial resistance detection has been incremental during this period. The full potential of FC technology still remains unrealized despite recent innovations aimed at making this technology simpler, cheaper, and more accessible for routine use. This chapter provides an overview of recently published studies with an emphasis on FC method innovations and future directions for FC antimicrobial resistance detection.

V. Chaturvedi (✉)
Mycology Laboratory, Wadsworth Center, New York State Department of Health, 120 New Scotland Ave., Albany, NY 12208, USA

Department of Biomedical Sciences, University at Albany School of Public Health, Albany, NY, USA
e-mail: vishnu@wadsworth.org

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Flow Cytometry Setup

A majority of published cytometry-based resistance detection studies rely on large, complicated, and expensive FC setup (Table 5.1). The optics and electronics of these instruments have seen tremendous improvements over the years with six, nine, or more color probes being detected simultaneously [10]. While there are valid applications of these enhanced capabilities in certain areas of medicine, the instruments require trained operators and vendor-supplied software for data analysis. The continued reliance on these instruments might pose a problem for the introduction of cytometry-based detection assays for routine use in the clinical microbiology laboratories. Most of the hospital microbiology laboratories are physically not in the same locations as the other primary users of these equipment and they do not have the resources and personnel for the operation of stand-alone instruments. However, the limited access to instrumentation is being addressed by the introduction of a new generation of tabletop FC instruments, which are cheaper and easier to operate. A few such devices are commercially available in the USA (easyCyte benchtop flow cytometers, Millipore, Billerica, MA; BD FACSVerse flow cytometer, BD Biosciences, San Jose, CA; and Accuri'sC6 Flow Cytometer System, BD Accuri Cytometers Inc., Ann Arbor, MI). An interesting study describes the use of such a device for detection of vancomycin-resistant *Enterococcus faecalis* [11]. The compact FC device allowed monitoring of *E. faecalis* viability and other cellular parameters, independent confirmation of FC measurements with confocal microscopy, and a good correlation between FC test results with traditional macrodilution susceptibility test [11]. This report will likely become the touchstone for future studies that aim to provide practical protocols for the routine use of FC tests for antimicrobial resistance.

Antimicrobial Resistance Measurements

The measurement of cell viability upon exposure to antimicrobials remains a popular choice for the detection of resistance (Table 5.1). A number of fluorescent probes have been described for this purpose. These include well-known dyes such as propidium iodide and acridine orange and newly introduced stains Sybr Green I and SYTO 16. The newer dyes are either cell permeant or impermeant and offer a selection of live–dead or dead cells staining, all with large fluorescence enhancements. For example, these probes work well for the detection of vancomycin resistance in *E. faecalis* and *Staphylococcus aureus* and for the multidrug resistance testing of *Mycobacterium tuberculosis* [11, 12]. *Plasmodium falciparum* resistance to antimalarials and *Candida glabrata*, *C. krusei*, and *C. parapsilosis* resistance to antifungals can also be detected via the measurements of such probes [13, 14].

A notable innovation in the viability measurements is the recent use of autofluorescent protein reporters such as *g*reen *fl*uorescent *p*rotein (GFP), which can be monitored to determine drug resistance in *M. tuberculosis*, Hepatitis C virus, and

Table 5.1 A summary of recently published flow cytometry (FC) assays for the detection of antimicrobial resistance

Target	No. tested	Antimicrobials	Measurement	FC dye/instrument	Software/statistics	Assay time	Comparator	References
Bacteria								
Enteroroccus faecalis	3	Vancomycin Vancomyquine PA1409 Daptomycin Telavancin	Membrane permeability	$DiOC_6$, propidium iodide, SYTO9/ Guava EasyCyte Plus	Kruskal–Wallis, Mann–Whitney, regression analysis	60–180 min	Macrodilution	[11]
	21	Vancomycin	Cellular binding	Propidium iodide, Vancomycin@ FL/FACS Scan	ANOVA	60–120 min	E-test	[12]
	47	Penicillin	Penicillin-binding protein	Bocillin@FL/FACS Scan	ANOVA	30 min	Disk diffusion	[18]
Staphylococcus aureus	17	Penicillin	Penicillin-binding protein	Bocillin@FL/FACS Scan	ANOVA	30 min	Disk diffusion	[18]
	48	Methicillin	MRSA	Bocillin@FL/FACS Scan	NA	60–120 min	Disk diffusion, E-test	[19]
	56	Oxacillin	MRSA	Peptide Nucleic Acid probe/ MicroPRO	Receiver-operating characteristic curves	4 h	Vitek II	[17]
	5	Telavancin	MRSA, VISA, hVISA	DiOC2(3)/Epics XL	FCS Express/ Concentration–response curve	90–120 min	Microtiter plates	[27]
Mycobacterium tuberculosis	1	Rifampicin Streptomycin Isoniazid	*gfp* expression	GFP, ZsYellow, Fluoromyco-bacteriophages/ Beckman-Coulter CYAN ADP	Summit 4.3Software	<24 h	Fluorescent microscopy	[15]

(continued)

Table 5.1 (continued)

Target	No. tested	Antimicrobials	Measurement	FC dye/instrument	Software/statistics	Assay time	Comparator	References
	16	Rifampicin Streptomycin Isoniazid Ethambutol	Cell viability	SYTO 16–Beckman Coulter XL/ MCL	ANOVA	~72–78 h	BACTEC MGIT 960	[28]
	14	Rifampicin I Isoniazid Ethambutol Ofloxacin	MDR	Propidium iodide, SYTO16/ Beckman Coulter FC500	NA	~72–78 h	BACTEC MGIT 960	[29]
	36	Pyrazinamide	Pyrazinamide resistance	Paraformaldehyde/ FACSCaliber, Bryte HS	CellQuest, WinBryte/ analysis of variance, Fisher's test	~24 h	BACTEC TB 460, ACTEC MGIT 960	[30]
Viruses								
Human immuno-deficiency virus	1	AMD3100 C34 Zidovudine	HIV envelope function	Fluorescent beads/ FACScalibur	NA	24 h	MTT cytotoxicity assay	[31]
	4	Drugs against reverse transcriptase, protease, integrase, and viral entry	Viral production	Propidium iodide and EGFP/ FACSCantoII	NA	24–72 h	Fluorescence microscopy	[32]
Human cytomegalovirus	6	Ganciclovir	Immediate early gene expression	FITC-conjugated antibody/ FACScalibur	Cell Quest	2–3 weeks	Plaque Reduction Assay	[33]
Hepatitis C virus	1	Cyclosporine A	Live cell sorting of replicon	GFP/BD FACS Aria	NA	72 h	RT-PCR	[16]

Parasites								
Leishmania donovani	12	Antimony-derivatives	Depletion of cellular thiols	CMFDA/FACS Calibur	Cell Quest	30–60 min	Not applicable	[34]
Plasmodium falciparum	3	Chloroquine Mefloquine dihydroartemisinin	Cell viability	Sybr Green I–FACS Canto II	FACSDiva, FlowJo 8.7	48–54 h	Tritiated hypoxanthine assay, Lactate dehydrogenase assay, Sybr Green Assay	[13]
	1	Chloroquine, quinine Atovaquone, artemisinin, mefloquine, primaquine, piperaquine, pyronaridine	Gametocytogenesis	GFP/FACScaliber	CellQuest	48–96 h	None	[35]
	3	Chloroquine, mefloquine	Parasitemia	Hoechst, GFP/ Beckman Coulter Cell Lab Quanta	Quanta	48–72 h	Fluorescence microscopy	[36]
		Primaquine, dihydroartemisinin Atovaquone		SC				
	1	Artemisinin	Drug pressure tolerance	DAPI-BD LSRII	FACSDiva	24–96 h	Epifluorescence, viability test	[37]

(continued)

Table 5.1 (continued)

Target	No. tested	Antimicrobials	Measurement	FC dye/instrument	Software/statistics	Assay time	Comparator	References
	1	Artemisinin Mefloquine Chloroquine	Overall and life cycle stage inhibition	Hoechst 33342, Thiazole orange/B-D LSRII		48 h	Tritiated hypoxanthine assay	[38]
Fungi								
Candida glabrata, *C. krusei*, *C. parapsilosis*	60	Anidulafungin Caspofungin	Cell viability	Acridine orange/ FACSCalibur	CellQuest Pro	4–8 h	Broth Microdilution/ E-test	[14]
C. tropicalis	4	Clotrimazole, ketoconazole Fluconazole Voriconazole	Efflux pumps, respiration	Rhodamine 123, Rhodamine6G/ FACScan	NA	30–120 min	Disk diffusion/E-test	[39]

P. falciparum [13, 15, 16]. GFP and other color variants can be used as direct or indirect reporters depending upon whether the pathogen or, in case of HCV, the host cells are labeled. These reporters allow multiparametric measurements of resistance and do away with the use of extraneous dyes, which could potentially interact with the antimicrobials being tested. An ingenious adaptation of a peptide-nucleic acid probe, originally designed for the rapid identification by hybridization, led to rapid characterization of methicillin-resistant *S. aureus* [17]. This approach has the potential to provide rapid identification and susceptibility test results simultaneously although it does require a provisional knowledge of the target organism. Another innovative approach is to monitor the resistance by the use of fluorescence-labeled versions of the target drugs as has been shown with labeled penicillin bocillin for *E. faecalis* and *S. aureus* [18, 19]. Both the autofluorescent proteins and fluorescent-labeled drugs are not only efficient for resistance measurements, but have the potential to facilitate mechanistic studies of drug resistance involving target binding, uptake kinetics, intracellular localizations, and effects on structure–function indicators of the target cells.

Assay Turnaround Time

Cytometry assay turnaround times are expectedly shorter than more conventional assays and this has remained a significant FC advantage over many competing technologies. However, such comparisons often omit the upstream processing time required before the actual FC assays can be performed, which in some cases could be many hours to many days in length (Table 5.1). While this in itself is not a shortcoming of the current protocols, there still remains a need for further improvements in the processing of test strains before they are used for cytometry-based testing. This is urgent as other competing technologies have now come up with integrated systems that test clinical samples directly for HIV and syphilis diagnosis or for molecular detection of rifampin resistance and identification of tuberculosis [20, 21]. Thus, the development of protocols that directly work with the clinical samples would be the next challenge to further improve turnaround times if FC were to remain competitive with other platforms.

Comparator Methods

As protocols intended for testing of clinical samples, it is important that cytometry -based assays perform well in head-to-head comparison with more traditional methods. The majority of published cytometry resistance protocols have demonstrated high efficacies in such comparisons with susceptibility testing methods that rely on visual growth and/or metabolite measurements of target bacteria, fungi, viruses, and parasites (Table 5.1). Thus, the standard methods for bacterial and

fungal susceptibility testing based upon disc diffusions, broth dilutions, or Epsilometer (E-test) show excellent correlation with FC results. This is also true for traditional viral assays such as plaque reduction or cytotoxicity assays. A noticeable trend in recent studies is the dual use of labeled organisms to get both quantitative (FC) and visual (fluorescence microscopy) observation of drug resistance, which independently confirm FC efficacy from the same samples. Further applications along these lines when combined with confocal microscopy, autofluorescent probes, and labeled drugs would open up new avenues of investigations of mechanism of drug resistance.

Flow Cytometry Challenges

There are still significant challenges in realizing the promised potential of cytometry-based assays for the detection of antimicrobial resistance. Although there are accepted procedures for the calibration of FC, other variables are significant as published FC resistance studies do not use same probes, instruments, and test protocols [22]. Additionally, there are no multi-laboratory studies of these protocols to assess their performance in routine operations. Curiously, all published studies address one or two target organisms mostly within the same group (bacteria or fungi) without addressing the flexibility of the setup for a variety of different pathogens, which is the likely test practice in a busy hospital laboratory. Therefore, new studies are needed that will compare multiple published protocols to identify their relative efficacies under the same operator conditions. Even more desirable will be the development of integrated platforms that allow one-stop testing of bacteria, viruses, fungi, and parasites. It will be equally desirable to use the same instruments for the identification as well as resistance testing as is currently possible for some growth-based commercial instruments for bacteria and fungi and molecular assays for bacteria and viruses.

Conclusions and Future Prospects

The basis of antimicrobial resistance in bacteria, fungi, parasites, and viruses could be genetic, structural, or functional, but they can all be tested accurately and rapidly by cytometry-based assay. This versatility and accuracy are unmatched by many other methods. However, FC assays still remain in the realm of research applications and not a single assay has become a gold standard for testing. Therefore, the time has come to make this technology more accessible by using both step-down and step-up approaches. Less sophisticated and cheaper contraptions that can be easily set up have been described; they deliver many of the benefits of cytometry testing without the burden of cost and operator complexity associated with the current instrumentation [23]. This approach is especially suitable in resource-poor areas

suffering from high burden of major infectious diseases [24]. On the other hand, the step-up approach would leverage latest progress in optics, microfabrication, and fluidics to develop "Lab-on-a-chip" devices that would deliver price-competitive and time-sensitive test results [25, 26]. It is the latter approach that holds a great promise for meaningful collaborations among diagnostic laboratories and device manufacturers so that the long-promised potential of FC can be finally realized in the clinical microbiology laboratory.

References

1. Boye E, Steen HB, Skarstad K (1983) Flow cytometry of bacteria: a promising tool in experimental and clinical microbiology. J Gen Microbiol 129:973–980
2. Alvarez-Barrientos A, Arroyo J, Canton R, Nombela C, Sanchez-Perez M (2000) Applications of flow cytometry to clinical microbiology. Clin Microbiol Rev 13:167–195
3. McSharry JJ (2000) Analysis of virus-infected cells by flow cytometry. Methods 21:249–257
4. Shapiro HM (2001) Multiparameter flow cytometry of bacteria: implications for diagnostics and therapeutics. Cytometry 43:223–226
5. Chaturvedi V (2008) Role of flow cytometry in medical mycology for antifungal testing, identification, and characterization. Curr Fungal Infect Rep 2:143–148
6. Müller S, Nebe-von-Caron G (2010) Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. FEMS Microbiol Rev 34:554–587
7. Ernst D, Bolton G, Recktenwald D et al (2006) Bead-based flow cytometric assays: a multiplex assay platform with applications in diagnostic microbiology. In: Tang Y-W, Stratton CW (eds) Advanced techniques in diagnostic microbiology. Springer US, New York, NY, pp 427–443
8. Lin Y-C, Sheng W-H, Chang S-C et al (2008) Application of a microsphere-based array for rapid identification of *Acinetobacter* spp. with distinct antimicrobial susceptibilities. J Clin Microbiol 46:612–617
9. Corrie SR, Feng Q, Blair T, Hawes SE, Kiviat NB, Trau M (2011) Multiplatform comparison of multiplexed bead arrays using HPV genotyping as a test case. Cytometry A 79A:713–719
10. Sewell WA, Smith SABC (2011) Polychromatic flow cytometry in the clinical laboratory. Pathology 43:580–591
11. Chau F, Lefort A, Benadda S, Dubee V & Fantin B (2011). Flow cytometry as a tool to determine the effects of cell wall-active antibiotics on vancomycin-susceptible and resistant *Enteroroccus faecalis*. *Antimicrob Agents Chemother* 55, A395–398
12. Jarzembowski T, Jaawik A, Wianiewska K, Witkowski J (2010) Flow cytometry approach study of *Enterococcus faecalis*; vancomycin resistance by detection of vancomycin@FL binding to the bacterial cells. Curr Microbiol 61:407–410
13. Karl S, Wong R, St Pierre T, Davis T (2009) A comparative study of a flow-cytometry-based assessment of in vitro Plasmodium falciparum drug sensitivity. Malaria J 8:294
14. Vale-Silva L, Pinto P, Lopes V, Ramos H & Pinto E (2012). Comparison of the Etest and a rapid flow cytometry-based method with the reference CLSI broth microdilution protocol M27-A3 for the echinocandin susceptibility testing of Candida spp. *European Journal of Clinical Microbiology & Infectious Diseases* 31, 941–946
15. Piuri M, Jacobs WR Jr, Hatfull GF (2009) Fluoromycobacteriophages for rapid, specific, and sensitive antibiotic susceptibility testing of *Mycobacterium tuberculosis*. PLoS One 4:e4870
16. Robotham JM, Nelson HB, Tang H (2009) Selection and characterization of drug-resistant HCV replicons in vitro with a flow cytometry-based assay. Methods Mol Biol 510:227–242

17. Shrestha NK, Scalera NM, Wilson DA, Brehm-Stecher B, Procop GW (2011) Rapid identification of *Staphylococcus aureus* and methicillin resistance by flow cytometry using a peptide nucleic acid probe. J Clin Microbiol 49:3383–3385
18. Jarzembowski T, Wianiewska K, Jazwik A, Bryl E, Witkowski J (2008) Flow cytometry as a rapid test for detection of penicillin resistance directly in bacterial cells in *Enterococcus faecalis* and *Staphylococcus aureus*. Curr Microbiol 57:167–169
19. Jarzembowski T, Wianiewska K, Jaawik A, Witkowski J (2009) Heterogeneity of methicillin-resistant *Staphylococcus aureus* strains (MRSA) characterized by flow cytometry. Curr Microbiol 59:78–80
20. Boehme CC, Nabeta P, Hillemann D et al (2010) Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med 363:1005–1015
21. Chin CD, Laksanasopin T, Cheung YK & other authors (2011). Microfluidics-based diagnostics of infectious diseases in the developing world. *Nat Med* 17, 1015–1019
22. Mittag A, Tárnok A (2009) Basics of standardization and calibration in cytometry – a review. J Biophotonics 2:470–481
23. Shapiro HM (2011) The cytometric future: it ain't necessarily flow! Methods Mol Biol (Clifton, NJ) 699:471
24. Jani IV, Janossy G, Brown DW, Mandy F (2002) Multiplexed immunoassays by flow cytometry for diagnosis and surveillance of infectious diseases in resource-poor settings. Lancet Infect Dis 2:243–250
25. Ateya D, Erickson J, Howell P, Hilliard L, Golden J, Ligler F (2008) The good, the bad, and the tiny: a review of microflow cytometry. Anal Bioanal Chem 391:1485–1498
26. Cheung KC, Di Berardino M, Schade-Kampmann G et al (2010) Microfluidic impedance-based flow cytometry. Cytometry A 77A:648–666
27. Lunde CS, Hartouni SR, Janc JW, Mammen M, Humphrey PP, Benton BM (2009) Telavancin disrupts the functional integrity of the bacterial membrane through targeted interaction with the cell wall precursor Lipid II. Antimicrob Agents Chemother 53:3375–3383
28. Pina-Vaz C, Costa-de-Oliveira S, Rodrigues AG (2005) Safe susceptibility testing of *Mycobacterium tuberculosis* by flow cytometry with the fluorescent nucleic acid stain SYTO 16. J Med Microbiol 54:77–81
29. Govender S, du Plessis SJ, van de Venter M, Hayes C (2010) Antibiotic susceptibility of multi-drug resistant *Mycobacterium tuberculosis* using flow cytometry. Medical Technology, SA
30. Fredricks BA, DeCoster DJ, Kim Y, Sparks N, Callister SM & Schell RF (2006). Rapid pyrazinamide susceptibility testing of Mycobacterium tuberculosis by flow cytometry. J Microbiol Methods 67, 266–272
31. Blanco J, Clotet-Codina I, Bosch B, Armand-Ugon M, Clotet B, Este JA (2005) Multiparametric assay to screen and dissect the mode of action of anti-human immunodeficiency virus envelope drugs. Antimicrob Agents Chemother 49:3926–3929
32. Covens K, Dekeersmaeker N, Schrooten Y et al (2009) Novel recombinant virus assay for measuring susceptibility of Human Immunodeficiency Virus Type 1 Group M subtypes to clinically approved drugs. J Clin Microbiol 47:2232–2242
33. Lee G-C, Lee D-G, Choi S-M et al (2005) Use of time-saving flow cytometry for rapid determination of resistance of Human Cytomegalovirus to ganciclovir. J Clin Microbiol 43:5003–5008
34. Sarkar A, Mandal G, Singh N, Sundar S, Chatterjee M (2009) Flow cytometric determination of intracellular non-protein thiols in *Leishmania promastigotes* using 5-chloromethyl fluorescein diacetate. Exp Parasitol 122:299–305
35. Peatey CL, Skinner-Adams TS, Dixon MWA, McCarthy JS, Gardiner DL, Trenholme KR (2009) Effect of antimalarial drugs on *Plasmodium falciparum* gametocytes. J Infect Dis 200:1518–1521
36. Buchholz K, Burke TA, Williamson KC, Wiegand RC, Wirth DF, Marti M (2011) A high-throughput screen targeting malaria transmission stages opens new avenues for drug development. J Infect Dis 203:1445–1453

37. Witkowski B, Lelievre J, Lopez Barragan MJ et al (2010) Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. Antimicrob Agents Chemother 54:1872–1877
38. Grimberg BT, Jaworska MM, Hough LB, Zimmerman PA, Phillips JG (2009) Addressing the malaria drug resistance challenge using flow cytometry to discover new antimalarials. Bioorg Med Chem Lett 19:5452–5457
39. Vandeputte P, Larcher G, Berges T, Renier G, Chabasse D, Bouchara J-P (2005) Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. Antimicrob Agents Chemother 49:4608–4615

Chapter 6
Biochemical Profile-Based Microbial Identification Systems

Nyasha O. Bullock and Jaber Aslanzadeh

Introduction

The first step in microbial identification is the phenotypic assessment of the growing colony. In many cases, colonial morphology such as color, shape, size, hemolytic reaction, and growth characteristics on various selective and differential media can place an organism in a single family, genus, or even species level. In fact, assessing the ability of an organism to grow on various laboratory media along with its oxygen requirements coupled with Gram stain morphology as well as a few rapid tests such as catalase, oxidase, coagulase, and indole often provides preliminary identification for many clinically significant isolates. For example, it is very likely that an organism that grows on MacConkey agar plate and ferments lactose is a member of the family *Enterobacteriaceae* or an oxidase-positive non-lactose fermenting Gram-negative rod that has distinct grape odor is likely to be *Pseudomonas aeruginosa.*

Overall the biochemical identification tests may be classified into two major groups: the conventional microbial identification systems and commercial microbial identification systems. The identification schemes used by various laboratories are not uniform in part due to the availability of numerous choices, varied complexity of the testing laboratories, volume, experience of technical staff, and cost. In general most laboratories rely on a combination of both conventional and commercial identification systems.

N.O. Bullock
Department of Pathology, Hartford Hospital and Clinical Laboratory partners, Hartford, CT 06102, USA

J. Aslanzadeh (✉)
Department of Pathology, Hartford Hospital and Clinical Laboratory partners, Hartford, CT 06102, USA

Division of Clinical Microbiology, Hartford Hospital and Clinical Lab. Partners, Hartford, CT 06102, USA
e-mail: Jaslanz@harthosp.org

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Conventional Microbial Identification Systems

Single Enzyme Rapid Tests

The single enzyme rapid tests are a group of tests that detect the presence or absence of a single enzyme or a biochemical reaction within seconds to minutes. These tests are fairly inexpensive, easy to perform, and often provide important initial information that is used to determine the subsequent steps in the microbial identification scheme. Rapid enzyme tests are an important part of both conventional as well as commercial microbial identification systems. In addition, these tests may be used for presumptive identification of certain organisms to the genus or even species level. For example, a positive catalase test can establish that a Gram-positive coccus is a staphylococcus; a subsequent positive coagulase test can then establish that the catalase-positive coccus is *Staphylococcus aureus.*

Catalase Test

Catalase, an enzyme within the cytochrome enzyme system, is responsible for the decomposition of hydrogen peroxide (H_2O_2) formed during aerobic respiration. All organisms using the cytochrome system of respiration will give a positive catalase reaction when tested. Those organisms using a different system will not produce catalase and will yield a negative reaction.

The mechanism of action is as follows:

$$H_2O_2 + \text{catalase} = H_2O + 1/2O_2$$

The possession of the catalase enzyme helps to distinguish staphylococci from streptococci and is useful in the identification of many other bacteria. A positive test is a rapid bubbling reaction caused by the release of O_2 from the H_2O_2 in the presence of catalase. A negative test is the absence of bubbling.

Despite the simplicity of the test, a false positive reaction is seen if the test is performed on colonies selected from blood agar plate (BAP); colonies that are selected from the first quadrant of a blood culture plate, or use of nickel loops to select colonies [1, 2].

Oxidase Test

The oxidase test is based on the production of the enzyme indophenol oxidase by organisms containing cytochrome *C*. Indophenol oxidase, in the presence of atmospheric oxygen, oxidizes a redox dye (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride) to form a dark-purple compound, indophenol.

Filter paper impregnated with the reagent is allowed to dry completely; the test is done by placing a loopful of bacteria from a nonselective plate onto the paper using a platinum loop or wooden applicator stick and then examining the paper for development of a violet or purple color immediately or up to 30s (positive reaction). No color change or a slight color change of a light pink/light purple after 30s indicates a negative result.

Steel loops, nichrome loops, or wire loops containing iron may give a false-positive reaction and reactions from weak oxidase-positive organisms, e.g., *Pasteurella* may be inaccurate. Colonies growing on selective media or differential media can carry over the indicator and thus cause inaccurate results. Colonies grown on media containing high glucose concentration cannot be used for oxidase determination, since fermentation inhibits indophenol oxidase activity resulting in false negative results [1, 3].

Spot Indole Test

The indole test is based on the ability of an organism to hydrolyze tryptophan to glycine and indole. Certain organisms are able to remove the glycine radical from tryptophan resulting in the production of indole. This test can be performed on organisms grown on a BAP after a 24-h incubation.

Filter paper is placed in a Petri plate and saturated with 3–4 drops of 1 % solution of *p*-dimethylaminocinnamaldehyde. An isolated colony from a 24-h-old culture grown on a BAP is rubbed into the filter paper using a wooden applicator stick or inoculating loop. Appearance of a blue color immediately or within 30 s of inoculation indicates a positive reaction; no blue color seen within 30 s indicates negative reaction.

The test must be performed from BAP. False negative results will occur from MacConkey agar and Triple Sugar Iron (TSI) slants since there is no sufficient source of tryptophan in these media. False positives will occur if indole-positive organisms are present in mixed cultures [4].

Slide Coagulase Test

Coagulase is a thermostable enzyme found primarily in *S. aureus* and is used to differentiate *S. aureus* from other commonly isolated staphylococci. Two forms of coagulase exist: one is bound to the bacterial cell wall, and one is liberated by the cell and is known as “free coagulase.” The slide coagulase test detects the bound coagulase (clumping factor), which acts directly on the fibrinogen in plasma and causes clumping of bacteria. Slide coagulase test results agree approximately 96 % with tube coagulase test results. Coagulase-positive organisms form clumps within 10 s but coagulase-negative organisms remain uniformly suspended.

The test is done as follows: Using a sterile pipette, a drop of sterile saline is placed on a glass slide. One to two colonies of the organism are emulsified in the

saline and tested for autoagglutination. A drop of rabbit plasma is placed on the slide and mixed for a few seconds and observe for clumping within 10 s.

A positive slide coagulase test result is valid only for strains of *Staphylococcus* sp. that are negative for autoagglutination or stickiness. Coagulase is also present in *S. intermedius* and *S. hyicus*, but these species are infrequent clinical isolates. Similarly, clumping factor is produced by *S. schleiferi* and *S. lugdunensis* and may give false positive reactions [1, 5].

Note: All negative slide tests must be confirmed using tube test.

Microdase

The Microdase disk is a reagent-impregnated disk used in the differentiation of *Staphylococcus* from *Micrococcus* by the detection of the oxidase enzyme. In the presence of atmospheric oxygen, the oxidase enzyme reacts with tetramethyl-*p*-phenylenediamine (TMPD) in the disk and cytochrome *C* in the organism to form a colored compound. All micrococci contain cytochrome *C*, whereas most staphylococci lack cytochrome *C*. The oxidase reagent substantiates the presence of type C cytochrome.

The Microdase disk is placed on a glass slide and inoculated with several colonies of an 18–24-h pure culture grown on BAP. The disk is examined for up to 2 min for development of a blue color to purple-blue (positive reaction). No color change or white to gray color after 2 min is considered a negative reaction.

Microdase is not designed for routine testing for oxidase activity in organisms other than *Staphylococcu*s and *Micrococcus*. *S. sciuri* is the only *Staphylococcus* species recognized to give a positive Microdase reaction [4, 6].

Bile Solubility Test

Gross morphology alone is often insufficient to differentiate between *Streptococcus pneumoniae* and alpha hemolytic streptococci spp. *S. pneumoniae* lyse when treated with a 10 % solution of sodium desoxycholate, while other streptococci and Gram-positive cocci are not bile soluble. Lysis occurs because bile-soluble organisms contain autolytic enzyme, an amidase that when activated by bile salts cleaves the bond between alanine and muramic acid in the cell wall.

One drop of desoxycholate is placed on a well-isolated 18–24-h culture of an alpha-hemolytic colony on a BAP and incubated at room temperature, agar side down for 15–30 min. The area where the reagent was applied is examined for evidence of colony disintegration or lysis. Dissolving of the colony is a positive test for *S. pneumoniae*. Colonies remaining intact indicates a negative test for *S. pneumoniae*.

False negative may occur when testing isolates older than 18–24 h. Occasionally, alpha-hemolytic colonies do not dissolve but merely lift off the surface of the agar, float away, and settle elsewhere on the plate. The plate should carefully be examined for evidence of this [5, 7].

PYR

PYR is a chromogenic substrate (L-pyrrolidonyl-β-naphthylamide or PYR) which when hydrolyzed by PYRase (L-pyrroglutamyl aminopeptidase) produces a red color upon the addition of *p*-dimethylaminocinnamaldehyde. PYR is a substrate that is hydrolyzed by 100 % of the enterococci and group A streptococci, but not by any other streptococcal species.

Two to four drops of a buffer reagent is applied to the PYR test strip circle. (Do not flood the disk.) The strip is then inoculated with 3–5 colonies of the organism (culture grown on BAP; 18–24 h old) and incubated at room temperature for 2 min. Two drops of *p*-dimethylaminocinnamaldehyde is applied to the test strip circle. An intense red color develops immediately around the colonies in the presence of hydrolyzed PYR. The PYR test is negative if no color, an orange color, or a weak pink color develops. Staphylococci may cause a positive PYR reaction [4–6].

Leucine Aminopeptidase Test

The leucine aminopeptidase (LAP) Test is a rapid assay for the detection of the enzyme leucine aminopeptidase in bacteria cultured on laboratory media. It is used as one of the tests for the presumptive identification of catalase-negative Gram-positive cocci. Leucine-β-naphthylamide impregnated disks serve as a substrate for the detection of leucine aminopeptidase. Following hydrolysis of the substrate by the enzyme, the resulting β-naphthylamine produces a red color upon the addition of cinnamaldehyde reagent.

A moistened LAP disk is placed on a glass slide or in a Petri dish and inoculated with isolated colonies of catalase-negative, Gram-positive cocci. The disk is incubated at room temperature for 5 min before a drop of the color developer is added and examined for up to 1 min for pink to red color development. Pink/red color indicates positive reaction. No color change/slight yellow indicates negative reaction.

The LAP test is only part of the overall scheme for identifying catalase-negative, Gram-positive cocci. Further biochemical characterization and serological grouping may be necessary for specific identification. False negatives may result from using too small an inoculum [8].

MUG Test

The MUG test is a rapid test used for the presumptive identification of *Escherichia coli*. *E. coli* produces the enzyme β-D-glucuronidase which hydrolyzes β-D-glucopyranoside-uronic derivatives to aglycons and D-glucuronic acid. The substrate 4-methylumbelliferyl-β-D-glucuronide is impregnated into a disk which, when hydrolyzed by the enzyme, yields the 4-methylumbelliferyl moiety which fluoresces blue under UV light.

Moisten a disk with water and with a wooden applicator stick, rub a portion of a colony from a pure culture onto the disk. Incubate in a close container at 35 °C for 2 h. Observe the disk under long-wavelength (366 nm) UV light. An electric blue fluorescence is a positive reaction [1].

Indoxyl Butyrate Disk

Moraxella catarrhalis produces the enzyme butyrate esterase. This property can be used as a rapid test in the identification of *M. catarrhalis*. Indoxyl is liberated from indoxyl butyrate by the enzyme butyrate esterase, forming an indigo color in the presence of oxygen.

Smear several colonies of oxidase-positive, Gram-negative diplococci across the disk surface using a loop or wooden applicator. Incubate at room temperature for 5 min and observe for a blue-green color development where the colonies were applied indicating a positive test for butyrate esterase production. A negative reaction is indicated by no color change.

Interpretation of results is based on testing only oxidase-positive, Gram-negative diplococci. Some strains of *Moraxella* sp. other than *M. catarrhalis* may produce a positive or weak positive reaction. *Acinetobacter*, *Staphylococcus*, and *Pseudomonas* may also yield a positive reaction [5, 6].

Chromogenic Enzyme Substrate Test

Chromogenic Enzyme Substrate Test is used for rapid identification of the different *Neisseria* species detected by colored end product after hydrolysis by bacterial enzymes. The two commercially available systems that use this approach include the Gonochek II and the Bacticard *Neisseria*.

Gonocheck II

The Gonocheck II consists of three synthetic chromogenic substrates contained in a single tube to detect preformed enzymes associated with different *Neisseria* species.

Oxidase-positive, Gram-negative diplococci from a pure culture growing on Martin-Lewis agar is emulsified in the tube with a wooden applicator. The tube is capped with a stopper and incubated for 30 min at 35 °C. Specific color reactions confirm the identity of *N. lactamica* (blue) and *N. meningitidis* (yellow). If neither color develops, the stopper is split apart and the top part is inserted into the tube. The tube is inverted so that the suspension comes in contact with the diazo dye coupler (*o*-aminoazotoluene diazonium) on the stopper. Development of a pink-red color indicates that the isolate is *N. gonorrhoeae*; absence of a colored product, or a pale yellow color, is presumptive for *M. catarrhalis*. The identification of

M. catarrhalis can be confirmed by a positive *M. catarrhalis* butyrate test. The active chemical ingredients used in the tube and the enzymatic reactions detected are the following:

(a) 5-Bromo-4-chloro-indoyl-β-D-galactopyranoside. Hydrolysis of the β-D-galactoside bond by β-galactosidase yields a blue color from the colorless substrate.
(b) Gamma-glutamyl-*para*-nitroanilide. Hydrolysis of this substrate by gamma-glutamyl aminopeptidase releases yellow *p*-nitroaniline from the colorless substrate.
(c) L-Proline-beta-naphthylamide. Hydrolysis of this substrate by hydroxylprolyl-aminopeptidase releases colorless free beta-naphthylamine derivative. Coupling of the beta-naphthylamine derivative with a diazo dye coupler (*o*-aminoazotoluene diazonium salt—Fast Garnet, GBC Salt) produces a pink to red color.

The Gonocheck II should be used on Gram-negative diplococci isolated from media such as Martin-Lewis agar. Do not use on isolates only grown on nonselective media such as chocolate agar since other *Neisseria* species (*N. sicca*, *N. mucosa*) may grow and lead to incorrect results. Similarly, *Kingella* species may be found on Martin-Lewis medium. It is essential to perform a Gram stain prior to selecting organisms for identification by NET. If the morphology of the organism selected is questionable, it is suggested that a catalase test be performed. *Kingella* species are catalase negative, and *Neisseria* and *Moraxella* species are catalase positive. *N. cinerea* will be pink after the addition of PRO reagent [9].

Bacticard *Neisseria*

This test uses an identification strip that contains the four chromogenic enzyme substrate test for the identification of the different *Neisseria* species. The four test circles are rehydrated with buffer solution, and growth from a selective media is applied to each of the four test circles area. If a blue-green color develops in the IB (butyrate esterase) within 2 min, the organism is identified as *M. catarrhalis*. If no color develops, the strip is incubated for another 13 min. If a blue-green color develops in the BGAL (β-galactosidase) the organism is *N. lactamica*. If the strip still remains colorless in that time, a single drop of color-developing reagent is added to the PRO (prolyl aminopeptidase) and GLUT (gamma glutamyl aminopeptidase) test area. The development of a red color in the PRO test area identifies the isolate as *N. gonorrhea* while similar color change in the GLUT test area identifies the organism as *N. meningitidis*.

Hippurate

The Hippurate hydrolysis test may be used to identify *Campylobacter jejuni*, *Gardnerella vaginalis*, and *Listeria monocytogenes* or differentiate *Streptococcus agalactiae* from other beta hemolytic streptococci. The assay is based on hydrolysis

of the sodium hippurate by the enzyme hippuricase to sodium benzoate and glycine. Glycine is detected by oxidation with ninhydrin reagent that results in production of a deep purple color.

Hippurate tubes are inoculated with a heavy suspension of the organism or a hippurate disk could be added to the suspension and incubated at 35 °C for 2 h. The tube is than inoculated with 0.2 ml of ninhydrin and re-incubated for additional 15–30 min. The presence of deep purple color indicates a positive hippurate and no color change indicates negative hippurate. A light inoculum or use of old culture may give false negative results [4, 6].

Lysostaphin

The Lysostaphin test is used to differentiate members of *Staphylococcus* sp. from *Micrococcus* sp. and is based on the activity of Lysostaphin as an endopeptidase, which cleaves the glycine-rich pentapeptide bridges of peptidoglycan. These cross bridges are found in all *Staphylococcus* sp. but not in *Micrococcus* sp. or *Stomatococcus* sp.

A suspension of the organism equivalent to a 3.0 McFarland is prepared and 0.2 ml of the working Lysostaphin solution is added to the tube and mixed. The tube is allowed to stand undisturbed for 2 h at 35 °C. Clearing of the solution indicates susceptibility to lysostaphin. Turbid Solution indicates resistance to lysostaphin. *Micrococcus*, *Stomatococcus*, and *Streptococcus* spp. are resistant to lysostaphin. Reading the test beyond the 2-h incubation may result in false positive tests. Lysostaphin susceptibility can also be determined using the disk diffusion method. A plate of Mueller Hinton agar is inoculated and a lysostaphin disk (10 μg) is placed on the plate. The plate is incubated for 24 h at 35 °C. A zone of inhibition of 10–16 mm indicates a *Staphylococcus* species. Micrococcus and related species will show no zones.

To obtain optimal result the organism must be grown in media containing beef-peptone rather than casein-peptone as the glycine content of the media is crucial [1].

CLO Test

The CLO test is a rapid test for identification of *Helicobacter pylori*. The test uses a sealed plastic slide holding an agar gel that contains urea, phenol red, buffers, and bacteriostatic agents. If the urease enzyme of *H. pylori* is present in the inserted gastric tissue biopsy, the urea in the gel is degraded resulting in an increased pH, and the color of the gel changes from yellow to a bright magenta.

Inoculate the CLO test slide with the specimen and incubate at 37 °C in the non-CO_2 incubator for 3 h. The slide is examined for color change from yellow to magenta pink after 1 h of incubation and again at 2 and 3 h. A magenta pink color indicates positive reaction. If the biopsy contains urease, the change first appears around the sample and eventually colors all of the gel. The pH change in a positive test is first seen at the interface of the gel and the biopsy. If a significant amount of urease is

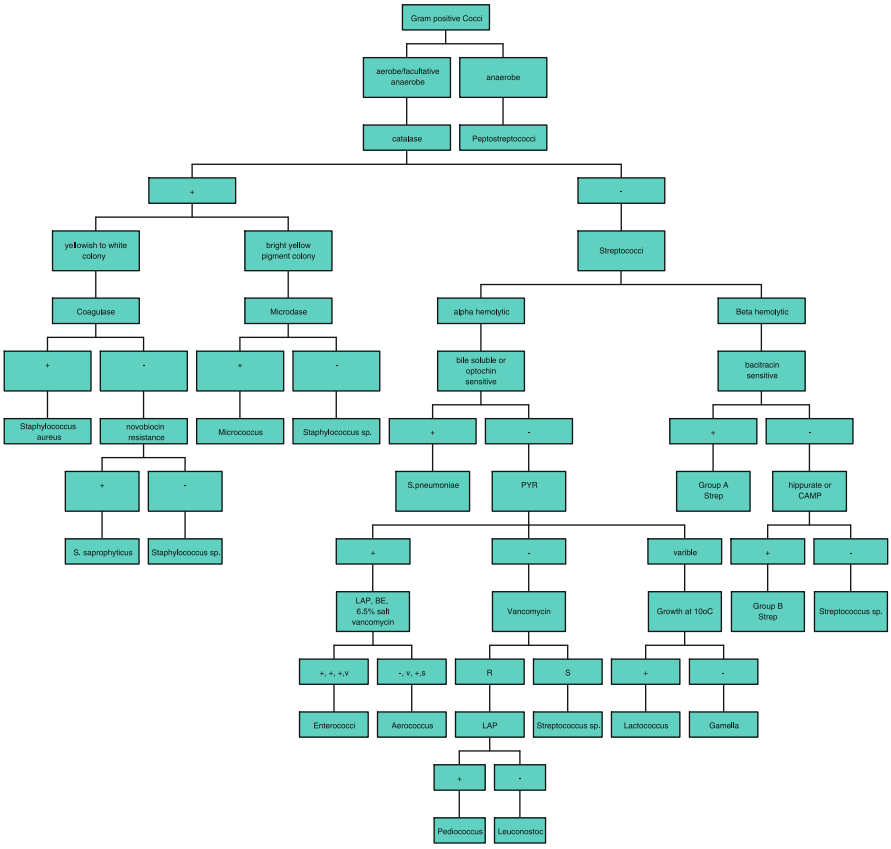

Fig. 6.1 Flowchart for presumptive identification of Gram-positive cocci

present, the visible change is rapid. Any color change of the whole gel to a shade other than yellow (i.e., red, magenta, pink, deep orange) indicates the presence of *H. pylori*. The test is considered negative if the medium remains yellow 24 h after insertion of the biopsy.

False negative CLO tests may occur when very low numbers of *H. pylori* are present or if the bacteria are focally distributed. False positive CLO tests can occur in patients with achlorhydria. This is because commensal organisms such as *Proteus* spp. that also produce urease will grow in the absence of acid [10] (Fig. 6.1–6.6).

Overnight Biochemical Tests

The overnight biochemical tests are a group of tests that require inoculating one or more culture media containing specific substrates and chemical indicators that

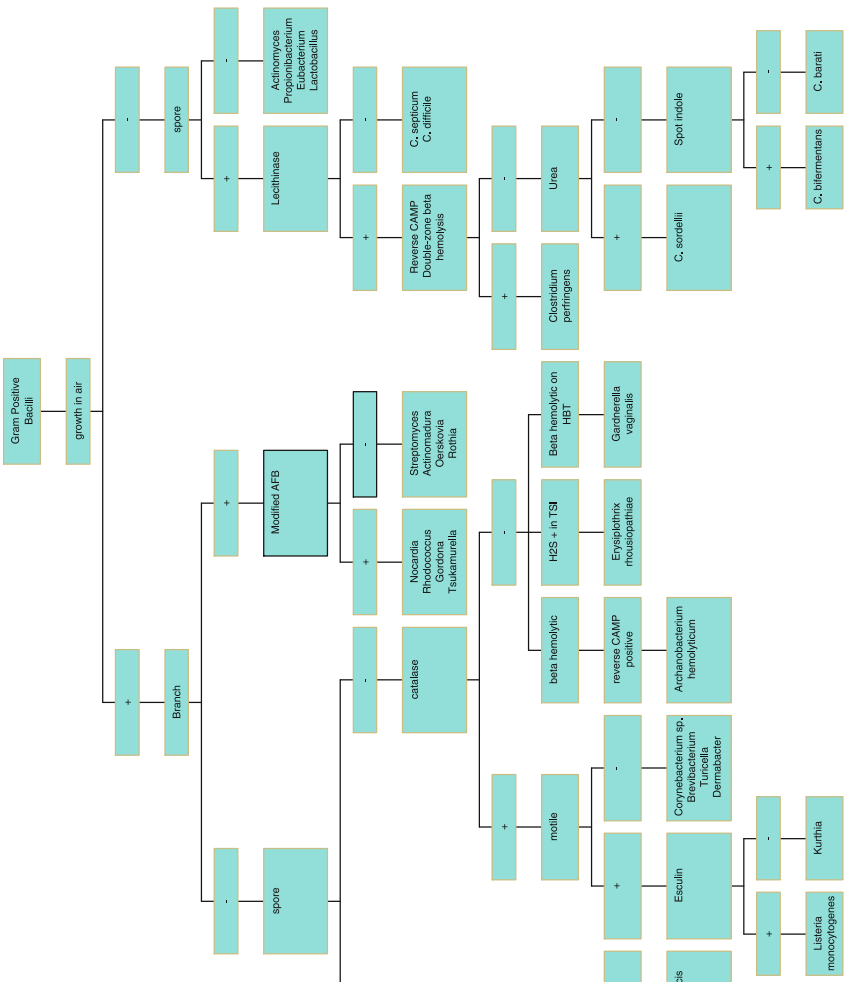

Fig. 6.2 Flowchart for presumptive identification of Gram-positive bacilli

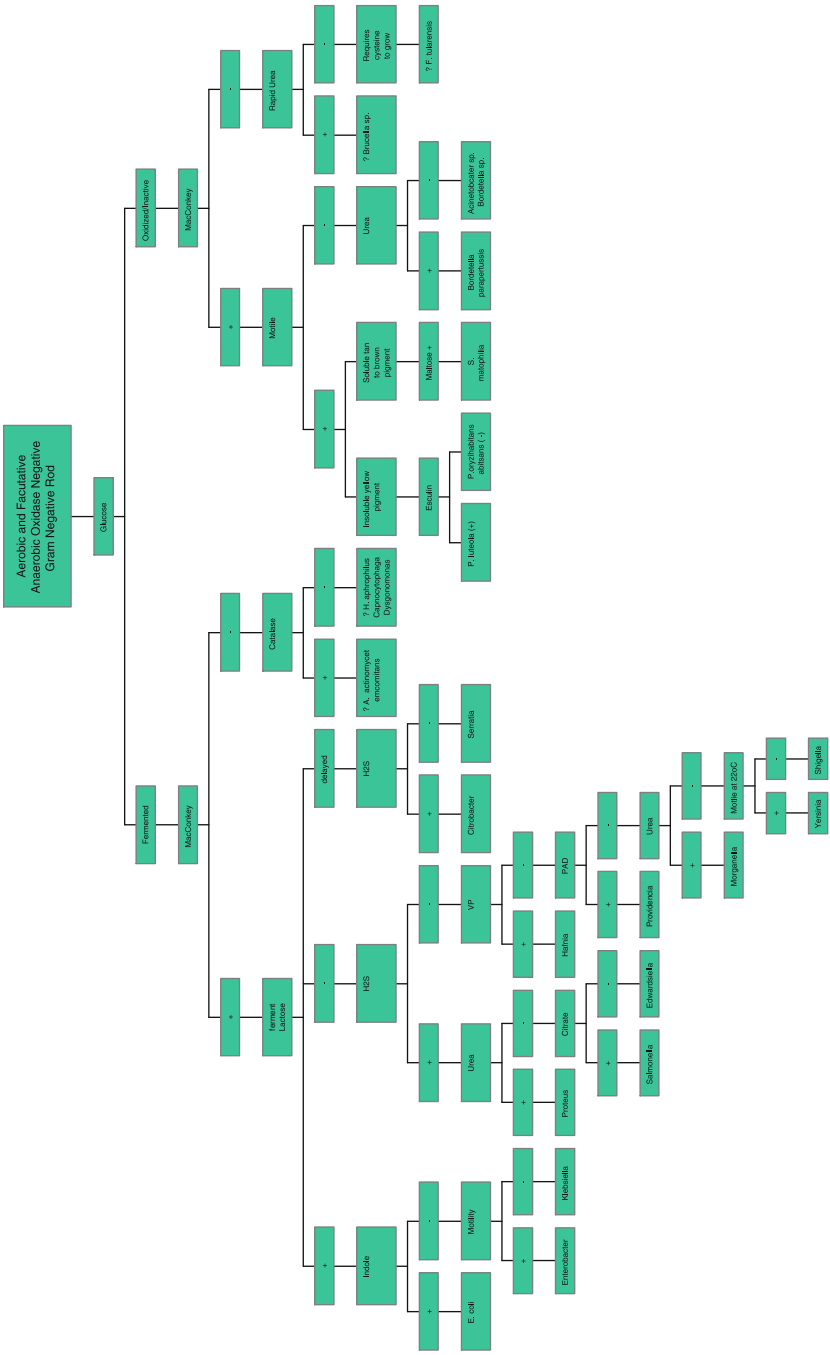

Fig. 6.3 Flowchart for presumptive identification of aerobic and facultative anaerobic oxidase-negative Gram-negative rod

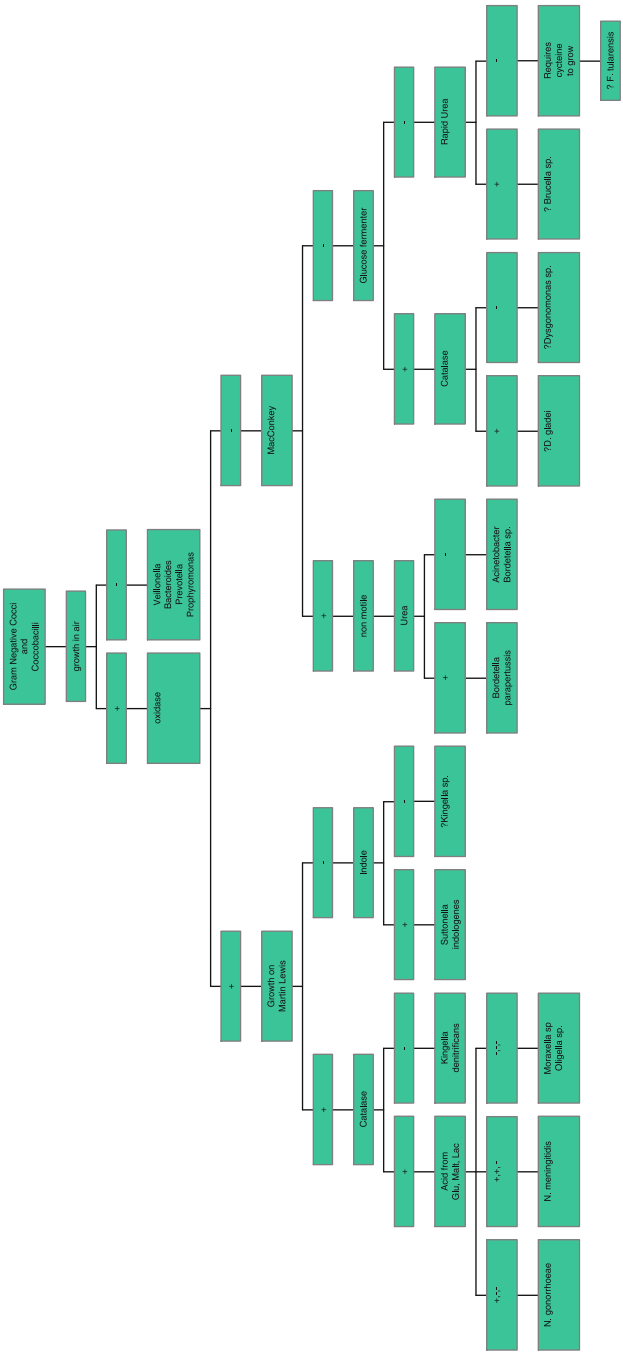

Fig. 6.4 Flowchart for presumptive identification of Gram-negative cocci and coccobacilli

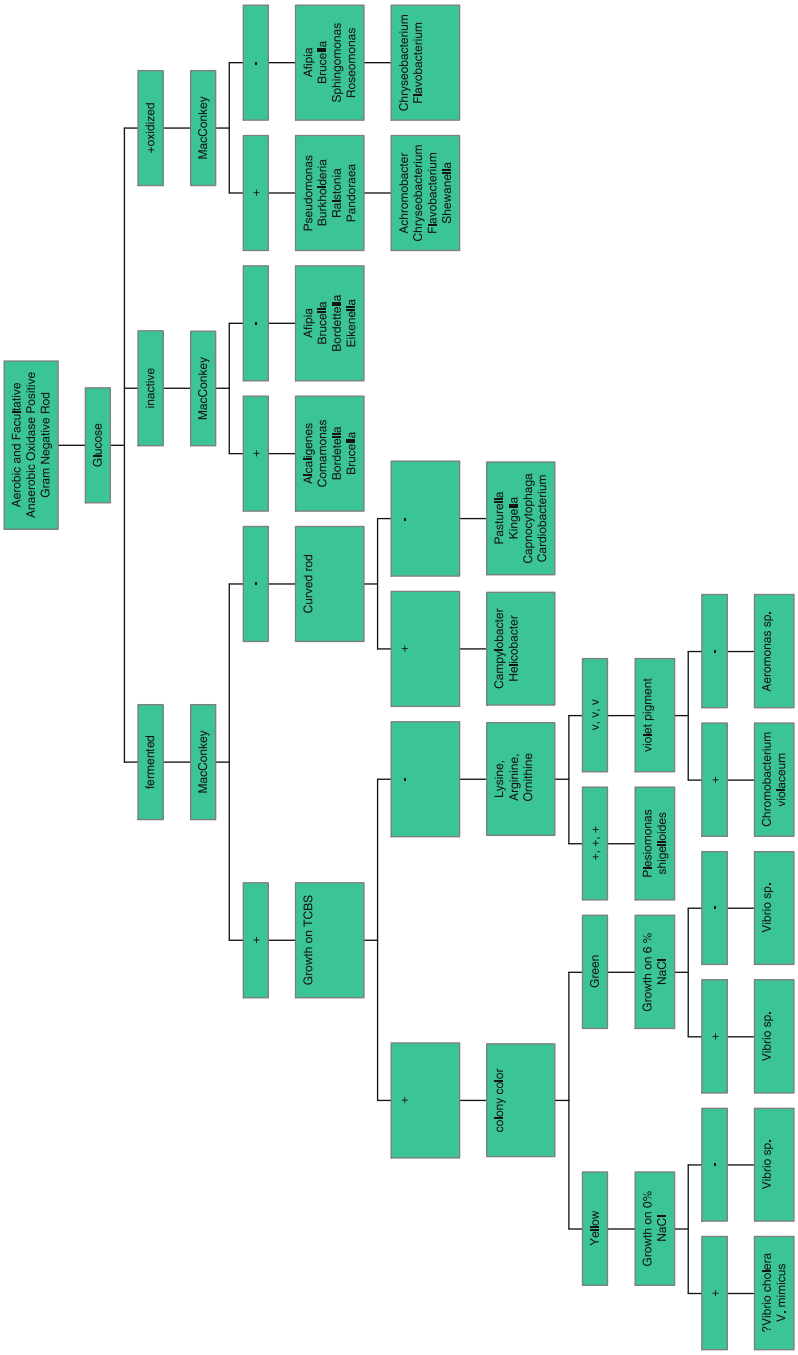

Fig. 6.5 Flowchart for presumptive identification of aerobic and facultative anaerobic oxidase-positive Gram-negative rod

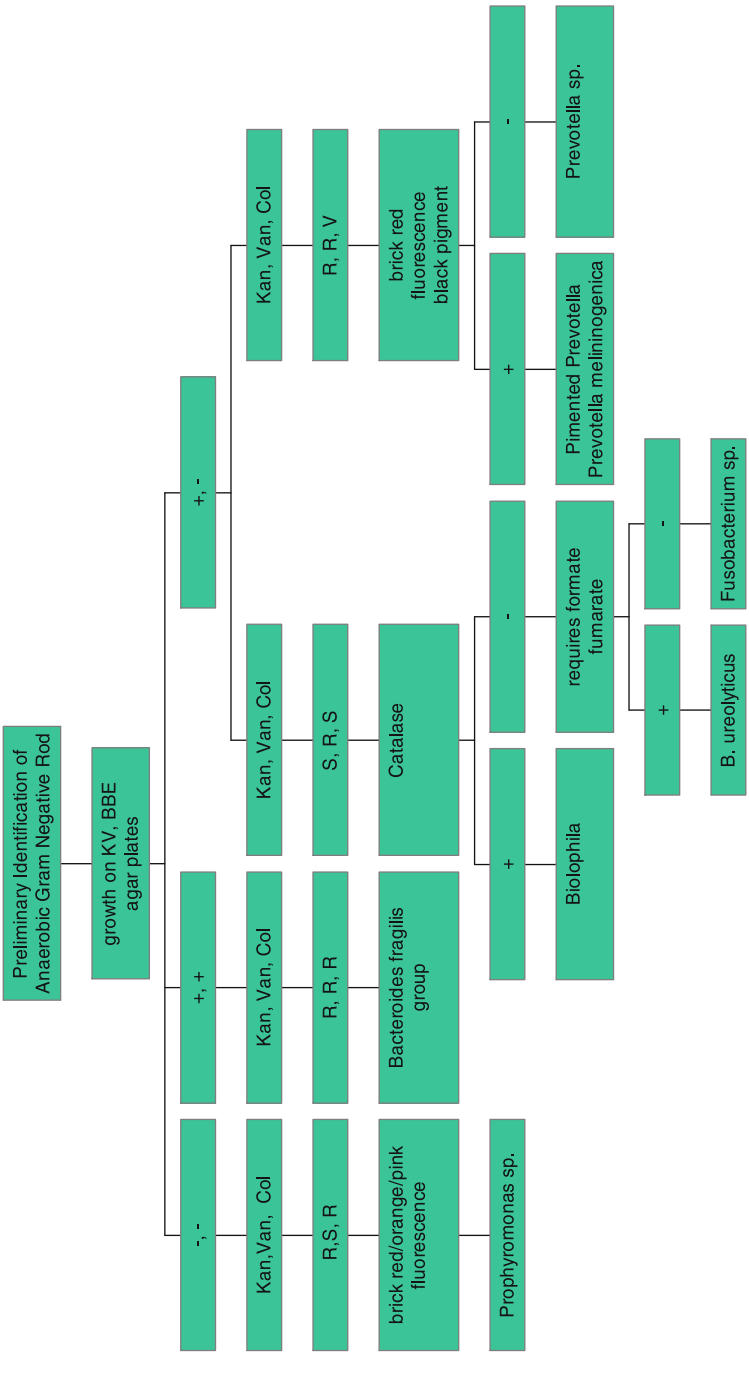

Fig. 6.6 Flowchart for presumptive identification of anaerobic Gram-negative rod

detect pH change or specific microbial by-products. Similar to rapid tests, the choice of overnight tests is based on Gram stain morphology and the results of preliminary testing with rapid enzyme tests. These tests are also inexpensive and easy to perform and may be used in three different ways. They may be used to obtain important initial information with respect to the identity of an unknown organism, such as the Motile-Indole-Lysine-Sulfide test, which is used to screen for the presence of enteric pathogens. They may be used to verify the result of a preliminary positive/negative test result or they may be used to assess an indeterminate finding. For example Taxo P is an overnight test that will demonstrate if an isolate with an equivocal bile solubility result is a *S. pneumoniae*. Similarly, a tube coagulase test will substantiate if a suspicious isolate that is slide coagulase negative is truly a coagulase-negative staphylococci. Finally these tests may be used as the sole identification system (classical biochemical identification) to identify an unknown organism. This is generally labor intensive and requires the technologist to inoculate, incubate, read, interpret, and chart a number of biochemical reactions over several days. This is then followed by using various identification schemes or flowcharts to generate final identification. As a rule, the classical biochemical identification system is used to identify fastidious or slow-growing organisms in the reference laboratories. These isolates are by and large rare biotypes that are not part of the commercial identification system's database. Table 6.1 depicts the list of biochemical tests that are commonly used to identify Gram-negative bacilli [11].

Tube Coagulase Test

The Tube coagulase test detects free coagulase (liberated by the cell) that forms a complex with coagulase-reacting factor found in plasma. The complex reacts with fibrinogen to form a fibrin clot.

Several colonies of staphylococcus are emulsified in 0.5 ml of rabbit plasma (with EDTA) to give a milky suspension and then incubated at 35 °C for 4 h. Examine for the presence of a clot. If negative for clot, re-incubate the tube and reexamine at 24 h. Any degree of clot formation at 4 or 24 h is considered positive reaction. No clot formation at 24 h is considered negative coagulase reaction [1, 4, 6].

DNA Hydrolysis

The DNA Hydrolysis test detects the presence of enzyme, Deoxyribonuclease (DNase), in an organism. Using this media, DNase-positive Coagulase-positive staphylococci are differentiated from other *Staphylococcus* sp. The media contains either toluidine blue or methyl green which upon hydrolysis of the incorporated DNA turns colorless if methyl green is used in the media or pink if toluidine blue is used instead.

The media is inoculated with the organism (generally, the organisms are boiled as some *S. epi* have DNase, but the DNase is not heat stable) and incubated overnight

Table 6.1 Commonly used biochemical test for identification of a Gram-negative organism

Date	1	2	3	4	5	6	7
Biochemicals							
Motility							
OF glucose (oxid)							
Of glucose (Ferm)							
Xylose							
Mannitol							
Lactose							
Suctose							
Maltose							
Catalase							
Oxidase							
MacConkey							
Citrate							
Sodium acetate							
Urea							
Nitrate							
Nitrate to gas							
Indole							
TSI slant							
TSI butt							
H_2S (TSI butt)							
H_2S (Pb ac paper)							
Gelatin							
Pigment							
Arginine							
Lysine							
Growth at 42 °C							

at 35 °C. The plate is examined for evidence of growth and loss of color or a pink color around the inoculum (positive reaction). No color change indicates negative reaction [6].

Vancomycin Disk Test

The vancomycin disk test is performed as a susceptibility procedure to help differentiate the Gram-positive, catalase-negative cocci. *Aerococcus, Gemella, Lactococcus, Streptococcus,* and some enterococci are susceptible to vancomycin. *Leuconostoc*, *Pediococcus*, *Lactobacillus,* and some enterococci are resistant to vancomycin.

A 0.5 McFarland suspension of the organism is prepared in sterile saline. Using a sterile swab, the bacterial suspension is inoculated onto a BAP. A vancomycin disk is placed in the center of the inoculated plate and incubated at 35 °C in a CO_2 incubator for 18–24 h. The plate is observed for the presence of a zone of inhibition

around the vancomycin disk. *Leuconostoc* sp., *Pediococcus* sp., *Lactobacillus* sp., and some *Enterococcus* sp. are resistant to vancomycin with growth to the edge of the disk ≤9 mm. *Aerococcus* sp., *Gemella* sp., *Lactococcus* sp., *Streptococcus* sp., and some *Enterococcus* sp. are susceptible to vancomycin and produce a zone of inhibition ≥12 mm [1, 6].

Bacitracin Inhibition Test (Taxo A Disk)

The Bacitracin Inhibition Test presumptively differentiates *Streptococcus pyogenes*, Group A streptococci, from other beta hemolytic streptococci. The bacitracin at concentration of 0.04 units will selectively inhibit growth of GAS. While there are rare strains of GAS that are bacitracin resistant, approximately 5–10 % of strains of non-group A beta hemolytic streptococci (C, F, and G) are bacitracin susceptible.

Using a pure culture of the test organism inoculate a BAP with the bacterial suspension. Using a sterile forceps, place a bacitracin disk in the first quadrant (the area of heaviest growth of the inoculated BAP) and incubate at 35 °C for 18–24 h. Any zone of inhibition around the bacitracin disk is considered positive test. Uniform lawn of growth right up to the rim of the disk indicates negative bacitracin inhibition test [1, 5, 6].

Bacitracin and STX Susceptibility Test

Susceptibility to low concentrations of bacitracin and sulfonamide trimethoprim–sulfamethoxazole (SXT) is a relatively inexpensive method for the presumptive identification of both Group A and Group B β-hemolytic streptococci. Group A streptococci are susceptible to bacitracin but resistant to SXT and Group B streptococci are resistant to both antibiotics. Other β-hemolytic streptococci may show varying susceptibility to bacitracin but are also susceptible to STX, so the combination increases the sensitivity and predictive value of the bacitracin test.

Streak three or four isolated colonies of β-hemolytic streptococci down the center of a BAP. Using a loop, spread the inoculum across the plate to create a confluent lawn. Place a Taxo A bacitracin disk and an SXT disk on the inoculated area. Incubate the plate in ambient air at 35 °C for 18–24 h. Susceptibility is defined as any zone around either disk while resistant is growth up to the edge of the disk [1].

Taxo P Disks (Optochin)

Ethylhydrocupreine hydrochloride (optochin) at the concentration 5.0 μg or less selectively inhibits the growth of *S. pneumoniae*, but not of other streptococci. *S. pneumoniae* may, therefore, be differentiated from other alpha-hemolytic streptococci by the formation of a zone of inhibition around a disk impregnated with this compound.

Three or four well-isolated colonies of alpha-hemolytic *Streptococcus* isolate are streaked onto one half of a BAP plate. Using a flamed forceps, place a Taxo P disk (optochin) firmly in the upper one-third of the streaked areas and incubate the plate aerobically at 35 °C for 24 h in 5–7 % CO_2. If using a 6 mm disk, a zone of inhibition of 14 mm or greater is considered sensitive. With a 10 mm disk, a zone of 16 mm or greater is sensitive. Other organisms may show zone sizes less than 14 mm in diameter. A diameter between 6 and 14 mm is questionable for *S. pneumoniae* and the strain should be tested for bile solubility [6].

CAMP Test

The CAMP Test is based on the fact that group B streptococci produce an extracellular protein known as the CAMP factor that acts synergistically with a staphylococcal beta-hemolysin (β-Lysin) on sheep erythrocytes to produce an enhanced zone of hemolysis.

Streak a loopful of β toxin-producing *S. aureus* in a straight line across the center of a BAP. Streak a loopful of group B streptococci perpendicular to and nearly touching the streak line of the staphylococci (positive control). Streak a loopful of group A streptococci perpendicular to and nearly touching the streak line of the staphylococci (negative control). Streak a loopful of unknown isolate perpendicular to and nearly touching the streak line of the staphylococci and incubate the plate at 35 °C for 18–24 h in the aerobic non-CO_2 incubator. Following the incubation if the unknown isolate demonstrates an arrowhead zone of enhanced hemolysis, the isolate is identified as group B streptococci. If the unknown isolate does not demonstrate an arrowhead of enhanced hemolysis, the isolate is not a group B streptococci.

Do not incubate the CAMP test plate in the presence of 5–10 % CO_2 incubator as this can cause false-positive results with non-group B streptococcus spp.

Reverse CAMP Test

The Reverse CAMP Test is based on the fact that some organisms such as *Arcanobacterium haemolyticum* completely inhibit the effect of *S. aureus* B-hemolysin on sheep erythrocytes. The β-hemolysin inhibition zone in the form of a triangle is formed.

A loopful of β toxin-producing *S. aureus* is streaked in a straight line across the center of a BAP. Group B streptococci and group A streptococci are streaked perpendicular to and nearly touching the streak line of the staphylococci. Similarly, *A. haemolyticum* and the test isolate are streaked perpendicular to and nearly touching the line of the staphylococci. The plate is incubated at 35 °C for 24 h in the aerobic non-CO_2 incubator. Following the incubation if the test isolate demonstrates a triangular shaped inhibition of β-hemolysis, it is reverse camp test positive. If the test isolate does not demonstrate a triangle-shaped inhibition of β-hemolysis, it is reverse camp test negative.

Do not incubate the Reverse CAMP test plate in the 5–10 % CO_2 incubator. This may result in an incorrect interpretation [5, 12].

Bile Esculin Agar Slant

Group D streptococci (including *Enterococcus* spp.) and a few other bacteria, such as *Listeria* spp., can grow in the presence of 40 % bile and produce esculinase, which hydrolyze esculin to esculetin. Esculetin reacts with ferric ions, supplied by ferric citrate in the agar medium, to form a diffusible black complex. Most strains of viridans streptococci that are capable of hydrolyzing esculin will not grow in the presence of 40 % bile.

Streak the surface of the bile esculin agar slant with several colonies of the organism to be tested. Incubate at 35 °C in non-CO_2 for 24–48 h. A diffuse blackening of more than half of the slant within 24–48 h is considered positive. No growth or growth without blackening of the medium after 48 h is considered negative test.

If the inoculum is too heavy, viridans streptococci may give a false positive test result. Approximately 3 % of viridans streptococci are able to hydrolyze esculin in the presence of bile. Growth in the presence of 6.5 % salt is used to differentiate enterococci from non-enterococcal group D streptococci [1, 5].

6.5 % Salt Broth

Heart infusion broth is a general-purpose medium for the cultivation of both fastidious and non-fastidious organisms. With the addition of 6.5 % sodium chloride, the medium can be used to differentiate between salt-tolerant and salt-intolerant organisms. It is especially useful for distinguishing *Enterococcus* spp., which are salt tolerant, from non-enterococcal group D streptococci, such as *S. gallolyticus* (previously known as *S. bovis*) and *S. equinus*. This broth contains small amount of glucose and a bromcresol purple as an indicator of acid production.

Inoculate the tube containing 6.5 % sodium chloride with the organism and incubate at 35 °C in non-CO_2 for 24–48 h. A visible turbidity with or without color change is considered positive and no growth or color change is considered negative.

If the medium is inoculated too heavily, the inoculum may be interpreted as growth, resulting in a false positive reaction. *Aerococci*, *Pediococci*, *Staphylococci*, and up to 80 % of group B *Streptococci* can grow in 6.5 % salt broth. In addition, *Aerococci* may also be bile esculin positive [1, 5].

Indole Test

Indole, a benzyl pyrrole, is one of the metabolic degradation products of the amino acid tryptophan. Bacteria that possess the enzyme tryptophanase are capable of hydrolyzing and deaminating tryptophan with the production of indole, pyruvic acid,

and ammonia. The indole test is based on the formation of a red color complex when indole reacts with the aldehyde group of *p*-dimethylaminobenzaldehyde, the active chemical in Kovac's or Ehrlich's reagent. In order to perform this test, the organism must be grown on a medium rich in tryptophan such as indole nitrate broth.

Inoculate the Indole Nitrate broth medium with 2–3 colonies of the organism to be tested. Incubate the tubes at 35 °C in a non-CO_2 incubator for 18–24 h. Examine the tubes for growth. When the broth is visibly turbid, using a sterile pipette, transfer 3 ml into a sterile tube. Add 0.5 ml of Kovac's reagent to tube and observe for the development of a bright fuscia red color at the interface between the reagent and the broth. If Ehrlich's reagent is used instead, add 1 ml of xylene to the contents of the tube, which extracts the indole, if present, from the broth into the xylene. Wait for 1–2 min, and add 0.5 ml Ehrlich's reagent and observe for the production of a pink to red color in the xylene layer. A pink to red color change after addition of either reagent indicates positive reaction. No color change indicates negative reaction [1].

Nitrite Test

Organisms that reduce nitrate have the ability to extract oxygen from nitrates to form nitrites and other reduction products. The presence of nitrites in the medium is detected by the formation of a red diazonium dye, *p*-sulfobenzeneazo-α-naphthylamine, following the addition of α-naphthylamine and sulfanilic acid. If no color develops after adding the reagents, this indicates that nitrates have not been reduced (a true negative reaction) or that they have been reduced beyond the oxidation level of nitrite to products such as ammonia, nitrogen gas (denitrification), nitric oxide (NO), or nitrous oxide (N_2O) and hydroxylamine. Since the test reagents detect only nitrites, the latter process would lead to a false-negative result. Therefore, it is necessary to add a small amount of zinc dust to all negative reactions. Because zinc ions reduce nitrates to nitrites, the development of a red color after adding zinc dust indicates the presence of nitrates and confirms a true negative reaction.

Using a sterile inoculating loop, an Indole Nitrate Broth medium is inoculated with 2–3 colonies of the organism to be tested and incubated at 35 °C in a non-CO_2 incubator for 18–24 h. When the broth is visibly turbid 3 ml of the broth culture is transferred into a sterile tube and 1 ml of α-naphthylamine (Nitrate Reagent A) is added to the broth. One milliliter of sulfanilic acid (Nitrate Reagent B) is then added to the broth and observed for the production of a pink to red color within 30 s. If no color change occurs within 30 s a small amount of zinc dust is added and looked for the production of a pink to red color within 10 min [1].

ALA (*Haemophilus influenzae* Porphyrin Test)

The porphyrin test is used in direct assessment of the ability of *Haemophilus* to synthesize protoporphyins intermediates in the production of hemin (Factor X) from substrate, δ-aminolevulenic acid. *Haemophilus* species (*H. parainfluenzae* and

H. parahaemolyticus) that produce the enzyme porphobilinogen synthase have the ability to synthesize heme (factor X) and therefore do not require an exogenous source of factor X for growth. Porphobilinogen and porphyrin, precursors in heme synthesis, can be detected by inoculation of the *Haemophilus* strain in δ-aminolevulenic acid (which can be incorporated in a disk, agar, or liquid) and by the addition of Kovac's reagent or by examination with a Wood's lamp.

In the tube method, a loopful of organism is suspended in 0.5 ml of δ-aminolevulenic acid. Incubate at 35 °C for 4 h if the suspension is heavy or 18–24 h if the suspension is light. After incubation add an equal volume of Kovac's reagent and vortex the mixture. Allow substrate and reagent to separate. After the addition of Kovac's reagent, a red (pink) color will form in the aqueous phase, indicating the presence of porphobilinogen, and therefore a positive test for *Haemophilus* sp. not requiring factor X. Alternatively, a Wood's lamp can be used to detect fluorescence in the reagent phase, indicating the presence of porphyrins, also a positive test. No coloration or fluorescence indicates a factor X-dependent *Haemophilus* spp. and a negative test. In the disk method, the ALA impregnated disk is moistened with water and a loopful of organism is gently rubbed onto the disk. The disk is incubated for 4 h and observed under ultraviolet light for pink fluorescence [1, 13].

Motility Indole Lysine Sulfide (MILS) Medium

MILS Medium is a semisolid medium useful in the identification of members of the *Enterobacteriaceae*, specifically for screening suspicious colonies from stool cultures for potential pathogens.

It is used to demonstrate motility, indole production, lysine decarboxylase and deaminase activity, and hydrogen sulfide production. A small amount of agar is added to the media for demonstration of motility along a stab line of inoculation. Growth of motile organisms extends out from the line of inoculation, while nonmotile organisms grow along the stab line.

The pH indicator bromcresol purple is used to facilitate detection of decarboxylase activity. When inoculated with an organism that ferments dextrose, acids are produced that lower the pH, causing the indicator in the medium to change from purple to yellow. The acidic pH also stimulates enzyme activity. Organisms that possess a specific decarboxylase degrade the amino acid provided in the medium, yielding a corresponding amine. Lysine decarboxylation yields cadaverine. The production of these amines elevates the pH and causes the medium in the bottom portion of the tube to return to a purple color. The medium in the upper portion of the tube remains acidic because of the higher oxygen tension. Lysine deamination produces a color change in the upper portion of MILS Medium. Oxidative deamination of lysine yields a compound that reacts with ferric ammonium citrate, producing a burgundy red color in the top of the medium. (The bottom portion of the medium remains acidic.) This reaction can only be detected if lysine decarboxylation is not produced, which is the case with *Proteus*, *Morganella*, and *Providencia* species.

Indole is produced in MILS Medium by organisms that possess the enzyme tryptophanase. Tryptophanase degrades the tryptophan present in the casein peptone, yielding indole. Indole can be detected in the medium by adding Kovac's reagent to the agar surface. MILS Medium is also used in the demonstration of hydrogen sulfide production. Hydrogen sulfide, which is produced by some enteric organisms from sulfur compounds contained in the medium, reacts with ferric ion, producing a characteristic black precipitate. (Note that Kovac's reagent is not added until the final lysine carboxylation, lysine deamination, and motility results are interpreted.) [14].

O-Nitrophenyl-β-D-Galactopyranoside Test

In order for an organism to ferment lactose it must have enzymes permease to transport the lactose inside the cell and beta-galactosidase to cleave the transported sugar. Some organisms (delayed lactose fermenters), though posses beta-galactosidase, do not have the enzyme permease. These organisms can utilize the enzyme beta-galactosidase to hydrolyze *O*-Nitrophenyl-β-D-Galactopyranoside (ONPG). ONPG is a colorless compound similar to lactose. In the presence of beta-galactosidase ONPG is hydrolyzed to galactose and a yellow compound *o*-nitrophenyl.

Bacteria grown on lactose-rich medium such as KIA and TIA gives optimal results with the ONPG test. A loopful of bacteria is suspended in 0.5 ml of saline to produce a heavy suspension. Add an equal amount of buffered OPNG solution to the suspension and incubate at 37 °C. Periodically examine the color change for up to 24 h. Yellow color indicates positive reaction and no color change indicates negative reaction. Alternatively an OPNG disk can be added to suspension and observe for color change [6].

Methyl Red Test

This assay determines if an organism metabolizing glucose utilizes mixed acid fermentation pathway and produces strong acid end products (lactic, acetic, formic) that are detected by the indicator methyl red.

A 5 ml MR-VP broth tube is inoculated with the organism and incubated at 35 °C for 48–72 h. 2.5 ml of the broth culture is transferred to a fresh tube and inoculated with five drops of methyl red indicator. Positive MR is indicated if the methyl red reagent remains red. Negative result is indicated if the reagent turns yellow-orange [1, 6].

Voges–Proskauer Test

Organisms such as *Klebsiella*, *Enterobacter*, *Hafnia*, and *Serratia* sp. that utilize the butylene glycol fermentation pathway produce acetoin, an intermediate in the fermentation of butylene glycol. The VP test detects the production of acetoin by these organisms. In the presence of air and potassium hydroxide, acetoin is oxidized to

diacetyl, which produces a red-colored complex. The addition of α-naphthol increases the sensitivity of the test.

A 5 ml MR-VP broth tube is inoculated with pure culture of organism and incubated at 35 °C for 18–24 h. 2.5 ml of the broth culture is transferred to a fresh tube and inoculated with six drops of α-naphthol followed by three drops of KOH. Shake the tube gently to expose the suspension to oxygen and leave undisturbed for 10–15 min. Positive result is indicated by the presence of red color that develops within 15 min. No color change indicates negative VP [1, 6].

Pseudosel Agar Slant

Pseudosel agar is a medium used for the identification of *Pseudomonas aeruginosa*. Magnesium chloride and potassium sulfate in the medium enhance the production of pyocyanin, a blue-green, water-soluble, nonfluorescent phenazine pigment. *P. aeruginosa* is the only Gram-negative rod known to excrete pyocyanin. In addition to the promotion of pyocyanin production, Pseudosel agar also enables the detection of fluorescent products by some *Pseudomonas* species other than *P. aeruginosa*. Streak the surface of the Pseudosel agar slant, and incubate at 35 °C in non-CO_2 for 18–24 h. A blue-green pigmentation surrounding the growth on the agar slant indicates positive reaction. No pigmentation indicates negative reaction.

Negative Pseudosel slants should be examined under short-wavelength (254 nm) ultraviolet light to check for fluorescent products produced by some *Pseudomonas* species. *P. aeruginosa* typically produces fluorescein as well as pyocyanin [15].

Urea Agar Slant

Microorganisms that possess the enzyme urease are capable of hydrolyzing urea, which releases ammonia. This reaction raises the pH of the medium and is detected by phenol red, which turns pink-red above pH 8.0. The color change first appears in the slant since the oxidative decarboxylation of amino acids in the air-exposed portion of the medium enhances the alkaline reaction. The color change eventually spreads deeper into the medium. Stuart's urea broth and Christensen's urea agar are the two most common media used in the detection of urease activity.

Streak the surface of the urea agar slant with a heavy inoculum of a pure culture. Incubate at 35 °C in non-CO_2 for 18–24 h. Production of intense pink-red color on the slant, which may penetrate into the butt, is considered positive reaction. No color change (yellow) indicates negative reaction.

The medium is not specific for urease. The utilization of peptones or other proteins in the medium by some urease-negative organisms may raise the pH due to protein hydrolysis and release of amino acid residues, resulting in false positive reactions [1, 15].

Citrate Agar Slant

Some organisms have the ability to utilize citrate, an intermediate metabolite in the Krebs cycle, as the sole external source of carbon. These organisms also utilize inorganic ammonium salts in the medium as the sole source of nitrogen. The resulting production of ammonia creates an alkaline environment that turns the bromthymol blue indicator to an intense blue.

Using an inoculating loop, select a well-isolated colony and streak the surface of the Simmons's citrate slant (do not stab the agar). Incubate at 35–37 °C in non-CO_2 incubator and examine daily for up to 7 days. Growth with an intense blue color on the agar slant indicates positive reaction and no growth and no color change (green) indicate negative reaction.

Luxuriant growth on the slant without an accompanying color change may indicate a positive test. This should be confirmed by incubating the tube for an additional 24 h. The biochemical reaction requires oxygen. Therefore, the medium should not be stabbed, and the cap must be kept loose during incubation. Carryover of protein and carbohydrate substrates from previous media may provide additional sources of carbon and therefore, cause false-positive reactions [15].

Cetrimide Agar

Cetrimide agar is a selective differential medium used for the identification of *P. aeruginosa*. The principle of the test is to determine the ability of an organism to grow in the presence of cetrimide. Cetrimide acts as a detergent and inhibits the growth of most other organisms. The magnesium chloride and potassium sulfate of the medium stimulate the production of pyocyanin and pyoverdin (fluorescein).

Using an inoculating loop, select a well-isolated colony and streak the surface of the cetrimide slant (do not stab the agar). Incubate at 35 °C in non-CO_2 incubator and examine daily for up to 7 days. Growth on the agar slant indicates positive reaction and no growth indicates negative reaction [4, 15].

Gelatin

The gelatin test is used to identify bacteria that produce the proteolytic enzyme, gelatinase. Organisms that produce gelatinase are capable of hydrolyzing gelatin and cause it to lose its gelling characteristics. Inoculate several well-isolated colonies deep into the gelatin and repeat to inoculate heavily. Incubate the inoculated tube and an uninoculated control at 35 °C in ambient air. The tubes are then removed daily and incubated at 4 °C to check for liquefaction. Liquefaction is determined only after the control has hardened. Alternatively, strips of exposed but undeveloped X-ray film are placed in the bacterial suspension of equivalent to at least 2.0 McFarland standard and incubate at 35 °C in a non-CO_2 incubator for 48 h. Prepare an uninoculated tube as control. The strip is examined after 24 and 48 h for loss of gelatin coating that leaves the X-ray clear [6].

Acetate Utilization

Some organisms have the ability to utilize acetate as a sole external source of carbon. Acetate slants contain a mixture of salts and sodium acetate in a medium without organic nitrogen. Organisms that can utilize acetate as a sole carbon source break down sodium acetate causing the pH of the medium to shift toward the alkaline range, turning the bromthymol blue indicator blue. Organisms that cannot utilize acetate as a sole carbon source do not grow on the medium. Acetate differential agar is useful in the differentiation of *Shigella* spp. and *E. coli.*

Streak the surface of the acetate differential agar slant (do not stab the agar) with a colony and cap the tube loosely. Incubate at 35 °C in non-CO_2 and examine daily for up to 7 days. Growth with an intense blue color on the agar slant indicates positive test and no growth or no color change (green) indicates negative test.

Luxuriant growth on the slant without an accompanying color change may indicate a positive test. This should be confirmed by incubating the tube for an additional 24 h. The biochemical reaction requires oxygen. Therefore, the medium should not be stabbed, and the cap must be kept loose during incubation. Carryover of protein and carbohydrate substrates from previous media may provide additional sources of carbon and therefore, cause false-positive reactions [15].

Lead Acetate for Hydrogen Sulfide Detection

Some organisms are capable of enzymatically liberating sulfur from sulfur-containing amino acids or inorganic sulfur compounds. The released hydrogen sulfide reacts with lead acetate to yield lead sulfide, an insoluble black precipitate. Lead acetate is the most sensitive H_2S indicator reagent and is useful with organisms that produce trace amounts of H_2S, especially organisms that are not in the family *Enterobacteriaceae.* Inoculate a TSI medium with the isolate. (Stab once through the center of the into the butt of the tube to within 3–5 mm of the bottom, withdraw the inoculating needle, and streak the surface of the TSI agar slant.) Place the lead acetate strip so that it hangs down approximately 1 in. inside the TSI tube. Incubate at 35°C in non-CO_2 for 18–24 h. A brownish-black coloration of the paper strip indicates positive reaction. No coloration of the strip indicates negative reaction.

Lead acetate is toxic to bacterial growth. Do not allow the strip to touch the medium. The TSI medium must support the growth of the test organism for H_2S production to occur [1, 6].

Lysine Iron Agar

Lysine Iron Agar (LIA) is a differential medium used for the identification of enteric bacilli based on their ability to decarboxylate or deaminate lysine and produce hydrogen sulfide. Dextrose serves as a source of fermentable carbohydrate. The pH indicator, bromcresol purple, is changed to a yellow color at or below pH 5.2 and is

purple at or above pH 6.8. Ferric ammonium citrate and sodium thiosulfate are indicators of hydrogen sulfide formation. Lysine serves as the substrate for detecting the enzymes lysine decarboxylase and lysine deaminase. LIA is designed for use with TSI Agar for the identification of enteric pathogens.

Using a sterile inoculating needle stab the butt of the LIA slant twice and then streak back and forth along the surface of the agar with the organism. Incubate at 35 ± 2 °C in non-CO_2 for 18–24 h.

Alkaline (purple) reaction in butt indicates Lysine decarboxylation; red slant indicates Lysine deamination and black precipitate indicates H_2S production. H_2S may not be detected in this medium by organisms, which are negative for lysine decarboxylase activity since acid production in the butt may suppress H_2S formation. For this reason, H_2S-producing *Proteus* species do not blacken this medium [15].

Triple Sugar Iron Agar Slant

TSI agar is a medium that differentiates Gram-negative bacilli on the basis of the ability to ferment carbohydrates and liberate hydrogen sulfide (H_2S). The medium contains one part glucose to ten parts each of lactose and sucrose. Phenol red serves as an indicator to detect pH change, and ferrous sulfate detects the formation of H_2S. If the organism ferments glucose, the butt and slant of the agar will become acidic and turn yellow. If the organism ferments lactose and/or sucrose, the slant will remain acidic (yellow). If the organism is unable to ferment lactose or sucrose, the slant will revert to alkaline (red) when the glucose is used up and alkaline amines are produced in the oxidative decarboxylation of peptides (derived from protein in the medium) near the surface of the agar. Organisms unable to ferment glucose will not change the pH of the medium or will produce alkaline products, and the TSI tube will remain red. Blackening of the medium indicates H_2S production. Gas production is indicated by splits or cracks in the butt of the agar. Gas may also push the agar up the tube.

Using a sterile inoculating needle stab the butt of the TSI slant twice and then streak back and forth along the surface of the agar with the organism. Incubate at 35 ± 2 °C in non-CO_2 for 18–24 hours. If acid slant–acid butt (yellow–yellow): glucose and sucrose and/or lactose fermented. If alkaline slant–acid butt (red–yellow): glucose fermented only. If alkaline slant–alkaline butt (red–red): glucose not fermented. The presence of black precipitate (butt) indicates hydrogen sulfide production and presence of splits or cracks or air bubbles indicates gas production.

Early readings may result in false acid–acid results, while delayed readings may result in false alkaline–alkaline results. Copious amounts of H_2S may mask the glucose reaction. If this occurs, glucose has been fermented even if it is not observable. The utilization of sucrose may suppress the enzyme mechanism that results in the production of H_2S. Trace amounts of H_2S may not be detectable with the ferrous sulfate indicator in the agar [1, 15].

Phenylalanine Deaminase

This assay is used to detect the ability of an organism to oxidatively deaminate phenylalanine to phenylpyruvic acid. The phenylpyruvic acid is detected by adding a few drops of 10 % ferric chloride.

Inoculate a phenylalanine agar slant with the organism and incubate at 35 °C in non-CO_2 incubator for 18–24 h with the cap loose. Following the incubation, add 4–5 drops of 10 % ferric chloride solution to the slant. The development of green color on the surface of the slant indicates positive reaction. No color change indicates negative reaction [4, 6].

Decarboxylase (Moeller's Method)

Decarboxylases are a group of substrate-specific enzymes that are capable of decarboxylate (or hydrolyze) amino acids to form alkaline-reacting amines. Each decarboxylase enzyme is specific for an amino acid. Lysine, ornithine, and arginine are the three amino acids used routinely in the identification of *Enterobacteriaceae*, Aeromonas*, Plesiomanas,* and *Vibrio* species. The decarboxylation of lysine and ornithine yields cadaverine and putrescine, respectively. Arginine is converted to citrulline by a dihydrolase reaction. A control tube containing the base without an added amino acid to verify that the organism utilizes glucose must accompany all decarboxylase tests. Since decarboxylation is an anaerobic reaction, the tubes must be overlaid with mineral oil prior to incubation. If the organism is viable, both the control and the test tube with amino acid should turn yellow because of fermentation of the small amount of glucose in the medium. If the amino acid is decarboxylated, the alkaline amines cause the indicator (bromcresol purple) in the acid medium to revert back to its original purple color.

Inoculate a Moeller decarboxylase broth containing ornithine, lysine, and/or arginine. Overlay the contents of all tubes with 1 ml of sterile mineral oil and incubate in a non-CO_2 incubator at 35 °C for 18–24 h. Examine for a color change. Negative reactions are examined daily for no more than 4 days [15].

OF Glucose Medium

Bacteria can utilize glucose and other carbohydrates by using various metabolic cascades. Some are fermentative routes; others are oxidative. Oxidation–fermentation (OF) medium permits classification of gram-negative bacilli by a simple method that differentiates aerobic and anaerobic degradation of carbohydrates. The low protein-to-carbohydrate ratio in the medium prevents the neutralization of weak acids by the alkaline products if the protein is utilized, thus allowing small quantities of acid to be detected. Acid production results in a pH shift that changes the color of the bromthymol blue indicator from green to yellow.

Using inoculating needle, two tubes of OF glucose medium are stabbed, inoculating halfway to the bottom of the tubes. The content of one tube is overlaid

with 1 ml of sterile mineral oil. Both tubes are incubated at 35 °C in non-CO_2, and examine daily for 72 h or longer for slow-growing organisms. Yellow color indicates the production of acid. Acid production in tube without oil overlay is considered Oxidative reaction. Acid production in both tubes is considered fermentative. No acid production in either tube is considered non-saccharolytic. Non-saccharolytic organisms produce slight alkalinity (blue-green color) in the tube without oil overlay, but the tube with oil will not exhibit a color change and will remain green [15].

OF Sugars

OF basal medium, when supplemented with an appropriate carbohydrate, is used to determine an organism's ability to utilize sugars such as Lactose, Xylose, Sucrose, Maltose, or Mannitol. The low protein-to-carbohydrate ratio in OF basal medium prevents the neutralization of small quantities of weak acids by the alkaline products of protein metabolism, which makes this medium ideal for determining carbohydrate utilization. Acid production from carbohydrate metabolism results in a pH shift that changes the color of the bromthymol blue indicator from green to yellow. Yellow color indicates carbohydrate metabolism.

Using an inoculating needle, touch the center of one colony and stab the OF medium with the appropriate carbohydrate once halfway to the bottom of the tube. Cap the tubes loosely and incubate at 35 °C in non-CO_2, examining the tube daily for 72 h or longer for slow-growing organisms. A yellow color indicates carbohydrate utilization and no color change (green) or blue color indicates no carbohydrate utilization. The acid reaction produced by oxidative organisms is detected first at the surface and gradually extends throughout the medium. When oxidation is weak or slow, it is common to observe an initial alkaline reaction at the surface of the tube that may persist for several days. This must not be mistaken for a negative test. If the organism is unable to grow in the OF medium, add either 2 % serum or 0.1 % yeast extract prior to inoculation [15].

Commercial Microbial Identification System

The commercial microbial identification system is the backbone of microbial identification in the clinical microbiology laboratories. It provides an advantage over conventional identification systems by requiring little storage space and having an extended shelf life, rapid turnaround, low cost, standardized quality control, and ease of use. They range from manual to semi-automated to fully automated systems. These systems require simultaneous inoculation and incubation of a series of miniaturized biochemical reactions which are either based on detecting bacterial enzymes or cellular products that do not require microbial growth and have fairly rapid turnaround time (2–4 h) or based on metabolic activity that requires microbial growth and requires several hours to overnight incubation. In either case, the

Table 6.2 Commercial systems commonly used in clinical laboratories

Product	Manufacturer	TAT
API Systems	bioMerieux Inc.	2 h to overnight
BBL Crystal Systems	Becton Dickinson	4 h to overnight
BBL Phoenix Systems	Becton Dickinson	2 h to overnight
Vitek	bioMerieux Inc.	2 h to overnight
MicroScan	Dade International	2 h to overnight
MIdI Sherlock	MIDI	Overnight
Sensititer AP80	Trek	5 h to overnight
Biolog Micro Plate	Biolog	Overnight

enzymatic or biochemical end results are combined, and using the Bayer's theorem with the aid of a computer program the identity of the test organism is determined. The majority of metabolic based automated commercial identification systems also incorporate antimicrobial susceptibilities testing. In fact, over the years, the growing numbers of clinically significant pathogens and their rapidly emerging resistance to various antimicrobial agents have led to innovation of several commercial identification (ID) and antimicrobial susceptibility testing (AST) systems. For the most part, these systems have a fairly extensive database. They are fast, accurate, and have significantly improved turnaround time for ID and AST of the common organisms. Despite their extensive database, they remain less than optimal in identifying fastidious slow-growing esoteric organisms. Table 6.2 presents the list of most commonly used commercial identification systems.

API Identification System

The API identification systems (bioMerieux Inc., Hazelwood, MO) consist of series of microcapsules on a plastic strip that contains dehydrated substrates for the demonstration of enzymatic activity or the fermentation of carbohydrates. Depending on type of the organism and the API strip utilized, it may or may not require microbial growth. API systems are manual and do not incorporate AST [16, 17].

API Gram-Negative Identification

1. API 20E is a 24-h identification test for identification of Enterobacteriaceae and group/species of non-fermenting gram-negative rods.
2. API Rapid 20E is a 4-h identification test for identification of Enterobacteriaceae.
3. API 20NE is a 24–48-h identification test for identification of gram-negative non-Enterobacteriaceae.
4. API NH is a 2-h test for identification of Neisseria, Haemophilus, and Moraxella.

API Gram-Positive Identification

1. API Staph is an overnight test for identification of clinical staphylococci and micrococci.
2. RAPIDEC Staph is a 2-h identification of the commonly occurring staphylococci.
3. API 20 Strep is a 4- or 24-h test for identification of streptococci and enterococci.
4. API Coryne is a 24-h test for identification of Corynebacteria and coryne-like organisms.

API Anaerobe Identification

1. API 20A is a 24-h test for identification of anaerobic organisms.
2. Rapid ID 32 is a 4-h test for identification of anaerobes.

BBL™ Crystal™ Identification System

The BBL™ Crystal™ System (Becton Dickinson, Cockeysville, MD) is a manual method that utilizes miniaturized fluorogenic and or chromogenic linked substrates to detect enzymes that microbes use to metabolize a variety of substrates. These kits consist of BBL Crystal panel lids, bases, and inoculum fluid tubes. A suspension of the test organism is prepared in the inoculum fluid and then used to fill the reaction wells in the base. The substrates are rehydrated when the base and lid are aligned and snapped into place. Following the recommended incubation time, the wells are manually examined for color changes or the presence of fluorescence. The resulting pattern of positive and negative test scores is the basis for identification [17, 18].

1. BBL™ Crystal™ Enteric/Nonfermenter (E/NF) Identification System is an overnight identification method utilizing modified conventional and chromogenic substrates. The E/NF identifies clinically significant aerobic gram-negative *Enterobacteriaceae* isolates and non-fermenting gram-negative rod.
2. The BBL™ Crystal™ Rapid Stool/Enteric (RS/E) Identification System is a miniaturized 3-h identification method employing modified conventional and chromogenic substrates. It is intended for the identification of clinically significant aerobic gram-negative bacteria that belong to the family *Enterobacteriaceae* as well as most pathogens isolated from stool specimens.
3. The BBL™ Crystal™ *Neisseria/Haemophilus* (N/H) Identification System is a miniaturized 4-h identification method employing modified conventional, fluorogenic, and chromogenic substrates. It is intended for the identification of *Neisseria, Haemophilus, Moraxella, Gardnerella vaginalis,* as well as other fastidious bacteria.
4. The BBL™ Crystal™ Gram-Positive ID System is a miniaturized 18-h identification method employing modified conventional, fluorogenic, and chromogenic substrates. It is intended for the identification of both gram-positive cocci and bacilli.

5. The BBL™ Crystal™ Rapid Gram-Positive ID System is a miniaturized 4-h identification method employing modified conventional, fluorogenic, and chromogenic substrates. It is intended for the identification of gram-positive bacteria isolated from clinical specimens.
6. The BBL™ Crystal™ Anaerobe ID kit is a miniaturized 4-h identification method employing modified conventional, fluorogenic, and chromogenic substrates to identify clinically significant anaerobic organisms.

BBL Phoenix Identification and Susceptibility System

The BBL Phoenix™ (Becton Dickinson, Cockeysville, MD) is an automated identification and susceptibility system that can identify clinically significant Gram-negative or gram-positive microorganisms. The Phoenix ID panel utilizes a series of conventional, chromogenic, and fluorogenic biochemical tests to determine the identification of the organism. Both growth-based and enzymatic substrates are employed to cover the different types of reactivity. The tests are based on microbial utilization and degradation of specific substrates detected by various indicator systems. Acid production is indicated by a change in phenol red indicator when an isolate is able to utilize a carbohydrate substrate. Chromogenic substrates produce a yellow color upon enzymatic hydrolysis of either *p*-nitrophenyl or *p*-nitroanilide compounds. Enzymatic hydrolysis of fluorogenic substrates results in the release of a fluorescent coumarin derivative. Organisms that utilize a specific carbon source reduce the resazurin-based indicator. The AST method is a broth-based microdilution test. The system utilizes a redox indicator for the detection of organism growth in the presence of an antimicrobial agent. Continuous measurements of changes to the indicator as well as bacterial turbidity are used in the determination of bacterial growth. Each AST panel configuration contains several antimicrobial agents with a wide range of two-fold doubling dilution concentrations. Organism identification is used in the interpretation of the MIC values of each antimicrobial agent resulting in Susceptible, Intermediate, or Resistant (S, I, R) result classification.

The system includes an inoculation station for panel setup and an incubator/reader carousel module. The carousel houses four horizontal tiers of 26 panel carriers to accommodate a tier-specific Normalizer and 25 Phoenix Panels. Phoenix Panel utilizes up to 51 micro-wells for ID and up to 85 micro-wells for AST. A bacterial inoculum concentration approximately equivalent to a 0.5 McFarland Standard is required for the identification of either gram-negative or gram-positive bacteria. Susceptibility testing is performed with an inoculum concentration of $3–7 \times 10^5$ cfu/ml. Kinetic measurements of bio-reactivity within individual micro-wells via red, green, blue, and fluorescence readings are collected and comparatively analyzed with the Phoenix database [18].

VITEK and VITEK 2 Identification System

The Vitek (bioMerieux Inc., Hazelwood, MO) is an automated ID and AST system that utilizes identification cards with miniaturized wells. The system is fairly automated. It requires the user to prepare a suspension of the isolate in saline and verify the organism concentration with a densitometer. The inoculum tube is then placed into a rack, called the cassette. The sample identification number is entered into the Carrier via barcode or keypad and electronically linked to the supplied barcode on each test card. ID and AST test cards can be mixed and matched in the cassette. All information entered at the bench is then transported to the instrument in a memory chip attached to the cassette.

VITEK 2 is the fully automated version that all processing steps are completely autonomous including test setup verification, AST inoculum dilution test inoculation, card sealing, incubator loading, optical reading and data transmission, and card disposal. The VITEK 2 optical system reads all the wells every 15 min. There are several card that are designed for ID and susceptibility testing with these systems, including Vitek GPI (Vitek 1), Vitek GPC (Vitek 2), Vitek EPS, GNI Plus, UID and UID, Vitek 2 ID-GNB, and Vitek NHI and AST panels for Gram-positive and Gram-negative organisms [16].

MicroScan WalkAway

The MicroScan WalkAway (Dade MicroScan Inc., West Sacramento, CA) is an automated ID and AST system that requires the ID and/or AST panels (96-well plates) be manually inoculated with bacteria isolated from clinical specimens and inserted into the WalkAway System. The panels are then incubated at 35 °C for 16–42 h, depending on panel, organism type, and results of readings. At the appropriate time, the WalkAway System automatically dispenses reagents into the appropriate biochemical wells and incubates the panels for an additional period of time (approximately 2–20 min, depending on the panel type). The WalkAway System then reads the panels. The identification of bacteria is based on measuring a series of biochemicals contained in panels designed for the speciation of most medically significant bacteria. The panels contain identification media consisting of substrates and/or growth inhibitors, which, depending on the species of the bacteria present, will exhibit color changes or increases in turbidity after incubation.

The panel may also contain series of antibiotic that are present in specified concentrations in the wells of applicable MicroScan panels. The WalkAway System reads the minimum inhibitory concentrations (MICs) and certain biochemicals and, if the criteria are met for adding reagents, reagents are added. The panel is then incubated for an additional period of time (approximately 5–30 min) depending on the panel type. The readings for the biochemicals needing no reagents and MIC wells (for Combo panels) are stored prior to reagent addition. If additional incubation

is necessary for the biochemicals, the susceptibilities and certain biochemicals will be read first and stored. The reagents will not be added until after additional incubation, at which time biochemicals not previously read will be determined. The following is the list of commonly used MicroScan panels: MicroScan Gram Pos ID panel, MicroScan Rapid Gram Pos ID panel, MicroScan Neg Type 2, MicroScan Rapid Neg ID Types 2 and 3, and MicroScan NHID [19].

Sensititre® Microbiology Systems

The Sensititre ARIS 2X (TREK Diagnostic Systems, Inc., Cleveland, OH) is an automated ID and AST system. The Sensititre ID and AST panels (96-well plates) may be inoculated manually or with an autoinoculator that is designed to automatically deliver inoculum in multiples of 50 μl to the 96-well sensititre plate. The Sensititre ID system is based on 32 biochemical tests pre-dosed and dried in the Sensititre plate that are formulated to allow fluorometric reading along with unique fluorescent tests. The AST plate may be read manually or using the automated system. The automated system is fluorescent based and detects bacterial growth by monitoring the activity of specific surface enzyme produced by the test organism. Growth is determined by generating a fluorescent product from a nonfluorescent substrate. Presumptive ID of Gram-negative organisms can be obtained in five hours; identification to species level for both Gram-negatives and Gram-positives can be obtained after overnight incubation. The Sensititre ARIS 2X is a combined incubation and reading system that fits onto an autoreader. Sensititre uses an internal barcode scanner to identify each plate type, assign the appropriate incubation time, and, when this assigned time has elapsed, transport the plate to the autoreader for fluorescence measurement. The system has capacity to accommodate up to 64 ID or AST plates. The following is the list of ID and AST plates with this system: GNID (AP80) for Gram-negative, GPID for Gram-positive Identification, Gram-positive and Gram-negative MIC plates, Sensititre *Haemophilus influenzae* or *Streptococcus pneumoniae* susceptibility Plates, Anaerobe MIC Plate, EBSL Confirmatory MIC plate, and *S. pneumoniae* MIC plate [17, 20].

MIDI Sherlock

The MIDI Sherlock ID system (MIDI, Inc., Newark, DE) is based on Gas Chromatographic (GC) analysis of the bacterial fatty acids. Branched-chain acids are known to predominate in most Gram-positive bacteria, while short-chain hydroxy acids often characterize the lipopolysaccharides of the Gram-negative organisms. The system is fairly labor intensive and is designed for use in reference laboratories to identify isolates that are not easily identified by the routine identification systems. The Sherlock system detects the presence or absence of more than 300 fatty acids

and related compounds (9–20 carbons in length) as well as quantity of these compounds. The peaks are automatically named and quantified by the system.

Initially the organism undergoes saponification, methylation, extraction, and base wash before GC analysis. A GC with phenyl methyl silicone fused silica capillary column is injected with the final prep. The temperature program in GC ramps from 170 to 270 °C at 5 °C/min. Following the analysis, a ballistic increase to 300 °C allows cleaning of the column. The electronic signal from the GC detector is then passed to the computer where the integration of peaks is performed. The electronic data is stored on the hard disk and the fatty acid methyl ester composition of the sample is compared to a stored database using the Sherlock pattern recognition software [2, 17].

BioLog ID System

The Biolog Micro Plate ID System (Biolog, Inc., Hayward, CA) relies on carbon source utilization test methodology in a 96-well format. The system is based on 95 reactions from six to eight different classes of carbon sources with redox indicator (tetrazolium dye) and one negative control well with no carbon source. The isolate is inoculated to the micro-well plate and incubated. If the isolate oxidizes any of the carbon sources the net electron will reduce the tetrazolium to highly colored formazin (purple color). The carbon source utilization produces a characteristic pattern or "fingerprint" that is then compared to the Biolog database for identification. The system can identify environmental as well as fastidious organisms. In addition to the original Microlog manual and a semi-automated version, the manufacturer has recently introduced a fully automated version (OmniLog) with a database to identify over 700 species of Gram-positive and Gram-negative organisms [17, 21].

References

1. Winn WC Jr et al (eds) (2005) Koneman's color atlas and textbook of diagnostic microbiology, 6th edn. J.B. Lippincott Co., Philadelphia, PA
2. MIDI Inc. (2004) http://www.midi-inc.com
3. Oxidase Reagent Package Insert. Becton Dickinson Microbiology Systems. Cockeysville, MD. Date of issue, July 2003
4. Forbes BA, Sahm DF, Weissfeld AS (eds) (2007) Bailey and Scott's diagnostic microbiology, 12th edn. Mosby, St. Louis, MO
5. Isenberg HD (1992) Clinical microbiology procedures handbook. American Society for Microbiology, Washington, DC
6. Versalovic J, Carroll KX, Gunde G, Jorgensen JH, Landry ML, Warnock DW (eds) (2011) Manual of clinical microbiology, 10th edn. American Society for Microbiology, Washington, DC
7. York M, Traylor MM, Hardy J, Henry M (2004) Biochemical tests for the identification of aerobic bacteria: Bile solubility tests. In: Isenberg HD (ed) Clinical microbiology procedures handbook, 2nd edn. American Society for Microbiology, Washington, DC, p 3.17.6
8. Coleman G, Ball LC (1984) Identification of streptococci in the medical laboratory. J Appl Microbiol 57:1–14

9. D'Amato RF et al (1978) Rapid identification of *Neisseria gonorrhoeae* and *Neisseria meningitidis* by using enzymatic profiles. J Clin Microbiol 7:77–81
10. CLO Test Package Insert (2001) Delta West Ply Ltd., Bentley, Western Australia
11. Weyant RS, Moss CW, Weaver RE et al (eds) (1996) Identification of unusual pathogenic gram-negative aerobic and facultatively anaerobic bacteria, 2nd edn. Williams & Wilkins Co, Baltimore, MD
12. Wilkinson HW (1977) Camp-disk test for presumptive Identification of group B streptococci. J Clin Microbiol 6:42
13. Killian M (1974) A rapid method of differentiation of *Haemophilus* strains-the porphyrin test. Acta Pathol Microbiol Scan (B) 82:835–842
14. Difco and BBL Manual (2009): Manual of Microbiological Culture Media. BD Diagnostic Systems, Sparks MD
15. Quality Control and Product Information Manual for Tubed Media (1992) BD Microbiology Systems, Cockeysville, MD
16. bioMerieux Inc. (2004) http://www.biomerieux-usa.com
17. Traunt AL (ed) (2002) Manual of commercial methods in clinical microbiology. American Society for Microbiology, Washington, DC
18. Becton Dickinson Inc. (2004) http://www.bd.com
19. Dade Behring MicroScan Dried Gram Negative and Dried Gram Positive Procedure Manual package insert. April 1998
20. TREK Inc. (2004) http://www.trekds.com
21. Biolog, Inc. (2004) http://www.biolog.com

Chapter 7
Infectious Disease Biomarkers: Non-Antibody-Based Host Responses

Audrey N. Schuetz

Introduction

Biomarkers, or biologic markers, are measurable substances which are produced by the body in response to a particular infection, or a recent change in status of the patient. They are in vivo host responses which are indicative of the overall biological state of the patient at the time. According to the Definitions Biomarkers Working Group, a biomarker is "… a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [1]. Usually quantifiable, biomarkers include both antibody- and non-antibody-based host responses. This chapter focuses on the assessment and interpretation of non-antibody-based host responses which are produced by the patient in response to changes in homeostasis due to infection.

The study of biomarkers has become popular in recent years due to the rapid information they can provide, beyond the routine clinical and laboratory data which have traditionally been available to the caregiver. Specifically, serum biomarker concentrations have been targeted as possible alternative markers of sepsis. In addition, biomarkers can provide information about a wide variety of disease states, including but not limited to ventilator-associated pneumonia (VAP), community-acquired pneumonia (CAP), lower respiratory tract infections, bloodstream infections in the absence of sepsis, abdominal infections, urinary tract infections, aspergillosis, and infectious hepatitis [2–9].

Biomarkers have many potential uses. They can aid in diagnosis or prognosis, as well as in therapeutic decisions, such as monitoring response to interventions or

A.N. Schuetz (✉)
Departments of Pathology & Laboratory Medicine, and Medicine,
Weill Cornell Medical College/The NewYork-Presbyterian Hospital,
New York, NY 10065, USA
e-mail: ans9112@med.cornell.edu

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guiding further antibiotic treatment [10]. Levels of some biomarkers change dynamically as the patient responds or fails to respond to treatment and so may be useful to measure over time. They may be used to screen patients who have not yet developed clinically significant disease but may be at risk of doing so; or, they may be used in risk stratification. Some studies have analyzed the use of biomarkers in the identification of patients who are at increased risk of poor outcomes or who may need further diagnostic testing. Other studies have assessed the role of biomarkers in indicating which patient may benefit from therapeutic interventions.

Although the study of non-antibody-based host responses is a rapidly growing field, there are few consensus guidelines regarding the use of these tests. In general, biomarker results are helpful to use in conjunction with culture or other diagnostic markers for the diagnosis and management of disease states. This chapter focuses primarily on the potential uses of biomarkers in infectious disease critical care medicine but also discusses the roles of novel biomarkers in the diagnosis and management of infectious diseases in general. Procalcitonin (PCT) is perhaps the most widely studied of biomarkers in recent years for its role in the diagnosis of sepsis; however, its role in diagnosis and management of other disease states will also be covered. PCT and other biomarkers, such as C-reactive protein (CRP), cytokines (such as interleukins [ILs] and interferons), cell markers, and other miscellaneous biomarkers related to inflammatory states will be discussed. Refer to Table 7.1 for an outline of biomarkers covered in this chapter. The chapter will conclude with highlights of potential applications of novel and promising biomarkers.

Procalcitonin

PCT is a peptide precursor of the hormone calcitonin and is released by parenchymal C cells of the thyroid in response to toxins produced by microorganisms. PCT may also be synthesized in extrathyroidal neuroendocrine tissues during bacterial infection [11]. Bacteremia appears to lead to higher PCT levels than does localized infection, viral infection, or infection with intracellular organisms, such as *Mycoplasma pneumoniae* [12, 13]. Gram-negative bacteremia leads to higher rises in PCT levels than gram-positive bacteremia [14]. Steroid use does not significantly affect PCT levels [15].

PCT is rapidly upregulated and sustained in the serum during infection [16]. It rises quickly within 2–4 h after the onset of infection and peaks between 6–8 h and 24 h [11, 17–19]. The half-life of PCT is 24 h [11]. It is present at very low concentrations of 0.033 ng/mL in the serum of healthy subjects and increases up to 1,000 times during periods of inflammation [18, 20–22]. Serum PCT levels rise after exercise [23]. In neonates, serum PCT levels rise to 2–3 ng/mL within 24 h after birth and return to normal by 72 h [18]. As a stable molecule, PCT is not significantly affected by varying blood preparation methods and different freezing or storage conditions [24–26].

Table 7.1 Types and specific examples of biomarkers evaluated for the diagnosis and/or management of infectious diseases

Acute phase protein biomarkers
- Lipopolysaccharide-binding protein
- Serum amyloid A

Coagulation biomarkers
- Activated partial thromboplastin time waveform

Biomarkers related to vascular endothelial damage
- Angiopoietins
- Neopterin

Receptor biomarkers
- Soluble urokinase-type plasminogen activator receptor (suPAR)
- Soluble form of the receptor for advanced glycation end products (sRAGE)
- Toll-like receptor-2 (TLR-2)
- Triggering receptor expressed on myeloid cells-1 (TREM-1)

Cell marker biomarkers
- CD64
- HLA-DR
- Presepsin, or soluble CD14 subtype

Cytokine/chemokine biomarkers
- Various interferons
- Various interleukins

Biomarkers related to vasodilation
- Proadrenomedullin

Other biomarkers
- Alpha-1 antitrypsin
- Gelsolin
- Interferon-gamma-inducible protein-10 (IP-10)
- Neutrophil distribution width
- Regulatory T lymphocytes

In surgical patients, PCT can increase after trauma, particularly after abdominal surgery or pancreatitis, but the levels usually rise transiently and decrease to normal levels after 12–24 h [11, 27]. In contrast, CRP can remain elevated for several days after surgery [11]. Mildly to moderately elevated PCT levels have also been reported in association with major abdominal or thoracic surgery and cardiac surgery [28, 29]. Patients with renal impairment have also shown elevated PCT levels in the absence of infection [30]. When PCT levels remain elevated, a persistent infection or ongoing sepsis may be suspected in a patient without renal impairment [20].

The diagnostic accuracy of PCT is high, with sensitivities ranging from 74.8 to 100%, specificities 70–100%, positive predictive values (PPVs) 55–100%, and negative predictive values (NPVs) 56.3–100% [17, 21, 24, 31–35]. Variations in diagnostic accuracies of tests are due to differences in cutoff values, testing platform used, and study designs [16]. Most studies have employed 0.5 ng/mL cutoff values, but cutoff values reported ranges from 0.3 to 8.05 ng/mL [16].

Many assays have been developed for PCT measurement, and most of them are manufactured by BRAHMS (part of Thermo Fisher Scientific, Berlin, Germany). Refer to Table 7.2 for a list of PCT tests. The first commercial PCT assay was a

Table 7.2 Procalcitonin assays

Assay	Principle	Specimen tested	Measuring range	Time to result	US FDA-approved	References
BRAHMS PCT LIA (BRAHMS USA, Inc., Middletown, VA, USA)	Sandwich-type luminescence immunoassay	Serum or plasma	Quantitative, 0.1–500 ng/mL	3 h	Yes	[36]
BRAHMS PCT Kryptor (BRAHMS USA, Inc., Middletown, VA, USA)	TRACE technology[a]	Serum or plasma	Quantitative, 0.02–5,000 ng/mL	25–40 min	Yes	[36]
VIDAS BRAHMS PCT (bioMérieux, Marcy l'Etoile, France)	Enzyme-linked fluorescent assay	Serum or plasma	Quantitative, 0.09–200 ng/mL	20 min	Yes	[36]
PCT-Q (BRAHMS Diagnostica, Henningdorf BEI, Berlin, Germany)	Immunochromato-graphic assay	Serum or plasma	Semiquantitative, reported as <0.5, ≥0.5, ≥2, or ≥10 ng/mL	30 min	No	[36]
PCT Liaison (BRAHMS Diagnostica, Henningdorf BEI, Berlin, Germany)	Immunolumino-metric assay	Serum or plasma	0.1–500 ng/mL	20 min	No	[36]
ADVIA Centaur CP BRAHMS PCT (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA)	Immunoassay	Serum or plasma	Quantitative, 0.02–75 ng/mL	26 min	No	[37]

[a]TRACE = time resolved amplified cryptate emission. See text for details
FDA = Food and Drug Administration

manual immunometric assay named the LUMItest, now the PCT LIA assay (BRAHMS USA, Inc., Middletown, VA, USA). The PCT LIA measures PCT as well as calcitonin and calcitonin-carboxyl-peptide-I (CCP-I), with a measuring range between 0.1 and 500 ng/mL [20]. The second generation assay approved by the US Food and Drug Administration was the PCT Kryptor Sensitive immunoassay (BRAHMS USA, Inc., Middletown, VA, USA) which is based on TRACE technology (time resolved amplified cryptate emission) and has a measuring range between 0.02 and 5,000 ng/mL [38]. The Kryptor assay measures PCT and CCP-I. The TRACE technology uses both the anti-katacalcin and anti-calcitonin antibodies labeled with different fluorescent tracers. If PCT is present within the patient sample, it is sandwiched between the two tracers in close proximity, and a transfer of energy results between the tracers. The intensity of the energy transfer signal is proportional to the amount of PCT in the specimen. None of the currently available PCT assays measure PCT levels alone. The PCT-Q Rapid assay (BRAHMS Diagnostica, Henningdorf BEI, Berlin, Germany) is a semiquantitative immunochromatographic kit, with a band which turns red in color after application of the patient specimen [39]. The intensity of the color is read against a reference card, and concentrations are reported as <0.5, ≥0.5, ≥2, or ≥10 ng/mL. However, interpretation of this test can be difficult, given that the test results are somewhat subjective in nature [26]. In one study, there was only moderate agreement between the results of the Kryptor assay and the PCT-Q assay when used in a clinical setting [26]. The LIA and LIA Sensitive assays are manual two-step sandwich luminescence immunoassays, while the Liaison PCT assay (BRAHMS Diagnostica, Henningdorf BEI, Berlin, Germany) is a two-site immunoluminometric assay. Advantages of the random access automated platforms include less manual handling and shorter incubation times (19 min incubation for Kryptor Sensitive and 30 min for Liaison, compared to 1 h for the LIA and 2.5 h for the LIA Sensitive) [40]. Although turnaround times vary from laboratory to laboratory based on testing platform and staffing, the average turnaround time is approximately 3 h for quantitative tests performed in the clinical laboratory [11].

Although cutoff values vary according to disease state, PCT levels of 0.25–0.5 ng/mL generally suggest bacterial infection, in the absence of other factors which may lead to increased PCT levels [41]. If PCT levels are less than 0.25 ng/mL, sepsis and severe bacterial infection are unlikely [41]. Higher cutoff values may be required for use in patients with renal disease [42].

The Roles of PCT and Other Biomarkers in Sepsis

Sepsis is one of the leading causes of death in critically ill patients worldwide, with mortality rates ranging from 20 to 50% [43, 44]. Sepsis is currently defined as a systemic inflammatory response to bacterial, fungal, or viral infections [45]. It is an innate physiologic response by the immune system to infection, involving complex pathophysiologic processes with many different disease mechanisms, including

coagulation, inflammation, complement activation, and apoptosis in many different organ systems in the body [46]. The septic response to infection is a complex chain of events involving many different arms of the immune and circulatory systems [43]. Early in the disease course, a massive release of inflammatory mediators is in part responsible for organ hypoperfusion and dysfunction [47]. The innate immune response directly or indirectly results in the release of thousands of endogenous mediators of inflammation and coagulation [48]. Compensatory mechanisms are then adapted in order to dampen the immune response. There are many underlying causes of sepsis. Likewise, clinical management and prognosis are equally as variable. Treatment of sepsis focuses on administration of broad-spectrum antimicrobials, with stabilization of the circulatory system.

The traditional diagnosis of sepsis based on physical findings and conventional laboratory methods is complicated by the nonspecific signs and symptoms and high variability of presentation from patient to patient [49]. Methods of diagnosis include culture, but diagnosis by culture is prolonged due to length of time to grow the microorganism. In addition, cultures may be insensitive, as some organisms may not grow under certain circumstances, and others may only be present in very low concentrations below the threshold of sensitivity for the blood culture system used [50]. Hence, a definitive microbiological diagnosis can only be made in two-thirds of patients with clinical sepsis [51]. Leukocytosis and band counts of peripheral blood also have low diagnostic accuracy for sepsis [52].

Rapid diagnosis of sepsis is important in order to start appropriate antimicrobial and other therapy, because even a 1-h delay in beginning antibiotic therapy increases mortality of infection and sepsis by 5–10% [41]. Alternative diagnostic methods, such as molecular-based testing, increase sensitivity and specificity and decrease time to results.

Biomarkers can also decrease the time to results, as the assays are more typically rapid than conventional cultures. Biomarkers have been studied for their roles in many different aspects of sepsis, such as distinguishing sepsis from non-septic patients with systemic inflammatory response syndrome (SIRS). The role of biomarkers in determining the severity of sepsis for prognostic purposes has been assessed, in addition to the differentiation of bacterial from viral or other causes of infection [20, 53]. Biomarkers have also been used to guide antibiotic therapy, to differentiate gram-negative from gram-positive microorganisms as the cause of sepsis, and to evaluate response to therapy [20, 54].

Investigators have studied hundreds of biomarkers in an effort to identify sensitive, specific, and rapid markers of sepsis, more so than in many other disease processes [55]. Of the available biomarkers, acute phase proteins are the best studied, particularly PCT in recent years. Many potential biomarkers of sepsis have been proposed, but PCT has been the most frequently assessed and has been integrated into practice guidelines and routine testing in some clinical laboratories [56]. C-reactive protein has been utilized as a biomarker for many years, but its specificity is relatively low [57–59]. In their review of 3,370 studies on biomarkers of sepsis, Pierrakos and Vincent found that 178 different biomarkers were evaluated amongst the studies [55].

Some of these biomarkers were evaluated only in experimental studies, and some were evaluated in clinical studies. An overview of the most commonly reported biomarkers will be presented, as will some novel biomarkers which appear promising for future use in clinical diagnostic laboratories.

The Roles of PCT in Sepsis

Studies have shown that PCT is generally a useful and accurate biomarker for bacterial infection [18, 20]. Several studies have reported significantly elevated PCT levels in patients with sepsis, compared to patients without sepsis [18, 20, 21, 31]. However, most studies have been somewhat small in number with fewer than 200 patients. The studies have used different methodologies and different cutoff values for PCT. Uzzan and colleagues performed a meta-analysis of 25 PCT studies, with a subanalysis on 15 studies to compare PCT to CRP [60]. In that meta-analysis, the sensitivities of PCT for the diagnosis of systemic infection ranged from 42 to 100%; the sensitivities for CRP ranged from 35 to 100%. The global diagnostic accuracy odds ratio for the diagnosis of systemic infection for PCT was 15.7 (95% confidence interval [CI] 9.1–27.1), while the odds ratio was 5.4 for CRP (95% CI 3.2–9.2). Uzzan et al. concluded that PCT was a helpful diagnostic marker for sepsis. However, results reported by Tang et al. in their meta-analysis suggested that PCT did not perform as well, with mean specificity and sensitivity of 71% [61]. The authors concluded that PCT could not reliably distinguish sepsis from noninfectious causes of SIRS in adults.

PCT levels appear to rise with increasing severity of sepsis. In a large prospective multicenter observational study, blood was collected within 24 h of onset of sepsis in 1,156 hospitalized patients [62]. Among patients in the intensive care unit (ICU), mortality was significantly higher for patients with PCT concentrations of at least 0.85 ng/mL.

Several studies have demonstrated that PCT is a useful prognostic marker of response to treatment and is associated with improved outcomes [18, 54, 63–66]. A rising PCT level may be indicative of an infectious process which is not resolving, as suggested by Giamarellos-Bourboulis et al. [53]. Nobre and colleagues performed a small, randomized open-label study comparing PCT-guided antibiotic duration against usual care [54]. The authors found a significant reduction in antibiotic treatment duration among patients whose therapy was guided by PCT levels. The use of PCT levels to follow respiratory tract infections and sepsis appears to reduce antibiotic exposure without worsening mortality rate [67]. As shown by Reinhart and Hartog in their review of 11 randomized controlled trials, the use of PCT resulted in 35–70% reduction in antibiotic use [68]. The systematic review by Agarwal and Schwartz which evaluated the efficacy and safety of PCT-guided antimicrobial therapy among ICU patients showed that PCT was associated with significant reductions in antimicrobial therapy in five of six studies [69]. On the other hand, infection

relapse and mortality were not changed in any of the studies. The PRORATA trial (PROcalcitonin to Reduce Antibiotic Treatments in Acutely ill patients) was the largest randomized PCT trial to date which compared PCT-guided antibiotic therapy to usual care for patients with bacterial sepsis [70]. Predefined algorithms for initiation or discontinuation of antibiotics based on serum PCT levels were used for the 621 ICU patients in the PRORATA trial. The duration of antibiotic treatment decreased for those patients in the PCT-guided group, without apparent detrimental patient outcomes.

Not all studies have appeared as promising as those mentioned above. The Procalcitonin and Survival Study (PASS) group demonstrated that PCT-guided antimicrobial escalation in the ICU did not improve survival and, in fact, led to organ-related damage and prolonged stay in the ICU [71].

Some studies have attempted to utilize PCT to distinguish between gram-negative and gram-positive infections, but the data have not been conclusive [20, 33, 63]. PCT has also been used in an attempt to distinguish viral and fungal infections from bacterial infection. PCT levels during viral infections remain at low levels, similar to those measured in healthy persons [19, 20, 65]. Fungal infections, on the other hand, tend to cause mild elevations in PCT levels, as compared to bacterial infections [19–21, 32, 63].

Systemic reviews have shown that PCT is superior to CRP in sensitivity [60]. In comparison to CRP, PCT is also a more specific marker of bacterial infection [72, 73]. The levels of PCT rise earlier than CRP and seem to correlate more closely with disease severity [74]. PCT may be a more specific biomarker than CRP, but some investigators have challenged its prognostic value [21, 61, 75]. Some studies suggest that PCT is not necessarily a more reliable marker of sepsis than CRP [61, 76, 77]. PCT appears to be an advance over CRP in the diagnosis of sepsis. However, given that healthcare providers have had experience with CRP in the past, and that many know it to be a relatively nonspecific marker of inflammation, some investigators believe that insufficient evidence exists to rely solely upon PCT for the initial diagnosis of sepsis [11].

In summary, PCT may result in false negative results at admission, particularly if specimens are collected too early in the course of infection or sepsis [78]. In such situations, a repeat test may be worthwhile at 6–12 h [13]. However, if all microbial cultures are negative and a clear source of infection has not manifested by 24 h, a repeat low PCT level, in conjunction with clinical judgment, may be helpful in guiding discontinuation of antibiotics or in searching for another source of the infection [11]. PCT and CRP have been helpful in the identification of serious infections in febrile pediatric patients, but different cutoff values for the assays should be further studied in order to increase the yield of these assays in children [79]. PCT appears to be an improvement over CRP and may be better used to rule out infection than to rule in systemic sepsis in a critically ill patient, due to its relatively low specificity [11].

The Roles of CRP in Sepsis

C-reactive protein is a general acute phase protein of the family of calcium-dependent ligand-binding plasma proteins. It is synthesized primarily by hepatocytes in response to stimulation by cytokines such as IL-6 and is a marker of inflammation. Serum CRP levels vary with age in healthy individuals and children [39, 80]. The plasma half-life of CRP is approximately 19 h [81]. In healthy adults, the plasma concentration of CRP is approximately 0.8 mg/dL [82, 83]. During infection or acute inflammation, CRP values rise over 10,000-fold [84]. CRP rises in response to infection after a delay of 12–24 h and peaks at 36 h [13, 23, 85, 86]. Since it rises relatively slowly, it may not be a highly sensitive marker of infection during initial assessment. Yet, CRP is more sensitive as a marker of sepsis than peripheral white blood cell count or body temperature, but it lacks specificity [87]. Increased levels of CRP have been associated with many other conditions, including ankylosing spondylitis, psoriatic arthritis, polymyalgia rheumatica, and rheumatoid arthritis [81]. CRP levels can overlap between infected and noninfected patients; therefore, the absolute levels may not be as important as the trend of CRP levels over time. Since CRP is produced by the liver, CRP levels do not always increase in patients with sepsis and concurrent fulminant liver failure [88].

Elevated CRP levels have been correlated with an increased risk of organ failure and death [89]. Sequential measurement has been utilized to evaluate response to therapy in septic patients [58]. CRP does not appear to be helpful in differentiating gram-negative from gram-positive infections [90]. Other authors have used CRP to distinguish between bacterial and fungal infections, with levels above 100 mg/L as indicative of bacterial infection [32, 91]. Most data suggest that CRP alone is an unreliable marker for viral infection [92, 93].

CRP may be more helpful in ruling out than ruling in sepsis [81]. The wide range of diagnostic accuracy reported in the literature is mainly due to the wide range of cutoff values reported in different studies [16]. There are several different assays and platforms which are currently available for CRP measurement with unique detection limits.

The main advantages of CRP testing include its availability, low cost, and familiarity of practitioners with its use. However, it is not specific for sepsis, as CRP levels may be increased in other inflammatory conditions.

The Roles of CRP and PCT in Infectious States Other than Sepsis

PCT also shows potential as a biomarker for other disease processes, including pneumonia, abdominal infections, urinary tract infections, and lower respiratory tract infections [2, 3, 6, 7, 10]. Chen et al. evaluated the role of PCT in the early detection of central venous catheter-related bloodstream infections in patients after

orthotopic liver transplantation [5]. Their study revealed that PCT may be a rapid, useful tool for the diagnosis of bacteremia while awaiting blood culture results, in order to facilitate earlier removal of the central venous catheter if PCT levels are elevated. Some other studies have shown that PCT may be useful as a biomarker to diagnose healthcare-associated infection in critically ill patients [94, 95]. Dallas et al. conducted a study of antimicrobial therapy in patients with hospital-acquired pneumonia (HAP) or VAP. They found that PCT measurements had little diagnostic value in such situations [96]. Blood PCT measurements may aid in differentiating viral from mixed viral–bacterial pneumonia in patients during influenza season [97]. For neonates, serum PCT at onset of sepsis appears to show good accuracy [98].

Some studies have demonstrated that PCT levels may be associated with improved outcomes in acute respiratory infections [99]. Agapakis and colleagues demonstrated that CRP levels of 100 mg/L were prognostic in determining the severity of CAP and the need for hospitalization [100]. PCT has been well studied for its use in the decision-making process of administration of antibacterial agents in patients presenting with pneumonia. In two studies, clinicians were encouraged to avoid prescription of antimicrobials in patients with PCT levels of <0.1 mcg/L but to prescribe antimicrobials if PCT levels measured >0.25 mcg/L [101, 102]. PCT guidance in lower respiratory tract infections substantially reduced antibiotic use, with an adjusted relative risk of antibiotic exposure of 0.49 in the PCT group. Several large, randomized controlled studies have shown significant decreases in antibiotic use without obvious harm when daily or serial PCT measurements were used to guide antibiotic treatment for patients with infections. Such studies have included patients with lower respiratory tract infections, exacerbations of chronic obstructive pulmonary disease (COPD), and CAP [4, 102–104]. In patients with VAP, Stolz and colleagues found that PCT use increased the number of antibiotic-free days alive, with an overall reduction in antibiotic exposure of 27% [2].

The Roles of Miscellaneous and Novel Biomarkers in Infectious States

Other biomarkers have been examined for a variety of roles in the diagnosis and/or management of infectious diseases.

Serum Amyloid A

Serum amyloid A (SAA) is an apolipoprotein with a potential for use in sepsis diagnosis. SAA is expressed at levels 1,000 times higher within 8–24 h after the onset of sepsis [17, 105]. SAA levels of less than 15 mg/L for elderly persons and less than 10 mg/L for other adults and newborns are seen in healthy individuals [80]. Compared to CRP, SAA levels rise faster and higher after the onset of sepsis and

remain higher for a longer period of time [106]. Similar to PCT and CRP, SAA has demonstrated a variety of diagnostic accuracies and cutoff values based on the assays used.

Lipopolysaccharide-Binding Protein

Lipopolysaccharide-binding protein (LBP) is an acute phase protein synthesized by the liver which rises in concentration 20–40 times within 24 h of an inflammatory response [107]. LBP levels increase during bacterial and fungal infections but not during viral infections [108]. In a pediatric study, LBP showed the highest combination of sensitivity and specificity in differentiating sepsis from noninfectious SIRS, as compared to PCT, CRP, and soluble CD14 in children and neonates younger than 48 h [108]. In a prospective cross-sectional study of prognostic markers of sepsis in adults, LBP showed higher specificity in patients with sepsis than PCT and CRP, as compared to healthy controls [109].

Coagulation Markers

Certain coagulation markers, such as activated partial thromboplastin time (aPTT) waveform analysis, offer some promise as biomarkers of sepsis. The optical transmission biphasic waveform is related to calcium-dependent formation of complexes between very low density lipoprotein and CRP. In a study of 331 ICU patients, a biphasic waveform was a highly specific measure of sepsis (92–98% specific) when measured on admission to the ICU [110]. Chopin et al. showed that a biphasic waveform was more useful than PCT or CRP in distinguishing severe sepsis and septic shock from SIRS and sepsis [111].

Angiopoietins

Angiopoietins (Angs) comprise a family of vascular growth factors which act on endothelial cells. Ang-1 and Ang-2 are secreted from endothelial cells. Ang-1 stabilizes the endothelium, preventing vascular leakage, inflammation, and the recruitment and transmigration of leukocytes [112]. Ang-2 promotes leaky endothelium. Ang-1 exerts its action by binding to the Tie2 receptor (tyrosine kinase receptor with immunoglobulin and epidermal growth factor domains). Ang-2 is stored with von Willebrand factor in platelets and is released from monocytes and endothelial cells in septic shock [113]. The actions of Ang-1 and Ang-2 antagonize each other, and Ang-2 competes with Ang-1 for binding to the Tie2 receptor [114]. In animals, infusion of lipopolysaccharide stimulates the expression of Ang-2 and attenuates gene expression of Ang-1 [115].

Numerous studies have shown that Ang-2 expression is increased in sepsis [116, 117]. Some studies have demonstrated increased Ang-2/Ang-1 ratios in patients who did not survive sepsis [118, 119]. Ricciuto and colleagues assessed the ability of a panel of biomarkers including Angs to predict outcome in sepsis [120]. The panel included Ang-1, Ang-2, von Willebrand factor, soluble intercellular adhesion molecule-1, and E-selectin. The panel was successful in predicting mortality. Ang-1 has itself been an independent factor related to unfavorable outcome in infection [113]. Activated protein C, which is used in the treatment of sepsis, increases the level of Ang-1 and decreases the level of Ang-2 in vitro [121]. One study of Angs in patients with invasive streptococcal infections demonstrated greater dysregulation of Angs in patients with shock than in those without shock [122].

Neopterin

Neopterin is a mediator of cell immunity against intracellular pathogens. It has been used to discriminate between bacterial and viral origins of lower respiratory tract infections [123, 124]. Neopterin correlates with severity of infection in patients with CAP [125]. In patients with exacerbations of COPD, lower levels of neopterin were measured when patients had respiratory cultures positive for pathogenic bacteria, as opposed to those who had cultures growing only normal respiratory flora [126].

Toll-Like Receptor-2

Toll-like receptor-2 (TLR-2) is present on cell surfaces and is upregulated on monocyte cell surfaces during infection. Kajiya et al. found significantly elevated TLR-2 expression in viral and bacterial infections [92]. However, the role of TLR-2 as a biomarker of infections remains poorly understood.

Triggering Receptor Expressed on Myeloid Cells-1

The plasma concentration of triggering receptor expressed on myeloid cells-1 (TREM-1), a member of the immunoglobulin superfamily, is upregulated in the presence of bacterial products but not during noninfectious inflammatory disease states [64]. TREM-1 is increased in patients with sepsis and appears to be both sensitive and specific for the diagnosis of sepsis [127, 128]. Gibot et al. reported significantly high plasma TREM-1 levels in patients with sepsis as compared to patients with non-septic SIRS [129]. Serial monitoring of TREM-1 may also be helpful in following the course of infection [127, 128]. TREM-1 measurements in

bronchoalveolar lavage fluid are accurate for the diagnosis of pneumonia [130]. However, TREM-1 has not been as extensively studied as PCT and CRP.

Soluble Urokinase-Type Plasminogen Activator Receptor

Urokinase-type plasminogen activator receptor (UPAR) is expressed on neutrophils, lymphocytes, macrophages, endothelial cells, and malignant cells. The soluble form of UPAR, or soluble urokinase-type plasminogen activator receptor (suPAR) has been reported as a potential biomarker for infectious diseases [131, 132]. Yilmaz et al. assessed the abilities of suPAR, CRP, and PCT to diagnose and provide prognostic information for patients with SIRS [131]. SuPAR possessed high sensitivity and specificity in the diagnosis of SIRS; additionally, high levels were shown to be poorly prognostic [131]. SuPAR shows potential prognostic value over a wide range of infectious diseases, including viral, bacterial, and parasitic diseases [133].

Soluble Receptor for Advanced Glycation End Products

The soluble form of the receptor for advanced glycation end products (sRAGE) has been used as a biomarker in patients with acute lung injury [134]. RAGE is a transmembrane pattern-recognition receptor of the immunoglobulin superfamily. It is constitutively expressed in low levels in all cells but most abundantly in the lung [135]. When RAGE is activated, cell signaling pathways result in an inflammatory response. In a acute respiratory distress syndrome study, plasma levels of sRAGE correlated with severity of acute lung injury [136]. The elevated levels of sRAGE measured during acute lung injury were independent of associated sepsis [134]. Sepsis has also been associated with increased levels of plasma sRAGE [137].

CD64

Neutrophil CD64 (nCD64) is a highly sensitive and specific marker of systemic inflammation and sepsis [138]. CD64 expression on neutrophils is present at low levels in the normal state [47]. When tissue injury occurs in the setting of inflammation, nCD64 expression increases [139]. nCD64 has been used as a biomarker for bacterial infection, but its expression does not differ between systemic and local bacterial infections [140, 141]. nCD64 is helpful in guiding antibiotic therapy; if nCD64 levels are negligible within 24 h of infection, antibiotics may be discontinued [142]. nCD64 may be measured in ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood and is stable at least 24 h at room temperature [47].

HLA-DR

HLA-DR expression is a marker of monocytes which generate proinflammatory cytokines in response to a stimulus [114]. HLA-DR is strongly expressed on monocyte surfaces at baseline. Monocytes with diminished HLA-DR expression have decreased antigen-presenting ability and cannot produce inflammatory mediators in response to a stimulus [44, 143]. In septic patients, decreased cell surface expression of HLA-DR has been observed in circulating monocytes [44]. Low levels of monocyte HLA-DR have been associated with adverse outcomes and septic complications in critically ill patients, particularly when the levels are persistently low [44, 144–147]. In injured patients who recover, monocyte HLA-DR rapidly returns to normal levels, generally in less than 1 week.

Presepsin

Presepsin, also known as sCD14-subtype, is believed to be a soluble fraction of CD14 circulating in blood and is the receptor for the LBP complex [148]. CD14 is present in macrophages, monocytes, and granulocytes and is thought to be responsible for intracellular transduction of endotoxin signals [148]. It is produced in response to bacterial infection. A study has shown that presepsin increased proportionally to the severity of sepsis [149]. Presepsin performed well as compared to other diagnostic markers of sepsis, including PCT, CRP, and IL-6.

Cytokines

The cytokines comprise a group of compounds which are currently well studied as potential biomarkers of sepsis. As important mediators in the complex pathway of sepsis, they are produced early after the onset of sepsis [55]. However, blood cytokine measurements can be erratic, which render interpretation difficult [150]. Circulating cytokines have short half-lives, which can result in false negative results [18, 106]. Various interleukins have been proposed as potential biomarkers [151]. Measurement of IL-6 levels has not proven to be useful in identifying patients who might benefit from treatment [152]. The cytokines IL-1β, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor-α (TNF-α), and interferon-(IFN)-γ were compared in one study to several other biomarkers, including PCT, soluble CD14, heparin-binding protein (HBP), cortisol, and monocyte expression of TLR-2, TLR-4, HLA-DR, and CD14 [7]. The study included 54 adult patients with bacterial infections, 21 patients with viral infections, and patients with acute CAP, pyelonephritis, and urosepsis. Serum PCT, HBP, IL-6, and cortisol showed the highest sensitivity and specificity for bacterial infection. Serum levels of IFN-γ and cortisol were higher and IL-8 levels lower in patients with pneumonia, as compared to patients with pyelonephritis

and urosepsis. In another study, Zetioun et al. reported that IL-10 may be helpful in the diagnosis of early- and late-onset sepsis in neonates [85]. Ng and colleagues reported sensitivity and specificity of >80% for IL-10 in the diagnosis of late-onset sepsis in very low birth weight infants [153]. Increases in TNF and IL-6 were seen within the first 24 h after patient admission to the ICU due to sepsis [150]. Serum cytokines have also been assessed for their roles in the diagnosis and outcome assessment of invasive aspergillosis [8, 154].

Proadrenomedullin

Proadrenomedullin (pro-ADM) belongs to the calcitonin peptide superfamily and exerts various vasodilatory, immune modulatory, and bactericidal effects [99]. Guignant et al. showed that pro-ADM and pro-vasopressin (copeptin), when measured within the first week after the onset of shock, were significantly elevated in patients who died [155]. Another group of investigators successfully utilized pro-ADM with the CURB65 risk assessment score (a severity assessment score for CAP) to predict adverse events and mortality in patients with CAP and with non-CAP lower respiratory tract infections [156].

Alpha-1 Antitrypsin and Other Hepatitis Biomarkers

One of the most common protease inhibitors in human serum, alpha-1 antitrypsin, is significantly elevated in serum samples from patients with severe chronic hepatitis [157]. Other potential biomarkers which have been examined for their roles in infectious hepatitis include plasma soluble human leukocyte antigen-G and serum microRNA 122 (miR-122) [9, 158].

Gelsolin

A plasma protein, gelsolin is an actin scavenger which severs and caps actin filaments which are released during tissue injury [114]. Gelsolin also binds to inflammatory mediators. In critically ill surgical patients, plasma gelsolin levels remained low compared to control patients [159].

Interferon-Gamma-Inducible Protein-10

Interferon-gamma-inducible protein-10 (IP-10) is a proinflammatory chemokine which is a promising biomarker for the diagnosis of viral infection [153]. IP-10 is released in response to rhinovirus, respiratory syncytial virus, hepatitis B and C

viruses, and H5N1 influenza virus [160, 161]. IP-10 may also correlate with severity of infection [153]. It has been used as a prognostic marker to guide treatment of patients with hepatitis C [162].

Neutrophil Distribution Width

Increased neutrophil distribution width (NDW) has been associated with left-shifted and reactive neutrophils [163]. NDW was compared to CRP and PCT in the detection of early sepsis in the ICU [164]. In that study, NDW was found on multivariate analysis to be helpful in the diagnosis of early sepsis in adults.

Regulatory T Lymphocytes (Treg)

During severe sepsis, T lymphocyte dysfunction leads to an anergic state, with decreased proliferation to mitogens, increased Th2 cytokine secretion, apoptosis, and increased percentage of CD4+CD25+ regulatory T lymphocytes (Treg) [43, 44, 165, 166]. An increased percentage of Treg has been described in patients with septic shock as compared to healthy states [165, 167]. CD127 gating on the CD4+CD25+ cell population allows differentiation of regulatory from activated T cells. A significant increase in the percentage of circulating CD4+CD25+CD127(low) Treg has been demonstrated in septic shock patients in comparison to healthy individuals [168].

Summary of Miscellaneous Biomarkers

Pierrakos and Vincent performed a review of the roles of over 178 biomarkers in sepsis diagnosis [55]. The authors described five biomarkers with sensitivities and specificities of over 90% which may be particularly helpful for the early diagnosis of sepsis. The five biomarkers and their roles include: (1) IL-12 in newborns, which showed higher levels in patients with sepsis than in patients without sepsis [169]; (2) IP-10 in neonates, which showed higher levels in patients with sepsis and necrotizing enterocolitis than in patients who had necrotizing enterocolitis without sepsis [153]; (3) phospholipase group II (PLA2-II), which was useful in the diagnosis of bacteremia in critically ill adult patients within 1 day of admission [170]; (4) CD64, useful in the early diagnosis of sepsis in adults [141]; and (5) neutrophil CD11b, which was helpful in pediatric patients in distinguishing culture-positive sepsis from patients with a septic clinical picture but who were culture-negative [171].

The remaining 11 biomarkers in their review which were assessed in various studies specifically for the early diagnosis of sepsis had reported sensitivities and specificities of less than 90%. Pierrakos and Vincent also assessed the prognostic ability of the reviewed biomarkers to differentiate those patients who are likely to survive from those who are likely to die [55]. However, according to the authors, none of the biomarkers in their opinion had sufficient ability to predict mortality with adequate (>90%) sensitivity and specificity [55].

Certain biomarkers can be helpful in ruling out sepsis due to high NPVs. Three biomarkers seem particularly useful in this regard: (1) PCT with 99% NPV when a cutoff of 0.2 ng/mL is employed [172]; (2) aPTT biphasic waveform with 96% NPV [173]; and (3) fibrin degradation products, with 100% NPV for gram-negative sepsis [174].

Since the use of a single biomarker in the septic patient is limited by either test availability or performance characteristics, it may be helpful to measure several biomarkers together in combination. A clinical trial showed that the combination of aPTT waveform and PCT increased the specificity of the aPTT waveform in the diagnosis of sepsis [173]. Other studies employing a variety of panels of biomarkers have shown some success in the use of a biomarker panel [175–177].

Newer Platforms for Measurement of Biomarkers

Microarrays may aid in understanding the complexity of the host response to sepsis at the level of transcription of messenger RNA (mRNA), by measuring many different potential biomarkers at one time [178, 179]. The microarray platform is a system whereby various patterns of upregulated or downregulated mRNA levels can be compared [179]. Microarray studies have shown that apoptotic genes are highly expressed in inflammatory states, and that proinflammatory response genes decrease over time in sepsis [114]. A microarray panel of 42 gene expression markers representing many different immunologic and cellular response pathways was found in one study to be helpful in the early diagnosis of sepsis [180]. Difficulties inherent to the use of an mRNA microarray system include the marked variation within the same individual of gene expression over time, the interindividual variation due to complexities of the host inflammatory response, and the differences in gene expression in different areas of the body.

Functional proteomics is the study of synthesized proteins and their relationship to health and disease. Two systems currently studied for a role in identification of biomarkers in disease states include matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and surface-enhanced laser desorption/ionization coupled to time-of-flight mass spectrometry (SELDI-TOF MS) [181, 182].

Conclusions and Future Directions

Numerous studies exist which assess the roles of non-antibody-based host response biomarkers in disease. The most commonly studied biomarkers include PCT and CRP, but cytokines and other markers related to endothelial dysfunction and inflammation are also under investigation. Despite ongoing research, there are problems inherent to the study of biomarkers. For example, sensitivities and specificities are difficult to establish, as a gold standard of sepsis diagnosis against which to measure performance characteristics is not available. Typically, the efficacy of a biomarker must be compared to current methods of diagnosis, which is usually a combination of clinical signs and traditional laboratory values. Small sample sizes, heterogeneous population types, and differences in assay equipment are also problematic in biomarker research. Importantly, cutoff values for biomarkers differ greatly from study to study. Therefore, direct comparison of testing methods and results between studies can be difficult. Of the many assays available to measure biomarkers, only a few are currently applicable for use in the clinical laboratory. Despite the large number of biomarker publications, differentiation of sepsis from other noninfectious causes of systemic inflammatory responses continues to remain problematic.

Further studies relating specifically to test costs, reliability of testing, and rapidity of testing for biomarkers are needed. Standardization of assay methodologies and systematic comparison of different systems should also be performed. Additionally, published reports should provide more detailed information about the platforms employed in analyte measurement and calibration characteristics. Given the potential uses of biomarkers, cost-effectiveness studies of their applications would be helpful. Development of multiplex point-of-care biomarker testing for triaging patients in emergency care or ICU settings is needed. Biomarker research of non-antibody-based host responses will continue to grow. However, the exact roles of biomarkers in the management of septic patients and patients in a wide variety of infections remain undefined (55). Clear consensus guidelines concerning the use of such testing would be invaluable in defining applications of biomarkers in the future.

References

1. Biomarkers Definitions Working Group (2001) Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 69:89–95
2. Stolz D, Smyrnios N, Eggimann P et al (2009) Procalcitonin for reduced antibiotic exposure in ventilator-associated pneumonia: a randomized study. Eur Respir J 34:1364–1375
3. Masia M, Gutierrez F, Shum C et al (2005) Usefulness of procalcitonin levels in community-acquired pneumonia according to patients outcome research team pneumonia severity index. Chest 128:2223–2229
4. Schuetz P, Christ-Crain M, Thomann R et al (2009) Effect of procalcitonin-based guidelines vs standard guidelines on antibiotic use in lower respiratory tract infections: the ProHOSP randomized controlled trial. JAMA 302:1059–1066

5. Chen J, Wang Y, Shen Z, Zhu Z, Song Y, Han R (2011) Early diagnostic value of plasma PCT and BG assay for CRBSI after OLT. Transplant Proc 43:1777–1779
6. Tsangaris I, Plachouras D, Kayatha D et al (2009) Diagnostic and prognostic value of procalcitonin among febrile critically ill patients with prolonged ICU stay. BMC Infect Dis 9:213–221
7. Chalupa P, Beran O, Herwald H, Kasprikova N, Holub M (2011) Evaluation of potential biomarkers for the discrimination of bacterial and viral infections. Infection 39:411–417.
8. Chai LA, Netea MG, Teerenstra S et al (2010) Early proinflammatory cytokines and C-reactive protein trends as predictors of outcome in invasive aspergillosis. J Infect Dis 202: 1454–1462
9. Bihrer V, Friedrich-Rust M, Kronenberger B et al (2001) Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. Am J Gastroenterol 106:1663–1669
10. Marshall JC, Reinhart K (2009) Biomarkers of sepsis. Crit Care Med 37:2290–2298
11. Kibe S, Adams K, Barlow G (2011) Diagnostic and prognostic biomarkers of sepsis in critical care. J Antimicrob Chemother 66:ii33–40
12. Dahaba AA, Metzler H (2009) Procalcitonin's role in the sepsis cascade. Is procalcitonin a sepsis marker or mediator? Minerva Anestesiol 75:447–452
13. Shehabi Y, Seppelt I (2008) Pro/con debate: Is procalcitonin useful for guiding antibiotic decision making in critically ill patients? Crit Care 12:211–216
14. Charles PE, Ladoire S, Aho S et al (2008) Serum procalcitonin elevation in critically ill patients at the onset of bacteremia caused by either gram negative or gram positive bacteria. BMC Infect Dis 8:38
15. Perren A, Cerutti B, Lepori M et al (2008) Influence of steroids on procalcitonin and C-reactive protein in patients with COPD and community-acquired pneumonia. Infection 36:163–166
16. Chan T, Gu F (2011) Early diagnosis of sepsis using serum biomarkers. Expert Rev Mol Diagn 11:487–496
17. Cetinkaya M, Ozkan H, Koksal N, Akaci O, Ozgur T (2009) Comparison of serum amyloid A concentrations with those of C-reactive protein and procalcitonin in diagnosis and follow-up of neonatal sepsis in premature infants. J Perinatol 20:225–231
18. Arnon S, Litmanovitz I (2008) Diagnostic tests in neonatal sepsis. Curr Opin Infect Dis 31:223–227
19. Deis JN, Creech CB, Estrada CM, Abramo TJ (2010) Procalcitonin as a marker of severe bacterial infection in children in the emergency department. Pediatr Emerg Care 26:51–63
20. Becker KL, Snider R, Nylen ES (2008) Procalcitonin assay in systemic inflammation, infection, and sepsis: clinical utility and limitations. Crit Care Med 36:941–952
21. Nakamura A, Wada H, Ikejiri M et al (2009) Efficacy of procalcitonin in the early diagnosis of bacterial infections in a critical care unit. Shock 31:586–591
22. Dong H, Shu W, Liu T et al (2010) Targeting procalcitonin with novel murine monoclonal antibodies. Hybridoma 29:189–194
23. Limper M, de Kruif MD, Duits AJ, Brandjes DP, van Gorp EC (2010) The diagnostic role of procalcitonin and other biomarkers in discriminating infectious from non-infectious fever. J Infect 60:409–416
24. Lacour AG, Gervaix A, Zamora SA et al (2011) Procalcitonin, IL-6, IL-8, IL-1 receptor antagonist and C-reactive protein as identificators of serious bacterial infections in children with fever without localizing signs. Eur J Pediatr 160:95–100
25. Schuetz P, Christ-Crain M, Huber AR, Muller B (2010) Long-term stability of procalcitonin in frozen samples and comparison of Kryptor and VIDAS automated immunoassays. Clin Biochem 43:341–344
26. Manzano S, Bailey B, Girodias JB, Cousineau J, Delvin E, Gervaix A (2009) Comparison of procalcitonin measurement by a semi-quantitative method and an ultra-sensitive quantitative method in a pediatric emergency department. Clin Biochem 42:1557–1560

27. Mimoz O, Benoist JF, Edouard AR, Assicot M, Bohuon C, Samii K (1998) Procalcitonin and C-reactive protein during the early posttraumatic systemic inflammatory response syndrome. Intensive Care Med 24:185–188
28. Meisner M, Tschaikowsky K, Hutzler A, Schick C, Schuttler J (1998) Postoperative plasma concentrations of procalcitonin after different types of surgery. Intensive Care Med 24:680–684
29. Hensel M, Volk T, Docke WD et al (1998) Hyperprocalcitonemia in patients with noninfectious SIRS and pulmonary dysfunction associated with cardiopulmonary bypass. Anesthesiology 89:93–104
30. Opatrna S, Klaboch J, Opatrny K Jr et al (2005) Procalcitonin levels in peritoneal dialysis patients. Perit Dial Int 25:470–472
31. Cardelli P, Ferraironi M, Amode R et al (2008) Evaluation of neutrophil CD64 expression and procalcitonin as useful markers in early diagnosis of sepsis. Int J Immunopathol Pharmacol 21:43.49
32. Martini A, Gottin L, Menestrina N, Schweiger V, Simion D, Vincent JL (2010) Procalcitonin levels in surgical patients at risk of candidemia. J Infect 60:425–430
33. Kim KE, Han JY (2010) Evaluation of the clinical performance of an automated procalcitonin assay for the quantitative detection of bloodstream infection. Korean J Lab Med 30:153–159
34. Galetto-Lacour A, Zamora SA, Gervaix A (2003) Bedside procalcitonin and C-reactive protein tests in children with fever without localizing signs of infection seen in a referral center. Pediatrics 112:1054–1060
35. Enguix A, Rey C, Concha A, Medina A, Coto D, Diequez MA (2001) Comparison of procalcitonin with C-reactive protein and serum amyloid for the early diagnosis of bacterial sepsis in critically ill neonates and children. Intensive Care Med 27:211–215
36. http://www.procalcitonin.com. Accessed 12 September 2011
37. http://www.medical.siemens.com. Accessed 12 September 2011
38. Morgenthaler NG, Struck J, Fischer-Schulz C, Bergmann A (2002) Sensitive immunoluminometric assay for the detection of procalcitonin. Clin Chem 48:788–790
39. Fioretto JR, Martin JG, Kurokawa CS et al (2010) Comparison between procalcitonin and C-reactive protein for early diagnosis of children with sepsis or septic shock. Inflamm Res 59:581–586
40. Schneider HG, Lam QT (2007) Procalcitonin for the clinical laboratory: a review. Pathology 39:383–390
41. Reinhart K, Meisner M (2011) Biomarkers in the critically ill patient: procalcitonin. Crit Care Clin 27:253–263
42. De Waele JJ, Hoste E, Blot S, Vogelaers D (2008) The value of procalcitonin to diagnose infection in critically ill patient: caveat emptor! [letter to editor]. Crit Care Med 36:3121
43. Hotchkiss RS, Karl IE (2003) The pathophysiology and treatment of sepsis. N Engl J Med 348:138–150
44. Monneret G, Venet F, Pachot A, Lepap A (2008) Monitoring immune dysfunction in the septic patient: a new skin for the old ceremony. Mol Med 14:64–78
45. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (1992) Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Crit Care Med 20:864–874
46. Hunter P (2006) Sepsis under siege—a new understanding of sepsis might lead to development of therapies to treat septic shock. EMBO Rep 7:667–669
47. Venet F, Guignant C (2011) Monneret. Flow cytometry developments and perspectives in clinical studies: examples in ICU patients. Methods Mol Biol 761:261–275
48. Calvano SE, Xiao W, Richards DR et al (2005) A network-based analysis of systemic inflammation in humans. Nature 437:1032–1037
49. Lever A, Mackenzie I (2007) Sepsis: definition, epidemiology, and diagnosis. BMJ 335: 879–883

50. Maubon D, Hamidfar-Roy R, Courby S et al (2010) Therapeutic impact and diagnostic performance of multiplex PCR in patients with malignancies and suspected sepsis. J Infect 61:335–342
51. Vincent J-L, Sakr Y, Sprung CL et al (2006) Sepsis in European intensive care units: results of the SOAP study. Crit Care Med 34:344–353
52. Davis GH, Bigelow NC (2005) Comparison of neutrophil CD64 expression, manual myeloid immaturity counts, and automated hematology analyzer flags as indicators of infection or sepsis. Lab Hematol 11:137–147
53. Giamarellos-Bourboulis EJ, Mega A, Grecka P et al (2002) Procalcitonin: a marker to clearly differentiate systemic inflammatory response syndrome and sepsis in the critically ill patient? Intensive Care Med 28:1351–1356
54. Nobre V, Harbarth S, Graf JD, Rohner P, Pugin J (2008) Use of procalcitonin to shorten antibiotic treatment duration in septic patients: a randomised trial. Am J Respir Crit Care Med 177:498–505
55. Pierrakos C, Vincent JL (2010) Sepsis biomarkers: a review. Crit Care 14:R15
56. O'Grady NP, Barie PS, Bartlett JG et al (2008) Guidelines for evaluation of new fever in critically ill adult patients: 2008 update from the American College of Critical Care Medicine and the Infectious Diseases Society of America. Crit Care Med 36:1330–1349
57. Povoa P, Coehlo L, Almeida E et al (2005) C-reactive protein as a marker of infection in critically ill patients. Clin Microbiol Infect 11:101–108
58. Schmit X, Vincent JL (2008) The time course of blood C-reactive protein concentrations in relation to the response to initial antimicrobial therapy in patients with sepsis. Infection 36:213–219
59. Clyne B, Olshaker JS (1999) The C-reactive protein. J Emerg Med 17:1019–1025
60. Uzzan B, Cohen R, Nicolas P, Cucherat M, Perret GY (2006) Procalcitonin as a diagnostic test for sepsis in critically ill adults and after surgery or trauma: a systematic review and meta-analysis. Crit Care Med 34:1996–2003
61. Tang BMP, Eslick GD, Craig JC, McLean AS (2007) Accuracy of procalcitonin for sepsis diagnosis in critically ill patients: systematic review and meta-analysis. Lancet Infect Dis 7:210–217
62. Giamarellos-Bourboulis EJ, Tsangaris I, Kanni T et al (2011) Procalcitonin as an early indicator of outcome in sepsis: a prospective observational study. J Hosp Infect 77:58–63
63. Sakr Y, Sponholz C, Tuche F, Brunkhorst F, Reinhart K (2008) The role of procalcitonin in febrile neutropenic patients: review of the literature. Infection 36:396–407
64. Schultz MJ, Determann RM (2008) PCT and sTREM-1: the markers of infection in critically ill patients? Med Sci Monit 14:RA241–247
65. Gilbert DN (2010) Use of plasma procalcitonin levels as an adjunct to clinical microbiology. J Clin Microbiol 48:2325–2329
66. Tang H, Huang T, Jing J, Shen H, Cui W (2009) Effect of procalcitonin-guided treatment in patients with infections: a systematic review and meta-analysis. Infection 37:497–507
67. Schuetz P, Chiappa V, Briel M, Greenwald JL (2011) Procalcitonin algorithms for antibiotic therapy decisions: a systematic review of randomized controlled trials and recommendations for clinical algorithms. Arch Intern Med 171:1322–1331
68. Reinhart K, Hartog CS (2010) Biomarkers as a guide for antimicrobial therapy. Int J Antimicrob Agents 36S:S17–S21
69. Agarwal R, Schwartz DN (2011) Procalcitonin to guide duration of antimicrobial therapy in intensive care units: a systematic review. Clin Infect Dis 53:379–387
70. Bouadma L, Luyt CE, Tubach F et al (2010) Use of procalcitonin to reduce patients' exposure to antibiotics in intensive care units (PRORATA trial): a multicentre randomised control trial. Lancet 375:463–474
71. Jensen JU, Hein L, Lundgren B et al (2011) Procalcitonin-guided interventions against infections to increase early appropriate antibiotics and improve survival in the intensive care units: a randomized trial. Crit Care Med 39:2048–2058

72. Muller B, Becker KL, Schachinger H et al (2000) Calcitonin precursors are reliable markers of sepsis in a medical intensive care unit. Crit Care Med 28:977–983
73. Rey C, Los Arcos M, Concha A et al (2007) Procalcitonin and C-reactive protein as markers of systemic inflammatory response syndrome severity in critically ill children. Intensive Care Med 33:477–484
74. Claeys R, Vinken S, Spapen H et al (2002) Plasma procalcitonin and C-reactive protein in acute septic shock: clinical and biological correlates. Crit Care Med 30:757–762
75. Luzzani A, Polati E, Dorizzi R, Rungatscher A, Pavan R, Merlini A (2003) Comparison of procalcitonin and C-reactive protein as markers of sepsis. Crit Care Med 31:1737–1741
76. Daniels JM, Schoorl M, Snijders D et al (2010) Procalcitonin versus C-reactive protein as predictive markers of response to antibiotic therapy in acute exacerbations of COPD. Chest 138:1108–1115
77. Gaini S, Koldkjaer OG, Pedersen C, Pedersen SS (2006) Procalcitonin, lipopolysaccharide-binding protein, interleukin-6 and C-reactive protein in community-acquired infections and sepsis: a prospective study. Crit Care 10:R53
78. Koeze J, Hendrix MG, van den Bergh FA, Brouwer RM, Zijlstra JG (2011) In critically ill patients the procalcitonin level can be misleading. Crit Care 15:422
79. Van den Bruel A, Thompson MJ, Haj-Hassan T et al (2011) Diagnostic value of laboratory tests in identifying serious infections in febrile children: systematic review. BMJ 342:d3082
80. Lannergard A, Friman G, Ewald U, Lind L, Larsson A (2005) Serum amyloid A (SAA) protein and high-sensitivity C-reactive protein (hsCRP) in healthy newborn infants and healthy young through elderly adults. Acta Paediatr 94:1198–1202
81. Vincent JL, Donadello K, Schmit X (2011) Biomarkers in the critically ill patient: C-reactive protein. Crit Care Clin 27:241–251
82. Shine B, De Beer FC, Pepys MB (1981) Solid phase radioimmunoassay for human C-reactive protein. Clin Chim Acta 117:12–23
83. Raitakari M, Mansikkaniemi K, Marniemi J, Viikari JS, Raitakari OT (2005) Distribution and determinants of serum high-sensitive C-reactive protein in a population of young adults: the cardiovascular risk in young Finns study. J Intern Med 258:428–434
84. Pepys MB, Hirschfield GM (2003) C-reactive protein: a critical update. J Clin Invest 111:1805–1812
85. Zeitoun AAH, Gad SS, Attia FM, Abu Maziad AS, Bell EF (2010) Evaluation of neutrophilic CD64, interleukin 10 and procalcitonin as diagnostic markers of early- and late-onset neonatal sepsis. Scand J Infect Dis 42:299–305
86. Reinhart K, Karzai W, Meisner M (2000) Procalcitonin as a systemic inflammatory response to infection. Intensive Care Med 26:1193–1200
87. Povoa P, Almeida E, Moreira P et al (1998) C-reactive protein as an indicator of sepsis. Intensive Care Med 24:1052–1056
88. Silvestre JP, Coelho LM, Povoa PM (2010) Impact of fulminant hepatic failure in C-reactive protein? J Crit Care 25(657):e7–e12
89. Lobo SM, Lobo FR, Bota DP (2003) C-reactive protein levels correlate with mortality and organ failure in critically ill patients. Chest 123:2043–2049
90. Oude Nijhuis CS, Vellenga E, Daenen SM et al (2003) Lipopolysaccharide-binding protein: a possible diagnostic marker for gram-negative bacteremia in neutropenic cancer patients. Intensive Care Med 29:2157–2161
91. Petrikkos GL, Christofilopoulou SA, Tentolouris NK, Charvalos EA, Kosmidis CJ, Daikos GL (2005) Value of measuring serum procalcitonin, C-reactive protein, and mannan antigens to distinguish fungal from bacterial infections. Eur J Clin Microbiol Infect Dis 24:272–275
92. Kajiya T, Orihara K, Hamasaki S et al (2008) Toll-like receptor 2 expression level on monocytes in patients with viral infections: monitoring infection severity. J Infect 57:249–259
93. Quint JK, Donaldson GC, Goldring JJ, Baghai-Ravary R, Hurst JR, Wedzicha JA (2010) Serum IP-10 as a biomarker of human rhinovirus infections at exacerbation of COPD. Chest 137:812–822

94. Charles PE, Kus E, Aho S et al (2009) Serum procalcitonin for the early recognition of nosocomial infection in the critically ill patients: a preliminary report. BMC Infect Dis 9:49–58
95. Luyt CE, Combes A, Reynaud C et al (2008) Usefulness of procalcitonin for the diagnosis of ventilator-associated pneumonia. Intensive Care Med 34:1434–1440
96. Dallas J, Brown SM, Hock K et al (2011) Diagnostic utility of plasma procalcitonin for nosocomial pneumonia in the intensive care unit setting. Respir Care 56:412–419
97. Cuquemelle E, Soulis F, Villers D et al (2011) Can procalcitonin help identify associated bacterial infection in patients with severe influenza pneumonia? A multicentre study. Intensive Care Med 37:796–800
98. Vouloumanou EK, Plessa E, Karageorgopoulos DE, Mantadakis E, Falagas ME (2011) Serum procalcitonin as a diagnostic marker for neonatal sepsis: a systematic review and meta-analysis. Intensive Care Med 37:747–762
99. Briel M, Schuetz P, Mueller B et al (2008) Procalcitonin-guided antibiotic use vs a standard approach for acute respiratory tract infections in primary care. Arch Intern Med 168:2000–2007
100. Agapakis DI, Tsantilas D, Psarris P et al (2010) Coagulation and inflammation biomarkers may help predict the severity of community-acquired pneumonia. Respirology 15:796–803
101. Christ-Crain M, Jaccard-Stolz D, Bingisser R et al (2004) Effect of procalcitonin-guided treatment on antibiotic use and outcome in lower respiratory tract infections: cluster-randomised, single-blinded intervention trial. Lancet 363:600–607
102. Christ-Crain M, Stolz D, Bingisser R et al (2006) Procalcitonin guidance of antibiotic therapy in community-acquired pneumonia: a randomized trial. Am J Resp Crit Care Med 174:84–93
103. Burkhardt O, Ewig S, Haagen U et al (2010) A simple procalcitonin-guided strategy results in safe reductions of antibiotic use in patients with symptoms of acute respiratory tract infections in primary care. Eur Resp J 36:601–607
104. Stolz D, Christ-Crain M, Bingisser R et al (2007) Antibiotic treatment of exacerbations of COPD: a randomized, controlled trial comparing procalcitonin-guidance with standard therapy. Chest 131:9–19
105. Cetinkaya M, Ozkan H, Koksal N, Akaci O, Ozgur T (2010) The efficacy of serial serum amyloid A measurements for diagnosis and follow-up of necrotizing enterocolitis in premature infants. Pediatr Surg Int 26:835–841
106. Arnon S, Litmanovitz I, Regev RH, Bauer S, Shainkin-Kestenbaum R, Dolfin R (2007) Serum amyloid A: an early and accurate marker of neonatal early-onset sepsis. J Perinatol 27:297–302
107. Schumann RR, Zweigner J (1999) A novel acute-phase marker: lipopolysaccharide binding protein (LBP). Clin Chem Lab Med 37:271–274
108. Pavcnik-Arnol M, Hojker S, Derganc M (2007) Lipopolysaccharide-binding protein, lipopolysaccharide, and soluble CD14 in sepsis of critically ill neonates and children. Intensive Care Med 33:1025–1032
109. Mierzchala M, Krzystek-Korpacka M, Gamian A, Durek G (2011) Quantitative indices of dynamics in concentrations of lipopolysaccharide-binding protein (LBP) as prognostic factors in severe sepsis/septic shock patients—comparison with CRP and procalcitonin. Clin Biochem 44:357–363
110. Dempfle CE, Lorenz S, Smolinksi M et al (2004) Utility of activated partial thromboplastin time waveform analysis for identification of sepsis and overt disseminated intravascular coagulation in patients admitted to a surgical intensive care unit. Crit Care Med 32:520–524
111. Chopin N, Floccard B, Sobas F et al (2006) Activated partial thromboplastin time waveform analysis: a new tool to detect infection? Crit Care Med 34:1654–1660
112. Gamble JR, Drew J, Trezise L et al (2000) Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res 87:603–607
113. Giamarellos-Bourboulis EJ (2011) Angiopoietins in sepsis: biomarkers or effector molecules? Crit Care Med 39:890–891
114. LaRosa SP, Opal SM (2011) Biomarkers: the future. Crit Care Clin 27:407–419

115. Mofarrahi M, Nouh T, Qureshi S, Guillot L, Mayahi D, Hussain SN (2008) Regulation of angiopoietin expression by bacterial lipopolysaccharide. Am J Physiol Lung Cell Mol Physiol 294:L955–L963
116. Giuliano JS, Lahni PM, Harmon K et al (2007) Admission angiopoietin levels in children with septic shock. Shock 28:650–654
117. Orfanos SE, Kotanidou A, Glynos C et al (2007) Angiopoietin-2 is increased in severe sepsis: correlation with inflammatory mediators. Crit Care Med 35:199–206
118. Giamarellos-Bourboulis EJ, Kanellakopoulou K, Pelekanou A et al (2008) Kinetics of angiopoietin-2 in serum of multi-trauma patients: correlation with patient severity. Cytokine 44:310–313
119. Van der Heijden M, Pickkers P, van Nieuw Amerongen GP et al (2009) Circulating angiopoietin-2 levels in the course of septic shock: Relation with fluid balance, pulmonary dysfunction and mortality. Intensive Care Med 35:1567–1574
120. Ricciuto DR, dos Santos CC, Hawkes M et al (2011) Angiopoietin-1 and angiopoietin-2 as clinically informative prognostic biomarkers of morbidity and mortality in severe sepsis. Crit Care Med 39:702–710
121. Minhas N, Xue M, Fukudome K, Jackson CJ (2010) Activated protein C utilizes the angiopoietin/Tie2 axis to promote endothelial barrier function. FASEB J 24:873–881
122. Page AV, Koth M, McGeer A, Low DE, Kain KC, Liles WC (2011) Systemic dysregulation on angiopoietin-1/2 in streptococcal toxic shock syndrome. Clin Infect Dis 52:e157–e161
123. Berdowska A, Zwirska-Korczala K (2001) Neopterin measurement in clinical diagnosis. J Clin Pharm Ther 26:319–329
124. Ip M, Rainer TH, Lee N et al (2007) Value of serum procalcitonin, neopterin, and C-reactive protein in differentiating bacterial from viral etiologies in patients presenting with lower respiratory tract infections. Diagn Microbiol Infect Dis 59:131–136
125. Prat C, Dominguez J, Andreo F et al (2006) Procalcitonin and neopterin correlation with aetiology and severity of pneumonia. J Infect 52:169–177
126. Lacoma A, Prat C, Andreo F et al (2011) Value of procalcitonin, C-reactive protein, and neopterin in exacerbations of chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 6:157–169
127. Gibot S, Cravoisy A, Kolopp-Sarda MN et al (2005) Time-course of sTREM (soluble triggering receptor expressed on myeloid cells)-1, procalcitonin, and C-reactive protein concentrations during sepsis. Crit Care Med 33:792–796
128. Gibot S, Le Renard PE, Bollaert PE et al (2005) Surface triggering receptor expressed on myeloid cells-1: expression patterns in septic shock. Intensive Care Med 31:594–597
129. Gibot S, Kolopp-Sarda MN, Bene MC et al (2004) Plasma level of a triggering receptor expressed on myeloid cells-1: its diagnostic accuracy in patients with suspected sepsis. Ann Intern Med 141:9–15
130. Gibot S, Cravoisy A, Levy B, Bene MC, Faure G, Bollaert PE (2004) Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. N Engl J Med 350:451–458
131. Yilmaz G, Koksal I, Karahan SC, Mentese A (2011) The diagnostic and prognostic significance of soluble urokinase plasminogen activator receptor in systemic inflammatory response syndrome. Clin Biochem 44:1227–1230
132. Kofoed K, Andersen O, Kronborg G et al (2007) Use of plasma C-reactive protein, procalcitonin, neutrophils, macrophage migration inhibitory factor, soluble urokinase-type plasminogen activator receptor, and soluble triggering receptor expressed on myeloid cells-1 in combination to diagnose infections: a prospective study. Crit Care 11:R38
133. Eugen-Olsen J (2011) SuPAR—a future risk marker in bacteremia. J Intern Med 270:29–31
134. Jabaudon M, Futier E, Roszyk L et al (2011) Soluble form of the receptor for advanced glycation end products is a marker of acute lung injury but not of severe sepsis in critically ill patients. Crit Care Med 39:480–488
135. Creagh-Brown BC, Burke-Gaffney A, Evans TW (2011) sRAGE: a useful biomarker in acute lung injury? Crit Care Med 39:589–590

136. Calfee CS, Ware LB, Eisner MD et al (2008) Plasma receptor for advanced glycation end products and clinical outcomes in acute lung injury. Thorax 63:1083–1089
137. Bopp C, Hofer S, Weitz J et al (2008) sRAGE is elevated in septic patients and associated with patients outcome. J Surg Res 147:79–83
138. Groselj-Grenc M, Ihan A, Pavcnik-Arnol M, Kopitar AN, Gmeiner-Stopar T, Derganc M (2009) Neutrophil and monocyte CD64 indexes, lipopolysaccharide-binding protein, procalcitonin and C-reactive protein in sepsis of critically ill neonates and children. Intensive Care Med 35:1950–1958
139. Davis BH, Olsen SH, Ahmad E, Bigelow NC (2006) Neutrophil CD64 is an improved indicator of infection or sepsis in emergency department patients. Arch Pathol Lab Med 130:654–661
140. Nuutila J (2010) The novel applications of the quantitative analysis of neutrophil cell surface FcγRI (CD64) to the diagnosis of infectious and inflammatory diseases. Curr Opin Infect Dis 23:268–274
141. Nuutila J, Hohenthal U, Laitinen L et al (2007) Simultaneous quantitative analysis of FcγRI (CD64) expression on neutrophils and monocytes: a new, improved way to detect infections. J Immunol Methods 328:189–200
142. Hoffmann JJ (2009) Neutrophil CD64: a diagnostic marker for infection and sepsis. Clin Chem Lab Med 47:903–916
143. Docke WD, Hoflich C, Davis KA et al (2005) Monitoring temporary immunodepression by flow cytometric measurement of monocytic HLA-DR expression: a multicenter standardized study. Clin Chem 51:2341–2347
144. Venet F, Tissot S, Debard AL et al (2007) Decreased monocyte human leukocyte antigen-DR expression after severe burn injury: correlation with severity and secondary septic shock. Crit Care Med 35:1910–1917
145. Landelle C, Lepape A, Voirin N et al (2010) Low monocyte human leukocyte antigen-DR is independently associated with nosocomial infections after septic shock. Intensive Care Med 36:1859–1966
146. Lukaszewicz AC, Grienay M, Resche-Rigon M et al (2009) Monocytic HLA-DR expression in intensive care patients: interest for prognosis and secondary infection prediction. Crit Care Med 37:2746–2752
147. Volk HD, Reinke P, Krausch D et al (1996) Monocyte deactivation-rationale for a new therapeutic strategy in sepsis. Intensive Care Med 22:S474–S481
148. Shozushima T, Takahashi G, Matsumoto N, Kojika M, Okamura Y, Endo S (2011) Usefulness of presepsin (sCD14-ST) measurements as a marker for the diagnosis and severity of sepsis that satisfied diagnostic criteria of systemic inflammatory response syndrome. J Infect Chemother 17:764–769
149. Yaegashi Y, Shirakawa K, Sato N et al (2005) Evaluation of a newly identified soluble CD14 subtype as a marker for sepsis. J Infect Chemother 11:234–238
150. Pinksy MR, Vincent JL, Deviere J, Alegre M, Kahn RJ, Dupont E (1993) Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality. Chest 103:565–575
151. Harbarth S, Holeckova K, Froidevaux C et al (2001) Diagnostic value of procalcitonin, interleukin-6 and interleukin-8 in critically ill patients with suspected sepsis. Am J Resp Crit Care Med 164:396–402
152. Panacek EA, Marshall JC, Albertson TE et al (2004) Efficacy and safety of the monoclonal anti-TNF antibody $F(ab')_2$ fragment afelimomab in patients with severe sepsis stratified by IL-6 level. Crit Care Med 32:2173–2182
153. Ng PC, Li K, Chui KM et al (2007) IP-10 is an early diagnostic marker for identification of late-onset bacterial infection in preterm infants. Pediatr Res 61:93–98
154. Kontoyiannis DP (2011) Are serum cytokines sensitive and specific enough to prognosticate in aspergillosis? J Infect Dis 203:1503
155. Guignant C, Voirin N, Venet F et al (2009) Assessment of pro-vasopressin and pro-adrenomedullin as predictors of 28-day mortality in septic shock patients. Intensive Care Med 35:1859–1867

156. Albrich WC, Dusemund F, Ruegger K et al (2011) Enhancement of CURB65 score with proadrenomedullin (CURB65-A) for outcome prediction in lower respiratory tract infections: Derivation of a clinical algorithm. BMC Infect Dis 11:112
157. Tan XF, Wu SS, Li SP, Chen Z, Chen F (2011) Alpha-1 antitrypsin is a potential biomarker for hepatitis B. Virol J 8:274
158. Shi WW, Lin A, Xu DP et al (2011) Plasma soluble human leukocyte antigen-G expression is a potential clinical biomarker in patients with hepatitis B virus infection. Hum Immunol 72:1068–1073
159. Lee PS, Drager LR, Stossel TP, Moore FD, Rogers SO (2006) Relationship of plasma gelsolin levels to outcomes in critically ill surgical patients. Ann Surg 243:399–403
160. Sumino KC, Walter MJ, Mikols CL et al (2010) Detection of respiratory viruses and the associated chemokine responses in serious acute respiratory illness. Thorax 65:639–644
161. Wang J, Wang PP, Xiang GJ, Hu XB (2010) Relationship between the expression of IP-10 and IP-10 mRNA in peripheral blood and HBV DNA level in patients with cirrhosis. Hepatobiliary Pancreat Dis Int 9:280–286
162. Lagging M, Romero AI, Westin J et al (2006) IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. Hepatology 44:1617–1625
163. Bagdasaryan R, Zhou Z, Tierno B, Rosenman D, Xu D (2007) Neutrophil VCS parameters are superior indicators for acute infection. Lab Hematol 13:12–16
164. Charafeddine KM, Youssef AM, Mahfouz RA, Sarieddine DS, Daher RT (2011) Comparison of neutrophil volume distribution width to C-reactive protein and procalcitonin as a proposed new marker of acute infection. Scand J Infect Dis 43:777–784
165. Venet F, Chung CS, Monneret G et al (2008) Regulatory T cell populations in sepsis and trauma. J Leukoc Biol 83:523–535
166. Remick DG (2007) Pathophysiology of sepsis. Am J Pathol 170:1435–1444
167. Monneret G, Debard AL, Venet F et al (2003) Marked elevation of human circulating CD4+CD25+ regulatory T cells in sepsis-induced immunoparalysis. Crit Care Med 31:2068–2071
168. Venet F, Chung CS, Kherouf H et al (2009) Increased circulating regulatory T cells (CD4(+) CD25(+)CD127(−)) contribute to lymphocyte anergy in septic shock patients. Intensive Care Med 35:678–686
169. Sherwin C, Broadbent R, Young S et al (2008) Utility of interleukin-12 and interleukin-10 in comparison with other cytokines and acute-phase reactants in the diagnosis of neonatal sepsis. Am J Perinatol 25:629–636
170. Rintala EM, Aittoniemi J, Laine S, Nevalainen TJ, Nikoskelainen J (2001) Early identification of bacteremia by biochemical markers of systemic inflammation. Scand J Clin Lab Invest 61:523–530
171. Nupponen I, Andersson S, Jarvenpaa AL, Kautiainen H, Repo H (2001) Neutrophil CD11b expression and circulating interleukin-8 as diagnostic markers for early-onset neonatal sepsis. Pediatrics 108:E12
172. Liaudat S, Dayer E, Praz G, Bille J, Troillet N (2001) Usefulness of procalcitonin serum level for the diagnosis of bacteremia. Eur J Clin Microbiol Dis 20:524–527
173. Zakariah AN, Cozzi SM, Van Nuffelen M, Clausi CM, Pradier O, Vincent JL (2008) Combination of biphasic transmittance waveform with blood procalcitonin levels for diagnosis of sepsis in acutely ill patients. Crit Care Med 36:1507–1512
174. Deitcher SR, Eisenberg PR (1993) Elevated concentrations of cross-linked fibrin degradation products in plasma. An early marker of gram-negative bacteremia. Chest 103:1107–1112
175. Bozza FA, Salluh JI, Japiassu AM et al (2007) Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. Crit Care 11:R49
176. Kofoed K, Schneider UV, Scheel T, Andersen O, Eugen-Olsen J (2006) Development and validation of a multiplex add-on assay for sepsis biomarkers using xMAP technology. Clin Chem 52:1284–1293

177. Shapiro NI, Trzeciak S, Hollander JE et al (2009) A prospective multicenter derivation of a biomarker panel to assess risk of organ dysfunction, shock, and death in emergency department patients with suspected sepsis. Crit Care Med 37:96–104
178. Cobb JP, Laramie JM, Stormo GD et al (2002) Sepsis gene expression profiling: murine splenic compared with hepatic responses determined by using complementary DNA microarrays. Crit Care Med 30:2711–2721
179. Tang BM, McLean AS, Dawes IW, Huang SJ, Lin RC (2009) Gene-expression profiling of peripheral blood mononuclear cells in sepsis. Crit Care Med 37:882–888
180. Sutherland A, Thomas M, Brandon RA (2011) Development and validation of a novel molecular biomarker diagnostic test for the early detection of sepsis. Crit Care 15:R149
181. Paugam-Burtz C, Albuquerque M, Baron G et al (2010) Plasma proteome to look for diagnostic biomarkers of early bacterial sepsis after liver transplantation: a preliminary study. Anesthesiology 112:926–935
182. Service RF (2008) Proteomics. Will biomarkers take off at last? Science 321:1760

Chapter 8
Functional Assessment of Microbial and Viral Infections by Real-Time Cellular Analysis System

Xiao Xu and Min Zheng

Introduction

Microbes and viruses infect their host cells and in doing so alter the physiological functions of the host cells leading to diseases. Analysis of pathogen-infected cells provides critical information with regards to functional assessment of microbe–host and virus–host interactions in addition to serving as an important diagnosis for infection. As early as 1950s, hemagglutination assay using red blood cells (RBC) were employed to detect the presence of hemagglutinin antigen-bearing virus particles [1]. Recently this assay was also used for bacterial detection [2]. Some viral families and many bacteria have envelope or surface proteins, which are able to agglutinate human or animal RBC and bind to *N*-acetylneuraminic acid. As each of the agglutinating molecule attaches to multiple RBCs, a lattice structure will form, allowing for visual inspection.

Mammalian cell culture has long been used to detect microbial and viral infections. The viral infection process of the host cells is complex involving many key steps required for productive infection and culminates in many changes in the host cells, collectively referred to as cytopathic effect (CPE). Such changes include altered shape, detachment from substrate, lysis, membrane fusion, altered membrane permeability, inclusion bodies, and apoptosis. Assessment of virus-induced CPE in host cells can provide a diagnostic tool for assessment of a given virus infection.

X. Xu (✉)
ACEA Biosciences, Inc., 6779 Mesa Ridge Rd. #100, San Diego, CA 92121, USA
e-mail: xxu@aceabio.com

M. Zheng
State Key Laboratory of Diagnostic and Treatment for Infectious Diseases,
First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qingchun Road,
Hangzhou, Zhejiang, PR 310006, China
e-mail: minzheng@zju.edu.cn

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For example, human rhinovirus infection of HeLa cells results in the cells changing shape, becoming round and more refractile (brighter) under phase contrast microscopy [3]. Some infected cells detach from the tissue culture flask and float in the medium, which can be measured by colorimetric cell viability assays. In addition, plaque formation assays are routinely being used for quantitative assessment of virus particles where the plaques resulting from virus-induced cytolytic effect can be seen and counted in cell culture lawns after staining.

Cellular analysis has also been used for assessing bacteria and host cell interaction and bacteria toxin detection. In order to induce an infection, bacteria must first colonize their host. Bacterial pathogens express various molecules or structures able to promote attachment to host cells [4]. These adhesins rely on interactions with host cell surface receptors or soluble proteins acting as a bridge between bacteria and host. Adhesion is a critical first step prior to invasion and/or secretion of toxins, thus it is a key event to be studied in bacterial pathogenesis. Furthermore, adhered bacteria often induce exquisitely fine-tuned cellular responses, the studies of which have given birth to the field of "cellular microbiology" [5]. Robust assays for bacterial adhesion to host cells and their invasion therefore play key roles in bacterial pathogenesis studies and have long been used in many pioneer laboratories [6–8]. In another example, the bacterium *Chlamydia trachomatis* causing a common sexually transmitted infection (STI) in humans is naturally found living only inside host cells. Cell culture using McCoy cells has been the gold standard assay for the diagnosis of genital chlamydial infections [9] [10]. The infection of Chlamydia in McCoy cells forms specific inclusion bodies which can be recognized by microscopy.

These cell based assays are now conducted on routine basis by most laboratories working on bacterial pathogenesis. In many other cases, bacterial toxins are recognized as virulent factors produced by pathogenic bacterial infections. These toxins are very potent and require only a relative small number of molecules to affect cells through endocytotic pathways mediated by cell surface receptors. Many of these toxins such as *Clostridium difficile* toxin, *Clostridium botulinum* toxin, and diphtheria toxin are lethal to the host cells. Taken together, bacterial host interaction is a multistep process which ultimately culminates in infection of the host cell. The precise mechanism of infection is specific to the bacterial strain and the host cell being infected. In terms of designing cell-based assays for bacterial infection, each step of the infection process can potentially lend itself to design of specific assays. It is imperative for diagnostic purposes that the assay and the detection methods should be extremely sensitive and functional. It has been estimated that Vero cell express approximately 150,000 receptor molecules for diphtheria toxin with the K*a* of 10^{-9} M for binding to the receptor. The abundance of toxin-specific receptor with high binding affinity has allowed these cells to be extremely sensitive to bacterial toxin. Therefore, cell-based assays can serve as sensitive and functional measurements of bacterial toxin.

Although a variety of diagnostic assays based on cellular analysis has been developed for microbial infections, very few platform technologies have been employed specifically for this purpose thus far. The majority of assays are still built on conventional optical-based technologies, which use different staining processes

including fluorescent and colorimetric dyes. Experienced staffs are often required to perform such assays and readouts are descriptive and often nonquantitative. With evolution of much easier, faster, and more quantitative molecular tests in clinical diagnostics such as PCR, EIAs, and sequencing, the crucial challenge is how to improve the performance of traditional cellular analysis while keeping the irreplaceable nature of its disease relevance and sensitivity in infectious disease diagnostics. Development of a quantitative, functional, faster, affordable, and yet easy to use cellular analysis system is urgently needed. In this chapter we will introduce an emerging technology termed real-time cellular analysis (RTCA) and its utility in microbial and viral infections including bacterial toxin detection, analyzing bacterial and host cell interaction, virus-induced CPE quantification, neutralizing antibody detection, parasite infective activity assessment, as well as functional detection and monitoring of host immune system.

RTCA and Its Principle of Detection Using Impedance Microelectrode Sensor Arrays

The RTCA utilizing cell-electrode subtract impedance readout to noninvasively quantify cellular status in real time was first reported by Giaever and Keese more than two decades ago [11–13]. This approach has gained considerable traction recently and the driving force mainly stems from significant progresses in cell research and understanding the value of physiologic relevance of cellular analysis in diagnosis of microbial and viral infections [14–19]. The RTCA system which is codeveloped by ACEA Biosciences and Roche Applied Sciences uses impedance sensor technology at its core. The system is comprised of three components, an electronic analyzer, an E-Plate station, and a microelectronic plate (E-Plate) (Fig. 8.1). Microelectrode sensor arrays are fabricated on glass slides with lithographical microfabrication methods and the electrode-containing slides are assembled to plastic trays to form electrode-containing wells (Fig. 8.1). The E-Plate station receives the E-Plate and is capable of electronically switching any one of the wells to the sensor analyzer for impedance measurement. In the operation mode, the E-Plates with cells cultured in the wells are connected to the E-Plate station which is connected to the sensor analyzer. Under the RTCA software control, the sensor analyzer can automatically select wells to be measured and continuously conduct impedance measurements. The impedance data from the analyzer is transferred to a computer, analyzed, and processed by the integrated software. Impedance measured between electrodes in an individual well depends on electrode geometry, ionic concentration in the well and whether there are cells attached to the electrodes. In the absence of the cells, electrode impedance is mainly determined by the ionic environment both at the electrode/solution interface and in the bulk solution. In the presence of the cells, cells attached to the electrode sensor surfaces will alter the local ionic environment at the electrode/solution interface, leading to an increase in the impedance (Fig. 8.2). The more cells there are on the electrodes, the larger the

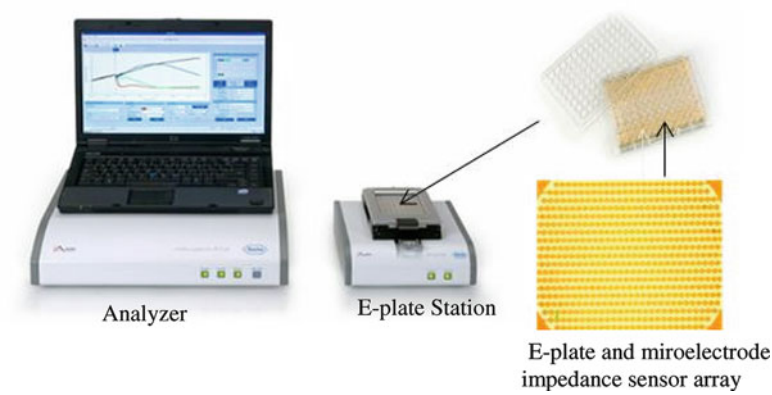


Fig. 8.1 RTCA system (xCELLigence, Roche Applied Sciences). The system is configured to contain three elements, the analyzer, the E-plate station and the microelectrode-integrated 96-well microtiter E-plate. The impedance sensor array shown is interdigitated microelectrodes

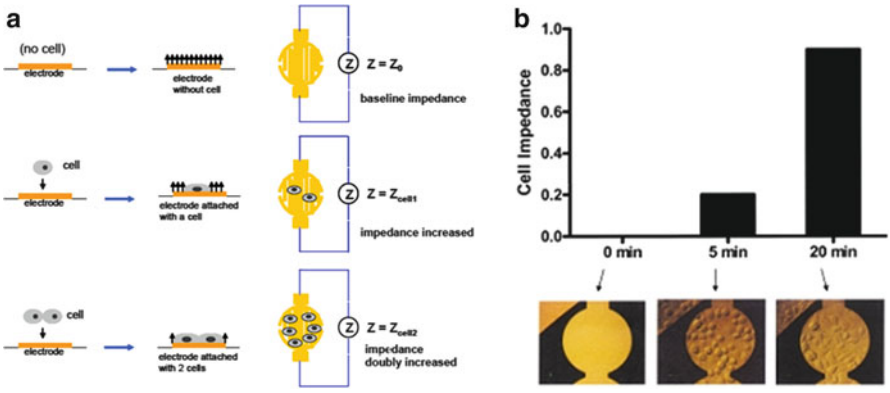


Fig. 8.2 Schematic representation of impedance measurement. (**a**) No cell is on an electrode surface, and there is baseline impedance (*top panel*). A cell attaches to the electrode surface and blocks partially the electrical current in the circuit, leading to an increase in the electrode impedance (*middle panel*). Two cells attach to the electrode surface and reduce even further the electrical current, leading to doubly increased impedance as compared with the *top* and *middle panels* (*bottom panel*). (**b**) The electrode impedance was plotted against time after cell addition to the wells (*top panel*). (With time, cells attach and spread on the electrodes. For the same amount of cells on the electrodes, better cell attachment and more cell spread onto the electrodes lead to a larger increase in the electrode impedance *bottom panel*) [13]

increase in cell-electrode impedance. Furthermore, the impedance change also depends on cell morphology and the extent to which cells attach to the electrodes. To quantify cell status based on the measured cell-electrode impedance, a parameter termed the cell index (CI) was derived to represent cell status based on the change of the electrode impedance for the wells with the cells relative to that of control wells without the cells (Fig. 8.2). The frequency-dependent electrode impedance

(resistance) without or with cells present in the wells was represented as $R_b(f)$ and $R_{cell}(fi)$, respectively, and the CI calculated as

$$\mathrm{CI} = \max_{i=1\ldots,N} = 1\left[\frac{R_{cell}(fi)}{R_b(fi)} - 1\right]$$

where N is the number of the frequency points at which the impedance is measured. Several features of the CI can be derived: (1) under the same physiological conditions, if more cells attach to the electrodes, the impedance value will increase. If no cells are present on the electrodes or if cells are detached from the electrodes, $R_{cell}(f)$ will be the same as $R_b(f)$, leading to CI = 0. Therefore, CI is a quantitative measure of the number of cells attached to the sensors. (2) For the same number of cells attached to the sensors, changes in cell status will lead to changes in CI. For example, an increase in cell adhesion or cell spread leading to a large cell/electrode contact area will lead to an increase in $R_{cell}(f)$ and therefore an increase in CI. In contrast, cell death or microbe- or virus-induced cell detachment or cell rounding will lead to a smaller $R_{cell}(f)$ and therefore a lower CI. In the current study, for display of RTCA response curves, a normalized cell index (nCI) generally was used, where cell index is normalized at the last time point before the microbial infection or the chemical/toxin treatment.

The impedance readout harnesses and quantifies these unique changes in cell number, morphology, and adhesion allowing for an unbiased detection of specific cellular processes in real time. Therefore, label-free, real-time cell-based technologies have recently received considerable attention for implementation in cellular analysis. As the name implies, the preclusion of label allows for assessment of cells in their native and physiologically relevant environment circumventing the potential negative impact of label on cellular processes. The inclusion of certain labels and reporters, particularly labels for live cells has been shown to impact various aspects of cellular behavior. For example, it has been shown that that the live cell fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF-AM) and rhodamine 6G (R6G) can dose-dependently block the migration of macrophage and mononuclear cells, respectively, urging caution when utilizing these dyes for cell-based assays. Label-free technologies have the added advantage of being non-invasive and therefore live cells present in tissue culture wells can be continuously interrogated (Fig. 8.3). This feature directly leads to one of the main advantages of label-free technologies, which is real-time kinetic measurement [12, 20–23]. Real-time monitoring of cellular processes offers distinct and important advantages over traditional end-point assays (Fig. 8.3). First, comprehensive representation of the entire length of the assay is possible, allowing the user to make informed decisions regarding timing of manipulations or treatments. Second, the actual kinetic response of cells provides important information regarding the biological status of the cells such as growth, arrest, and morphological changes (Fig. 8.3). This technology has been applied in a number of cell-based assays including cytotoxicity, cell adhesion and spreading, functional monitoring of receptor-mediated signaling and cell invasion and migration, and stem cell-derived cardiomyocyte beating [24–28].

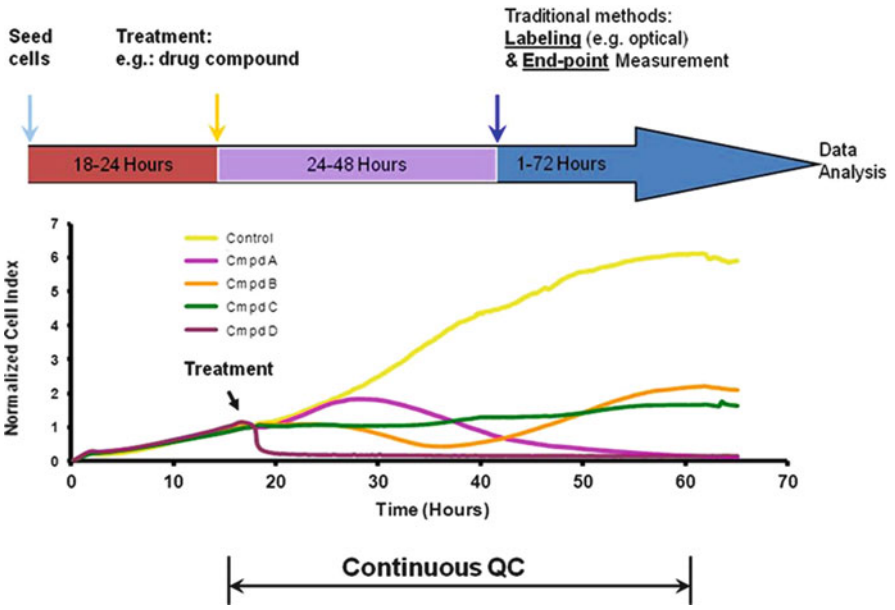


Fig. 8.3 Dynamic proliferation curves of A549 cells (a lung cancer cell line) in response to treatment of anticancer compounds having distinct mechanisms of action: control, DNA-damaging (**a**), antimitotic (**b**), cytostatic (**c**), and cytoskeletal (**d**). In contrast to conventional endpoint cell analysis, the RCTA allows real-time monitoring entire cell processes in response to compounds from same wells and continuous QC of the assay

Analysis of Host Cell Responses to Bacterial Infections on RTCA System

The RTCA system can measure and monitor minute changes in cell morphology, adhesion, and cell number which are the very parameters that are affected by bacterial infections including through release of bacterial toxins or direct cell–cell contact. Therefore, this system is well suited to serve as a functional assay for bacterial detection. To demonstrate the utility of the system for functional detection of toxin effect and host cell response to bacteria–host cell interaction, the system was used to conduct in vitro testing of cellular processes after exposure to microbial toxins including *Clostridium difficile* (*C. difficile*) toxin and *Clostridium botulinum* (*C. botulinum*) toxin or infected with *Neisseria meningitidis*.

Detection of Clostridium difficile Toxin

C. difficile is recognized as the leading cause of infectious diarrhea that develops in patients after hospitalization and/or in patients receiving antibiotic treatment

[29, 30]. A recently emerged, highly virulent strain BI-Nap1-027 has been associated with increased morbidity and mortality [16, 31]. A definitive diagnosis depends on the detection *C. difficile*-specific toxin B production in the laboratory, which allows for prompt treatment and isolation procedures to prevent further nosocomial spread of the infection. The well-accepted standard for *C. difficile* diagnosis is cytotoxigenic culture, which is conducted by culturing *C. difficile* from the stool and then performing a cytotoxic assay on the isolates on cultured cells [32]. Cytotoxigenic assay is a "gold standard" assay for *C. difficile* toxin detection even though it is labor-intensive, subjective, and time-consuming, and therefore is not widely used in the clinical setting [29, 32]. Recently, several enzyme immunoassays (EIAs) and PCR-based toxin gene detection have been used in clinical laboratory [33, 34]. Although promising performance has been reported, the low assay sensitivity (EIAs) and functional relevance between toxin gene detection by PCR and disease onset are still a matter of debate. The use of RTCA for quantitative detection of *C. difficile* toxin has been reported recently [14, 35]. He et al. described an ultrasensitive cell-based immunocytotoxicity assay for detecting *C. difficile* toxin A and B on RTCA system. The assay utilized a cloned CHO cell line expressing a high level of Fcγ receptor. By adding a specific antibody to *C. difficile* toxin, the toxin–antibody complex could be captured by Fcγ receptor residing on the surface of the cells triggering the endocytotic process. The cytotoxic activity of *C. difficile* toxin was then monitored and quantified using the RTCA system in real time. The detection sensitivity was shown to be 1 pg/ml for *C. difficile* toxin in this assay. While the *C. difficile* toxin used in the above-mentioned report was recombinant, it has been reported to behave similarly to the native toxin [18]. Whether the detection and the sensitivity can be achieved in real clinical patient samples remained to be tested. Recently, a clinical study using RTCA technology for *C. difficile* toxin detection was reported on the quantitative capability of the toxin detection. The authors used a human fibroblast cell line, HS27, which is commonly used for conventional cytotoxicity assessment for *C. difficile* toxin [14]. The toxin caused cytotoxic effects on the cells, resulting in a dose-dependent and time-dependent decrease in cellular impedance (Fig. 8.4). The RTCA assay displayed an analytical sensitivity of 0.2 ng/ml for *C. difficile* toxin B with no cross-reactions with other enterotoxins, nontoxigenic *C. difficile*, and other *Clostridium* species [14].

To quantify the toxin concentration from a given sample, a calibration curve was established by monitoring the cell response to toxin treatment at a number of known concentrations and plotting the time for normalized nCI to drop by 50 % versus the toxin concentration (Fig. 8.4). Using this calculation curve, toxin concentrations from the cell response curves for all the specimens assayed can be calculated based on the time taken for a sample to result in a 50 % drop in nCI. Clinical validation was performed on 300 consecutively collected stool specimens from patients with suspected *C. difficile* infection (CDI). A positive *C. difficile* toxin was called in a representative patient sample (sample 14, Fig. 8.5) when a sample resulted in a time-dependent drop in nCI and when such an nCI drop could be rescued by the addition of the toxin-specific neutralizing antibody. A negative *C. difficile* toxin result (sample I4, Fig. 8.5) was called when sample treatment with or without

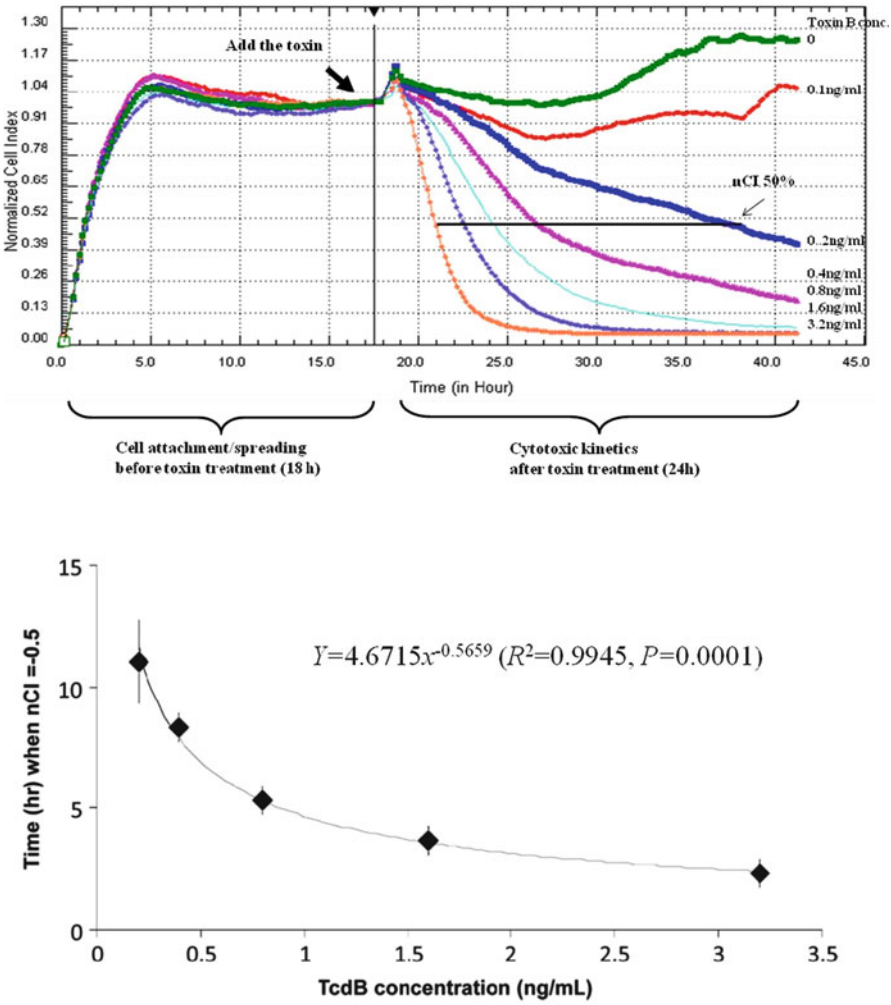
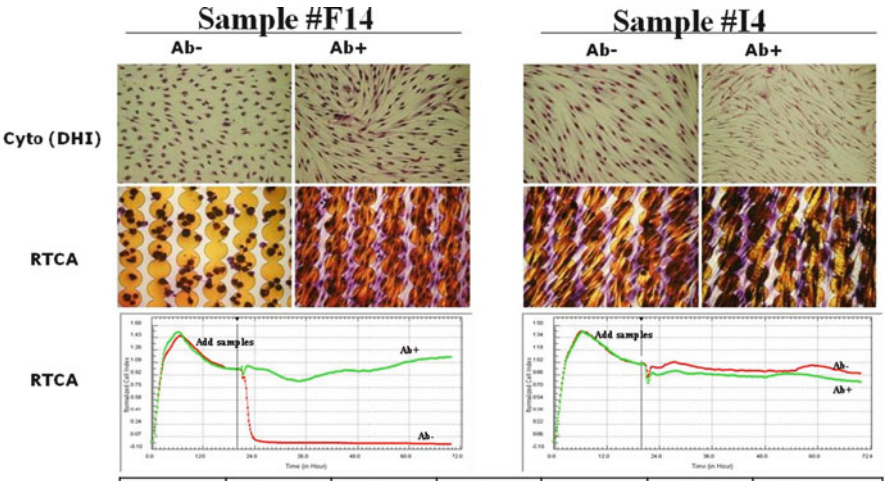


Fig. 8.4 Quantitative detection of *C. difficile* toxin on RTCA system. HS27 cells (5,000 cells/well) were cultivated in E-plate, and cell status (attachment and spreading) were monitored on the system in real time for 18 h before the toxin treatment as indicated in the *top panel*. The cells then were treated with *C. difficile* toxin (TcdB) at different concentrations as indicated in the *top panel* followed continuous monitoring of the cytotoxic effect of the toxin for 24 h. The vertical line indicates the time point $t=0$, when the tested toxin was added and the horizontal line indicates the nCI 50 % cutoff values. The dependency of the time for a 50 % drop in nCI (y axis) in a TcdB-treated well on the toxin concentration (x axis) (*bottom panel*). A nonlinear regression of the data provided a formula of $y=4.6715x^{-0.5659}$ ($R^2=0.9945$, $p=0.0001$)

neutralizing antibody would not cause a drop in nCI. The nCI drop on RTCA showed very good correlation with cytotoxicity imaged under microscope (Fig. 8.5). The performance of *C. difficile* toxin detection was also compared with conventional EIAs and PCR-based assays.



Sample#	Toxin B (Premier)	GDH (Wampole)	Toxin B (Wampole)	qPCR (BD)	RTCA (ACEA)	Cytotoxic Assay (DHI)
F14	P	P	P	P	P (1:5) (20 min)	P (1:5) (48 hr)
I4	N	N	N	P	N (48 hr)	N (48 hr)

Fig. 8.5 Detection of *C. difficile* toxin in stool samples from a *C. difficile* infected patient (sample #14) and a uninfected patient (sample #14) on RTCA system comparing with conventional methods including EIAs (Premier, Wampole), qPCR (BD), and conventional cytotoxic assay (DHI). The cytotoxic effect can be seen in the sample from the infected patient but not the uninfected patient and the toxic effect can be neutralized in the presence of the specific neutralizing antibody (*top panel*, DHI and *middle panel*, RTCA). The kinetic curves were recorded by real-time monitoring of cytotoxic effect of *C. difficile* toxin on RTCA system (RTCA). The fast drop of nCI can be seen in the patient stool sample in the absence of the neutralizing antibody (Ab^-) but not in the presence of the neutralizing antibody (Ab^+). The detection results for two representative patient samples are summarized in the table. Note that the qPCR shows the false positive for the sample I4 from uninfected patient. P = positive, N = negative, 1:5 = dilution of stool samples. The time in the parlance = at the time when a definitive call was given

The detection of 300 clinical stool specimens on RTCA system resulted in a sensitivity of 87.6 %, a specificity of 99.6 %, a positive predictive value (PPV) of 96.5 % and a negative predictive value of 98.5 % (Table 8.1). In addition, the RTCA assay allowed for quantification of toxin protein concentration in a given specimen. Among RTCA-positive specimens collected prior to treatment with metronidozole and/or vancomycin, a significant correlation between toxin protein concentrations and clinical CDI severities was observed ($R^2 = 0.732$, $p = 0.0004$). Toxin concentrations after treatment (0.89 ng/ml) were significantly lower than those prior to the treatment (15.68 ng/ml, Wilcoxon $p = 0.01$) [14]. In one case (Fig. 8.6), the toxin level in the patient stool sample detected on RTCA system was correlated very well with the efficacy of the treatment and clinical symptom. The study demonstrates for the first time that the RTCA assay provides a functional tool for the potential assessment of CDIs, including determining clinical CDI severity prior to treatment as well as monitoring therapeutic intervention.

Table 8.1 Correlation between disease severity and concentration of *C. difficile* toxin in the stool samples

No. of toxin-positive specimens detected	CDI severity	Stool toxin concentration (ng/ml)[a]					
		Mean	Geometric mean	Median	Minimum	Maximum	SD
9	None	0.27	0.17	0.19	0.03	0.89	3.0
1	Mild	1.32	1.32	1.32	1.32	1.32	NA
4	Mild to moderate	33.49	11.51	10.11	2.21	111.53	5.6
2	Moderate	15.68	15.68	15.68	15.68	15.68	0
2	Moderate to severe	58.04	22.52	58.04	4.55	111.53	9.6
1	Severe	111.53	111.53	111.53	111.53	111.53	NA

[a]The toxin concentration for a specimen is calculated based on the time for a 50 % nCI drop after addition of specimen to the cells and the formula $y=4.6715x-0.5659$ (Fig. 8.4). *NA* not applicable. Cell responses were monitored on the RTCA system for up to 48 h after addition of processed specimen

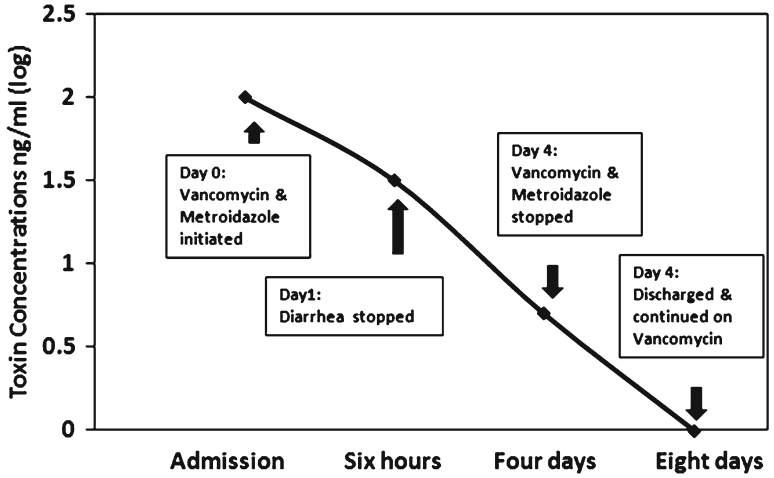


Fig. 8.6 Monitoring treatment of *C. difficile* infected patient by quantitatively detection of *C. difficile* toxin. Stool samples were collected from a *C. difficile* infected patient at the time before the treatment was initiated (day 0), 6 h, 4 days, and 8 days after the treatment. The *C. difficile* toxin was detected and quantified on RTCA system. The *y*-axis shows the toxin concentration and the *x* axis shows the time points when the sample collected

Detection of Clostridium botulinum Toxin

Botulinum neurotoxin produced by neurotoxigenic clostridia are the most potent naturally occurring toxin known [36]. Based on their antigenic specificity, the toxins, zinc-containing metalloproteins, are distinguished into seven serotypes, with type A, B, and E accounting for nearly all recorded cases of human botulism. The toxin appears to target neuronal cells, especially motor neurons. Endocytotic internalization

of the toxin through the binding of the heavy chain of the toxin to the cell surface receptor leads to the release of the light chain of the toxin inside the cells which targets the SNAP-25, VAMP/synaptobrevin, and syntoxin 1 and as a consequence, blocks the release of neurotransmitters [37–39]. Animal study is the only standard assay for functional assessment of botulium toxin. In vitro assays including the mouse diaphragm assay, EIAs, immunoprecipitation assay, radioimmunoassays, and enopeptidase assays have been developed to replace the animal study. However, due to the drawback of nonfunctional nature of these assays, it is difficult to predict whether the toxin is functional or nonfunctional inside the cells [40].

Attempts have been made to develop reliable cell-based assays to conduct functional assessment. Preliminary cell detection was performed on RTCA system [41]. In this study, cytological effect of botulinum toxin on CNS cell lines, A172 glioblastoma cell line, and SH-SY5Y neuroblastoma cell line can be detected. Cells were treated with botulinum toxin at a concentration of 6.67 μg/ml, and the effect was continuously monitored on RTCA system. Since botulinum toxin can have a complex effect on the cells, including binding to cell surface receptors, uptake, processing, and prevention of synaptic vesicle anchoring to the cell membrane, the underlying cellular mechanisms responsible for the cellular processes after toxin treatment remain unknown and need to be studied further. We believe that the quantitative detection of botulinum toxin effect on CNS cells by the RTCA system has the potential of replacing the required animal test and further studies are warranted to improve the assay sensitivity and determine the extent of correlation with LD_{50} data derived from animal testing.

Detection of Meningococcal Infection

Interaction of meningococcus (*Neisseria meningitidis*) with host cells leads to physiological and pathological changes of the host cells, some of which are critical and required for bacterial infection. For example, the infection of *N. meningitidis* results in brain microvascular endothelia cell detachment from matrix leading to disruption of the blood–brain barrier (BBB), and interestingly, the direct cell–cell contact is required for *N. meningitidis* to induce such host cell response [42]. The bacterial cell surface proteins such as the lipopolysaccharide (LPS), the polysaccharide capsule and the outer membrane protein Opc are considered to be the virulence factors. Slanina et al. recently reported using the RTCA system to measure responses of brain endothelial cells infected both by pathogenic *N. meningitidis* and apathogenic *Nesseria* spp. strains [43]. In this study, human brain microvascular endothelial cells (HBMEC) were seeded into gelatin-coated wells (50,000 host cells/well) of the E-Plate, and cells were then infected with the bacteria at different doses (multiplicity of infection or MOI) followed by real-time monitoring of cellular responses to the infection. A unique kinetic response curve was reported, which showed a significant increase of nCI immediately after addition of the bacteria followed by a dramatic drop of nCI. The peak of nCI increase and the onset of nCI drop resulting

from cells rounding up or detachment were correlated well with infection dose of *N. meningitidis*. The higher the MOI the higher the nCI peak is and faster nCI drops. The unique kinetic pattern resulted specifically from HBMEC in response to *N. meningitidis* infection but not the bacterial cells since the bacteria only in the wells did not produce such pattern. To address whether the kinetic pattern was correlated with the pathogenesis of *N. meningitidis*, the authors tested 14 apathogenic strains and found that cellular responses were minor, and kinetic patterns were very different from the pathogenic strain. Therefore, kinetic pattern in response to *N. meningitidis* generated from RTCA system may provide virulence assessment of *N. meningitidis* infection.

Virus-Induced CPE and Neutralizing Antibody Detection on RTCA System

Microscopically visible changes on the cells after virus infection include cell shrinkage or enlargement, deterioration, cell fusion, and the formation of small particles called inclusion bodies. Not all viruses cause CPE in their host cell, but when they do, it can be a useful tool for identifying or diagnosing a virus in the laboratory. Typically, a thin, single layer of cells, called a monolayer, is inoculated with a virus specimen and observed for morphological changes. The unique adaptation of the RTCA system to CPE assay was described in recent studies to quantitatively monitor virus-induced CPEs and neutralizing antibodies specific to viruses [15–17]. The examples given below include those applications for West Nile virus, St. Louis encephalitis, influenza virus, and Hand-foot-mouth virus, as well as specific neutralizing antibodies.

West Nile Virus and St. Louis Encephalitis Virus CEP and Neutralizing Antibody Detection

West Nile virus (WNV) and Louis encephalitis virus (SLEV) are closely related antigenically and classified within the Japanese encephalitis virus serocomplex within the genus Flavivirus in the family Flaviviridae. Both viruses are maintained and amplified within Culex-passerine bird cycle that intermittently spills over to induce equines and humans that suffer variable symptoms and disease, but are dead-end hosts for these viruses [44, 45]. Human disease caused by these two viruses varies clinically and is frequently confused with influenza viruses. Disease onset typically occurs after peak viremia, making clinical diagnosis difficult and requiring laboratory confirmation by serology [46, 47]. Both viruses grow well on a variety of mosquito, avian, and mammalian cell cultures, and measurement of viral concentration is quantified by counting plaque forming units (PFU) on cell monolayers of African green monkey kidney or Vero cells. This method is slow, time-consuming,

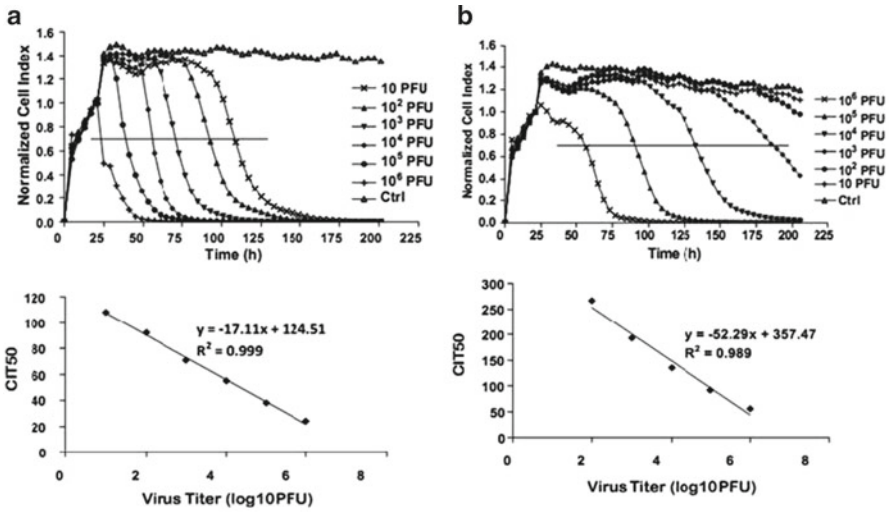


Fig. 8.7 Real-time monitoring of (**a**) WNV and (**b**) SLEV-induced CPE on Vero cells. Normalized cell index plotted as a function of time in hours post infection for E-wells inoculated with different plaque forming units (PFU) of virus and control (ctrl) wells without virus addition. The curve is an average of two independent replicate wells. The horizontal line depicts the 50 % point in the decline of the CI value. The intersect of the horizontal line and cell index response curve corresponds to the CIT50. CIT50 values were regressed as a function infectious dose in log 10 PFU. Goodness of regression fit to experimental data is shown by R^2

and difficult to measure in real time. RTCA system was used to monitor CPE in Vero cell cultures induced by West WNV and SLEV at infectious doses ranging from 10^1 to 10^6 PFU of virus. A kinetic parameter characterizing virus-induced CPE, CIT_{50}, time to 50 % decrease in cell impedance, was inversely proportional to virus infectious dose. In WNV-infected cells, the onset and rate of CPE was earlier and faster than in SLEV-infected cells, which was consistent with viral cytolytic activities (Fig. 8.7). A mathematical model simulating impedance-based CPE kinetic curves indicated that the replication rate of WNV was about three times faster than SLEV [15]. The RTCA system also was used for quantifying the level of cell protection by specific neutralizing antibodies against WNV and SLEV. The onset of WNV or SLEV-induced CPE was delayed in the presence of specific antisera and the delay in the CIT_{50} was well correlated with the titer of the neutralizing antibody as measured independently by plaque reduction neutralization test (PRNT) (Fig. 8.8). This assay was also employed to detect serum samples from infected birds in the field and very good correlation was reported between the result obtained from RTCA system and conversion PRNT. In this study, the authors also used the time of the delay of the onset of CPEs (CIT50) to a standard antibody dose curve and determined the neutralization antibody titer without performing serum titration [15]. The results demonstrated that RTCA system can provide a high-throughput and quantitative method for real-time monitoring of viral growth in cell culture and its inhibition by neutralizing antibodies.

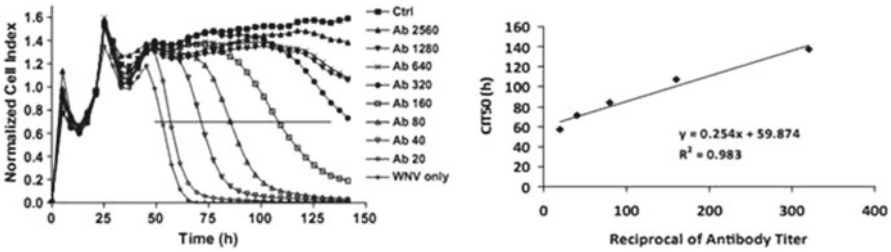


Fig. 8.8 Quantitative detection of WNV neutralizing antibody titer using real-time monitoring of virus-induced CPE. Virus-induced CPE kinetic pattern of Vero cells presented with 106 PFU of WNV incubated with different titers of conspecific neutralizing antibody; control (ctrl), no infection. The *cross line* indicates the 50 % decline of CI value. Linear regression between CIT50 value and the WNV PRNT antibody titer was significant between 1:20 and 1:320. Antibody values are the reciprocal of the endpoint PRNT titers

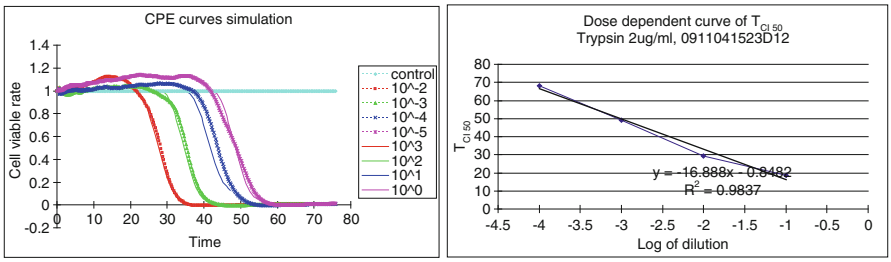


Fig. 8.9 Quantitative detection of H1N1 virus-induced CPEs in MDCK cells. The kinetic curves recorded by real-time monitoring of H1N1 virus-induced CPEs on MDCK cells (*left panel*) at different concentrations as indicated. Normalized cell index plotted as a function of time in hours after infection of the virus. The solid line curves are the curves recorded from infected cells, the dot line curves are the curves simulated from a mathematic model created to quantify the infection of virus-induced CPEs on RTCA system [22]. The logarithmic numbers indicate the $TCID_{50}$ units of virus used for infection. The control (ctrl) indicates the cells without viral infection. The good linearity of quantitative measurement of H1N1-induced CPE was calculated by using the time at 50 % drop of CI (*right panel*)

Influenza Virus and Neutralizing Antibody Detection

RTCA was also used for influenza A virus detection [48]. Recently, this technology was successfully applied for quantitative measurement of influenza A H1N1 strain-induced CPE , which is a pandemic influenza strain first isolated in Mexico and that caused a global outbreak in 2009. To conduct the RTCA on H1N1 virus-induced CPE on MDCK cells using RTCA system, 10,000 cells (early stationary phase) were infected with H1N1 virus isolated from a H1N1-infected patient. The concentration of the virus was determined by real-time PCR. A decline of CI indicated the cells dying as a consequence of H1N1 virus replication in the cells and onset of CPE (the time of cell index drop in the infected cells) was linearly correlated well with infection doses of H1N1 virus (Fig. 8.9). Furthermore, consistent with WNV infection, onset of H1N1-induced CPE can be delayed in the presence of neutralizing antibody

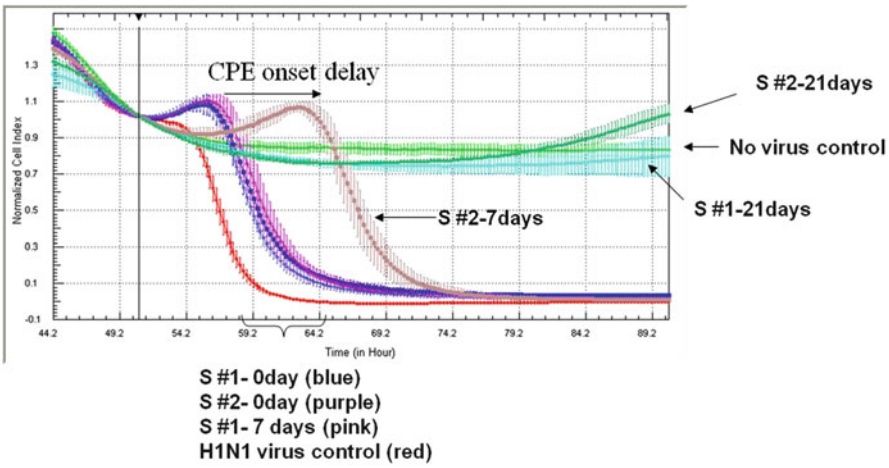


Fig. 8.10 Neutralizing antibody activity measurement in H1N1-vaccinated human subjects using real-time monitoring of virus-induced CPE on RTCA system. Samples of two human subjects from different days after vaccination were tested for neutralizing activity against H1N1 virus challenge. In samples from day 0 (before vaccination), no protection can be seen showing the CPE onset similar to virus control. Sample #2 (S#2) at day 7 showed significant delay of H1N1-induced CPE indicating the presence of specific neutralizing antibody against H1N1 virus, and in contrast, Sample #1 (S#1) at day 7 showed no effect on the onset of H1N1 virus-induced CPE. At the day 21, both samples (S#1 and S#2) showed complete protection against H1N1-induced CPE indicating both subjects developed neutralizing antibody against H1N1 virus after vaccination

specific to H1N1, and such delay was also quantitatively correlated with antibody concentration or titers. The higher the titer or activity of the neutralizing antibody the more profound the delay of the onset of H1N1 virus-induced CPE. The ability to quantitatively measure neutralizing antibody activity on RTCA system can allow for evaluating the success of vaccination of H1N1 vaccine in human population. Samples of 123 human subjects vaccinated with the virus strain A/California/07/2009 (H1N1) were tested on RTCA system. For each subject, three samples from different times (day 0, day 7, and day 21) were collected and the seroconversion and neutralizing activity against the H1N1 virus were monitored on the RTCA system (Fig. 8.10) [16]. According to hemagglutinin-inhibition antibody titers of 1:40 or more, 67.48 % of vaccinated subjects showed neutralizing antibody positive on day 7 post-vaccination, and on day 21, 82.11 % of subjects scored positive for the neutralizing antibody [17]. For neutralization assays, all subjects could be ensured that they obtained the immune protection against wide influenza virus. Moreover, the rates of seroconversion, as measured using hemagglutinin-inhibition assays and neutralization assays, were 73.98 and 91.87 % of subjects, respectively. This result indicates that the vaccination was successful. Although, the result obtained from the neutralizing antibody test agreed with that from HI test, the seroconversion rate using neutralizing antibody detection is slightly lower than the seroconversion rate using standard HI test, indicating that the HI activity against H1N1 might not be necessarily related to the neutralizing activity.

Hand-Foot-Mouth Virus Detection

Hand, foot, and mouth disease (HFMD) is a common viral illness in infants and children. The disease causes fever and blister-like eruptions in the mouth and/or a skin rash. HFMD is caused by viruses that belong to the enterovirus genus (group). This group of viruses includes polioviruses, coxsackieviruses, echoviruses, and enteroviruses [49]. Enteroviruses, including enterovirus 71 (EV71), have also been associated with HFMD and with outbreaks of the disease [50, 51]. As described above, we evaluated RTCA system for detection of EV71-induced CPE of in RD cells (Fig. 8.11). The RD cells cultivated in E-Plates were infected with EV71 at serially diluted concentrations ranging from $1{:}10^2$ to 10^8 dilution of a viral stock. The EV71-induced CPEs were then monitored in real time on the RTCA system as shown in the Fig. 8.11, and an infected dose-dependent kinetic curve can be clearly seen. The higher the dilution of the viral stock is, the longer the onset of CPE (nCI drop). Interestingly, a good quantitative curve can also been generated by using the time of 50 % drop of nCI (Fig. 8.11).

Helminth Motility and Egg Hatching Activity on RTCA System

Billions of people are infected with helminths in developing countries, resulting in many thousands of deaths annually. Parasitic worms cause untold morbidity and mortality of billions of people and livestock worldwide [52]. Drugs are available but resistance is problematic in livestock parasites and is a looming threat for human helminths. Currently, new drug discovery and resistance monitoring is hindered as drug efficacy is assessed by observing motility or development of parasites using laborious, subjective, low-throughput methods evaluated by using low throughput techniques such as visualization by light microscopy. Recently, a group of Australian researchers utilized the RTCA system to develop a novel application that can simply and objectively assess real-time antiparasite efficacy of drugs on eggs, larvae, and adults in a fully automated, label-free, high-throughput fashion (Fig. 8.12). This technique overcomes the current low-throughput bottleneck in anthelmintic drug development and resistance-detection pipelines. The widespread use of this device to screen for new therapeutics or emerging drug resistance will be an invaluable asset in the fight against human, animal, and plant parasitic helminths and other pathogens that plague our planet.

The motility of all helminth species and developmental stages were assessed using an RTCA system that monitors cellular events in real time without the incorporation of labels by measuring electrical impedance across interdigitated microelectrodes integrated on the bottom of tissue culture E-Plates [19].

Because the RTCA system measures changes in worm motility with a high level of precision, it is widely applicable to a range of helminth species and developmental stages. While this technique was used only for the species herein (Table 8.2), it is

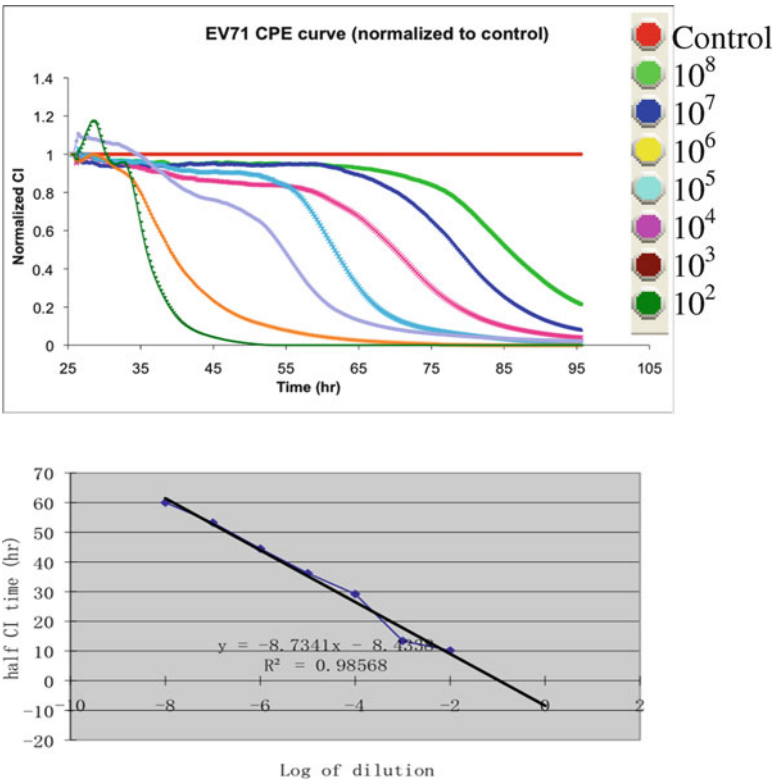


Fig. 8.11 Quantitative detection of EV71 virus-induced CPEs in RD cells. The kinetic curves obtained by real-time monitoring of EV71-induced CPEs in RD cells (*top panel*) on RTCA system. Normalized cell index was plotted as a function of time in hours after infection of EV71 virus at different concentrations as indicated by logarithm of $TCID_{50}$ units and control is the cells without infection. The good linearity (R^2=0.986) of quantitative measurement of EV71-induced CPE was calculated by using the time at 50 % drop of CI (*bottom panel*)

highly likely that any motile developmental stage from any species that will rest at the bottom of a 96-well microtiter plate can be monitored using minor adaptations of the techniques that we describe here. The ability to directly assess multiple developmental stages for susceptibility to a drug or other intervention is a distinct advantage. For example, PZQ is much more effective against the adult stage of *S. mansoni* than it is against the schistosomulum, the developmental stage that is usually the focus of in vitro drug assessments. *H. contortus* displays drug susceptibility differences between infective larval and adult stages, which poses a problem for drug screening and resistance detection that can be overcome by utilizing the RTCA assay for assessing motility of adult worms.

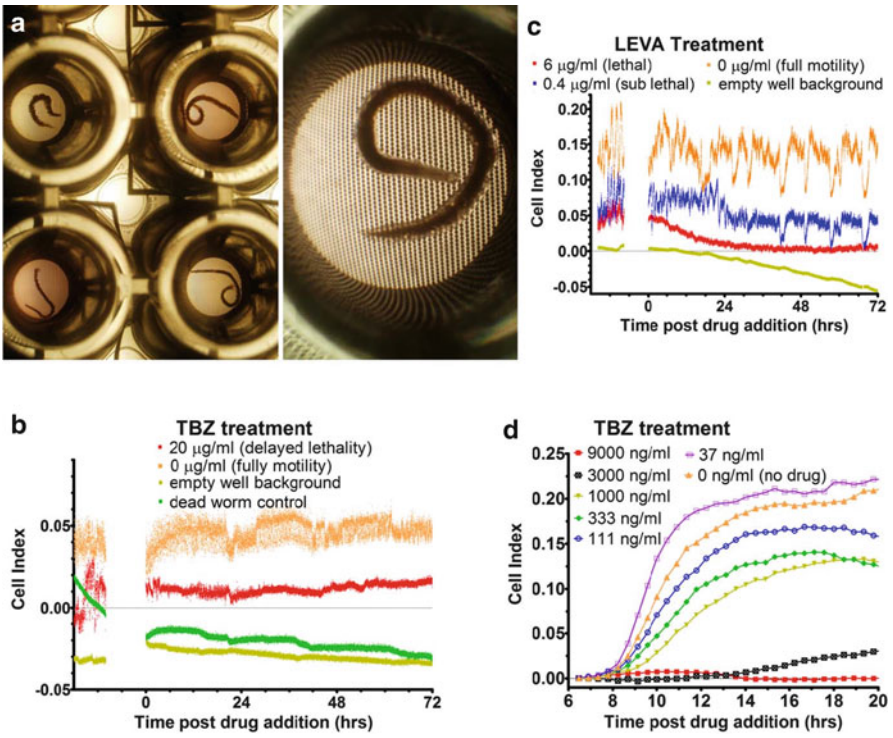


Fig. 8.12 RTCA unit differentiates between live and dead parasites from different developmental stages using the cell index readout. Panel (**a**): Micrograph of adult *Ancylostoma caninum* hookworms—females in the top two wells and magnified image on the *left*, and males in the bottom two wells. Note the gold circular electrodes covering the base of the E-Plate in the magnified image. Panel (**b**): Cell index output generated by a single adult female *A. caninum* with and without exposure to thiabendazole (TBZ)*. Panel (**c**): *Haemonchus contortus* L3 cell index output in the presence of varying amounts of levamisole (LEVA)*. Panel (**d**): *H. contortus* egg hatching in the presence of varying amounts of TBZ—curves show the average of duplicate experiments. Note that increasing drug concentrations result in less egg hatching and a corresponding lesser cell index output. *The cell index numerical value is not relevant to this analysis—the curves have been manually repositioned to assist with visualization of the data. The amplitude within each curve is the important feature of the data for this experiment

Host Immune Function Assessment on RTCA: Nature Killer Cells and T Cells

Natural Killer Cell Function Assessment

Natural killer (NK) cells are considered the major cytotoxic effector cells of innate immunity. NK cells are defined by their ability to kill cells displaying a foreign Ag (e.g., tumor cells or cells infected with virus or microbes) regardless of MHC type and regardless of previous sensitization (exposure) to the antigens. Some NK cells are

Table 8.2 Summary of IC50 values for a range of drugs and developmental stage of parasitic helminthes as measured by RTCA

Parasite	Drug	IC_{50} (ng/ml)	95 % CI (ng/ml)	Time to stable IC_{50} (h)	Previous data (95 % CI) (ng/ml)
Female *A. caninum*[a]	TBZ	13.4	6.3–28.5	6	Unknown
S. mansoni adult pairs	PZQ	188	161–221	48	Varies depending on methodology
S. ratti L_3	IVM	174	103–296	4	1,032 (946–1,204) (Kotze et al. 2004)
H. contrortus L_3 IVM-resistant strain	IVM	310	240–390	24[b]	2,950 (1,910–4,550) (pers. Comm. Andrew Kotze 2010)
IVM-sensitive strain		280	230–330		1,040 (860–1,250) (Kotze et al. 2006)
LEVA-resistant strain	LEVA	1,710	1,480–1,990	6[c]	24,000 (1,810–3,200) (pers. Comm. Andrew Kotze 2010)
LEVA-sensitive strain		410	380–440		20,000 (1,890–2,110) (pers. Comm. Andrew Kotze 2010)
H. contortus eggs (TBZ-resistant strain)	TBZ	704	525–946	12	4,400 (3,980–4,870) (Kotze et al. 2009)

[a]Male hookworms were successfully tested but too few worms were available to calculate IC_{50} values

[b]IVM-resistant strain does not show significant IC_{50} difference after stabilization (24 h), but does show a significant difference up to 12 h (minimum $p < 0.01$)

[c]Significantly different to sensitive strain at all time points (minimum $p < 0.01$)

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probably derived from TC cells (CTLs), but they do not display T cell markers [53]. NK cells can be activated by IL-2 and gamma IFN [54]. Natural Killers lyse cells in the same manner as CTLs, which require cell-to-cell contact mediated by the pair-wise recognition between multiple NK cell receptors present on the surfaces of effector and target cells [55]. In other cases, some NK cells have receptors for the Fc domain of IgG and so are able to bind to the Fc portion of IgG antibody on the surface of a target cell and release cytolytic components that kill the target cell. This mechanism of killing is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells play an important role in defending against microbial and viral infections and therefore functional assessment of NK cell activity greatly helps in the diagnosis and prognosis of microbial and viral infections. Various methodologies have been developed to measure cell-mediated cell death. Most in vitro assays are based on quantifying cytoplasmatic constituents (labels or naturally occurring compounds) that are released to the extracellular compartment as a result of plasma membrane disintegration. The prototypic experimental setup employs targets cells loaded with a compound that is retained in the cytoplasm. The ^{51}Cr release assay is widely used for in vitro measurement of cell-mediated cytotoxicity [56], but the radioactive materials and non-kinetic readouts of the analysis have significantly limited the applications for functional assessment in clinical laboratories.

RTCA system has been used to detect NK cells-mediated cytotoxic effect [57, 58]. Unlike the conventional assay, the assay developed on the RTCA system does not require any labeling process and dynamically monitors the cytotoxic effect of NK cells in target cells. In the reports by Glamann et al. [57], a human breast cancer cell line, MCF-7, was used as the target cell for measuring NK-mediated cell killing. MCF-7 cells are adherent cells, and the cell attachment on the electrodes leads to increase in CI. In contrast to the adherent cells, the suspended NK cells cause minimum CI increase when they were added to the wells in the E-plate where the target cells (MCF-7 cells) were cultured. Therefore, the CI changes after addition of effector, NK cells are mainly contributed by target cells. There was a clear discrepancy between CI and number of viable cells during the first hours of NK cell killing. At 2.5 h the CI decreased by 60 % (effector:target cell ratio 5:1) and 80 % (effector:target cell ratio 20:1), whereas the [3H]thymidine amount was lowered only by 10–15 % for both ratios as determined. The loss of radioactivity did approach the decline of CI with time, but it took many hours (6 h) before there was a match, indicating the RTCA assay was more sensitive than a [3H] thymidine-based cell viability assay. In another study [58], a murine NK cell line and a murine target cell line, NIH 3T3 were used. The target cells were seeded in the wells of 96-well E-plate at 5,000 cells/well and cell growth was continuously monitored on RTCA system. When the cell growth reached the logarithmic phase, the effector cells added at different E:T ratios (30:1, 15:1, and 7.5:1) and the NK cell-mediated cytolysis was dynamically monitored on the RTCA system A significant decline of nCI was seen in the wells where the mNK cells were added (Fig. 8.13). In this study, a control cell line, Yac cell line was also used to determine the specificity of NK cell killing. When the Yac cells, a T lymphocyte line without cytolysis function, were added to the target cells, no significant cytolysis effects were seen in E:T ratio at 15:1 and 7.5:1. However, when the Yac cells were overloaded to the well (30:1), the decline of CI can be seen only after 20 h, and such decline of CI mainly resulted from the nutrition depletion in the well (Fig. 8.11). Therefore, the appropriate E:T ratios are needed to be selected before the assay can be used for NK cell function assessment. RTCA system also could be used to measure ADCC. A431 is a known ADCC target using human antibodies specific for the EGF-R. The values of CI 10 h after addition of NK cells were used to construct a dose–response curve, which gave a 50 % effective concentration for Erbitux of 1.3 ng/ml. This result is consistent with previous report [59] with the 51Cr labeling assay in the same cell line with another human antibody against EGF-R.

T Cell Surface Receptor Detection

The importance of antigen-specific T cells for proper immune function is widely recognized [60, 61]. T cell receptor numbers and ratios are commonly used to assess disease status and progression as well as monitor treatment [62]. In certain disease states where nonfunctional T cells are present [63, 64], enumeration of

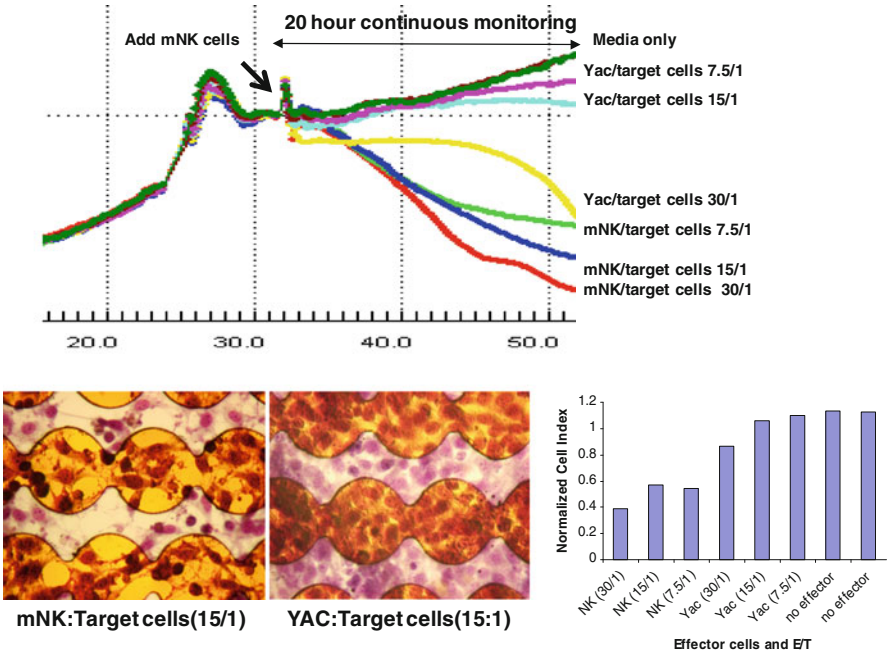

Fig. 8.13 Dynamic monitoring of mouse NK cell (mNK) cytolysis of target cells using the RTCA system. NIH3T3 cells (target cells) were seeded in the wells of E-plate and mouse NK cells, or Yac cells (T cells without NK function) and media alone were added at the indicated time point (arrow) at different E/T ratios as shown. The viability of target cells was continually monitored using the RTCA system (Top panel) in the wells in the presence of NK, Yac, media control for 20 h in real time. At the end of the experiment (20 h), the wells of the E-plates were washed, fixed in methanol, and stained with Giemsa dye, and the target cells. The wells were visualized and photographed using a light microscope connected to CCD camera. The *bar graph* shows the Normalized Cell Index at 14 h of treatment

antigen-specific T cells may yield only a partial picture of the disease. A cell-based assay is an added tool to define functional T cells. Flow cytometry is a standard clinically approved method used to quantitate antigen-specific T cells and can also be used for a functional readout (i.e., cytokine production). However, this assay still requires lysis/fixation, permeabilization, and labeling steps. We demonstrated that by coating the wells of the E-Plates with functional anti-CD3 or anti-CD28 antibodies, activation of the T cell receptor complex, which leads to cell adhesion, was reflected as an increase in cell index (Fig. 8.14). Co-stimulatory molecules such as CD28 activate secondary signaling cascades, allowing the cell to proliferate through release of interleukin-2 [65]. Upon co-stimulation of the T cell receptor complex with CD28, there was an increase in cell index over that of either anti-CD3 or anti-CD28 antibody alone (Fig. 8.14). Note that the peak response does not occur at the same time and is readily distinguished and quantitated in a real-time format with kinetic monitoring.

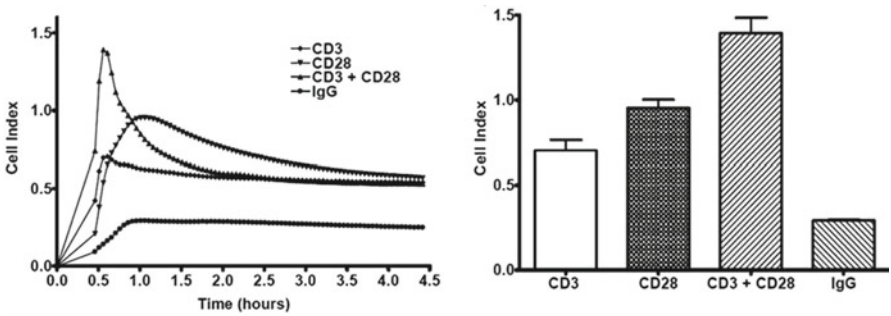

Fig. 8.14 T Cell activation and adhesion using the RTCA system. Wells of the E-Plate were coated with functional antibodies for CD3, CD28, or IgG overnight at 4 °C. After washing the E-Plates with PBS, media was added for background measurement, followed by the addition of 250,000 Jurkat cells per well. Activation and adhesion of Jurkat cells was monitored every 3 min for 4 h (*left panel*). Peak Cell Index responses upon activation by CD3, CD28, CD3 + CD28, or IgG are summarized from the data in *right panel*

Perspectives

Cellular analysis is not a new laboratory procedure. Clinical diagnostics using cells and tissues is one of the oldest laboratory medicine disciplines which had changed little until recently. What is new, however, is the myriad of combinations of cell-based assays and technologies. As discussed in this chapter, a microelectronic sensor-based cellular analysis technology was developed allowing for real-time monitoring kinetic cellular processes. Notably, the kinetic responses of cells to the microbial and viral infections make the quantitative and functional assessment possible and can hardly be achieved by conventional cell-based assay systems. Although the clinical applications of RTCA system and related platform technologies remain to be further explored, the value in diagnostics of the microbial and viral infections and guidance of treatments for complex diseases in a functionally relevant setting have been demonstrated both for pathogen detection and host immune system assessment. Assays based on RTCA technology may become widely utilized in clinical diagnostics of microbial and viral infections and in assessment of host immune functions. Furthermore, a new map of cell diagnostics based on RTCA technology will be created to cover the assay systems that are highly diversified and involve several laboratory disciplines, including not only microbiology but also hematology, histology, cytology, tissue transplant medicine, and most recently cell therapeutics. The evolution of cell research and cellular analysis technologies has turned cell-based diagnostics into a melting pot of clinical diagnostics and technologies.

References

1. Donald HB, Isaacs A (1954) Counts of influenza virus particles. J Gen Microbiol 10:457–464
2. Chen X, Xu J, Shuai J, Chen J, Zhang Z, Fang W (2007) The S-layer proteins of Lactobacillus crispatus strain ZJ001 is responsible for competitive exclusion against Escherichia coli O157:H7 and Salmonella typhimurium. Int J Food Microbiol 115:307–312
3. Leland DS, Ginocchio CC (2007) Role of cell culture for virus detection in the age of technology. Clin Microbiol Rev 20:49–78
4. Kline KA, Falker S, Dahlberg S, Normark S, Henriques-Normark B (2009) Bacterial adhesins in host-microbe interactions. Cell Host Microbe 5:580–592
5. Cossart P, Boquet P, Normark S, Rappuoli R (1996) Cellular microbiology emerging. Science 271:315–316
6. Elsinghorst EA (1994) Measurement of invasion by gentamicin resistance. Methods Enzymol 236:405–420
7. Pizarro-Cerda J, Lecuit M, Cossart P (2002) In: Sansonetti PJ, Zychlinsky A (eds) Measuring and analyzing invasion of mammalian cells by bacterial pathogens: The Listeria monocytogenes system in Molecular Cellular Microbiology. Methods in Microbiol, Academic Press, London, 31:161–177
8. Srivastava A, Isberg RR (2002) In: Sansonetti PJ, Zychlinsky A (eds) Measurement of bacterial uptake by cultured cells in Molecular Cellular Microbiology. Methods in Microbiol, Academic Press, London, 31:179–188
9. Ridgway GL, Oriel JD, Mumtaz G, Mellars B (1986) Comparison of methods for detecting Chlamydia trachomatis. J Clin Pathol 39:232–233
10. Belland R, Ojcius DM, Byrne GI (2004) Chlamydia. Nat Rev Microbiol 2:530–531
11. Giaever I, Keese CR (1986) Use of electric fields to monitor the dynamical aspect of cell behavior in tissue culture. IEEE Trans Biomed Eng 33:242–247
12. Giaever I, Keese CR (1993) A morphological biosensor for mammalian cells. Nature 366:591–592
13. Solly K, Wang X, Xu X, Strulovici B, Zheng W (2004) Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. Assay Drug Dev Technol 2:363–372
14. Ryder AB, Huang Y, Li H et al (2010) Assessment of Clostridium difficile infections by quantitative detection of tcdB toxin by use of a real-time cell analysis system. J Clin Microbiol 48:4129–4134
15. Fang Y, Ye P, Wang X, Xu X, Reisen W (2011) Real-time monitoring of flavivirus induced cytopathogenesis using cell electric impedance technology. J Virol Methods 173:251–258
16. Lu HZ, Xu X (2011) Label-free real-time cell based assay system for evaluating H1N1 vaccination success. Asia Pacific Biotech News 14:15–17
17. Sun F, Zhang Y, Tian D et al (2011) Responses after one dose of a monovalent influenza A (H1N1) 2009 inactivated vaccine in Chinese population—a practical observation. Vaccine 29:6527–6531
18. Yang G, Zhou B, Wang J et al (2008) Expression of recombinant Clostridium difficile toxin A and B in Bacillus megaterium. BMC Microbiol 8:192
19. Smout MJ, Kotze AC, McCarthy JS, Loukas A (2010) A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. PLoS Negl Trop Dis 4:e885
20. Cooper MA (2006) Non-optical screening platforms: the next wave in label-free screening? Drug Discov Today 11:1068–1074
21. McGuinness R (2007) Impedance-based cellular assay technologies: recent advances, future promise. Curr Opin Pharmacol 7:535–540
22. Fang Y (2006) Label-free cell-based assays with optical biosensors in drug discovery. Assay Drug Dev Technol 4:583–595
23. Atienza JM, Yu N, Kirstein SL et al (2006) Dynamic and label-free cell-based assays using the real-time cell electronic sensing system. Assay Drug Dev Technol 4:597–607

24. Abassi YA, Xi B, Zhang W et al (2009) Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. Chem Biol 16:712–723
25. Atienza JM, Yu N, Wang X, Xu X, Abassi Y (2006) Label-free and real-time cell-based kinase assay for screening selective and potent receptor tyrosine kinase inhibitors using microelectronic sensor array. J Biomol Screen 11:634–643
26. Xing JZ, Zhu L, Jackson JA et al (2005) Dynamic monitoring of cytotoxicity on microelectronic sensors. Chem Res Toxicol 18:154–161
27. Xi B, Yu N, Wang X, Xu X, Abassi YA (2008) The application of cell-based label-free technology in drug discovery. Biotechnol J 3:484–495
28. Ke N, Wang X, Xu X, Abassi YA (2011) The xCELLigence system for real-time and label-free monitoring of cell viability. Methods Mol Biol 740:33–43
29. Bartlett JG (2006) Update in infectious diseases. Ann Intern Med 144:49–56
30. Kelly CP, LaMont JT (2008) Clostridium difficile—more difficult than ever. N Engl J Med 359:1932–1940
31. McDonald LC, Killgore GE, Thompson A et al (2005) An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med 353:2433–2441
32. Chang TW, Lauermann M, Bartlett JG (1979) Cytotoxicity assay in antibiotic-associated colitis. J Infect Dis 140:765–770
33. Planche T, Aghaizu A, Holliman R et al (2008) Diagnosis of Clostridium difficile infection by toxin detection kits: a systematic review. Lancet Infect Dis 8:777–784
34. Peterson LR, Manson RU, Paule SM et al (2007) Detection of toxigenic Clostridium difficile in stool samples by real-time polymerase chain reaction for the diagnosis of C. difficile-associated diarrhea. Clin Infect Dis 45:1152–1160
35. He X, Wang J, Steele J et al (2009) An ultrasensitive rapid immunocytotoxicity assay for detecting Clostridium difficile toxins. J Microbiol Methods 78:97–100
36. Johnson EA (ed) (2005) Clostridium botulinum and Clostridium tetani, 8th edn. Arnold Hodder, London
37. Blasi J, Chapman ER, Link E et al (1993) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature 365:160–163
38. Binz T, Blasi J, Yamasaki S et al (1994) Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J Biol Chem 269:1617–1620
39. Neale EA, Bowers LM, Jia M, Bateman KE, Williamson LC (1999) Botulinum neurotoxin A blocks synaptic vesicle exocytosis but not endocytosis at the nerve terminal. J Cell Biol 147:1249–1260
40. Lindstrom M, Korkeala H (2006) Laboratory diagnostics of botulism. Clin Microbiol Rev 19:298–314
41. O'Connell J, Abassi YA, Xi B, Wang X, Xu X (2008) Real-time cell-based toxicology testing might replace animal testing for product release and drug safety. Biochemica 4:11–13
42. Schubert-Unkmeir A, Konrad C, Slanina H, Czapek F, Hebling S, Frosch M (2010) Neisseria meningitidis induces brain microvascular endothelial cell detachment from the matrix and cleavage of occludin: a role for MMP-8. PLoS Pathog 6:e1000874
43. Slanina H, Konig A, Claus H, Frosch M, Schubert-Unkmeir A (2011) Real-time impedance analysis of host cell response to meningococcal infection. J Microbiol Methods 84:101–108
44. Kramer LD, Styer LM, Ebel GD (2008) A global perspective on the epidemiology of West Nile virus. Annu Rev Entomol 53:61–81
45. Reisen WK, Chiles RE, Martinez VM, Fang Y, Green EN (2003) Experimental infection of California birds with western equine encephalomyelitis and St Louis encephalitis viruses. J Med Entomol 40:968–982
46. Oceguera LF 3rd, Patiris PJ, Chiles RE, Busch MP, Tobler LH, Hanson CV (2007) Flavivirus serology by Western blot analysis. Am J Trop Med Hyg 77:159–163
47. Patiris PJ, Oceguera LF 3, Peck GW, Chiles RE, Reisen WK, Hanson CV (2008) Serologic diagnosis of West Nile and St. Louis encephalitis virus infections in domestic chickens. Am J Trop Med Hyg 78:434–441

48. McCoy MH, Wang E (2005) Use of electric cell-substrate impedance sensing as a tool for quantifying cytopathic effect in influenza A virus infected MDCK cells in real-time. J Virol Methods 130:157–161
49. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA (1999) Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol 73:1941–1948
50. Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH (2010) Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis 10:778–790
51. Wong SS, Yip CC, Lau SK, Yuen KY (2010) Human enterovirus 71 and hand, foot and mouth disease. Epidemiol Infect 138:1071–1089
52. Albonico M, Allen H, Chitsulo L, Engels D, Gabrielli AF, Savioli L (2008) Controlling soil-transmitted helminthiasis in pre-school-age children through preventive chemotherapy. PLoS Negl Trop Dis 2:e126
53. Robertson MJ, Ritz J (1990) Biology and clinical relevance of human natural killer cells. Blood 76:2421–2438
54. Frey JR, Kamber M, Peck R (1987) Recombinant interferons or interleukin-2 increase cytotoxicity by human monocytes and NK cells. Lymphokine Res 6:215–227
55. Lanier LL (2005) NK cell recognition. Annu Rev Immunol 23:225–274
56. Brunner KT, Mauel J, Cerottini JC, Chapuis B (1968) Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology 14:181–196
57. Glamann J, Hansen AJ (2006) Dynamic detection of natural killer cell-mediated cytotoxicity and cell adhesion by electrical impedance measurements. Assay Drug Dev Technol 4:555–563
58. Zhu J, Wang X, Xu X, Abassi YA (2006) Dynamic and label-free monitoring of natural killer cell cytotoxic activity using electronic cell sensor arrays. J Immunol Methods 309:25–33
59. Bleeker WK, Lammerts van Bueren JJ, van Ojik HH et al (2004) Dual mode of action of a human anti-epidermal growth factor receptor monoclonal antibody for cancer therapy. J Immunol 173:4699–4707
60. Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S (2002) Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 20:621–667
61. Wange RL, Samelson LE (1996) Complex complexes: signaling at the TCR. Immunity 5:197–205
62. Messele T, Roos MT, Hamann D et al (2000) Nonradioactive techniques for measurement of in vitro T-cell proliferation: alternatives to the [(3)H]thymidine incorporation assay. Clin Diagn Lab Immunol 7:687–692
63. Sieg SF, Harding CV, Lederman MM (2001) HIV-1 infection impairs cell cycle progression of CD4(+) T cells without affecting early activation responses. J Clin Invest 108:757–764
64. Zajac AJ, Blattman JN, Murali-Krishna K et al (1998) Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med 188:2205–2213
65. Salazar-Fontana LI, Barr V, Samelson LE, Bierer BE (2003) CD28 engagement promotes actin polymerization through the activation of the small Rho GTPase Cdc42 in human T cells. J Immunol 171:2225–2232
66. Kotze AC, LeJambre LF, O'Grady J (2006) A modified larval migration assay for detection of resistance to macrocyclic lactones in *Haemonchus contortus*, and drug screening with Trichostrongylidae parasites. Veterinary Parasitology 137:294–305
67. Kotze AC, Lowe A, O'Grady J, Kopp SR, Behnke JM (2009) Dose-response assay templates for *in vitro* assessment of resistance to benzimidazole and nicotinic acetylcholine receptor agonist drugs in human hookworms. Am J Trop Med Hyg 81:163–170

Chapter 9
Cellular Fatty Acid-Based Microbial Identification and Antimicrobial Susceptibility Testing

Nicole Parrish and Stefan Riedel

Introduction

Bacteria produce a wide array of complex lipids and fatty acids. Both fatty acids and complex lipids can vary in chain length, number and position of functional groups, and number and position of double bonds. As early as 1963, the analysis of cellular fatty acid (CFA) for the purpose of bacterial identification was introduced by Abel et al. and Kaneda [1, 2]. The variability of fatty acid structure in bacteria has led to the characterization of over 300 bacterial fatty acids and related compounds [3]. These fatty acids and related compounds range from relatively polar lipids found in bacterial membranes to more complex lipids found in lipooligosaccharides and lipopolysaccharides. Other fatty acids are components of highly specialized lipids, such as the mycolic acids, found in *Mycobacteria*, *Nocardia*, and related genera. Specialized methods have been developed for detailed analysis of fatty acids and related lipids. These methods have aided efforts in bacterial classification yielding both qualitative and quantitative analysis of specific fatty acids and their constituent components. Such methods have provided the basis for bacterial identification and taxonomic classification and continue to be used by many laboratories as an inexpensive and rapid screening tool or as an ancillary test to other standard bacterial identification methods. More recently, CFA analysis has been used to identify bacterial agents of bioterrorism [4].

The following sections describe the basic principles of CFA analysis, currently available testing platforms for bacterial identification, specific identification of bioterror agents, use of mycolic acid analysis for mycobacterial identification, potential for rapid susceptibility testing, and advantages and disadvantages versus other identification technologies currently in use.

N. Parrish (✉) • S. Riedel
Division of Medical Microbiology, Department of Pathology,
Johns Hopkins School of Medicine, 600 North Wolfe Street,
Meyer Building B1-193, Baltimore, MD 21287, USA
e-mail: nparrish@jhmi.edu

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General Principles and Methods of Cellular Fatty Acid Analysis

In bacteria, fatty acids are primarily found in the phospholipids of the cell membrane, the Lipid A component of LPS (gram-negative organisms), lipoteichoic acid (gram-positive organisms), or mycolic acids in aerobic Actinomycetes. So specific are the fatty acids contained within some of the larger lipids that the presence or absence of a particular chemical species can be indicative of a gram-negative or gram-positive organism. For instance, hydroxy fatty acids, such as 10:0 3OH, 12:0 3OH, and/or 14:0 3OH, are derived from LPS, which indicates a gram-negative organism, whereas the absence of LPS and hence the absence of hydroxylated fatty acids are indicative of a gram-positive organism [5].

The vast majority of bacteria synthesize fatty acids with chain lengths ranging from 10 to 19 carbons in length, with hexadecanoic acid being the predominant type. This 16 carbon, saturated fatty acid is highly conserved among bacteria. Higher organisms tend to have polyunsaturated fatty acids not found in bacteria. The fatty acids of gram-negative bacteria tend to have a higher proportion of straight-chain, saturated and monounsaturated cellular fatty acids comprising even numbers of carbon atoms. Gram positives (e.g., *Bacillus* spp. and *Staphylococci*) tend to have saturated, branched-chain cellular fatty acids consisting of odd numbers of carbon atoms with overall lower amounts of straight-chain, saturated fatty acids. Other organisms have both straight-chain and unsaturated cellular fatty acids (*Streptococci* and coryneforms). In contrast, eukaryotic cells of humans and fungi typically lack the branched-chain and cyclopropane-containing fatty acids found in bacteria [5].

Preparation of samples for CFA analysis begins with hydrolysis of whole-cell fatty acids forming sodium salts followed by methylation of the CFA esters. Although various procedures have been utilized over time, the most commonly used procedure involves a four-step process in sample preparation. Initially, cells are harvested (approximately 20 mg wet weight) off specific solid media from an area of the plate which represents log phase growth. Typically plates are incubated for 24 h (±2 h). It is important to note that the quantitative CFA composition of bacteria can vary depending on both culture conditions, including temperature and the type of media used [6–8]. Thus when utilizing such methods for routine bacterial identification, it is important to insure that standardized and validated growth conditions including the use of commercially available media are met before undertaking any CFA analysis. Once harvested, fatty acids are saponified using a sodium hydroxide–methanol solution for 30 min at 100 °C. The saponification breaks covalently linked fatty acids from cellular lipids and the application of heat accelerates the process. The free fatty acids are then methylated using hydrochloric acid and methanol at 80 °C for 10 min followed by extraction into an organic solution of hexane and methyl *t*-butyl ether for 10 min. Once these steps have been completed, extracts are washed with aqueous sodium hydroxide for 5 min and transferred to vials for analysis by gas liquid chromatography (GLC). Sample preparation requires roughly an hour.

CFA Testing Platforms for Bacterial Identification

CFA analysis has been in use for more than 50 years for bacterial identification [9]. Initial systems relied on manual rather than automated operation. The most common GLC technology currently in use is fully automated and utilizes fused silica capillary columns which allow for extremely small injection volumes (Sherlock® Microbial Identification System, MIDI, Newark, Delaware). Fatty acids ranging from 9 to 20 carbons (9:0 to 20:0) are identified and quantified. A calibration standard (MIDI) consisting of saturated (9–20 carbons) and additional hydroxy fatty acids is used to monitor the quality control and performance characteristics of the assay. For each peak eluting from the column, retention times are calculated and converted to equivalent chain length (ECL) which is used in turn for fatty acid naming. Each fatty acid profile is then compared to a library database containing greater than 100,000 entries from strain collections obtained from around the world [10]. This is an important consideration since geographic bias is possible given that microbes occupy a wide variety of environmental conditions. In addition, for each species or subspecies, approximately 20 or more strains of each species tested were included in the analysis to allow for strain-to-strain variation [10, 11]. The particular fatty acid profile of an unknown organism is compared with the mean fatty acid composition of the strains used to create the library entry or entries listed as its match. The resulting similarity index represents the relative distance from the population mean for a given species. Thus, an exact match between two profiles would yield a similarity index of 1.0. Currently available libraries include a large number of bacterial genera and species including aerobic bacteria (192 genera; >700 species), anaerobic bacteria (92 genera; >500 species), *Mycobacteria* (>31 species), and bacterial agents of bioterrorism (five genera; six species) [10]. In addition, a rapid sample preparation method is also available designed to identify environmental aerobes and yeast in less than 15 min from pure culture [10].

Library Expansion and Fatty Acid Polymorphisms

As our ability to recover and identify new organisms or subspecies or strain variations within the same genus increases, it will become extremely important that standardized culture conditions are being used to routinely expand current library entries and databases now in use for identification. It is important to recognize that discrepancies in identification may occur as a result of variation in the similarity index, and may also reflect poorly defined library entries (i.e., too few representative strains). Discrepancies in identification may also occur due to organisms which are very closely related biochemically and genetically.

However, the fatty acid composition of all strains within a species is not necessarily homogeneous. For example, the CFA profile of *Francisella* spp. is distinctive. The predominant fatty acids are C10:0 (22 %), C14:0 (15 %), C16:0 (18 %), 3 OH

C18:0 (17 %), and the somewhat unusual C22:0, C24:0, and C24:10 [12]. In a study conducted by Clarridge et al., all strains analyzed were named *Franciscella tularensis* by the database in use, yet the profiles were different enough to separate the strains into three closely related groups [13]. A second study illustrated this same finding with Yersinia spp. in which all species tested had the following fatty acids: 12:0, 14:0, 3-OH-14:0, 16:0, 16:1v9cis, 17:0-cyc, and 18:1v9trans indicating that the overall fatty acid composition between strains and species was very homogeneous [14]. However, when ratios for specific fatty acids were examined more closely (12:0/16:0 and 14:0/16:0) it was determined that the genus *Yersinia* could be separated into three distinct clusters, the last of which contained *Y. pestis* and *Y. pseudotuberculosis* [14]. Findings such as these indicate that just like genetic polymorphisms, fatty acid polymorphisms may occur within each genus and species and also between strains. The presence of such polymorphisms may lead to difficulties in interpretation if they are not represented in the currently used database. Such issues can be overcome by inclusion of additional strains of a given species which may broaden the fatty acid profile and provide more precise separation from other taxonomically similar organisms.

Identification of Agents of Bioterrorism and Other Select Agents

The recent threat of bioterrorism has resulted in development of technologies which can rapidly detect and identify select bacterial agents. With regard to CFA analysis, it was recognized early on that specific select agents had unique fatty acid profiles versus other species within the same genus [15, 16]. In fact, CFA analysis was used to identify *Burkholderia mallei* in a laboratory-acquired infection in a microbiologist [17].

Separation of organisms suitable for biowarfare and bioterrorism from related nonpathogenic species within the same genus is based upon unique fatty acid profiles between the two. For instance, in one study, 95 % of *B. pseudomallei* strains tested were positive for 2-hydroxymyristic acid (14:0 2OH), as compared with 0 % of the avirulent *B. thailandensis* strains [18]. Similarly, other agents of bioterrorism, such as *B. mallei and B. pseudomallei*, *Y. pestis*, *F. tularensis*, and *Brucella* spp., can also be differentiated from each other and from other members within each respective genus based on CFA analysis [13, 15–17, 19]. It is very difficult, for example, to differentiate the two closely related *Yersinia* species, *Y. pestis* and *Y. pseudotuberculosis*, by phenotypic means alone. The fact that these two organisms are genetically very close also poses difficulties for the use of molecular test methods. CFA has been shown to be a useful and alternate test method to reliably differentiate between these two species [14, 20]. Currently, the MIDI Sherlock system and the MIDI BIOTER database are approved by the AOAC International Official Methods of Analysis for confirmatory identification of *B. anthracis* [10, 15]. The assay was also granted FDA 510(k) clearance for confirmation of *B. anthracis* which can be differentiated from *B. cereus*. Furthermore, with the

increase in outbreaks of food-borne illnesses, CFA has been shown to be a valuable asset to laboratories for the identification of organisms associated with food-borne illnesses (e.g., *Listeria*, *Bacillus*, *Salmonella*, *Vibrio*, *Escherichia coli*) as well as for identification of endospore-forming aerobic bacilli [21].

A disadvantage to the use of CFA analysis for identification of bioterror agents is the requirement for isolates to be grown in pure culture to insure accuracy and reproducibility. This necessarily exposes laboratory personnel to potentially dangerous pathogens; thus proper training and handling of the organism(s) are of paramount importance.

Mycolic Acid Analysis for Identification of Mycobacteria

Mycobacteria produce unique fatty acids known as mycolic acids. Mycolic acids are high-molecular-weight α-alkyl, β-hydroxy fatty acids containing an α-branch of 20–25 carbons, and a primary or meromycolate chain of 50–60 carbons. The total combined size of these fatty acids ranges from 70 to 90 carbons. In addition, these large fatty acids contain a variety of functional groups and can vary in both qualitative and quantitative characteristics between species. This variety provides the basis for separation and identification of a large number of mycobacterial species using high-performance liquid chromatography (HPLC). HPLC rather than GLC is necessary for separation of mycolic acids due to their large size and complexity which requires the use of different columns and solvents. Initial methods required manual interpretation of chromatograms with eventual development of automated systems [22, 23].

Mycolic acids are covalently linked to the cell wall arabinogalactan matrix. Removal of the mycolic acids requires saponification with potassium hydroxide (50 % w/v) which is often performed in an autoclave to accelerate the process and provide for the safety of laboratory personnel working with Biosafety Level III mycobacterial species. Once autoclaved, the organisms are killed by the procedure and the mycolic acids released from the cell wall. As in the case of GLC and bacteria, standardized culture conditions must be used in order to permit accuracy and reproducibility of chromatograms. Mycobacteria are typically grown on solid medium such as Middlebrook 7 H10 or 7 H11 at 35–37 °C until growth is visible. Currently available databases have been developed which incorporate mycobacterial species which require different growth conditions such as *M. haemophilum* and *M. marinum* (30 °C) [24]. Once autoclaving has been completed, samples are cooled to room temperature, acidified, and extracted into chloroform. Subsequently, fluorescently labeled derivatives of the mycolic acid methyl esters are prepared using a combination of 4-bromomethyl 1-6, 7-dimethoxycoumarin and crown ether. The fluorescently labeled mycolic acids are then analyzed via HPLC using a reverse-phase column (C18) and a fluorescent detector. Both quantitative and qualitative data are captured by the instrument. The amount of mycolic acid present is related to the amount of light emitted and the structure of the mycolic acid is related to the

time of elution off the column (retention time). Analysis is performed by the software which utilizes retention time, peak width, and peak amount to provide a peak name which can then be compared to a library database in order to provide an identification [24].

HPLC-based identification of the *Mycobacteria* provides an alternative to other more technically demanding and often time-consuming methodologies such as DNA sequencing for those laboratories which frequently encounter the species contained in the current library database. If the isolate cannot be identified using the currently available database, the testing algorithm may proceed to alternative methods such as DNA sequencing for identification. Some species of mycobacteria cannot be separated based on mycolic acid profiles as their fatty acids are too similar. This also occurs with other technologies such as DNA sequencing in which the genetic sequences are too similar to allow for separation and identification. In such cases, identification is often reported to the species-complex level. Very often, these species are typically closely related in terms of both DNA and fatty acid homology and often share clinical significance. Common examples include the *M. tuberculosis complex* and the *M. avium* complex. Other species which cannot be reliably separated at this time due to the closely related characteristics of the mycolic acids are *M. abscessus*/*M. chelonae* and *M. fortuitum/M. peregrinum.* Simple ancillary biochemical tests can often confirm the species identification. For instance, a positive citrate test would be confirmation of *M. chelonae* versus *M. abscessus* [25].

HPLC and Susceptibility Testing

Quantitative data extracted from the current HPLC-based identification platform has enabled development of a more rapid, phenotypically based susceptibility testing method for *M. tuberculosis* and *M. avium* [26–29]. The principle of this method rests on the fact that nearly 30–50 % of the dry weight of the mycobacterial cell comprises mycolic acids. As a result, this large proportion of mycolic acids is linearly related to the number of colony-forming units (CFUs) per ml of culture. The method, which has been standardized for all first-line drugs (isoniazid, rifampin, ethambutol, and pyrazinamide), is performed much as the standard identification procedure with a few modifications. The entire incubation required for all drugs considered together is 72 h versus the typical days to weeks for other automated platforms and solid media. Hence the common name for this method is termed "RAM," *r*apid *a*nalysis of *m*ycolic acids. For each test, a standardized inoculum is prepared, and used to inoculate control and paired drug containing broth cultures. Samples are subsequently incubated for 72 h, at which time the cultures are harvested and subjected to the standard HPLC extraction protocol using equal volumes from control and treated cultures. Once the HPLC is run the resulting chromatogram provides a quantitative measure of the total peak height/area response of the mycolic acid peaks (TMAP). Values from control versus drug-treated cultures are acquired with ChemStation software and processed using Sherlock software for calculation of the total mycolic

acid profile. TMAP is determined for each isolate–drug combination and compared to the corresponding untreated control. The resulting ratio is plotted relative to a standardized breakpoint of 30 % which is used for all further data analysis [26, 28].

In one study, test agreement with the reference method was 99.5 % for INH, EMB, and PZA, and 98.7 % for RIF. Inter- and intra-assay reproducibility varied by drug with an average precision of 13.4 % [28]. Discernible differences in TMAP reductions were also noted based on the mechanism of action of individual drugs. For instance, the most rapid decrease in TMAP occurred in MTB treated with INH. This is not surprising since the INH-specific mechanism of action in MTB is inhibition of mycolic acid synthesis. Thus, with a mycolic acid-specific inhibitor such as INH, significant differences in CFU/ml are not necessary to detect susceptibility or resistance. However, for other drugs which do not directly target mycolic acid synthesis such as RIF or PZA, or are bacteriostatic as in the case of EMB, a significant difference in CFU/ml is required to determine drug susceptibility using TMAP, requiring longer incubation times to maximize differences in CFU/ml [28].

Subsequent studies have been conducted in which this same methodology has been applied to other mycobacterial species of medical importance including *M. avium*. In this study, clarithromycin susceptibility testing was performed using a modification of the standard HPLC method previously described for *M. tuberculosis*. As with *M. tuberculosis*, clarithromycin susceptibility and resistance was determined following 72-h incubation versus the standard 7–14 days required for the reference method, microbroth dilution [26, 30]. RAM concordance with the reference method for claritrhomycin susceptibility was 98 % (254/258) and 100 % for clarithromycin resistance (72/72). The four discordant results occurred with two strains, which demonstrated intermediate resistance with an MIC of 16 μg/ml [26]. These studies demonstrate that RAM-based susceptibility testing for determination of first-line drugs in *M. tuberculosis* and clarithromycin resistance in *M. avium* is both rapid and accurate. In both cases, a significant reduction in turnaround time from days/weeks to hours was realized. Currently, the RAM assay is performed using growth in pure culture. Smaller studies have been conducted which demonstrate that direct susceptibility testing from smear-positive sputum is possible with this assay [31]. Additional improvements in mycolic acid extraction, derivatization, and sensitivity should streamline the assay and improve this possibility.

Advantages and Disadvantages of Fatty Acid-Based Identification Methods

Technological advances continue to move the field of microbiology forward with improvements in both detection and identification of organisms. Molecular-based methods such as DNA sequencing of various targets are now competing with other techniques for detection and identification of microorganisms. However, the single most perfect diagnostic method remains elusive and more often than not, final identification requires a combination of methods. For this reason, many clinical

laboratories continue to use CFA analysis in conjunction with other methods such as biochemical testing and DNA sequencing for identification of bacterial unknowns. Many laboratories find CFA analysis especially helpful in identification of gram-negative, non-fermentative organisms for which biochemical testing is not routinely performed.

As with any testing platform, there are advantages and disadvantages to be considered. CFA analysis requires relatively low technical proficiency and can accommodate large numbers of unknowns per day (~40 to ~200), depending upon the capability of the laboratory. A technician averages about 5 min per sample to prepare a batch of 30 samples and because nonsubjective offline tests or gram stains are required, the naming is highly objective and reproducible.

In addition, CFA analysis is relatively inexpensive to perform at a cost of less than $3.00 per test versus an average of >$100.00 per test for many molecular assays. Although the list of consumables needed for sample preparation and chromatographic analysis is quite lengthy, they are all stored at room temperature and though the initial investment for the instrument itself is not trivial, it is comparable to that of other identification systems on the market.

CFA analysis requires growth in pure culture using standardized conditions negating the possibility of direct testing of patient specimens. In addition, numerous steps are required for sample preparation including harvesting of the bacterial unknown which can result in unintentional laboratory exposures. Laboratory directors must consider all aspects of their particular testing needs when considering implementation of not only existing technologies but emerging ones as well.

References

1. Abel K, Deschmertzing H, Peterson JI (1963) Classification of microorganisms by analysis of chemical composition. I. Feasibility of utilizing gas chromatography. J Bacteriol 85:1039–1044
2. Kaneda T (1963) Biosynthesis of branched chain fatty acids. I. Isolation and identification of fatty acids from *Bacillus subtilis* (ATCC 7059). J Biol Chem 238:1222–1228
3. VanDamme P (2007) Taxonomy and classification of bacteria. In: Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, Murray P (eds) Manual of clinical microbiology. ASM, Washington, DC, pp 543–572
4. Lim DV, Simpson JM, Kearns EA, Kramer MF (2005) Current and developing technologies for monitoring agents of bioterrorism and biowarfare. Clin Microbiol Rev 18:583–607
5. Kunitsky C, Osterhout J, Sasser M (2006) Identification of microorganisms using fatty acid methyl ester (FAME) analysis and the MIDI Sherlock® microbial Identification System. In: Miller MJ (ed) Encyclopedia of rapid microbiological methods, vol 3. DHI Publishing, LLC, River Grove, IL, pp 1–56
6. Drucker DB (1974) Chemotaxonomic fatty-acid fingerprints of some streptococci with subsequent statistical analysis. Can J Microbiol 20:1723–1728
7. Knivett VA, Cullen J (1965) Some factors affecting cyclopropane acid formation in *Escherichia coli*. Biochem J 96:771–776
8. Marr AG, Ingraham JL (1990) Effect of temperature on the composition of fatty acids in *Escherichia coli*. J Bacteriol 84:1260–1267
9. Welch DF (1991) Applications of cellular fatty acid analysis. Clin Microbiol Rev 4:422–438

10. www.midi-inc.com
11. O'Hara C (2005) Manual and automated instrumentation for identification of*Enterobacteriaceae* and other aerobic gram-negative bacilli. Clin Microbiol Rev 18:147–162
12. Jantzen E, Berdal BP, Omland T (1979) Cellular fatty acid composition of *Francisella tularensis*. J Clin Microbiol 10:928–930
13. Clarridge J 3, Raich T, Sjosted A et al (1996) Characterization of two unusual clinically significant *Franciscella tularensis* strains. J Clin Microbiol 34:1995–2000
14. Leclerq A, Guiyoule A, El Lioui M, Carniel E, Decallone J (2000) High homogeneity of the Yersinia pestis fatty acid composition. J Clin Microbiol 38:1545–1551
15. AOAC International (2004) Initiative yields effective methods for anthrax detection; RAMP and MIDI, Inc., methods approved. Inside Lab Manage 10:3
16. Song Y, Yang R, Guo Z, Zhang M, Wang X, Zhou F (2000) Distinctness of spore and vegetative cellular fatty acid profiles of some aerobic endospore-forming bacilli. J Microbiol Methods 39:225–241
17. Srinivasan A, Kraus CN, DeShazer D et al (1999) Glanders in a military research biologist. N Engl J Med 345:256–258
18. Timothy J, Inglis J, Aravena-Roman M et al (2003) Cellular fatty acid profile distinguishes *Burkholderia pseudomallei* from avirulent *Burkholderia thailandensis*. J Clin Microbiol 41:4812–4814
19. Khan AS, Ashford DA (2001) Ready or not-preparedness for bioterrorism. N Engl J Med 345:287–289
20. Tan Y, Wu M, Liu H et al (2010) Cellular fatty acids as chemical markers for differentiation of *Yersinia pestis* and *Yersinia pseudotuberculosis*. Lett Appl Microbiol 50:104–11
21. Whittaker P, Fry FS, Curtis SK et al (2005) Use of fatty acid profiles to identify food-borne bacterial pathogens and aerobic endospore-forming bacilli. J Agric Food Chem 53:3735–3742
22. Butler WR, Jost KC Jr, Kilburn JO (1991) Identification of mycobacteria by high-performance liquid chromatography. J Clin Microbiol 29:2468–2472
23. Centers for Disease Control (1996) Standardized method for HPLC identification of mycobacteria. CDC, Atlanta, GA
24. Mycobacteria Identification System Operating Manual Version 1.0 (2003) MIDI incorporated, Newark, DE
25. Pfyffer G (2007) *Mycobacterium:* general characteristics, laboratory detection, and staining procedures. In: Murray P, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA (eds) Manual of clinical microbiology. ASM, Washington, DC, pp 543–572
26. Flauta V, Osterhout G, Ellis B et al (2010) Use of the "RAM" susceptibility testing method for rapid detection of clarithromycin resistance in the *Mycobacterium avium* complex. Diagn Microbiol Infect Dis 67(1):47–51
27. Garza-Gonzales E, Guerrero-Olazaran M, Tijerina-Menchaca R, Viader-Salvado J (1997) Determination of drug susceptibility of *Mycobacterium tuberculosis* through mycolic acid analysis. J Clin Microbiol 35:1287–1289
28. Parrish N, Osterhout G, Dionne K et al (2007) A rapid, standardized, susceptibility method for *Mycobacterium tuberculosis* using mycolic acid analysis. J Clin Microbiol 45:3915–3920
29. Viader-Salvado JM, Garza-Gonzalez E, Valdez-Leal R, Bosque-Moncaya M, Tijerina-Menchaca R, Guerrero-Olazaran M (2001) Mycolic acid index susceptibility method for *Mycobacterium tuberculosis*. J Clin Microbiol 2001(39):2642–2645
30. Clinical and Laboratory Standards Institute (CLSI) (2008) Laboratory detection and identification of mycobacteria. approved guideline
31. Dionne K, Carroll K, Parrish N (2011) "RAM"-based rapid identification and determination of resistance in *Mycobacteria* direct from sputum. An abstract presented at the 111th ASM General Meeting, New Orleans

Chapter 10
MALDI-TOF Mass Spectrometry-Based Microbial Identification

Alexander Mellmann and Johannes Müthing

Introduction

Rapid and accurate species identification of bacteria, fungi, and viruses is a fundamental requirement in clinical and food microbiology and other fields of microbiology diagnostics. Whereas virus recognition is usually achieved within hours by either serological tests or genotyping approaches using various nucleic acid detection systems, the conventional identification of bacteria and fungi still mainly relies on methods that include laborious and time-consuming initial cultivation and ensuing isolation of the microorganism. This approach is therefore dependent on the generation time (growth) of the particular microorganism, resulting in assay durations of 16–24 h minimum, e.g., in the case of *Enterobacteriaceae* or other fast-growing prokaryotes, and up to several weeks in the case of slow-growing mycobacteria and some fungi. Though species identification of a pure culture is achievable within 24–48 h with various (semi-)automated systems, additional isolation steps are frequently necessary, which can extend the time until diagnosis by days, e.g., if the potential pathogen must be separated from the physiological background flora. Realistically species assignment of a putative pathogen from a nonsterile specimen takes at least 2–3 days. In many areas of patient care, elapsed time until diagnosis may considerably reduce the therapeutic quality of care due to a lack of information about the infecting pathogen. Therefore, a rapid species diagnosis is of high priority as a focused therapy might be lifesaving for the patient [1, 2]. Similarly, a timely diagnosis is imperative for surveillance studies or screenings with particular demands during outbreak situations of foodborne pathogens or preadmission screening to detect multiresistant bacteria in the hospital setting [3, 4]. Both species identification

A. Mellmann (✉) • J. Müthing
Institute of Hygiene, University Hospital Münster,
Robert-Koch-Str. 41, 48149 Münster, Germany
e-mail: mellmann@uni-muenster.de

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and resistance testing are of equal importance; however, this chapter focuses primarily on species identification.

In addition to the time required to identify an unknown species, some bacterial species or groups are still difficult to differentiate. During the last decade molecular studies have raised doubts about traditional genus and species assignments, resulting in profound reclassifications of numerous bacterial genera and species as well as the discovery of a large number of novel species. Furthermore, these investigations demonstrated substantial limitations of previously employed methods and the urgent need for the development of more reliable techniques [5, 6]. Finally, in some bacterial species, such as within the diverse group of gram-negative, nonfermenting rods, extensive reclassification as well as their nonreactive biochemical behavior and different colony morphologies pose further challenges in unequivocal species identification [7].

Great efforts have been made to enhance the accuracy and the speed of species identification. In addition to genotypic methods that rely on DNA sequencing of discriminatory regions, for example 16S rRNA encoding genes in prokaryotes [8, 9], various phenotype-related procedures have been developed such as cell wall analysis or determination of fatty acid and protein profiles [10–12], enabling a robust species identification that is independent from the bacterial metabolism or regulatory phenomena. In addition to the ameliorated species identification, the expense per assay is a key issue and has to be considered. The applicability to automation plays a pivotal role in modern clinical laboratories and must be taken into account in addition to the hands-on/turn-around time and assay costs. Finally, ease and robustness of procedures are prerequisites for their implementation in the clinical laboratory. In this context, reproducibility of results and acceptance by both the client and regulatory authority are essential for the establishment in a clinical laboratory.

Here, the power of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is demonstrated as a redevelopment that has evolved to revolutionize the identification of prokaryotic and eukaryotic pathogens in microbial laboratories during recent years. This chapter starts with a short technical overview about the principles of mass spectrometry, in particular MALDI-TOF MS. Speed, accuracy, and reproducibility of MS techniques will be compared with customary methods and their current limitations will be discussed. Additionally, an alternative MS strategy based on electrospray ionization (ESI), also suitable for species identification, will be introduced.

General Remarks on Mass Spectrometry

Mass spectrometry is an emerging technique that has been developed into a very useful tool to structurally analyze biomolecules of various substance classes, such as nucleic acids [13], (glyco)proteins [14], (glyco)lipids [15], and others. The primary objective of MS lies in identification of the exact molecular mass of an isolated biomolecule or individual molecules such as proteins in mixture. The applicability of

mass spectrometry to the analysis of complex biomolecules has been greatly improved by introducing two soft ionization techniques—MALDI and ESI MS. Both methods ionize large molecules, which tend to be fragile and fragment when more conventional ionization methods are applied. MALDI and ESI MS can be easily implemented in a straightforward diagnostic procedure to reliably identify the genus, species, and, in some cases, subspecies of bacteria [16].

Generally, a typical mass spectrometer is built up from three components: an ion source, a mass analyzer, and a detector. The ion source produces ions from the sample, the mass analyzer separates ions with different mass-to-charge ratios (m/z), and the numbers of different ions are detected by the detector. The resulting output is a mass spectrum which is displayed as a graph of the ion intensities versus m/z values and consists of a number of mass spectral peaks, forming a unique pattern. The majority of ions generated by MALDI contain only one charge and only one peak is shown for each individual compound in the spectrum, facilitating data interpretation. Ions produced by ESI may be multiply charged resulting in considerably more complex mass spectra than MALDI. Notably, signal intensities do not necessarily reflect the quantities of different sample molecules.

MALDI and ESI MS

In the past decades, several different MALDI- and ESI-based methods were developed, a few of which are nowadays applied in systems used for microbial identification purposes. Both methods are highly advantageous as the analyte structure is preserved due to the use of soft ionization. MALDI, invented in the 1980s [17, 18], is based on UV laser ionization of the analyte (any substance up to whole bacterial or fungal cells), which is embedded in an appropriate matrix on a target plate. Matrix molecules fulfill several requirements that are crucial for ionization of the investigated biomolecules. They are of low molecular weight and low volatility preventing vaporization during sample preparation. Acidic matrices are useful as they act as proton donors that are essential for ionization of the analyte. Furthermore, they not only possess polar groups that enable use in aqueous solutions but also exhibit strong optical absorption in the UV range, so that they efficiently absorb the energy from laser irradiation. In most cases small organic molecules, such as 2,5-dihydroxy benzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA), are used for UV MALDI and mixed in a 1,000–10,000-fold excess to the analyte [17, 19]. Cocrystallization of the analyte with the matrix is a another key issue in selecting a proper matrix to obtain a good quality mass spectrum of the analytes of interest. A pulsed laser-beam, usually a nitrogen UV laser ($\lambda = 337$ nm), is fired within a vacuum at the matrix/analyte crystals that are within the dried-droplet spot on the target plate. The laser energy is absorbed by the matrix, which in turn is desorbed in an expanding plume and ionized by addition of a proton [20]. Charging of the analyte occurs through the transfer of protons or sodium ions to the sample molecules and quasimolecular singly charged ions are formed, e.g., $[M+H]^+$ or

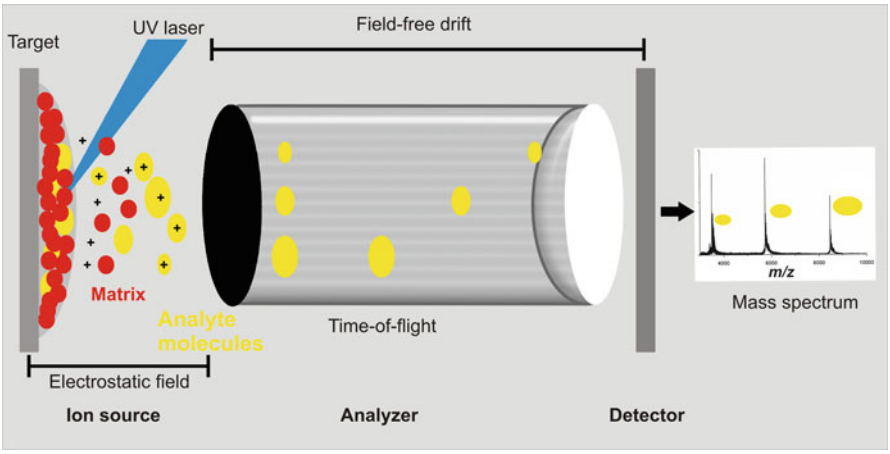

Fig. 10.1 Schematic illustration of a linear UV laser MALDI-TOF mass spectrometer. The matrix-analyte mixture is shot with an UV laser leading to desorption of the analyte and matrix and to a transfer of protons from the matrix to the analyte molecules. Ions are subsequently accelerated in an electric field, separated during their travel in a field-free flight tube according to their mass-to-charge (*m*/*z*) ratio, and finally detected with the detector. The resulting mass spectrum is displayed as a graph of the ion intensities versus *m*/*z* values

$[M+Na]^+$, respectively (Fig. 10.1). After the sample and matrix molecules have entered the gas phase of the vacuum environment, the newly generated ions are accelerated in an electric field of known strength in the time-of-flight (TOF) analyzer. The TOF analyzer is a field-free flight tube, where the ions can travel in a strait and linear direction to the detector (Fig. 10.1). A linear TOF spectrum is limited in resolution and cannot distinguish ions with similar *m*/*z* values. This can be partially corrected in a reflectron TOF analyzer in which ions are reflected by an "iron mirror" using an electric field, thereby doubling the ion flight path and increasing the resolution (not shown). The velocity of the ions depends on the mass and the degree of ionization, i.e., the *m*/*z* ratio, and is measured as the time it takes for the ions to fly from one end of the analyzer to the other and strike the detector. The flying speeds of ions are proportional to their *m*/*z* ratio and the *m*/*z* values versus signal intensity are then finally drawn as the mass spectrum where the *x*-axis depicts the *m*/*z* value and the *y*-axis the intensity [19]. Figure 10.1 displays a schematic graphical representation of a linear TOF MALDI mass spectrometer and an example mass spectrum of a sample composed of three different mass components.

The mathematical basis of the mass determination is the following equation:

$$E_{kin} = \tfrac{1}{2} m v^2 = z e U$$

with E_{kin} as the kinetic energy of the ions after acceleration within the electric field with the voltage U. The speed v is determined indirectly via the TOF and the

covered distance, and e is the elementary electric charge. Altogether, this enables the exact calculation of the m/z values. For further characterization of biomolecules, modern mass spectrometers can also be equipped with a collision chamber filled with an inert gas, e.g., argon. Collision with these molecules leads to fragmentation of the analyte ions and assists in full structural characterization of the unknown analyte molecules.

In contrast to MALDI, in ESI the sample is solubilized in volatile organic solvent (e.g., methanol, acetonitrile) prior to ionization. The analyte is then nebulized, together with the solvent, as a fine spray through a very small, charged and usually metal or glass capillary equipped with a stainless steel needle into the electric field at atmospheric pressure. The resulting charged droplets of the analyte are then subjected to a TOF analyzer for generation of a mass spectrum. During the spraying process, the solvent continuously evaporates leading to an increase of the charge density. At the Raleigh-limit the electrostatic repulsive forces exceed the surface tension and the droplets divide into smaller subunits (Coulomb explosion). The smaller droplets continue to evaporate and the process is repeated again ultimately resulting in charged analyte molecules which enter the mass analyzer. As whole bacterial or fungal cells cannot be solubilized sufficiently, ESI is mainly used for analysis of cellular components or other soluble analytes such as polymerase chain reaction (PCR)-amplified microbial nucleic acids providing an alternative biomarker analysis [21, 22]. As mentioned above, a general advantage of MALDI-TOF MS is the soft ionization of the embedded analyte without destruction of the analyte, which makes it especially suitable in the structural characterization of intact biomolecules. Moreover, only a few microliters of the matrix–analyte mixture are required for placement onto the target plate. Preparation of this mixture is usually very simple and requires only a few minutes to complete [19].

Species Identification Using MALDI-TOF MS

Species identification of intact microorganisms, taken directly from culture, by means of MALDI-TOF MS has been firstly described for various gram-positive and gram-negative bacteria by Claydon et al. in 1996 and the general applicability has been reviewed, including intact viruses, spores, and fungi, by Fenselau and Demirev in 2001 [23, 24]. The spectra obtained allowed identification of microorganisms from different genera, different species, and from different strains of the same species [23]. Assignment was realized with whole cell extracts by the exact mass determination of desorbed peptides and small proteins of the cell wall resulting in a unique mass spectral fingerprint of the microorganism under investigation. At that time it was assumed that the measured masses were unique and representative for individual microorganisms forming the basis of applications of mass spectrometry in microbiology without knowing the detailed characterization of each component [24–26]. Figure 10.2 illustrates the workflow for species identification using MALDI-TOF MS.

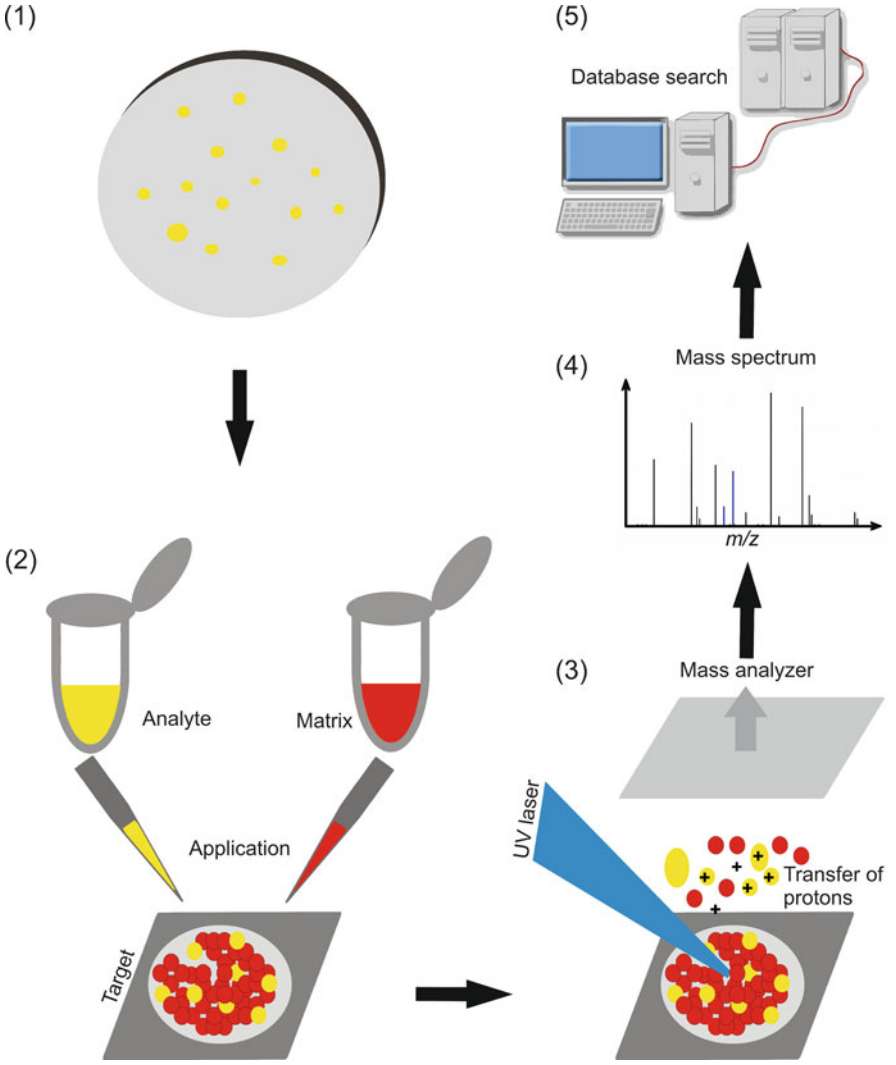

Fig. 10.2 Principal workflow of MALDI-TOF MS-based species identification. (*1*) Bacterial or fungal colonies on an agar plate. (*2*) Mixing of matrix and analyte for sample preparation and subsequent cocrystallization of the matrix–analyte mix by gentle air-drying. (*3*) Laser desorption, ionization, and mass analysis of the analyte molecules. (*4*) Generation of the mass spectrum. (*5*) Computer-assisted data analysis and comparison of acquired spectra with reference database entries

Shortly after the first description [23] it became apparent that the poor reproducibility of measurements was mainly due to (1) shortcomings in sample preparation, especially in gram-positive bacteria [27], (2) inconsistencies among different MALDI-TOF mass spectrometers employed, and (3) variability in bacterial culture conditions [28]. Moreover, until the middle of the last decade, there was still a lack

of a urgently needed sophisticated software and data analysis tools that were essential for the integration of MALDI-TOF MS outcomes into the clinical laboratory. A valid identification of bacterial and fungal species was further hampered by the deficit in central spectra databases that comprised fingerprint libraries derived from well-characterized reference strains required for comparison with newly generated mass spectra of unknown composition. For these reasons microbial MALDI-TOF MS at its infancy was neither well accepted nor implemented for routine application into the microbial and/or clinical laboratory.

To address poor reproducibility and serious inconsistency that could be attributed to different culture conditions and resulting alteration of the microorganisms' metabolism, the investigation of proteins was shifted to a higher *m*/*z* range. Whereas the first publications reported on measured mass ranges from 550 to 2,000 Da to identify a species [23], current systems encompass a mass range of 2,000–20,000 Da. This mass range mainly measures ribosomal proteins [29, 30], conserved proteins that are highly abundant in any type of prokaryotic and eukaryotic cells. This approach warrants a relatively high robustness against variability of metabolic products and fluctuation of other cell components that may occur by varying culture conditions. Additionally, ribosomal proteins are positively charged, which facilitates MALDI-TOF MS [31]. This improved stability and reproducibility has been recently demonstrated for some prokaryotic microorganisms, e.g., nonfermenting bacteria or staphylococci [31, 32]. For other groups of pathogens, a strict adherence to certain culture conditions is strongly recommended as accumulation of metabolites [33], sporulation (e.g., *Bacillus* spp.) or autolytic processes due to long-term storage (e.g., *Streptococcus* spp.) might cause sizeable changes in the mass pattern.

Technical advancements in the last decade have eliminated the production of diverging spectra due to operation on individual instruments (Fig. 10.3) [31]. Additionally, optimization and standardization of sample preparation protocols now allow for rapid processing of any type of bacteria within minutes [27, 34].

To further enhance the quality of MALDI-TOF mass spectra for use in species identification, new algorithms have been developed which aim at better and more valid comparison of spectra from unknown microorganisms against a database harboring spectra from well-characterized reference strains [35–39]. In combination with continuously growing reference databases, nowadays commercially available systems produce a correct species identification in most instances. The following two prerequisites should be fulfilled for a reference database: First, the database should contain spectra from well-characterized culture collection strains that have been cultured under optimal and standardized conditions. These strains should be relevant for the specific diagnostic questions that may differ significantly between various disciplines (e.g., medical microbiology, food microbiology, or environmental monitoring). Second, the reference database should include not only the prototypical strain of a certain species but also as many other strains as possible from the same species to both determine and compensate for the naturally occurring intraspecies variability. In general, these two core conditions are essential for any diagnostic procedure that is based on comparisons against a reference [8, 40]. Similar to other identification strategies that rely on phenotype-related fingerprint libraries, the assignment is usually supplemented by calculation of an error probability or result

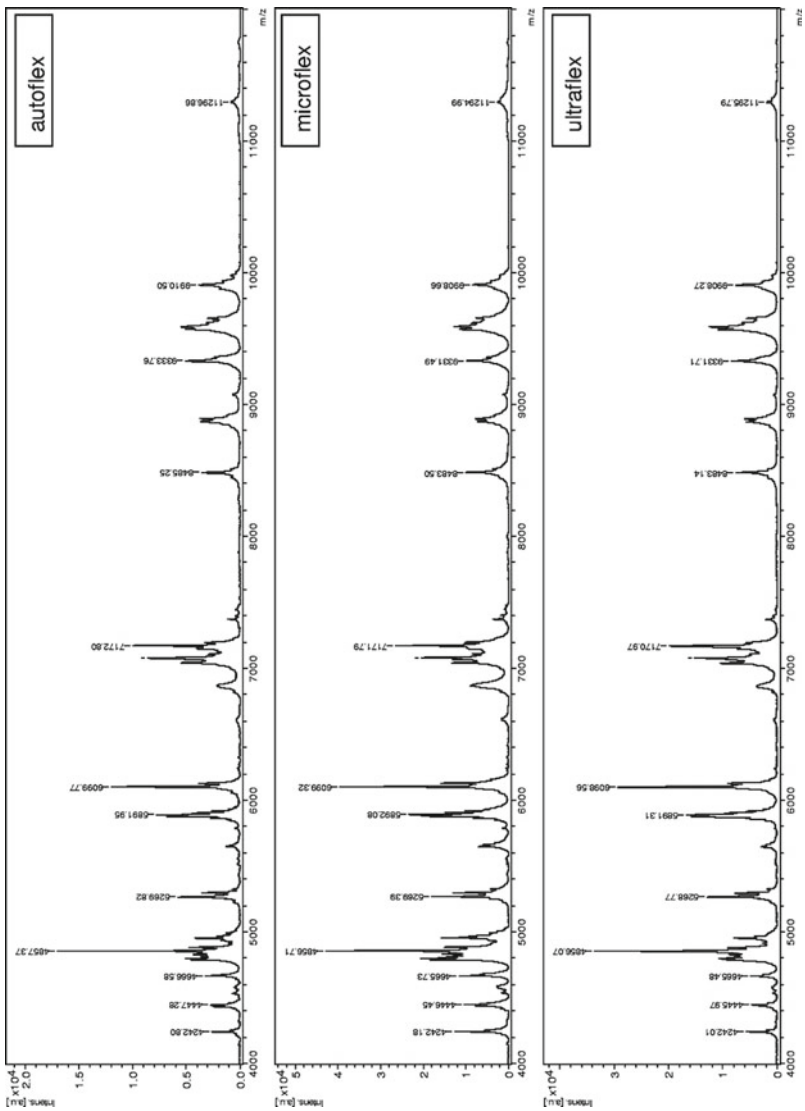

Fig. 10.3 Reproducibility of MALDI-TOF mass spectra. Spectra were directly obtained from bacterial colonies for species identification using three different MALDI-TOF mass spectrometers: autoflex, microflex, and ultraflex (all from Bruker Daltonics). Masses are depicted in the *m/z* range from 4,000 to 12,000 Da. Intens. [a.u.], intensity (in arbitrary units). J Clin Microbiol 2008;46:1946–1954; doi:10.1128/JCM.00157-08

validity extrapolated from the quality and quantity of matched peaks within the newly generated and the reference MALDI pattern [29].

Tables 10.1 and 10.2 provide an overview about the studies focusing on mass spectrometry derived identification of bacterial and fungal species. Most of the studies were performed within the last 5 years indicating the growing use of MALDI-TOF MS in the field of microbe identification. Systematic comparisons between MALDI-TOF MS and classical genotypic and phenotypic methods for species identification demonstrate not only the capability but also comparability of MALDI to provide reliable results [41–45]. In many studies, MALDI-TOF MS has impressively shown its potential to further differentiate species to the subspecies or even clonal level. For example, it is now possible to differentiate the *Burkholderia cepacia*-complex into its species by mass spectrometry [31, 46, 47] while in the past this issue required great efforts using conventional phenotypic or even genotypic methods. This aspect is of high relevance in medical microbiology due to the distinct outcome and infection control measures related to the different species. Other impressive examples of the enhanced discriminatory power (see also Tables 10.1 and 10.2) are the differentiation of *Salmonella* spp. [48] or *Listeria* spp. [49]. Although differentiation of diverse clonal lineages within the species *Staphylococcus aureus* could be attained, attempts to discriminate methicillin-resistant *S. aureus* (MRSA) from methicillin-susceptible *S. aureus* were unsuccessful [50].

After the major obstacles impending reproducibility and accuracy have been resolved, the use of MALDI-TOF MS exhibits its outstanding advantage over all other identification methods: its rapidness of identification. Starting from a single colony, it has now become an easy task to identify an unknown species within only a few minutes [31, 51, 52]. In contrast to the majority of the conventional procedures, a pure culture is no longer needed for MALDI-TOF MS, allowing tedious, laborious, and time-consuming isolation steps can be skipped. First studies have even demonstrated the proof of concept to identify species within a mixed culture [52]. While the computer-assisted spectral analysis works within a time span of a few seconds or minutes, the sample preparation prior to the MALDI-TOF MS analysis represents the most time-consuming step. However, sample extraction is not required for all organisms. For example, in gram-negative bacteria analysis the whole bacterial cell suspension can be directly mixed with the matrix. Still, for organisms with a more refractory cell wall, such as gram-positive bacteria or fungi, the extraction step is required before mixing the sample with the matrix. Regardless of the analyte, a dedicated extraction step prior to MALDI-TOF MS increases the quality of spectra [53, 54]. Ultimately, the operator must decide if an analyte pretreatment is always necessary or whether preceding extraction should be performed only in situations where the analysis of a directly spotted sample failed. Depending on the specification of the mass spectrometer, high-throughput analyses of more than 100 identifications per hour are achievable.

Further advantages of the MALDI-TOF MS based strategy of microbial identification are the relatively low consumable costs which include the expenses for the matrix and chemicals needed for sample extraction. Still, the financial investment for installation of a mass spectrometer and the running costs of the reference

Table 10.1 Synopsis of MALDI-TOF MS studies focusing on bacterial identification (as of September 2011)

Organism(s)	References
Aeromonas spp.	Donohue et al. [76]
Anaerobes	Grosse-.Herrenthey et al. [77], La Scola et al. [78], Nagy et al. [79], Shah et al. [80], Stingu et al. [81], Veloo et al. [82–84]
Arthrobacter spp.	Vargha et al. [33]
Bacillus spp.	Hotta et al. [85]
Bartonella spp.	Fournier et al. [86]
Brucella spp.	Ferreira et al. [87]
Campylobacter/*Helicobacter* spp.	Alispahic et al. [88], Bessède et al. [89], Mandrell et al. [90], Winkler et al. [91]
Cardiobacterium hominis	Wallet et al. [92]
Clavibacter spp.	Zaluga et al. [93]
Corynebacterium spp.	Konrad et al. [94]
Coxiella burnetii	Hernychova et al. [95]
Enterobacteriaceae	Conway et al. [96], Lynn et al. [97], Saffert et al. [98]
Gallibacterium spp.	Alispahic et al. [99]
HACEK group	Couturier et al. [100]
Haemophilus spp.	Haag et al. [101]
Foodborne pathogens	Mazzeo et al [102].
Legionella spp.	Gaia et al. [103], He et al. [104], Moliner et al. [105]
Listeria spp.	Barbuddhe et al. [49]
Mycobacteria spp.	Bouakaze et al. [106], Hettick et al. [107], Lotz et al. [108], Pignone et al. [109], Saleeb et al. [110]
Nocardia spp.	Verroken et al. [111]
Nonfermenters	Degand et al. [46], Jacquier et al. [112], Mellmann et al. [31], Vanlaere et al. [47]
Pantoea spp.	Rezzonico et al. [113]
Plesiomonas spp.	Kolinska et al. [114]
Salmonella spp.	Dieckmann et al. [48], Dieckmann and Malorny [115], Sparbier et al. [116]
Staphylococcus spp.	Bergeron et al. [117], Bernardo et al. [50], Carbonnelle et al. [32], Carpaij et al. [118], Decristophoris et al. [119], Dubois et al. [120], Dupont et al. [121], Rajakaruna et al. [122]
Stenotrophomonas spp.	Vasileuskaya-Schulz et al. [123]
Streptococcus spp.	Cherkaoui et al. [124], Friedrichs et al. [41], Hinse et al. [125], Lartigue et al. [126], Rupf et al. [127]
Vibrio spp.	Dieckmann et al. [128]
Yersinia spp.	Ayyadurai et al. [129], Lasch et al. [130], Stephan et al. [131]

database and maintenance of the system are not irrelevant and should also be taken into account, when comparing novel MALDI-TOF MS with traditional methods.

There are currently two commercial systems available that use MALDI-TOF MS for either bacterial or fungal species identification: the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) and the VITEK® MS system (bioMérieux,

Table 10.2 Synopsis of MALDI-TOF MS studies focusing on fungal identification (as of September 2011)

Organism(s)	References
Aspergillus spp.	Alanio et al. [132], De Carolis et al. [133], Hettick et al. [134]
Candida spp.	Quiles-Merelo et al. [135]
Cryptococcus spp.	McTaggart et al. [136]
Fusarium spp.	De Carolis et al. [133], Kemptner et al. [137]
Dermatophytes	Erhard et al. [138]
Mucorales spp.	De Carolis et al. [133]
Penicillium spp.	Hettick et al. [139]
Pseudoallescheria/*Scedosporium* complex spp.	Coulibaly et al. [140]
Various yeasts/fungi	Bader et al. [141], Dhiman et al. [142], Kaleta et al. [143], Marklein et al. [144], Putignani et al. [145],Qian et al. [146], Stevenson et al. [147], Van Herendal et al. [148]

Marcy l'Etoile, France). The MALDI Biotyper system has already proven to be comparable, even superior, to classical identification methods in a plethora of peer-reviewed publications. For the VITEK® MS system, launched in 2010 and consisting of a mass spectrometer from the Shimadzu Corporation (Kyoto, Japan) and a database application acquired from AnagnosTec GmbH (Zossen, Germany), a few study reports are already available (see Tables 10.1 and 10.2). Interestingly, a recent study has compared the capability of both systems and found similar results obtained from both platforms [55].

Limitations and Shortcomings of MALDI-TOF MS

Despite the impressive improvements of MALDI-TOF MS, some limitations still exist regarding species identification of microorganisms. The system is thus far of limited use in species identification and not universally usable, for instance, for determination of the antibiotic resistance pattern, which is an essential part of the diagnosis, especially in clinical microbiology. Currently, the antibiotic resistance has to be determined by means of different, mostly phenotypic approaches, such as agar diffusion or using automated systems. Matching MALDI-TOF MS and resistance testing faces the most ambitious challenge to validate species identification and the resistance profile of the species, e.g., to reflect intrinsic resistance. Early studies have demonstrated the feasibility of MALDI-TOF MS to detect certain resistance determinants [56–58]; however, most assays are still on an experimental level or suffer from insufficient robustness for routine applications. Interestingly, very recent novel approaches detecting antibiotics, e.g., carbapenems, and their degradation products, were able to validly determine specific resistance determinants such as extended-spectrum beta-lactamases by measuring the presence of the intact or degraded antibiotic [59, 60].

A concern for clinical practices is the lack of external quality assurance tests and standardization of laboratory procedures which would to ensure interlaboratory reproducibility. This issue may only be valid for a limited time as an international ring trial has already approved the high reproducibility of MALDI-TOF MS based identification [61]. Although MALDI-TOF MS is a rapid method that is both similar to and in competition with molecular methods like real-time-PCR assays, it is still classified as a phenotypic method as whole cell extracts are analyzed that do not require any template-driven amplification steps of molecular techniques. So far, either a single colony on solid medium or an aliquot of a pure liquid culture with a minimal number of 1,000–5,000 cells is necessary for a valid identification [62, 63].

A further issue that currently still limits MALDI-TOF MS use is the inability to directly investigate samples from primarily sterile body sites, e.g., urine or cerebrospinal fluid, or from a positive blood culture. In the past, malfunctions using such materials were due to the interference of the MS-based approach by mass signals of proteins or other molecules derived from human cells or body fluids. Currently, different approaches, mainly based on differential centrifugation and absorption of disturbing proteins or other components, have demonstrated the general applicability for direct use of such samples in MALDI-TOF MS [54, 64–71]. This is of high importance in the clinical microbiological laboratory, where the timely diagnosis of pathogens has a great impact on the success of therapy.

Finally, the quality of entries in the reference database is of crucial importance for valid species identification. During the last several years, commercially available MALDI-TOF MS systems have constantly updated and extended their reference databases to overcome this problem. Several evaluation studies (Tables 10.1 and 10.2) have addressed this issue; however, it seems that even comprehensive databases are not able to differentiate all species with the necessary precision. Examples are the differentiation of pneumococci and members of the *Streptococcus mitis*/*oralis*-group [71, 72] or the delineation of some *Enterobacter* species [73], where their close relationship likely hampers the valid species identification. Therefore, like the users of customary laboratory methods, MALDI-TOF mass spectrometer users need to be aware of the boundaries of the system to warrant an accurate species identification of microorganisms.

Further Challenges Approaching MALDI-TOF MS for Species Diagnosis

In addition to the discussed species identification using protein-based MALDI-TOF MS spectra, alternative techniques have been reported combining mass spectrometry with genotyping methods. Such technique relies upon DNA sequence determination of informative PCR products by MALDI-TOF MS and subsequent comparison with database-deposited sequences. Here, MALDI-TOF MS employs a variation of DNA sequencing that uses the attained spectra for comparison to known in silico-spectra [74]. Alternatively, mass spectrometry is able to determine the DNA base

composition by identifying the exact masses of the PCR products which are subsequently compared to reference PCR products [75]. This method is discussed in detail and extensively in Chap. 25 later in this book. Both systems are already offered by commercial suppliers: the MassARRAY® System by SEQUENOM Inc. (San Diego, California, USA) and the PLEX-ID by Abbott Laboratories (Abbott Park, Illinois, USA). However, these systems are quite expensive and labor-consuming as compared to less costly approaches based on phenotyping mass spectrometry.

Conclusion

Collectively, MALDI-TOF mass spectrometry has successfully entered the microbiological laboratory and initiated a still ongoing revolution for prokaryotic and eukaryotic species identification. MALDI-TOF MS has notably come of age as a fast, accurate, and robust method for reliable species identification suitable for implementation into the routine laboratory workflow. To which extent MALDI-TOF MS will replace classical methods in microbial laboratories is very likely dependent on whether separate identification of species and determination of their resistance pattern is accepted by users. Clients will at least be easily convinced of a fast species result, which allows for an empiric antibiotic therapy based on local resistance data in clinical microbiology.

References

1. Park BS, Park YJ, Kim YJ et al (2008) A case of disseminated *Nocardia farcinica* diagnosed through DNA sequencing in a kidney transplantation patient. Clin Nephrol 70:542–545
2. O'Riordan P, Schwab U, Logan S et al (2008) Rapid molecular detection of rifampicin resistance facilitates early diagnosis and treatment of multi-drug resistant tuberculosis: case control study. PLoS One 3:e3173
3. Kumar A, Roberts D, Wood KE et al (2006) Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med 34:1589–1596
4. Harbarth S, Masuet-Aumatell C, Schrenzel J et al (2006) Evaluation of rapid screening and pre-emptive contact isolation for detecting and controlling methicillin-resistant *Staphylococcus aureus* in critical care: an interventional cohort study. Crit Care 10:R25
5. Bergey DH, Garrity GM (2005) Volume 2: the proteobacteria. Springer, Berlin (ISBN no. 978-0-387-95040-2)
6. O'Hara CM (2005) Manual and automated instrumentation for identification of *Enterobacteriaceae* and other aerobic gram-negative bacilli. Clin Microbiol Rev 18:147–162
7. Gilligan PH (1991) Microbiology of airway disease in patients with cystic fibrosis. Clin Microbiol Rev 4:35–51
8. Clarridge JE (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 17:840–862

9. Harmsen D, Karch H (2004) 16S rDNA for diagnosing pathogens: a living tree. ASM News 70:19–24
10. Groth I, Schumann P, Martin K et al (1999) *Ornithinicoccus hortensis* gen. nov., sp. nov., a soil actinomycete which contains L-ornithine. Int J Syst Bacteriol 49:1717–1724
11. Miller LT (1982) Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. J Clin Microbiol 16:584–586
12. Stackebrandt E, Koch C, Gvozdiak O, Schumann P (1995) Taxonomic dissection of the genus *Micrococcus: Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. Int J Syst Bacteriol 45:682–692
13. Huber CG, Oberacher H (2001) Analysis of nucleic acids by on-line liquid chromatography-mass spectrometry. Mass Spectrom Rev 20:310–343
14. Wang H, Hanash S (2005) Intact-protein based sample preparation strategies for proteome analysis in combination with mass spectrometry. Mass Spectrom Rev 24:413–426
15. Müthing J, Distler U (2009) Advances on the compositional analysis of glycosphingolipids combining thin-layer chromatography with mass spectrometry. Mass Spectrom Rev 29(3): 425–479 [Epub ahead of print]
16. Ho YP, Reddy PM (2011) Advances in mass spectrometry for the identification of pathogens. Mass Spectrom Rev 30(6):1203–1224 [Epub ahead of print]
17. Karas M, Hillenkamp F (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 60:2299–2301
18. Tanaka K, Waki H, Ido Y, Akita S, Yishida Y, Yshida T (1988) Protein and polymer analyses up to m/z 100,000 by laser ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2:151–153
19. Hillenkamp F, Peter-Katalinic J (2007) MALDI MS. A practical guide to instrumentation, methods and applications. Wiley-VCH, Weinheim, ISBN no. 3-527-31440-7
20. Dreisewerd K (2003) The desorption process in MALDI. Chem Rev 103:395–426
21. Hall TA, Budowle B, Jiang Y et al (2005) Base composition analysis of human mitochondrial DNA using electrospray ionization mass spectrometry: a novel tool for the identification and differentiation of humans. Anal Biochem 344:53–69
22. Muddiman DC, Anderson GA, Hofstadler SA, Smith RD (1997) Length and base composition of PCR-amplified nucleic acids using mass measurements from electrospray ionization mass spectrometry. Anal Chem 69:1543–1549
23. Claydon MA, Davey SN, Edwards-Jones V, Gordon DB (1996) The rapid identification of intact microorganisms using mass spectrometry. Nat Biotechnol 14:1584–1586
24. Fenselau C, Demirev PA (2001) Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev 20:157–171
25. Holland RD, Wilkes JG, Rafii F et al (1996) Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10:1227–1232
26. Krishnamurthy T, Ross PL, Rajamani U (1996) Detection of pathogenic and non-pathogenic bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10:883–888
27. Smole SC, King LA, Leopold PE, Arbeit RD (2002) Sample preparation of Gram-positive bacteria for identification by matrix assisted laser desorption/ionization time-of-flight. J Microbiol Methods 48:107–115
28. Wang Z, Russon L, Li L, Roser DC, Long SR (1998) Investigation of spectral reproducibility in direct analysis of bacteria proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 12:456–464
29. Maier T, Kostrzewa M (2007) Fast and reliable MALDI-TOF MS-based microorganism identification. Chem Today 25:68–71
30. Suh M, Hamburg D, Gregory ST, Dahlberg AE, Limbach PA (2005) Extending ribosomal protein identifications to unsequenced bacterial strains using matrix-assisted laser desorption/ionization mass spectrometry. Proteomics 5:4818–4831

31. Mellmann A, Cloud J, Maier T et al (2008) Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J Clin Microbiol 46:1946–1954
32. Carbonnelle E, Beretti J, Cottyn S et al (2007) Rapid identification of Staphylococci isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 45:2156–2161
33. Vargha M, Takats Z, Konopka A, Nakatsu CH (2006) Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. J Microbiol Methods 66:399–409
34. Liu H, Du Z, Wang J, Yang R (2007) Universal sample preparation method for characterization of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 73:1899–1907
35. Arnold RJ, Reilly JP (1998) Fingerprint matching of *E. coli* strains with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells using a modified correlation approach. Rapid Commun Mass Spectrom 12:630–636
36. Bright JJ, Claydon MA, Soufian M, Gordon DB (2002) Rapid typing of bacteria using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry and pattern recognition software. J Microbiol Methods 48:127–138
37. Jarman KH, Cebula ST, Saenz AJ et al (2000) An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry. Anal Chem 72:1217–1223
38. Jarman KH, Daly DS, Petersen CE, Saenz AJ, Valentine NB, Wahl KL (1999) Extracting and visualizing matrix-assisted laser desorption/ionization time-of-flight mass spectral fingerprints. Rapid Commun Mass Spectrom 13:1586–1594
39. Pineda FJ, Lin JS, Fenselau C, Demirev PA (2000) Testing the significance of microorganism identification by mass spectrometry and proteome database search. Anal Chem 72:3739–3744
40. Harmsen D, Rothgänger J, Frosch M, Albert J (2002) RIDOM: ribosomal differentiation of medical micro-organisms database. Nucleic Acids Res 30:416–417
41. Friedrichs C, Rodloff AC, Chhatwal GS, Schellenberger W, Eschrich K (2007) Rapid identification of viridans streptococci by mass spectrometric discrimination. J Clin Microbiol 45:2392–2397
42. Neville SA, Lecordier A, Ziochos H et al (2011) Utility of matrix-assisted laser desorption ionization-time of flight mass spectrometry following introduction for routine laboratory bacterial identification. J Clin Microbiol 49:2980–2984
43. Benagli C, Rossi V, Dolina M, Tonolla M, Petrini O (2011) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the identification of clinically relevant bacteria. PLoS One 6:e16424
44. Seng P, Rolain J, Fournier PE, La Scola B, Drancourt M, Raoult D (2010) MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiol 5:1733–1754
45. Carbonnelle E, Mesquita C, Bille E et al (2011) MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin Biochem 44:104–109
46. Degand N, Carbonnelle E, Dauphin B et al (2008) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of nonfermenting gram-negative bacilli isolated from cystic fibrosis patients. J Clin Microbiol 46:3361–3367
47. Vanlaere E, Sergeant K, Dawyndt P et al (2008) Matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry of intact cells allows rapid identification of *Burkholderia cepacia* complex. J Microbiol Methods 75:279–286
48. Dieckmann R, Helmuth R, Erhard M, Malorny B (2008) Rapid classification and identification of *Salmonellae* at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74:7767–7778
49. Barbuddhe SB, Maier T, Schwarz G et al (2008) Rapid identification and typing of *Listeria* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74:5402–5407

50. Bernardo K, Pakulat N, Macht M et al (2002) Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics 2:747–753
51. Ruelle V, El Moualij B, Zorzi W, Ledent P, Pauw ED (2004) Rapid identification of environmental bacterial strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 18:2013–2019
52. Warscheid B, Fenselau C (2004) A targeted proteomics approach to the rapid identification of bacterial cell mixtures by matrix-assisted laser desorption/ionization mass spectrometry. Proteomics 4:2877–2892
53. Alatoom AA, Cunningham SA, Ihde SM, Mandrekar J, Patel R (2011) Comparison of direct colony method versus extraction method for identification of gram-positive cocci by use of Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49:2868–2873
54. Ferreira L, Sanchez-Juanes F, Munoz-Bellido JL, Gonzalez-Buitrago JM (2011) Rapid method for direct identification of bacteria in urine and blood culture samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: intact cell vs. extraction method. Clin Microbiol Infect 17:1007–1012
55. Cherkaoui A, Hibbs J, Emonet S et al (2010) Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. J Clin Microbiol 48:1169–1175
56. Camara JE, Hays FA (2007) Discrimination between wild-type and ampicillin-resistant *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Bioanal Chem 389:1633–1638
57. Du Z, Yang R, Guo Z, Song Y, Wang J (2002) Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 74:5487–5491
58. Edwards-Jones V, Claydon MA, Evason DJ, Walker J, Fox AJ, Gordon DB (2000) Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. J Med Microbiol 49:295–300
59. Hrabak J, Walkova R, Studentova V, Chudackova E, Bergerova T (2011) Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49:3222–3227
60. Burckhardt I, Zimmermann S (2011) Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours. J Clin Microbiol 49:3321–3324
61. Mellmann A, Bimet F, Bizet C et al (2009) High interlaboratory reproducibility of matrix-assisted laser desorption ionization-time of flight mass spectrometry-based species identification of nonfermenting bacteria. J Clin Microbiol 47:3732–3734
62. Hathout Y, Demirev PA, Ho YP et al (1999) Identification of *Bacillus* spores by matrix-assisted laser desorption ionization-mass spectrometry. Appl Environ Microbiol 65:4313–4319
63. Tao L, Yu X, Snyder AP, Li L (2004) Bacterial identification by protein mass mapping combined with an experimentally derived protein mass database. Anal Chem 76:6609–6617
64. Schubert S, Weinert K, Wagner C et al (2011) Novel, improved sample preparation for rapid, direct identification from positive blood cultures using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. J Mol Diagn 13(6):701–706 [Epub ahead of print]
65. Kok J, Thomas LC, Olma T, Chen SCA, Iredell JR (2011) Identification of bacteria in blood culture broths using matrix-assisted laser desorption-ionization sepsityper and time of flight mass spectrometry. PLoS One 6:e23285
66. Kroumova V, Gobbato E, Basso E, Mucedola L, Giani T, Fortina G (2011) Direct identification of bacteria in blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: a new methodological approach. Rapid Commun Mass Spectrom 25:2247–2249

67. Schmidt V, Jarosch A, Marz P, Sander C, Vacata V, Kalka-Moll W (2011) Rapid identification of bacteria in positive blood culture by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Eur J Clin Microbiol Infect Dis 31(3):311–317 [Epub ahead of print]
68. Romero-Gomez MP, Mingorance J (2011) The effect of the blood culture bottle type in the rate of direct identification from positive cultures by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry. J Infect 62:251–253
69. Moussaoui W, Jaulhac B, Hoffmann A et al (2010) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry identifies 90% of bacteria directly from blood culture vials. Clin Microbiol Infect 16:1631–1638
70. Ferreira L, Sanchez-Juanes F, Porras-Guerra I et al (2011) Microorganisms direct identification from blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Microbiol Infect 17:546–551
71. Drancourt M (2010) Detection of microorganisms in blood specimens using matrix-assisted laser desorption ionization time-of-flight mass spectrometry: a review. Clin Microbiol Infect 16:1620–1625
72. van Veen SQ, Claas ECJ, Kuijper EJ (2010) High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. J Clin Microbiol 48:900–907
73. Risch M, Radjenovic D, Han JN, Wydler M, Nydegger U, Risch L (2010) Comparison of MALDI TOF with conventional identification of clinically relevant bacteria. Swiss Med Wkly 140:w13095
74. Honisch C, Chen Y, Mortimer C et al (2007) Automated comparative sequence analysis by base-specific cleavage and mass spectrometry for nucleic acid-based microbial typing. Proc Natl Acad Sci U S A 104:10649–10654
75. Ecker DJ, Sampath R, Blyn LB et al (2005) Rapid identification and strain-typing of respiratory pathogens for epidemic surveillance. Proc Natl Acad Sci U S A 102:8012–8017
76. Donohue MJ, Smallwood AW, Pfaller S, Rodgers M, Shoemaker JA (2006) The development of a matrix-assisted laser desorption/ionization mass spectrometry-based method for the protein fingerprinting and identification of *Aeromonas* species using whole cells. J Microbiol Methods 65:380–389
77. Grosse-Herrenthey A, Maier T, Gessler F et al (2008) Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Anaerobe 14:242–249
78. La Scola B, Fournier P, Raoult D (2011) Burden of emerging anaerobes in the MALDI-TOF and 16S rRNA gene sequencing era. Anaerobe 17:106–112
79. Nagy E, Maier T, Urban E, Terhes G, Kostrzewa M (2009) Species identification of clinical isolates of *Bacteroides* by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. Clin Microbiol Infect 15:796–802
80. Shah HN, Keys CJ, Schmid O, Gharbia SE (2002) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and proteomics: a new era in anaerobic microbiology. Clin Infect Dis 35:S58–S64
81. Stingu CS, Rodloff AC, Jentsch H, Schaumann R, Eschrich K (2008) Rapid identification of oral anaerobic bacteria cultivated from subgingival biofilm by MALDI-TOF-MS. Oral Microbiol Immunol 23:372–376
82. Veloo ACM, Welling GW, Degener JE (2011) The identification of anaerobic bacteria using MALDI-TOF MS. Anaerobe 17:211–212
83. Veloo AC, Knoester M, Degener JE, Kuijper EJ (2011) Comparison of two matrix-assisted laser desorption ionisation-time of flight mass spectrometry methods for the identification of clinically relevant anaerobic bacteria. Clin Microbiol Infect 17(10):1501–1506 [Epub ahead of print]
84. Veloo ACM, Erhard M, Welker M, Welling GW, Degener JE (2011) Identification of Gram-positive anaerobic cocci by MALDI-TOF mass spectrometry. Syst Appl Microbiol 34:58–62

85. Hotta Y, Sato J, Sato H, Hosoda A, Tamura H (2011) Classification of the genus *Bacillus* based on MALDI-TOF MS analysis of ribosomal proteins coded in S10 and spc operons. J Agric Food Chem 59:5222–5230
86. Fournier P, Couderc C, Buffet S, Flaudrops C, Raoult D (2009) Rapid and cost-effective identification of *Bartonella* species using mass spectrometry. J Med Microbiol 58:1154–1159
87. Ferreira L, Vega Castano S, Sanchez-Juanes F et al (2010) Identification of Brucella by MALDI-TOF mass spectrometry. Fast and reliable identification from agar plates and blood cultures. PLoS One 5:e14235
88. Alispahic M, Hummel K, Jandreski-Cvetkovic D et al (2010) Species-specific identification and differentiation of *Arcobacter, Helicobacter* and *Campylobacter* by full-spectral matrix-associated laser desorption/ionization time of flight mass spectrometry analysis. J Med Microbiol 59:295–301
89. Bessede E, Solecki O, Sifre E, Labadi L, Megraud F (2011) Identification of *Campylobacter* species and related organisms by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Clin Microbiol Infect 17(11):1735–1739 [Epub ahead of print]
90. Mandrell RE, Harden LA, Bates A, Miller WG, Haddon WF, Fagerquist CK (2005) Speciation of *Campylobacter coli, C. jejuni, C. helveticus, C. lari, C. sputorum,* and *C. upsaliensis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 71:6292–6307
91. Winkler MA, Uher J, Cepa S (1999) Direct analysis and identification of *Helicobacter* and *Campylobacter* species by MALDI-TOF mass spectrometry. Anal Chem 71:3416–3419
92. Wallet F, Loiez C, Decoene C, Courcol R (2011) Rapid identification of *Cardiobacterium hominis* by MALDI-TOF mass spectrometry during infective endocarditis. Jpn J Infect Dis 64:327–329
93. Zaluga J, Heylen K, Van Hoorde K et al (2011) *gyrB* sequence analysis and MALDI-TOF MS as identification tools for plant pathogenic *Clavibacter*. Syst Appl Microbiol 34:400–407
94. Konrad R, Berger A, Huber I et al (2010) Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry as a tool for rapid diagnosis of potentially toxigenic *Corynebacterium* species in the laboratory management of diphtheria-associated bacteria. Euro Surveill 15:pii: 19702
95. Hernychova L, Toman R, Ciampor F et al (2008) Detection and identification of *Coxiella burnetii* based on the mass spectrometric analyses of the extracted proteins. Anal Chem 80:7097–7104
96. Conway GC, Smole SC, Sarracino DA, Arbeit RD, Leopold PE (2001) Phyloproteomics: species identification of *Enterobacteriaceae* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Mol Microbiol Biotechnol 3:103–112
97. Lynn EC, Chung MC, Tsai WC, Han CC (1999) Identification of *Enterobacteriaceae* bacteria by direct matrix-assisted laser desorptiom/ionization mass spectrometric analysis of whole cells. Rapid Commun Mass Spectrom 13:2022–2027
98. Saffert RT, Cunningham SA, Ihde SM, Jobe KEM, Mandrekar J, Patel R (2011) Comparison of Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometer to BD Phoenix automated microbiology system for identification of gram-negative bacilli. J Clin Microbiol 49:887–892
99. Alispahic M, Christensen H, Hess C, Razzazi-Fazeli E, Bisgaard M, Hess M (2011) Identification of *Gallibacterium* species by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry evaluated by multilocus sequence analysis. Int J Med Microbiol 301:513–522
100. Couturier MR, Mehinovic E, Croft AC, Fisher MA (2011) Identification of HACEK clinical isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49:1104–1106
101. Haag AM, Taylor SN, Johnston KH, Cole RB (1998) Rapid identification and speciation of *Haemophilus* bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Mass Spectrom 33:750–756

102. Mazzeo MF, Sorrentino A, Gaita M et al (2006) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the discrimination of food-borne microorganisms. Appl Environ Microbiol 72:1180–1189
103. Gaia V, Casati S, Tonolla M (2011) Rapid identification of *Legionella* spp. by MALDI-TOF MS based protein mass fingerprinting. Syst Appl Microbiol 34:40–44
104. He Y, Chang TC, Li H, Shi G, Tang Y (2011) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry and database for identification of *Legionella* species. Can J Microbiol 57:533–538
105. Moliner C, Ginevra C, Jarraud S et al (2010) Rapid identification of *Legionella* species by mass spectrometry. J Med Microbiol 59:273–284
106. Bouakaze C, Keyser C, Gonzalez A et al (2011) Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry-based single nucleotide polymorphism genotyping assay using iPLEX gold technology for identification of *Mycobacterium tuberculosis* complex species and lineages. J Clin Microbiol 49:3292–3299
107. Hettick JM, Kashon ML, Slaven JE et al (2006) Discrimination of intact mycobacteria at the strain level: a combined MALDI-TOF MS and biostatistical analysis. Proteomics 6:6416–6425
108. Lotz A, Ferroni A, Beretti J et al (2010) Rapid identification of mycobacterial whole cells in solid and liquid culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 48:4481–4486
109. Pignone M, Greth KM, Cooper J, Emerson D, Tang J (2006) Identification of mycobacteria by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. J Clin Microbiol 44:1963–1970
110. Saleeb PG, Drake SK, Murray PR, Zelazny AM (2011) Identification of *Mycobacteria* in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49:1790–1794
111. Verroken A, Janssens M, Berhin C et al (2010) Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of *Nocardia* species. J Clin Microbiol 48:4015–4021
112. Jacquier H, Carbonnelle E, Corvec S et al (2011) Revisited distribution of nonfermenting Gram-negative bacilli clinical isolates. Eur J Clin Microbiol Infect Dis 30(12):1579–1586
113. Rezzonico F, Vogel G, Duffy B, Tonolla M (2010) Application of whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification and clustering analysis of *Pantoea* species. Appl Environ Microbiol 76:4497–4509
114. Kolinska R, Drevinek M, Aldova E, Zemlickova H (2010) Identification of *Plesiomonas* spp.: serological and MALDI-TOF MS methods. Folia Microbiol (Praha) 55:669–672
115. Dieckmann R, Malorny B (2011) Rapid screening of epidemiologically important *Salmonella enterica* subsp. enterica serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 77:4136–4146
116. Sparbier K, Weller U, Boogen C, Kostrzewa M (2011) Rapid detection of *Salmonella* sp. by means of a combination of selective enrichment broth and MALDI-TOF MS. Eur J Clin Microbiol Infect Dis 31(5):767–773 [Epub ahead of print]
117. Bergeron M, Dauwalder O, Gouy M et al (2011) Species identification of staphylococci by amplification and sequencing of the *tuf* gene compared to the *gap* gene and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Eur J Clin Microbiol Infect Dis 30:343–354
118. Carpaij N, Willems RJL, Bonten MJM, Fluit AC (2011) Comparison of the identification of coagulase-negative staphylococci by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and tuf sequencing. Eur J Clin Microbiol Infect Dis 30:1169–1172
119. Decristophoris P, Fasola A, Benagli C, Tonolla M, Petrini O (2011) Identification of *Staphylococcus intermedius* group by MALDI-TOF MS. Syst Appl Microbiol 34:45–51
120. Dubois D, Leyssene D, Chacornac JP et al (2010) Identification of a variety of *Staphylococcus* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 48:941–945

121. Dupont C, Sivadon-Tardy V, Bille E et al (2010) Identification of clinical coagulase-negative staphylococci, isolated in microbiology laboratories, by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and two automated systems. Clin Microbiol Infect 16:998–1004
122. Rajakaruna L, Hallas G, Molenaar L et al (2009) High throughput identification of clinical isolates of *Staphylococcus aureus* using MALDI-TOF-MS of intact cells. Infect Genet Evol 9:507–513
123. Vasileuskaya-Schulz Z, Kaiser S, Maier T, Kostrzewa M, Jonas D (2011) Delineation of Stenotrophomonas spp. by multi-locus sequence analysis and MALDI-TOF mass spectrometry. Syst Appl Microbiol 34:35–39
124. Cherkaoui A, Emonet S, Fernandez J, Schorderet D, Schrenzel J (2011) Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification of Beta-hemolytic streptococci. J Clin Microbiol 49:3004–3005
125. Hinse D, Vollmer T, Erhard M et al (2011) Differentiation of species of the *Streptococcus bovis/equinus*-complex by MALDI-TOF Mass Spectrometry in comparison to *sodA* sequence analyses. Syst Appl Microbiol 34:52–57
126. Lartigue M, Kostrzewa M, Salloum M et al (2011) Rapid detection of 'highly virulent' Group B *Streptococcus* ST-17 and emerging ST-1 clones by MALDI-TOF mass spectrometry. J Microbiol Methods 86:262–265
127. Rupf S, Breitung K, Schellenberger W, Merte K, Kneist S, Eschrich K (2005) Differentiation of mutans streptococci by intact cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Oral Microbiol Immunol 20:267–273
128. Dieckmann R, Strauch E, Alter T (2010) Rapid identification and characterization of *Vibrio* species using whole-cell MALDI-TOF mass spectrometry. J Appl Microbiol 109:199–211
129. Ayyadurai S, Flaudrops C, Raoult D, Drancourt M (2010) Rapid identification and typing of *Yersinia pestis* and other *Yersinia* species by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. BMC Microbiol 10:285
130. Lasch P, Drevinek M, Nattermann H et al (2010) Characterization of *Yersinia* using MALDI-TOF mass spectrometry and chemometrics. Anal Chem 82:8464–8475
131. Stephan R, Cernela N, Ziegler D et al (2011) Rapid species specific identification and subtyping of *Yersinia enterocolitica* by MALDI-TOF Mass spectrometry. J Microbiol Methods 87(2):150–153 [Epub ahead of print]
132. Alanio A, Beretti J, Dauphin B et al (2011) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for fast and accurate identification of clinically relevant *Aspergillus* species. Clin Microbiol Infect 17:750–755
133. De Carolis E, Posteraro B, Lass-Florl C et al (2011) Species identification of *Aspergillus*, *Fusarium* and *Mucorales* with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect 18(5):475–484 [Epub ahead of print]
134. Hettick JM, Green BJ, Buskirk AD et al (2008) Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Anal Biochem 380:276–281
135. Quiles-Melero I, Garcia-Rodriguez J, Gomez-Lopez A, Mingorance J (2011) Evaluation of matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry for identification of *Candida parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*. Eur J Clin Microbiol Infect Dis 31(1):67–71 [Epub ahead of print]
136. McTaggart LR, Lei E, Richardson SE, Hoang L, Fothergill A, Zhang SX (2011) Rapid identification of *Cryptococcus neoformans* and *Cryptococcus gattii* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49:3050–3053
137. Kemptner J, Marchetti-Deschmann M, Mach R, Druzhinina IS, Kubicek CP, Allmaier G (2009) Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 23:877–884

138. Erhard M, Hipler U, Burmester A, Brakhage AA, Wostemeyer J (2008) Identification of dermatophyte species causing onychomycosis and tinea pedis by MALDI-TOF mass spectrometry. Exp Dermatol 17:356–361
139. Hettick JM, Green BJ, Buskirk AD et al (2008) Discrimination of *Penicillium* isolates by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Rapid Commun Mass Spectrom 22:2555–2560
140. Coulibaly O, Marinach-Patrice C, Cassagne C, Piarroux R, Mazier D, Ranque S (2011) *Pseudallescheria/Scedosporium* complex species identification by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry. Med Mycol 49:621–626
141. Bader O, Weig M, Taverne-Ghadwal L, Lugert R, Gross U, Kuhns M (2011) Improved clinical laboratory identification of human pathogenic yeasts by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect 17:1359–1365
142. Dhiman N, Hall L, Wohlfiel SL, Buckwalter SP, Wengenack NL (2011) Performance and cost analysis of matrix-assisted laser desorption ionization-time of flight mass spectrometry for routine identification of yeast. J Clin Microbiol 49:1614–1616
143. Kaleta EJ, Clark AE, Cherkaoui A et al (2011) Comparative analysis of PCR-electrospray ionization/mass spectrometry (MS) and MALDI-TOF/MS for the identification of bacteria and yeast from positive blood culture bottles. Clin Chem 57:1057–1067
144. Marklein G, Josten M, Klanke U et al (2009) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. J Clin Microbiol 47:2912–2917
145. Putignani L, Del Chierico F, Onori M et al (2011) MALDI-TOF mass spectrometry proteomic phenotyping of clinically relevant fungi. Mol Biosyst 7:620–629
146. Qian J, Cutler JE, Cole RB, Cai Y (2008) MALDI-TOF mass signatures for differentiation of yeast species, strain grouping and monitoring of morphogenesis markers. Anal Bioanal Chem 392:439–449
147. Stevenson LG, Drake SK, Shea YR, Zelazny AM, Murray PR (2010) Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically important yeast species. J Clin Microbiol 48:3482–3486
148. Van Herendael BH, Bruynseels P, Bensaid M et al (2011) Validation of a modified algorithm for the identification of yeast isolates using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Eur J Clin Microbiol Infect Dis 31(5):841–848 [Epub ahead of print]

Chapter 11
Nucleic Acid Extraction Techniques

Jeong Hwan Shin

Introduction

Since thermostable *Taq* DNA polymerase was discovered in 1987, nucleic acid amplification techniques have made great strides and contributed greatly to progress in the life sciences. These techniques were introduced into the clinical laboratory and have produced great changes in diagnostic tools and tests. In particular, there have been many innovative molecular testing developments in the field of diagnostic microbiology.

Culture methods of bacterial identification are labor-intensive and time-consuming. However, they are simple, cheap, and remain the gold standard. It also is possible to perform antimicrobial susceptibility testing on cultured isolates, so conventional culture methods with biochemical phenotyping are still the most common procedures performed in clinical microbiology laboratories [1]. To further assist in microbial identification, nucleic acid amplification has been introduced in the clinical microbiology laboratory. Such testing was initially done for viruses, allowing detection of small amounts of viral nucleic acid quickly. Similar tests also have been applied to bacteria, especially those that require cell cultures, are difficult to grow on routine culture media, or are slow growing such as *Chlamydia*, *Neisseria gonorrhoeae*, and *Mycobacterium*. In addition, there are ongoing attempts to apply these new techniques for routine clinical microbiology testing, including the diagnosis of sepsis [1].

The development of nucleic acid amplification has proceeded at an unprecedented pace and achieved higher sensitivity and specificity [2]. However, in order to

J.H. Shin (✉)
Department of Laboratory Medicine, Busan Paik Hospital,
Inje University College of Medicine, 633-165, Gaegeum-dong Busanjin-gu,
Busan 614-735, Korea
e-mail: jhsmile@inje.ac.kr

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obtain satisfying results with this new technique, the testing must go through several important steps. Preanalytical testing variables comprise sample collection and preparation, specimen transport and storage, stability of the nucleic acid in the samples, and nucleic acid extraction [3, 4].

Nucleic acid extraction is the first step of any amplification experiment no matter what kind of amplification is used to detect a specific pathogen [1, 5]. It is a crucial preanalytic step in the development and performance of any successful molecular diagnostic method and ensures a reliable result [3, 4]. We must pay attention to the technical progress of the nucleic acid extraction as well as to the method for amplification and detection of nucleic acids in order to obtain satisfactory results. Nucleic acid extraction consists of three major processes: isolation, purification, and concentration. Commercial extraction kits are commonly used in the clinical microbiology laboratory [2]. These kits provide the essential requirements for nucleic acid extraction. These essential requirements have been well described by Boom et al. [6, 7] as follows: Extraction should be simple, rapid, and should show high sensitivity and specificity. It is preferred that there be no requirements for specialized equipment or special knowledge and skills. The final nucleic acid should be pure and easy to modify for various amplification techniques. The reagents and their product should be harmless, and the process of preparation should resist contamination with other specimens. If the final volume of eluate is small, detection limits are maximized. When we deal with clinical specimens, we also should consider the elimination of potential inhibitors of the DNA polymerase and the removal of pathogenicity from hazardous pathogens as well as good target recovery and establishment of the integrity of nucleic acid targets [2]. Ideally, the final target is pure nucleic acid without amplification inhibitors or contaminants such as protein, carbohydrate, and other nucleic acids [8].

There are a few points to be specially considered when we consider the use of nucleic acid extraction in the field of clinical microbiology. Usually, we extract DNA or RNA, and some times, we may extract both, depending on the circumstances. The targets for nucleic acid extraction are diverse. They can be the cultured bacterial isolates themselves. Alternatively, we can use culture media, including blood culture bottles or various clinical specimens such as sputum, stool, urine, tissue, or cerebrospinal fluid [1]. In terms of nucleic acid extraction, they may target the same nucleic acid, but they have different implications for the extraction procedure itself. Nucleic acid extraction from cultured bacteria is relatively simple because they are pure colonies and they contain large numbers of organisms. However, we need to recognize that gram-positive bacteria have thick walls, such that nucleic acid extraction is more difficult than it is from gram-negative bacteria, which have thinner walls [5]. For clinical specimens, the details of the method depend on the characteristics of each specimen. It is important to remember that the subject is nucleic acid, not of humans, but of bacteria, virus, or fungus. If we extract from clinical specimens containing human cells, we cannot help mixing human DNA and sometimes, recovery of microbial DNA can be diminished by the presence of human DNA.

Nucleic Acid Extraction Techniques

Cesium Chloride/Ethidium Bromide Density Gradient Centrifugation

Since 1950, density gradient centrifugation using cesium chloride (CsCl)/ethidium bromide (EtBr) has been used as for DNA extraction method and has become standard in research laboratories [9, 10]. The basic principle of this method is to use the difference in density between the cesium ion and water and intercalation of EtBr, which shows good results for separation of various DNAs and the procurement of high-yield DNA [11]. For example, each DNA can be separated as independent bands as a result of the differences in each DNA's density in the gradient by the intercalation of EtBr [7]. However, it has important limitations in that it requires an expensive ultracentrifuge and considerable time, it is difficult to perform, and EtBr is harmful [7, 8]. Consequently, this method is not suitable for clinical microbiology and has not been used in the clinical laboratory.

Phenol–Chloroform Extraction

Phenol–chloroform extraction is widely used. The process consists of vigorous mixing of phenol–chloroform solution and sample followed by centrifugation [7]. Phenol does not completely inhibit RNase activity, and this characteristic enables isolation of nucleic acid by combination with chloroform and alcohol [12]. After centrifugation, the upper (aqueous) phase containing the DNA can be separated from the lower (organic) phase containing denatured proteins, and DNA can be precipitated by adding ethanol or isopropanol with a high concentration of salt [8]. After washing with 70% ethanol to remove any remaining ethanol or isopropanol, the final target DNA is collected by dissolving it in TE buffer or sterile distilled water [13]. This method is also used for RNA extraction by concomitant use of guanidinium isothiocyanate. This combination can overcome the limitation of RNA extraction using the guanidinium isothiocyanate itself, so RNA could be isolated conveniently using a single-step technique by Chomczynski et al. [12, 14]. Total RNA is recovered by precipitation with isopropanol after separation of the upper phase containing the total RNA from the lower phase containing DNA and proteins [12, 14]. Although the phenol–chloroform method is relatively easy compared with CsCl/EtBr and is very useful for the extraction of nucleic acids, it is problematic for the clinical microbiology laboratory because phenol has important limitations due to it being toxic, caustic, and flammable [5, 15, 16].

Solid-Phase Extraction

McCormick et al. introduced a new DNA extraction method involving solid-phase extraction in 1989 [17]. They used an insoluble siliceous core particle rather than liquid phenol. The function of this siliceous core particle is similar to that of phenol, but it has a few advantages in that it is safer, and cross-contamination can be reduced. It is well known that the precipitation in the phenol/chloroform method causes DNA loss, and Meijer et al. could reduce it by replacing the precipitating step with silica particles [18]. Solid-phase nucleic acid extraction was incorporated into many commercial kits, and it still is the basis of many extraction methods, although siliceous core particles have been replaced by other materials such as silica matrices, glass particles, diatomaceous earth, and anion-exchange carriers (Fig. 11.1) [7].

Solid-phase extraction uses a spin column operated by centrifugal force allowing DNA to be purified rapidly and efficiently without the limitations of liquid extraction, including incomplete phase separation [8]. Solid-phase extraction using silica now is one of the most common methods for nucleic acid extraction. Silica that possesses a positive charge combines strongly with DNA, which possesses a negative charge, so it can enable rapid, pure, and quantitative purification [7]. In 1990, Boom et al. [6] used an innovative approach in which diatomaceous earth served as a matrix for solid-phase extraction. The principle of this method is that it immobilizes DNA onto its particles in the presence of a chaotropic agent. The technique can purify rRNA as well as single-stranded and double-stranded DNA. It takes only a short time and can be applied to clinical specimens as well as to DNA and bacteria. The process of solid-phase extraction involves cell lysis, nucleic acid adsorption, washing, and elution [7, 8]. Column conditioning is obtained using a buffer at a particular pH [19]. The nucleic acid will be released after cell lysis and decanting of lysis buffer into the column. Nucleic acid adsorption is completed in a chaotropic salt solution [19]. Washing buffer contains a competitive agent and can remove contaminants such as proteins and salts. In elution, TE buffer is applied to the column so that purified nucleic acid will be released [19].

Magnetic Bead Method

There is another important modification of solid-phase extraction, that is, the magnetic bead method. The beads have a negative surface charge and bind proteins and cellular debris selectively [7]. So, DNA can be isolated easily from specimens by removing proteins and cellular debris on the beads. This has the potential advantages of removing the need for repeated centrifugation, vacuum filtration, and column separation for washing and elution as well as organic solvents [7, 8]. The magnetic bead method is very simple and convenient; so many commercial kits are available for this method [8]. Some manufacturers combined the techniques of solid-phase extraction using silica and magnetic beads, which satisfies the customers'

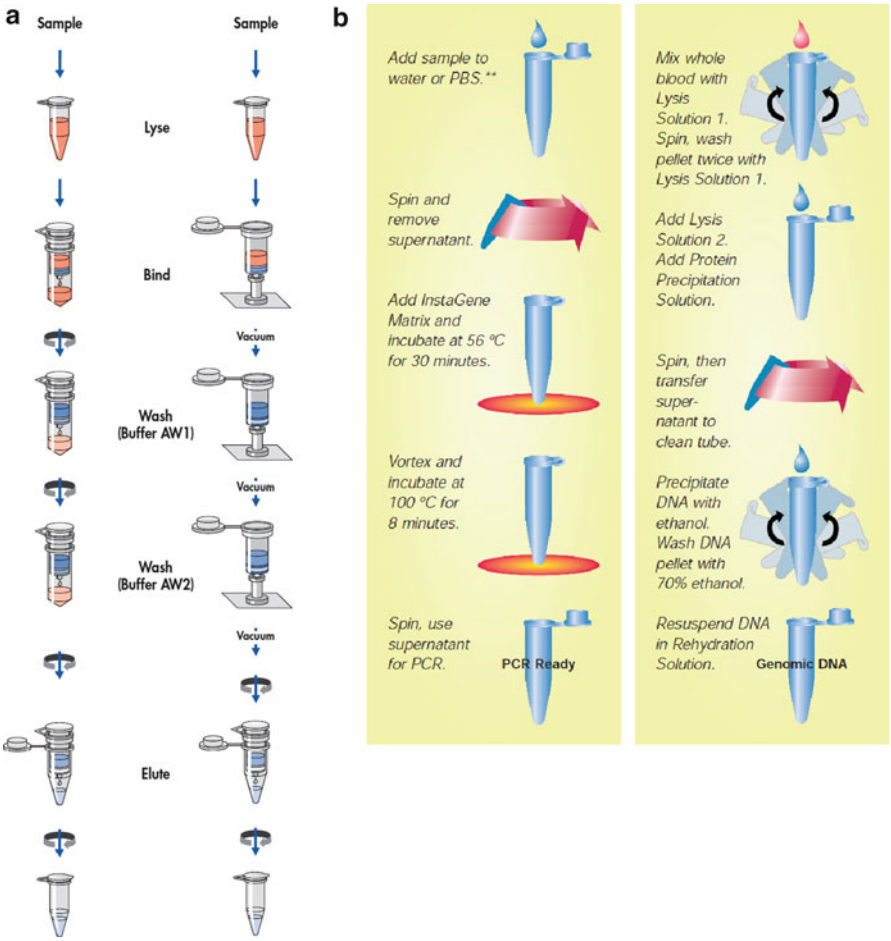

Fig. 11.1 Schematic diagrams of (**a**) solid-phase, spin-column nucleic acid extraction method (QIAamp DNA Mini kit from Qiagen, http://www.qiagen.com) and (**b**) liquid-based nucleic acid extraction method (InstaGene Matrix and Genomic DNA kit from Bio-Rad, http://www.bio-rad.com)

requests for time- and labor-effectiveness and efficiency (Fig. 11.2). This method is commonly used in automated extraction methods such as miniMag (bioMerieux) and MagNA Pure (Roche). In terms of new technology, additional commercial kits using this new technique are being launched into the market. The enzymatic method is an example of these new extraction methods [20]. These new methods help investigators by giving advantages of more convenience, requirement for only small volumes of specimen, and enhancement of DNA recovery.

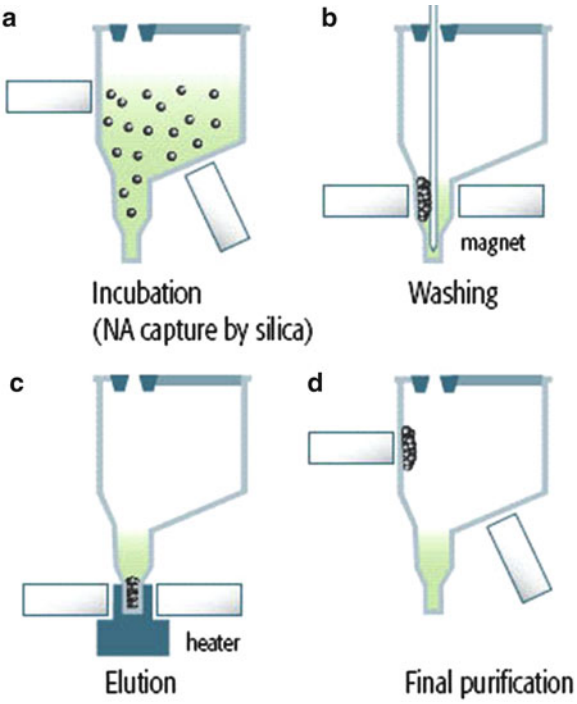

Fig. 11.2 Extraction principles using magnetic silica particles. (**a**) During incubation of the lysed samples, all the target nucleic acid is captured by magnetic silica particles. (**b**) The NucliSENS easy MAG magnetic device attracts all the magnetic silica, enabling the system to purify the nucleic acids through several washing steps. (**c**) The heating step releases the nucleic acids from the silica. (**d**) In the final step, the magnetic silica particles are separated from the eluate by the magnetic device (NucliSENS easyMAG from biomerieux, http://www.biomerieux-diagnostics.com)

Applications to Clinical Specimens

Nucleic acid extraction from clinical specimens is quite different from that from cultured isolates of bacteria or fungi. This extraction step can influence the subsequent performance of the diagnostic tests; the efficiency of nucleic acid extraction is related directly to the sensitivity of the final test results [21]. Each clinical specimen has diverse characteristics. Blood and stool are composed of many substances, and among these, heme and bile act as inhibitors of amplification and should be removed [5]. We can find the comparison results for nucleic acid extraction; however, this cannot ensure that we can adapt this result to different specimens and pathogens. In previous reports, herpes simplex virus DNA was isolated relatively easily from genital swabs [5, 22, 23], but obtaining bacterial DNA from stool samples was more complex [5, 24].

To overcome these limitations, the extraction method must be evaluated before routine testing of specific pathogens from specific specimens. For detection of clinically important viruses, extraction efficiency was evaluated in various specimens, including serum, urine, and cerebrospinal fluid, and good performance was confirmed [2, 25, 26]. However, we should not extrapolate these specific results to all types of virus and specimens.

Cellular Component

Tissue is an important clinical specimen for diagnosing localized cytomegalovirus infections in a transplanted organ as biopsy is a common method used to evaluate potential CMV infection [27]. However, tissue specimens have problems because the large amount of human tissue contains cellular DNA, proteins, and other materials [28]. So, a more complex step to extract the nucleic acid of the microbial pathogen is needed. Most commercial kits for tissue specimens extract human nucleic acid also. In recent years, we have been able to extract the viral nucleic acid from clinical specimens having cellular components, and there have been a few trials of these kits to detect various clinically important viruses [29–31]. There is one other report concerning the extraction of six viruses from clinical cellular specimens, and the investigators compared four commercial extraction methods [28]. The viruses included in this study are BK virus, cytomegalovirus, Epstein–Barr virus, human herpesvirus 8, herpes simplex virus, and varicella-zoster virus. All four kits could extract DNA from all six viruses.

Stool is an important clinical specimen for the detection of viruses causing diarrheal illnesses. The results can be affected by the efficiency of nucleic acid extraction from stool, because stool is a mixture of many unrecognized materials, including bacteria, protein, and other cellular materials. So, stool specimens are considered one of the most difficult specimens for nucleic acid extraction in the clinical laboratory. In one report by Peiris et al. [32], the positive rate was 97% for severe acute respiratory syndrome coronavirus in stool samples. However, the detection rates were quite different in another report: only 26.2 and 68.0% between 1 and 2 weeks after disease onset [33]. It is suggested that the difference in positive rates is a consequence of variations in the RNA extraction method [34].

Serum, Plasma, and Whole Blood

Clinicians place great emphasis on the detection of bacteria and fungi in blood. Therefore, nucleic acid extraction from blood has become very important. Many researchers have found that there are numerous PCR inhibitors in blood culture bottles such as sodium polyanetholesulfonate (SPS) and hemins [35]. Millar and collaborators compared several commercial and in-house extraction methods used to detect bacteria and fungi in BacT/Alert blood culture bottles [36]. To reduce the detection time, the serum, plasma, or whole blood is used as a main specimen for detection of bacteria and fungi. Serum or plasma is more efficient and convenient than whole blood because whole blood includes many PCR inhibitors [37]. Most commercial kits showed a high recovery rate of pathogen DNA, but only those methods that used heat lysis with an alkali wash could remove PCR inhibitors. Detection of brucellosis was highly sensitive even though *Brucellae* are facultative intracellular pathogens [38]. Similarly, kits containing proteinase K showed better yield of *Brucella* in serum specimens [39]. However, to enhance the sensitivity of

PCR amplification, whole blood is considered as a final target because it contains more pathogens than serum or plasma [40]. Although the nucleic acid kits were not developed to extract microbial DNA from whole blood, all commercial kits are able to do so [21]. In recent years, we have been able to use several automated systems to extract bacterial or fungal DNA from whole blood [30, 41, 42], although they are expensive. They are suitable for high-throughput detection [43].

It also is important to consider the concentrations of pathogens. The recovery of *Toxoplasma gondii* was similar for two DNA extraction techniques when using various PCR methods [44]. However, the results were different when there were low concentrations of tachyzoites in blood [45] vs. amniotic fluid [44]. We can see the similar result in *Chlamydia pneumoniae* detection from stools [34]. The positive rates were lowered when the RNA concentrations dropped and this confirms the clinical importance of the extraction methods used for stool samples.

The use of dried blood spots (DBSs) is an alternative to whole blood and is an important and common specimen used for the diagnosis of congenital infections [46]. It is different from whole blood in that the tiny specimen may contain very few causative pathogens. At present, many kinds of commercial or noncommercial DBSs are in use. Because the amount of blood is small, inadequate DNA extraction can be a problem and result in a low sensitivity. Several investigators attempted to detect CMV DNA in DBS using several extraction methods and found that the recovery of CMV DNA differed according to the extraction method used [46–50]. The lysis buffer used also can affect the yield of RNA from DBS, and column extraction methods revealed significant loss in RNA recovery [51].

Influence of Specific Pathogen

Even when we use clinical specimens to extract nucleic acid, we should recognize that recovery is influenced by the physical properties of the pathogen [44]. The effect will be greater if the method does not include proteinase K in the lysis step. The *Apicomplexa* phylum including *Toxoplasma* is well known to be resistant to detergent lysis [52].

Fungi are problematic when attempting to extract nucleic acid because it is difficult to break their cell walls in order to release the DNA [15, 42]. Moreover, the detection rates in certain clinical specimens such as whole blood are low because of the very low loads of fungal cells [53–55]. So the extraction once again is a crucial step and can determine the sensitivity of a particular PCR assay [56, 57]. To recover small amounts of fungal DNA from clinical specimens, a protocol should be established for an optimal extraction method. The QIAamp DNA blood kit was successful in extracting *Candida* DNA and was suitable to use with a *Taq*Man-based PCR assay, whereas all other kits tested failed to detect low amounts of *Candida* DNA [15]. In another study, the investigators were successful with all of the extraction methods used in their study, even though those kits were not specifically designed for the extraction of fungal DNA from whole blood [58]. We encounter a similar

difficulty in extracting nucleic acid from *Mycobacteria*. Many researchers have tried to find optimal extraction methods for most clinically important specimens; the most appropriate method for each laboratory's situation should be applied [59].

PCR Inhibitors

There are many factors that affect the efficiency of nucleic acid extraction, an important one being the presence of many kinds of inhibitors. It is well known that bile salts, hemoglobin, and polysaccharides can inhibit PCR [60, 61]. There also may be contaminating bacterial or fungal DNA in the reagents [39, 58, 62]. Most PCR assays can be influenced by reaction inhibitors and other contaminants, especially when the various clinical specimens containing these inhibitors are used as samples, and nucleic acid extraction thus becomes the crucial step that determines their influence [63]. In previous reports [29, 64], the presence of inhibitors was confirmed when the MagNA Pure Compact system was used for a principal nucleic acid extraction. In one other report [45], the authors compared the MagNA Pure Compact system and QIAamp DNA minikit for the detection of *Toxoplasma* DNA from blood. The sensitivity of PCR using the MagNA Pure Compact system was lower than that of the QIAamp DNA minikit, so the presence of inhibitors may have been responsible for the difference of sensitivity between the two methods. Moreover, the combination of the extraction kit and the master mix can make a difference in PCR performance in terms of inhibition [44]. We should also consider the fact that many human DNAs are mixed with relatively rare pathogen DNAs in clinical specimens, meaning abundant human DNA will be obtained during extraction for pathogen detection [65].

Measurement of DNA Quality

The classical method to check DNA purity is to measure the adsorption of UV light at 260 and 280 nm. The DNA content is proportional to the adsorption of UV light at 260, and adsorption at 280 nm reflects protein contamination. So, we can easily calculate the DNA purity using the OD260/OD280 ratio. In recent years, newly developed methods such as PicoGreen have been introduced and are becoming more popular in clinical laboratories, although the spectrophotometric method does have many advantages [66]. PicroGreen is based on the use of fluorescence and needs only a minute volume of sample.

Comparison of Nucleic Acid Extraction Methods

The method used for nucleic acid extraction differs greatly in clinical microbiology laboratories. When we deal with cultured bacteria to get genomic DNA, it is common to use simple heating, but this has many limitations and is not appropriate for

use directly on clinical specimens. There are many reports comparing various extraction methods, including commercial kits, from various specimens for bacteria, virus, and fungi [21, 25, 26, 29, 30, 39, 42, 44, 67–71]. The methods can be divided into solution or column based according to differences of their principles, and most commercial extraction kits we use can be divided the same way. DNA recovery was better when a spin column method was used for extraction of *C. pneumoniae* DNA from vascular tissue [72]. However, DNA recovery ability can differ among kits even though all use the spin-column method as the principal tool [73]. So the method itself does not give assurance, and we should keep in mind that the DNA recovery can be different among various kits that use the similar principles.

Regardless of specific kits, specific companies, and their protocols, they have common steps in their procedures for optimal extraction [8]. Cell lysis must be the first step. After nucleoprotein complexes have been denatured, nucleases are inactivated. The contaminants are removed, and nucleic acid is purified. Even though these basic steps are not changed, there has been a vast alteration in nucleic acid extraction, namely, development of automated instrumentation. The method for the nucleic acid extraction can be divided into manual or automated, and this is an important point in the classification of nucleic acid extraction methods.

Manual Method

Many commercial kits have been developed for nucleic acid extraction. These kits are composed of a few reagents and are designed primarily for manual extraction. These kits are suitable for use in clinical laboratories and have replaced older in-house methods (Table 11.1). There are many publications that have evaluated the performance of these commercial nucleic acid extraction methods and compared them with conventional methods such as phenol–chloroform and the alkali wash/heat lysis [15, 36, 39, 58]. These manual commercial extraction kits show good performance for nucleic acid extraction compared with in-house methods.

Their ability to recover pure DNA and to remove the contaminants, including proteins, is of great importance, but there are also important differences in cost, time demands, labor intensity, and principles of each method. Given these differences, there are numerous choices available; the most appropriate method for a particular laboratory should be selected. Both liquid- and column-based methods are commonly used at present.

Most of these kits use noncorrosive agents, so they are safe and easy to deal with. However, there still are some pitfalls. Although the entire extraction procedure is standardized by the manufacturer's manual, the process is still complicated and is performed manually. Therefore, problems with reproducibility by different persons can be seen. To minimize such reproducibility problems, it is necessary to provide continuous training and quality control [5]. Ethanol is used for precipitation of the nucleic acid in some manual kits, and the inhibition of PCR can occur when this ethanol is not completely removed [74]. The manual extraction method has been

Table 11.1 Manual methods of nucleic acid extraction and purifications for rapid real-time PCR assays

Kit/manufacturer/ homepage	Technologic principle	Specimen throughput	Specimen type
High Pure Roche Applied Science www.roche-applied-science.com	Nucleic acid capture by glass fiber fleece immobilized in a special plastic filter tube and subjected to centrifugation	24 samples in 1 h	Serum, whole blood, plasma, urine, stool, sterile body fluids, respiratory tract specimens, swabs (genital, dermal)
QIAamp Qiagen www.qiagen.com	Nucleic acid capture by silica gel membrane placed in tube column and subjected to centrifugation or vacuum conditions	24 samples in 1 h for DNA 24 samples in 1.5 h for RNA	Respiratory tract specimens, plasma, stool, serum, whole blood, urine, sterile body fluids, swabs (nasal, fecal)
IsoQuick Orca Research www.bioexpress.com	Nucleic acid is partitioned into an aqueous phase and then precipitated with ethanol and resuspended in water or buffer	24 samples in 1 h for DNA 24 samples in 2 h for RNA	Plasma, whole blood, stool, respiratory tract specimens, sterile body fluids, swabs (dermal, fecal, genital)
IsoCode Stix Schleichar & Schuell www.whatman.com	DNA bound to matrix and released by simple water and heat elution	Processed individually	Whole blood

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designated as a high-complexity test according to Clinical Laboratory improvement Amendments of 1988 (CLIA '88) regulations (http://www.cms.hhs.gov/clia/), so only trained laboratory personnel can perform it. Moreover, the number of specimens for molecular testing is increasing, which places more stress on the technologists who are processing these specimens. This can affect the accuracy of tests as a result of a processing mistake or by contamination attributable to the complex processing procedure.

Automatic Methods

The introduction of commercial manual extraction kits was a valuable adjunct for molecular testing in the clinical microbiology laboratory. However, the manual extraction method is still labor-intensive and time-consuming and requires a well-trained technologist. There were also reports of outbreaks of cross contamination when the samples were treated at the same time [39, 75]. In recent years, many manufacturers developed and launched various automated extraction instruments;

Table 11.2 Automated nucleic acid extraction methods and their characteristics

Manufacturer	Instrument	Technologic principle	Specimens/run (batch size)
BioMerieux	Easy MAG	Magnetic bead extraction	24
BioMerieux	MiniMAG	Magnetic bead extraction	12
Roche	MagNA Pure LC	Magnetic bead extraction	32 (1–32)
Roche	MagNA Pure Compact	Magnetic bead extraction	8 (1–8)
QIAGEN	BioRobot Universal/ BioRobot MDx	Vacuum-based and/or magnetic bead extraction	96 (8)
QIAGEN	QIAsymphony SP	Magnetic bead extraction	96 (1–24)
QIAGEN	QIAxtractor	Vacuum-based extraction	96 (8–96)
bioneer	ExiPrep 16 Plus, ExiPrep 16 Pro	Magnetic bead extraction	16 (1–16)
Abbott Molecular	M2000sp	Magnetic bead extraction	96 (24)
Eppendorf	epMotion 5070/ epMotion 5075	Vacuum-based and/or magnetic bead extraction	384 (1–384)
Fisher Scientific	Thermo KingFisher Flex	Magnetic bead extraction	1–96 (1–96)
Beckman Coulter	Biomek NXp Span 8/ Biomek NXp Multichannel 96	Magnetic bead extraction	96 (8)

these instruments vary in principle, procedure time, cost, and size (Table 11.2). The automated extraction instruments are easily divided by their workload capacity; the most appropriate instrument thus can be selected according to each laboratory's workload situation from low-throughput instruments such as MagNA Pure compact, NucliSens miniMAG, and BioRobot EZ1 systems to medium- to high-throughput instruments such as the MagNA Pure LC and BioRobot M48/9604 [76].

The automated extraction methods have many advantages compared with manual methods, and these instruments have proven to be very useful adjuncts for PCR testing. The steps proceed automatically with fast turnaround. This reduces the working time and avoids mistakes such as pipetting error. Many specimens can be analyzed at the same time [43]. It provides constant reproducibility for recovery of nucleic acid, avoiding person-to-person variations seen with manual extraction methods. It can also diminish cross contamination by reducing unnecessary handling steps and avoiding mistakes by personnel [5]. It has an additional advantage for quality control monitoring, whereas the manual method needs intensive work for quality control monitoring [77].

Since many automated extraction instruments and kits have been developed, numerous evaluations have been reported [67, 78–81]. These studies included various kinds of extraction kits, clinical specimens, and pathogens. Even though some reports showed high detection rates with manual extraction method, the results of automated extraction methods were similar to or better than those of manual methods in most of these studies [28, 29, 45, 64, 67, 78–81].

For example, Cook et al. [28] evaluated the performance of four commercial automated extraction kits for the detection of viruses using clinical specimens.

They compared viral yield using cultured cells containing CMV, EBV, HSV, BK, VZV, or HHV-8. The procedures of the kits were similar, and DNA extraction was successful with all kits. There were some variations of viral yields, which were only 50% compared with those of the manual kits. The differences of yields are not of great significance if we consider the biologic range of viral loads in clinical practice as the manufacturer's recommendation. In the study of multiplex molecular detection of infection in septic patients using automated extraction, the recovery of DNA was similar to that of the conventional manual method at the point of maximal binding surface of MagNA pure nanoparticles [76]. Many leukocytes are present in the blood sample, so the final DNA amounts recovered by the manual method can be about three times those obtained by MagNA pure extraction [82]. We can face different results for the evaluation of automated extraction instrument. The efficiency of the NucliSens easyMAG was low for CMV [49] and respiratory viruses [29]; however, in another study, the NucliSens miniMAG showed the best results for the isolation of polyomavirus BK virus and the human beta-actin gene from urine specimens [2] and severe respiratory syndrome coronavirus RNA in stool samples [34].

The most important drawback we must consider is the economic aspect of the automated methods. To use such a system, an expensive instrument and extraction reagents, including disposables, are needed. Sometimes, this increased cost for the automated system precludes its use. However, the influenza A (H1N1) pandemic in 2009 demonstrated the value of an automated extraction system. At that time, requests for influenza A (H1N1) identification were increasing rapidly, and many laboratories could not perform all the requested tests because of limited personnel. This made clear the usefulness of the automated extraction system. Although the detection rates and yield recovery are the most important factors in selecting commercial extraction methods, other factors, including ease of use and cost per extraction, also must be considered [28].

Conclusion

In recent years, advanced molecular tests have come to occupy an important position in the diagnosis of infectious diseases because of their high sensitivity and specificity [83, 84]. Efficient nucleic acid extraction is essential. The optimal extraction method should fulfill the following conditions: speed, short working time, cost-effectiveness, high sensitivity and specificity, good reproducibility, and safety [1]. It will be ideal for use with all kinds of specimens and pathogens. However, at present, there is no one extraction method that satisfies all these conditions. On the contrary, there are significant differences between extraction kits because nucleic acids can be different in specific clinical specimens. So, it is important to carefully evaluate the performance of any extraction method used in the clinical microbiology laboratory.

References

1. Mancini N, Carletti S, Ghidoli N, Cichero P, Burioni R, Clementi M (2010) The era of molecular and other non-culture-based methods in diagnosis of sepsis. Clin Microbiol Rev 23:235–251
2. Tang YW, Sefers SE, Li H, Kohn DJ, Procop GW (2005) Comparative evaluation of three commercial systems for nucleic acid extraction from urine specimens. J Clin Microbiol 43:4830–4833
3. Anwar A, Wan G, Chua KB, August JT, Too HP (2009) Evaluation of pre-analytical variables in the quantification of dengue virus by real-time polymerase chain reaction. J Mol Diagn 11:537–542
4. Lippi G, Guidi GC, Mattiuzzi C, Plebani M (2006) Preanalytical variability: the dark side of the moon in laboratory testing. Clin Chem Lab Med 44:358–365
5. Espy MJ, Uhl JR, Sloan LM et al (2006) Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 19:165–256
6. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J (1990) Rapid and simple method for purification of nucleic acids. J Clin Microbiol 28:495–503
7. Price CW, Leslie DC, Landers JP (2009) Nucleic acid extraction techniques and application to the microchip. Lab Chip 9:2484–2494
8. Tan SC, Yiap BC (2009) DNA, RNA, and protein extraction: the past and the present. J Biomed Biotechnol 2009:574398
9. Meselson M, Stahl FW, Vinograd J (1957) Equilibrium sedimentation of macromolecules in density gradients. Proc Natl Acad Sci U S A 43:581–588
10. Radloff R, Bauer W, Vinograd J (1967) A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc Natl Acad Sci U S A 57:1514–1521
11. Carr SM, Griffith OM (1987) Rapid isolation of animal mitochondrial DNA in a small fixed-angle rotor at ultrahigh speed. Biochem Genet 25:385–390
12. Chomczynski P, Sacchi N (2006) The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat Protoc 1:581–585
13. Buckingham L, Flaws MI (2007) Molecular diagnostics; fundamentals, methods, & clinical applications. F.A. Davis, Philadelphia, PA
14. Sambrook J, Russel D (2001) Molecular cloning: a laboratory manual, vol. 3, 3rd edn. Cold Spring Harbor Laboratory Press, New York
15. Maaroufi Y, Ahariz N, Husson M, Crokaert F (2004) Comparison of different methods of isolation of DNA of commonly encountered *Candida* species and its quantitation by using a real-time PCR-based assay. J Clin Microbiol 42:3159–3163
16. Randhawa P, Ho A, Shapiro R et al (2004) Correlates of quantitative measurement of BK polyomavirus (BKV) DNA with clinical course of BKV infection in renal transplant patients. J Clin Microbiol 42:1176–1180
17. McCormick RM (1989) A solid-phase extraction procedure for DNA purification. Anal Biochem 181:66–74
18. Meijer A, van der Vliet JA, Schouls LM, de Vries A, Roholl PJ, Ossewaarde JM (1998) Detection of microorganisms in vessel wall specimens of the abdominal aorta: development of a PCR assay in the absence of a gold standard. Res Microbiol 149:577–583
19. Gjerse DT, Hoang L, Hornby D (2009) RNA purification and analysis; sample preparation, extraction, chromatography, 1st edn. Wiley-VCH, Weinheim
20. Coolbear T, Whittaker JM, Daniel RM (1992) The effect of metal ions on the activity and thermostability of the extracellular proteinase from a thermophilic Bacillus, strain EA.1. Biochem J 287(Pt 2):367–374
21. Smith K, Diggle MA, Clarke SC (2003) Comparison of commercial DNA extraction kits for extraction of bacterial genomic DNA from whole-blood samples. J Clin Microbiol 41:2440–2443

22. Espy MJ, Ross TK, Teo R et al (2000) Evaluation of LightCycler PCR for implementation of laboratory diagnosis of herpes simplex virus infections. J Clin Microbiol 38:3116–3118
23. Espy MJ, Uhl JR, Mitchell PS et al (2000) Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. J Clin Microbiol 38:795–799
24. Sloan LM, Uhl JR, Vetter EA et al (2004) Comparison of the Roche LightCycler vanA/vanB detection assay and culture for detection of vancomycin-resistant enterococci from perianal swabs. J Clin Microbiol 42:2636–2643
25. Beuselinck K, van Ranst M, van Eldere J (2005) Automated extraction of viral-pathogen RNA and DNA for high-throughput quantitative real-time PCR. J Clin Microbiol 43:5541–5546
26. Dundas N, Leos NK, Mitui M, Revell P, Rogers BB (2008) Comparison of automated nucleic acid extraction methods with manual extraction. J Mol Diagn 10:311–316
27. Gerna G, Lilleri D (2006) Monitoring transplant patients for human cytomegalovirus: diagnostic update. Herpes 13:4–11
28. Cook L, Atienza EE, Bagabag A, Obrigewitch RM, Jerome KR (2009) Comparison of methods for extraction of viral DNA from cellular specimens. Diagn Microbiol Infect Dis 64:37–42
29. Chan KH, Yam WC, Pang CM et al (2008) Comparison of the NucliSens easyMAG and Qiagen BioRobot 9604 nucleic acid extraction systems for detection of RNA and DNA respiratory viruses in nasopharyngeal aspirate samples. J Clin Microbiol 46:2195–2199
30. Loens K, Bergs K, Ursi D, Goossens H, Ieven M (2007) Evaluation of NucliSens easyMAG for automated nucleic acid extraction from various clinical specimens. J Clin Microbiol 45:421–425
31. Mengelle C, Legrand-Abravanel F, Mansuy JM, Barthe C, Da Silva I, Izopet J (2008) Comparison of two highly automated DNA extraction systems for quantifying Epstein-Barr virus in whole blood. J Clin Virol 43:272–276
32. Peiris JS (2003) Severe acute respiratory syndrome (SARS). J Clin Virol 28:245–247
33. Chan PK, To WK, Ng KC et al (2004) Laboratory diagnosis of SARS. Emerg Infect Dis 10:825–831
34. Petrich A, Mahony J, Chong S et al (2006) Multicenter comparison of nucleic acid extraction methods for detection of severe acute respiratory syndrome coronavirus RNA in stool specimens. J Clin Microbiol 44:2681–2688
35. Fredricks DN, Relman DA (1998) Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor sodium polyanetholesulfonate. J Clin Microbiol 36:2810–2816
36. Millar BC, Jiru X, Moore JE, Earle JA (2000) A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material. J Microbiol Methods 42:139–147
37. Queipo-Ortuno MI, Garcia-Ordonez MA, Colmenero JD, Morata P (1999) Hydrogen peroxide improves the efficiency of a peripheral blood PCR assay for diagnosis of human brucellosis. Biotechniques 27:248–250, 252
38. Zerva L, Bourantas K, Mitka S, Kansouzidou A, Legakis NJ (2001) Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. J Clin Microbiol 39:1661–1664
39. Queipo-Ortuno MI, Tena F, Colmenero JD, Morata P (2008) Comparison of seven commercial DNA extraction kits for the recovery of Brucella DNA from spiked human serum samples using real-time PCR. Eur J Clin Microbiol Infect Dis 27:109–114
40. Ohlin A, Backman A, Bjorkqvist M, Molling P, Jurstrand M, Schollin J (2008) Real-time PCR of the 16S-rRNA gene in the diagnosis of neonatal bacteraemia. Acta Paediatr 97:1376–1380
41. Klingspor L, Jalal S (2006) Molecular detection and identification of Candida and Aspergillus spp. from clinical samples using real-time PCR. Clin Microbiol Infect 12:745–753
42. Loeffler J, Schmidt K, Hebart H, Schumacher U, Einsele H (2002) Automated extraction of genomic DNA from medically important yeast species and filamentous fungi by using the MagNA Pure LC system. J Clin Microbiol 40:2240–2243
43. Stormer M, Kleesiek K, Dreier J (2007) High-volume extraction of nucleic acids by magnetic bead technology for ultrasensitive detection of bacteria in blood components. Clin Chem 53:104–110
44. Yera H, Filisetti D, Bastien P, Ancelle T, Thulliez P, Delhaes L (2009) Multicenter comparative evaluation of five commercial methods for toxoplasma DNA extraction from amniotic fluid. J Clin Microbiol 47:3881–3886

45. Edvinsson B, Jalal S, Nord CE, Pedersen BS, Evengard B (2004) DNA extraction and PCR assays for detection of Toxoplasma gondii. APMIS 112:342–348
46. de Vries JJ, Claas EC, Kroes AC, Vossen AC (2009) Evaluation of DNA extraction methods for dried blood spots in the diagnosis of congenital cytomegalovirus infection. J Clin Virol 46(Suppl 4):S37–S42
47. Barbi M, Binda S, Primache V et al (2000) Cytomegalovirus DNA detection in Guthrie cards: a powerful tool for diagnosing congenital infection. J Clin Virol 17:159–165
48. Scanga L, Chaing S, Powell C et al (2006) Diagnosis of human congenital cytomegalovirus infection by amplification of viral DNA from dried blood spots on perinatal cards. J Mol Diagn 8:240–245
49. Soetens O, Vauloup-Fellous C, Foulon I et al (2008) Evaluation of different cytomegalovirus (CMV) DNA PCR protocols for analysis of dried blood spots from consecutive cases of neonates with congenital CMV infections. J Clin Microbiol 46:943–946
50. Vauloup-Fellous C, Ducroux A, Couloigner V et al (2007) Evaluation of cytomegalovirus (CMV) DNA quantification in dried blood spots: retrospective study of CMV congenital infection. J Clin Microbiol 45:3804–3806
51. Monleau M, Montavon C, Laurent C et al (2009) Evaluation of different RNA extraction methods and storage conditions of dried plasma or blood spots for human immunodeficiency virus type 1 RNA quantification and PCR amplification for drug resistance testing. J Clin Microbiol 47:1107–1118
52. Mann T, Gaskins E, Beckers C (2002) Proteolytic processing of TgIMC1 during maturation of the membrane skeleton of *Toxoplasma gondii*. J Biol Chem 277:41240–41246
53. Ahmad S, Khan Z, Mustafa AS, Khan ZU (2002) Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection, and biochemical methods for species identification. J Clin Microbiol 40:2483–2489
54. Challier S, Boyer S, Abachin E, Berche P (2004) Development of a serum-based Taqman real-time PCR assay for diagnosis of invasive aspergillosis. J Clin Microbiol 42:844–846
55. White PL, Shetty A, Barnes RA (2003) Detection of seven *Candida* species using the LightCycler system. J Med Microbiol 52:229–238
56. Bretagne S, Costa JM (2005) Towards a molecular diagnosis of invasive aspergillosis and disseminated candidosis. FEMS Immunol Med Microbiol 45:361–368
57. Fredricks DN, Smith C, Meier A (2005) Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J Clin Microbiol 43:5122–5128
58. Metwally L, Fairley DJ, Coyle PV et al (2008) Improving molecular detection of *Candida* DNA in whole blood: comparison of seven fungal DNA extraction protocols using real-time PCR. J Med Microbiol 57:296–303
59. Hosek J, Svastova P, Moravkova M, Vavlik I, Bartos M (2006) Methods of mycobacterial DNA isolation from different biological material: a review. Vet Med 51:180–192
60. Monteiro L, Bonnemaison D, Vekris A et al (1997) Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J Clin Microbiol 35:995–998
61. Widjojoatmodjo MN, Fluit AC, Torensma R, Verdonk GP, Verhoef J (1992) The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. J Clin Microbiol 30:3195–3199
62. Radstrom P, Knutsson R, Wolffs P, Lovenklev M, Lofstrom C (2004) Pre-PCR processing: strategies to generate PCR-compatible samples. Mol Biotechnol 26:133–146
63. Bastien P, Procop GW, Reischl U (2008) Quantitative real-time PCR is not more sensitive than "conventional" PCR. J Clin Microbiol 46:1897–1900
64. Schuurman T, de Boer R, Patty R, Kooistra-Smid M, van Zwet A (2007) Comparative evaluation of in-house manual, and commercial semi-automated and automated DNA extraction platforms in the sample preparation of human stool specimens for a *Salmonella enterica* 5′-nuclease assay. J Microbiol Methods 71:238–245
65. Handschur M, Karlic H, Hertel C, Pfeilstocker M, Haslberger AG (2009) Preanalytic removal of human DNA eliminates false signals in general 16S rDNA PCR monitoring of bacterial pathogens in blood. Comp Immunol Microbiol Infect Dis 32:207–219

66. Ahn SJ, Costa J, Emanuel JR (1996) PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. Nucleic Acids Res 24:2623–2625
67. Knepp JH, Geahr MA, Forman MS, Valsamakis A (2003) Comparison of automated and manual nucleic acid extraction methods for detection of enterovirus RNA. J Clin Microbiol 41:3532–3536
68. Loffler J, Hebart H, Schumacher U, Reitze H, Einsele H (1997) Comparison of different methods for extraction of DNA of fungal pathogens from cultures and blood. J Clin Microbiol 35:3311–3312
69. Mygind T, Ostergaard L, Birkelund S, Lindholt JS, Christiansen G (2003) Evaluation of five DNA extraction methods for purification of DNA from atherosclerotic tissue and estimation of prevalence of *Chlamydia pneumoniae* in tissue from a Danish population undergoing vascular repair. BMC Microbiol 3:19
70. Verheyden B, Thielemans A, Rombaut B, Kronenberger P (2003) RNA extraction for quantitative enterovirus RT-PCR: comparison of three methods. J Pharm Biomed Anal 33:819–823
71. Wilson D, Yen-Lieberman B, Reischl U, Warshawsky I, Procop GW (2004) Comparison of five methods for extraction of *Legionella pneumophila* from respiratory specimens. J Clin Microbiol 42:5913–5916
72. Berg HF, Maraha B, Bergmans AM, van der Zee A, Kluytmans JA, Peeters MF (2003) Extraction of Chlamydia pneumoniae DNA from vascular tissue for use in PCR: an evaluation of four procedures. Clin Microbiol Infect 9:135–139
73. Read SJ (2001) Recovery efficiencies on nucleic acid extraction kits as measured by quantitative LightCycler PCR. Mol Pathol 54:86–90
74. Valentine-Thon E (2002) Quality control in nucleic acid testing—where do we stand? J Clin Virol 25(Suppl 3):S13–S21
75. Schabereiter-Gurtner C, Nehr M, Apfalter P, Makristathis A, Rotter ML, Hirschl AM (2008) Evaluation of a protocol for molecular broad-range diagnosis of culture-negative bacterial infections in clinical routine diagnosis. J Appl Microbiol 104:1228–1237
76. Regueiro BJ, Varela-Ledo E, Martinez-Lamas L et al (2010) Automated extraction improves multiplex molecular detection of infection in septic patients. PLoS One 5:e13387
77. Garrett PE (2001) Quality control for nucleic acid tests: common ground and special issues. J Clin Virol 20:15–21
78. Espy MJ, Rys PN, Wold AD et al (2001) Detection of herpes simplex virus DNA in genital and dermal specimens by LightCycler PCR after extraction using the IsoQuick, MagNA Pure, and BioRobot 9604 methods. J Clin Microbiol 39:2233–2236
79. Germer JJ, Lins MM, Jensen ME et al (2003) Evaluation of the MagNA pure LC instrument for extraction of hepatitis C virus RNA for the COBAS AMPLICOR Hepatitis C Virus Test, version 2.0. J Clin Microbiol 41:3503–3508
80. Gobbers E, Oosterlaken TA, van Bussel MJ, Melsert R, Kroes AC, Claas EC (2001) Efficient extraction of virus DNA by NucliSens Extractor allows sensitive detection of hepatitis B virus by PCR. J Clin Microbiol 39:4339–4343
81. Shi PY, Kauffman EB, Ren P et al (2001) High-throughput detection of West Nile virus RNA. J Clin Microbiol 39:1264–1271
82. Miller DN, Bryant JE, Madsen EL, Ghiorse WC (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol 65:4715–4724
83. Bretagne S, Costa JM (2006) Towards a nucleic acid-based diagnosis in clinical parasitology and mycology. Clin Chim Acta 363:221–228
84. Schuurman T, van Breda A, de Boer R et al (2005) Reduced PCR sensitivity due to impaired DNA recovery with the MagNA Pure LC total nucleic acid isolation kit. J Clin Microbiol 43:4616–4622

Chapter 12
Nonamplified Probe-Based Microbial Detection and Identification

Fann Wu, Tao Hong, and Phyllis Della-Latta

Introduction

Probe-based nonamplified molecular assays were first developed for detection of microorganisms decades ago. Over the years, the variety of molecular technologies for the laboratory diagnosis of infectious diseases has expanded greatly, largely due to the rapidly expanding field of sequenced microbial genomes. Probe-based hybridization assays remain a commonly used format in clinical microbiology laboratories due to its numerous advantages over routine culture-based methodologies. Conventional phenotypic methods of bacterial identification which include the Gram stain, culture, and biochemical reactions, contain three major challenges. First, nonviable or nonculturable organisms simply cannot be identified due to growth restrictions. Second, some microbial strains may exhibit atypical biochemical characteristics that do not match established patterns routinely used for identification. Third, slow-growing or fastidious organisms require a prolonged time to identification. In contrast, probe-based assays bypass many of the limitations of phenotypic methods and provide accurate pathogen identification in a clinically relevant timeframe. A variety of commercial assays are available to identify pathogens from culture, and in addition there are several assays that can detect infectious agents directly from specimen. This chapter presents an overview of the design and clinical applications of prominent nonamplified probe-based methods commonly used in clinical microbiology laboratories to identify pathogens.

F. Wu (✉) • P. Della-Latta
Clinical Microbiology Services, Department of Pathology,
Columbia University Medical Center, New York-Presbyterian Hospital,
622 West 168th Street, C.H.C. 3-325, New York, NY 10032, USA
e-mail: faw9001@nyp.org

T. Hong
Clinical Microbiology Laboratory, Department of Pathology,
Hackensack University Medical Center, Hackensack, NJ 07601, USA

Y.-W. Tang and C.W. Stratton (eds.), *Advanced Techniques in Diagnostic Microbiology*,
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Probe-Based Assay Design

A probe is a single-stranded segment of DNA or RNA specifically designed to hybridize to its complementary sequence in the microorganism to be identified. The nucleic acid probe is labeled by a variety of reporter molecules that can be chemiluminescent, fluorescent, enzymatic, or antigenic in order to detect the double-stranded hybrids. There are a variety of probes and targets that are carefully selected when designing diagnostic assays.

Target Selection

Since the sensitivity of a probe-based assay is primarily determined by the abundance of the target in the original sample, ribosomal RNA (rRNA) has been selected as a favorite target sequence. Ribosomes are highly conserved and essential organelles responsible for protein synthesis and are therefore present in all living cells in high quantity. The presence of as many as $10^{4–5}$ copies of 5S, 16S, and 23S rRNA per cell makes them excellent targets compared to DNA targets which usually have only very few copies per cell [1, 2]. The nucleotide sequence of rRNA is well conserved within a species, yet quite variable among different microbial species, making rRNA an ideal target for species identification for medically important organisms. The 16S and 23S rRNA molecules contain variable sequence motifs that reflect their phylogenetic origins. The sequence variability allows the design of species specific probes for organism identification. Other RNA targets for in situ hybridization (ISH) have also been applied to identify or differentiate bacteria, such as tmRNA, a RNA molecule of 363 nucleotides with combined properties of tRNA and mRNA [3], and mRNA [4].

Probe Selection

Probe selection and labeling have direct impact on hybridization assay efficiency. The ideal probe is single-stranded, lacks secondary structure, and does not self anneal. It typically is either a short DNA or RNA oligonucleotide that is usually 15–25 bases long. Most DNA probes are generated by oligonucleotide synthesis, whereas in vitro transcription is used for RNA probes. A critical feature of probe selection is the careful choice of probe sequence that is complementary to the sequence of the target of interest. In order to achieve high stability and to optimize base pair binding, a probe should contain greater than 50% guanine and cytosine (or GC-content). Under optimal conditions, the specific probe should hybridize to single-stranded nucleic acid (DNA or RNA) to generate a probe-target base paired double-stranded hybrid.

In the past, probes were commonly labeled with radioactive molecules; however, these have now been replaced by a broad range of reporter molecules that are safer to use in a clinical laboratory, such as alkaline phosphatase, antibodies, chemiluminescence, and fluorescence dyes [5]. Currently, nucleic acid probe hybrids are detected by incorporating pre-labeled probes in the methodology. The most commonly used probe labels to detect DNA or RNA within tissue or cells are enzymatically linked antibody-based reporter molecules like digoxigenin. DNA sequences or RNA transcripts that have high sequence similarity to the probe are then detected by visualizing the hybridized probe via imaging techniques. The application of these probes to tissue sections allows DNA or RNA to be localized within tissue regions and cell types. Fluorescent-labeled probes offer the advantages of producing strong signals with less background, but have the disadvantages of poor fluorescent signal stability and the purchase of a fluorescence microscope with appropriate filters.

Nucleic Acid Hybridization Formats

Nucleic acid probe hybridization assays commonly used in diagnostics occur in three major formats: in solution or liquid-phase, solid-phase, and ISH. With the liquid-phase format, both probe and target rapidly interact in a homogeneous solution, as exemplified by the hybridization protection assay (HPA). The HPA method utilizes short oligonucleotide probes covalently labeled with a chemiluminescent acridinium ester (AE) [6]. Solid-phase hybridizations, such as line probe or dot blot assays, occur on a solid surface (nylon membranes) to which the nucleic acid probe is bound. In general, hybridizations assays on solid-support platforms are not as sensitive as those liquid-phase formats due to the lack of exposure to all target sequences. ISH is a type of solid-phase format that fixes intact cells or tissues onto glass slides for detection of target nucleic acid. The controlled enzymatic digestion of cellular membranes and other proteins allow the probes to gain access to the target sequences. The labels for the nucleic acid probes, which can be biotin or digoxigenin, incorporate a signal compound, such as a colorimetric or a fluorescent compound. In the fluorescent model, the signal probe hybridizes with the target to produce fluorescence, as exemplified by the peptide nucleic acid fluorescence in situ hybridization (PNA FISH) assays. Other formats have been designed to combine solution and solid-phased hybridization. In this model, the capture probes, which are coated on the metal beads, hybridize with the target nucleic acid in solution. A magnet is applied to the reaction tube, to separate the hybrids from the rest of the reaction and washing steps remove unbound probes and other unrelated molecules. In this solid support format, called "sandwich hybridization," the signal probe will remain with the reaction only if the target is hybridized with both signal and capture probes.

The sensitivity and specificity of probe hybridization formats are highly influenced by hybridization stringency conditions that occur during the reaction, such as the temperature settings, washing conditions, and formamide, pH, or salt buffer concentrations. Stringency conditions define the number of base pair mismatches that

can be tolerated during the hybridization reaction. Assays with high stringency parameters are characterized by increased assay specificity and decreased sensitivity. Therefore high stringency parameters predict few hybrid mismatches and few false-positive results. In contrast, less stringent reactions increase the sensitivity of the assay at the risk of detecting unwanted, nonspecific results. In order to maximize assay performance characteristics, the stringency conditions for hybridization need to be optimized. Although most commercial hybridization assays are highly standardized, a laboratory developed assay, may need to adjust hybridization conditions to achieve the level of stringency that fits their needs.

Clinical Application of Nonamplified Probe-Based Assays

Probe-based assays have been developed to identify microbial nucleic acid targets from culture or directly from specimen. The accuracy, simplicity of use, and rapid turnaround time to results are advantages that have been applied to diagnostic test platforms, where they have been developed for the rapid identification of a wide range of infectious agents, thus facilitating appropriate patient management and optimal treatment.

Gen-Probe Nucleic Acid Detection Methods

Gen-Probe AccuProbe and PACE-2 assays (Gen-Probe, Inc., San Diego, CA) were among the first companies to utilize nonamplified nucleic acid hybridization for routine identification of microorganisms [7–9]. The kits are nonisotopic, simple to use, and highly sensitive (92–100%) and specific (99–100%) (Table 12.1).

The principle of the AccuProbe is based on in-solution hybridization and the HPA. In this assay, rRNA molecules are released from the organisms by sonication. A single-stranded chemiluminescent acridinium ester-labeled DNA probe binds to a complementary 16S rRNA region of the target organism to form DNA:RNA hybrids [6, 10]. The acridinium ester of unstable hybrids and unattached probes are degraded by alkaline hydrolysis thus preventing chemiluminescence. However, stable DNA:RNA hybrids protect the chemiluminescent probe from hydrolytic degradation resulting in light emission, which is measured in a luminometer as relative light units (RLU).

The AccuProbe assay is marketed for use in clinical laboratories to identify and detect specific rRNA targets in infectious agents from culture and directly from specimen. Assays for culture identification of mycobacteria, include *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii*, *Mycobacterium gordonae*, *Mycobacterium avium* complex, and specific probes to differentiate *Mycobacterium intracellulare*, from *M. avium.* Probe assays are available for identification of the following systemic dimorphic fungi: *Blastomyces dermatitidis*, *Coccidioides immitis*,

Table 12.1 Gen-Probe AccuProbe assay sensitivity and specificity

	Sensitivity (%)	Specificity (%)
Mycobacterial identification		
Mycobacterium avium	99.3	100
Mycobacterium intracellulare	100	100
Mycobacterium avium complex	99.9	100
Mycobacterium gordonae	98.8	99.7
Mycobacterium kansasii	92.8	100
Mycobacterium tuberculosis complex	99.2	99.0
Fungal identification		
Blastomyces dermatitidis	98.1	99.7
Coccidioides immitis	98.8	100
Histoplasma capsulatum	100	100
Bacterial identification		
Campylobacter	100	99.7
Enterococcus	100	100
Group A streptococci (*Streptococcus pyogenes*)	99.0	99.7
Group B streptococci (*Streptococcus agalactiae*)	97.7	99.1
Haemophilus influenzae	97.1	100
Neisseria gonorrhoeae	100	100
Staphylococcus auresus	100	100
Listeria monocytogenes	100	99.7
Streptococcus pneumoniae	100	100

Information provided by Gen-Probe

and *Histoplasma capsulatum.* Gen-Probes assays are available for identification of *Campylobacter* sp., enterococci, Group B streptococci (*Streptococcus agalactiae*), *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Streptococcus pneumoniae.* In addition, tests for detection of the following bacteria directly from samples are available from Gen-Probe: *Chlamydia trachomatis*, *N. gonorrhoeae*, Group A streptococci (*Streptococcus pyogenes*) and *Legionella pneumophila.*

Hybrid Capture Assay

The hybrid capture (HC) assay was first designed by Digene (Gaithersburg, MD) in 1994 [11] as a relatively fast, liquid-based hybridization assay to detect specific viruses and bacteria. The HC system is a signal-amplification assay that combines antibody capture and chemiluminescent signal detection. The basic steps of the assay involves the combination of specific RNA probes to target DNA, generating RNA:DNA hybrids. These hybrids are captured onto a solid phase coated with universal antibodies that are specific for RNA:DNA nucleic acid hybrids. The RNA:DNA hybrids are detected with multiple signaling antibodies conjugated to alkaline phosphatase, resulting in chemiluminescence, which is measured in a luminometer.

HC technology has been applied for detection of *C. trachomatis* (CT), *N. gonorrhea*, and human papillomavirus (HPV). This signal amplification assay is widely used for the detection of 14 HPV types, some causing cervical cancer, from thin preparation, liquid-based cervical brushes [12]. Recently, the HC assay is readily applied to detect HPV from head and neck squamous cell carcinoma using fine-needle aspirate preparations [13]. This may eliminate the need to obtain tissue solely for the purpose of HPV testing, and provides broad application of the assay across specimen types.

Affirm DNA Assay

The DNA probe-based Affirm VPIII (BD Diagnostics, Sparks, MD), is a multiplex assay that detects complementary targeted DNA sequences of three major agents that can cause vaginitis/vaginosis: *Candida*, *Gardnerella*, and *Trichomonas*. The test is performed directly from a vaginal specimen without requiring nucleic acid amplification. The technology includes distinct single-stranded nucleic acid capture probes and color development probes that are complementary to unique genetic sequences of each target organism. The capture probes are immobilized on a bead embedded in a Probe Analysis Card, along with separate beads for each target organism. The color development probes are contained in a multiwell Reagent Cassette.

The Affirm VPIII Microbial Identification Test for *Candida* species can detect 1×10^4 CFU of *Candida* species in log phase per assay, for *G. vaginalis* can detect 2×10^5 CFU of *G. vaginalis* in log phase per assay and for *T. vaginalis* can detect 5×10^3 trichomonads per assay (ref, Affirm VPIII package insert). Since *Gardnerella* is present as normal vaginal flora, its detection does not necessarily indicate bacterial vaginosis (BV). However, there have been clinical correlations of BV with high number of *Gardnerella* in vaginal samples. To minimize false-positive results and possible inappropriate treatment, a high positive cut-off value of 2×10^5 was established for Affirm VPIII. Reports have indicated a high specificity (97.1%), and a sensitivity (89%) that correlated with a grade 3 Nugent Gram stain score [14]. Other studies have indicated that the Affirm VPIII is an excellent tool for the diagnosis or exclusion of BV [15] and is a more sensitive diagnostic predictor for symptomatic vaginitis/vaginosis than conventional clinical examination and wet mount testing [16].

Line Probe Assay

The line probe assay (LiPA), developed by Innogenetics (Innogenetics N.V., Ghent, Belgium) is a solid-phase assay based on the principle of reverse hybridization technology [17]. Biotinylated DNA amplicons hybridize to specific oligonucleotide probes that are immobilized as parallel lines on nitrocellulose membrane-based strips.

Each line represents a specific probe. After hybridization and stringent wash steps, specific hybrids can be detected by colorimetric reactions. By visually comparing the hybridization pattern on the strip to a reference read-out template, the test result can easily be interpreted. The advantages to this procedure include ease of use, no special instruments and it can be used to analyze amplicons resulting from polymerase chain reaction (PCR) technology. Although the LiPA test is not FDA cleared, many laboratories are utilizing the test after in-house validation due to its high sensitivity in the presence of multiple targets. The analytes it can detect include mycobacteria, hepatitis B virus, hepatitis C virus, and HPV genotyping. The reagents for test performance are available commercially through companies such as Siemens Healthcare Diagnostics, Dearfield, IL. The LiPA genotyping assays simultaneously detect mutations, wild types and allelic variant strains [18]. The emergence of variants resistant to the current antiviral therapy is the leading cause of treatment failure in patients with chronic HBV and HCV infections. Thus, the early identification of these mutations may allow a timely adjustment of therapy to avoid hepatitis progression [19].

In Situ Hybridization Assays

ISH is a solid-phase reaction in which labeled nucleic acid probes are used to detect specific DNA or RNA targets in tissue or cells that are often formalin-fixed or paraffin-embedded. ISH combines the specificity and sensitivity of nucleic acid hybridization with the ability to obtain histological and/or cytological information. Digoxigenin-labeled ISH probes are detected enzymatically with antigoxigenin antibodies conjugated with alkaline phosphatase or horseradish peroxidase. Biotin is another popular label that can be detected with enzyme conjugates of avidin, streptavidin, or antibiotin antibodies. These enzymes convert soluble substrates into insoluble precipitates that appear as dark, localized cellular or subcellular stains.

ISH is performed by transferring a small aliquot of a solution containing labeled probe (single-stranded or denatured double-stranded probes) to a protease-digested tissue section. Double-stranded targets are denatured prior to hybridization, which may enhance mRNA or rRNA hybridization by eliminating secondary structures. The stability of the hybridization between probe and target nucleic acid molecule is dependent on temperature, salt and formamide concentration, and length and GC content of the hybrid. Optimum conditions for a successful ISH should be developed by the laboratory performing the test.

ISH is clinically applied for detection or identification of viral nucleic acids associated with infectious disease and cancer. ISH has been used to detect the following viruses: *adenovirus, cytomegalovirus* [20], hepatitis, HPV, herpes simplex, JC, *Epstein–Barr virus* [21], HHV-8 [22]. ISH has been widely used to detect and differentiate HPV in cervical specimens. Dako Corporation (Carpinteria, CA) provides biotinylated DNA probes for HPV ISH, including probes for high risk genomic types associated with cervical cancer.

Peptide Nucleic Acid FISH Assays

FISH using PNA probes is a novel diagnostic technique combining the simplicity of traditional staining procedures with the unique performance of PNA probes to provide rapid and accurate detection of infectious agents [23]. PNA probes are synthetic compounds with nucleotide bases attached to a peptide backbone. They hybridize to targets with improved high sensitivity and specificity mainly due to electrical neutrality of its chemical structure. In addition, the relatively hydrophobic nature of PNA probes penetrates the hydrophobic cell wall easily, following preparation of a standard smear. These unique characteristics enable PNA probes to hybridize to highly structured targets such as rRNA in a quick and efficient manner.

PNA FISH probe-based assays by AdvanDx (AdvanDx, Woburn, MA) have been designed to detect yeast and bacteria directly from newly positive blood cultures expediting pathogen detection and targeted empiric therapy. The rapid diagnosis of bloodstream infections can significantly improve patient care, management, and treatment by reducing turnaround time to 90 min, compared to 1–3 days by traditional culture-based methods. Most of the assays are either FDA approved or pending approval. The first PNA FISH assay using specific PNA probes to target rRNA of *Candida albicans*, thereby differentiating this species from non-albicans *Candida.* The assay was intended for detection from newly positive blood culture bottles that were smear-positive for yeast [24]. Conventional culture-based identification methods can lead to the initial administration of costly and inappropriate antifungal therapy, whereas early species identification enables optimal initial drug selection. A second-generation dual color PNA FISH assay was designed to identify both *C. albicans*, which is susceptible to fluconazole in most instances, from *C. glabrata,* which can be resistant and may require echinocandin therapy [25]. The sensitivity of the AdvanDx test for *C. albicans*, and *C. glabrata* were 98.7% (78/79) and 100%, respectively, and the specificity of this assay was 100% [26]. A new Yeast Traffic Light (AdvanDx, Woburn, MA) is a tricolor-labeled PNA FISH assay intended for the identification of *C. albicans* and/or *C. parapsilosis*, *C. tropicalis* and *C. glabrata* and/or *C. krusei*.

The AdvanDx PNA FISH assays were expanded to include the detection of gram-positive and gram-negative bloodstream pathogens. An assay that identifies staphylococci from newly positive blood bottles with gram-positive cocci in clusters accurately differentiates S. aureus from coagulase-negative staphylococci [27]. The Enterococcus PNA FISH assay distinguishes *Enterococcus faecalis*, which is ampicillin and vancomycin susceptible, from non *E. faecalis* enterococci usually expressing vancomycin resistance [28]. The GNR Traffic Light PNA FISH is a tricolor multiplex assay that identifies *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* within 90 min of a positive blood culture [29, 30]. The impact of the GNR PNA FISH assay for detecting bacteremia is underscored by the multidrug resistance nature of gram-negative organisms, particularly *K. pneumoniae* and *P. aeruginosa.* Their rapid identification can influence empiric therapy. A PNA FISH assay is also available to identify *S. agalactiae* from turbid growth in Lim broth obtained from vaginal and rectal swabs of pregnant women between 35 and 37 weeks gestation [31]. Recently,

Table 12.2 Summary of PNA FISH assays

Intended use	Bloodstream pathogens identification				Vaginal/ rectal GBS[a] screening
Primary culture media	Blood culture bottles				Lim broth
Gram stain	Gram-positive cocci in clusters	Gram-positive cocci in chains	Gram-negative rods	Yeast	NA
Assay results					
Green fluorescing cells	*S. aureus*	*E. faecalis*	*E. coli*	*C. albicans* and/or *C. parapsilosis*	*S. agalactiae* (GBS[a])
Red fluorescing cells	Coagulase-negative staphylococci	Non-*faecalis* enterococci	*P. aeruginosa*	*C. glabrata* and/or *C. krusei*.	NA
Yellow fluorescing cells	NA	NA	*K. pneumoniae*	*C. tropicalis*	NA

[a]Group B streptococci

a new shortened PNA FISH procedure is being developed to reduce assay turnaround time to 30 min. A summary of PNA FISH assays used in clinical laboratories for rapid identification of organisms directly in positive blood cultures and Lim broth showed in Table 12.2.

Future Considerations

Nonamplified nucleic acid probes are successfully being used in clinical microbiology laboratories in many different formats. The technology will continue to expand due to their distinct advantages over culture-based tests, particularly in the detection and identification of poorly growing pathogens and those with long generation times. The commercially developed assays have the potential to become automated. The new probe-based assays under development will be designed to detect infectious agents directly from clinical specimens and fixed tissue.

References

1. DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 43(4896):1360–1363; Erratum in: Science 1989;245(4924):1312.

2. Kemp PF, Lee S, LaRoche J (1993) Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl Environ Microbiol 59:2594–2601
3. Schonhuber W, Le Bourhis G, Tremblay J, Amann R, Kulakauskas S (2001) Utilization of tmRNA sequences for bacterial identification. BMC Microbiol 1:20
4. Wagner M, Schmid M, Juretschko S, Trebesius KH, Bubert A, Goebel W, Schleifer KH (1998) In situ detection of a virulence factor mRNA and 16S rRNA in Listeria monocytogenes. FEMS Microbiol Lett 160:159–168
5. Anderson MLM (1999) Nucleic acid hybridization. Springer, New York
6. Arnold LJ Jr, Hammond PW, Weiss WA, Nelson NC (1989) Assay formats involving acridinium-ester labeled DNA probes. Clin Chem 35:1588–1594
7. Iwen PC, Blair TMH, Woods GL (1991) Comparison of the Gen-Probe PACE 2 Systems, direct fluorescent-antibody and cell culture for detecting *Chlamydia trachomatis* in cervical specimens. Am J Clin Pathol 95:578–582
8. Lumb R, Lanser JA, Lim IS (1993) Rapid identification of *Mycobacteria* by the Gen-Probe AccuProbe system. Pathology 25:313–315
9. Stockman L, Clark KA, Hunt JM, Roberts GD (1993) Evaluation of commercially available acridinium ester-labeled chemiluminescent DNA probes for culture identification of *Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans,* and *Histoplasma capsulatum*. J Clin Microbiol 31:845–850
10. Nelson NC (1998) Rapid detection of genetic mutations using the chemiluminescent hybridization protection assay (HPA): overview and comparison with other methods. Crit Rev Clin Lab Sci 35(5):369–414
11. Farthing A, Masterson P, Mason WP, Vousden KH (1994) Human papillomavirus detection by hybrid capture and its possible clinical use. J Clin Pathol 47:649–652
12. Clavel C, Masure M, Putaud I, Thomas K, Bory JP, Gabriel R, Quereux C, Birembaut P (1998) Hybrid capture II, a new sensitive test for human papillomavirus detection. Comparison with hybrid capture I and PCR results in cervical lesions. J Clin Pathol 51:737–740
13. Bishop JA, Maleki Z, Valsamakis A, Ogawa T, Chang X, Pai SI, Westra WH (2011) Application of the Hybrid Capture 2 assay to squamous cell carcinomas of the head and neck: A convenient liquid-phase approach for the reliable determination of human papillomavirus status. Cancer Cytopathol 120(1):18–25
14. Nugent RP, Krohn MA, Hillier SL (1991) Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. J Clin Microbiol 29: 297–301
15. Witt A, Petricevic L, Kaufmann U, Gregor H, Kiss H (2002) DNA hybridization test: rapid diagnostic tool for excluding bacterial vaginosis in pregnant women with symptoms suggestive of infection. J Clin Microbiol 40:3057–3059
16. Brown HL, Fuller DD, Jasper LT, Davis TE, Wright JD (2004) Clinical evaluation of affirm VPIII in the detection and identification of *Trichomonas vaginalis, Gardnerella vaginalis*, and *Candida* species in vaginitis/vaginosis. Infect Dis Obstet Gynecol 12:17–21
17. Smith GL, Socransky SS, Sansone C (1989) "Reverse" DNA hybridization method for the rapid identification of subgingival microorganisms. Oral Microbiol Immunol 4:141–145
18. Bouchardeau F, Cantaloube JF, Chevaliez S, Portal C, Razer A, Lefrère JJ, Pawlotsky JM, De Micco P, Laperche S (2007) Improvement of hepatitis C virus (HCV) genotype determination with the new version of the INNO-LiPA HCV assay. J Clin Microbiol 45:1140–1145
19. Mirandola S, Campagnolo D, Bortoletto G, Franceschini L, Marcolongo M, Alberti A (2011) Large-scale survey of naturally occurring HBV polymerase mutations associated with anti-HBV drug resistance in untreated patients with chronic hepatitis B. J Viral Hepat 18: e212–e216
20. Wu TC, Lee WA, Pizzorno MC, Au WC, Chan YJ, Hruban RH, Hutchins GM, Hayward GS (1992) Localization of the human cytomegalovirus 2.7-kb major early beta-gene transcripts by RNA in situ hybridization in permissive and nonpermissive infections. Am J Pathol 141: 1247–1254

21. Prange E, Trautmann JC, Kreipe H, Radzun HJ, Parwaresch MR (1992) Detection of Epstein–Barr virus in lymphoid tissues of patients with infectious mononucleosis by *in situ* hybridization. J Pathol 166:113–119
22. Li JJ, Huang YQ, Cockerell CJ, Friedman-Kien AE (1996) Localization of human herpes-like virus type 8 in vascular endothelial cells and perivascular spindle-shaped cells of Kaposi's sarcoma lesions by *in situ* hybridization. Am J Pathol 148:1741–1748
23. Perry-O'Keefe H, Stender H, Broomer A, Oliveira K, Coull J, Hyldig-Nielsen JJ (2001) Filter-based PNA *in situ* hybridization for rapid detection, identification and enumeration of specific micro-organisms. J Appl Microbiol 90:180–189
24. Kenneth O, Haase G, Kurtzman C, Hyldig-Nielsen JJ, Stender H (2001) Differentiation of *Candida albicans* and *Candida dubliniensis* by fluorescent in situ hybridization with peptide nucleic acid probes. J Clin Microbiol 39:4138–4141
25. Safdar A, Chaturvedi V, Koll BS, Larone DH, Perlin DS, Armstrong D (2002) Prospective, multicenter surveillance study of *Candida glabrata*: Fluconazole and Itraconazole susceptibility profiles in bloodstream, invasive, and colonizing strains and differences between isolates from three urban teaching hospitals in New York City (*Candida* susceptibility trends study, 1998 to 1999). Antimicrob Agents Chemother 46:3268–3272
26. Shepard JR, Addison RM, Alexander BD, Della-Latta P, Gherna M, Haase G, Hall G, Johnson JK, Merz WG, Peltroche-Llacsahuanga H, Stender H, Venezia RA, Wilson D, Procop GW, Wu F, Fiandaca MJ (2008) Multicenter evaluation of the *Candida albicans/Candida glabrata* peptide nucleic acid fluorescent in situ hybridization method for simultaneous dual-color identification of *C. albicans* and *C. glabrata* directly from blood culture bottles. J Clin Microbiol 46:50–55
27. Kenneth O, Brecher SM, Durbin A, Shapiro DS, Schwartz DR, Paola C, Girolami DE, Dakos J, Procop GW, Wilson D, Hanna CS, Haase G, Peltroche-Llacsahuanga H, Chapin KC, Musgnug MC, Levi MH, Shoemaker C, Stender H (2003) Direct identification of *Staphylococcus aureus* from positive blood culture bottles. J Clin Microbiol 41:889–891
28. Forrest GN, Roghmann MC, Toombs LS, Johnson JK, Weekes E, Lincalis DP, Venezia RA (2008) Peptide nucleic acid fluorescent in situ hybridization for hospital-acquired enterococcal bacteremia: delivering earlier effective antimicrobial therapy. Antimicrob Agents Chemother 52:3558–3563
29. Morgan M, Marlowe E, Della-Latta P, Salimnia H, Novak-Weekley S, Wu F, Crystal BS (2010) Multicenter evaluation of a new shortened peptide nucleic acid fluorescence in situ hybridization procedure for species identification of select Gram-negative bacilli from blood cultures. J Clin Microbiol 48:2268–2270
30. Della-Latta P, Salimnia H, Painter T, Wu F, Procop GW, Wilson DA, Gillespie W, Mender A, Crystal BS (2011) Identification of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aerugiosa* in blood cultures: a multicenter performance evaluation of a three-color peptide nucleic acid fluorescence *in situ* hybridization assay. J Clin Microbiol 49:2259–2261
31. Montague NS, Cleary TJ, Martinez OV, Procop GW (2008) Detection of group B streptococci in Lim broth by use of group B streptococcus peptide nucleic acid fluorescent in situ hybridization and selective and nonselective agars. J Clin Microbiol 46:3470–3472

Chapter 13
Molecular Typing Techniques: State of the Art

Richard V. Goering

Introduction

The treatment of infectious disease centers around the goals of both curing the patient and preventing or at least restricting the spread of disease. In a perfect world, health care professionals would know that these goals have been achieved when the patient's health is restored and there are no new occurrences of infected patients. However, the real world of infectious disease is far from perfect. The individual patient may present with evidence of recurring or additional infection by a pathogen (e.g., at a different body site). Different members of a patient population may yield cultures of the same organism. In both instances, the question commonly asked is whether multiple isolates of a given pathogen represent the same strain. In the individual patient, this question commonly relates to issues of therapeutic efficacy while in a patient population the concern is infection control. However, in both settings, the resolution of these questions is aided by specific epidemiological assessment. In the past, a variety of methods based on phenotypic characteristics have been used for this purpose including biotype, serotype, susceptibility to antimicrobial agents, or bacteriophages, etc. [1–4]. However, in the 1970s, techniques developed for the recombinant DNA laboratory began to find application in the molecular characterization of clinical isolates. These included comparing protein molecular weight distributions by polyacrylamide gel electrophoresis, relative mobility of specific enzymes by starch-gel electrophoresis (multi-locus enzyme electrophoresis), specific antibody reactions with immobilized cellular proteins (immunoblotting), and cellular plasmid content (i.e., plasmid fingerprinting) [2, 5, 6]. However, by the 1980s it was clear that comparisons at the genomic level would provide the most fundamental measure of epidemiological relatedness. Thus, molecular typing was born.

R.V. Goering (✉)
Department of Medical Microbiology and Immunology,
Creighton University School of Medicine, Omaha, NE 68178, USA
e-mail: rgoeri@creighton.edu

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While a wide range of etiological agents are of clinical concern, this review focuses on molecular approaches to the epidemiological analysis of bacterial pathogens.

What Does "State of the Art" Mean?

In any area of scientific investigation, state of the art methodology may be viewed from two different perspectives. There are cutting-edge techniques requiring specialized equipment and expertise that perform remarkably well but are of limited availability to many investigators. Alternatively, there are functional state of the art approaches, meaning that one is using the best method available within the prevailing (financial, geographic, technical expertise, etc.) environment. In this context, it is important to recognize that while one may not have access to the most recently published sophisticated methods, from an epidemiological standpoint, it is better to do something rather than nothing. Thus, this review begins with examples of established molecular typing techniques which, depending on the (financial, geographic, scientific) environment, may still be viewed as state of the art while also considering more recently described cutting-edge approaches.

The Ultimate Foundation for Epidemiological Comparison: The Bacterial Genome

Advances in DNA sequencing have shown that what was once thought of as the bacterial chromosome is actually a core genome plus a variety of inserted mobile genetic elements [7–9]. Nevertheless, the totality of these sequences makes the cell a specific strain of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, etc. Thus, the bacterial genome represents the most fundamental molecule of identity in the cell and the common goal of molecular typing approaches is to provide a measure of isolate genomic relatedness [10]. While the methodological aspects of these techniques differ, they can generally be grouped into two categories of data output, either electrophoretic "bands" or DNA sequences.

Methods with Electrophoretic Output

Restriction Enzyme-Based

Chromosomal Restriction Enzyme Analysis

The ubiquitous presence of chromosomal DNA in all bacterial pathogens made restriction enzyme analysis (REA) an attractive early approach to molecular strain typing. While all bacterial cells can theoretically be analyzed by such a process, the

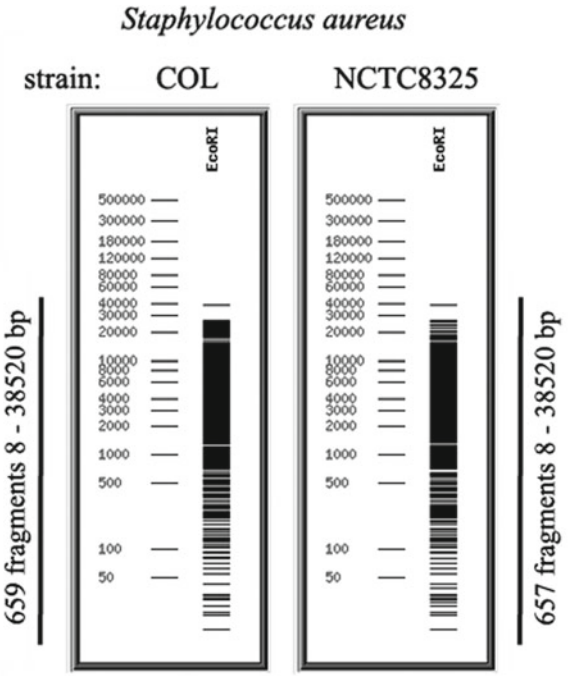

Fig. 13.1 Diagrammatic representation of REA with chromosomal DNA from *S. aureus* strains COL and NCTC8325 digested with the restriction enzyme *Eco*RI. Data were generated using the Comprehensive Microbial Resource of the J. Craig Venter Institute Web site: http://cmr.jcvi.org/cgi-bin/CMR/shared/Menu.cgi?menu=genome

DNA sequences recognized by common restriction enzymes such as *Eco*RI, *Hind*III, etc., are abundantly dispersed (e.g., on average >600 copies) throughout a typical 2–3 Mb bacterial chromosome. This is illustrated in Fig. 13.1 with a comparison of *Eco*RI REA for *S. aureus* strains COL and NCTC8325. Thus, the resulting challenge is to accurately compare electrophoretic patterns that comprise hundreds of restriction fragments, often co-migrating in clusters of similar size, and potentially including resident plasmid DNA [11]. Consequently, at the present time this method continues to be recommended only for use with *Clostridium difficile* [12].

Since the mid 1970 Southern hybridization [13] has been a staple of molecular biology, and its power to probe for specific DNA sequences soon began to find clinical application. For diagnostic purposes, tests to detect the presence or absence of clinically relevant sequences (e.g., related to organism identification, antibiotic resistance) began to be developed. For epidemiological analysis, probes specific for sequences found at multiple chromosomal locations can be hybridized against chromosomal restriction enzyme fragments which have been electrophoretically separated. The resulting hybridization patterns (restriction fragment length polymorphisms (RFLPs)) provide an indication of chromosomal relatedness between different bacterial isolates. However, at present this approach is not widely used for epidemiological analysis with the exception of probes for the insertion sequence IS*6110* in the RFLP analysis of *Mycobacterium tuberculososis* [14, 15].

Pulsed-Field Gel Electrophoresis

In contrast to conventional REA, rare-cutting restriction enzymes cleave the bacterial chromosome into a relatively small number of fragments (e.g., 10–30) due to the length and/or DNA base composition of their recognition sequences,. However, electrophoretic analysis of the megabase-size restriction fragments generated is complicated by their size-independent migration during conventional agarose-gel electrophoresis [16, 17]. In 1980, alternative electrophoretic approaches were developed based on the principal of periodic reorientation of the electric field (and DNA migration) relative to the direction of the gel. The pulsed electric field separates DNA fragments over a wide range of sizes from kilobytes to megabytes (Fig. 13.2)

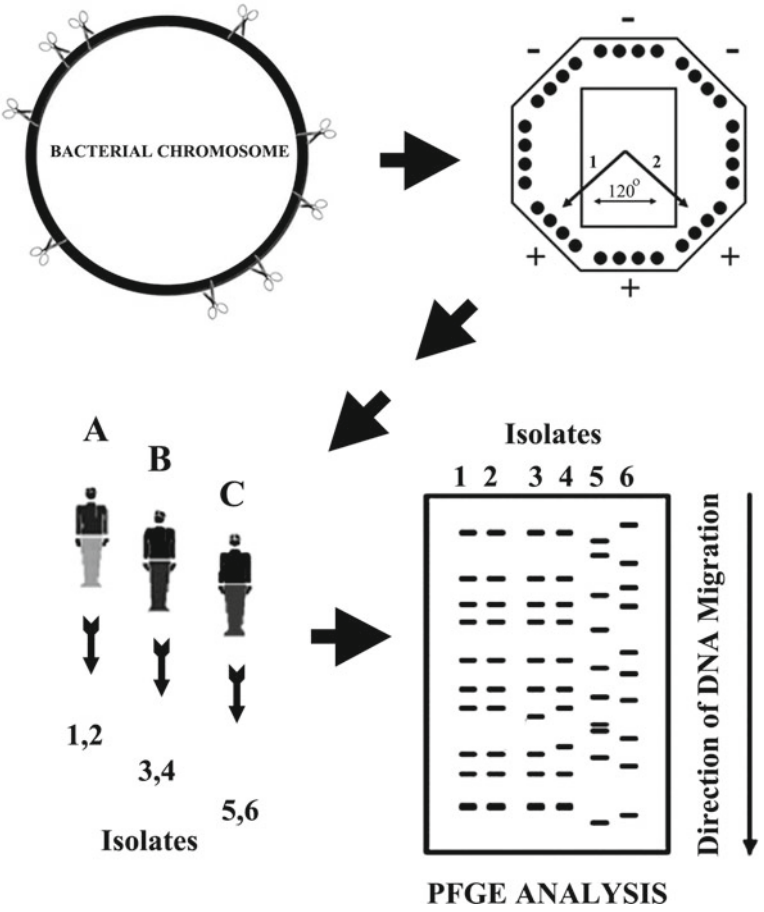

Fig. 13.2 Illustration of PFGE workflow moving from chromosomal digestion with rare-cutting restriction enzymes to macro-restriction fragment separation by PFGE to the final analysis of fragment patterns from different (patient) sources

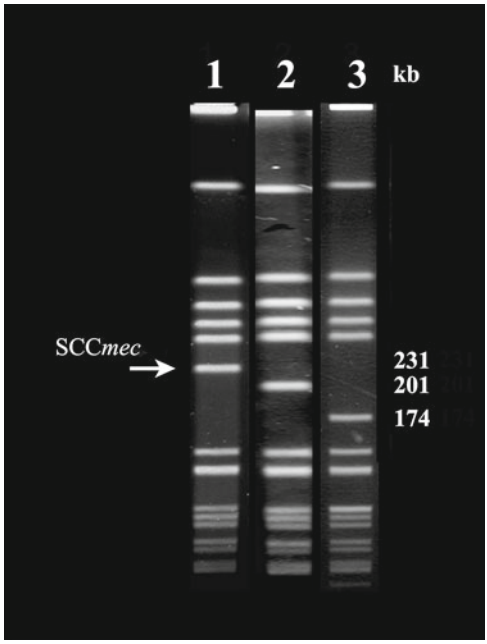

Fig. 13.3 *Sma*I-digested chromosomal DNA from USA300 *S. aureus* isolates which are (*lane 1*) methicillin resistant and PCR positive for the adjacent ACME *arcA* gene (*lane 2*), methicillin susceptible due to loss of SCC*mec* but *arcA* positive, or (*lane 3*) negative for both SCC*mec* and *arcA*. (Modified from Goering et al. [21])

thus allowing a more manageable comparison of isolate patterns. The usefulness of pulsed-field gel electrophoresis (PFGE) for molecular typing has been recently reviewed [18, 19]. However, it is important to note that while the method is far from new, PFGE has exhibited enormous staying power as a valuable method of genomic analysis and comparison. This is especially true for molecular typing where for the majority of bacterial pathogens it remains the acknowledged "gold standard" for assessing isolate interrelationships. The reason for this longevity is multifold. Overall, the method for chromosomal DNA isolation (i.e., the in situ lysis of bacterial cells encased in agarose plugs) requires only minor variation with different bacterial species. A wide range of bacterial pathogens can be analyzed using a small number of different restriction enzymes (commonly *Sma*I and *Xba*I for gram-positive and -negative isolates, respectively). Despite the fact that PFGE obviously does not detect every genetic change and macro-restriction fragment, for most organisms analyzed the sum of the visible fragment sizes represents greater than 90% of the chromosome. This visual sense of global chromosomal monitoring can be highly informative not only for isolate comparisons, but also in associating characteristic PFGE patterns with specific (e.g., internationally recognized) bacterial strains [20]. In addition, the chromosomal overview provided by PFGE allows visualization of genomic rearrangements as in the case of *S. aureus* strain USA300 where changes in PFGE patterns can be specifically associated with loss of the staphylococcal chromosomal cassette encoding methicillin resistance (SCC*mec*) or the adjacent arginine catabolic mobile element (ACME) [21] (Fig. 13.3).

Optical Mapping

Optical mapping (OM) is an interesting variation of the REA-PFGE method with potential epidemiological application. Similar to PFGE, high molecular weight genomic DNA is obtained by agarose-encased lysis of cells. As illustrated in Fig. 13.4, single DNA molecules are then electrostatically fixed to the surface of material amenable to scanning by fluorescent microscopy. The DNA molecules are exposed to restriction endonuclease digestion but the order of the resulting fragments is maintained since each molecule is immobilized. After staining with a fluorescent dye, fluorescent microscopy coupled with appropriate software converts the optical image to a digital format producing restriction maps of the individual molecules. The overlapping maps are then assembled to produce an ordered restriction map of the entire chromosome. Overall hands-on time of a few hours with final genomic data output in less than 2 days from start to finish makes OM an interesting technology which has shed recent light on a variety of microbial strain interrelationships [22, 23]. However, a per-isolate cost of several thousand dollars (total instrumentation list price >$200,000) currently makes OM impractical for infection control surveillance or routine multi-isolate epidemiological analysis.

PCR-Based

Amplified Fragment-Length Polymorphism

Amplified fragment-length polymorphism (AFLP) remains in current use as an interesting approach that combines the use of restriction enzymes and PCR to potentially analyze a wide range of bacterial pathogens [24]. The process involves creation of typing patterns based on PCR amplification of a subset of chromosomal restriction fragments (Fig. 13.5). This is accomplished by digesting isolated DNA with two different restriction endonucleases, usually chosen so that one cuts more frequently than the other (e.g., *Eco*RI and *Mse*I). While a large group of restriction fragments are initially created, only specific subsets are utilized for isolate comparison. Adapters specific for the cleaved restriction-sites are ligated to the fragment ends thus extending the length of the known end sequences and serving as primer binding sites for PCR. The adapter design includes extra nucleotides beyond the restriction-site sequence allowing only a subset of fragments to be amplified. Using labeled primers the specificity of the process may be further controlled, ultimately leading to an electrophoretic pattern of amplified products that becomes the basis for assessing isolate interrelationships. Recent AFLP improvements have included multiple enzyme–adapter combinations and either fluorescent or radioactively labeled primers, allowing high-throughput analysis to be achieved using an automated DNA sequencer, phosphoimager, etc. [24, 25]. However, issues regarding data analysis and inter-laboratory sharing, and the specialized equipment required for electrophoresis have limited the use of this method in the clinical setting.

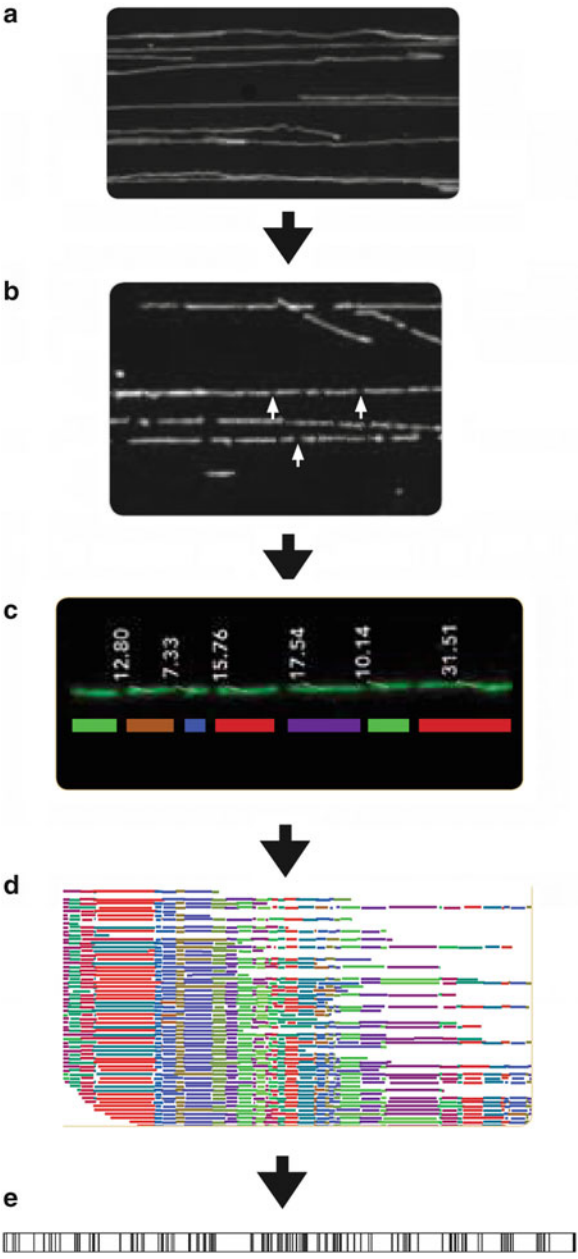

Fig. 13.4 Protocol for optical mapping. (**a**) DNA is electrostatically immobilized, (**b**) digested, DNA and fluorescently imaged. (**c**) Restriction fragments are sized and (**d**) assembled into a (**e**) consensus optical map. (Modified from the OptiGen®, LLC Web site http://www.optigen.com)

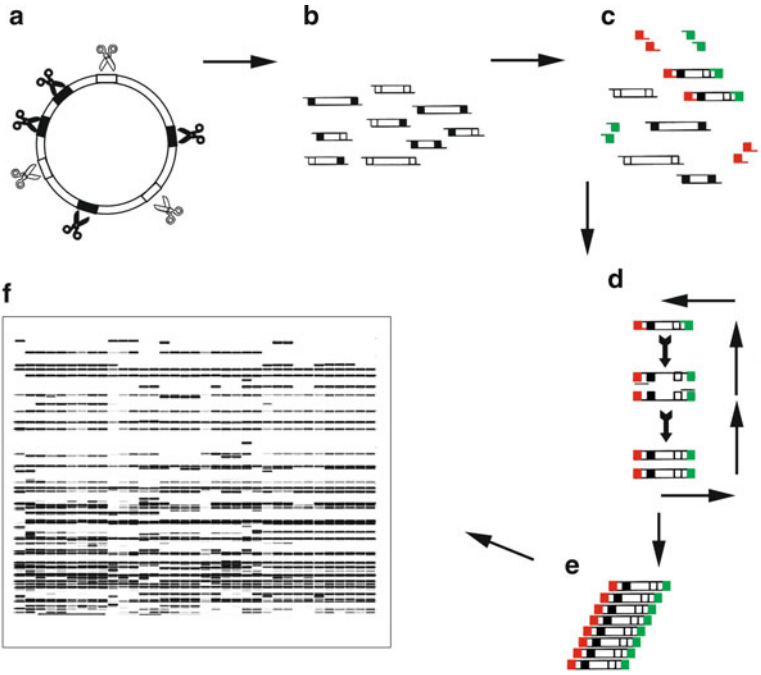

Fig. 13.5 AFLP protocol. (**a**) Genomic DNA is restricted using two different enzymes to yield fragments (**b**) with a mixture of restriction sequence ends. (**c**) Restriction-site specific adapters are ligated to the fragment ends. (**d**) PCR primers complementary to the adapters with additional bases at their 3′ ends restrict amplification to a subset of fragments (**e**) the sizes of which are then analyzed by electrophoresis (**f**). (Modified from Rademaker and Savelkoul [84])

Repetitive Sequence-Based PCR

Well before our current level of technology and understanding regarding bacterial genomics, specific DNA sequences were known to be repeated at multiple chromosomal sites in a variety of clinically important pathogens. Enterobacteria were found to contain several hundred copies of repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences [26]. Repeated BOX element sequences were observed in the chromosome of *Streptococcus pneumoniae* [27]. Multiple copies of IS*256* were found in staphylococcal genomes [28]. These and other repeat elements represent genomic landmarks of known sequence to which PCR primers may be specifically anchored in an outwardly oriented direction. The resulting amplicons represent inter-repeat distances that do not exceed the capability of the *Taq* polymerase (Fig. 13.6). Thus, strain typing by repetitive sequence-based PCR (rep-PCR) is accomplished by comparing the chromosomal distribution of such repeated sequences as reflected by the resulting pattern of amplicon sizes. Performed under relatively stringent conditions, rep-PCR is much more reproducible than other more generic PCR approaches such as

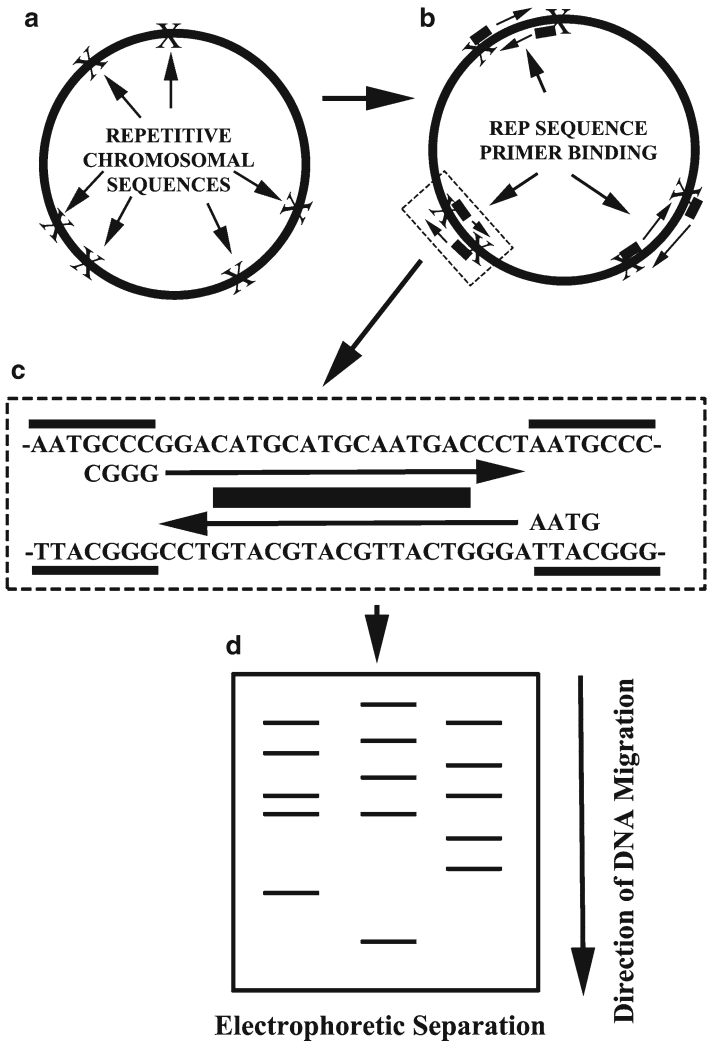

Fig. 13.6 Illustration of rep-PCR. (**a**) Repetitive sequences in the bacterial chromosome are recognized by outwardly directed primers (**b**) allowing PCR amplification of the inter-repetitive regions (**c**) when are then analyzed by electrophoresis (**d**)

randomly amplified polymorphic DNA (RAPD) and arbitrarily primed-PCR (AP-PCR) which are not considered here [1, 5]. Initial "home brew" efforts at rep-PCR encountered issues such as appropriate primer combinations, PCR conditions, and optimum visualization of amplicon fragment patterns by agarose gel electrophoresis [29]. However, the process has become highly reproducible via commercial automation. The DiversiLab System (bioMérieux) employs optimized protocols, separation of PCR products in a charged microfluidic field (i.e., on a chip) rather

than by conventional agarose gel electrophoresis, and software for data analysis. While in some instances less discriminatory than PFGE [30, 31], rep-PCR remains an interesting typing method although issues regarding database libraries and inter-laboratory data sharing [4] as well as costs associated with the commercial approach are factors to be considered.

PCR Ribotyping

Bacteria typically contain multiple chromosomal copies of rRNA genes. Conventional ribotyping exploits the fact that strain-to-strain differences in the chromosomal regions flanking rRNA genes affect restriction enzyme recognition sites producing different RFLP hybridization patterns with rRNA probes [32]. However, this approach is no longer considered state of the art. PCR-ribotying, based on primers amplifying polymorphisms in the 16–23S intergenic spacer region, continues to be used as an important tool in the epidemiological monitoring of *C. difficile* [33]. However, it is important to note that the amplicons generated typically include a variety of similar sizes which are a challenge to separate by agarose gel electrophoresis. Nevertheless, the patterns obtained are amenable to databasing and inter-laboratory comparison especially with regard to highly toxigenic strains such as *C. difficile* ribotype 027 [34–36].

Staphylococcal Cassette Chromosome *mec* Typing

Staphylococci resistant to the antibiotic methicillin, especially *S. aureus* (MRSA), represent an infectious disease problem of global concern. Central to this issue is the mobile genetic element staphylococcal cassette chromosome *mec* (SCC*mec*) encoding the altered penicillin-binding protein (known as PBP2a or PBP2') responsible for resistance [37]. Increased understanding of staphylococcal genomics has revealed SCC*mec* variations (termed SCC*mec* types) which differ with regard to their internal organization and total size (<30–>60 kb) [38]. A variety of multiplex PCR approaches have been developed with primers positioned to detect type-specific differences reflected by amplicon banding patterns in agarose gels [39–41]. However, SCC*mec* represents one of the most highly recombinogenic regions in the staphylococcal genome. This is reflected in the increasing complexity associated with newly described SCC*mec* types and subtypes and the multiplex PCR protocols required for their detection (http://www.sccmec.org/Pages/SCC_TypesEN.html) [38]. Nevertheless, SCC*mec* typing represents an important means of studying the element's organization, persistence, and movement in staphylococcal populations. In this context, SCC*mec* type has become a landmark trait in the definition of specific staphylococcal epidemic strains (especially MRSA). However, the method is not discriminating enough to stand alone as an approach to epidemiological monitoring and SCC*mec* differences do not significantly impact anti-staphylococcal chemotherapy [42].

Multiple-Locus VNTR Analysis

Similar to the repetitive sequences discussed earlier (i.e., rep-PCR), advances in bacterial genomics have revealed the presence of chromosomal regions consisting of tandemly repeated sequence "units" varying both in the number and sequence of the individual repeats (Fig. 13.7). These occur by slipped strand mispairing during chromosomal replication resulting in the insertion or deletion of repeat units [43–45]. Bacterial genomes may contain different variable number tandem repeats (VNTR) at multiple chromosomal sites. Properly designed multiplexed PCR primers thus produce multiple-locus VNTR analysis (MLVA) banding patterns by electrophoresis with potential application for strain typing [46]. Finding and validating the epidemiological usefulness of specific MLVA approaches is a deliberative process which varies depending on a number of factors including the degree of VNTR polymorphisms, the organism being analyzed, etc. [3, 46]. Nevertheless, MLVA strain typing has been described for a variety of clinically important bacterial pathogens including *Bacillus anthracis*, *Brucella* spp., *E. coli*, *Legionella pneumophila*, *Leptospira interrogans*, *Mycobacterium tuberculosis*, *P. aeruginosa*, *Yersitia pestis*, *Shigella* spp., *S. aureus*, and *S. pneumonia* (see [46] for a review). This trend has been facilitated by a number of advances including digitized MLVA pattern nomenclature based on VNTR repeat numbers, improved accuracy with pattern visualization by capillary, rather than agarose-gel, electrophoresis, and proper molecular size standards.

Overall, with some exceptions such as PFGE, electrophoretic-based typing methods tend to be relatively simple to perform and also benefit from the potential for decreased cost when agarose-gels are used for analysis. However, it is important to emphasize that strain typing based on electrophoretic banding patterns is primarily a comparison of chromosomal fragment sizes rather than specific genomic content. With the exception of PCR ribotyping and MLVA this is true for both restriction enzyme and PCR-based methods but is especially the case with the former where equivalent-sized fragments in different isolate patterns may or may not represent the same chromosomal sequence. Electrophoresis-based typing approaches are also challenged

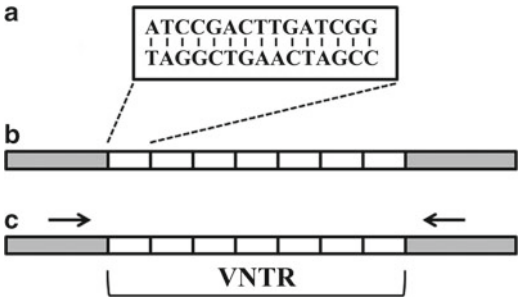

Fig. 13.7 Diagram of a chromosomal VNTR where (**a**) a sequence unit of "X" base pairs is (**b**) tandemly repeated "Y" number of times during chromosomal replication. PCR primers anchored to chromosomal regions adjacent to the VNTR (**c**) allow amplification with subsequent electrophoretic analysis to determine the VNTR "Y" repeat number

regarding issues of typing pattern nomenclature, databasing, and interlaboratory sharing. Nevertheless, as noted earlier, in the context of locally available economic and scientific resources these methods continue to remain of value as options for the epidemiological evaluation of problem bacterial pathogens.

DNA Sequence-Based Methods

Since the bacterial chromosome is the most fundamental molecule of identity in the cell, strain typing based on DNA sequence analysis is the most direct approach to assessing isolate relatedness. Sequence-based approaches have a number of additional advantages over electrophoresis-based typing methods including:

1. Simplicity and reproducibility.
   Older molecular methods for epidemiological analysis involve numerous experimental variables including types of equipment, reagents, experimental protocols, etc., all of which affect inter- and intra-laboratory reproducibility. With enough time and effort, any epidemiological method can be standardized as evidenced by classical bacteriophage typing of staphylococci [47] or the success of the nationwide PFGE Pulse-Net System for the investigation of foodborne outbreaks designed by the United Stated Centers for Disease Control [48]. However, DNA sequence analysis is a more straightforward process that can be performed in a more controlled, uniform, and reproducible manner with specific known chromosomal loci.
2. Data sharing and storage.
   Electronic storage and sharing of data from electrophoresis-based typing methods is accomplished using bitmapped (e.g., .tiff, .jpeg) computer images. However, the larger the number of isolates the more unwieldy the process can become. In addition, some form of nomenclature must be used to identify and interrelate isolate banding patterns. With large data sets, the use of appropriate computer software is essential to accomplish this process. However, the framework for data sharing, storage, and retrieval is necessarily based on visual images and the limits that format imposes. Conversely, nucleotide sequences represent simple, highly portable, quaternary data that can much more easily be shared, stored, and retrieved.
3. Data interpretation and detection of significant differences.
   As will be discussed more fully later, the most crucial aspect of any typing method is its ability to detect significant (epidemiologically-relevant) differences between isolates. While the goal of molecular typing is a comparison of chromosomal similarity, electrophoretic banding patterns only indirectly address this issue. Despite computer programs which can assist the process, there is always an element of end user judgment that can affect the final evaluation. In contrast, nucleotide sequence data allows direct and unambiguous genomic comparison.

Advances in DNA sequencing and the rapidly expanding database of sequenced microbial genomes have served as the foundation for a variety of typing approaches which can generally be categorized as single-locus, multiple-locus, or whole-genome sequence typing.

Single-Locus Sequence Typing

Since the genome of bacterial pathogens is mega-base in size, it is remarkable to think that a single locus of ca. 1,000 bases could contain sufficient information to be epidemiologically relevant. Nevertheless, three instances where this is the case are detailed below.

S. aureus Protein A Typing

The production of protein A is a hallmark characteristic of *S. aureus*. Thus, the gene for protein A (*S. aureus* protein A, *spa*) is found in all *S. aureus* strains. The 3′ end of the *spa* locus (i.e., the polymorphix "X" region) contains a 24-bp VNTR which can be amplified with appropriate primers (e.g., see Fig. 13.7) and sequenced to determine the specific *spa* type. Software packages such as StaphType (Ridom GmbH, Münster, Germany) and BioNumerics (Applied Maths NV, Sint-Martens-Latem, Belgium) are available to assist with the sequence analysis process. Numerous studies have shown that comparisons of *S. aureus spa* types, facilitated by an Internet-based *spa* server (http://spaserver.ridom.de), provide epidemiologically-relevant information that correlates well with other typing methods such as PFGE [42, 49–52]. In Europe this has led to the formally organized use of *spa* typing in the epidemiological monitoring of specific *S. aureus* strains (i.e., SeqNet; http://www.seqnet.org) involving 60 laboratories from 39 countries.

Strepcococcus pyogenes M Protein (*emm*) Typing

The cell surface M protein is an important virulence factor in *S. pyogenes* [53]. Genomic analysis has revealed that the M protein locus (*emm*) is variable and can encode at least 100 different M protein types which were initially detected and cataloged serologically. However, PCR primers flanking the hypervariable region of the *emm* gene allow direct sequencing to determine specific isolate *emm* types. As a result, sequence-based *emm* typing is currently the most widely used approach to group A streptococcal epidemiology [53–56]. As with *S. aureus spa* typing, *emm* typing is facilitated by an Internet-based server (hosted by the US Centers for Disease Control) which houses the *S. pyogenes emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm). This resource has allowed the CDC to follow specific *S. pyogenes* epidemiological trends such as the proportion

of *emm* types contributing to specific disease in different global regions (e.g., Africa, Asia, Latin America, Middle East, Australia/Pacific Island) (http://www.cdc.gov/ncidod/biotech/strep/emmtype_proportions.htm).

mec-Associated Direct Repeat Unit Typing

In 1991, Ryffell et al. [57] identified a cluster of repeated imperfect 40-bp sequences (i.e., direct repeat units, *dru*) adjacent to IS*431* within the SCC*mec* element of *S. aureus* isolates. While the *dru* VNTR is absent in a minority of MRSA isolates [58], its constant location in different SCC*mec* types of both coagulase-positive and -negative staphylococcal species represents a valuable and stable internal SCC*mec* characteristic [59]. As with staphylococcal *spa* typing, properly positioned PCR primers allow amplification and sequencing of the *dru* region. Software such as BioNumerics (Applied Maths NV, Sint-Martens-Latem, Belgium) and DruID (http://www.mystrains.com/druid) is available to assist with assignment of *dru* types the central repository for which is an Internet-based server (http://www.dru-typing.org). As with SCC*mec* typing, *dru* typing has become an increasingly important means of characterizing the persistence and movement of SCC*mec* in staphylococcal populations. While not discriminating enough to serve as a standalone approach to epidemiological monitoring, *dru* typing has proven helpful in assessing movement of SCC*mec* in staphylococcal populations and in subtyping highly clonal (i.e., difficult to differentiate) staphylococcal strains [58, 60, 61]. In addition, a combination of *dru* typing and analysis of SCC*mec* (*ccr*) recombinase genes, has proved highly informative with regard to the phylogeny of specific *S. aureus* MRSA strains [62, 63].

Multi-Locus Sequence Typing

Since its initial description in 1998 [64] multi-locus sequence typing (MLST) has become one of the most popular approaches to microbial strain typing with demonstrated utility for a wide range of clinically relevant pathogens (http://www.mlst.net/databases/default.asp). The method is based on PCR amplification and subsequent sequencing of the internal regions (450–500 bp) of multiple essential (i.e., housekeeping) genes. Seven genes are typically employed, the sequences of which are assigned numeric allelic designations (Fig. 13.8a). Individual strains are thus characterized by a seven digit MLST sequence type (ST). For a given organism, individual STs are interrelated based on an algorithm that identifies a parent or "founding" ST as that which has the greatest number of single-locus variants (SLV). Using online graphic tools (eBURST; http://saureus.mlst.net/eburst/) the STs can be further grouped into clonal complexes (CC) where members of the group share a minimum of five or six of the seven allellic designations (Fig. 13.8b). The highly portable nature of such data and availability of online databases has facilitated the use of MLST for global epidemiological analysis [65, 66] and long-term

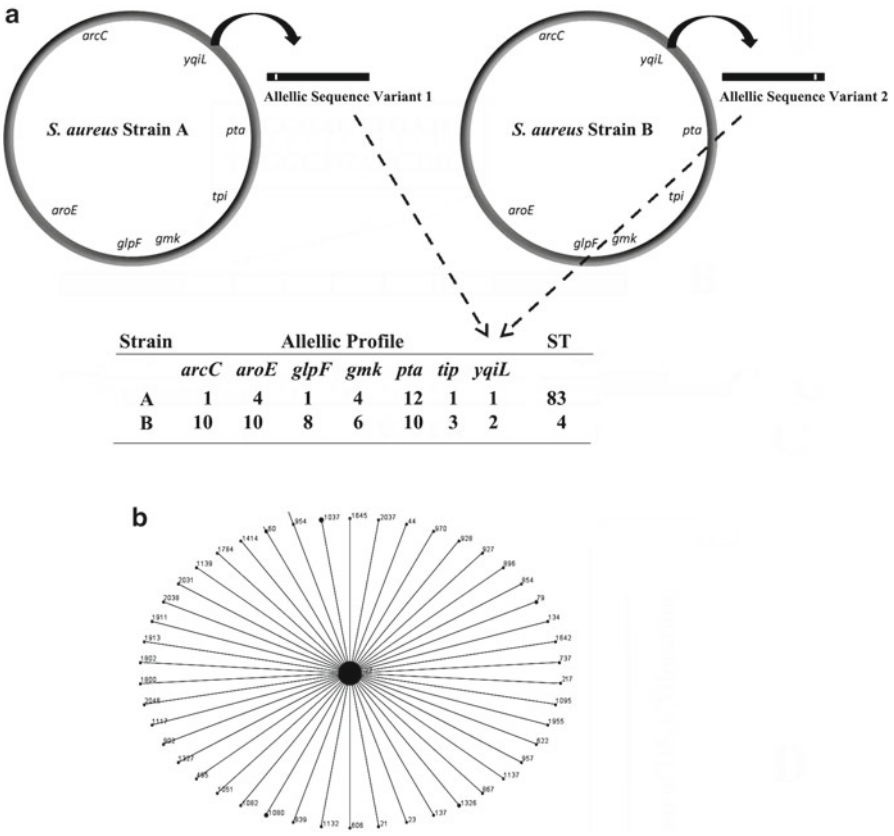

Fig. 13.8 Illustration of MLST with hypothetical *S. aureus* strains A and B depicting the seven chromosomal housekeeping genes with an example of allelic differences (e.g., in *yqiL*) constituting different STs (**a**). An eBURST example of a clonal complex with central founding ST and associated SLVs is also shown (**b**)

(i.e., phylogenetic) investigation of bacterial lineages [67–69]. However, the method has not found routine clinical application since MLST housekeeping gene sequences are too conserved to reliably differentiate the closely related isolates typically encountered during short-term outbreaks (e.g., MRSA and MSSA could both have the same ST). The time and cost associated with multiple-gene sequencing (a total of ca. 3–4 kb for 7 loci) has also been a disincentive to routine use.

Other Multi-Locus Approaches: Hybridization-Based Typing

As noted earlier, only a small number of loci may be simultaneously queried using DNA hybridization with restriction fragment-based typing. However, this is not the case with array-based methods where thousands of specific oligonucleotide probes

(e.g., representing species-specific, antimicrobial resistance, and virulence-associated genes) can be anchored to solid surfaces such as glass, plastic, or silicone chips. The hybridization pattern of labeled genomic DNA from isolates to be analyzed thus has the potential to provide a wealth of information regarding genomic content (e.g., the presence or absence of specific genes). Depending on the length of the anchored array sequences even minor sequence variations including insertions, deletions, or changes in a single base of a sequence (single nucleotide polymorphism, SNP) can be detected. The power of this approach has been applied to the characterization of a wide variety of clinically relevant organisms [3, 70–72]. However, while microarrays have the potential for high-throughput genomic analysis their use is not cost-effective for routine clinical use. In addition, a high level of technical expertise is required especially for data analysis which can be complicated by "background" noise due to partial hybridization, etc. An interesting variation on microarray analysis, developed by Luminex (Luminex, Austin), involves the use of flow cytometry to detect hybridization of test DNA to fluorochrome-labeled beads conjugated with specific sequence probes [73]. However, the utility of this suspension-based approach for strain typing remains to be thoroughly evaluated.

Whole Genome Sequence Typing

As noted earlier, the goal of molecular strain typing is epidemiological assessment based on the most fundamental molecule of identity in the cell—the bacterial chromosome. Thus, the ability to compare whole genome sequences (WGS) represents the ultimate molecular typing approach. While this was impossible with older dideoxy/chain termination sequencing technology [74], newer (i.e., next-generation sequencing, NGS) methods have made this goal a reality. The technology behind NGS is discussed in Chap. 37 of this book and is not considered further here. However, from a strain typing standpoint it is important to note that revolutionary developments in NGS have made (WGS) possible with benchtop instrumentation such as the Ion Torrent PGM (Life Technologies, Guilford) and the MiSeq (Illumina, San Diego). Such instrumentation now allows WGS to be completed in only a few hours with extensive multifold coverage allowing isolates to be compared down to the level of SNPs. However, for NGS, as for previous sequencing iterations, the critical issues are throughput, quality, read length and cost. All of these are currently in a state of flux as commercial technology improves and positions itself in the scientific marketplace. However, an example of these concerns is seen in the application of WGS to the analysis of a recent *E. coli* outbreak in Europe which claimed more than 50 lives. One report, based on sequencing with the Ion Torrent PGM, concluded that the outbreak strain and an older 2001 isolate arose from a common ancestor with the current outbreak resulting from gene loss [75]. However, another study, using single-molecule real-time (third-generation) DNA sequencing (Pacific Biosciences, Menlo Park), proposed that the outbreak strain evolved by acquiring the gene for Shiga toxin [76]. These conflicting reports underscore what will clearly become the greatest need as

WGS-based strain typing rapidly develops—bioinformatic data interpretation and analysis. Nevertheless, these are exciting "problems" to have and the scientific stage is clearly set for additional remarkable developments in this most fundamental approach to determine isolate epidemiological interrelationships.

Non-Sequence-Based Whole Cell Typing

While strain typing is firmly directed toward sequence-based analysis, two whole-cell methods deserve mention: Raman spectroscopy and MALDI-TOF mass spectrometry. Both technologies are not new but are finding renewed emphasis in applications for application of strain typing.

The SpectraCell RA Bacterial Strain Analyzer (River Diagnostics, Rotterdam) employs Raman spectroscopy for isolate characterization. Sir C.V. Raman received the Nobel Prize in Physics in 1930 for his discovery of this light-scattering technology. Since every molecule in the cell contributes to the generated spectrum of scattered laser light, in principle different bacterial strains would be expected to generate different Raman spectra while highly related isolates would not. Thus, the SpectraCell system seeks to accomplish strain typing based on the quantitation of these spectral measurements. Early reports suggest that the method has promise in the typing of problem pathogens such as *P. aeruginosa* and *S. aureus* [77, 78]. However, uniformity of pre-analysis bacterial growth conditions as well as method reproducibility and discriminatory power are key issues for the future of this approach to typing.

MALDI-TOF mass spectrometry is considered in detail in Chap. 10 of this book. The method has generated intense interest as a means of rapid microbial identification via the detection of unique cellular protein biomarkers. As a related issue, MALDI-TOF is also being investigated as an approach to bacterial strain typing [79–82]. However, as with Raman spectroscopy, experimental parameters (e.g., loading of the target plate, matrix composition) must be carefully controlled with optimized post-processing and analysis of the mass spectra. Nevertheless, as an adjunct to microbial identification, strain typing is a logical goal for MALDI-TOF technology which will most certainly see additional refinement and application in the future.

Strain Typing in the Context of the Epidemiological Window

In the final analysis, regardless of the quantity or quality of strain typing data, the issue ultimately comes down to data interpretation. In this context, it is important to note that while the term "molecular" epidemiology implies a precise process, this is not always the case regardless of the method employed since such investigations have an unavoidable context-driven component. A variety of environmental factors as well as interaction between the host and infectious agent may all influence the

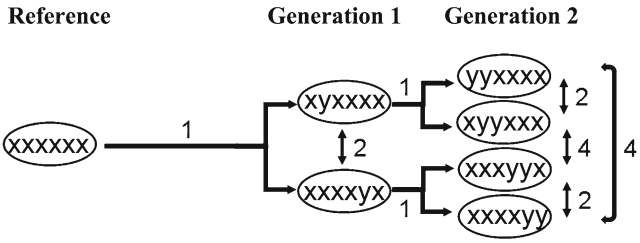

Fig. 13.9 Depiction of the interrelationships between a reference genome containing six characteristics (each designated "x") with two subsequent generations each experiencing sequential single genetic events (x→y). Within and between each generation, the number of resulting genetic differences is indicated

course of disease transmission. In addition, infectious disease issues benefiting from epidemiological evaluation are not typically given advance warning. Thus, identification of the index patient in a particular outbreak is a common problem in epidemiological analysis. Nevertheless, the analysis must be conducted in the context of the available isolate data (i.e., the epidemiological window [5, 83]) which, unfortunately, does not always include the outbreak source. Thus, the analytical process is commonly one of attempting to work backward in time which, depending on the available data, may necessitate conclusions based on probabilities rather than hard data. For this reason, regardless of the sophistication of the typing approach, epidemiological analysis commonly contains an element of educated guess. Nevertheless, for most clinical scenarios (i.e., outbreak investigation) the key issue is whether or not a series of bacterial isolates are the result of person-to-person transfer. At the heart of this question is the concept of significant difference which for chromosomal comparison relates to epidemiologically relevant genomic clock speed. In the absence of an index case or isolate, all strain typing methods are challenged as the opportunity for chromosomal change over time increases the potential for genetic distance between epidemiologically related isolates. As illustrated in Fig. 13.9, if one considers a simple reference genome of six characteristics ("x") evolving through two generations with sequential genetic events of unknown complexity (x→y), the resulting second-generation genomes would vary from each other by four differences. The potential complexity of the scenario obviously increases further over time. These issues underscore the potential difficulties one may encounter in attempting to discern lineages of infectious agent transmission, regardless of the typing method employed. Thus, for an optimum outcome (e.g., in an outbreak setting) analysis of strain typing data and its epidemiological relevance requires knowledge of (1) the limitations of the typing method, (2) the etiological agent (e.g., genomic clock speed), and (3) the clinical setting within which the issue is being studied.

For the future, it is exciting to consider the advances in strain typing that will continue to be made. The persistence and spread of problem pathogens in patient populations will obviously continue to occur. Thus, perhaps the most important point of all is to emphasize that, more than ever before, strain typing and epidemiological

analysis benefit from communication. It is when all interested parties participate (e.g., physician, nurse, infection control specialist, laboratory) that the epidemiological educated guess is most likely to be correct, and that most certainly is a key goal for the treatment of infectious disease both now and in the future.

References

1. Tenover FC, Arbeit RD, Goering RV (1997) How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Infect Control Hosp Epidemiol 18(6):426–439
2. Riley LW (2004) Molecular epidemiology of infectious disease: principles and practices. ASM, Washington, DC
3. Chen Y, Brown E, Knabel SJ (2011) Molecular Epidemiology of Foodborne Pathogens. In: Wiedmann M, Zhang W (eds) Genomics of foodborne bacterial pathogens. Springer, New York, pp 403–453
4. Van Belkum A, Tassios PT, Dijkshoorn L et al (2007) Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect 13(Suppl 3):1–46
5. Goering RV (2000) The molecular epidemiology of nosocomial infection: past, present, and future. Rev Med Microbiol 11:145–152
6. Goering RV (2000) Molecular strain typing for the clinical laboratory: current application and future direction. Clin Microbiol News 22:169–173
7. Halpern D, Chiapello H, Schbath S et al (2007) Identification of DNA motifs implicated in maintenance of bacterial core genomes by predictive modeling. PLoS Genet 3(9):1614–1621
8. Lindsay JA, Moore CE, Day NP et al (2006) Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. J Bacteriol 188(2):669–676
9. Lindsay JA (2010) Genomic variation and evolution of *Staphylococcus aureus*. Int J Med Microbiol 300(2–3):98–103
10. Goering RV (2002) The influence of genomics on the molecular epidemiology of nosocomial pathogens. In: Shaw KJ (ed) Pathogen genomics: impact on human health. Humana, Totowa, pp 113–131
11. Bialkowska-Hobrzanska H, Jaskot D, Hammerberg O (1990) Evaluation of restriction endonuclease fingerprinting of chromosomal DNA and plasmid profile analysis for characterization of multiresistant coagulase-negative staphylococci in bacteremic neonates. J Clin Microbiol 28:269–275
12. Tenover FC, Akerlund T, Gerding DN et al (2011) Comparison of strain typing results for *Clostridium difficile* isolates from North America. J Clin Microbiol 49(5):1831–1837
13. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98(3):503–517
14. Van Belkum A (2003) High-throughput epidemiologic typing in clinical microbiology. Clin Microbiol Infect 9:86–100
15. Thorne N, Borrell S, Evans J et al (2011) IS6110-based global phylogeny of *Mycobacterium tuberculosis*. Infect Genet Evol 11(1):132–138
16. Schwartz DC, Saffran W, Welsh J, Haas R, Goldenberg M, Cantor CR (1983) New techniques for purifying large DNA's and studying their properties and packaging. Cold Spring Harbor Symp Quant Biol 47:189–195
17. Schwartz DC, Koval M (1989) Conformational dynamics of individual DNA molecules during gel electrophoresis. Nature 338:520–522

18. Goering RV, Ribot EM, Gerner-Smidt P (2011) Pulsed-field gel electrophoresis: laboratory and epidemiologic considerations for interpretation of data. In: Persing DH, Tenover FC, Tang YW, Nolte FS, Hayden RT et al (eds) Molecular microbiology, 2nd edn. ASM, Washington, DC, pp 167–177
19. Goering RV (2010) Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. Infect Genet Evol 10(7):866–875
20. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC (2003) Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol 41(11):5113–5120
21. Goering RV, McDougal LK, Fosheim GE, Bonnstetter KK, Wolter DJ, Tenover FC (2007) Epidemiologic distribution of the arginine catabolic mobile element among selected methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates. J Clin Microbiol 45(6):1981–1984
22. Schwan WR, Briska A, Stahl B et al (2010) Use of optical mapping to sort uropathogenic *Escherichia coli* strains into distinct subgroups. Microbiology 156(Pt 7):2124–2135
23. Petersen RF, Litrup E, Larsson JT et al (2011) Molecular characterization of *Salmonella typhimurium* highly successful outbreak strains. Foodborne Pathog Dis 8(6):655–661
24. Goering RV, Stemper ME, Shukla SK, Foley SL (2011) Restriction analysis techniques. In: Foley SL, Chen AY, Simjee S, Zervos MJ (eds) Molecular techniques for the study of hospital acquired infection. Wiley-Blackwell, Hoboken, N.J., pp 135–144
25. Melles DC, Schouls L, Francois P et al (2009) High-throughput typing of *Staphylococcus aureus* by amplified fragment length polymorphism (AFLP) or multi-locus variable number of tandem repeat analysis (MLVA) reveals consistent strain relatedness. Eur J Clin Microbiol Infect Dis 28(1):39–45
26. Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in Eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19: 6823–6831
27. Van Belkum A, Sluijter M, De Groot R, Verbrugh H, Hermans PWM (1996) Novel BOX repeat PCR assay for high-resolution typing of *Streptococcus pneumoniae* strains. J Clin Microbiol 34:1176–1179
28. Deplano A, Vaneechoutte M, Verschraegen G, Struelens MJ (1997) Typing of *Staphylococcus aureus* and *Staphylococcus epidermidis* strains by PCR analysis of Inter-IS256 spacer length polymorphisms. J Clin Microbiol 35:2580–2587
29. Frye SR, Healy M (2011) Repetitive sequence-based PCR typing of bacteria and fungi. In: Persing DH, Tenover FC, Tang YW, Nolte FS, Hayden RT, Van Belkum A (eds) Molecular microbiology: diagnostic principles and practice. ASM, Washington, DC, pp 199–212
30. Ross TL, Merz WG, Farkosh M, Carroll KC (2005) Comparison of an automated repetitive sequence-based PCR microbial typing system to pulsed-field gel electrophoresis for analysis of outbreaks of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 43(11): 5642–5647
31. Roussel S, Felix B, Colaneri C et al (2010) Semi-automated repetitive-sequence-based polymerase chain reaction compared to pulsed-field gel electrophoresis for *Listeria monocytogenes* subtyping. Foodborne Pathog Dis 7(9):1005–1012
32. Bouchet V, Huot H, Goldstein R (2008) Molecular genetic basis of ribotyping. Clin Microbiol Rev 21(2):262–273
33. Tenover FC, Novak-Weekley S, Woods CW et al (2010) Impact of strain types on detection of toxigenic *Clostridium difficile*: comparison of molecular diagnostic and enzyme immunoassay approaches. J Clin Microbiol 48(10):3719–3724
34. Valiente E, Dawson LF, Cairns MD, Stabler RA, Wren BW (2011) Emergence of new PCR-ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. J Med Microbiol 61 (Pt 1):49–56
35. Solomon K, Fanning S, McDermott S et al (2011) PCR ribotype prevalence and molecular basis of macrolide-lincosamide-streptogramin B (MLSB) and fluoroquinolone resistance in Irish clinical *Clostridium difficile* isolates. J Antimicrob Chemother 66(9):1976–1982

36. Gerding DN (2010) Global epidemiology of *Clostridium difficile* infection in 2010. Infect Control Hosp Epidemiol 31(Suppl 1):S32–S34
37. Katayama Y, Ito T, Hiramatsu K (2000) A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 44:1549–1555
38. IWG-SCC (2009) Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother 53(12): 4961–4967
39. Kondo Y, Ito T, Ma XX et al (2007) Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. Antimicrob Agents Chemother 51(1):264–274
40. Milheirico C, Oliveira DC, De Lencastre H (2007) Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome mec type IV in methicillin-resistant *Staphylococcus aureus*: 'SCCmec IV multiplex'. J Antimicrob Chemother 60(1):42–48
41. Oliveira DC, De Lencastre H (2002) Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 46(7):2155–2161
42. Deurenberg RH, Stobberingh EE (2008) The evolution of *Staphylococcus aureus*. Infect Genet Evol 8:747–763
43. Van Belkum A, Scherer S, van Alphen L, Verbrugh H (1998) Short-sequence DNA repeats in prokaryotic genomes. Microbiol Mol Biol Rev 62:275–293
44. Lindstedt BA (2005) Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. Electrophoresis 26(13):2567–2582
45. Hammerschmidt S, Muller A, Sillmann H et al (1996) Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol Microbiol 20(6):1211–1220
46. Pourcel C, Vergnaud G (2011) Strain typing using multiple "variable number of tandem repeat" analysis and genetic element CRISPR. In: Persing DH, Tenover FC, Tang YW, Nolte FS, Hayden RT, Van Belkum A (eds) Molecular microbiology: diagnostic principles and practice, 2nd edn. ASM, Washington, DC, pp 179–197
47. Bannerman TL, Hancock GA, Tenover FC, Miller JM (1995) Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. J Clin Microbiol 33:551–555
48. Swaminathan B, Barrett TJ, Hunter SB, Tauxe RV (2001) PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg Infect Dis 7:382–389
49. Harmsen D, Claus H, Witte W et al (2003) Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J Clin Microbiol 41:5442–5448
50. Church DL, Chow BL, Lloyd T, Gregson DB (2011) Comparison of automated repetitive-sequence-based polymerase chain reaction and spa typing versus pulsed-field gel electrophoresis for molecular typing of methicillin-resistant *Staphylococcus aureus*. Diagn Microbiol Infect Dis 69:30–37
51. Frenay HME, Bunschoten AE, Schouls LM et al (1996) Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. Eur J Clin Microbiol Infect Dis 15:60–64
52. Shopsin B, Gomez M, Montgomery SO et al (1999) Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J Clin Microbiol 37: 3556–3563
53. Bessen DE (2009) Population biology of the human restricted pathogen, *Streptococcus pyogenes*. Infect Genet Evol 9:581–593
54. Steer AC, Law I, Matatolu L, Beall BW, Carapetis JR (2009) Global *emm* type distribution of group A streptococci: systematic review and implications for vaccine development. Lancet Infect Dis 9:611–616

55. Wajima T, Murayama SY, Sunaoshi K, Nakayama E, Sunakawa K, Ubukata K (2008) Distribution of *emm* type and antibiotic susceptibility of group A streptococci causing invasive and noninvasive disease. J Med Microbiol 57:1383–1388
56. Tanaka D, Gyobu Y, Kodama H et al (2002) *emm* typing of group A streptococcus clinical isolates: identification of dominant types for throat and skin isolates. Microbiol Immunol 46(7):419–423
57. Ryffel C, Bucher R, Kayser FH, Berger-Bächi B (1991) The *Staphylococcus aureus mec* determinant comprises an unusual cluster of direct repeats and codes for a gene product similar to the *Escherichia coli sn*-glycerophosphoryl diester phosphodiesterase. J Bacteriol 173:7416–7422
58. Ionescu R, Mediavilla JR, Chen L et al (2010) Molecular characterization and antibiotic susceptibility of *Staphylococcus aureus* from a multidisciplinary hospital in Romania. Microb Drug Resist 16:263–272
59. Goering RV, Morrison D, Al-Doori Z, Edwards GF, Gemmell CG (2008) Usefulness of *mec*-associated direct repeat unit (*dru*) typing in the epidemiological analysis of highly clonal methicillin-resistant *Staphylococcus aureus* in Scotland. Clin Microbiol Infect 14:964–969
60. Fessler A, Scott C, Kadlec K, Ehricht R, Monecke S, Schwarz S (2010) Characterization of methicillin-resistant *Staphylococcus aureus* ST398 from cases of bovine mastitis. J Antimicrob Chemother 65:619–625
61. Shore AC, Rossney AS, Kinnevey PM et al (2010) Enhanced discrimination of highly clonal ST22-methicillin-resistant *Staphylococcus aureus* IV isolates achieved by combining *spa*, *dru*, and pulsed-field gel electrophoresis typing data. J Clin Microbiol 48(5):1839–1852
62. Smyth DS, Wong A, Robinson DA (2011) Cross-species spread of SCC*mec* IV subtypes in staphylococci. Infect Genet Evol 11:446–453
63. Smyth DS, McDougal LK, Gran FW et al (2010) Population structure of a hybrid clonal group of methicillin-resistant *Staphylococcus aureus*, ST239-MRSA-III. PLoS One 5:e8582
64. Maiden MCJ, Bygraves JA, Feil E et al (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A 95:3140–5145
65. Feil EJ, Spratt BG (2001) Recombination and the population structures of bacterial pathogens. Annu Rev Microbiol 55:561–590
66. Aanensen DM, Spratt BG (2005) The multilocus sequence typing network: mlst.net. Nucleic Acids Res 33(Web Server issue):W728–W733
67. Yan Y, Cui Y, Han H et al (2011) Extended MLST-based population genetics and phylogeny of *Vibrio parahaemolyticus* with high levels of recombination. Int J Food Microbiol 145: 106–112
68. Ch'ng SL, Octavia S, Xia Q et al (2011) Population structure and evolution of pathogenicity of *Yersinia pseudotuberculosis*. Appl Environ Microbiol 77:768–775
69. Litrup E, Torpdahl M, Malorny B, Huehn S, Christensen H, Nielsen EM (2010) Association between phylogeny, virulence potential and serovars of *Salmonella enterica*. Infect Genet Evol 10:1132–1139
70. Miller MB, Tang YW (2009) Basic concepts of microarrays and potential applications in clinical microbiology. Clin Microbiol Rev 22:611–633
71. Musser JM, Shelburne SA III (2009) A decade of molecular pathogenomic analysis of group A Streptococcus. J Clin Invest 119:2455–2463
72. Li W, Raoult D, Fournier PE (2009) Bacterial strain typing in the genomic era. FEMS Microbiol Rev 33:892–916
73. Dunbar SA (2006) Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. Clin Chim Acta 363:71–82
74. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74:5463–5467
75. Mellmann A, Harmsen D, Cummings CA et al (2011) Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. PLoS One 6:e22751

76. Rasko DA, Webster DR, Sahl JW et al (2011) Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany. N Engl J Med 365:709–717
77. Willemse-Erix DF, Jachtenberg JW, Schut TB et al (2010) Towards Raman-based epidemiological typing of *Pseudomonas aeruginosa*. J Biophotonics 3:506–511
78. Wulf MW, Willemse-Erix D, Verduin CM, Puppels G, van Belkum A, Maquelin K (2012) The use of Raman spectroscopy in the epidemiology of methicillin-resistant *Staphylococcus aureus* of human- and animal-related clonal lineages. Clin Microbiol Infect 18(2):147–152
79. Moura H, Woolfitt AR, Carvalho MG et al (2008) MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive *Streptococcus pyogenes* isolates. FEMS Immunol Med Microbiol 53:333–342
80. Wolters M, Rohde H, Maier T et al (2011) MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. Int J Med Microbiol 301:64–68
81. Williamson YM, Moura H, Woolfitt AR et al (2008) Differentiation of *Streptococcus pneumoniae* conjunctivitis outbreak isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74:5891–5897
82. Dieckmann R, Malorny B (2011) Rapid screening of epidemiologically important Salmonella enterica subsp. enterica serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 77(12):4136–4146
83. Goering RV (1998) The molecular epidemiology of nosocomial infection: an overview of principles, application, and interpretation. In: Specter S, Bendinelli M, Friedman H (eds) Rapid detection of infectious agents, 1st edn. Plenum, New York, pp 131–157
84. Rademaker JL, Savelkoul P (2004) PCR amplification-based microbial typing. In: Persing DH, Tenover FC, Versalovic J, Tang YW, Unger ER, Relman DA et al (eds) Molecular microbiology: diagnostic principles and practice. ASM, Washington, DC, pp 197–221

Chapter 14
An Introduction to In Vitro Nucleic Acid Amplification Techniques

Haijing Li and Yi-Wei Tang

A molecular microbiology detection procedure consists of three basic components: (1) nucleic acid extraction, (2) amplification, and (3) detection/identification. Over the past two decades, the development and optimization of a series of in vitro nucleic acid amplification (NAA) technologies has opened new avenues in diagnostic microbiology for the detection, identification, and characterization of pathogenic organisms [1–3]. These techniques promise to replace traditional culture-based biological replication of live microbial pathogens by enzymatic amplification of specific nucleic acid sequences. These techniques have reduced the dependency of the clinical microbiology laboratory on cultured-based methods and created new opportunities for the field of microbiology to enhance patient care.

Based on theoretical aspects for each method, NAA techniques can be placed into three broad categories: (1) target amplification systems, (2) probe amplification systems, and (3) signal amplification systems. All of these categories share certain advantages over traditional methods, particularly for the detection of fastidious, unculturable, and/or highly contagious organisms (Table 14.1). Experience from our and other groups has demonstrated that application of NAA techniques enhances the speed, sensitivity, and sometimes the specificity of an etiologic diagnosis [4–10]. Each of these categories is discussed in detail.

H. Li
Department of Pathology, Vanderbilt University School of Medicine, Nashville, TN, USA

Y.-W. Tang (✉)
Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center,
New York, NY 37212, USA
e-mail: tangy@mskcc.org

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Table 14.1 In vitro, enzyme-mediated nucleic acid amplification methods

Amplification method	Amplification category	Manufacturer/license (trade mark)[a]	Enzymes used	Temperature requirement	Main references
Polymerase chain reaction	Target	Roche Molecular System, Inc., Branchburg, NJ (Cobas)	*Taq* DNA polymerase	Thermal cycler	[12, 13]
Transcription-mediated amplification	Target	Gen-Probe, Inc., San Diego, CA (APTIMA)	Reverse transcriptase, RNA polymerase, RNase H	Isothermal	[23, 25]
Nucleic acid sequence-based amplification	Target	bioMerieux, Durham, NC (Nuclisens)	Reverse transcriptase, RNA polymerase, RNase H	Isothermal	[22, 63]
Strand displacement amplification	Target	Becton-Dickinson, Sparks, MD (ProbTec)	Restrictive endonuclease, DNA polymerase	Isothermal	[31, 33]
Loop-mediated isothermal amplification	Target	Meridian Bioscience, Inc., Cincinnati, OH (Illummigene)	DNA polymerase	Isothermal	[39, 40]
Helicase-dependent amplification	Target	BioHelix Corp., Beverly, MA (IsoAmp)	Helicase, DNA polymerase	Isothermal	[35, 37]
Cycling probe technology	Probe	ID Biomedical Corp., Vancouver, Canada	Rnase H	Isothermal	[48, 50]
Ligase chain reaction	Probe	Abbott Molecular, Abbott Park, IL (LCx)	DNA ligase	Thermal cycler	[42, 44]
Invader technology	Signal	Hologic, Madison, WI (Cervista)	Cleavase	Isothermal	[56, 57]
Hybrid capture system	Signal	Qiagen, Inc., Silver Spring, MD (HC2)	None	Isothermal	[53, 54]
Branched DNA	Signal	Siemens, Emeryville, CA (Versant)	None	Isothermal	[61, 62]

[a]PCR-based devices are also commercially available from Abbott Molecular, Becton Dickinson, Cepheid, Prodesse/Gen-Probe, and Eragen/Luminex

Target Amplification Systems

Target amplification systems are defined as NAA procedures in which many copies (i.e., amplicons) of the nucleic acid targets are made. Target amplification systems include polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), transcription-medicated amplification (TMA), loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA), and helicase-dependent amplification (HDA). Among them, PCR and PCR-derived techniques are the best-developed and most widely used methods of NAA [11–13]. Real-time PCR-based platforms have been widely incorporated into numerous commercial instruments manufactured by Roche, Abbott Molecular, Becton Dickinson, Cepheid, Prodesse/Gen-Probe and Eragen/Luminex (Table 14.1). Repetitive sequence-based PCR techniques, exampled by the bioMeriuex Bacterial Barcode system, have been used in the clinical setting for rapid microbial strain typing and subtyping [14, 15]. Broad-ranged PCR-based microbial ribosomal RNA gene amplification has been recognized as a rapid and accurate tool for microbial pathogen identification [16, 17]. Several novel multiplex PCR techniques including dual priming oligonucleotide [18] and target-enriched multiplexing systems [19] have been developed and commercialized for detection and characterization of a panel of microbial pathogens [18–21].

Transcription-based, RNA-targeting amplification techniques are another example of NAA procedures within the target amplification category. Transcription-mediated amplification (TMA) and NASBA involve several enzymes and a complex series of reactions which all take place simultaneously at the same temperature and in the same buffer [22, 23]. The advantages include very rapid kinetics and no requirement for a thermocycler due to the reactions taking place under isothermal conditions. Moreover, isothermal conditions in a single tube with a rapidly degradable product (RNA) help minimize (but may not eliminate) contamination risks. Amplification of RNA not only makes it possible to detect RNA viruses, but also increases the sensitivity of detecting bacterial and fungal pathogens by targeting high copy number RNA templates. Several TMA-based system manufactured by GenProbe Inc. has been used to detect *Mycobacterium tuberculosis* in smear-positive sputum specimens, to confirm *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection as well as to screen human immunodeficiency virus (HIV)-1 RNA in donor blood specimens [24–26]. NASBA system-based products are commercially available from bioMérieux, which have been used for the detection of enteroviruses in cerebrospinal fluid and influenza virus and respiratory syncytial virus in respiratory specimens [27, 28] as well as for the quantification of hepatitis C virus (HCV) levels in serum [29, 30].

Several isothermal amplification-based target amplification techniques have been well developed in the diagnostic microbiology field. SDA uses specific primers, a DNA polymerase, and restriction endonuclease to achieve exponential amplification of the target [31]. The key technology behind SDA is the generation of site-specific nicks by the restriction endonuclease. Commercial devices have

been available from Becton Dickinson for diagnosis and monitoring of *C. trachomatis*, *N. gonorrhea*, and *M. tuberculosis* infections [32–34]. HDA is a unique isothermal DNA amplification technique in which a DNA helicase enzyme is employed to unwind double-stranded DNA [35]. Commercial devices, which incorporate HDA amplification with a disposable device for amplification product detection, are commercially available in BioHelix for rapid detection of *Staphylococcus aureus*, *Clostridium difficile* and herpes simplex viruses [36–38]. As another simple, rapid, and specific NAA method, LAMP has been used as one major user-developed system for the detection and identification of microbial pathogens in Asia [39]. Commercial devices incorporating LAMP technology have not been available in the United States until recently when a Meridian device (Illumigene) became commercially available for detection of *C. difficile* in stool specimens [40].

Probe Amplification Systems

In probe amplification systems, many copies of the probe that hybridizes the target nucleic acid are made [41]. The ligase chain reaction (LCR) and cycling probe technology (CPT) have been fully developed and have been applied in diagnostic microbiology [42]. A gapped LCR procedure, which is similar in design to a target amplification method, such as PCR, can be sensitive and useful for the detection of point mutations [43]. Although convenient and readily automated, one potential drawback of LCR is the difficult inactivation of post-amplification products. The nature of the technique does not allow for the most widely used contamination control methods to be applied. A combination LCR kit for the detection of both *C. trachomatis* and *N. gonorrhea* was at one time widely used in the routine clinical service [44]. The inclusion of a real-time identification system within the same reaction tube (closed reaction systems) would significantly decrease the possibility of contamination which is associated with the opening of reaction tubes. The LCR products are no longer commercially available.

Multiplex ligation-dependent probe amplification (MLPA) incorporates both ligation and PCR to generate conditions compatible with multiplex detection and analysis [45]. Each MLPA assay is divided into three basic steps: (1) annealing of probes to their target sequences, (2) ligation of the probes, and (3) PCR amplification of ligated probes using universal primers. An MLPA technology–based RespiFinder assay is commercially available in Europe and is able to detect 15 respiratory viruses simultaneously in one reaction [46]. The RespiFinder assay has demonstrated satisfactory specificity and perfect sensitivity for AdV, hMPV, Flu-A, PIV 1–3, RhV, and RSV. Use of the RespiFinder assay has resulted in a 24.5% increase in the diagnostic yield compared with cell culture [46]. This assay is being extended to cover four additional bacterial pathogens that cause respiratory tract infections: *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Bordetella pertussis*. A similar, MLPA-based device (MeningoFinder) was reported for simultaneous detection of six virus species causing central nervous system infections [47].

Another similar system, cycling probe technology, utilizes a unique chimeric DNA–RNA–DNA probe sequence that provides an RNase H sensitive scissile link when hybridized to a complementary target DNA sequence [48]. The CPT reaction occurs at a constant temperature, which allows the probe to anneal to the target DNA. RNase H cuts the RNA portion of the probes, allowing the cleaved fragments to dissociate from the target DNA. A cycling probe designed for detection of a specific sequence with the *mecA* and *vanA/B* genes, and the former one has been cleared for in vitro diagnostic use by the Food and Drug Administration as a culture confirmation assay for methicillin-resistant *S. aureus* [49–52].

Signal Amplification Systems

Signal amplification is an NAA procedure in which a signal or reporter molecule attached to the probe is detected, and the signal is amplified enormously. Signal amplification methods are designed to strengthen a signal by increasing the concentration of label attached to the target nucleic acid. Unlike procedures which increase the concentration of the probe or target, signal amplification increases the signal generated due to a fixed amount of probe hybridized to a fixed amount of specific target. The fact that signal amplification procedures do not involve a nucleic acid target or probe amplification is a theoretical advantage because of lower susceptibility to contamination problems inherent in enzyme-catalyzed NAA. Sensitivity, however, compared to target NAA techniques, may be a limiting factor.

Currently, three diagnostic companies have their signal amplification products available for diagnostic microbiology purposes. The Qiagen/Digene hybrid capture system is widely used to determine human papillomavirus (HPV) infection and viral types in cervical swabs or fresh cervical biopsy specimens as well as other diagnostic targets [53]. Persistent high-risk HPV infection detected by the Qiagen/Digene hybrid capture system represents a reliable tool to select populations at risk for the development of high-grade cervical lesions [53, 54]. Limitations of the hybrid capture system are the need of relatively high volume of clinical specimens as well as indeterminant results that are probably a result of nonspecific binding of reporter probes [55].

The homogenous invader technology relies on cleavase enzymes, which cleaves the 5′ end single-stranded flap of a branched base-pair duplex [56]. The characteristics of the technique make it a powerful tool for genetic analysis of single nucleotide polymorphisms in both microorganisms and hosts which are associated with specific diseases. Detection is accomplished through a fluorescence resonance energy transfer mechanism [56]. In addition to its wide application in molecular genetics, the technology has been used in diagnostic microbiology to detect, identify, and genotype several microbial pathogens [57, 58]. Among them, the Cervista HPV device is commercially available from Hologic for HPV detection and partial genotyping [59, 60].

Another signal amplification-based product is the branched DNA (bDNA) probe, which uses multiple specific synthetic oligonucleotides that hybridize to the target and thus capture the target onto a solid surface [61]. Synthetic bDNA amplifier molecules, which are enzyme conjugated, branched oligonucleotide probes, subsequently are added. Hybridization proceeds between the amplifier and the immobilized hybrids. After addition of a chemiluminescent substrate, light emission is measured and may be quantified. This technique represents an excellent method for quantification and therapeutic response monitoring of HCV and HIV-1 [62, 63]. The new Siemens VERSANT HIV-1 assay also provides good reproducibility since no amplification variation is expected [64, 65].

Currently, commonly used in vitro NAA techniques are categorized and summarized in Table 14.1. Each of the three categories is discussed in the following several chapters; this discussion is followed by a closer look at individual techniques and includes the principles as well as applications in the diagnostic microbiology.

References

1. Tang YW, Persing DH (2009) Diagnostic microbiology. In: Schaechter M (ed) Encyclopedia of microbiology, 3rd edn. Elsevier, Oxford, pp 308–320
2. Read RC, Cornaglia G, Kahlmeter G (2011) Professional challenges and opportunities in clinical microbiology and infectious diseases in Europe. Lancet Infect Dis 11(5):408–415
3. Nolte FS, Caliendo AM (2011) Molecular microbiology. In: Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW (eds) Manual of clinical microbiology, 10th edn. American Society for Microbiology, Washington, DC, pp 27–59
4. Tang YW, Procop GW, Persing DH (1997) Molecular diagnostics of infectious diseases. Clin Chem 43(11):2021–2038
5. Tang YW, Mitchell PS, Espy MJ, Smith TF, Persing DH (1999) Molecular diagnosis of herpes simplex virus infections in the central nervous system. J Clin Microbiol 37(7):2127–2136
6. Yolken RH (2002) Nucleic acid amplification assays for microbial diagnosis: challenges and opportunities. J Pediatr 140(3):290–292
7. Smalling TW, Sefers SE, Li H, Tang YW (2002) Molecular approaches to detecting herpes simplex virus and enteroviruses in the central nervous system. J Clin Microbiol 40(7):2317–2322
8. Miller MB, Tang YW (2009) Basic concepts of microarrays and potential applications in clinical microbiology. Clin Microbiol Rev 22(4):611–633
9. Wu W, Tang YW (2009) Emerging molecular assays for detection and characterization of respiratory viruses. Clin Lab Med 29(4):673–693
10. Tang YW, Stratton CW (2010) *Staphylococcus aureus*: an old pathogen with new weapons. Clin Lab Med 30(1):179–208
11. Eisenstein BI (1990) The polymerase chain reaction. A new method of using molecular genetics for medical diagnosis. N Engl J Med 322(3):178–183
12. Mullis KB (1990) The unusual origin of the polymerase chain reaction. Sci Am 262(4):56–61, 4–5
13. Ou CY, Kwok S, Mitchell SW et al (1988) DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 239(4837):295–297
14. Healy M, Huong J, Bittner T et al (2005) Microbial DNA typing by automated repetitive-sequence-based PCR. J Clin Microbiol 43(1):199–207

15. Tenover FC, Gay EA, Frye S, Eells SJ, Healy M, McGowan JE Jr (2009) Comparison of typing results obtained for methicillin-resistant *Staphylococcus aureus* isolates with the DiversiLab system and pulsed-field gel electrophoresis. J Clin Microbiol 47(8):2452–2457
16. Tang YW, Ellis NM, Hopkins MK, Smith DH, Dodge DE, Persing DH (1998) Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli. J Clin Microbiol 36(12):3674–3679
17. Hall L, Wohlfiel S, Roberts GD (2004) Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of filamentous fungi encountered in the clinical laboratory. J Clin Microbiol 42(2):622–626
18. Kim JK, Lee HJ, Lee YJ et al (2008) Direct detection of lamivudine-resistant hepatitis B virus mutants by a multiplex PCR using dual-priming oligonucleotide primers. J Virol Methods 149(1):76–84
19. Han J, Swan DC, Smith SJ et al (2006) Simultaneous amplification and identification of 25 human papillomavirus types with Templex technology. J Clin Microbiol 44(11):4157–4162
20. Bibby DF, McElarney I, Breuer J, Clark DA (2011) Comparative evaluation of the Seegene Seeplex RV15 and real-time PCR for respiratory virus detection. J Med Virol 83(8):1469–1475
21. Li H, McCormac MA, Estes RW et al (2007) Simultaneous detection and high-throughput identification of a panel of RNA viruses causing respiratory tract infections. J Clin Microbiol 45(7):2105–2109
22. Compton J (1991) Nucleic acid sequence-based amplification. Nature 350(6313):91–92
23. Kwoh DY, Davis GR, Whitfield KM, Chappelle HL, DiMichele LJ, Gingeras TR (1989) Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci U S A 86(4):1173–1177
24. Gaydos CA, Quinn TC, Willis D et al (2003) Performance of the APTIMA Combo 2 assay for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in female urine and endocervical swab specimens. J Clin Microbiol 41(1):304–309
25. La Rocco MT, Wanger A, Ocera H, Macias E (1994) Evaluation of a commercial rRNA amplification assay for direct detection of *Mycobacterium tuberculosis* in processed sputum. EurJ Clin Microbiol Infect Dis 13(9):726–731
26. Ren A, Louie B, Rauch L et al (2008) Screening and confirmation of human immunodeficiency virus type 1 infection solely by detection of RNA. J Med Microbiol 57(Pt 10):1228–1233
27. Moore C, Hibbitts S, Owen N et al (2004) Development and evaluation of a real-time nucleic acid sequence based amplification assay for rapid detection of influenza A. J Med Virol 74(4):619–628
28. Deiman B, Schrover C, Moore C, Westmoreland D, van de Wiel P (2007) Rapid and highly sensitive qualitative real-time assay for detection of respiratory syncytial virus A and B using NASBA and molecular beacon technology. J Virol Methods 146(1–2):29–35
29. Hollingsworth RC, Sillekens P, van Deursen P, Neal KR, Irving WL (1996) Serum HCV RNA levels assessed by quantitative NASBA: stability of viral load over time, and lack of correlation with liver disease. J Hepatol 25(3):301–306
30. Landry ML, Garner R, Ferguson D (2003) Comparison of the NucliSens basic kit (nucleic acid sequence-based amplification) and the Argene Biosoft enterovirus consensus reverse transcription-PCR assays for rapid detection of enterovirus RNA in clinical specimens. J Clin Microbiol 41(11):5006–5010
31. Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP (1992) Strand displacement amplification—an isothermal, in vitro DNA amplification technique. Nuc Acids Res 20(7):1691–1696
32. Little MC, Andrews J, Moore R et al (1999) Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. Clin Chem 45(6 Pt 1):777–784
33. Hellyer TJ, Fletcher TW, Bates JH et al (1996) Strand displacement amplification and the polymerase chain reaction for monitoring response to treatment in patients with pulmonary tuberculosis. J Infect Dis 173(4):934–941

34. Spears PA, Linn CP, Woodard DL, Walker GT (1997) Simultaneous strand displacement amplification and fluorescence polarization detection of *Chlamydia trachomatis* DNA. Anal Biochem 247(1):130–137
35. An L, Tang W, Ranalli TA, Kim HJ, Wytiaz J, Kong H (2005) Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification. J Biol Chem 280(32):28952–28958
36. Chow WH, McCloskey C, Tong Y et al (2008) Application of isothermal helicase-dependent amplification with a disposable detection device in a simple sensitive stool test for toxigenic *Clostridium difficile*. J Mol Diagn 10(5):452–458
37. Goldmeyer J, Li H, McCormac M et al (2008) Identification of *Staphylococcus aureus* and determination of methicillin resistance directly from positive blood cultures by isothermal amplification and a disposable detection device. J Clin Microbiol 46(4):1534–1536
38. Kim HJ, Tong Y, Tang W et al (2011) A rapid and simple isothermal nucleic acid amplification test for detection of herpes simplex virus types 1 and 2. J Clin Virol 50(1):26–30
39. Notomi T, Okayama H, Masubuchi H et al (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28(12):E63
40. Lalande V, Barrault L, Wadel S, Eckert C, Petit JC, Barbut F (2011) Evaluation of a loop-mediated isothermal amplification assay for diagnosis of *Clostridium difficile* infections. J Clin Microbiol 49(7):2714–2716
41. Birkenmeyer LG, Mushahwar IK (1991) DNA probe amplification methods. J Virol Methods 35(2):117–126
42. Wu DY, Wallace RB (1989) The ligation amplification reaction (LAR)—amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 4(4):560–569
43. Osiowy C (2002) Sensitive detection of HBsAg mutants by a gap ligase chain reaction assay. J Clin Microbiol 40(7):2566–2571
44. Carroll KC, Aldeen WE, Morrison M, Anderson R, Lee D, Mottice S (1998) Evaluation of the Abbott LCx ligase chain reaction assay for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urine and genital swab specimens from a sexually transmitted disease clinic population. J Clin Microbiol 36(6):1630–1633
45. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30(12):e57
46. Reijans M, Dingemans G, Klaassen CH et al (2008) RespiFinder: a new multiparameter test to differentially identify fifteen respiratory viruses. J Clin Microbiol 46(4):1232–1240
47. Wolffs PF, Vink C, Keijdener J et al (2009) Evaluation of MeningoFinder, a novel multiplex ligation-dependent probe amplification assay for simultaneous detection of six virus species causing central nervous system infections. J Clin Microbiol 47(8):2620–2622
48. Duck P, Alvarado-Urbina G, Burdick B, Collier B (1990) Probe amplifier system based on chimeric cycling oligonucleotides. Biotechniques 9(2):142–148
49. Beggs ML, Cave MD, Marlowe C, Cloney L, Duck P, Eisenach KD (1996) Characterization of *Mycobacterium tuberculosis* complex direct repeat sequence for use in cycling probe reaction. J Clin Microbiol 34(12):2985–2989
50. Cloney L, Marlowe C, Wong A, Chow R, Bryan R (1999) Rapid detection of mecA in methicillin resistant *Staphylococcus aureus* using cycling probe technology. Mol Cell Probes 13(3):191–197
51. Fong WK, Modrusan Z, McNevin JP, Marostenmaki J, Zin B, Bekkaoui F (2000) Rapid solid-phase immunoassay for detection of methicillin-resistant *Staphylococcus aureus* using cycling probe technology. J Clin Microbiol 38(7):2525–2529
52. Modrusan Z, Marlowe C, Wheeler D, Pirseyedi M, Bryan RN (2000) CPT-EIA assays for the detection of vancomycin resistant vanA and vanB genes in enterococci. Diagn Microbiol Infect Dis 37(1):45–50
53. Brown DR, Bryan JT, Cramer H, Fife KH (1993) Analysis of human papillomavirus types in exophytic condylomata acuminata by hybrid capture and Southern blot techniques. J Clin Microbiol 31(10):2667–2673

54. Schiffman MH, Kiviat NB, Burk RD et al (1995) Accuracy and interlaboratory reliability of human papillomavirus DNA testing by hybrid capture. J Clin Microbiol 33(3):545–550
55. Muldrew KL, Beqaj SH, Han J et al (2007) Evaluation of a Digene-recommended algorithm for human papillomavirus low-positive results present in a "retest zone". Am J Clin Pathol 127(1):97–102
56. Lyamichev V, Mast AL, Hall JG et al (1999) Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. Nature Biotechnol 17(3):292–296
57. Cooksey RC, Holloway BP, Oldenburg MC, Listenbee S, Miller CW (2000) Evaluation of the invader assay, a linear signal amplification method, for identification of mutations associated with resistance to rifampin and isoniazid in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 44(5):1296–1301
58. Sreevatsan S, Bookout JB, Ringpis FM et al (1998) Algorithmic approach to high-throughput molecular screening for alpha interferon-resistant genotypes in hepatitis C patients. J Clin Microbiol 36(7):1895–1901
59. Day SP, Hudson A, Mast A et al (2009) Analytical performance of the Investigational Use Only Cervista HPV HR test as determined by a multi-center study. J Clin Virol 45(Suppl 1):S63–S72
60. Belinson JL, Wu R, Belinson SE et al (2011) A population-based clinical trial comparing endocervical high-risk HPV testing using hybrid capture 2 and Cervista from the SHENCCAST II Study. Am J Clin Pathol 135(5):790–795
61. Urdea MS, Horn T, Fultz TJ et al (1991) Branched DNA amplification multimers for the sensitive, direct detection of human hepatitis viruses. Nucleic Acids Symp Ser 24:197–200
62. Lau JY, Davis GL, Kniffen J et al (1993) Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 341(8859):1501–1504
63. Revets H, Marissens D, de Wit S et al (1996) Comparative evaluation of NASBA HIV-1 RNA QT, AMPLICOR-HIV monitor, and QUANTIPLEX HIV RNA assay, three methods for quantification of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 34(5):1058–1064
64. Church D, Gregson D, Lloyd T et al (2011) Comparison of the RealTime HIV-1, COBAS TaqMan 48 v1.0, Easy Q v1.2, and Versant v3.0 assays for determination of HIV-1 viral loads in a cohort of Canadian patients with diverse HIV subtype infections. J Clin Microbiol 49(1):118–124
65. Holguin A, Lopez M, Molinero M, Soriano V (2008) Performance of three commercial viral load assays, Versant human immunodeficiency virus type 1 (HIV-1) RNA bDNA v3.0, Cobas AmpliPrep/Cobas TaqMan HIV-1, and NucliSens HIV-1 EasyQ v1.2, testing HIV-1 non-B subtypes and recombinant variants. J Clin Microbiol 46(9):2918–2923

Chapter 15
PCR and Its Variations

Michael Loeffelholz and Jianli Dong

PCR: The Quintessential Nucleic Acid Amplification Method

The polymerase chain reaction (PCR) is an in vitro technique used to replicate, or amplify, a specific region of DNA billions-fold in a few hours or less [1–3]. The amplification is primer directed; oligonucleotide primers anneal to and flank the DNA region to be amplified. PCR is utilized in diagnostic and research laboratories to generate sufficient quantities of DNA to be adequately tested, analyzed, or manipulated. Because of the exquisite sensitivity it offers, PCR has rapidly become a standard method in diagnostic microbiology. More recently, reagent kits and various instrument platforms have added speed, flexibility, and simplicity [4–10]. How significant is the contribution of PCR to the field of biomedicine? A PubMed search in 2005 for the first edition of this book using the key word "PCR" produced 214,352 hits [11]. A search conducted in June 2011 using the same key word produced 259,042 hits.

PCR was conceived in 1983 by Kary Mullis, an achievement that earned him the Nobel Prize in chemistry in 1993 [12]. The first practical application of PCR was described by Saiki and colleagues in 1985 [2], and less than 10 years later the US Food and Drug Administration (FDA) cleared the first PCR-based test for diagnosis of an infectious disease [9]. The 1990s saw the birth of a number of alternative nucleic acid amplification methods, including Qβ replicase, ligase chain reaction, strand-displacement amplification, transcription-mediated amplification, and others. Some of these methods are discussed elsewhere in this text. Research and diagnostic applications of PCR continued to be developed during the 1990s. In an incredibly short period of time, PCR revolutionized the field and became a staple on the clinical microbiologist's menu of tests. Indeed, molecular diagnostics is now a recognized

M. Loeffelholz (✉) • J. Dong
Department of Pathology, University of Texas Medical Branch,
301 University Blvd., Galveston, TX 77555-0740, USA
e-mail: mjloeffe@utmb.edu; jidong@utmb.edu

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subspecialty within clinical microbiology. The 2000s could be characterized as the decade of PCR instrument platforms, with the availability of numerous commercial instruments for real-time and multiplexed PCR that provided significant advances in speed and automation [13–16]. Lacking however, in 2011 is a broad menu of FDA-cleared or approved PCR assays for infectious diseases. Clinical microbiologists still rely heavily on laboratory-developed PCR assays for the detection of many important infectious agents.

Principles of PCR

Enzymatic Amplification of DNA: Components of the PCR

Two early innovations responsible for making PCR a practical research and diagnostic tool are thermal stable DNA polymerase and the thermal cycler. The thermal cycler is discussed later in this chapter. PCR was first performed using heat labile DNA polymerase. This necessitated manual replenishment of enzyme that was destroyed by the high temperatures of every cycle. Heat stable DNA polymerase was isolated from the bacterium *Thermus aquaticus,* which inhabits hot springs where temperatures exceed 90°C. This enzyme, called *Taq* DNA polymerase, remains active despite repeated heating during many cycles of amplification.

In addition to DNA polymerase, the essential components of the PCR include oligonucleotide primers, deoxynucleoside triphosphates (dNTPs), a divalent cation such as magnesium chloride, template or target DNA, and buffer (usually Tris). Primers are oligonucleotides, generally 20–25 bases long. They are designed to recognize specific sequences of the intended target, and define the amplified region. At temperatures appropriate for annealing, the two primers bind to opposite ends of this region, each to a complementary strand of target DNA. Primers must be designed carefully to avoid self-annealing or dimerization (Appendix). The length and sequence of the primer determine its melting temperature, and hence, annealing temperature. Once annealed to target DNA, primers create a binding site for DNA polymerase, which requires a double-stranded DNA template. This short double-stranded section primes the DNA replication or amplification process. As stated at the beginning of the chapter, PCR is a primer-directed amplification of DNA. *Taq* DNA polymerase is the enzyme responsible for synthesizing or extending the new DNA strand. Complementary base pairing creates a new strand, which is in essence the mirror image of the template strand. dNTPs are the building blocks for the new DNA strands, or amplicons. The dNTP mixture includes dATP, dCTP, dGTP, and dTTP, generally at equimolar concentrations. If the enzyme uracil *N*-glycosylase (UNG) is used in the PCR to prevent carry-over contamination, dUTP is added in place of or in combination with dTTP. Magnesium is the co-factor most commonly used in PCRs, and is required for *Taq* DNA polymerase activity. Magnesium concentration must be carefully optimized, as the window of optimal activity is rather narrow.

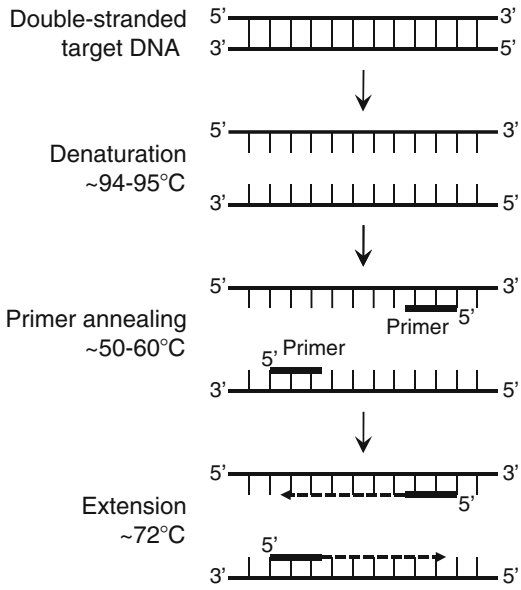

Fig. 15.1 PCR cycling steps

The PCR Cycle

Traditionally, PCR consists of three steps: denaturation, primer annealing, and extension (Fig. 15.1). One round of these three steps is referred to as a PCR cycle. These steps require different temperatures. This is accomplished using an automated thermal cycler, which can heat and cool tubes rapidly. While most PCR protocols use three different temperatures for each step, two-temperature PCR cycles, where primer annealing and extension occur at the same temperature, are frequently used for faster cycling. Generally, 30–40 rounds of temperature cycling are required to generate a sufficient amount of PCR product (amplicons).

Denaturation

At a temperature generally between 94 and 95°C, the two strands of the DNA target are separated, or denatured. At this temperature, all enzymatic reactions, such as the extension from a previous cycle, stop.

Annealing

Following denaturation, the temperature of the reaction is reduced to allow strands of DNA with complementary sequence to anneal. The annealing temperature varies, depending on the sequence, and hence, melting temperature of the oligonucleotide

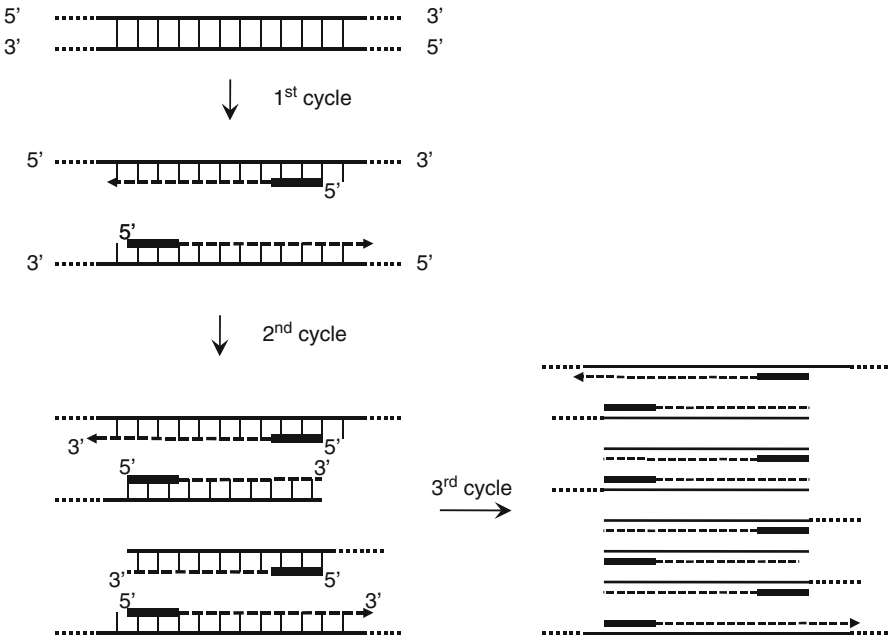

Fig. 15.2 Exponential amplification of DNA by PCR. Primers are represented by *bold lines*, newly replicated DNA strands are represented by long *dashes*, and template DNA strands by *solid lines*

primers, but is often between 50 and 60°C. At annealing temperature, the primers are in movement, caused by Brownian motion. Ionic bonds are constantly formed and broken between the single-stranded primer and DNA target. When primers come in contact with a perfectly complementary target sequence, the bond that forms is sufficiently stable to allow DNA polymerase to sit and initiate DNA synthesis at the 3′ end of each primer.

Extension

Extension of the primers generally occurs at 72°C, the temperature at which *Taq* DNA polymerase is most active. As bases are added to the 3′ end of the primer, and the double-stranded section lengthens, the resulting ionic bond is greater than the forces that break these attractions.

Each round of temperature cycling theoretically doubles the amount of DNA. After several rounds of temperature cycling, the amount of short double-stranded DNA product (flanked by sequence complementary to the primers) vastly exceeds the amount of the original target DNA. As a result, amplicons accumulate geometrically. After the first PCR cycle, a single starting piece of double-stranded DNA becomes two, after two cycles there are four copies, after three cycles, eight copies, and so on (Fig. 15.2). As stated, 30–40 rounds of PCR are generally required to produce

detectable amounts of amplicon. Due to the presence of inhibitory substances in the PCR and other factors, amplification efficiency probably never reaches 100%. While the analytical sensitivity of PCR is theoretically at the single copy level [4, 17], sampling variability and lower amplification efficiency generally prevent reliable detection of less than 10–20 target copies per PCR.

The entire procedure is carried out in a programmable thermal cycler—a computer-controlled cycling system with heating and cooling parameters. Many techniques for thermoregulation are used in the designs of thermal cyclers. These include the Peltier effect [18], heated and chilled air-streams [19, 20], and a continuous flow manner [21]. In this last design, heat from one side of a semiconductor is transferred to another, heating or cooling the overall temperature of the system. This design is much more effective than traditional designs of thermoregulation, which requires the use of refrigerants and compressors. Other approaches for thermoregulation include the use of continually circulating air-streams, water baths, or a combination of Peltier and convective technologies. When choosing a thermal cycler, functions that should be considered (depending on a particular laboratory's needs) include temperature accuracy and consistency across all PCR positions, maintenance costs, throughput, menu of FDA-cleared or approved assays, and the ability to add user-defined cycling protocols for laboratory-developed assays.

Detection and Analysis of the PCR Product

The PCR product should be a fragment or fragments of DNA of defined length. Before the PCR product is used in further applications, it should be analyzed. First, reactions should be examined to ensure product is actually formed. This seems intuitive, but when amplicon is detected with a probe, unexpectedly negative results could be due to either lack of amplification, or failure of the probe to hybridize and produce a detectable signal. Causes of unsuccessful PCR amplification include poor quality of target DNA, too little (or too much) target DNA, lack of sequence homology between primers and the intended target, and failure to optimize PCR conditions. PCR product must also be the expected length and sequence. Unexpected amplicon length or melting curve indicate that the target region itself is different than expected, that the target sequence is shared, or that amplification conditions are suboptimal and allow nonspecific annealing. PCR product should also be evaluated to ensure that the correct number of distinct products is produced. In most diagnostic applications, a single amplicon is generated by one primer pair. Additional, unintended product is usually produced as a result of suboptimal amplification conditions (poor primer design, *Taq* or $MgCl_2$ concentration too high, annealing temperature not optimized).

Conventional PCR

In conventional PCR, end-point product is analyzed after PCR is completed, by electrophoresis in agarose gels and visualization with ethidium bromide. DNA fragment

size is determined by comparison with known molecular weight markers. Agarose gel electrophoresis is also a PCR detection format, but is not recommended as a stand-alone method, as amplicon sequence cannot be confirmed.

Real-Time PCR

In real-time PCR, product is detected by the production of fluorescence, usually as cycling is occurring (hence, in real-time). Amplicons are detected by the intercalation of nonspecific fluorescent dyes, or by hybridization of sequence-specific probes that are labeled with a fluorescent reporter dye. Depending on the detection method used, amplicons can be further analyzed by subjecting PCR to a melting curve analysis, and determining the temperature at which the double-stranded product disassociates. PCR detection formats are discussed in detail in another chapter of this text.

PCR Variations

Allele-Specific PCR

Allele-specific PCR (AS-PCR) is used as a diagnostic technique to identify or utilize single nucleotide polymorphisms (SNPs), which in turn can distinguish closely related bacterial species [22]. The 3′-end of the primer is essential in the extension of the primer in a PCR. Selective amplification is usually achieved by designing a primer such that the primer will match/mismatch one of the target sequences at the 3′-end of the primer. Therefore, AS-PCR does require prior knowledge of the target sequences. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with a SNP-specific primer signals the presence of the specific SNP in a sequence. Sometimes, an additional mismatch is deliberately introduced close to the targeted SNP to further decrease the melting temperature of the double-stranded DNA product, and enhance sequence discrimination. Several detection methods can be used in AS-PCR. The most commonly used detection method involves fluorescence-labeled, dideoxynucleotide terminators that stop the chain extension. Alternatively, the primer can be labeled and extension products separated by electrophoresis. The single base extension product can also be broken down into smaller pieces and measured by mass spectrometry.

Hot-Start PCR

Hot-start PCR was first described in the literature in 1991 [23] and practical applications were demonstrated in 1992 [24]. Hot-start PCR techniques focus on the inhibition

of DNA polymerase activity during reaction set up. By limiting polymerase activity prior to the elevated temperatures of PCR, nonspecific amplification is reduced and target yield is increased. This is accomplished by physically separating or chemically inactivating one or more of the reaction components until high temperature triggers mixing or reactivation to give a complete reaction mixture.

In manual hot-start PCR, reactions lacking one essential component (usually DNA polymerase) are prepared and held at a temperature above the threshold of nonspecific binding of primer to template. Just prior to cycling, the missing component is added to allow the reaction to take place at the higher temperature. This procedure limits nonspecific annealing of the primers, and generally improved yield of the desired amplicon. This manual method is tedious and ungainly, as the tubes must be kept at 95–100°C. At this temperature, tubes are uncomfortable to handle. Opening the tubes to add the final reagent increases the chance of introducing contamination.

Hot-start PCR is also accomplished by creating a physical barrier between the essential components, such as primers and template, or enzyme and magnesium chloride. This barrier can be created by adding wax over an incomplete PCR mixture in a tube [25–27]. The wax can be preformulated for PCRs, or in bulk form, such as paraffin. The remaining PCR component(s) is placed on top of the wax layer. During the first denaturation step, the wax barrier melts and convection currents mix the essential PCR components.

Additional hot-start methods include chemically modified *Taq* DNA polymerase and an antibody-inhibited *Taq* DNA polymerase [28, 29]. The antibody is directed against the active site of the enzyme, preventing DNA replication until the high temperature of the denaturation step disassociates the antibody [30]. These modified enzyme preparations require a longer initial denaturation step than standard *Taq* DNA polymerase.

Similar to controlled temperature and physical separation of PCR components, cosolvents and enzymes have also been used to reduce or eliminate nonspecific annealing of primers [31]. Cosolvents such as dimethyl sulfoxide (DMSO) and formamide increase stringency by changing the melting temperature of the primer-template hybrid. Glycerol is believed to function similar to cosolvents. Cosolvents have various effects on the polymerase enzyme. Glycerol increases the temperature stability of *Taq* DNA polymerase, while formamide lowers it. The enzyme UNG is used in PCR as part of a system to degrade dUTP-containing product carried over from previous PCRs [32, 33]. Another benefit of UNG is that it degrades PCR product formed during the PCR setup process, prior to the high temperatures of cycling that provide specificity. In this role, UNG essentially provides an enzymatic hot-start PCR.

Touchdown PCR

Unlike a standard PCR program that utilizes a constant annealing temperature, touchdown PCR incorporates a range of annealing temperatures. The earliest cycles of touchdown PCR have high annealing temperatures. In subsequent cycles, the

annealing temperature is decreased by small increments (usually 1°C) every several cycles to a final "touchdown" annealing temp which is then used for the remaining ten or so cycles. This gradual decrease in annealing temperature selects for the most complementary primer/target binding in early cycles. This is most likely the sequence of interest. As the annealing temperature decreases, primers will anneal to nonspecific sequences; however, amplification of these products will lag behind that of the specific product. This favors synthesis of intended product over any nonspecific products [34]. Touchdown PCR was originally utilized to simplify the process of determining optimal PCR annealing temperatures.

Degenerate PCR

Degenerate PCR is a procedure that intentionally lowers analytical specificity, to allow divergent sequences to be detected in spite of sequence variation in the primer binding region. Rather than using a single primer pair with a specific sequence, degenerate primer sets may contain several primers that vary at one or more nucleotide positions, or a primer containing a nonspecific base, such as inosine, at a divergent position. There are circumstances in diagnostic microbiology when greater inclusivity is useful. For example, the genus *Norovirus* (family Caliciviridae) comprises dozens of distinct strains with relatively high genetic diversity [35]. A single, standard primer pair would lack sufficient complementarity to detect most strains, and have little diagnostic value. Degenerate reverse transcription (RT)-PCR has been used successfully to detect a broad range of noroviruses [35].

Nested and Heminested PCR

Nested and heminested PCR are designed to increase the sensitivity of PCR by directly reamplifying the product from a primary PCR with a second PCR. Nested PCR uses two sets of amplification primers and two separate stages of PCR [36, 37]. The second (nested) set of primers anneal to a sequence internal to the region flanked by the first set. In heminested PCR, the second stage of PCR utilizes one of the first-stage primers and one new, internal primer. The advantage of nested PCR is increased sensitivity and specificity of the reaction, since the internal primers anneal only if the amplicon has the corresponding, expected sequence. The second-stage PCR is usually free of inhibitory substances that can reduce the efficiency and sensitivity of the first-stage PCR. Disadvantages of nested PCR include additional time and cost associated with two stages of PCR, and the increased risk of contamination incurred during transfer of first-stage amplification products to a second tube. The physical separation of amplification mixtures with wax or oil [38], using an integrated PCR system in a cartridge or pouch [5, 6], and designing the second primer set with a higher annealing temperature are variations used to reduce the potential for contamination.

Multiplex PCR

In multiplex PCR, two or more unique DNA sequences in the same specimen are amplified simultaneously [10, 39]. Primers used in multiplex reactions must be designed carefully to have similar annealing temperatures and to lack complementarity, to avoid dimerization. Primers are designed such that each amplification product has a unique size, melting temperature, or probe binding sequence. This allows the detection and identification of different microorganisms in the same specimen. Multiplex PCR requires careful optimization of annealing conditions for maximal amplification efficiency. Commercial kits have been shown to efficiently amplify different sequences under single annealing conditions due to careful design of primer sequences and the use of buffer conditions that widen the annealing temperature window [40]. Multiplex PCR assays frequently incorporate an internal control to monitor every step of the procedure. Multiplex PCR has been well demonstrated to accurately and reliably detect multiple targets in a single PCR [6, 13, 15, 41–43]. Highly multiplexed PCR assays (generally detect more than six targets) are based on conventional PCR, as multiplexing by real-time PCR is limited by the emission spectra and overlapping absorption ("bleed-through") of reporter dyes.

Reverse Transcription-PCR

RT-PCR is a technique used to amplify RNA targets. Because DNA polymerase requires a double-stranded DNA template, RNA must be transcribed into complementary (c) DNA prior to PCR by the enzyme reverse transcriptase. The cDNA then serves as the template for the first PCR temperature cycle (Fig. 15.3). The combined use of RT and PCR with thermostable DNA polymerase to amplify RNA targets was first described in 1988 [3]. Reviews describing the numerous applications of RT-PCR are available [44]. RT-PCR is an important technique in the diagnosis of infectious diseases, given the large number of clinically significant RNA viruses.

Two reverse transcriptase enzymes commonly used are Moloney-murine leukemia virus (M-MuLV) reverse transcriptase and avian myeloblastosis virus (AMV) reverse transcriptase. Both enzymes have the same fundamental activities, but differ in some characteristics, including temperature and pH optima. In addition to M-MuLV and AMV, other variants of this enzyme are available for use in the molecular diagnostic laboratory. These enzymes are available in pre-optimized RT-PCR kits.

In vitro reverse transcription is primer directed: a single primer is used to generate cDNA, and can be one of the primers used in the subsequent PCR (sequence-specific), or a random oligonucleotide. Specificity is not required of reverse transcription. Random oligonucleotides are convenient in that one RT kit or reaction can be used for all RNA targets. When initially described, the RT step was performed in a separate tube containing only components necessary for reverse transcription.

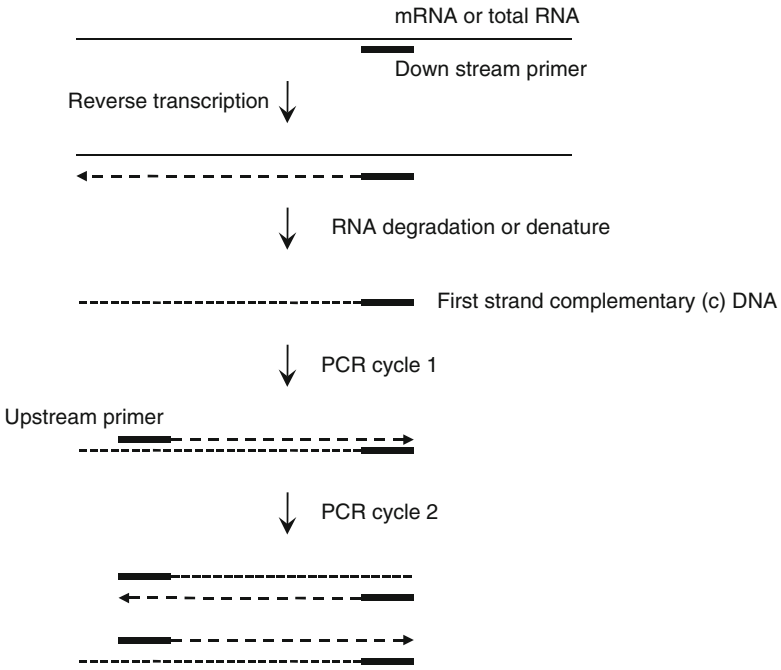

Fig. 15.3 Amplification of RNA by reverse transcription-PCR. Primers are represented by *bold lines*, newly replicated DNA strands are represented by *long dashes*, and template DNA strands by *short dashes*

Following RT, an aliquot is removed, added to a PCR tube, and subjected to amplification. Drawbacks of the separate tube method include inconvenience and cross-contamination risk. Currently, single tube RT-PCR assays, either two enzyme or single enzyme, are the norm. A DNA polymerase isolated from *Thermus thermophilus* (*Tth*) is a thermostable enzyme that replicates DNA at approximately 74°C in the presence of magnesium, and the polymerization of cDNA using an RNA template in the presence of manganese. This enzyme is used extensively for PCR amplification of RNA targets [45].

Quantitative PCR

A variety of quantitative PCR assays have been developed to accurately quantify nucleic acid targets in clinical specimens [8, 46–48]. In addition to PCR, other molecular techniques such as branched (b) DNA provide accurate quantification of nucleic acids. While these methods determine the amount of DNA or RNA template in a clinical specimen, the results can be easily extrapolated to organism equivalents, hence the use of terms bacterial load, viral load, and so forth. The clinical value

of quantitative PCR has led to commercialization of tests for such viruses as human immunodeficiency virus (HIV), cytomegalovirus, hepatitis C virus, and hepatitis B virus. Quantitative PCR results have become a valuable tool for guiding antiviral therapy, monitoring clinical course, and predicting outcome from a variety of infectious diseases [8, 49–51].

Nucleic acids can be quantified using an absolute standard in order to generate concrete numbers, or a relative standard to give comparative data. Absolute standards can be used whenever definite numbers are needed. Relative standards are useful when absolute quantities are less important than knowing how a sample differs from a control. Fundamental PCR quantification strategies are relative, competitive, and comparative.

Relative quantitative PCR compares nucleic acid amount across a number of serial dilutions of a sample, using a co-amplified internal control for sample normalization. Results are expressed as ratios of the sequence-specific signal to the internal control signal. This yields a corrected relative value for the sequence-specific product in each sample. Relative PCR uses primers for an internal control that are multiplexed in the same PCR with the target specific primers. Internal control and target specific primers must be compatible—that is, they must not produce additional bands or hybridize to each other. The signal from the internal control is used to normalize sample data to account for variation in RT or amplification efficiency. Common internal controls include the house keeping genes (or their mRNAs) β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S rDNA.

Competitive PCR provides absolute quantification of a nucleic acid target in a sample. An internal control or quantification standard is added at a known concentration to samples, and co-amplified with the target sequence. Addition of the internal control to the sample prior to processing monitors for nucleic acid recovery during this step. The internal control is often a synthetic RNA or DNA with the same primer binding sequence (hence the term, competitive PCR) but designed to produce an amplicon slightly different in size than the target amplicon, or with a unique internal sequence allowing detection with a different probe. Following amplification and detection, the amount of product or signal generated by the internal control is equated to its known input copy number. This relationship is then used to determine the copy number of the target sequence.

The availability of real-time PCR has allowed comparative quantification comparing PCR results to an external standard curve to determine target copy number. A separate internal control of an unrelated sequence should be incorporated into each PCR to monitor all steps of the procedure. Because measurements are taken at each cycle, during the exponential phase of PCR, efficiency is consistent between samples. Conventional PCR measures product only at the endpoint, when the effects of inhibitors are significant. Because PCR product is measured during the exponential cycles, quantification by real-time PCR is more accurate and precise over a greater range than conventional PCR. Real-time PCR offers dynamic range of up to 10^7-fold, compared to 1,000-fold in conventional PCR. External standards are used to create a standard curve across the dynamic range of the PCR assay. Real-time PCR generates a crossing threshold (C_T) or crossing point (C_p) cycle for each sample.

This is the point where product (fluorescence) crosses a predetermined threshold. The higher the amount of starting target, the lower the C_T. The C_T for an unknown patient sample is analyzed against the standard curve to yield a target DNA or RNA copy number.

Finally, digital PCR is a new approach to absolute nucleic acid quantification that directly counts the number of target molecules, rather than relying on reference standards or endogenous controls [52].

Real-Time PCR

Real-time PCR, also known as kinetic PCR, detects and measures amplification as it occurs [53], compared to conventional PCR where the product of amplification is detected after the reaction is complete. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle. During amplification, fluorescence increases in direct proportion to the amount of PCR product (Fig. 15.4). During the exponential phase of amplification, the increase in the amount of PCR product correlates to the initial amount of target template. Hence, real-time PCR is well suited for quantitative PCR, as already described in this chapter.

The two common methods for detection of real-time PCR products are: (1) DNA binding agents (nonspecific fluorescent dyes such as SYBR Green) that intercalate nonspecifically with double-stranded DNA, and (2) sequence-specific DNA probes labeled with a reporter which fluoresces only after hybridization of the probe with its complementary DNA target. Among the sequence-specific probes, there are hybridization probes and hydrolysis probe [54]. All methods require a thermal cycler equipped with optics that monitor the fluorescence in each PCR at frequent intervals.

Since SYBR green binds to nonspecifically to double-stranded DNA, fluorescence will be produced by any amplification product, including primer-dimer. This necessitates the post-amplification analysis of PCR product- melting curve analysis. Dissociation, or "melting" of double-stranded DNA during heating is measurable by the large reduction in fluorescence that results. The melting temperature of DNA is very exact, and depends on the base composition (and length if it is very short). In addition to its use with SYBR green real-time PCR, melting curve analysis is often performed when using hybridization probes. The temperature at which the probes dissociate from amplicons can be used to distinguish SNPs, and other minor differences such as those of amplicons generated from the glycoprotein D gene of Herpes simplex virus types 1 and 2 [55]. In real-time PCR, amplification and amplicon analysis are performed in the same tube, and do not require the transfer or opening of PCR vessels. The melting curve analysis is performed automatically as per a predefined program, immediately upon completion of amplification. A graph of the negative first derivative of the temperature of dissociation (defined as 50% dissociation), produces distinct peaks (Fig. 15.5).

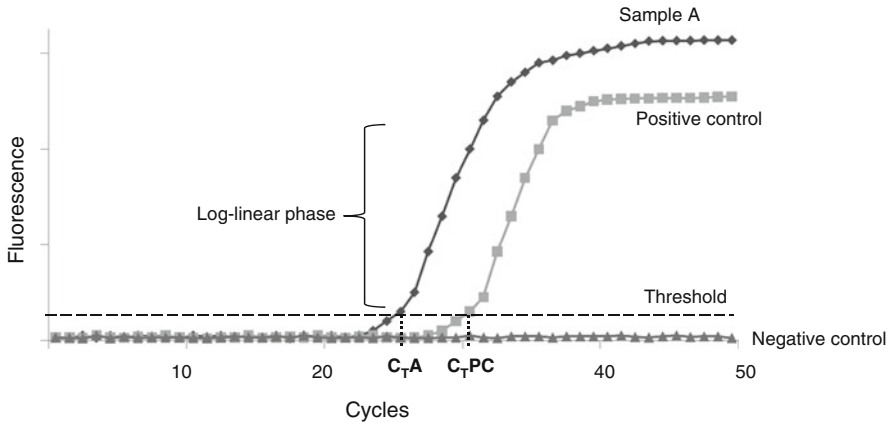

Fig. 15.4 Real-time PCR amplification plot

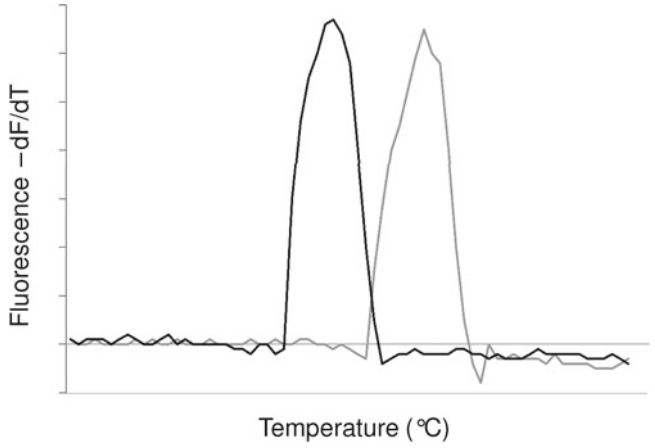

Fig. 15.5 Real-time PCR melting curve

PCR-Based Strain Typing Techniques

PCR-based strain typing techniques are designed to generate multiple bands that provide a unique fingerprint for a particular species or strain of microorganism [56, 57]. Unlike diagnostic tests that determine the presence or absence of a microorganism (or its nucleic acid) in a specimen, these procedures are used to differentiate epidemiologically unrelated organisms at the species or subspecies level. They must generally produce multiple DNA bands to provide sufficient discrimination power, and these banding patterns must be reproducible run-to-run and among isolates of the same predefined group, while clearly distinguishing isolates that epidemiologically or phenotypically fall outside of that group.

Arbitrarily Primed-PCR and Random Amplified Polymorphic DNA

Arbitrarily primed-PCR (AP-PCR) and random amplified polymorphic DNA (RAPD) are methods of creating genomic fingerprints from species, even if little is known about the target sequence to be amplified [58–63]. Strain-specific arrays of amplicons (fingerprints) are generated by PCR amplification using arbitrary, or random sequence oligonucleotides that are often less than ten nucleotides in length, and low temperature annealing. A single primer is often used, since it will anneal in both orientations. Detectable PCR product is generated when the primers anneal at the proper orientation, and within a reasonable distance of one another. In spite of the arbitrary nature of the assay, and amplification conditions that are relatively nonspecific, these methods have been shown to generate reproducible DNA banding patterns. These same characteristics make these methods suitable for a wide range of bacteria.

Amplified Fragment-Length Polymorphism

Amplified fragment-length polymorphism (AFLP) involves the restriction of genomic DNA, followed by ligation of adapters or linkers containing the restriction sites to the ends of the DNA fragments. The linkers and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Selective nucleotides extending into the restriction fragments are added to the 3′ ends of the PCR primers such that only a subset of the restriction fragments are recognized. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. The amplified fragments are visualized by means of autoradiography, phospho-imaging, or other methods. Like AP-PCR and RAPD, AFLP can be applied to organisms without previous knowledge of genomic sequence.

ERIC-, Rep-, BOX-, IS-, and VNTR-PCR

Enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive element (Rep)-PCR, insertion sequence (IS)-PCR and variable number tandem repeat (VNTR)-PCR are examples of PCR-based typing methods that target repetitive, conserved sequences found in bacteria and in some cases, fungi. In a seminal 1991 paper, Versalovic et al. described the presence of repetitive sequences in a wide range of bacterial species and demonstrated their use to directly fingerprint bacterial

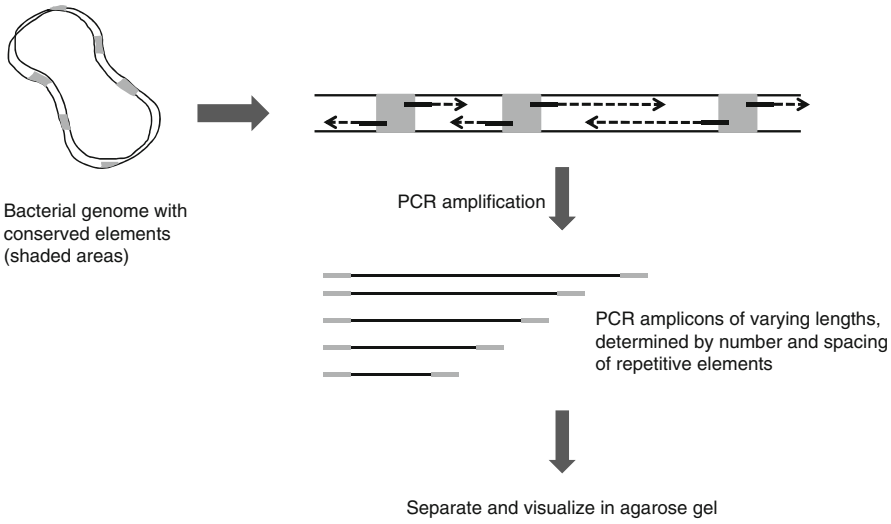

Fig. 15.6 Repetitive element (Rep)-PCR

genomes (Fig. 15.6) [64]. Specific repetitive sequences include the 124–127 base pair ERIC sequence, the 154 base pair BOX sequence, and the 35–40 base pair repetitive extragenic palindromic sequence. These sequences are located intergenically throughout the chromosome. Some repetitive sequences translocate to new locations in the genome, and are called transposons or insertion sequences. Some ISs are species-specific, while others have no species restriction. VNTRs are repeated sequences of noncoding DNA. Whether ERIC, IS, VNTR, or other repetitive element or sequence, the basis of the strain typing is the same. The ability of repetitive element-based PCR methods to distinguish unrelated strains or species is based on the random distribution of elements within the genome, and the time required for these to become established. That is, bacteria associated with a common source outbreak are highly unlikely to have any differences in the number or location of repetitive elements, while bacteria that are geographically, temporally, and epidemiologically unrelated are more likely to have experienced mutational events. Repetitive element-based PCR assays are designed so that primers anneal to the specific sequence in an outward orientation, so that DNA between the repeated elements is amplified. Variability between unrelated organisms is due to the random number and location of the elements on the genome. The utility of this PCR-based fingerprinting method is demonstrated by its successful commercialization. A kit includes reagents to perform Rep-PCR and electrophoresis, a fingerprint library, and software for data analysis for a variety of bacteria and fungi [65].

Appendix

Primer Design Resources

There are a number of primer design programs and related resources available for free on the world wide web. Following is a brief sampling of primer design programs. (URLs accessed on June 15, 2011).

For information on	URL
General resource site	http://www.med.nyu.edu/rcr/rcr/course/primer.html
Primer design	http://biotools.umassmed.edu/bioapps/primer3_www.cgi
BLAST search for primer design	http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=NcbiHomeAd
Real-time PCR (Taqman) primer design	https://www.genscript.com/ssl-bin/app/primer
Degenerate primer design	http://bibiserv.techfak.uni-bielefeld.de/genefisher2/
RAPD-PCR (fingerprinting) primer design	http://www2.uni-jena.de/biologie/mikrobio/tipps/rapd.html
General resource site	http://molbiol-tools.ca/PCR.htm
Real-time PCR primer design	http://www.uic.edu/depts/rrc/cgf/realtime/primer.html

References

1. Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155:335–350
2. Saiki RK, Scharf S, Faloona F et al (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350–1354
3. Saiki RK, Gelfand DH, Stoffel S et al (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491
4. Fredricks DN, Relman DA (1999) Application of polymerase chain reaction to the diagnosis of infectious diseases. Clin Infect Dis 29:475–486
5. Marlowe EM, Novak-Weekley SM, Cumpio J et al (2011) Evaluation of the Cepheid Xpert MTB/RIF assay for direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens. J Clin Microbiol 49:1621–1623
6. Rand KH, Rampersaud H, Houck HJ (2011) A comparison of two multiplex methods for the detection of respiratory viruses: FilmArray RP and xTAG RVP. J Clin Microbiol 49: 2449–2453
7. Selvaraju SB, Wurst M, Horvat RT, Selvarangan R (2009) Evaluation of three analyte-specific reagents for detection and typing of herpes simplex virus in cerebrospinal fluid. Diagn Microbiol Infect Dis 63:286–291
8. Sire JM, Vray M, Merzouk M et al (2011) Comparative RNA quantification of HIV-1 group M and non-M with the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 v2.0 and Abbott Real-Time HIV-1 PCR assays. J Acquir Immune Defic Syndr 56:239–243
9. Tang YW, Procop GW, Persing DH (1997) Molecular diagnostics of infectious diseases. Clin Chem 43:2021–2038

10. Tang Y, Persing DH (1999) Molecular detection and identification of microorganisms. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (eds) Manual of clinical microbiology, 7th edn. ASM, Washington, DC
11. Loeffelholz M, Deng H (2006) PCR and its variations. In: Tang YW, Stratton CW (eds) Advanced techniques in diagnostic microbiology, 1st edn. Springer, New York
12. Mullis KB (1990) The unusual origin of the polymerase chain reaction. Sci Am 262:56–61, 64-65
13. Balada-Llasat JM, LaRue H, Kelly C, Rigali L, Pancholi P (2011) Evaluation of commercial ResPlex II v2.0, MultiCode-PLx, and xTAG respiratory viral panels for the diagnosis of respiratory viral infections in adults. J Clin Virol 50:42–45
14. Kost CB, Rogers B, Oberste MS et al (2007) Multicenter beta trial of the GeneXpert enterovirus assay. J Clin Microbiol 45:1081–1086
15. Selvaraju SB, Selvarangan R (2010) Evaluation of three influenza A and B real-time reverse transcription-PCR assays and a new 2009 H1N1 assay for detection of influenza viruses. J Clin Microbiol 48:3870–3875
16. Stevenson JB, Hymas WC, Hillyard DR (2011) A novel capillary electrophoresis-based multiplex PCR assay for detection of respiratory pathogens. Ann Clin Lab Sci 41:33–38
17. White TJ, Madej R, Persing DH (1992) The polymerase chain reaction: clinical applications. Adv Clin Chem 29:161–196
18. Collasius M, Falk H, Ciesler C, Valet G (1989) How to build an inexpensive cyclotherm instrument for automated polymerase chain reaction. Anal Biochem 181:163–166
19. Wittwer CT, Fillmore GC, Hillyard DR (1989) Automated polymerase chain reaction in capillary tubes with hot air. Nucleic Acids Res 17:4353–4357
20. Wittwer CT, Hillyard DR, Ririe KM (1995) US Patent 5455175
21. Martin UK, de Mello AJ, Andreas M (1998) Chemical amplification: continuous-flow PCR on a chip. Science 280:1046–1048
22. Espinosa de los Monteros LE, Galan JC, Gutierrez M et al (1998) Allele-specific PCR method based on pncA and oxyR sequences for distinguishing *Mycobacterium bovis* from *Mycobacterium tuberculosis*: intraspecific *M. bovis* pncA sequence polymorphism. J Clin Microbiol;36:39–242
23. Mullis KB (1991) The polymerase chain reaction in an anemic mode: how to avoid cold oligodeoxyribonuclear fusion. PCR Methods Appl 1:1–4
24. Chou Q, Russell M, Birch DE, Raymond J, Bloch W (1992) Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res 20:1717–1723
25. Bassam BJ, Caetano-Anolles G (1993) Automated "hot start" PCR using mineral oil and paraffin wax. Biotechniques 14:30–34
26. Horton RM, Hoppe BL, Conti-Tronconi BM (1994) AmpliGrease: "hot start" PCR using petroleum jelly. Biotechniques 16:42–43
27. Riol H, Levesque G, Murthy MR (1994) A method of using heavy mineral oil for performing "hot-start" amplification of rare nucleic acids. Anal Biochem 221:210–212
28. Birch DE (1996) Simplified hot start PCR. Nature 381:445–446
29. Kebelmann-Betzing C, Seeger K, Dragon S et al (1998) Advantages of a new Taq DNA polymerase in multiplex PCR and time-release PCR. Biotechniques 24:154–158
30. Kellogg DE, Rybalkin I, Chen S et al (1994) TaqStart Antibody': "Hot Start" PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase. BioTechniques 16:1134–1138
31. Rasmussen HN, Rasmussen OF, Andersen JK, Olsen JE (1994) Specific detection of pathogenic *Yersinia enterocolitica* by two-step PCR using hot-start and DMSO. Mol Cell Probes 8:99–108
32. Pang J, Modlin J, Yolken R (1992) Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Mol Cell Probes 6:251–256

33. Udaykumar J, Epstein JS, Hewlett IK (1993) A novel method employing UNG to avoid carry-over contamination in RNA-PCR. Nucleic Acids Res 21:3917–3918
34. Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008
35. Moe CL, Gentsch J, Ando T et al (1994) Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. J Clin Microbiol 32:642–648
36. Haqqi TM, Sarkar G, David CS, Sommer SS (1988) Specific amplification with PCR of a refractory segment of genomic DNA. Nucleic Acids Res 16:11844
37. Schmidt B, Muellegger RR, Stockenhuber C et al (1996) Detection of *Borrelia burgdorferi*-specific DNA in urine specimens from patients with erythema migrans before and after antibiotic therapy. J Clin Microbiol 34:1359–1363
38. Whelen AC, Felmlee TA, Hunt JM et al (1995) Direct genotypic detection of *Mycobacterium tuberculosis* rifampin resistance in clinical specimens by using single-tube heminested PCR. J Clin Microbiol 33:556–561
39. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16:11141–11156
40. Rossister BJF, Grompe M, Caskey CT (1991) Detections of deletions and point mutations. In: McPherson MJ, Quirke P, Taylor GR (eds) PCR: a practical approach. Oxford University Press, New York
41. Bej AK, Mahbubani MH, Miller R, DiCesare JL, Haff L, Atlas RM (1990) Multiplex PCR amplification and immobilized capture probes for detection of bacterial pathogens and indicators in water. Mol Cell Probes 4:353–365
42. Geha DJ, Uhl JR, Gustaferro CA, Persing DH (1994) Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. J Clin Microbiol 32:1768–1772
43. Roberts TC, Storch GA (1997) Multiplex PCR for diagnosis of AIDS-related central nervous system lymphoma and toxoplasmosis. J Clin Microbiol 35:268–269
44. Larrick JW (1992) Message amplification phenotyping (MAPPing)—principles, practice and potential. Trends Biotechnol 10:146–152
45. Myers TW, Gelfand DH (1991) Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. Biochemistry 30:7661–7666
46. Boyer N, Marcellin P (2000) Pathogenesis, diagnosis and management of hepatitis C. J Hepatol 32:98–112
47. Clarke JR, McClure MO (1999) HIV-1 viral load testing. J Infect 38:141–146
48. Mylonakis E, Paliou M, Rich JD (2001) Plasma viral load testing in the management of HIV infection. Am Fam Physician 63:483–490, 495–486
49. Hodinka RL (1998) The clinical utility of viral quantitation using molecular methods. Clin Diagn Virol 10:25–47
50. Jung R, Soondrum K, Neumaier M (2000) Quantitative PCR. Clin Chem Lab Med 38: 833–836
51. Orlando C, Pinzani P, Pazzagli M (1998) Developments in quantitative PCR. Clin Chem Lab Med 36:255–269
52. Zhong Q, Bhattacharya S, Kotsopoulos S et al (2011) Multiplex digital PCR: breaking the one target per color barrier of quantitative PCR. Lab Chip 11:2167–2174
53. Higuchi R, Dollinger G, Walsh PS, Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. Biotechnology (NY) 10:413–417
54. Nolte FS, Caliendo AM (2011) Molecular microbiology. In: Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW (eds) Manual of clinical microbiology, 10th edn. ASM, Washington, DC, pp 27–59
55. Whiley DM, Mackay IM, Syrmis MW, Witt MJ, Sloots TP (2004) Detection and differentiation of herpes simplex virus types 1 and 2 by a duplex LightCyler PCR that incorporates an internal control PCR reaction. J Clin Virol 30:32–38
56. Fernandez-Cuenca F (2004) Applications of PCR techniques for molecular epidemiology of infectious diseases. Enferm Infecc Microbiol Clin 22:355–360

57. Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 37:1661–1669
58. Grattard F, Berthelot P, Reyrolle M, Ros A, Etienne J, Pozzetto B (1996) Molecular typing of nosocomial strains of *Legionella pneumophila* by arbitrarily primed PCR. J Clin Microbiol 34:1595–1598
59. MacGowan AP, O'Donaghue K, Nicholls S, McLauchlin J, Bennett PM, Reeves DS (1993) Typing of *Listeria* spp. by random amplified polymorphic DNA (RAPD) analysis. J Med Microbiol 38:322–327
60. Matsui C, Pereira P, Wang CK et al (1998) Extent of laminin-5 assembly and secretion effect junctional epidermolysis bullosa phenotype. J Exp Med 187:1273–1283
61. van Belkum A, Kluytmans J, van Leeuwen W et al (1995) Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. J Clin Microbiol 33:1537–1547
62. Welsh EA, Clark HH, Epstein SZ, Reveille JD, Duvic M (1994) Human leukocyte antigen-DQB1*03 alleles are associated with alopecia areata. J Invest Dermatol 103:758–763
63. Woods JP, Kersulyte D, Tolan RW Jr, Berg CM, Berg DE (1994) Use of arbitrarily primed polymerase chain reaction analysis to type disease and carrier strains of *Neisseria meningitidis* isolated during a university outbreak. J Infect Dis 169:1384–1389
64. Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19:6823–6831
65. Wise MG, Siragusa GR, Plumbee J, Healy M, Cary PJ, Seal BS (2009) Predicting *Salmonella enterica* serotypes by repetitive sequence-based PCR. J Microbiol Methods 76:18–24

Chapter 16
Non-PCR Target Amplification Techniques

Rosemary C. She and Elizabeth M. Marlowe

Introduction

In 1983 while driving up a mountain road, Dr Kary Mullis envisioned the concept of the polymerase chain reaction (PCR). These scientific "driving" thoughts completely revolutionized biology and bolstered an entire biotechnology industry, resulting in the creation of new biotechnology companies and jobs. As with any good idea, PCR was quickly patented by Cetus and then sold to Hoffman La Roche for $300 million [1].

In the business of clinical diagnostics, the ability to compete with PCR has propelled new technology. Since the advent of PCR, several other patents have been filed relating to non-PCR-mediated target amplification techniques. Today, companies like Gen-Probe, Becton Dickinson, bioMerieux, Meridian Biosciences, and BioHelix all have commercially available diagnostic products that are based on non-PCR-mediated target amplification techniques (Table 16.1).

Non-PCR-mediated target amplification techniques are generally based on isothermal amplification. While various names of isothermal amplification have appeared in the literature throughout the years, the primary focus of this chapter is to discuss the commercially available non-PCR techniques. For an historical perspective and more in-depth technical review of non-PCR-mediated target amplification techniques see this chapter in the first edition of this book. Mimicking cellular or viral replication of DNA or RNA is the basis of these techniques [2]. For example, they may consist of transcription-based amplification which generates an RNA product, or strand displacement-based amplification which generates a DNA product [2].

R.C. She (✉) • E.M. Marlowe
Kaiser Permanente, SCPMG Regional Reference Labs,
11668 Sherman Way, North Hollywood, CA 91605, USA
e-mail: rosemary.c.she@kp.org; elizabeth.m.marlowe@kp.org

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Table 16.1 Commercially available non-PCR target amplification assays for the detection of microorganisms

Manufacturer	Technology	Targets	URL
Gen-Probe	TMA	Diagnostic *Mycobacterium tuberculosis* *C. trachomatis/N. gonorrhoeae* *Trichomonas vaginalis* HIV-1 HCV Blood screening HIV, HCV, HBV, WNV	www.gen-probe.com
bioMerieux	NASBA	Enterovirus MRSA KPC[a] HIV-1 v. 2.0[a] HPV[a]	www.biomerieux.com
Becton Dickinson	SDA	*C. trachomatis/N. gonorrhoeae* HSV 1/2	www.bd.com
Meridian Biosciences	LAMP	*Clostridium difficile* HSV-1/HSV-2	www.meridianbiosciences.com

[a]Research use only

The advantage of using non-PCR-mediated target amplification techniques is that they can be very robust because of the amount of target that is generated in a very short amount of time. They also avoid the royalty costs associated with PCR. However, like any amplification technology, they can also be subject to contamination. Thus adherence to good molecular practices still applies when using non-PCR methods. Given the ease of designing and ordering PCR primers and probes, PCR still remains a common practice for many research and clinical laboratories.

Isothermal Transcription-Based Amplification

Isothermal transcription-based amplification (ITA) is available under several commercial names. These names include transcription-mediated amplification (TMA) and nucleic acid sequence-based amplification (NASBA). The basic concept of ITA is born from retroviral amplification which relies on three key enzymatic reactions. Once RNA is present in the reaction the first primer attaches to its complementary site at the 3′ end of the template. The reverse transcriptase (RT) enzyme is used to make the complementary DNA (cDNA) of the RNA target. RNAse H then degrades the initial RNA template only with RNA-DNA hybrids, but not single-stranded RNA. The second primer attaches to the 5′ end of the DNA strand and the T7 RNA

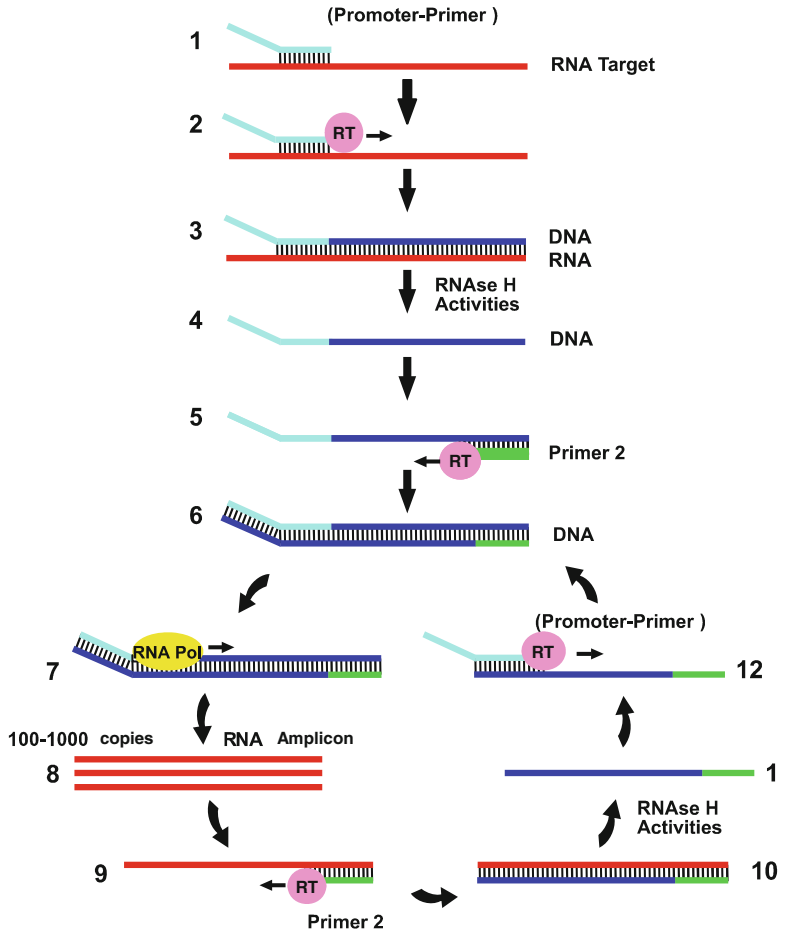

Fig. 16.1 Transcription-mediated amplification (TMA) (Image courtesy of Gen-Probe, Incorporated)

polymerase produces a complementary RNA strand which can be used again in the initial step of the reaction [2]. The process repeats automatically, resulting in an exponential amplification of the original target that can produce over a billion copies of amplicon in less than 30 min (http://gen-probe.com/science/).

The crux of Gen-Probe's (San Diego, CA) amplification technology is TMA. Like PCR, this isothermal technology relies on target amplification with complementary oligonucleotide primers to specifically anneal and allow enzymatic amplification of the target nucleic acid strands (Fig. 16.1) (http://gen-probe.com/science/). The TMA bacterial assays replicate specific regions of ribosomal RNA. Thus the assays are already targeting a template that is in abundant quantity, making the TMA assays very robust [3].

TMA is also used to detect viral targets. The PROCLEIX® assays (offered by Gen-Probe in collaboration with Novartis Vaccines and Diagnostics, Inc., Cambridge, MA) is used to screen blood for human immunodeficiency virus-1 (HIV-1), hepatitis B virus (HBV), and hepatitis C virus (HCV). Gen-Probe had the first FDA-approved nucleic acid test for screening donated human blood with the PROCLEIX® HIV-1/HCV assay [4]. This product was quickly followed by the PROCLEIX® West Nile virus (WNV) assay which has been used to look for WNV in blood donations [5, 6]. The Procleix® Ultrio® is also available and simultaneously detects HIV-1, HBV, and HCV [7].

TMA performed manually can still be labor intense and requires defined hands-on time. Gen-Probe has tried to overcome this by automating the process. The Tigris® DTS® system is a fully automated system for TMA assays. It is particularly useful in high volume laboratories because it allows for the automation of up to 450 specimens in 8 h or 1,000 specimens in 13.5 h (http://gen-probe.com). The Panther™ is the next automated system expected from Gen-Probe which will serve a smaller throughput laboratory and is expected to have a larger test menu than the Tigris®.

NASBA is also an isothermal reaction that uses three enzymes (avian myeloblastosis virus-RT (AMV-RT), RNase H, and T7 RNA polymerase) and target-specific oligonucleotides. NASBA, like TMA, has been introduced into medical diagnostics, where it has been shown to have a faster turn-around time than PCR, and it can also be more sensitive [8]. After its introduction, NASBA was used for quantification of HIV-1 in human sera [9]. However, the HIV-1 assay was never FDA-approved and is only available in the United States as a research use only (RUO) product from bioMerieux (Marcy l'Etoile, France). bioMerieux has marketed NASBA under the NucliSENS® name. NucleiSENS Easy Q® Enterovirus was the first FDA-approved real-time NASBA assay (Fig. 16.2) used to detect viral RNA utilizing a florescent beacon [10]. An expanded menu is available and includes methicillin-resistant *Staphylococcus aureus* (MRSA). Additional analytes are available as RUO assays (Table 16.1).

TMA and NASBA are very similar technologies. The differences between them are the specific enzymes used in reaction and their detection systems. For reverse transcription, TMA utilizes Moloney murine leukemia virus-RT (MMLV-RT) and NASBA utilizes AMV-RT [11]. To date, end point detection for all FDA-cleared TMA assays use Gen-Probe's hybridization protection technology with chemiluminescent DNA probes. Currently available NASBA assays use real-time-based detection with molecular beacons. For a list of the targets for which there are commercially available products, see Table 16.1.

Strand Displacement Amplification

Strand displacement amplification (SDA) is an isothermal amplification technique that was first described in 1992 [12]. Rather than using thermocycling to facilitate repeating rounds of DNA synthesis as in PCR, SDA relies upon the activities of a

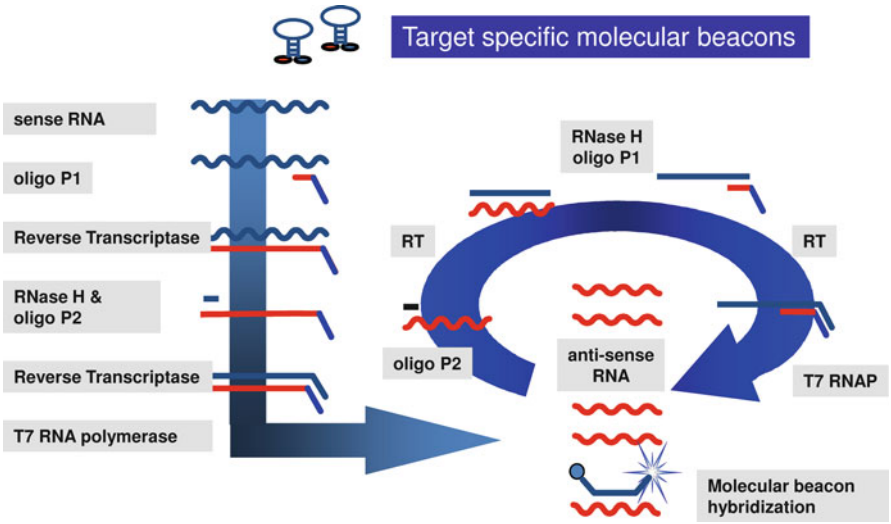

Fig. 16.2 Real-time nucleic acid sequence-based amplification (NASBA). (Image courtesy of bioMerieux)

restriction endonuclease and an exonuclease-deficient DNA polymerase to continually and isothermally amplify a DNA target. Inner primers are designed to have a restriction enzyme recognition sequence within their 5′ overhang. Flanking outer primers bind upstream of the inner primer targets. Extension of all four primers by DNA polymerase results in amplicons incorporating restriction enzyme sites (Fig. 16.3a). A restriction endonuclease added to the reaction creates a single-stranded nick at these sites, where DNA polymerase can then initiate replication and displace the annealed strand (Fig. 16.3b). The substitution of a dNTP with a modified form containing a 5′ [alpha-thio] triphosphate, typically dATPαS, allows for incorporation of a hemiphosphorothioate linkage at the restriction site during amplification. This permits single-stranded nicking by the restriction enzyme.

The DNA polymerase that is used in SDA must be able to initiate polymerization at a nick site and must lack exonuclease activity. Such polymerases include exo$^-$ Klenow, exo-*Bst*, and exo-*Bca* polymerases [12, 13]. The restriction endonuclease must be able to create a single-stranded nick at a hemiphosphorothioated recognition site, dissociate quickly to allow the polymerase to act, and repeatedly perform rounds of nicking and dissociation. Restriction enzymes which meet these criteria include *Hinc*II, *Bso*BI, *Ava*I, *Nci*I, and *Fnu*4HI [12, 14].

The maximum target length is influenced by the stringency of the reaction conditions, the processivity of the DNA polymerase, and the frequency of recognition sites of the restriction enzyme surrounding the target sequence. Amplicons are generally 50–120 bp long, and SDA is less well suited for target sequences longer than 1,000 bp [14]. At 37°C, exo-Klenow polymerase demonstrates decreased

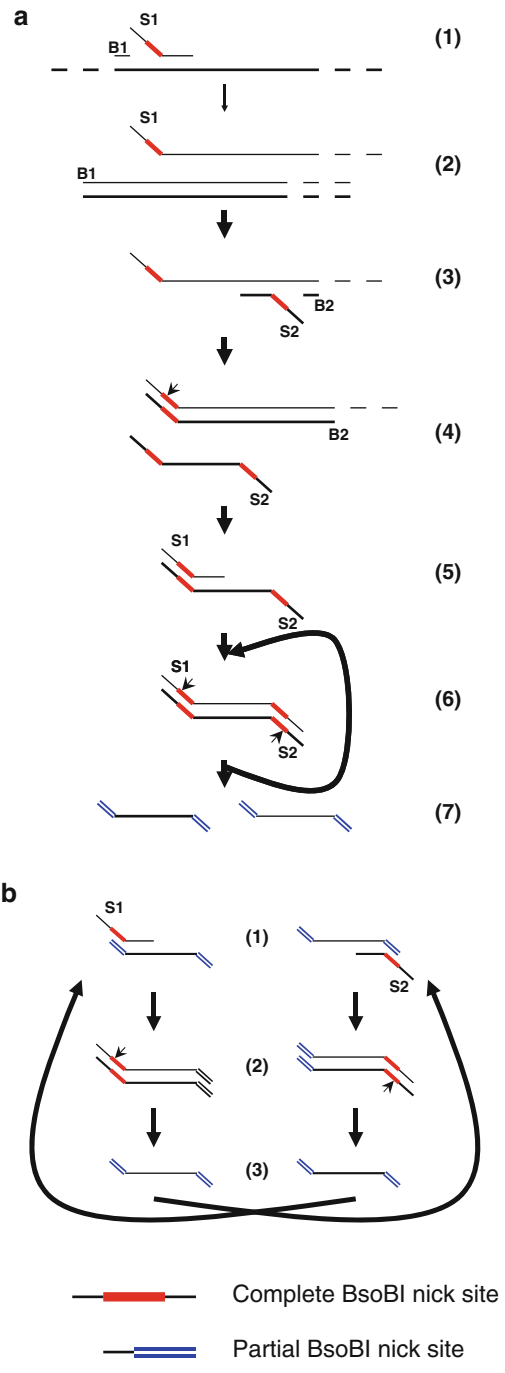

Fig. 16.3 Strand displacement amplification (SDA). See detailed descriptions in texts. (Image courtesy of Beckton Dickinson)

amplification efficiency with target lengths greater than 50 bp. At this temperature, the stringency of the reaction is relatively low with increased background amplification. *Bso*BI restriction endonuclease and exo-*Bst* polymerase, derived from the thermophilic bacterium *Bacillus stearothermophilus*, function well at relatively high temperatures (50–60°C). This higher temperature enables increased stringency of primer hybridization and improved reaction kinetics [15]. Doubling time is approximately 30 s, and up to 10^{10} amplicons can be generated within 15 min [13]. In SDA, target detection may be accomplished in real-time with the use of fluorescently labeled probes (Fig. 16.4), and if desired, produce quantitative results [16, 17]. Use of dUTP instead of dTTP to enable contamination control with uracil-DNA glycosylase has been described [13].

Initial studies focused on the application of SDA to the detection of *Mycobacterium tuberculosis* [13, 17, 18]. A BDProbeTec™ ET assay (Becton Dickinson, Franklin Lakes, NJ) to aid in the identification of *M. tuberculosis* complex from colony growth on solid media was cleared by the FDA in 2001 but is no longer available in the United States. Nonetheless, studies have shown this assay to perform comparably to other amplified detection assays for *M. tuberculosis* directly from respiratory specimens [19–21]. Currently, FDA-cleared assays which utilize SDA include the BD ProbeTec™ ET assay for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (CT/GC) from a variety of genitourinary sources, and for HSV-1 and HSV-2 from clinician-collected external anogenital specimens from adults. These assays generate qualitative results using real-time detection and employ *Bso*BI restriction endonuclease and exo-*Bst* polymerase [16]. Additional reagents to monitor amplification inhibition are available and this reaction would be performed in a separate well.

The BD ProbeTec™ ET assay has comparable performance to the other FDA-cleared amplified assays for *C. trachomatis* and *N. gonorrhoeae* for a variety of specimen sources [22–25]. However, for self-collected vaginal specimens, sensitivity of the ProbeTec™ assay may be somewhat lower [26]. Performance of the assay is suitable in a low to medium volume laboratory. For higher volume laboratories, the assay can be fully automated with the inclusion of front-end specimen processing on the BD Viper™ system. One current advantage of the BD ProbeTec™ ET CT/GC is that it is the only FDA-cleared target amplification assay for endocervical samples collected in SurePath media for cytological screening.

Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) was developed by Eiken Chemical Co. (Tokyo, Japan) and the method was first published in 2000 [27]. Like SDA, LAMP requires a DNA polymerase with high strand displacement activity, e.g., *BstI* polymerase. Also like SDA, two inner and two outer primers are used in an isothermal reaction (Fig. 16.5a). The inner primers incorporate a sequence in their 5′ overhang that is complementary to the region that will be amplified just downstream of the primer. After the outer primers anneal upstream to the inner primers and initiate

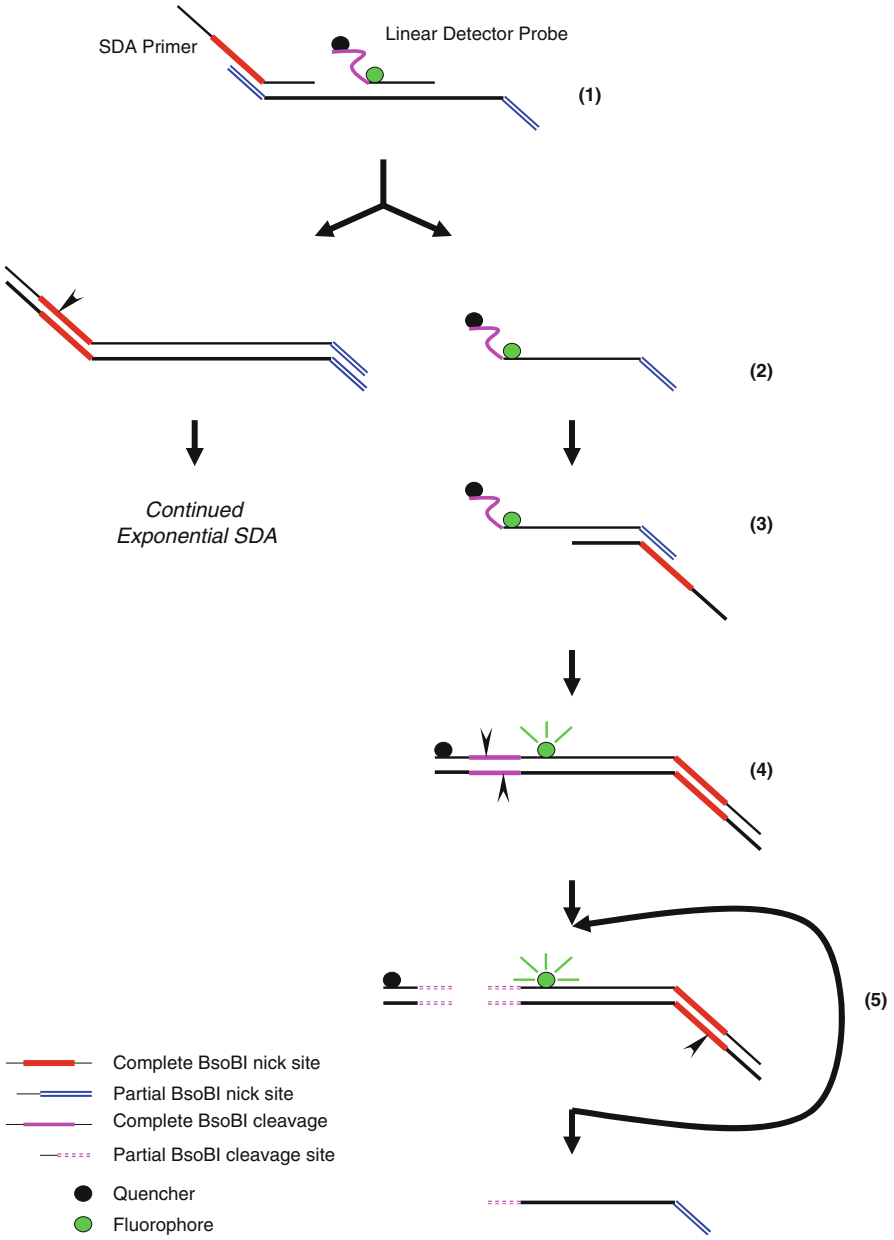

Fig. 16.4 Real-time strand displacement amplification (SDA) (Image courtesy of Beckton Dickinson)

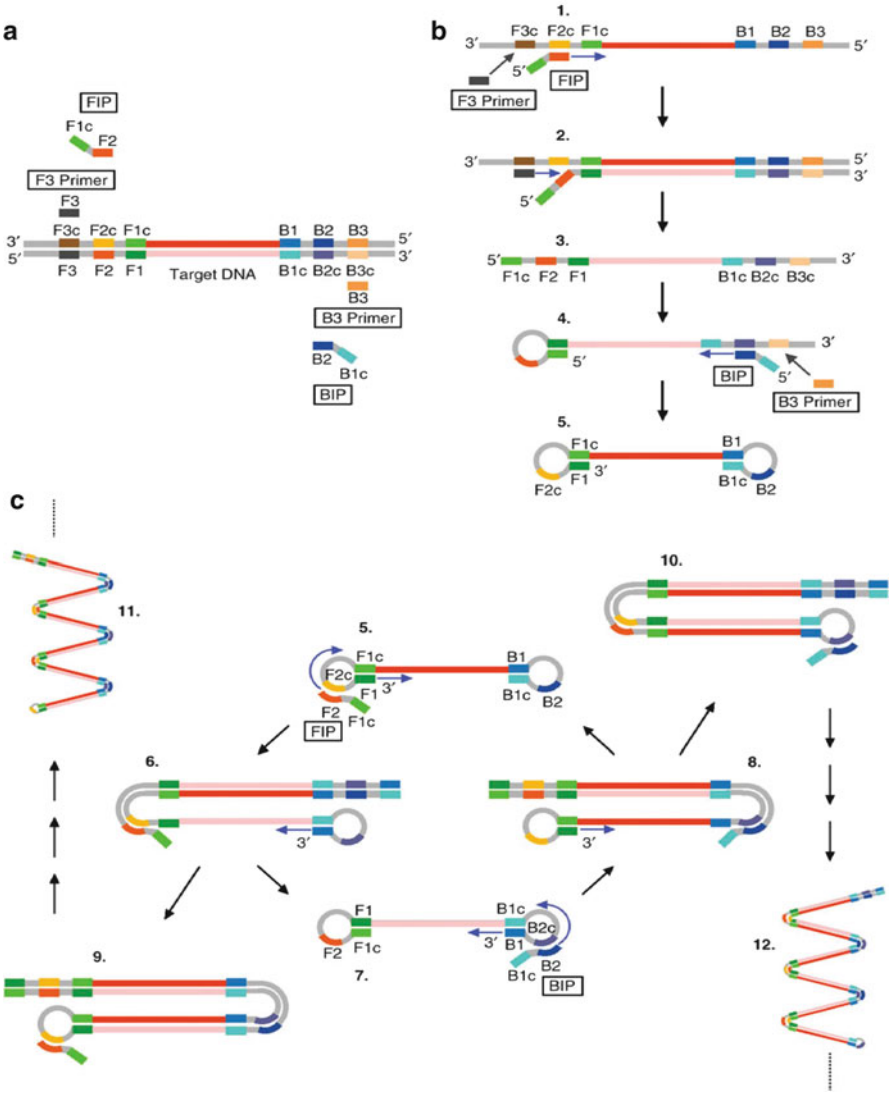

Fig. 16.5 Loop-mediated isothermal amplification (LAMP). See detailed descriptions in texts. (Image courtesy of Eiken Chemical Co.)

strand displacement, the complementary regions of the displaced strand can then anneal and form a loop structure at its 3′ end (Fig. 16.5b). Subsequent amplification may occur as self-primed DNA synthesis off of the 3′ end of the loop, or as primers annealed to the single-stranded area of the loop. A variety of stem-loop structures of varying sizes are formed in the process (Fig. 16.5c). Amplicons may be detected by

measuring change in turbidity from the formation of magnesium pyrophosphate salt [28]. This change is turbidity can also be directly visualized. Other methods of detection, such as fluorescent probes and bioluminescence, have been described [29, 30]. While detection of precipitated magnesium phosphate is not specific for the target of interest, specificity is achieved by the use of four primers targeting six regions of DNA during the reaction process.

Reverse-transcription LAMP has also been developed for detection of RNA targets and can be accomplished in a one-step, isothermal reaction with the use of AMV-RT [31]. Use of dUTP instead of dTTP to enable contamination control with uracil-DNA glycosylase has also been shown to be feasible [32]. One study demonstrated that LAMP is less inhibited by urine and stool than PCR, suggesting that purification of nucleic acids may not be necessary prior to LAMP reactions on certain human specimens [33].

The LAMP technology has been licensed by Meridian Biosciences, Inc. (Cincinnati, OH). The first FDA-cleared test utilizing LAMP for clinical diagnostics is the *illumi*gene® *Clostridium difficile* assay. Specimens undergo a heat lysis rather than purified nucleic acid extraction, thus decreasing the complexity and turnaround time of the assay. Inhibition control is assayed in a separate reaction tube. The reaction occurs at 60–65°C and can be completed in less than 1 h within a closed system. Detection of product is by turbidimetric readings on a luminometer. According to manufacturers' data, analytical sensitivity of this assay (4–64 colony forming units/reaction) is roughly the same as for other FDA-cleared *C. difficile* assays that are PCR-based. Likewise, initial studies have shown this LAMP-based method to have excellent performance characteristics compared to PCR-based assays using clinical specimens [34, 35].

Other analytes, such as Group B *Streptococcus*, are being evaluated on the *illumi*gene® LAMP platform and will likely be available in the near future. Currently, the *illumi*gene® assay can test up to ten samples per instrument and the pipetting steps are manually performed. This format would seem to be most beneficial for small- to medium-sized laboratories that desire to perform molecular testing using a simple protocol.

Helicase-Dependent Amplification

This amplification technique was developed by BioHelix (Beverly, MA) and first described in 2004 [36]. Unlike PCR which relies upon heat to denature double-stranded DNA, helicase-dependent amplification (HDA) relies on helicase activity to separate double-stranded DNA or RNA-DNA hybrid strands isothermally. Similar to PCR, oligonucleotide primers flanking a target region result in an amplicon of expected base pair length after extension by DNA polymerase. The ease of primer design provides an advantage over other isothermal methods already discussed.

Use of a thermophilic helicase, such as Tte-UvrD, allows the reaction to occur at a higher temperature. This in turn results in greater assay specificity and sensitivity and also may obviate the need for accessory proteins in the reaction [37]. A variety of strand-displacing DNA polymerases may be used and have included *Bst* exo-polymerase, exo- Klenow fragment, and GST large fragment polymerase [36–38]. HDA is compatible with many methods of amplicon detection. Colorimetric detection, intercalating DNA dyes, hybrid capture with fluorescence end-point detection, and real-time detection by TaqMan probes have all been performed successfully [38–41]. One-step reverse-transcription HDA has also been proven to be feasible, highly efficient, and amenable to real-time detection of human pathogens [40].

In 2011, the FDA cleared an HDA assay for the detection of HSV-1 and HSV-2 from oral and genital lesions (IsoAMP® HSV Assay, BioHelix). After specimen preparation, samples undergo a 1-h amplification step at 64°C which generates amplicons labeled with biotin and either dioxygenin or fluorescein. This dual-labeling system enables differential detection of HSV (if present) and a built-in internal control. After the amplification product is applied to a vertical-flow test strip within a self-contained cassette, the control and test lines are interpreted visually. This format has been shown to have comparable performance to PCR for detection of HSV from clinical specimens [42]. It is suitable for small volume laboratories because it is simple, self-contained, low-throughput, and low cost in terms of capital equipment.

Conclusions

In recent years, we have seen the progressive introduction of various isothermal amplification methods to the clinical microbiology marketplace. Non-PCR amplification technologies that are currently commercially available for infectious disease testing offer numerous advantages over PCR. These non-PCR molecular assays have also become more accessible to smaller laboratories. Isothermal amplification techniques can offer flexibility in terms of inexpensive capital equipment, decreased complexity, and rapid turn-around time. Laboratories now have more FDA-cleared options to choose from, and the menu promises to expand with time. Automation has also increased the capabilities of integrating these assays into the larger clinical laboratory. Contained, automated systems obviate the need for separate workspaces for individual steps in performing the assay. For quantitative nucleic acid testing, however, real-time PCR is still the best studied and most highly utilized method. Competition among quantitative assay platforms may soon increase as more non-PCR real-time assays are introduced. As with any technology that is introduced for patient testing, each laboratory will have to consider its patient population, test volume, and resources to decide which molecular platform best fits its needs.

References

1. Mullis K (2000) Dancing naked in the mind field, 1st edn. Vintage, New York
2. Pendrak ML, Yan SS (2006) Non-polymerase chain reaction mediated target amplification techniques. In: Tang Y, Sutton C (eds) Advanced techniques in diagnostic microbiology, 1st edn. Springer, New York, pp 505–523
3. Hill CS (2001) Molecular diagnostic testing for infectious diseases using TMA technology. Expert Rev Mol Diagn 1(4):445–455
4. McAuley JD, Caglioti S, Williams RC et al (2004) Clinical significance of nondiscriminated reactive results with the Chiron Procleix HIV-1 and HCV assay. Transfusion 44(1):91–96
5. Linnen JM, Deras ML, Cline J et al (2007) Performance evaluation of the PROCLEIX West Nile virus assay on semi-automated and automated systems. J Med Virol 79(9):1422–1430
6. Ziermann R, Sanchez-Guerrero SA (2008) PROCLEIX West Nile virus assay based on transcription-mediated amplification. Expert Rev Mol Diagn 8(3):239–245
7. Sgourou A, Karakantza M, Theodori E et al (2008) Procleix Ultrio transcription-mediated amplification vs. serological blood screening in south-western Greece. Transfus Med 18(2): 104–111
8. Schneider P, Wolters L, Schoone G et al (2005) Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. J Clin Microbiol 43(1):402–405
9. Kievits T, van Gemen B, van Strijp D et al (1991) NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J Virol Methods 35(3):273–286
10. Capaul SE, Gorgievski-Hrisoho M (2005) Detection of enterovirus RNA in cerebrospinal fluid (CSF) using NucliSens EasyQ Enterovirus assay. J Clin Virol 32(3):236–240
11. Compton J (1991) Nucleic acid sequence-based amplification. Nature 350(6313):91–92
12. Walker GT, Little MC, Nadeau JG, Shank DD (1992) Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. Proc Natl Acad Sci U S A 89(1):392–396
13. Spargo CA, Fraiser MS, Van CM (1996) Detection of *M. tuberculosis* DNA using thermophilic strand displacement amplification. Mol Cell Probes 10(4):247–256
14. Hellyer TJ, Nadeau JG (2004) Strand displacement amplification: a versatile tool for molecular diagnostics. Expert Rev Mol Diagn 4(2):251–261
15. Little MC, Andrews J, Moore R et al (1999) Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. Clin Chem 45(6 Pt 1):777–784
16. Nadeau JG, Pitner JB, Linn CP, Schram JL, Dean CH, Nycz CM (1999) Real-time, sequence-specific detection of nucleic acids during strand displacement amplification. Anal Biochem 276(2):177–187
17. Walker GT, Linn CP (1996) Detection of *Mycobacterium tuberculosis* DNA with thermophilic strand displacement amplification and fluorescence polarization. Clin Chem 42(10):1604–1608
18. Walker GT, Nadeau JG, Spears PA, Schram JL, Nycz CM, Shank DD (1994) Multiplex strand displacement amplification (SDA) and detection of DNA sequences from *Mycobacterium tuberculosis* and other mycobacteria. Nucleic Acids Res 22(13):2670–2677
19. Mazzarelli G, Rindi L, Piccoli P, Scarparo C, Garzelli C, Tortoli E (2003) Evaluation of the BDProbeTec ET system for direct detection of *Mycobacterium tuberculosis* in pulmonary and extrapulmonary samples: a multicenter study. J Clin Microbiol 41(4):1779–1782
20. Iinuma Y, Senda K, Fujihara N et al (2003) Comparison of the BDProbeTec ET system with the Cobas Amplicor PCR for direct detection of *Mycobacterium tuberculosis* in respiratory samples. Eur J Clin Microbiol Infect Dis 22(6):368–371
21. Goessens WH, de Man P, Koeleman JG et al (2005) Comparison of the COBAS AMPLICOR MTB and BDProbeTec ET assays for detection of *Mycobacterium tuberculosis* in respiratory specimens. J Clin Microbiol 43(6):2563–2566

22. Chan EL, Brandt K, Olienus K, Antonishyn N, Horsman GB (2000) Performance characteristics of the Becton Dickinson ProbeTec System for direct detection of Chlamydia trachomatis and *Neisseria gonorrhoeae* in male and female urine specimens in comparison with the Roche Cobas System. Arch Pathol Lab Med 124(11):1649–1652
23. Gaydos CA, Theodore M, Dalesio N, Wood BJ, Quinn TC (2004) Comparison of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* in urine specimens. J Clin Microbiol 42(7):3041–3045
24. Ota KV, Tamari IE, Smieja M et al (2009) Detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in pharyngeal and rectal specimens using the BD Probetec ET system, the Gen-Probe Aptima Combo 2 assay and culture. Sex Transm Infect 85(3):182–186
25. Van Der Pol B, Ferrero DV, Buck-Barrington L et al (2001) Multicenter evaluation of the BDProbeTec ET System for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urine specimens, female endocervical swabs, and male urethral swabs. J Clin Microbiol 39(3):1008–1016
26. Masek BJ, Arora N, Quinn N et al (2009) Performance of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by use of self-collected vaginal swabs obtained via an Internet-based screening program. J Clin Microbiol 47(6):1663–1667
27. Notomi T, Okayama H, Masubuchi H et al (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28(12):E63
28. Mori Y, Nagamine K, Tomita N, Notomi T (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun 289(1):150–154
29. Aoi Y, Hosogai M, Tsuneda S (2006) Real-time quantitative LAMP (loop-mediated isothermal amplification of DNA) as a simple method for monitoring ammonia-oxidizing bacteria. J Biotechnol 125(4):484–491
30. Gandelman OA, Church VL, Moore CA et al (2010) Novel bioluminescent quantitative detection of nucleic acid amplification in real-time. PLoS One 5(11):e14155
31. Francois P, Bento M, Hibbs J et al (2011) Robustness of loop-mediated isothermal amplification reaction for diagnostic applications. FEMS Immunol Med Microbiol 62(1):41–48
32. He L, Xu HS, Wang MZ, Rong HN (2010) Development of rapid detection of infectious hypodermal and hematopoietic necrosis virus by loop-mediated isothermal amplification. Bing Du Xue Bao 26(6):490–495
33. Ren X, Li P (2011) Development of reverse transcription loop-mediated isothermal amplification for rapid detection of porcine epidemic diarrhea virus. Virus Genes 42(2):229–235
34. Lalande V, Barrault L, Wadel S, Eckert C, Petit JC, Barbut F (2011) Evaluation of a loop-mediated isothermal amplification (LAMP) assay for the diagnosis of *Clostridium difficile* infections. J Clin Microbiol 49(7):2714–2716
35. Noren T, Alriksson I, Andersson J, Akerlund T, Unemo M (2011) Rapid and sensitive loop-mediated isothermal amplification test for *Clostridium difficile* detection challenges cytotoxin B cell test and culture as gold standard. J Clin Microbiol 49(2):710–711
36. Vincent M, Xu Y, Kong H (2004) Helicase-dependent isothermal DNA amplification. EMBO Rep 5(8):795–800
37. An L, Tang W, Ranalli TA, Kim HJ, Wytiaz J, Kong H (2005) Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification. J Biol Chem 280(32):28952–28958
38. Doseeva V, Forbes T, Wolff J et al (2011) Multiplex isothermal helicase-dependent amplification assay for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. Diagn Microbiol Infect Dis 71(4):354–365
39. Gill P, Alvandi AH, Abdul-Tehrani H, Sadeghizadeh M (2008) Colorimetric detection of *Helicobacter pylori* DNA using isothermal helicase-dependent amplification and gold nanoparticle probes. Diagn Microbiol Infect Dis 62(2):119–124

40. Goldmeyer J, Kong H, Tang W (2007) Development of a novel one-tube isothermal reverse transcription thermophilic helicase-dependent amplification platform for rapid RNA detection. J Mol Diagn 9(5):639–644
41. Tong Y, Tang W, Kim HJ, Pan X, Ranalli T, Kong H (2008) Development of isothermal TaqMan assays for detection of biothreat organisms. Biotechniques 45(5):543–557
42. Kim HJ, Tong Y, Tang W et al (2011) A rapid and simple isothermal nucleic acid amplification test for detection of herpes simplex virus types 1 and 2. J Clin Virol 50(1):26–30

Chapter 17
Probe Amplification Technologies

Fei Ye, Miao Cui, Tao Feng, Ivy Lee, Josephine Wu, Bingjiao Yin, and David Zhang

Introduction

Oligonucleotide probes provide a useful tool for the detection of target nucleic acids by the formation of a double-helical structure between complementary sequences. The stringent requirements of Watson–Crick base pairing make hybridization extremely specific. However, the detection of target sequence by hybridization is often insensitive due to the limited number of signal molecules that can be labeled on the probe. In general, the analytical sensitivity of probe hybridization is in the order of 10^6 molecules. Therefore, it cannot meet the needs of most clinical diagnostic applications. Many technologies have been developed to improve the detection sensitivity by amplifying the probe sequence bound to the target. All probe amplification technologies are developed based on the recent advancement in molecular biology and the understanding of in vivo nucleic acid synthesis, i.e., ligation, polymerization, transcription, digestion/cleavage, etc.

A fundamental advantage of probe amplification technologies ascribes to their isothermal nature, i.e., accomplishing amplification at a constant temperature with the exception of LCR which requires temperature cycling. Isothermal amplification allows the test to be done using a simple instrument and makes quality control of the instrument easier. In order for the probe to be amplified, the probes have to be specially designed or synthesized. For example, in rolling circle amplification (RCA), a circularized probe is used while the Invader assay employs an overlapping structure within the probes. Finally, maximum amplification is achieved by generating new DNA products (RCA, RAM, SMART, MLPA, etc.), although some of the technologies (i.e., LCR and CPT) use existing DNA primers without a net increase of DNA products.

F. Ye • M. Cui • T. Feng • I. Lee • J. Wu • B. Yin • D. Zhang (✉)
Molecular Pathology Laboratory, Department of Pathology,
Mount Sinai School of Medicine, New York University,
One Gustave Levy Place, Box 1122, New York, NY 10029, USA
e-mail: David.Zhang@MSSM.edu

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In addition to amplification of probe sequence to achieve a desired sensitivity, each technology has its unique features, thus, unique clinical applications. For example, Invader technology is very useful for single-nucleotide polymorphism (SNP) scoring due to specific recognition by the enzyme cleavase to the overlapping structure of two probes. On the other hand, RCA is probably the only technology that can be used for on-chip amplification due to the attachment of product to the primer sequence linked on the chip surface. In addition, multiplex ligation-dependent probe amplification (MLPA), a variation of the ligation-dependent PCR, permits amplification of multiple targets with a single primer pair. Therefore, in order to select a technology for a particular application, one has to understand the principle of the technology and address the need of the clinical problem accordingly.

This chapter reviews the most common probe amplification technologies and presents some of their applications with primary focus on microorganism diagnosis in clinical laboratory. For more in-depth discussion of clinical applications, the readers should refer to other excellent chapters in this book.

Rolling Circle Amplification

Circularizable probe (C-probe or padlock probe) is a uniquely designed oligonucleotide probe that contains three regions: two target complementary sequences located at the 5′ and 3′ termini and an interposed generic linker region [1, 2]. Once the C-probe hybridizes to its target, the 5′ and 3′ ends are juxtaposed (Fig. 17.1a). A closed circular molecule is then generated following incubation of the C-probe–target complex with a DNA ligase. The resulting closed circular molecule is helically twisted around the target strand [1]. The permanently locked C-probe permits stringent washing for the removal of unbound components, thereby enhancing assay signal-to-noise ratios.

The unique design of the C-probe allows its amplification by a rolling circle (RCA) mechanism as observed in in vivo bacteriophage replication (Fig. 17.1b) [3–5]. In this scheme, a single forward primer complementary to the linker region of the C-probe and a DNA polymerase bearing strand displacement activity are employed. The polymerase extends the bound primer along the closed C-probe for many revolutions and displaces upstream sequences, producing a long single-stranded DNA (ssDNA) of multiple repeats of the C-probe sequence that can be as long as 0.5 Mb [3]. This type of amplification, however, only results in linear growth of the products with up to several thousandfold amplification [3]. Some of the properties of RCA are summarized in Table 17.1.

Recently, Murakami et al. [6] described a modified RCA method, termed primer generation-RCA (Fig. 17.2) and demonstrated that under the optimized condition, this method can detect 84.5 fmol (50.7 molecules) of synthetic sample DNA and 0.163 pg (~60 molecules) of genomic DNA from *Listeria monocytogenes*, indicating an excellent sensitivity of the modified RCA for the detection of microorganism in clinical laboratory.

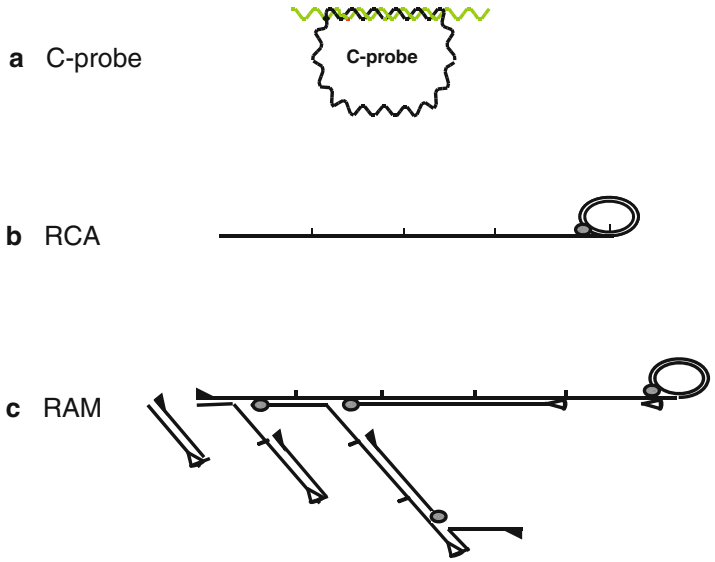

Fig. 17.1 Schematic representation of C-probe, RCA, and RAM. (**a**) A C-probe hybridizes to its target through its complementary regions and helical turns formed between C-probe and target result in the locking of C-probe onto the target. The sequence between the target-binding regions is generic for the binding of primers. (**b**) A DNA polymerase (*gray circle*) extends a bound primer along a closed C-probe for five rounds through rolling circle amplification (RCA). (**c**) A forward primer (*filled pointer*) bound to a C-probe is extended by DNA polymerase (*gray circle*), generating a long ssDNA. Multiple reverse primers (*unfilled pointer*) bind to the nascent ssDNA as their binding sites become available. Each bound reverse primer extends and displaces the upstream primers and their extended products. The forward primer binding sites of the displaced ssDNA are then available for the forward primers to bind and extend similarly, thus forming a large ramifying DNA complex (RAM)

Table 17.1 Comparison of Probe amplification technology

Property	RCA	RAM	SMART	Invader	MLPA	CPT
Amplification capability	1^u	2^u	2^n	1^u	2^n	1^u
Temperature alteration	–	–	–	–	–	–
Detection of DNA target	+	+	+	+	+	+
Detection of RNA target	+	+	+	±	+	–
Detection of protein target	+	±	–	–	–	–
Real time	+	+	+	+	±	+
Enzyme used	DNA pol	DNA pol	DNA–RNA pol	Cleavase	Ligase	RNase H
On-surface amplification	+	±	–	–	–	–
Multiplexing	+	+	±	+	+	+
SNP detection	+	+	±	+	+	±

RCA rolling circle amplification; *RAM* ramification amplification; *SMART* signal-mediated amplification of RNA technology; *MLPA* multiplex ligation-dependent probe amplification; *CPT* cycling probe technology; *u* number of rounds accomplished by DNA polymerase along a C-probe; *n* number of cycle; *SNP* single-nucleotide polymorphism; *DNA pol* DNA polymerase; *RNA–RNA pol* RNA-directed RNA polymerase; *DNA–RNA pol* DNA-directed RNA polymerase

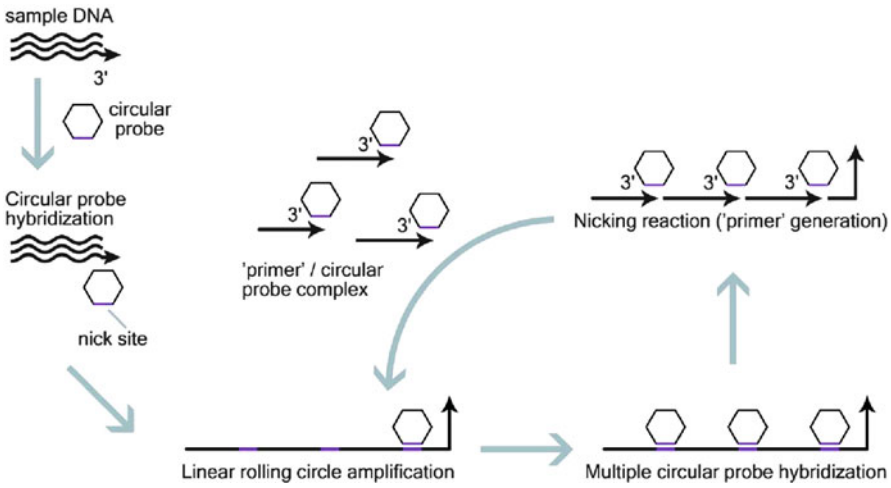

Fig. 17.2 Schematic representation of primer-generating RCA. PG-RCA initiates from hybridization between a sample DNA and a circular probe (Step 1). Once a sample DNA and circular probe form a complex, DNA polymerase synthesizes a long concatenated sequence copy of the circular probe through linear rolling circle amplification (LRCA) (Step 2). Then, multiple circular probes hybridize to multiple sites of the LRCA product, and nicking enzyme recognition sequences of the LRCA product are activated by double-strand formation (Step 3). Nicking enzyme recognizes and cleaves the recognition sequences and produces multiple complexes of a "primer" and circular probe (Step 4). Each complex can initiate the next round of PG-RCA

Since the product of RCA remains attached to the primer, RCA is amenable to an on-chip probe amplification system (Fig. 17.3a). In this way, the target molecule can be recognized, amplified, and detected directly on a solid support, such as a microarray platform. With RCA, Nallur et al. [7] reported in 2001 that they were able to detect 480 fM (150 molecules) of spotted primers, corresponding to an 8,000-fold increase in detection sensitivity over hybridization under the same conditions. The levels of amplification by RCA on microarray were comparable to those achieved in solution-phase format, indicating that RCA can function with virtually 100% efficacy when used on microarray. Thus, combination of RCA and DNA microarray allows the real-time detection of multiple targets with great sensitivity and specificity [8].

Recently, an RCA-based protein detection method, referred to as immuno-RCA, has been developed [9–12]. In this scheme, a primer is linked to an antibody and the signal is amplified by RCA (Figs. 17.2b and 17.3b). Detection of allergen-specific IgE in blood samples using this approach was demonstrated in a microarray format [13, 14]. Wiltshire et al. [14] printed several allergen extracts, including cat dander, house dust mites, and peanuts onto a glass slide, which was then incubated with 10 μL of patient's serum to allow anti-allergen antibody to bind. After washing, an anti-IgE antibody tagged with an RCA primer complexed with its complementary precircularized C-probe was added to the slide. The RCA products were visualized with a microarray scanner after hybridization with fluorescence-labeled probe complementary to RCA products. With this system, the authors tested 30 patients whose

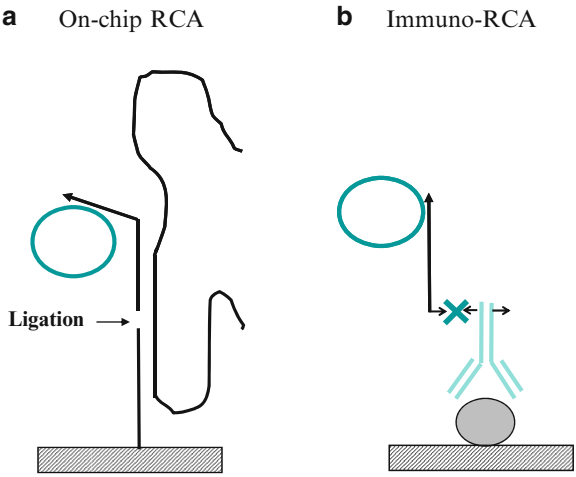

Fig. 17.3 Schematic representation of on-chip RCA and immuno-RCA. (**a**) A probe with a portion of the sequence complementary to a target is spotted onto a support. An RCA primer contains a 5′ region complementary to the target sequence adjacent to the spotted probe and a 3′ region complementary to C-probe. In the presence of target, the RCA primer links to the spotted probe by ligation. The C-probe is amplified by RCA and the resulting single-stranded DNA is linked to the probe spotted on the support. (**b**) An antibody tagged with an RCA primer binds to a protein spotted onto a support. The RCA primer links to an antibody through the interaction of biotin–avidin–biotin. The bound C-probe is amplified by RCA and the resulting single-stranded DNA remains linked to the antibody

allergen status has been confirmed by a skin prick test. The authors compared the assay with a commercially available kit, autoCAP (Pharmacia), and found that immuno-RCA was more sensitive than autoCAP for peanuts and cat dander but not house dust mites. The specificity of immuno-RCA was above 90%, which was more superior to that of autoCAP. Although relatively small group of allergens were tested on a small number of patients, the study showed that immuno-RCA on microarray holds great promise for allergen testing.

Ramification Amplification

Ramification amplification (RAM) [2, 15], also referred to as hyperbranched RCA [16] or cascade RCA [17], is a novel, isothermal DNA amplification that amplifies a C-probe exponentially through the mechanism of primer extension, strand displacement, and ramification. In contrast to RCA, the RAM assay utilizes two primers, one complementary to the C-probe (forward) and the other identical in sequence to a second binding site in the C-probe (reverse). As with RCA, the initial rolling circle primer extension process generates a long ssDNA. However, as the ssDNA molecule expands, multiple reverse primers are able to bind to the growing ssDNA and initiate a second “round” of primer extension templated by the initial “rolling

circle" products. Once a downstream primer encounters a bound upstream primer, the polymerase displaces the upstream bound primer along with any extended sequence that may be attached to it. The displaced ssDNAs serve as templates for further primer extension and amplification (Fig. 17.1c). Like the constant unfurling of streamers, multiple primer extensions take place simultaneously, resulting in a large ramified complex. Since the displaced DNAs are single stranded, the binding of primers occurs at a constant temperature, thus obviating the need for thermocycling to generate ssDNA, as in the case for LCR primers. Some of the properties of RAM are summarized in Table 17.1.

The practical use of RAM has been shown in several studies for detecting target nucleic acids in clinical samples. Zhang et al. [18] were able to detect *Chlamydia trachomatis* in cervical specimens collected in PreservCyt cytological solution. Thirty clinical specimens were tested using the RAM assay and the assay conferred accurate detection of all the positive samples that were confirmed by PCR and LCx. The RAM assay can detect as few as ten *C. trachomatis* elementary bodies in less than 2 h, similar to the lower limit of detection for Amplicor PCR and LCx. Therefore, the RAM assay can serve as a feasible alternative to PCR and LCx for the detection of sexually transmitted infectious agents owing to its simplicity and isothermal amplification conditions. Yi et al. [19] reported the incorporation of molecular zipper into the RAM assay, and they were able to detect as few as 10 molecules within 90-min reaction. A linear relationship was observed between initial input of targets and threshold time ($R^2 = 0.985$).

The RAM assay was also used in the identification of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) in food and human samples. Combining magnetic bead-based DNA isolation, amplification of an *stx2*-specific C-probe by RAM, and real-time fluorescence detection, Li and colleagues [20] accurately identified all 27 pathogenic *E. coli* isolates producing Shiga toxin 2 from food and human samples, as previously confirmed by PCR using primers specific for the *stx2* gene. One *Shigella dysenteriae* and three nonpathogenic *E. coli* were found negative by RAM assay. With respect to such application, the RAM assay provides a simple yet sensitive method that can be readily employed in clinical laboratories for the detection of food-borne pathogens and in meat product inspections.

Rector et al. reported the use of a modified RAM technique, multiple-primed RCA with phi29 DNA polymerase, for the amplification of circular virus and cloning the sequence [21] (Fig. 17.4). They employed an isothermal RAM protocol and random hexamer primers to amplify the complete genomes of papillomaviruses without the need for prior knowledge of their DNA sequences. They were able to clone the complete HPV-16 genome from the RAM product. The optimized protocol was subsequently applied to a bovine fibropapillomatous wart tissue sample. Whereas no papillomavirus DNA could be detected by restriction enzyme digestion of the original sample, multiply-primed RCA enabled us to obtain a sufficient amount of papillomavirus DNA for restriction enzyme analysis, cloning, and subsequent sequencing of a novel variant of bovine papillomavirus type 1. Whole genomic amplification of viral sequences using random-primed RAM allows unbiased representation of the viral genome [22].

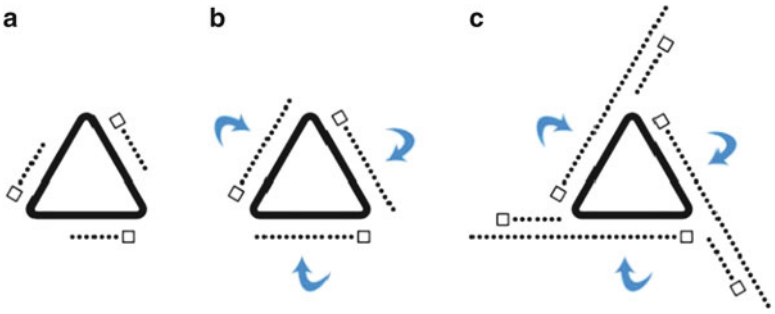

Fig. 17.4 Schematic representation of the random-primed RAM method for amplification of the circular viral DNA genomes. Exonuclease-protected random hexamer primers (—) anneal to multiple sites on the template DNA, after which the φ29 DNA polymerase (•) binds (**a**) and isothermally extends these primers at the 3′ end (→) (**b**). Strand displacement synthesis occurs when the DNA polymerase reaches a downstream extended primer, and hexamer primers can anneal to the displaced single-stranded product strands and will again be elongated by the φ29 DNA polymerase (**c**). Continuation of this process results in exponential amplification of the template DNA, generating linear double-stranded, high-molecular-weight repeated copies of the complete PV genome. Digestion of this multiply-primed RCA product with a restriction enzyme which has only a single recognition site in the viral genome resulted in multiple double-stranded, linear copies of the complete genomic DNA, which can be cloned into a vector for downstream analysis

Signal-Mediated Amplification of RNA Technology

Signal-mediated amplification of RNA technology (SMART) is a novel isothermal amplification technology which utilizes a three-way junction (3WJ) structure to facilitate target-dependent production of multiple copies of an RNA product [23, 24]. The 3WJ structure is composed of two target-specific ssDNA probes (the "template" probe and the "extension" probe) and a target sequence. Both probes have a longer region that hybridizes to the target at adjacent sites and a shorter region that only hybridizes to each other in the presence of the target, thus forming the 3WJ structure (Fig. 17.5a). In addition, the template probe also contains a nonfunctional single-stranded T7 RNA polymerase promoter sequence. Following 3WJ formation and addition of *Bst* DNA polymerase, the polymerase extends the short probe (extension probe) along the single-stranded template probe to form a functional double-stranded promoter for T7 RNA polymerase. In the presence of T7 RNA polymerase, multiple copies of RNA can be synthesized (Fig. 17.5b). Both *Bst* DNA polymerase and T7 RNA polymerase can function under the same reaction condition, hence the reaction can be performed in a single tube. In order to further improve the signal, a second template oligonucleotide (probe for RNA amplification) containing a second T7 promoter sequence can be added to the reaction to allow the RNAs generated from 3WJ to bind, which, in turn, allows its extension by *Bst* DNA polymerase and generation of secondary RNAs by T7 RNA polymerase, ultimately leading to a further increase in RNA yield (Fig. 17.5b). The RNA product can be measured by an enzyme-linked oligosorbent assay. This assay is capable of generating

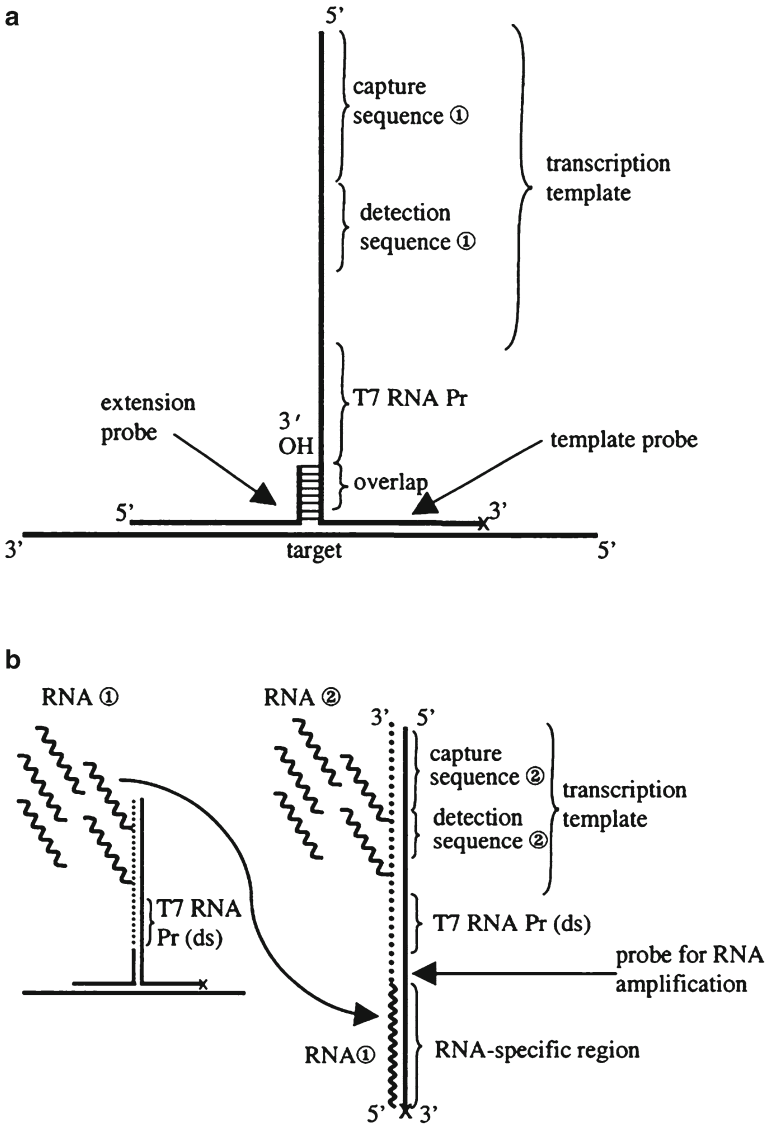

Fig. 17.5 Schematic representation of the SMART assay. (**a**) Formation of a 3WJ. Extension and template probes anneal to the target, and only then they can hybridize with each other. The short extension probe has a free 3′-OH to allow extension. The template probe includes a single-stranded (nonfunctional) T7 RNA polymerase promoter (Pr) and sequences to allow the capture and detection of the RNA signal. The 3′ end of the template probe is blocked (x) by phosphorylation to prevent extension. (**b**) Extension and transcription generate an RNA signal. *Bst* DNA polymerase extension of the extension probe generates a double-stranded (ds), hence functional, T7 RNA polymerase promoter (Pr), allowing transcription of multiple copies of an RNA signal (RNA1) by T7 RNA polymerase. If required, RNA1 anneals to a second template (probe for RNA amplification), leading to further extension and transcription by the DNA and RNA polymerases to generate increased amounts of a second RNA signal (RNA2)

a detectable signal from 50 nmol single-stranded synthetic target, 10 ng bacterial genomic DNA, or 0.1 ng total bacterial RNA (or 10^4 bacteria) [23]. Some of the properties of SMART are summarized in Table 17.1.

Hall et al. demonstrated that SMART can detect both synthetic oligonucleotide targets and genomic cyanophage DNA and specific signals were obtained for each cyanophage strain (S-PM2 and S-BnMI). Nonspecific genomic DNA did not produce false signals or inhibit the detection of a specific target. Therefore, SMART assay can be used to discriminate two similar target sequences [24]. Later, the same group reported that SMART was suitable for differentiating between virus-infected and noninfected cells and for detecting virus gene expression: the first report of using this technology for such application [25].

Levi et al. evaluated the SMART assay (CytAMP assay kit, Cytocell Ltd., Adderbury, Oxford, UK) for the rapid detection of methicillin (oxacillin)-resistant *Staphylococcus aureus* (MRSA) [26]. Two sets of probes were designed against the *coa* (coagulase) and *mecA* (methicillin resistance) genes; hence, simultaneous identification of *S. aureus* and methicillin (oxacillin) resistance is possible. The detection limit of the assay was 2×10^5 and 10^6 CFU/assay for *mecA* and *coa*, respectively. When tested with *S. aureus* isolates, the assay detected 113 MRSA among 396 *S. aureus* with 100% sensitivity and specificity, compared with a *mecA–femB* PCR assay. When 100 enrichment broths containing sets of screening swabs from individual patients were tested, the presence of MRSA was detected in 19, 24, and 31 enrichment broths by SMART assay, conventional culture, and *mecA–femB* PCR, respectively. Six enrichment broths were found negative by SMART assay but positive by both PCR and culture. Five of these contained an equivalence of 10^2–10^5 CFU/assay (below the predicted detection limit of 2×10^5 CFU/assay for SMART assay), and the sixth contained an equivalence of 10^6 CFU/assay. Overall, culture and SMART had similar sensitivities and specificities relative to those of PCR.

Invader Assay

The Invader assay is a unique, isothermal amplification technology that can detect DNA or RNA with high specificity and sensitivity. The basis for the Invader assay is the cleavage of a unique secondary structure formed by two partially overlapping oligonucleotides (an allele-specific primary probe and an invader probe) that hybridize to a target sequence to create a "flap" [27] (Fig. 17.6). Cleavase VIII (Flap endonuclease I from *Archaeoglobus fulgidus*) recognizes this 3D structure as a specific substrate and cleaves the 5′ flap of the primary probe. The flap initiates a secondary reaction in which the released 5′-flap serves as an invader probe on a fluorescence resonance energy transfer (FRET) cassette to create another overlapping tertiary structure that is, in turn, recognized and cleaved by the Cleavase® enzyme (Fig. 17.6a). The Invader assay is optimal with a high concentration of primary probe and at temperatures near its melting temperature (60°C) at which the primary

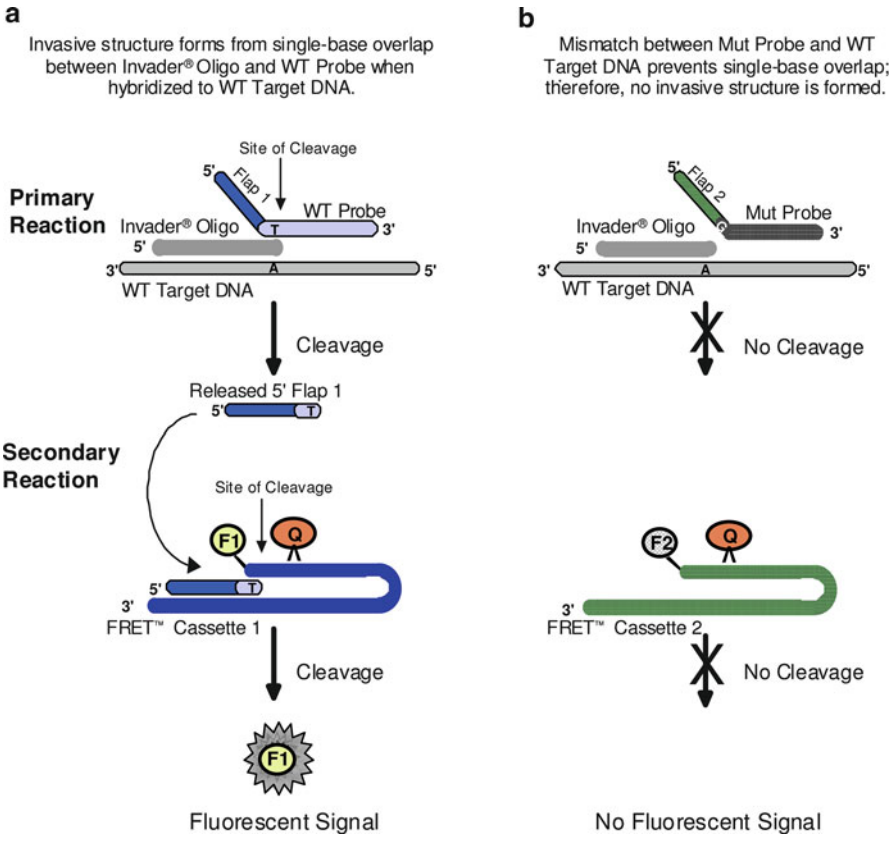

Fig. 17.6 Schematic representation of the Invader assay. During an initial reaction, a discriminatory primary probe (WT probe (**a**); Mut probe (**b**)) and an invader oligo hybridize to the target, overlapping at the SNP position and forming a 3D flap structure that is recognizable by the cleavase enzyme at this site. The flap subsequently anneals to an FRET cassette in a separate reaction and initiates secondary cleavage that releases a fluorescent dye detectable by a fluorometer. Fluorescence is detectable only when a match occurs **(a)**; if the primary probe is mismatched, cleavase remains inactive and no fluorescence is detected **(b)**

probe can easily cycle on and off the target for cleavage. When the FRET cassette is cleaved, a fluorophore dissociates from the quencher labeled on the FRET cassette, emitting a detectable fluorescence signal proportional to the target sequence. Some of the properties of Invader assay are summarized in Table 1.

Wong et al. [28] utilized the Invader assay to detect hepatitis B virus (HBV) in patients' serum and liver biopsies. Three different viral DNA structures occur in HBV life cycle: linear double-stranded DNA (non-replicative), relaxed circle DNA, and covalently closed circular DNA (cccDNA) which serves as the template for the production of viral and pregenomic messenger RNA. Because the specific 3D structure is required for cleavase, the Invader assay is an ideal method to detect various

forms of HBV as well as HBV viral load. Wong et al. [28] designed two sets of Invader probes targeting direct repeat 2 region, in which the negative and positive strands anneal together to bring both ends of the linear form of HBV DNA together to form a relaxed circle. Both Invader probe signals should be detected if cccDNA is present, one probe signal for relaxed circle DNA and one for linear DNA. In their study, the lower limit of detection was 50 copies/assay or 0.0002 copies/cell for hepatic tissue or 10^4 copies/ml for serum with a dynamic range of 5 orders of magnitude. cccDNA was detected in liver biopsy tissue in 16 hepatitis B e antigen (HBeAg)-positive and 36 antibody-to-HBeAg-positive (anti-HBe-positive) chronic hepatitis B patients and these results correlated positively with the total intrahepatic HBV DNA. Anti-HBe-positive patients had lower median total intrahepatic HBV DNA and intrahepatic cccDNA levels than HBeAg-positive patients. However, the proportion of intrahepatic HBV DNA in the form of cccDNA was inversely related to the amount of total intrahepatic HBV DNA. A small amount of cccDNA was detected in 39 of 52 (75%) serum samples. Anti-HBe-positive patients had lower median serum cccDNA levels than HBeAg-positive patients. Serum HBV DNA correlated positively with intrahepatic total HBV DNA and intrahepatic cccDNA. Serum and intrahepatic total HBV DNA and cccDNA levels diminish as the disease progresses from HBeAg-positive to anti-HBe-positive phase, with cccDNA becoming the predominant form of intrahepatic HBV DNA.

More recently, the United States Food and Drug Administration (FDA) approved the Third Wave/Hologic Invader HPV high-risk test (rebranded as Cervista™ HPV HR Test). The HPV HR test applies Invader Chemistry Platform to detect 14 HPV high-risk DNAs, and the specific HPV16 and 18 genotyping, which can be used as a reflex to the initial high-risk screen test. The Cervista™ HPV HR test performs in a manner comparable to the Qiagen (Digene) hc2 test, which is a widely used FDA-approved HPV test [29, 30].

The Invader assay could be a sensitive method for detecting certain mutations associated with drug resistance in microbial pathogens. Cooksey et al. [31] applied the Invader assay to detect mutations associated with resistance to rifampicin (RIF) and isoniazid (INH) in *Mycobacterium tuberculosis*. Nine pairs of probes, five for mutations in *rpoB* gene (resistance to RIF) and *katG* gene (resistance to INH) and 4 for the corresponding wild-type (drug-susceptible) alleles, were synthesized. Each allele-specific primary probe had a different length of 5′ flap (from 4 to 13 nucleotides) and was labeled with different fluorophores. The PCR-amplified DNA fragments were tested and the fluorescence-labeled cleavage products were resolved by denaturing polyacrylamide (20–24%) gel electrophoresis. All 9 alleles could be identified and differentiated on the basis of product size. Multiple mutations of the *rpoB* gene in PCR products could be identified, as could mutants that were present at ≥0.5% of the total population of PCR products.

In addition, the Invader platform was used in the International HapMap Project, a multinational research collaboration to develop a freely available haplotype map of the human genome. Invader was one of the five platforms used for high-throughput genotyping of over one million SNPs [32] and was used to generate the haplotypes for 25% of the human chromosomes.

Multiplex Ligation-Dependent Probe Amplification

MLPA is a multiplex PCR method detecting abnormal copy numbers of up to 50 different genomic DNA or RNA sequences and is able to distinguish sequences differing in only one nucleotide [33]. MLPA is a variation of the polymerase chain reaction that permits multiple targets to be amplified with only a single primer pair [34]. Each MLPA probe consists of a two oligonucleotides which recognize adjacent target sites on the DNA. One probe oligonucleotide consists of a 5′ fluorescent label, a universal forward primer binding site, and a target-specific recognition sequence at the 3′ end, whereas the other probe oligonucleotide consists of a target-specific recognition sequence at the 5′ end, a nonspecific stretch of DNA of defined length ("stuffer" sequence), and a universal reverse primer binding site at the 3′ end. Only when both probe oligonucleotides are hybridized to their respective targets, can they be ligated into a complete probe. The advantage of splitting the probe into two parts is that only the ligated oligonucleotides, but not the unbound probe oligonucleotides, are amplified. If the probes were not split in this way, the primer sequences at either end would cause the probes to be amplified regardless of their hybridization to the template DNA, and the amplification product would not be dependent on the number of target sites present in the sample DNA. Each complete probe has a unique length because of varying the length of "stuffer" sequence for each set of probes, so that its resulting amplicons can be separated and identified by (capillary) electrophoresis. The MLPA platform features greatly reduced probe concentrations and longer hybridization periods to generate conditions compatible with multiplex analysis [35]. MLPA is of low cost and easy to use. However, it requires a capillary electrophoresis equipment which is very expensive. MLPA-based techniques have proved sufficiently sensitive, reproducible, and sequence specific for use in screening human DNA. Some of the properties of MLPA are summarized in Table 17.1.

The MLPA reaction can be divided into four major steps: (a) DNA denaturation and hybridization of MLPA probes; (b) ligation reaction; (c) PCR; and (d) separation of amplification products by (capillary) electrophoresis. During the first step, the DNA is denatured and incubated overnight with a mixture of MLPA probes. Each set of probe oligonucleotides hybridize to immediately adjacent target sequences (Fig. 17.7, step a). Only when the two probe oligonucleotides are both hybridized to their adjacent targets can they be ligated during the ligation reaction (Fig. 17.7, step b). The ligated probes will be exponentially amplified during the subsequent PCR (Fig. 17.7, step c). The amplification products are separated using capillary electrophoresis (Fig. 17.7, step d). Probe oligonucleotides that are not ligated only contain one primer sequence. As a consequence, they cannot be amplified exponentially and will not generate a signal. The removal of unbound probes is therefore unnecessary in MLPA and makes the MLPA method easy to perform.

Since its introduction in 2002, MLPA has gained widespread clinical acceptance for the identification of gene copy number changes in a broad range of predominantly genetic diseases [36–38]. MLPA assay was used for the detection and identification of several pathogenic microorganisms, including rapid characterization of

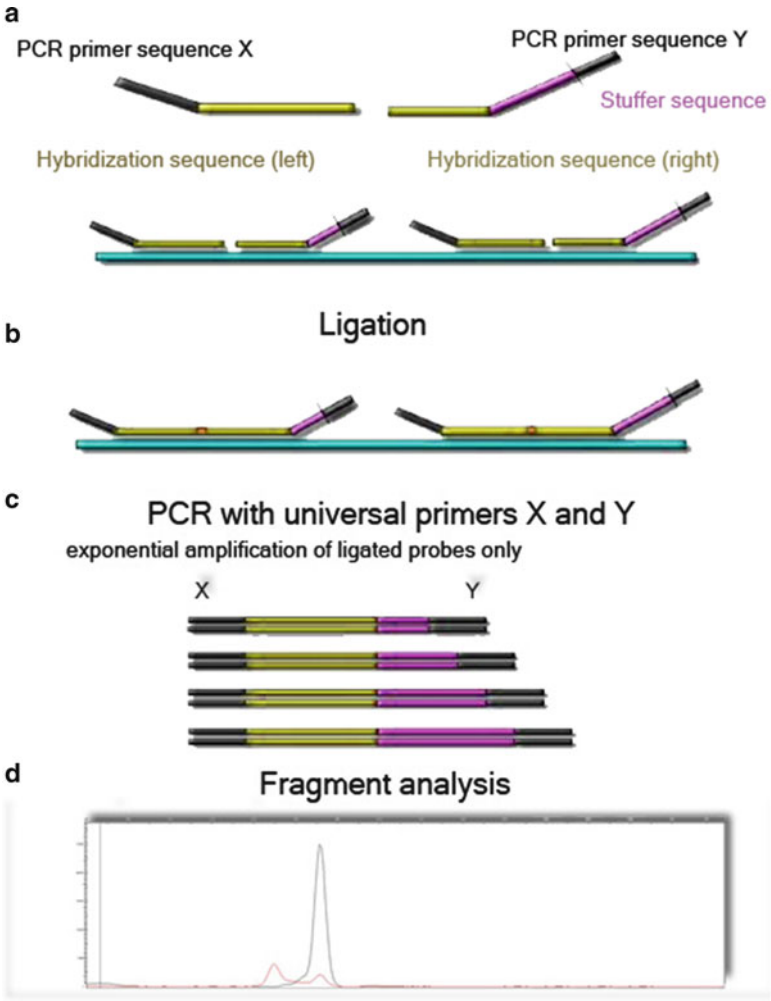

Fig. 17.7 Schematic representation of MLPA method. The MLPA reaction can be divided into four major steps: (**a**) DNA denaturation and hybridization of MLPA probes; (**b**) ligation reaction; (**c**) PCR; and (**d**) separation of amplification products by (capillary) electrophoresis

M. tuberculosis [39], and relative quantification of targeted bacterial species in oral microbiota [40]. Recently, the MLPA technology was applied for the first time for detection of infectious agents causing respiratory tract infections [41]. Reijans and colleagues described an MLPA-based RespiFinder assay to detect 15 respiratory viruses simultaneously in one reaction. In this case, the MLPA reaction was preceded by a preamplification step that ensured detection of both RNA and DNA viruses with the same specificity and sensitivity as individual monoplex real-time RT-PCR assays. The RespiFinder assay showed satisfactory specificity and sensitivity for adenovirus, human metapneumovirus (hMPV), Flu-A, parainfluenza virus

(PIV) types 1 and 3, rhinovirus (RhV), and RSV. Use of the RespiFinder assay resulted in a 24.5% increase in the diagnostic yield compared with cell culture.

Cycling Probe Technology

Cycling probe technology (CPT) is an isothermal probe amplification method [42] (Fig. 17.8). The probe is a single-stranded oligonucleotide, approximately 25–30 bases in length, containing a short run of four to six ribonucleotides flanked by deoxynucleotides (i.e., chimeric DNA–RNA–DNA). The CPT reaction is carried out at a single elevated temperature (55–65°C) in the presence of thermostable RNase H, an enzyme that degrades RNA portion of the probe–target hybrid. The DNA portions of the probe have lower thermal stability (melting temperature) than those of the intact probe. At the reaction temperature, the probe fragments dissociate from the target sequence, leaving the target free to hybridize to another probe molecule. The cleaved products can be observed using a variety of methods, most commonly by gel electrophoresis. The assay is a linear reaction with analytical sensitivity of 6×10^5 copies/reaction [43, 44]. Although the scale of amplification is limited, this assay does provide an easy means of quantitating target DNA with the aid of fluorescence labeling. Some of the properties of CPT are summarized in Table 17.1.

CPT assay in combination with a lateral-flow strip was used to detect the *mecA* gene from MRSA in cultures [45]. The *mecA* probe was labeled with fluorescein at the 5′ terminus and biotin at the 3′ terminus. The nitrocellulose was impregnated with streptavidin and immunoglobulin G antibody. In the absence of the *mecA* gene, the uncut probe is bound to an antifluorescein–gold conjugate and subsequently captured by streptavidin to form a test line. In the presence of the *mecA* gene, the probe is cut

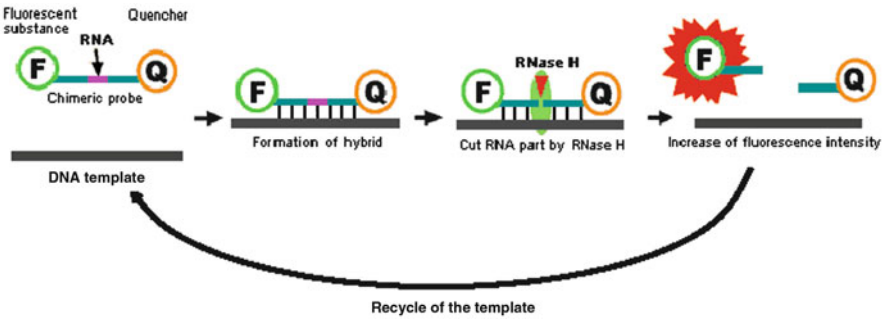

Fig. 17.8 Cycling probe technology. A sequence-specific single-stranded probe (approx. 25–30 nucleotides) contains an internal stretch of 4–6 ribonucleotides (RNA) flanked by deoxyribonucleotides (DNA). The probe is labeled with a fluorophore and a quencher, which hybridizes to the target sequence. Thermostable RNase H binds to the RNA/DNA duplex region and cleaves the RNA segment. Since thermal stability of the resulting cleaved products is lower than the intact probe, the products dissociate from the target sequence and the target sequence then becomes available to hybridize with another intact CPT probe. The cleaved probe emits fluorescence and is detected by a fluorometer

and no test line is formed on the strip. A screen of 324 *S. aureus* clinical isolates by CPT-strip assay revealed a 99.4% sensitivity and a 100% specificity compared to the results of PCR for the detection of the *mecA* gene. The assay takes 1.5 h, starting from a primary culture to the time of detection of the *mecA* gene in *S. aureus* isolates.

Most recently, a modified CPT technique called isothermal target and signaling probe amplification (iTPA) was introduced in 2010 [46] and is a combination of novel isothermal chain amplification (ICA) and FRET CPT for simultaneous target and signal amplification and the quantitative detection of nucleic acids. The essential issue in isothermal amplification methods is the achievement of denaturation of double-stranded DNA under isothermal conditions. The strategy devised for this purpose relies on two main displacement events that are mediated by the RNA degrading activity of RNase H within an RNA/DNA heteroduplex and the strand displacement activity of DNA polymerase during DNA extension. Since the two distinct displacement events are designed to take place simultaneously and lead to two different amplification routes, they can produce remarkably high amplification efficiencies even under isothermal conditions. The detailed mechanism and reaction steps of ICA are illustrated in Fig. 17.9. Through these automatically repeated cycles, highly enhanced amplification of double-stranded DNA, including the target region, can occur. In the rationally designed, isothermal autoamplification system, three differently sized amplification products are generated. The main product is double-stranded DNA including either the forward inner primer or the reverse inner primer (blue orbit in Fig. 17.9). Besides the main amplification product, two other double-stranded DNAs are also expected to form, one that includes both the forward and the reverse inner primers (green orbit in Fig. 17.9) and the other that is composed of only the 3′ end DNA region inside both the forward and reverse inner primers (purple orbit in Fig. 17.9). By simultaneously utilizing the dual amplification powers of the target DNA and FRET probes, iTPA can be used to quantitatively detect a model *C. trachomatis* gene down to single copy level.

Summary and Future Direction

In the past decade, probe amplification technologies have advanced significantly, from the initial description of Q-beta replicase amplification in 1986 [47] to the most recently introduced RAM [2] and iTPA [46]. It is expected that more probe amplification methods will be invented in the next 10 years and the applications of the current probe amplification methods will become more diversified. Homogeneous and real-time monitoring of amplification will be devised to probe amplification technologies to reduce detection time and improve quantification capability of the assay. Additional technologies will be developed to be used for the detection of RNA, DNA, and protein (antigen/antibody) on a single platform, which will further enhance the detection sensitivity and specificity. Finally, the applications of these technologies will become broader as the fields of genomics, proteomics, and pharmacogenomics advance. Therefore, a technology that offers in situ detection and amplification, microarray, immunoassay, real-time monitoring, whole genome amplification, and SNP detection

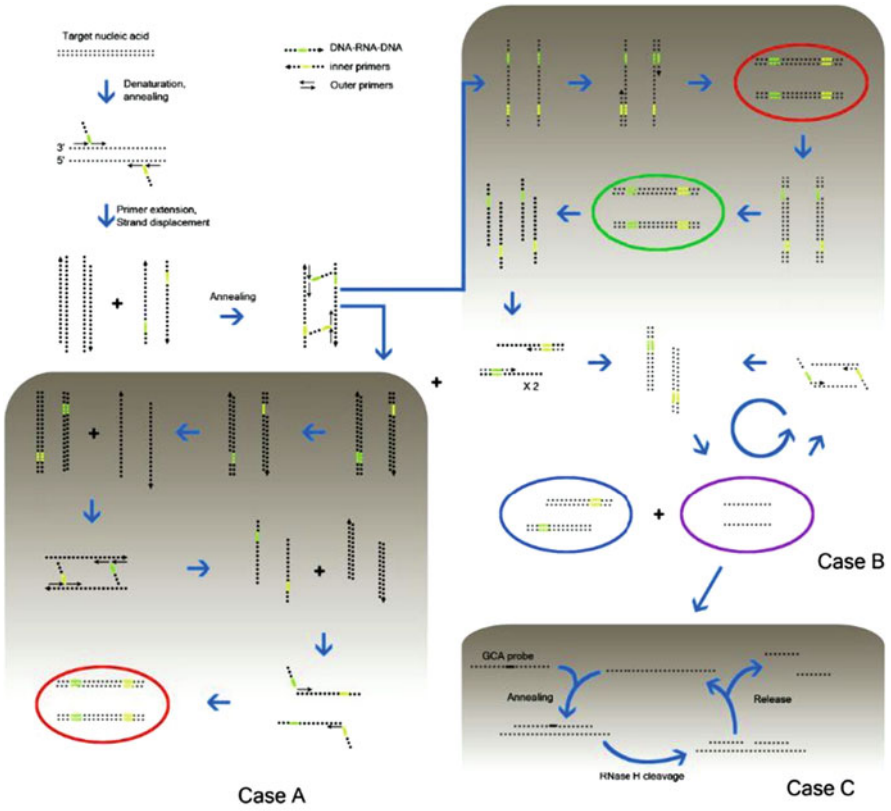

Fig. 17.9 Overview of the isothermal target and signaling probe amplification (iTPA) system. The first step in the scheme involves denaturation of the target DNA mixed with an outer primer and a DNA–RNA–DNA chimeric inner primer set. The double-stranded DNA resulting from the extension of the outer primer follows Case A while the released single-stranded DNA follows Case B shown in Fig. 17.9. In case A, the RNA region at the 5′ end of the inner primer-containing strand of the double-stranded DNA, formed in the previous step, is degraded by RNase H. As the primer annealing, extension, and strand displacement steps progress, specific DNA/RNA heteroduplex forms are generated, as depicted in the red orbit in Fig. 17.9. As represented by Case B, simultaneously, the inner primers are annealed to the inner primer-containing single-stranded DNA generated in the previous step. The annealed primers are extended to form the same DNA/RNA heteroduplex structures as those shown in the red orbit. In a way, represented by the last part, an auto-cycling route, which includes RNA digestion, DNA extension, strand displacement, and primer annealing, followed by the extension, is derived from the heteroduplex forms shown in the red orbit for both cases A and B

will be more favorable. However, no single technology can meet all of these requirements and possible combination of these technologies may be the answer. Also, PCR, the dominant amplification technology, cannot fulfill all these needs and ample room is available for probe amplification technology to grow.

On the other hand, the instrumentation for probe amplification will change significantly in the next 10 years. Fluorescence-based real-time detection instrument will be widely used in diagnostic laboratory which will certainly improve throughput. Miniaturized microfluidic assay format will soon be available in clinical laboratory that will significantly reduce sample volume. Automation and miniaturization of the instrument will make molecular diagnosis at doctor's office and bedside possible. It is expected that the array-based assay and instrument will be significantly improved and the cost will be reduced to an affordable level. Given the advantages of probe amplification (isothermal, multiplex, on-chip amplification, etc.), probe-based amplification could be easily adapted in these formats and will become the dominant technologies in clinical diagnostic applications.

However, most described probe amplification technologies are still at the early stage of development. Most publications only demonstrated the feasibility in clinical diagnosis and their clinical performance has not yet been demonstrated in large clinical trials. It is anticipated that some of these technologies may not meet the clinical diagnostic requirements and will consequently be lost in market competition. For example, Q-beta replicase technology did not reach the clinical laboratory even after initial favorable clinical trial and the LCx assay (LCR technology) for Chlamydia was voluntarily withdrawn by Abbott in 2003 due to significant reproducibility problems [48]. Therefore, it is expected that more changes (exciting or disappointing) will happen in the field of probe-based amplification technologies in the next 10 years.

References

1. Nilsson M, Malmgren H, Samiotaki M, Kwiatkowski M, Chowdhary BP, Landegren U (1994) Padlock probes: circularizing oligonucleotides for localized DNA detection. Science 265: 2085–2088
2. Zhang DY, Brandwein M, Hsuih TC, Li H (1998) Amplification of target-specific, ligation-dependent circular probe. Gene 211:277–285
3. Baner J, Nilsson M, Mendel-Hartvig M, Landegren U (1998) Signal amplification of padlock probes by rolling circle replication. Nucleic Acids Res 26:5073–5078
4. Fire A, Xu SQ (1995) Rolling replication of short DNA circles. Proc Natl Acad Sci U S A 92: 4641–4645
5. Zhang DY, Zhang W, Li X, Konomi Y (2001) Detection of rare DNA targets by isothermal ramification amplification. Gene 274:209–216
6. Murakami T, Sumaoka J, Komiyama M (2009) Sensitive isothermal detection of nucleic-acid sequence by primer generation-rolling circle amplification. Nucleic Acids Res 37:e19
7. Nallur G, Luo C, Fang L et al (2001) Signal amplification by rolling circle amplification on DNA microarrays. Nucleic Acids Res 29:E118
8. Mayer-Enthart E, Sialelli J, Rurack K, Resch-Genger U, Koster D, Seitz H (2008) Toward improved biochips based on rolling circle amplification – influences of the microenvironment on the fluorescence properties of labeled DNA oligonucleotides. Ann N Y Acad Sci 1130:287–292
9. Schweitzer B, Wiltshire S, Lambert J et al (2000) Immunoassays with rolling circle DNA amplification: a versatile platform for ultrasensitive antigen detection. Proc Natl Acad Sci U S A 97:10113–10119

10. Schweitzer B, Roberts S, Grimwade B et al (2002) Multiplexed protein profiling on microarrays by rolling-circle amplification. Nat Biotechnol 20:359–365
11. Terletskaia-Ladwig E, Leinmuller M, Schneider F, Meier S, Enders M (2007) Laboratory approaches to the diagnosis of adenovirus infection depending on clinical manifestations. Infection 35:438–443
12. Kobori T, Matsumoto A, Takahashi H, Sugiyama S (2009) Rolling circle amplification for signal enhancement in ovalbumin detection. Anal Sci 25:1381–1383
13. Kim TE, Park SW, Cho NY et al (2002) Quantitative measurement of serum allergen-specific IgE on protein chip. Exp Mol Med 34:152–158
14. Wiltshire S, O'Malley S, Lambert J et al (2000) Detection of multiple allergen-specific IgEs on microarrays by immunoassay with rolling circle amplification. Clin Chem 46:1990–1993
15. Zhang DY, Brandwein M, Hsuih T, Li HB (2001) Ramification amplification: a novel isothermal DNA amplification method. Mol Diagn 6:141–150
16. Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC (1998) Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet 19:225–232
17. Thomas DC, Nardone GA, Randall SK (1999) Amplification of padlock probes for DNA diagnostics by cascade rolling circle amplification or the polymerase chain reaction. Arch Pathol Lab Med 123:1170–1176
18. Zhang W, Cohenford M, Lentrichia B et al (2002) Detection of *Chlamydia trachomatis* by isothermal ramification amplification method: a feasibility study. J Clin Microbiol 40:128–132
19. Yi J, Zhang W, Zhang DY (2006) Molecular Zipper: a fluorescent probe for real-time isothermal DNA amplification. Nucleic Acids Res 34:e81
20. Li F, Zhao C, Zhang W et al (2005) Use of ramification amplification assay for detection of *Escherichia coli* O157:H7 and other E. coli Shiga toxin-producing strains. J Clin Microbiol 43:6086–6090
21. Rector A, Tachezy R, Van Ranst M (2004) A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. J Virol 78:4993–4998
22. Burtt NP (2011) Whole-genome amplification using Phi29 DNA polymerase. Cold Spring Harb Protoc 2011(1):pdb.prot5552
23. Wharam SD, Marsh P, Lloyd JS et al (2001) Specific detection of DNA and RNA targets using a novel isothermal nucleic acid amplification assay based on the formation of a three-way junction structure. Nucleic Acids Res 29:E54-4
24. Hall MJ, Wharam SD, Weston A, Cardy DL, Wilson WH (2002) Use of signal-mediated amplification of RNA technology (SMART) to detect marine cyanophage DNA. BioTechniques 32:604–606, 608–611
25. Wharam SD, Hall MJ, Wilson WH (2007) Detection of virus mRNA within infected host cells using an isothermal nucleic acid amplification assay: marine cyanophage gene expression within *Synechococcus* sp. Virol J 4:52
26. Levi K, Bailey C, Bennett A, Marsh P, Cardy DL, Towner KJ (2003) Evaluation of an isothermal signal amplification method for rapid detection of methicillin-resistant *Staphylococcus aureus* from patient-screening swabs. J Clin Microbiol 41:3187–3191
27. Lyamichev VI, Kaiser MW, Lyamicheva NE et al (2000) Experimental and theoretical analysis of the invasive signal amplification reaction. Biochemistry 39:9523–9532
28. Wong DK, Yuen MF, Yuan H et al (2004) Quantitation of covalently closed circular hepatitis B virus DNA in chronic hepatitis B patients. Hepatology 40:727–737
29. Kurtycz DF, Smith M, He R, Miyazaki K, Shalkham J (2010) Comparison of methods trial for high-risk HPV. Diagn Cytopathol 38:104–108
30. Stillman MJ, Day SP, Schutzbank TE (2009) A comparative review of laboratory-developed tests utilizing Invader HPV analyte-specific reagents for the detection of high-risk human papillomavirus. J Clin Virol 45(Suppl 1):S73–S77
31. Cooksey RC, Holloway BP, Oldenburg MC, Listenbee S, Miller CW (2000) Evaluation of the invader assay, a linear signal amplification method, for identification of mutations associated

with resistance to rifampin and isoniazid in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 44:1296–1301
32. Deloukas P, Bentley D (2004) The HapMap project and its application to genetic studies of drug response. Pharmacogenomics J 4:88–90
33. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30:e57
34. Park YN, Abe K, Li H, Hsuih T, Thung SN, Zhang DY (1996) Detection of hepatitis C virus RNA using ligation-dependent polymerase chain reaction in formalin-fixed, paraffin-embedded liver tissues. Am J Pathol 149:1485–1491
35. Wu W, Tang YW (2009) Emerging molecular assays for detection and characterization of respiratory viruses. Clin Lab Med 29:673–693
36. Bourdeaut F, Lequin D, Brugieres L et al (2011) Frequent hSNF5/INI1 germline mutations in patients with rhabdoid tumor. Clin Cancer Res 17:31–38
37. Pristauz G, Petru E, Stacher E et al (2010) Androgen receptor expression in breast cancer patients tested for BRCA1 and BRCA2 mutations. Histopathology 57:877–884
38. Damato B, Dopierala JA, Coupland SE (2010) Genotypic profiling of 452 choroidal melanomas with multiplex ligation-dependent probe amplification. Clin Cancer Res 16:6083–6092
39. Bergval IL, Vijzelaar RN (2008) Dalla Costa ER, et al. Development of multiplex assay for rapid characterization of Mycobacterium tuberculosis. J Clin Microbiol 46:689–699
40. Terefework Z, Pham CL, Prosperi AC et al (2008) MLPA diagnostics of complex microbial communities: relative quantification of bacterial species in oral biofilms. J Microbiol Methods 75:558–565
41. Reijans M, Dingemans G, Klaassen CH et al (2008) RespiFinder: a new multiparameter test to differentially identify fifteen respiratory viruses. J Clin Microbiol 46:1232–1240
42. Bekkaoui F, Poisson I, Crosby W, Cloney L, Duck P (1996) Cycling probe technology with RNase H attached to an oligonucleotide. Biotechniques 20:240–248
43. Dickinson Laing T, Mah DC, Poirier RT, Bekkaoui F, Lee WE, Bader DE (2004) Genomic DNA detection using cycling probe technology and capillary gel electrophoresis with laser-induced fluorescence. Mol Cell Probes 18:341–348
44. Modrusan Z, Bekkaoui F, Duck P (1998) Spermine-mediated improvement of cycling probe reaction. Mol Cell Probes 12:107–116
45. Fong WK, Modrusan Z, McNevin JP, Marostenmaki J, Zin B, Bekkaoui F (2000) Rapid solid-phase immunoassay for detection of methicillin-resistant *Staphylococcus aureus* using cycling probe technology. J Clin Microbiol 38:2525–2529
46. Jung C, Chung JW, Kim UO, Kim MH, Park HG (2010) Isothermal target and signaling probe amplification method, based on a combination of an isothermal chain amplification technique and a fluorescence resonance energy transfer cycling probe technology. Anal Chem 82: 5937–5943
47. Chu BC, Kramer FR, Orgel LE (1986) Synthesis of an amplifiable reporter RNA for bioassays. Nucleic Acids Res 14:5591–5603
48. Gronowski AM, Copper S, Baorto D, Murray PR (2000) Reproducibility problems with the Abbott laboratories LCx assay for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. J Clin Microbiol 38:2416–2418

Chapter 18
Signal Amplification Technologies

Ted E. Schutzbank

Introduction

The introduction of target nucleic acid amplification technologies, such as polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), and strand displacement amplification (SDA), was accompanied by a plethora of technology patents. This made it very difficult for companies without access to these amplification technologies to compete in the area of assay development for ultrasensitive infectious disease detection and quantification. One way around this intellectual property roadblock was the development of highly sensitive assays that depended not on target amplification, but signal amplification. Signal amplification technologies have one major advantage over target amplification in that the issue of contaminating one test run with previously amplified material from a previous run is not an issue. Also, the three signal amplification technologies that are discussed in this chapter are isothermal, meaning that unlike PCR, thermocycling instrumentation is not required. Lastly, assays can be designed to detect and or quantify specific DNA or RNA targets using each of these methods, and, in the case of RNA, without the need to first convert the RNA target to DNA via reverse transcription. Care must still be taken, however, to minimize cross-contamination between samples being tested due to the enhanced analytical sensitivity inherent in assays using signal amplification technologies. An inherent concern with signal amplification methods is that great care must be taken during the design of the assay to ensure that carryover of the different assay components, from one step to the next, is minimized to reduce background noise, which will degrade the analytical sensitivity of the test in question.

T.E. Schutzbank (✉)
Covance Central Laboratory Services Inc., 8211 SciCor Drive,
Indianapolis, IN 46214-2985, USA
e-mail: ted.schutzbank@covance.com

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Commercially developed clinical assays based on branched DNA (bDNA, Siemens Healthcare Diagnostics, Deerfield, IL), hybrid capture (HC, Qiagen Inc., Valencia, CA) and Invader™ (Hologic Inc., Bedford, MA) technologies have found broad usage in the clinical laboratory. Each one is discussed individually in this chapter.

Principles and Characteristics of Signal Amplification Technologies

Branched DNA Technology

bDNA is a sandwich nucleic acid hybridization procedure based on the use of a bDNA probe [1, 2]. This methodology relies on a series of hybridization steps that result in the formation of a sandwich complex of probes and target sequences. These are synthetic oligonucleotides that are designed to form a branched structure extending from a primary sequence [3].

The most current version 3 of bDNA, used in commercially available virus quantification assays, comprises the following steps. The first step in any bDNA assay is to ensure that the target organism is completely disrupted, and the target nucleic acid is exposed (Fig. 18.1a). If the target nucleic acid is DNA, a denaturation step must be included in the protocol. This is not necessary for such RNA targets such as human immunodeficiency virus type 1 (HIV)-1 or hepatitis C virus (HCV). The next step is to transfer the sample lysate to a microwell containing an immobilized target capture probe, and the addition of solution capture probes and target probes (Fig. 18.1b). The capture probes, which are in solution, have two "arms," one that binds with high specificity to the complementary sequences on the nucleic acid target, and the other that hybridizes only to the immobilized capture probe. The target probe is also designed to have two domains, one that binds to the target DNA or RNA being detected, and the other that binds specifically to the preamplifier probe that is added in step 3 (Fig. 18.1c). As shown in the figure, the preamplifier probe binds to two target probes in a cruciform-binding structure. This adds increased stability to the binding of the preamplifier probe, which allows for higher stringency washes to be used to remove any unbound probes, to minimize background noise. In step 4, amplifier probes are hybridized to the bound preamplifier probes, forming the bDNA complex that gives the methodology its name (Fig. 18.1d). After the wash step to remove unbound amplifier probes, the label probes are added (Fig. 18.1e). These are alkaline phosphatase-conjugated oligonucleotides that are complementary in sequence to the amplifier probes. Lastly, after the final wash step, a substrate is added (dioxytane) (Fig. 18.1f). Dioxytane (Lumi-Phos, Lumigen Inc., Southfield, MI) is cleaved by alkaline phosphatase, producing a chemiluminescent signal [4]. Light emissions resulting from the assay, measured as relative light units (RLUs), are directly proportional to the amount of target DNA or RNA present in

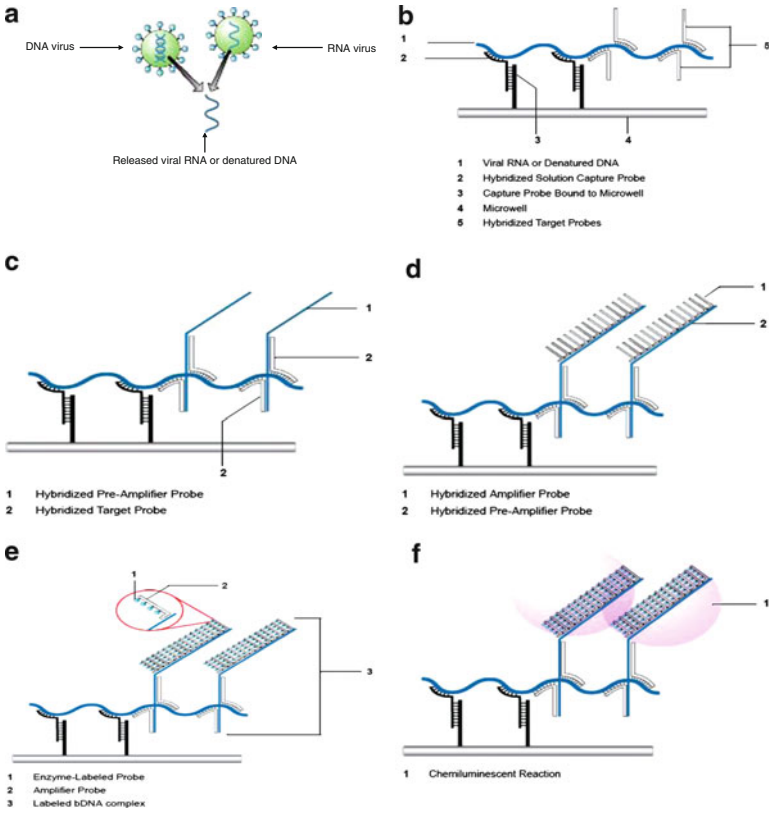

Fig. 18.1 Branched DNA technology. See text for details

the initial sample. The concentration of the viral nucleic acid is determined by a standard curve that is defined by standards processed in the assay along with the clinical samples.

The first generation of bDNA was introduced in the early 1990s. The current bDNA 3.0 version was designed with two new features to increase sensitivity. One of these, the formation of the cruciform hybridization of the preamplifier probes, has already been discussed. The second is the incorporation of two bases not found in nature, Iso5MeC and IsoG into the probe design. Isocytosine (Iso5MeC) and isoguanosine (IsoG) are isomers of cytosine (C) and guanosine (G) and participate in Watson–Crick base pairing with each other, but have unstable interactions with DNA sequences containing natural bases (C and G). Approximately every fourth nucleotide in all probes, except for the solution capture probe (Fig. 18.1b), is Iso5MeC or IsoG. Use of a six-base code allows the design of amplification sequences that do not interact with target sequences or other bDNA components [2]. Thus, the capture probes (on microwell or in solution) do not hybridize with the

amplification complex, therefore reducing nonspecific probe interactions. Using probes made with IsoC and IsoG increases specificity and sensitivity because higher concentrations of probes can be employed. With the amplification complex (preamplifier, amplifier, and AP-conjugated label probes), the potential of hybridization to nontarget nucleic acids is reduced, signal to noise ratio is increased significantly [2] and thus, signal amplification is improved, approaching that of some target amplification technologies such as PCR.

Hybrid Capture

Hybrid capture is a term that describes a signal amplification methodology incorporating synthetic RNA probes complementary to a specific target sequence (e.g., a viral or bacterial DNA target sequence), followed by capture and detection of the RNA/DNA hybrid molecules using an antibody specific for such RNA/DNA heteroduplexes. The production of anti-DNA:RNA antibodies actually dates back to the 1970s [5, 6]. These were polyclonal antibodies elicited by injection of poly (A) · poly (dT) hybrids into rabbits [7]. The development of monoclonal antibodies against heteropolymer duplexes for the detection of a naturally occurring target nucleic acid were elicited using the bacteriophage ΦX174 single-stranded genomic DNA genome hybridized with RNA transcribed in vitro from this template [8]. This same article described the use of such antibodies in the immunodetection of DNA:RNA hybrid molecules. The first report for the use of anti-DNA:RNA antibodies in a clinical assay was in 1993 by Carpenter et al. [9]. This paper described the development of novel oligonucleotide hairpin probe encoding a T7 RNA polymerase promoter. The hairpin probe, and an adjacently hybridizing biotinylated capture probe, were hybridized to target DNA (purified *Chlamydia trachomatis* DNA), and the duplex was captured onto streptavidin-coated magnetic particles. After ligation of the immobilized probes, which served to maintain specificity, the hairpin probe was transcribed by T7 RNA polymerase. The transcribed RNA product was hybridized to a biotinylated capture probe and bound to streptavidin-coated magnetic particles. The immobilized heteroduplex was detected with a DNA:RNA antibody-alkaline phosphatase conjugate. After a wash step to remove unbound antibody-enzyme conjugate the chemiluminescent substrate adamantyl-1,2-dioxetane phenyl phosphate was added, and chemiluminescence measured in a luminometer. This method was shown to detect down to 10 attomoles of *C. trachomatis* DNA in a background of 5 μg of background DNA. Signal amplification using this procedure was achieved both by the linear amplification of the ligatable probes through the RNA transcription process, followed by binding of the enzyme conjugated anti-DNA:RNA antibodies. This methodology, however, was never developed for commercial use.

The first use of anti-DNA:RNA antibodies in a commercial assay was the Hybrid Capture 1 (HC1) human papillomavirus test manufactured by Digene Corporation (now Qiagen Inc.) [10]. Since then, improvements to the methodology have been made with the introduction of Hybrid Capture 2 (HC2). The methodology can use

either a DNA probe to detect an RNA target, or an RNA probe to detect a DNA target. Similar to the bDNA assay already described, the first step in an HC2 test is lysis of the target organism, to release the target nucleic acid. If the target is DNA, a denaturation step is required. Therefore, the first step of an HC2 assay for the detection of a DNA-containing target organism is the addition of a lysis solution containing sodium hydroxide, which disrupts the virus or bacteria in the sample, releases the target DNA, and denatures the DNA into single strands, which are accessible for hybridization with a target-specific RNA probe. After hybridization to the probes (illustrated in Fig. 18.2a), the next step is to transfer the sample to a microwell containing an immobilized anti-DNA:RNA monoclonal antibody (Fig. 18.2b). During this step, the DNA:RNA duplexes are captured by the immobilized antibodies onto the surface of the microwell. A wash cycle removes any unbound material. The third step is the addition of an alkaline phosphatase-conjugated (AP) DNA · RNA monoclonal antibody. Several AP molecules are conjugated to each antibody, and multiple conjugated antibodies bind to each captured hybrid, which in turn results in signal amplification (Fig. 18.2). The fourth step is detection of amplified chemiluminescent signal. The container is washed to remove all of the unbound components while the RNA:DNA hybrids and the labeled antibody remain bound to the container. Similar to the bDNA methodology, the chemiluminescent (dioxytane) substrate is added, which is cleaved by the bound alkaline phosphatase to produce light [4] which in turn is detected and measured using a luminometer.

Invader Technology

The Invader® assay, originally developed by Third Wave Technologies (now Hologic Inc.) is a homogeneous, isothermal DNA probe-based system with broad utility, ranging from the detection of single base pair mutations to the qualitative and quantitative detection of specific DNA sequences. The Invader methodology relies on the linear amplification of a target-specific signal, but not the actual target itself. Specificity is achieved through a combination of sequence-specific oligonucleotide hybridization steps and structure-specific enzymatic cleavage of one of the oligonucleotides using the Cleavase® enzyme. A diagram of this process is illustrated in Fig. 18.3. Two synthetic oligonucleotides, an upstream Invader oligonucleotide (Invader oligo), and a downstream probe, both hybridize to the single-stranded target DNA to create the structure shown in Fig. 18.3. This structure contains a single base overlap precisely at the nucleotide being interrogated [11]. The 3′ section of the probe, the target-specific region (TSR) is complementary to the target. The non-specific 5′ domain remains un-hybridized and forms a flap upon hybridization. The tripartite structure formed by the hybridization of the Invader oligo and probe to the target is the substrate for the Cleavase® enzyme which recognizes this structure and specifically cleaves the probe, releasing the "flap." If, in the case of the structure shown in Fig. 18.3, the highlighted base was other than a "C," the base at the site of this mismatch on the TSR becomes part of the "flap." Cleavage does not occur because this structure is a poor substrate for Cleavase® [12].

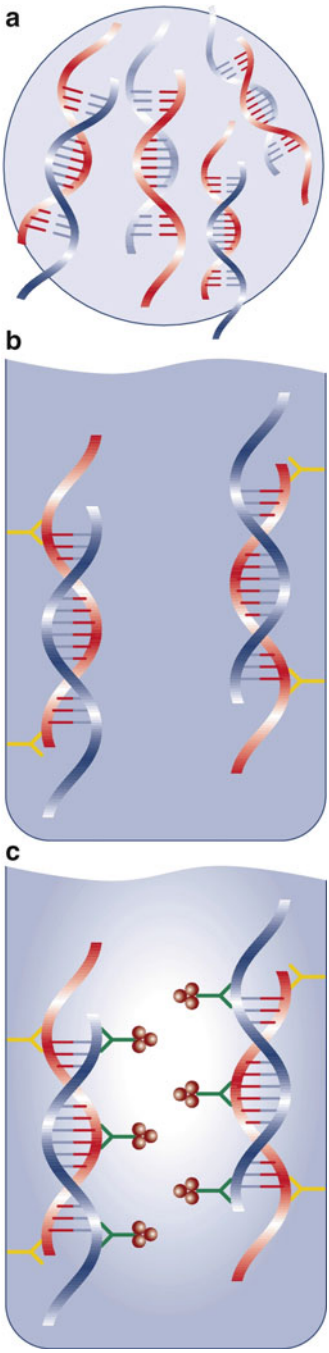

Fig. 18.2 Hybrid capture technology (**a**, **b**). See text for details

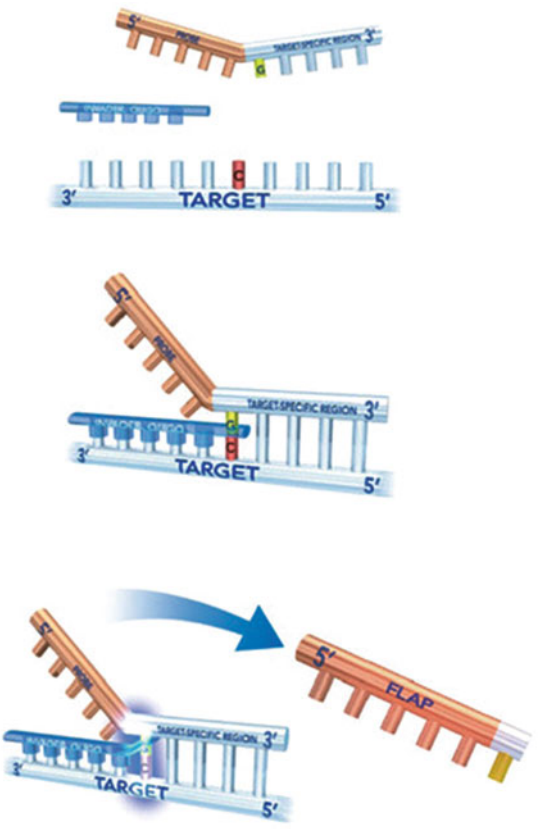

Fig. 18.3 Invader technology. See text for details

How this is all configured to create a signal amplification system is shown in Fig. 18.4. The signal generating reaction mixture contains all of the components just described, and shown in Fig. 18.3. The reactions are performed at temperatures very near the melting temperature (T_m) of the probe, which is present in molar excess. Each time an intact probe molecule binds to the target in the presence of the Invader oligo, a cleavage can occur. Therefore, multiple probes are cleaved per target molecule since the probes cycle rapidly on and off the target molecule. The signal generating component of the reaction is the fluorescence energy transfer (FRET) cassette composed of synthetic hairpin oligonucleotides containing a fluorescent dye (F), and a nonfluorescent quencher (Q) that acts as an FRET pair [12, 13]. The FRET cassette comprises two elements. The first is a region that is complementary to the flap sequence described above. The second is a self-complementary region that forms a hairpin structure that mimics the binding of an Invader probe to a target DNA molecule. Signal amplification occurs when the released flap from the first reaction hybridizes to the FRET cassette, forming the complex recognized by Cleavase®. Cleavage occurs

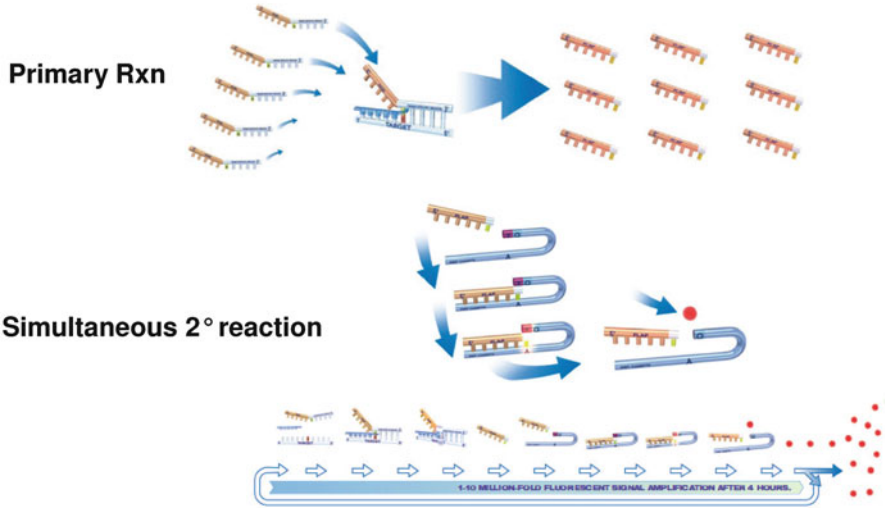

Fig. 18.4 Invader Biplex technology. See text for details

between the fluorescent dye and the quencher, releasing the dye from the quencher, permitting the formation of a fluorescent signal when excited by light at the appropriate wavelength in a fluorimeter. Each 5′ flap generates between 10^3 and 10^4 cleaved FRET cassettes per hour in a linear signal amplification reaction [14, 15].

Application of Signal Amplification Technologies in Diagnostic Microbiology

bDNA Assays

The application of bDNA technology for the development of molecular infectious disease assays has been limited due in large part to the complexity of the technology itself, and also because of the intellectual property surrounding bDNA technology. Through a series of corporate purchases, the original patents, held by the former Chiron Diagnostics, are now owned by Siemens Healthcare Diagnostics. Since the invention of bDNA technology in the late 1980s [8] methods have been developed targeting a broad range of infectious agents, including *Trypanosoma brucei* [16], cytomegalovirus (CMV) [17], the *Staphylococcus aureus mec*A gene [18], human papillomavirus [19], and hepatitis B virus [20]. However, with the exception of hepatitis B, none of these tests has been commercially developed, and are not available to the clinical laboratory. The majority of effort for bDNA research has

been focused on the development of quantitative tests for the detection and quantification of human immunodeficiency virus type-1 (HIV-1) and hepatitis C virus (HCV). The first-generation bDNA HIV-1 and HCV assays were developed in the mid 1990s [21, 22]. For HIV-1 the molecular target is the highly conserved HIV-1 polymerase gene. These assays were relatively insensitive compared to HIV-1 viral load assays in use today. The dynamic range of the first-generation HIV-1 Quantiplex method was 1×10^4–1.6×10^6 copies/mL [21]. Sensitivity of the HIV-1 assay was enhanced in the second-generation test by improving the capture probe design, and the addition of the preamplifier oligonucleotides [23, 24]. The improved design of the target and capture probes increased the stringency of hybridization, thereby decreasing background. Signal output was enhanced by increasing the number of signal generating probes that could bind to the preamplifier probes. These improvements reduced the lower limit of quantification (LLOQ) to 500 HIV-1 genome copies/mL for the second-generation HIV-1 bDNA Quantiplex assay. However, with the advent of effective antiretroviral drugs for the treatment of HIV-1 infections, the need for viral load assays that could detect even lower levels of virus became a requirement in order to determine the effectiveness of treatment. The major technological improvement in the third-generation bDNA HIV-1 viral load assay was the incorporation of the non-natural isoG and isoC bases as described earlier. A high speed centrifugation of the plasma being tested was also added to the protocol to concentrate the virus prior to sample processing. The initial description of this improved methodology demonstrated a new LLOQ of 50 copies/mL [2]. The current FDA approved Versant HIV-1RNA 3.0 assay has a stated dynamic range of 75–5×10^5 copies/mL; however, the European version of the same assay is approved for an LLOQ of 50 copies/mL. This compares very favorably to the current versions of both the Abbott Molecular Diagnostics RealTime HIV-1 assay, and the Roche Molecular Diagnostics Cobas Ampliprep/Cobas TaqMan HIV-1 version 2.0 tests, with lower limits of quantification of 40 and 20 copies/mL, respectively.

Only two other bDNA-based assays for infectious disease testing are commercially available. The Siemens Versant HCV version 3.0 kit targets both the core and the 5′ untranslated region of the HCV genome, and is approved for in vitro diagnostic (IVD) testing by the FDA. The dynamic range of this assay is 615–7.69×10^6 IU/mL of serum or plasma. Because of its relatively low sensitivity this test has not found broad usage for following patients for response to antiviral therapy. Both the Abbot Molecular and Roche Molecular real-time PCR assays have significantly lower LLOQs of 12 and 25 IU/mL, respectively, making the utility of any test that cannot approach these levels of analytical sensitivity questionable for following response to antiviral therapy.

The other bDNA-based viral load test marketed by Siemens is the VERSANT® HBV bDNA 3.0 Assay. This test is approved for use as an IVD assay in Europe (CE marked), but is labeled for research use only (RUO) in the United States. The dynamic range of the HBV bDNA 3.0 test is 2000–1×10^8 copies/mL. The two FDA approved PCR tests, the Roche Molecular Diagnostics COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 assay and the Abbott Molecular Diagnostics RealTi*m*e HBV test, have LLOQs of 20 and 10 IU/mL, respectively. As with both

HIV-1 and HCV viral load tests, viral load quantification assays for HBV are used predominantly for following response to antiviral therapy [25]. Therefore, the LLOQ of 2,000 copies/mL limits the utility of the bDNA-based assay.

As for the future of bDNA, recent improvements of the Versant bDNA assays have been more in the area of instrumentation than assay chemistry. The Versant HIV-1, HCV, and HBV 3.0 tests were originally designed to be run on the system 340 analyzer in conjunction with the proprietary Data Management Software. The 340 analyzer is a semi-automated instrument that can simultaneously process up to two 96 well microtiter plates. The newer Versant 440 platform is a fully automated instrument that was introduced in Europe in 2005 and in the United States in 2007. This is a fully automated instrument capable of processing up to 168 samples per run.

In the last several years, bDNA technology has been applied very broadly to the development of in situ hybridization (ISH) and fluorescent in situ hybridization (FISH) assays. One such application was the development of both ISH and FISH for the detection of HPV DNA sequences in infected cells [26]. Both HPV ISH and FISH were able to visualize, and distinguish, HPV types 16 and 18 DNA sequences in human cervical carcinoma cell lines. Under the trade names QuantiGene® ViewRNA, Panomix, a division of Affymetrix Inc. (Santa Clara, CA), has produced kits to allow investigators to design and develop FISH assays suited to their own areas of research [27–31]. Panomix has also applied bDNA technology for the detection and quantification of DNA, RNA, and protein targets based on the Luminex *x*MAP® (Luminex Inc., Riverside, CA) bead array technology that has seen broad usage as a research tool in the field of infectious disease research [32–35].

Hybrid Capture

Hybrid capture technology has been used to develop nucleic acid detection assays for HPV, CMV, *C. trachomatis* (CT), *Neisseria gonorrhoeae* (GC), HBV, and herpes simplex virus types 1 and 2.

From the public health perspective, cervical cancer is of great importance due to the fact that the causative agent, HPV [36–39], is sexually transmitted [40, 41]. HPV is also implicated in other types of cancer, such as anal cancer, vulvar cancer, penile cancer, and oral and laryngeal cancers. There are over 100 different types of HPV, only a subset of which is implicated in male and female genital infections. These can be differentiated into two groups, high-risk, and low-risk, referring to the predilection of infection by these viruses to progress to cancer. Digene Inc. (Beltsville, MD, now Qiagen Inc.) developed the first FDA approved diagnostic assays for the detection of HPV in cervical scrapings. This test is available in two different configurations. The digene HC2 HPV DNA Test employs probe cocktails to detect five low-risk HPV types, 6, 11, 42, 43, and 44, and 13 high-risk HPV types, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, which are associated with cervical cancer. The second configuration, the digene HC2 high-risk HPV DNA test, detects the 13 high-risk HPV types. The clinical utility of the digene HC2 HPV

DNA Test is questionable, since the most recent guidelines for HPV testing, published by the American Society for Colposcopy and Cervical Pathology [42] state that there is no clinical utility in testing for nononcogenic HPV types.

Samples for testing are collected with a proprietary collection device, available from Qiagen. Alternatively, cells collected in the liquid cytology media, Cytyc PreservCyt Solution (Hologic Inc. Bedford, MA), or from cervical biopsies are also approved for testing. Testing may be performed manually, or with the Rapid Capture System, a semi-automated pipetting and microwell plate handling system for high-volume sample-throughput testing.

The analytical sensitivity of the HC2 assays is ~5,000 HPV genome copies. On the surface, this may appear to be insensitive from a purely analytical perspective; however, this level of analytical sensitivity is ideal with respect to clinical sensitivity. Clinical utility of HPV screening is based on the association of detecting high-risk HPV in a clinical sample, and the association with the presence of cervical cancer determined by a PAP smear, not just simply the presence of the virus. Therefore, detection of very low levels of HPV by ultrasensitive methods, such as PCR, may have no clinical significance [43]. HPV detection assays based on PCF technology must be designed such that a positive result is clinically significant.

The hybrid capture assays are not cleared to be used independently as a screening assay for the general population [44]. As just mentioned, FDA approval of HPV detection assays such as the digene HC2 high-risk HPV DNA test are based not on sensitivity and specificity of detection of HPV, but the sensitivity and specificity, in conjunction with PAP smear results, for the risk of developing cervical cancer.

The indications for using this test are:

- To aid in the diagnosis of sexually transmitted HPV infections, and to differentiate between high-and low-risk HPV type infections.
- To screen patients with a Papinicolau smear (PAP smear) interpretation of atypical squamous cells of undetermined significance (ASCUS) to determine the need for referral to colposcopy.
- As a supplement to the PAP smear in women with low-grade squamous intraepithelial lesions (LSIL) high-grade squamous intraepithelial lesions (HSIL) results to assess the risk for developing cervical cancer.

The hc2 high-risk HPV DNA test is not intended for use as a screening test for PAP normal women under age 30, and is not meant to be a substitute for regular Pap screening.

There are some limitations to this assay, one of which is that cross-reactivity of the high-risk HPV probes with low-risk HPV DNA sequences has been observed which may lead to false-positive results [45, 46]. Neither of the HC-based assays have an internal positive control to differentiate true from false negative results; the latter have been reported with the hc2 HPV high-risk test [46, 47].

HC2 technology has also been used to develop assays for the detection of two other sexually transmitted organisms, *C. trachomatis* (CT) and *N. gonorrhoeae* (GC). These are the most common causes of bacterial infections of the lower genital tract. The FDA approved hc2 CT-ID DNA Test Version 2.0 is an IVD assay for the

qualitative detection of *C. trachomatis* DNA in cervical specimens collected using the hc2 DNA Collection Device (cervical brush and Specimen Transport Medium™ (STM)) and in cervical specimens collected using the Female Swab Specimen Collection Kit™ (Dacron swab and STM). The CT Probe Cocktail supplied with the hc2 CT-ID DNA Test is complementary to approximately 39,300 bp or 4% of the *Chlamydia* genomic DNA (1×10^6 bp). One probe is complementary to 100% of the cryptic plasmid of 7,500 bp. In studies comparing the sensitivity and specificity of this test to culture, the hc2 CT-ID DNA Test demonstrated very comparable results in symptomatic and asymptomatic women [48, 49]. When compared to PCR, the HC2 method, also gave very similar results, with PCR showing slightly higher sensitivity, as would be expected by a target amplification procedure [50]. The digene CT/GC Dual ID HC2 DNA test, which is also FDA approved, is designed to screen patient populations for CT and GC. The clinical performance of the NG component of this test also compares very favorably to culture [49].

Hybrid capture technology has also been applied to the detection of CMV in clinical specimens. Indeed, this was the first FDA approved nucleic acid detection assay for this purpose. When used solely as a diagnostic method to detect CMV disease, the performance of the Digene Hybrid Capture CMV DNA assay (version 2) was equivalent to [51] or superior [52], to that of the CMV antigenemia assay. Both of these studies demonstrated significant superiority of the HC2 method compared to culture. However, when compared with PCR-based methods, the analytical sensitivity of 2,600 copies/mL for the HC2 assay, as determined by probit analysis, put HC2 at a distinct disadvantage for detecting the onset of CMV infections in solid-organ transplant patients [51]. PCR-based assays detected CMV DNA earlier after transplant, and remained positive longer after initiation of ganciclovir therapy, than the HC2 assay [53]. The Digene Hybrid Capture CMV DNA assay is no longer being manufactured.

Lastly, the Digene HBV DNA test was designed to quantify hepatitis B DNA in human serum, and is specific for subtypes ad and ay. This test came in two versions, the standard assay, with a dynamic range of 1.42×10^5–1.7×10^9 copies/mL, based on an input sample volume of 30 mL of plasma [54], and an ultrasensitive test, with a dynamic range of 4.7×10^3–5.6×10^7 copies/mL [55]. The latter is based on a 1 mL sample volume with the virus concentrated by centrifugation. As stated earlier in the discussion of the bDNA-based HBV viral load assay, FDA approved real-time PCR assays are available with significantly lower analytical sensitivities. Since HBV viral load assays play a critical role in determining response to antiviral therapy, the relatively high LLOQ of the ultrasensitive HBV HC2 test makes it less useful for this purpose then the currently available real-time PCR assays.

What is the future of HC technology? Qiagen has announced the development of new platforms, the QIAensemble and QIAensemble Plus which integrate nucleic acid extraction and assay technology automation (Hybrid capture, PCR, and other target detection methods) to provide laboratories with mid- and high-throughput, modular, fully automated, molecular testing platforms. Improvements have also been announced regarding HC2 technology itself, moving from solid substrate, microwell assays to magnetic particle capture methodology. The first such test will

be the *digene*® HPV eHC (ensemble hybrid capture). Also announced was the development of HPV 16 and 18 genotyping tests. Qiagen's long-term strategy however, does not seem to rely on long-term investment in hybrid capture-based assays, but more reliance on traditional target amplification methodologies, such as real-time PCR, and newer technologies, such as isothermal helicase-dependent amplification (tHDA).

Invader Technology

As described above, the Invader® technology, originally developed at Third Wave Technologies, now Hologic Inc., is a homogeneous, DNA probe-based system that has been applied to a wide variety of applications. These include detection of single base-pair changes, insertions and deletions, determination of gene copy number, detection of infectious agents, and gene expression. The test can be performed on either DNA or RNA.

One of the earliest applications of Invader® technology was the development of assays for the detection of mutations in genes involved in hypercoagulability disorders, specifically factor V Leiden, factor II (prothrombin) and methylenetetrahydrofolate reductase (MTHFR) [56]. These assays were available initially as analyte-specific reagents, but have been recently approved by the FDA. All three tests are based on Invader Plus®. This process involves a PCR reaction, followed by an Invader reaction, all of which occurs in a single reaction, in a closed, single-tube format. This sequential process combines PCR-based target amplification with subsequent signal amplification through Invader® chemistry.

Another commercial application of Invader® technology was for the detection of mutations in the UGT1A1 gene that codes for the enzyme UDP-glucuronosyltransferase. This gene is responsible for the metabolism of certain drugs, including irinotecan, a chemotherapy agent commonly used to treat colorectal and lung cancer.

Detecting specific variations in the UGT1A1 gene predicts which patients are at an increased risk of toxicity from irinotecan [57, 58]. The Invader® UGT1A1 Molecular Assay (Hologic Inc., Bedford, MA) targets the *28 allele, which is a two base-pair insertion (TA) in the UGT1A1 promoter region. Individuals either homozygous or heterozygous for this allele are seven times more likely to demonstrate severe toxicity to irinotecan than patients homozygous for the wild-type allele [57]. This test received FDA approval in 2005.

Invader technology has also been successfully applied to the molecular diagnosis of cystic fibrosis. The InPlex® CF Molecular Test (Hologic Inc, Bedford, MA) is an IVD that tests for 23 separate mutations in the cystic fibrosis transmembrane receptor (CFTR) gene. The IVS8-5T/7T/9T markers are automatically reflexed as part of the test. All mutations contained in the assay are recommended for testing by the American College of Obstetricians and Gynecologists (ACOG) and the American College of Medical Genetics (ACMG). For the InPlex® technology, the appropriate genomic region is amplified by PCR using a limiting number of cycles. For the CF

test this is 13 cycles. The amplification products are injected into an assay-specific microfluidics card that has multiple chambers for analyzing each of the CFTR mutations. FDA clearance for this test was granted in 2008.

One of the flagship products marketed by Hologic is the Cervista® HPV HR test. This is an FDA approved assay that tests the for the 13 high-risky HPV types listed above for the HC2 method, but also includes HPV type 66. In addition, this test also includes an internal positive control to confirm adequacy of sample collection, and to indicate if inhibitory substances are present. Instrumentation choices available from Hologic to perform this test range from semi-automated to fully automated platforms, depending on the needs of the laboratory. Cells collected in Cytyc PreservCyt Solution (Hologic Inc., Bedford, MA) is the indicated sample type for this test, although cervical cells collected in SurePath preservative-fixative fluid (BD TriPath Imaging, Burlington, SC) have been shown to work with this test as well [59]. However, the use of any collection medium other than Cytyc PreservCyt Solution would be considered an off-label use of the assay.

Several studies have been published Comparing the Invader® HPV assay with the digene HC2 HPV DNA Test [46, 60–62]. Performance characteristics for the two methods are comparable in terms of clinical sensitivity. Three studies also concurred that the Invader® assay had improved specificity in terms of fewer false positive results. In addition, the Cervista® HPV HR test can detect infection by multiple HPV types, which is important since recent studies have suggested that the risk of progression toward cervical cancer can be as high for simultaneous infections with multiple high risk HPV types as it is for infection with just one of the highest risk types, HPV 16 or 18.

A companion test for the Cervista® HPV HR assay is the Cervista™ HPV 16/18 test. HPV types 16 and 18 are recognized as highly oncogenic and persistent, and are associated with 60 and 10% of cervical cancers, respectively. The test is intended to be used adjunctively with the Cervista® HPV HR test in combination with cervical cytology to assess the presence or absence of high-risk HPV types 16 and 18.

In addition to the commercially produced assays described above, Invader® technology has been applied widely in the development of laboratory developed tests for a broad range of clinical and nonclinical applications. The reason for this is the availability of Universal Invader™ reagents, and Universal Invader™ software for designing Invader®-based assays. Several Invader-based methods have been published for a variety of infectious disease-related applications, including detection assays for varicella-zoster [63] rifampin and isoniazid resistance of *Mycobacterium tuberculosis* [64], quantification of bacteria involved in periodontis [65], and hepatitis B genotyping for detection of drug-resistance mutations [66, 67]. A study published by Xie et al. [68] describes an Invader®-based immunoassay. Two different antibodies are employed in this method, one of which captures an antigen to a solid surface, and the other, which is biotinylated, is used as the detector. After the removal of unbound antibody via a wash step, streptavidin, and a biotinylated oligonucleotide are added to the reaction mixture. After washing away the unbound oligonucleotide, the remaining components required for the invader assay (see above and Fig. 18.4) are added. The authors report that this method could detect 0.1 pg/mL of the target

antigen (tumor necrosis factor-α), vs. 3.5 pg/mL for a commercially available standard enzyme immunoassay method using colorimetric detection.

In the future, it is expected that Hologic will continue commercializing applications for Invader® technology. Evidence for this is based on the recent FDA approval of the factor V Leiden, factor II, and MTHFR tests for determining an individual's risk for hypercoagulability disorders, as described above. In addition, Hologic is licensing the use of Invader® technology to other in vitro diagnostics, and biotechnology companies for new test development. Such an example is Exact Sciences (Madison, WI), which licensed Invader® technology for the development of cancer diagnostic and screening assays. In addition, Hologic has an AgBIO services division dedicated to working with the agriculture industry for custom assay development using Invader® technology, specifically in the areas of SNP genotyping analysis, copy number determination, marker-assisted selection, and animal diagnostics.

References

1. Horn T, Chang C, Urdea MS (1997) Chemical synthesis and characterization of branched oligodeoxyribonucleotides (bDNA) for use as signal amplifiers in nucleic acid quantification assays. Nucleic Acids Res 25:4842–4849
2. Collins ML (1997) Irvine, Tyner D, et al. A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/mL. Nucleic Acids Res 25:2979–2984
3. Horn T, Urdea M (1989) Forks and combs and DNA: the synthesis of branched oligodeoxyribonucleotides. Nucleic Acids Res 17:6959–6967
4. Beck S, Koster S (1990) Applications of dioxetane chemiluminescent probes to molecular biology. Anal Chem 62:2258–2270
5. Colby C, Stollar BD, Simon MI (1971) Interferon induction: DNA-RNA hybrid or double stranded RNA? Nat New Biol 229:172–174
6. Rudkin GT, Stoller BD (1977) High resolution detection of DNA-RNA hybrids in situ by indirect immunofluorescence. Nature 265:472–473
7. Stollar BD (1970) Double-helical polynucleotides: immunochemical recognition of differing conformations. Science 169:609–611
8. Boguslawski S, Smith DE, Michalak MA (1986) Characterization of monoclonal antibody to DNA· RNA and its application to immunodetection of hybrids. J lmmunol Methods 89:123–130
9. Carpenter WR, Schutzbank TE, Tevere VJ et al (1993) A transcriptionally amplified DNA probe assay with ligatable probes and immunochemical detection. Clin Chem 39:1934–1938
10. Schiffman MH, Kiviat NB, Burk RD et al (1995) Accuracy and interlaboratory reliability of human papillomavirus DNA testing by hybrid capture. J Clin Microbiol 33:545–550
11. Lyamichev V, Mast AL, Hall JG et al (1999) Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. Nat Biotechnol 17:292–296
12. Lyamichev VI, Kaiser MW, Lyamicheva NE et al (2000) Experimental and theoretical analysis of the invasive signal amplification reaction. Biochemistry 39:9523–9532
13. Stryer L (1978) Fluorescence energy transfer as a spectroscopic ruler. Ann Rev Biochem 47:819–846

14. Kwiatkowski RW, Lyamichev V (1999) de Arruda, et al. Clinical genetic and pharmacogenetic applications of the Invader Assay. Mol Diagn 4:353–364
15. Hall JG, Eis PS, Law SM et al (2000) Sensitive detection of DNA polymorphisms by the serial invasive signal amplification reaction. Proc Natl Acad Sci U S A 97(15):8272–8277
16. Harris E, Detmer J, Dungan J et al (1996) Detection of *Trypanosoma brucei* spp. in human blood by a nonradioactive branched DNA-based technique. J Clin Microbiol 34:2401–2407
17. Chernoff DN, Miner RC, Hoo BS et al (1997) Quantification of cytomegalovirus DNA in peripheral blood leukocytes by a branched-DNA signal amplification assay. J Clin Microbiol 35:2740–2744
18. Kolbert CP, Arruda J, Varga-Delmore P et al (1998) Branched-DNA assay for detection of *mecA* gene in oxacillin-resistant and oxacillin-sensitive staphylococci. J Clin Microbiol 36:2640–2644
19. Player AN, Shen L-P, Kenny D et al (2001) Single-copy gene detection using branched DNA (bDNA) in situ hybridization. J Histochem Cytochem 49:603–611
20. Hendricks DA, Stowe BJ, Hoo BS et al (1995) Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. Am J Clin Pathol 104:537–546
21. Pachl C, Todd JA, Kern DG et al (1995) Rapid and precise quantification of HIV-1 RNA in plasma using a branched DNA (bDNA) signal amplification assay. J Acquir Immune Defic Syndr Hum Retrovirol 8:446–454
22. Alter HJ, Sanchez-Pescador R, Urdea MS et al (1995) Evaluation of branched DNA signal amplification for the detection of hepatitis C virus RNA. J Viral Hepat 2:121–132
23. Kern D, Collins M, Fultz T et al (1996) An enhanced-sensitivity branched-DNA assay for quantification of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 34:3196–3202
24. Nolte FS, Boysza J, Thurmond C et al (1998) Clinical comparison of an enhanced-sensitivity branched-DNA assay and reverse transcription–PCR for quantitation of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 36:716–720
25. Keeffe EB, Dieterich DT, Han SH (2004) A treatment algorithm for the management of chronic hepatitis B virus infection in the United States. Clin Gastroenterol Hepatol 2:87–106
26. Player AN, Shen LP, Keny D et al (2001) Single-copy gene detection using branched DNA (bDNA) in situ hybridization. J Histochem Cytochem 49:603–612
27. Honkavuori KS, Shivaprasad HL, Briese T et al (2011) Novel picornavirus in Turkey poults with hepatitis, California, USA. Emerg Infect Dis 17:480–487
28. Lee K, Kunkeaw N, Jeon SH et al (2011) Precursor miR-886, a novel noncoding RNA repressed in cancer, associates with PKR and modulates its activity. RNA 17:1076–1089
29. Piotrowska J, Hansen SJ, Park N et al (2010) Stable formation of compositionally unique stress granules in virus-infected cells. J Virol 84:3654–3665
30. Ting DT, Lipson D, Suchismeta P et al (2011) Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers. Science 331:593–596
31. Uniacke J, Colon-Ramos D, Zerges W (2011) FISH and immunofluorescence staining in *Chlamydomonas*. Methods Mol Biol 714:15–29
32. Rutherford ST, van Kessel JC, Shao Y et al (2011) AphA and LuxR/HapR reciprocally control quorum sensing in vibrios. Genes Dev 25:397–408
33. Woodson SE, Freiberg AN, Holbrook MR (2011) Differential cytokine responses from primary human Kupffer cells following infection with wild-type or vaccine strain yellow fever virus. Virology 412:188–195
34. Cha W, Ma Y, Saif YM et al (2010) Development of microsphere-based multiplex branched DNA assay for detection and differentiation of avian influenza virus strains. J Clin Microbiol 48(7):2575–2577
35. Momose H, Mizukami T, Ochiai M et al (2010) A new method for the evaluation of vaccine safety based on comprehensive gene expression analysis. J Biomed Biotechnol 2010:361841
36. Munger K, Baldwin A, Edwards KM et al (2004) Mechanisms of human papillomavirus-induced oncogenesis. J Virol 78:11451–11460
37. Snijders PJ, Steen Bergen RD, Heideman DA et al (2006) HPV-mediated cervical carcinogenesis: concepts and clinical implications. J Pathol 208:152–164

38. zur Hausen H (1976) Condylomata acuminate and human genital cancer. Cancer Res 36 (2 pt 2):794
39. zur Hausen H (2002) Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2:342–350
40. Schiffman MH (1994) Epidemiology of cervical human papillomaviruses. In: zur Hausen H (ed) Human pathogenic papillomaviruses. Springer, Heidelberg
41. Munoz N, Bosch FX, de Sanjose S et al (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348:518–527
42. Wright T, Massad L, Dunton C et al (2007) 2006 Consensus guidelines for the management of women with abnormal cervical cancer screening tests. Am J Obstet Gynecol 197:340–345
43. Arney A, Bennett K (2010) Molecular diagnostics of human papillomavirus. Lab Med 41:523–530
44. Burd E (2003) Human papillomavirus and cervical cancer. Clin Microbiol Rev 16:1–17
45. Castle PE, Solomon D, Wheeler CM et al (2008) Human papillomavirus genotype specificity of hybrid capture 2. J Clin Microbiol 46:2595–2604
46. Schutzbank T, Jarvis C, Kahman N et al (2007) Detection of high-risk papillomavirus DNA with commercial invader-technology-based analyte-specific reagents following automated extraction of DNA from cervical brushings in ThinPrep Media. J Clin Microbiol 45:4067–4069
47. Munson E, Du Chateau BK, Bellerose B (2011) Clinical laboratory evaluation of Invader® chemistry and hybrid capture for detection of high-risk human papillomavirus in liquid-based cytology specimens. Diagn Microbiol Infect Dis 71:230–235
48. Schachter J, Hook EW, McCormack WM et al (1999) Ability of the Digene Hybrid Capture II Test to identify *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in cervical specimens. J Clin Microbiol 37:3668–3671
49. Darwin LH, Cullen AP, Crowe SR et al (2002) Evaluation of the Hybrid Capture 2 CT/GC DNA tests and the GenProbe PACE 2 tests from the same male urethral swab specimens. Sex Transm Dis 29:576–580
50. Quint K, Porras C et al (2007) Evaluation of a novel PCR-based assay for detection and identification of *Chlamydia trachomatis* serovars in cervical specimens. J Clin Microbiol 45:3986–3991
51. Mazzulli T, Drew LW, Yen-Lieberman B et al (1999) Multicenter comparison of the Digene Hybrid Capture CMV DNA Assay (Version 2.0) and the pp 65 antigenemia assay, and cell culture for detection of cytomegalovirus viremia. J Clin Microbiol 37:958–963
52. Walmsey S, O'Rourke K, Mortimer C et al (1998) Predictive value of cytomegalovirus (CMV) antigenemia and Digene Hybrid Capture DNA assays for CMV disease in human immunodeficiency virus-infected patients. Clin Infect Dis 27:573–581
53. Hanson KE, Reller LB, Kurtzberg J et al (2007) Comparison of the Digene Hybrid Capture System Cytomegalovirus (CMV) DNA (Version 2.0), Roche CMV UL54 analyte-specific-reagent, and QIAGE RealART CMV Light Cycler PCR reagent tests using AcroMetrix OptiQuant CMV DNA quantification panels and specimens from allogeneic-stem-cell transplant recipients. J Clin Microbiol 45:1972–1973
54. Yuan HJ, Yuen MF, Wong DKH et al (2004) Clinical evaluation of the Digene Hybrid Capture II Test and the COBAS AMPLICOR Monitor test for determination of hepatitis B virus DNA levels. J Clin Microbiol 42:3513–3517
55. Konnick EQ, Erali M, Ashwood ER et al (2005) Evaluation of the COBAS amplicor HBV monitor assay and comparison with the ultrasensitive HBV hybrid capture 2 assay for quantification of hepatitis B virus DNA. J Clin Microbiol 43:596–603
56. Ledford M, Friedman KID, Hessner MJ et al (2000) A Multi-site study for detection of the factor V (Leiden) mutation from genomic DNA using a homogeneous Invader microtiter plate fluorescence resonance energy transfer (FRET) assay. J Mol Diagn 2:97–104
57. Ando Y, Saka H, Ando M et al (2000) Polymorphisms of UDP-glucoruonosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. Cancer Res 60:6921–6926

58. Innocenti R, Undevia SD, Iyer L et al (2004) Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. J Clin Oncol 22:1382–1388
59. Harvey M, Stout S, Starkey CR et al (2009) The clinical performance of Invader® technology and SurePath® when detecting the presence of high-risk HPV cervical infection. J Clin Virol 51:S79–S83
60. Ginocchio CC, Barth D, Zhang F (2008) Comparison of the third wave invader human papillomavirus (HPV) assay and the Digene HPV Hybrid Capture 2 assay for detection of high-risk HPV DNA. J Clin Microbiol 46:1641–1646
61. Johnson LR, Starkey CR, Palmer J et al (2008) A comparison of two methods to determine the presence of high-risk HPV cervical infections. Am J Clin Pathol 130:401–408
62. Wong AK, Chan RCK, Nichols S et al (2008) Human papillomavirus (HPV) in atypical squamous cervical cytology: the Invader HPV test as a new screening assay. J Clin Microbiol 46:869–875
63. Tang YW, Allawi HT, DeLeon-Carnes M et al (2007) Detection and differentiation of wild-type vaccine mutant varicella-zoster viruses using an Invader Plus® method. J Clin Virol 40(2):129–134
64. Cooksey RC, Holloway BP, Oldenburg MC et al (2000) Evaluation of the Invader Assay, a linear signal amplification method, for identification of mutations associated with resistance to rifampin and isoniazid in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 44:1296–1301
65. Tadokoro K, Yamaguchi T, Kawamura K et al (2010) Rapid quantification of periodontitis-related bacteria using a novel modification of Invader PLUS technologies. Microbiol Res 165:43–49
66. Takodoro K, Kobayashi M, Yamaguchi T et al (2006) Classification of hepatitis B virus genotypes by the PCR-Invader method with genotype-specific probes. J Virol Methods 138:30–39
67. Tadokiro K, Suzuki F, Kobayashi M et al (2011) Rapid detection of drug-resistant mutations in hepatitis B virus by the PCR-Invader assay. J Virol Methods 171:67–73
68. Xie MJ, Fukui K, Horie M et al (2008) A novel sensitive immunoassay method based on the Invader technique. Anal Biochem 374:278–284

Chapter 19
Real-Time Nucleic Acid Quantification

Nima Mosammaparast and Alexander J. McAdam

Introduction

It is difficult to overstate the vital role of polymerase chain reaction (PCR) and its derivatives in the clinical microbiology laboratory. The ease of adapting PCR, combined with the availability of complete genomic sequences from various pathogens, has permitted the rapid development of commercial as well as laboratory-developed molecular techniques for diagnostic purposes. As its name implies, real-time PCR, a common PCR variant, has furthered clinical utility by permitting the detection and quantification of PCR products during the amplification process. In this chapter, we discuss the principles behind real-time PCR and its use in quantification of pathogens.

Conceptual Framework of Quantitative Real-Time PCR

If one could monitor a PCR reaction during every cycle of amplification, the quantity of product produced would approximate a logistic function, which follows a sigmoidal curve (Fig. 19.1). The prototypical application of the logistic function is its use for modeling population growth, where the rate of population growth depends on two factors: (1) the existing population and (2) the amount of available resources [1]. In such a model, the initial rate of population growth depends far more on the former factor, since available resources are not limiting. At this initial growth stage, the rate of population growth is nearly exponential. As the population grows, resources diminish, and the growth rate also diminishes; this continues until the

N. Mosammaparast • A.J. McAdam (✉)
Infectious Disease Diagnostics Division, Department of Laboratory Medicine
and Department of Pathology, Boston Children's Hospital, Harvard Medical School,
300 Longwood Avenue, Boston, MA 02115, USA
e-mail: Alexander.McAdam@childrens.harvard.edu

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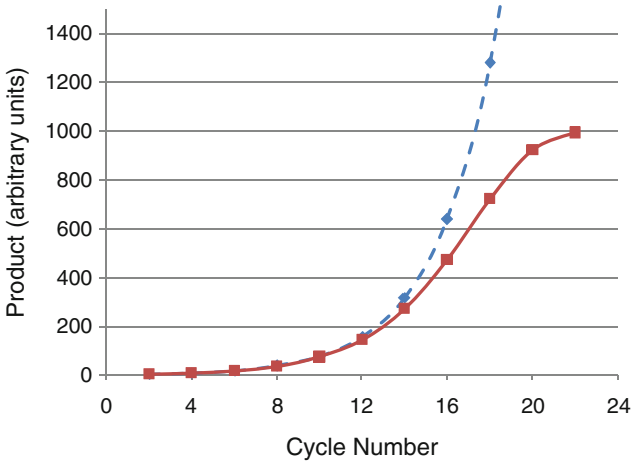

Fig. 19.1 A hypothetical PCR amplification curve represents a logistic function and follows a sigmoidal curve (*solid red line*). A purely exponential amplification is also shown for comparison (*dashed blue line*). Notice that during early cycles the two curves are nearly identical

growth rate decreases to zero and equilibrium is reached. The same basic concept applies to PCR reactions: after an initial lag phase, the rate of product formation is nearly exponential and depends primarily on the starting template concentration. Thus, under ideal conditions, the product is initially doubled after every cycle, until the reaction is inhibited by excessive product or limiting PCR reactants. Prior to this inhibition, the exponential phase of the PCR reaction may be described by the following equation:

$$P = T\left(1+E\right)^{n}$$

where P is the PCR product concentration, T is the initial template concentration, E is the efficiency of the PCR reaction, and n is the number of amplification cycles [2]. The efficiency E ranges between 0 and 1.0, with 1.0 (100 % efficiency) representing an ideal amplification reaction where the target molecule is doubled after every reaction cycle. Under such conditions, it would take 3.32 amplification cycles to increase the target PCR product tenfold (Fig. 19.2a). Thus, plotting the relationship between the number of amplification cycles necessary to reach a specified concentration of product (as determined by a set level of fluorescence) and the log of the starting concentration of the nucleic acid template gives a negative linear relationship (Fig. 19.2b). While in theory the product threshold set point can be any amount during the exponential phase of the reaction, in reality this threshold should be set at the lowest reliably detectable level of fluorescence above background, since this is where the reaction behaves most like to the exponential ideal (Fig. 19.1). The latter is defined as the crossing threshold (C_t) of the reaction [2]. Besides the threshold method, a number of other methods exist for determining the C_t; these have been described in greater detail elsewhere [3].

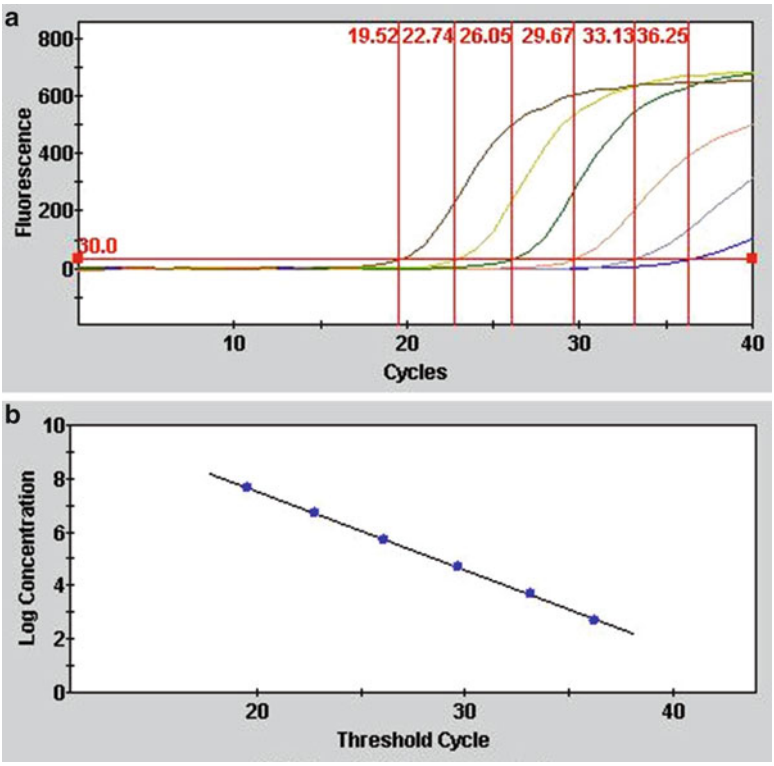

Fig. 19.2 Example of a real-time PCR standard curve. (**a**) Serial tenfold dilutions (ranging from 5 to 5×10^5 copies per reaction) of a plasmid including the hexon gene for adenovirus were used as template for detection in real-time PCR. Numbers on *top* indicate the threshold cycle (C_t) for each dilution. (**b**) Plot of the threshold cycle vs. log template concentration demonstrates an inverse linear curve

For any given PCR reaction, plotting C_t values of a serial dilution of template nucleic acid serves two purposes. First, one can empirically determine the slope of the C_t vs. log product curve, which may be used to determine the percent efficiency of the PCR reaction:

$$E = (10^{-\text{slope}} - 1) \times 100.$$

Suboptimal PCR conditions require, on average, more than one cycle to double the product, thus flattening the slope of the curve. As a general rule, a high PCR efficiency is desirable, primarily because the lower limit of detection will be otherwise compromised [2]. The second purpose in plotting such a curve is to create calibration or standard curves, which may be used to calculate the concentration of the target sequence in any given sample. The C_t value determined from the unknown sample may be applied using linear regression methods to the standard curve to determine the concentration of the target sequence. The use of such a method assumes that the amplification reaction in the unknown sample behaved identically

as the amplification reactions used to create the standard curve. The presence of PCR inhibitors in an unknown sample would, for example, negate this assumption and result in underestimation during quantification. We will consider PCR inhibitors later in this chapter.

Dynamic Range and Detection Limits

In theory, the upper limit of quantification in real-time PCR exceeds 10^{10} copies per reaction, and the lower limit of detection is one molecule of template [2]. Generally, the former is true in practice but is seldom approached in the clinical laboratory due to a lack of need for such a large dynamic range. The lower limit of detection is far more critical, especially for analysis of viral loads such as human immunodeficiency virus, and cannot reliably reach one copy of template per reaction. This is because, due to the Poisson distribution, it is impossible to be certain that one copy of template is present in every given reaction at such low concentrations [3]. For clinical purposes, the lower limit of detection is usually defined as the lowest quantity of analyte (nucleic acid in this case) with a 95 % probability of detection. For PCR this is theoretically possible with three copies of template. In reality this is not achievable due to losses during sample extraction and preparation, as well as the imperfect efficiency of the reverse transcription step if RT-PCR is being performed for RNA quantification. In our laboratory, we have been successful in validation of assays that detect as few as 5–10 copies of template per reaction, in agreement with other published validations [4–6]. In cases where it is desirable to further improve the lower limit of detection, clinical samples may be concentrated during the extraction step and the appropriate correction factor be implemented for quantification purposes.

Quantification Methodologies

How are PCR reactions monitored for product in real time? While this is considered in more detail in other chapters in this book, we will discuss differences in the techniques as they are relevant to quantitative testing here. In the vast majority of cases, a fluorescent marker that detects amplified product is included in the reaction, and the real-time PCR machine contains a fluorimeter calibrated to excite and detect the marker. Some years after the discovery of PCR, the addition of ethidium bromide (EtBr) to the PCR reaction was described as a method to detect and quantify the amplification product in real time [7]. EtBr is an intercalating agent that preferentially binds to double-stranded DNA and has since been replaced with the minor groove binding dye SYBR Green I. SYBR Green I and its derivatives distinguish double-stranded DNA from single-stranded DNA better than EtBr and are widely used in research laboratories for quantitative PCR and RT-PCR [2]. The use of DNA binding dyes in real-time PCR in the clinical laboratory is uncommon, in part

because of concerns about the accuracy of quantitative results obtained with these dyes. At higher concentrations, SYBR Green I can inhibit the PCR reaction, resulting in inaccurate quantitative results [8], although this can be overcome by using a lower concentration of the dye [9]. A greater concern is that the binding (and hence detection and quantification) of such dyes is not sequence specific, and thus specific and nonspecific amplification products, as well as primer dimers, will all contribute to detection of reaction signal. If such an approach is used clinically, it is imperative that signals due to these potential nonspecific products be recognized prior to test implementation and rectified wherever possible to avoid potential over-quantification and false positive results. With the use of such dyes, melting curve analysis will help to discriminate specific products from other contaminants [2].

An alternative method involves coupling of a fluorophore to one of the primers (e.g., Light upon extension, or Lux technology) [9]. The primer is designed to form a hairpin structure in the absence of product. In the hairpin conformation, the fluorophore is quenched but it fluoresces upon incorporation in the PCR product, which results in extension of the hairpin structure. Like DNA binding dyes, a primer-based detection method depends largely on the specificity of the primers, in contrast to probe-based detection methods, discussed below.

In place of a dye-conjugated primer or DNA binding dyes such as SYBR Green I, a sequence-specific fluorescent probe may be used for detection and quantification of PCR products. The main advantage of using any of these probe methods instead of dyes such as SYBR Green I or a primer-coupled fluorophore is that there is increased specificity due to independent confirmation of specific product formation, since the probe hybridization sequence does not overlap with the primers used for amplification. As a result of their sequence-specific binding, probes are unlikely to anneal to nonspecific amplification products and primer dimers, and so are predicted to give more accurate quantitative results.

External Quantification Controls

An important initial consideration in designing a quantitative PCR test is what nucleic acid material will be used for setting up the standard calibrator curve. This can be a synthetic oligonucleotide, a purified PCR product, recombinant plasmid, or quantified pathogen nucleic acid. All of these are reasonable choices for use as calibrators in laboratory-developed tests, although quantified pathogen nucleic acid is preferred if it is available, since it most closely resembles clinical specimens. One potential disadvantage of using a quantified pathogen DNA or RNA (such as extracted viral nucleic acid) is that they may only be available in relatively low concentrations, thus limiting the upper limit of quantification. We have found that engineering a plasmid using a common vector backbone (such as pBlueScript) to contain the target sequence of interest to be a useful and simple way to produce calibrator standards for a number of different assays. These can be easily purified from transformed bacteria when necessary using commercially available DNA purification

column kits, quantified using spectrophotometry, and then serially diluted to be used as calibrators described earlier. The quantitative test results using these synthetic standards should be compared to quantified pathogen whenever available (such as reference standards distributed by the World Health Organization), and adjusted if necessary to better reflect the result should a true biological standard have been used. While not available for all pathogens, use of such reference material is an important step towards the standardization of quantitative PCR results between different laboratories, since in their absence, quantification can vary widely, as much as four orders of magnitude [10, 11]. It is important to recognize that plasmid or synthetic oligonucleotide calibrators may not behave as patient-derived specimens, and this cause inaccurate quantitative measurements. Differences in quantification can be introduced with biological matrices such as urine, resulting in PCR inhibition [12]. In at least one published report, the commutability of a calibrator standard has been shown to be dependent on the type of matrix that is used [13]. Therefore, it is imperative that during test validation, the purified standard curve nucleic acid should be repurified and assayed after addition to the desired biological matrix to determine whether any matrix effect is observed.

Amplicon Selection

For certain pathogens, such as adenovirus or BK virus, choosing the target sequence for the assay amplification (the amplicon) is challenging, because numerous viral subtypes or common sequence variants abound, respectively [6, 14]. Careful sequence comparison to all known variants of a given pathogen deposited in genome databases is essential in choosing the appropriate amplicon sequence to avoid regions of high sequence variability. A single nucleotide mismatch in the primer or probe sequence can be sufficient to falsely reduce quantification several orders of magnitude or give a false negative result. A number of computer programs exist, such as Beacon Designer (Premier Biosoft), which can help the user in selection of the amplicon. On occasion, it may be impossible to find a specific pair of primers and a single probe that will work for all known sequence variants of a given pathogen. In these cases, it will be necessary to synthesize a small pool of primers or probes that will target a reasonably well-conserved region and cover all potential variants. It is important to realize that in the cases where combinations of primers and probes are pooled, colinearity of quantification between the variants may not always be the norm, although this has been reported for at least one quantitative PCR assay for adenovirus [14].

A number of other factors need to be considered when selecting the amplicon region. These include the primer and probe length, their melting temperatures (T_m), and the overall size of the amplicon. Longer amplicons are associated with lower PCR efficiencies and thus greater challenges in quantification, and therefore the amplicon size should be kept between 50 and 150 base pairs. The T_m of the primers should be between 56 and 62 °C, but more importantly the T_m of each of the primers

should be as similar as possible. If a Taqman probe is used, it is important for the T_m of the probe to be approximately 10 °C higher than the primers. This is because the probe needs to remain annealed during polymerase extension, and incomplete annealing during extension may result in under-quantification. Again, programs such as Beacon Designer help navigate these and other issues surrounding primer and probe design, such as G:C content.

Internal Controls and PCR Inhibitors

In the research context, real-time PCR methods are often used to quantify a gene product of interest relative to an internal reference, such as actin. In the clinical lab, such an internal reference gene is often not present in the clinical sample (e.g., in urine), or, even if present, may be widely variable due to a particular state of the patient (e.g., severe pancytopenia in the context of cancer chemotherapy). For these reasons, absolute quantification is preferred to relative quantification in the clinical setting. Nevertheless, use of an internal reference for amplification is vital in the clinical context. As mentioned earlier, the presence of PCR inhibitors or amplification failure for other reasons must be ruled out for any negative result. An internal reference, usually an unrelated plasmid or RNA for RT-PCR, is added into the amplification reaction to verify the amplification conditions. Internal references are available from many commercial sources and can be amplified separately using the identical reaction conditions as the target of interest, or can be implemented in a multiplex platform using a probe that is detected at a channel distinct from the target. The latter is preferred because the same reaction is used for quantification of the clinical sample and for control purposes. Care must be taken, however, that the presence of the internal control does not interfere with the amplification of the target itself. This is because certain components of the two simultaneous amplification reactions are shared (e.g., DNA polymerase), and under certain conditions may be present in relatively limiting quantities. For this reason, the internal control is added at a relatively low copy number to avoid potential competition for PCR reactants.

The internal reference can be added at two different steps prior to amplification: (1) directly to the clinical sample prior to sample extraction or (2) to the amplification reaction itself. In the latter case, any loss of amplification of the internal reference can be attributed to a PCR inhibitor or general amplification failure. In the former case, reduced extraction efficiency can also contribute to any observed reduction of internal reference amplification. Thus, the addition of an internal reference standard is an important consideration in setting up a quantitative PCR assay.

Variability in Quantitative PCR

As suggested earlier, there is great concern with regard to the variability inherent in quantitative PCR reactions. Analysis of the sources of this variability in gene expression analysis via quantitative reverse transcription PCR has yielded some insightful

results [15]. In general, the amplification reaction itself is not a major source of the variance. Rather, much of the variability is inherent in the sampling itself, although the reverse transcription step does make a significant contribution in certain cases. It is unclear whether such an analysis of gene expression variability fully applies to clinical microbiology samples. However, it is important to recognize the inherent variability of quantitative PCR technology and to communicate this to patient care providers who are interpreting such results that will impact management decisions.

Conclusions

Real-time PCR has made PCR-based nucleic acid quantification readily accessible to the clinical microbiology laboratory. The laboratory director must consider several factors when designing or validating a quantitative real-time PCR assay. The sensitivity of real-time PCR quantification depends on the efficiency of the PCR reaction as well as the efficiency of nucleic acid purification and, if used, reverse transcription. Accurate quantification depends on several factors that are important in assay design. These include the selection of an appropriate method for generation of fluorescence as the PCR product accumulates. Although dyes for double-stranded DNA and primer-based fluorescence are commonly used in research, the additional specificity provided by probe-based methods of amplicon detection makes these appropriate for clinical use. The selection of an external quantification control is an important step in designing a quantitative real-time PCR test, but this is an area in which further development is needed. Although it is desirable that the quantification control resemble (or be) quantified pathogen, this is not always practical, and purified nucleic acids are a reasonable alternative. When national or international quantification standards exist, these should be used if possible.

References

1. Verhulst PF (1838) Notice sur la loi que la population pousuit dans son accroissement. Correspondance mathematique et physique 10:113–121
2. Edwards K, Logan J, Saunders N (eds) (2004) Real-time PCR: an essential guide. Horizon Bioscience, Norfolk
3. Kochanowski B, Reischl U (eds) (1999) Quantitative PCR protocols. Humana, Totowa
4. Forman M, Wilson A, Valsamakis A (2011) Cytomegalovirus DNA quantification using an automated platform for nucleic acid extraction and real-time PCR assay setup. J Clin Microbiol 49:1703–1705
5. Huang ML, Nguy L, Ferrenberg J, Boeckh M, Cent A, Corey L (2008) Development of multiplexed real-time quantitative polymerase chain reaction assay for detecting human adenoviruses. Diagn Microbiol Infect Dis 62:263–271
6. Iwaki KK, Qazi SH, Garcia-Gomez J et al (2010) Development of a real-time quantitative PCR assay for detection of a stable genomic region of BK virus. Virol J 7:295
7. Higuchi R, Dollinger G, Walsh PS, Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y) 10:413–417

8. Karsai A, Muller S, Platz S, Hauser MT (2002) Evaluation of a homemade SYBR green I reaction mixture for real-time PCR quantification and gene expression. Biotechniques 32(790–2): 794–796
9. Buh Gasparic M, Tengs T, La Paz JL et al (2010) Comparison of nine different real-time PCR chemistries for qualitative and quantitative applications in GMO detection. Anal Bioanal Chem 396:2023–2029
10. Hayden RT, Hokanson KM, Pounds SB et al (2008) Multicenter comparison of different real-time PCR assays for quantitative detection of Epstein–Barr virus. J Clin Microbiol 46:157–163
11. Pang XL, Fox JD, Fenton JM, Miller GG, Caliendo AM, Preiksaitis JK (2009) Interlaboratory comparison of cytomegalovirus viral load assays. Am J Transplant 9:258–268
12. Behzadbehbahani A, Klapper PE, Vallely PJ, Cleator GM (1997) Detection of BK virus in urine by polymerase chain reaction: a comparison of DNA extraction methods. J Virol Methods 67:161–166
13. Caliendo AM, Shahbazian MD, Schaper C et al (2009) A commutable cytomegalovirus calibrator is required to improve the agreement of viral load values between laboratories. Clin Chem 55:1701–1710
14. Gu Z, Belzer SW, Gibson CS, Bankowski MJ, Hayden RT (2003) Multiplexed, real-time PCR for quantitative detection of human adenovirus. J Clin Microbiol 41:4636–4641
15. Tichopad A, Kitchen R, Riedmaier I, Becker C, Stahlberg A, Kubista M (2009) Design and optimization of reverse-transcription quantitative PCR experiments. Clin Chem 55:1816–1823

Chapter 20
An Introduction to Amplification–Production–Detection Techniques

Criziel Quinn, Charles W. Stratton, and Yi-Wei Tang

Introduction

This decade has seen the use of molecular detection techniques grow in popularity within the field of diagnostic microbiology. The importance of molecular methods in the detection of infectious disease agents has been recognized because of their abilities to detect targets that may be present in very low concentrations. Even though culture methods are still considered the gold standard for most laboratories, some fastidious bacteria, fungi, and viruses simply do not grow in culture media [1]. Improved speed, sensitivity, specificity, and ease of use compared to traditional culture methods for the detection of clinical pathogens have made molecular techniques an indispensable tool in the modern microbiology laboratory.

Polymerase chain reaction (PCR) has become the backbone of almost all molecular assays. With the use of PCR, it is possible to amplify a single or several copies of DNA or RNA and generate thousands to millions of copies of a particular sequence. After amplification is achieved, the presence or absence of a target is determined using a detection method of choice. For a PCR assay to be successful, a reliable method to detect the presence or absence of an amplified target is needed. Because of the ever-increasing interest in molecular-based assays, the development of amplification product detection methods available for use in diagnostic microbiology laboratory has greatly increased as well.

These detection methods can be classified as either qualitative or quantitative. They can range in complexity from being able to detect one or two analytes to being able to identify multiple targets like those seen in a multiplex PCR [2]. Based on the

C. Quinn (✉) • C.W. Stratton
Departments of Pathology and Medicine, Vanderbilt University Medical Center, Nashville, TN 37212, USA
e-mail: criziel.quinn@vanderbilt.edu

Y.-W. Tang
Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 37212, USA

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theoretical basis behind the technology used, these detection methods can be divided into two different categories: traditional and advanced.

Traditional Detection Methods

Traditional detection methods for the detection of amplification products are gel-based methods that do not have the sensitivity or specificity that most advanced methods have. They also require tedious blotting techniques to achieve desirable results. Agarose gel results are based on size discrimination and therefore can be subjective, leading to imprecise detection results. Ethidium bromide, which is used to visualize the PCR product on the gel, is also a known mutagen. Use and disposal of this dye require special training of laboratory personnel. Despite these disadvantages, some laboratories may still prefer to use gel electrophoresis as a gold standard to confirm amplification [3] or for troubleshooting purposes. Traditional techniques, although labor intensive, are still considerably cheaper than most advanced methods currently in use. Gel electrophoresis and Southern blotting will be discussed further in Chap. 21.

Gel Electrophoresis

Gel electrophoresis is an easy and cost-effective way to separate and visualize DNA fragments by size [4]. The principle behind electrophoresis is based on DNA being negatively charged at a neutral pH because of its phosphate backbone. When an electrical potential is placed on the DNA, it will move towards the positive pole or the anode. The speed of migration will depend on the size of the DNA fragment and the concentration of the gel matrix used. A loading buffer is added to the sample containing the nucleic acid to increase the density of the sample so that it stays at the bottom of the well [5]. A tracking dye also adds color to the sample to allow better visualization during electrophoresis.

To visualize the product, the gels are stained with Ethidium bromide and viewed under a UV transilluminator. A DNA molecular weight marker or a DNA ladder should be used as a standard for determining the sizes of the unknown fragments. For target detection, it is essential to know the amplicon size in order to confirm the presence or absence of an analyte (Table 20.1).

Southern Blot

This method involves the transfer of DNA fragments separated by gel electrophoresis onto a filter membrane. The procedure employs the use of restriction enzymes to cut the purified DNA into smaller pieces of different sizes. This step is followed by gel electrophoresis where these DNA fragments will be sorted by size.

Before blotting can occur, the gel can be treated with an acid (typically HCl) to depurinate the DNA fragments and render them single stranded. This increases the

Table 20.1 Comparison of current amplification–production–detection techniques

Detection method	Signal molecules	Test turnaround time including PCR amplification (hours)	Comments
Gel electrophoresis	Ethidium bromide	4–8	Labor intensive; subjective and insensitive results
Southern blot	Radioactive label or chemiluminescence	24–48	Tedious process, no longer routinely used in a clinical laboratory
ELISA	Enzyme	6–8	Highest sensitivity; contamination risks are higher
Real-time PCR	Fluorescence	2–4	Fastest test turnaround time; closed system
Direct sequencing	Fluorescence	24–48	Standard for amplicon identification; mostly accurate
DNA microarray	Fluorescence	4–6	Can detect and identify multiple targets simultaneously
Mass spectrometry	Ionic weight	6–12	Excellent discriminatory power for identification; expensive instrumentation; longest test turnaround time

efficiency of DNA transfer from the gel to the membrane. A piece of nitrocellulose or nylon membrane is then applied on the gel. Using an equal amount of pressure to ensure an even amount of contact between the gel and membrane, the DNA is then allowed to transfer to the membrane by capillary action. This can be achieved by using either a vacuum method [4] or a stack of paper towels with a weight on top. After transfer is complete, the membrane is baked to permanently attach the DNA to the sheet. To identify the DNA product of interest, the membrane must be probed with a DNA fragment complementary to the fragment of interest. This radioactive-labeled DNA probe is then incubated with the membrane, washed to prevent nonspecific binding, and visualized on an X-ray film by autoradiography [6]. Alternatively, the membrane is analyzed by color development on a membrane if a chromogenic dye was used to label the probe.

PCR-ELISA Detection Methods

PCR–enzyme-linked immunosorbent assay (ELISA), also sometimes referred to as PCR–enzyme-linked oligosorbent assay (ELOSA) [7], is a hybrid technique of using both PCR and ELISA to detect amplification products. Colorimetric titer plate detection methods were first described in the late 1980s and early 1990s and

have since been used as user-defined assays or as commercially available kits and analyte-specific reagents (ASRs). In this method, the nucleic acids are labeled (e.g., with digoxigenin as described in Tang et al. [8]) during the amplification step. A capture probe specific to the amplicon is used to immobilize it onto the microtiter well plate. Using ELISA, the label is quantified using an antibody (e.g., anti-digoxigenin) against the label. A spectrophotometer [9] can be used to read the colorimetric microtiter plate and quantitate the amount of product detected. The entire process of PCR and the ELISA step combined can be finished in 1 day and can be automated with the use of inexpensive equipment. The ability to run 96 or 384 samples at once makes it a high-throughput detection method.

Because of its speed, usability, and cost-effectiveness, it is considered a better alternative detection method compared to Southern blot and radioactive identification. Despite these advantages, PCR–ELISA can still be labor intensive because of the amount of manual pipetting that is required. Open-reaction vessels are also used posing a risk for carryover amplicon contamination [1].

Advanced Detection Methods

Over the past decade, PCR has become a mainstream technique used on a daily basis in clinical laboratories for the detection of pathogens. More advanced methods for amplicon detection have been developed in order to meet the needs of these laboratories as the demand for PCR-based assays increases. The quest for a faster, cheaper, and more specific platform is never-ending, and there will almost certainly be more novel technologies realized in the near future. Worth mentioning in this introduction are the following methodologies that offer better usability and have gained popular acceptance in diagnostic microbiology laboratories:

Real-Time PCR

Real-time PCR (also known as quantitative PCR or qPCR) is a very practical variation of the PCR process. Whereas traditional PCR allows end-point detection, this method detects the accumulation of amplification products as the reaction is taking place, hence the name “real-time.” Because most real-time PCR methods combine target amplification and fluorescent detection in a single reaction vessel, there is less potential for carryover contamination from previously amplified products. Rapid thermal cycling conditions offer shorter turnaround times and less hands-on time for laboratory personnel. Real-time PCR has proven to be an essential tool [10] for rapid identification and quantification in diagnostic molecular laboratories. In recent years, one important clinical application of real-time PCR has been in viral load measurement and in monitoring the efficacy of antiviral therapy [11].

Real-time PCR techniques can be placed under two categories:

Nonspecific Detection

A simple, less-specific, cheaper alternative for quantifying PCR products is the double-stranded DNA binding dye chemistry. This methodology quantitates amplification products with the use of nonspecific intercalator dyes like ethidium bromide, SYBR green, acridine orange [1], or pyrylium dye [12].

SYBR green is much more commonly used than the other dyes because it can discriminate between double strands and single strands better with more accuracy [1]. This double-stranded DNA-specific dye will bind to the minor groove of the DNA double helix. The amount of fluorescence emitted will be proportional to the amount of double-stranded DNA detected. However, because it detects any double-stranded product, it will also detect nonspecific amplicons as well as primer–dimer complexes. Because of the high potential for false positives, hot-start PCR techniques and follow-up testing are recommended (i.e., melting point or dissociation curve analysis) for amplicon determination [13]. Because of these disadvantages, it may not be as useful to a clinical microbiology laboratory as it might be to a research laboratory.

Specific Detection

The technology behind most of the specific detection methods available is based on fluorescent resonance energy transfer (FRET). FRET technology [14] involves the detection and quantitation of a fluorescent reporter. Although there are other probes that are commercially available in the market today which include the locked nucleic Acid (LNA) probes and the Scorpion® probes, only the Taqman®, Molecular Beacon®, and Lightcycler® probes are discussed in this introduction. Because of their known efficiency when compared to the Scorpion® probes [15] and the minor groove binder (MGB) probes [16], they are commonly favored for use in a clinical laboratory setting. Numerous instruments are also available for real-time PCR detection using FRET technology, making its use more attractive for clinical laboratories. Some of the more popular options include the LightCycler (Roche Applied Science, Indianapolis, IN), SmartCycler (Cepheid, Sunnyvale, CA), and the ABI 7900HT (Applied Biosystems, Foster City, CA).

TaqMan®

The TaqMan hydrolysis probe involves the measurement of fluorescent signals emitted by the quencher and reporter dyes as they are cleaved by the 5′ nuclease activity of the Taq polymerase. This method utilizes the use of hydrolysis (Taqman) probes. The Taq polymerase extends the primers and digests the probe, thereby releasing the reporter from the vicinity of the quencher [4]. Product amplification is detected by monitoring the increase in fluorescence of the reporter dye. The cleavage will only occur if the probe is hybridized; therefore only specific amplification

is detected. It also provides a closed system, therefore minimizing the likelihood for carryover contamination. Because of their rapid test turnaround time, versatility, simple design, and synthesis, Taqman probes have greatly increased in popularity in the molecular field.

Molecular Beacons®

Molecular beacons [17] are single-stranded oligonucleotides that contain a fluorescent dye (e.g., FAM, TAMRA, TET, or ROX) on one end and a quenching dye (usually DABCYL or BHQ) on the other end. Similar to TaqMan probes, molecular beacons also use FRET to detect and quantitate amplification products. When the beacon is free in solution or is not hybridized, it forms a hairpin-loop structure to bring the fluorescent dye and the quencher dye close together. The close proximity between the dyes in this hairpin configuration inhibits reporter fluorescence. If the target is present during the annealing step, the beacon will hybridize to the target and form a probe–target hybrid. Consequently, the beacon undergoes a conformational change that causes the reporter and the quencher dyes to move away from each other, therefore allowing reporter fluorescence to take place [17]. The hairpin structure of the molecular beacon allows it to discriminate single base-pair mismatches better in comparison to linear probes. Several uses of molecular beacons include SNP detection, real-time PCR quantification [18], allelic discrimination, and multiplex PCR assays [19].

Roche Lightcycler Probes®

Lightcycler probes are sequence-specific and highly sensitive fluorescent probes developed for use with the Roche Lightcycler®. The Lightcycler Probe system is made up of a pair of single-stranded fluorescent-labeled oligonucleotide. The first probe is labeled at the 3′ end with a donor fluorophore dye (i.e., FAM) and the second probe is labeled at the 5′ end with an acceptor fluorophore dye (i.e., 5′ Lightcycler Red 610, 640, 670, or 705). During the PCR reaction, these two probes bind to the specific amplicon sequence and bring the donor dye into close proximity with the acceptor dye. Once the donor dye is excited by light from the Lightcycler instrument, energy is transferred by FRET from the donor to the acceptor dye. This increase in fluorescence signal is directly proportional to the amount of amplicon present.

The Lightcycler probes are commonly used for Real-time quantitative PCR, SNP detection, Allelic discrimination, or End-point detection. Recent research has shown the importance of using the Roche lightcycler probes in Real-time PCR [20] and in multiplex PCR assays as well [21]. Because these probes have increased thermal stability and hybridization specificity, the probes are preserved during the reaction and offer greater accuracy for gene quantitation and allelic discrimination.

Capillary Electrophoresis and Direct Sequencing

Capillary electrophoresis was originally used to separate organic chemicals and inorganic ions and metals. This method is preferred over high-performance liquid chromatography (HPLC) because it is cheaper and results can be obtained faster [4]. It is increasingly being used for the separation and analysis of PCR products and nucleic acids because of its high sensitivity. It is a good alternative to gel electrophoresis because of its ability for automation. Manual pouring of slab-gels in traditional gel electrophoresis methods can cause inconsistencies in the matrix affecting results. Unlike traditional methods though, expensive instrumentation is usually needed to gather data and resolve sequences. Instruments like the Applied Biosystems 3130 and 3130xl Genetic Analyzers (Applied Biosystems, Foster City, CA) are very expensive but can run up to four 96- or 384-well plates at once. Both these instruments also have the ability to detect multiple fluorophores which enables better discrimination between similar sizes.

Direct amplicon sequencing is commonly used in the clinical laboratory to detect viral mutations (i.e., HIV, Hepatitis B and C) to monitor the development of antiviral drug resistance in chronically ill patients. Before genotyping can occur, the viral nucleic acid is extracted and amplified by PCR. The PCR products are then analyzed for mutations through sequencing [4]. Commercially available kits for genotyping have made it possible to obtain results within 48–72 h, thereby making this method more appealing for use in diagnostic molecular laboratories. An example is the Viroseq HIV-1 Genotyping system (Celera Diagnostics), which incorporates capillary electrophoresis and sequencing in its technology and is currently widely used in laboratories [22] to detect antiretroviral drug resistance mutations. Other commonly used instrumentation for sequencing include the ABI 3130 (Applied Biosystems, Foster City, CA) and the TruGene System (Siemens Healthcare Diagnostics, Deerfield, IL). Direct sequencing, along with deep sequencing (full genome sequencing), is commonly used as the gold standard comparator methods for the identification of viruses or bacteria that do not grow well using culture techniques. BK Polyomavirus does not grow well in culture, making comparison with traditional viral culture methods for a gold standard non-preferable [23]. Direct sequencing for amplification product detection and identification will be discussed in detail in Chap. 22.

Pyrosequencing

Pyrosequencing is a totally different approach to sequencing compared to other chain termination method. In brief, this method relies on detecting the activity of a DNA-synthesizing enzyme with another chemiluminescent enzyme rather than chain termination with dideoxynucleotides.

The reaction begins with a single-stranded sequencing template. Along with this template, the reaction mix also includes a sequencing primer, sulfurylase and

luciferase, and two substrates adenosine 5′ phosphosulfate (APS) and luciferin. Solutions of A, C, G, and T nucleotides are added and removed from the reaction. The generation of light indicates which nucleotide solution complements the first unpaired base of the template. In turn, the sequence of solutions which produced chemiluminescence determines the sequence of the template.

A huge advantage of this system is the absence of gels, fluorescent dyes, or ddNTPs. However, this method is limited to short sequence analysis (about 300–500 nucleotides) and is mostly used for mutation detection and infectious disease typing like in determining Hepatitis C virus genotypes [24], rather than for generating new sequences.

DNA Microarray

DNA microarray is a multiplex technology commonly used in the field of molecular biology. It is a collection of microscopic features (usually DNA) on a glass slide or similar medium that can be probed with target molecules [25] or fluorescent nucleotides that are used to label the test samples. It is also sometimes supplied with software for identifying the spots on the array by the array reader. Depending on the medium used, a microarray assay can either be solid or liquid. The traditional solid-phase array will have spots or features, each with a specific probe, attached to a solid surface such as nylon membranes, glass, or silicon biochip (DNA chip or genome chip). Other Microarray platforms such as seen in Illumina BeadChip Technology (Illumina, San Diego, CA) use microscopic beads instead of the large solid support, and are therefore referred to as liquid-phase microarrays. The Luminex liquid microarray system (Luminex, Austin, TX) employs the use of liquid beads as well instead of a solid medium.

Microarray technology has been used for over a decade in gene expression studies and is now gaining popularity in microbial identification and detection. Amplification by broad-range PCR [26] of microbial targets before performing microarray analysis has been considered a standard procedure.

Multiplex PCR amplification techniques have also been incorporated with microarray technology to develop assays to simultaneously detect and identify a panel of microbial pathogens in a single reaction. A couple of years ago, the ResPlex System I (Qiagen, Valencia, CA) was used to detect a group of bacterial targets with known relevance in cases of community-acquired pneumonia from tracheal aspirates collected from pediatric patients [27]. The favorable results obtained from this study increased interest in the use of multiplex PCR with microarray technology for the diagnosis of respiratory viruses.

Today, several commercial products that incorporate microarrays as the identification method are available in the market for the detection of a panel of respiratory viral pathogens [28–30]. Among them are the Resplex II assay (Qiagen, Valencia, CA), the MultiCode-PLx RVP (EraGen Biosciences, Madison, WI), and the xTAG RVP (Luminex, Austin, TX), all of which use a liquid-bead microarray system developed by Luminex [31]. The NGEN respiratory virus assay (Nanogen, San Diego, CA), however, uses a solid electronic microarray technology.

PCR-Mass Spectrometry

A novel approach to amplification product detection employs the use of PCR amplification in combination with mass spectrometry. This strategy allows multiplex analysis and simultaneous identification of a broad range of microorganisms in a given sample. The PLEX-ID system (Abbott Molecular, Des Plaines, IL) relies on PCR amplification and base-composition analysis using high-performance electrospray ionization mass spectrometry [32] (ESI-MS). Multiple pairs of broad-range primers are used to amplify highly conserved regions of bacterial, viral, or fungal genomes. Following PCR amplification, the samples are desalted and are analyzed on the PLEX-ID analyzer [33]. Using the masses of the base compositions of amplicons from all the primer pairs, the organisms present in the sample can be identified and quantified. The PLEX-ID system utilizes the IBIS Bioscience database (Abbott Molecular) of genomic information that correlates base composition to the identity of over 750,000 organisms.

With the results of recent published evaluation studies [34], there is no doubt that PCR-mass spectrometry (PCR-MS) can find a niche in the modern clinical microbiology laboratory in the future as a revolutionary microbial identification tool. However, even with the promise of faster turnaround time and accurate pathogen identification, it is not certain how these instruments will perform under diagnostic laboratory conditions. Cost-effectiveness and test complexity are also still open questions as well. Chapter 25 will explain more about PCR-MS and its applications.

References

1. Persing D, Tenover F, Tang Y-W, Nolte F, Hayden R, van Belkum A (eds) (2011) Molecular microbiology: diagnostic principles and practice, 2nd edn. ASM, Washington, DC
2. Tang YW, Duplex PCR (2009) assay simultaneously detecting and differentiating Bartonella quintana, B. henselae, and Coxiella burnetii in surgical heart valve specimens. J Clin Microbiol 47:2647–2650
3. Myoung Y, Shin JH, Lee JS et al (2011) Multilocus sequence typing for *Candida albicans* isolates from candidemic patients: comparison with Southern blot hybridization and pulsed-field gel electrophoresis analysis. Korean J Lab Med 31:107–114
4. Buckingham LaF M (ed) (2007) Molecular diagnostics: fundamentals, methods, and clinical applications, 1st edn. F.A. Davis, Philadelphia
5. Sambrook J, MacCullum P (eds) (2005) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor
6. Ohga S, Ishimura M, Yoshimoto G et al (2011) Clonal origin of Epstein–Barr virus (EBV)-infected T/NK-cell subpopulations in EBV-positive T/NK-cell lymphoproliferative disorders of childhood. J Clin Virol 51:31–37
7. Mallet F, Hebrard C, Brand D et al (1993) Enzyme-linked oligosorbent assay for detection of polymerase chain reaction-amplified human immunodeficiency virus type 1. J Clin Microbiol 31:1444–1449
8. Tang YW, Rys PN, Rutledge BJ, Mitchell PS, Smith TF, Persing DH (1998) Comparative evaluation of colorimetric microtiter plate systems for detection of herpes simplex virus in cerebrospinal fluid. J Clin Microbiol 36:2714–2717

9. Poljak M, Seme K (1996) Rapid detection and typing of human papillomaviruses by consensus polymerase chain reaction and enzyme-linked immunosorbent assay. J Virol Methods 56:231–238
10. Li H, Dummer JS, Estes WR, Meng S, Wright PF, Tang YW (2003) Measurement of human cytomegalovirus loads by quantitative real-time PCR for monitoring clinical intervention in transplant recipients. J Clin Microbiol 41:187–191
11. Mylonakis E, Paliou M, Rich JD (2001) Plasma viral load testing in the management of HIV infection. Am Fam Physician 63(483–90):95–96
12. Yamamoto N, Okamoto T (1995) A rapid detection of PCR amplification product using a new fluorescent intercalator; the pyrylium dye, P2. Nucleic Acids Res 23:1445–1446
13. Ririe KM, Rasmussen RP, Wittwer CT (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 245:154–160
14. Cardullo RA, Agrawal S, Flores C, Zamecnik PC, Wolf DE (1988) Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer. Proc Natl Acad Sci U S A 85:8790–8794
15. Reynisson E, Josefsen MH, Krause M, Hoorfar J (2006) Evaluation of probe chemistries and platforms to improve the detection limit of real-time PCR. J Microbiol Methods 66:206–216
16. Josefsen MH, Lofstrom C, Sommer HM, Diagnostic HJ (2009) Diagnostic PCR: comparative sensitivity of four probe chemistries. Mol Cell Probes 23:201–203
17. Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14:303–308
18. Lewin SR, Vesanen M, Kostrikis L et al (1999) Use of real-time PCR and molecular beacons to detect virus replication in human immunodeficiency virus type 1-infected individuals on prolonged effective antiretroviral therapy. J Virol 73:6099–6103
19. Vet JA, Majithia AR, Marras SA et al (1999) Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc Natl Acad Sci U S A 96:6394–6399
20. Stevens MP, Garland SM, Tabrizi SN (2008) Development and validation of a real-time PCR assay specifically detecting human papillomavirus 52 using the Roche LightCycler 480 system. J Virol Methods 147:290–296
21. Selvaraju SB, Wurst M, Horvat RT, Selvarangan R (2009) Evaluation of three analyte-specific reagents for detection and typing of herpes simplex virus in cerebrospinal fluid. Diagn Microbiol Infect Dis 63:286–291
22. Kazennova EV, Vasil'ev AV, Korovina GI et al (2009) [Comparative analysis of ViroSeq and Trugene HIV-1 genotyping systems in the application to the virus variants in Russia]. Klin Lab Diagn (12)46–51
23. Zhong S, Zheng HY, Suzuki M et al (2007) Age-related urinary excretion of BK polyomavirus by nonimmunocompromised individuals. J Clin Microbiol 45:193–198
24. Elahi E, Pourmand N, Chaung R et al (2003) Determination of hepatitis C virus genotype by pyrosequencing. J Virol Methods 109:171–176
25. Miller MB, Tang YW (2009) Basic concepts of microarrays and potential applications in clinical microbiology. Clin Microbiol Rev 22:611–633
26. Tang YW, Ellis NM, Hopkins MK, Smith DH, Dodge DE, Persing DH (1998) Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli. J Clin Microbiol 36:3674–3679
27. Deng J, Zheng Y, Zhao R, Wright PF, Stratton CW, Tang YW (2009) Culture versus polymerase chain reaction for the etiologic diagnosis of community-acquired pneumonia in antibiotic-pretreated pediatric patients. Pediatr Infect Dis J 28:53–55
28. Li H, McCormac MA, Estes RW et al (2007) Simultaneous detection and high-throughput identification of a panel of RNA viruses causing respiratory tract infections. J Clin Microbiol 45:2105–2109
29. Brunstein J, Thomas E (2006) Direct screening of clinical specimens for multiple respiratory pathogens using the Genaco Respiratory Panels 1 and 2. Diagn Mol Pathol 15:169–173
30. Krunic N, Yager TD, Himsworth D, Merante F, Yaghoubian S, Janeczko R (2007) xTAG RVP assay: analytical and clinical performance. J Clin Virol 40(Suppl 1):S39–S46

31. Dunbar SA (2006) Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. Clin Chim Acta 363:71–82
32. Ecker DJ, Sampath R, Li H et al (2010) New technology for rapid molecular diagnosis of bloodstream infections. Expert Rev Mol Diagn 10:399–415
33. Eshoo MW, Crowder CD, Li H et al (2010) Detection and identification of *Ehrlichia* species in blood by use of PCR and electrospray ionization mass spectrometry. J Clin Microbiol 48:472–478
34. Massire C, Ivy CA, Lovari R et al (2011) Simultaneous identification of mycobacterial isolates to the species level and determination of tuberculosis drug resistance by PCR followed by electrospray ionization mass spectrometry. J Clin Microbiol 49:908–917

Chapter 21
Gel Electrophoresis, Southern Blot, and Colorimetric Microwell Plate-Based System

Jie He, Michael J. Loeffelholz, and Jiang Fan

Introduction

Infectious disease-related illnesses are a significant threat to human health resulting in substantial morbidity and mortality, worldwide. Timely and accurate diagnostic tools are critical for patient treatment decisions and disease outcomes. Molecular diagnostics are revolutionizing the clinical practice of infectious disease. The various formats of nucleic acid amplification are the most frequently used molecular tests in the diagnosis of infectious diseases due to its exquisite sensitivity and specificity. Gel electrophoresis and Southern hybridization are two basic technologies that are used to display the specific amplification of targeted gene and are still used in the laboratories for diagnosis because it is such a powerful technique, and yet reasonably easy and inexpensive.

Due to significant advances in technology, the conventional gel electrophoresis and Southern hybridization are not mainstream methods in molecular diagnostic laboratories anymore. Instead, continued refinements in electrophoresis technology, such as improvements in automation and throughput have allowed this technology to be increasingly adapted and integrated into various currently used state of the art molecular technologies used in clinical and research laboratories for rapid, highly sensitive and specific and quantitative pathogen detection [1–9]. For example, the real-time PCR, which is the most commonly used molecular method, is the combination of the PCR amplification and nucleic acid hybridization with fluorescent

J. He • J. Fan (✉)
Department of Pediatrics, Medical College of Wisconsin,
8701 Watertown Plank road, Milwaukee, WI 53226, USA
e-mail: jhe@mcw.edu; jfan@mcw.edu

M.J. Loeffelholz
Department of Pathology, University of Texas Medical Branch,
301 University Blvd., Galveston, TX 77555-0740, USA
e-mail: mjloeffe@utmb.edu

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labeled probe; the sequencer is the capillary gel electrophoresis system with automatic analysis; and the microarray is a miniature and densified Southern hybridization array. Therefore, the gel electrophoresis and nucleic acid hybridization are the two basic technologies that are being used in most presently available advanced molecular diagnostic assays and systems. In addition, some complex electrophoresis methods, such as 2-D gel systems, have well developed and widely used in analyzing complex pathogenesis to get plenty of information and make molecular diagnosis even more powerful for clinicians providing better treatment and prevention. Thus, this section provides an up-to-date look at the general principles, diagnostic value, and the advances in development of the gel electrophoresis and Southern hybridization technology.

The Principles and Application of Gel Electrophoresis

Electrophoresis is a technique used to separate charged molecules in a gel matrix. The macromolecules, DNA, RNA, and protein are charged molecules. These charged molecules could be moved by electric current through a gel matrix. The types of gel most commonly used are agarose and polyacrylamide [10]. Agarose is a polysaccharide consisting mainly of long chain of galactopyranose residues. Dissolved agarose can polymerize into a semisolid matrix by cross-linking the sugar polymers with each other to form the gel matrix. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and *N,N*-methylene-bis-acrylamide. The polymerization is initiated by the addition of ammonium persulfate along with either dimethyl amino-propionitrile (DMAP) or *N,N,N,N*,-tetramethylethylenediamine (TEMED) [11]. The polyacrylamide gels are neutral, hydrophillic, 3-D networks of long hydrocarbons cross-linked by methylene groups. The separation of molecules within an agarose or polyacrylamide gel is determined by the relative size of the pores formed within the gel. For agarose gel, the pore size of a gel is determined by the concentration of agarose. The higher the concentration of agarose is, the smaller the pore size is. The pore size of a polyacrylamide gel is determined by two factors, the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the total amount of acrylamide increases, the pore size decreases. With cross-linking, 5 %C gives the smallest pore size. Any increase or decrease in %C increases the pore size. The total acrylamide is given as a % (w/v) of the acrylamide plus the bis-acrylamide.

In spite of the many physical arrangements for the apparatus, electrophoretic separations depend upon the charge distribution of the molecules being separated. DNA and RNA are negatively charged molecules, and can be moved by electric current toward the anode through a matrix of agarose. The migration of the DNA molecules is almost entirely dependent on their size when they are electrophoresed in the buffer system with certain concentrations of iron and pH. Proteins have different isoelectric point and could be positively or negatively charged depending on the PH of the buffer they were kept. When the detergent sodium dodecyl

sulfate (SDS) is used with proteins, all of the proteins become negatively charged by their attachment to the SDS anions. Therefore, all of the proteins can migrate toward the anode when separated on a polyacrylamide gel. The procedure is called sodium dodecyl sulfate-polyacrylamide gel electrophoresis abbreviated as SDS-PAGE. The technique has become a standard means for molecular weight determination [12–15].

Although the traditional gel electrophoresis was used in the molecular diagnosis in clinical genetics [10, 16], The traditional agarose or polyacrylamide gel electrophoresis are not the primary method anymore to detect the PCR amplification for the diagnosis purpose. The traditional electrophoresis process is time-consuming that do not fit the requirement of rapid molecular diagnosis. In addition, the size information of amplicons that gel electrophoresis acquired is not specific enough to determine etiological pathogen. Recently, automatic gel electrophoresis systems have been developed and released to the market [17, 18]. The ScreenTape system [17] automatically loads, runs, and analyzes sample of RNA, DNA, and protein. Each card-sized tape containing 16 microgels can analyze up to 16 DNA, RNA, or protein samples in 1 min per sample. The FT-2020A auto agarose gel electrophoresis analyzer [18] can automatically run up to 24 tests at the controlled temperature. This instrument can connect with auto sample processing system. These automatic gel electrophoresis systems dramatically reduced the running time and sample handling time, and were designed to coordinate with automatic sample preparation system. Automatic electrophoresis systems provide the potential to be applied in the automatic diagnostic device that integrates the nucleic acid extraction, amplification, and detection together. They might become the new detection choice of the rapid, automatic diagnosis system.

Electrophoresis can be 1-D or 2-D. 1-D electrophoresis separates macromolecules with the electric current from one direction and is used for most routine protein and nucleic acid separations. The gel result shows the bands of macromolecules on gel according to their size and can be integrated visually. The specific amplification of targeted pathogen gene can be detected by showing the specific size band on either agarose gel or 1-D polyacrylamide gel. The 1-D polyacrylamide gel can separate the proteins in a sample and then distinguish the presence of pathogen by hybridizing the protein band with specific antibody on the membrane.

2-D electrophoresis starts with 1-D electrophoresis, and then further separates molecules by the second direction current in a 90° from the first current. Therefore, molecules are more effectively separated in 2-D electrophoresis since complex protein mixtures within a sample (cell, pathogen, clinical specimen) can be resolved effectively according to their isoelectric points and molecular weights by 2-D polyacrylamide gel electrophoresis [19]. The "spot" of 2-D electrophoresis could be subject to mass spectrometry for identification and analyzed with the assistant of software [20] due to the complexity of the gel image.

2-D gel electrophoresis was a classic method used in the proteomics. 2-D gel electrophoresis can be combined with mass spectrometry techniques and genomic databases to determine the quality and quantity of proteins, and the information of protein–protein interaction [21]. By using these methods, the molecular diagnosis

of infection disease not only provides the etiologic information of infection diseases [22], but also investigates characterization of pathogenicity of a pathogen agent [23]; discovery of further putative virulence determinants assesses global cellular response toward infection and identifies potential anti-infection drug targets. A number of groups have applied proteomics to the study of fungal [24], bacterial, and viral infections [22, 25–27]. These researches develop the early diagnosis method for challenge pathogens (i.e., fungi *Candida albicans* and *Aspergillus fumigatus*) [22], investigate the molecular details of pathogenicity that are hardly understood and identify the cellular proteins that is responsible for infection.

The Principles of Southern Blotting

Southern blot is a molecular method used for detection of a specific DNA sequence in DNA samples. Southern blotting combines gel electrophoresis and probe hybridization together. Both fragment size and probe sequence are used to determine the specificity of a result [28]. DNA was run on an agarose gel to separate them by size. The negatively charged DNA band on gel was then transferred to positively charged membrane. Before transferring, the DNA gel sometimes needs to be treated with acid to break the DNA that is larger than 15 kb to smaller piece so that the transfer from the gel to membrane is more efficient. Some research also treats the DNA gel with an alkaline solution as they believe that an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, and also denature DNA into single strands for later hybridization. The transfer of the DNA to membrane is done by placing the gel on top of a buffer-saturated filter paper, then by laying a sheet of nitrocellulose or nylon membrane on top of the gel (or toward the positively charged direction if the electrical transfer is applied), and finally placing some dry filter papers on top of this membrane.

Pressure is applied evenly to the gel to ensure good and even contact between gel and membrane. Due to capillary action, the buffer moves from the bottom filter paper through the gel carrying with it the denatured DNA present in the gel. The DNA becomes trapped in the nitrocellulose or nylon membrane as the buffer phases through it. This process takes several hours to complete. The relative positions of the bands on the membrane remain the same as those in the gel and there is a minimal loss in their resolution. The membrane is then baked in a vacuum or regular oven at 80 °C for 2 h (standard conditions; nitrocellulose or nylon membrane) or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane. The radioactive or dye-labeled probe could bind to the free binding sites on the membrane because single-stranded DNA has a high affinity for nitrocellulose membrane. To prevent nonspecific binding, the baked membrane is treated, with a solution containing salmon or herring sperm DNA, 0.2 % each of Ficoll (an artificial polymer of sucrose), polyvinylpyrrolidone and bovine serum albumin. This pre-hybridization solution often also use for hybridization reaction. The probe DNA has labeled a single DNA fragment with a specific

sequence whose presence in the target DNA is to be determined so that it can be detected; the probe usually incorporates radioactivity or a fluorescent or chromogenic dye. While the membrane is incubated with the hybridization probe, the probe with a specific sequence binds to the DNA fragment that the sequence is complement to the probe sequence. Therefore, the labels of probe showed the position of a specific DNA fragment. To ensure the specificity of the binding of the probe to the sample DNA, most common hybridization methods use deionized formamide, and detergents such as SDS to reduce nonspecific binding of the probe. After hybridization, excess probe is washed from the membrane using SSC buffer, and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe or by the development of color on the membrane if a chromogenic detection method is used. The conventional Southern blot is a complex process that does not fit the need and trend of the rapid molecular diagnosis and has not been used for diagnosis anymore. But the DNA hybridization technology and concept have been used to develop many modern technologies, such as microplate assay that we introduce later in this chapter and microarray technology that is illustrated in another chapter.

Although the gel electrophoresis and Southern blot technology we discuss here are not commonly used techniques for molecular diagnosis anymore, these two technologies represent two basic principles that were applied in the development of many modern molecular diagnostic methods. It is helpful for clinicians and infectious disease specialists to understand the basic principles, procedures, and limitations of the diagnostic techniques to best interpret test results that use these methodologies.

Colorimetric Microwell Plate-Based System

Principle of Colorimetric Microwell Plate-based System for Amplicon Detection

The concept of colorimetric microwell plate-based system (CMPS) for amplicon detection is original from the immune assay, such as Elisa. However, the difference between Elisa and CMPS for amplicon detection is, for Elisa, which is protein–protein interaction, or antigen/antibody reaction; for amplicon detection, which is nucleic acid/nucleic acid interaction, or sequence reaction. These sequence-specific methods for the detection of the products of nucleic acid (amplicons or PCR products) have been developed for a variety of solid phases, including nylon membranes, microwell plates, microparticles, and most recently, microchips (oligonucleotide probe microarrays) [29–35]. Nucleic acid amplification assays (e.g., PCR) that employ microwell plate detection offer a number of advantages, including convenience (nonisotopic chemistry), simplicity, high throughput, and high specificity. This format is amenable to automation and has sensitivity comparable to that of

other detection systems and chemistries. The procedure has many similarities to enzyme-linked immunosorbent assays (ELISA); in fact, this amplicon detection method has been referred to as the enzyme-linked oligosorbent assay (ELOSA), and enzyme hybridization assay (EHA). The scope of this part is limited to procedures in which the molecules that capture amplicons are immobilized onto the surface of wells of microwell plates. As such, methods that capture amplicons on microparticles or microspheres (e.g., Luminex, Austin, Texas) are not discussed, even though the detection is carried out in microwell plates.

The detection of amplicons by CMPS were first described in the late 1980s [36–39] and were based on procedures for characterization of synthetic oligonucleotides by hybridization in microwell plates [40, 41]. Early microwell plate detection procedures used sandwich hybridization, in which two or more probes—a capture probe and a secondary labeled probe—were used to detect amplicons [37–39]. Typically, a single biotin-labeled probe with a sequence specific for the captured amplicon was used as the secondary probe [38]. In a variation of this procedure, two secondary probes were used to detect a hybridized amplicon: an initial probe containing an amplicon-specific sequence at the 5′ end and a 3′ poly(T) tail, and an additional probe containing a 5′ poly(A) tail and biotin substituents [39]. Sandwich hybridization was simplified to a direct hybridization procedure, in which the amplicon was labeled with biotin during the extension phase of the PCR cycle, thereby eliminating the need for a secondary probe [36, 42]. The direct hybridization procedure is easier to perform than the sandwich hybridization and has lower background levels [42].

CMPS can be divided into two formats based on the molecule used to capture amplicons: oligonucleotide probe (sequence-specific capture) and avidin (nonspecific capture). Formats in which an immobilized oligonucleotide probe is used as the capture agent rely on sequence-specific DNA–DNA hybridization of amplicons to the probe. Formats in which avidin (or streptavidin) is used as the capture agent rely on the high affinity of this chemical for biotin, in addition to the specificity of the amplicon-probe hybridization.

CMPS has been developed in-house [29, 36–39, 42–45] and are available commercially (Millipore, Billerica, Mass.; Roche Diagnostics, Indianapolis, IN; Argene, North Massapequa, NY) [46–49]. The Roche format is available only as part of complete kits (Amplicor) for detection of infectious agents, including *Chlamydia trachomatis*, human immunodeficiency virus, and hepatitis C virus. These kits include reagents for specimen processing and PCR amplification. The Millipore (formerly Chemicon) systems, OligoDetect and ChemFLASH, consist of universal detection reagents only. Argene provides both universal detection systems and kits for specific infectious agents. A line of microwell-based, multiplex PCR assays was available from Gen-Probe Prodesse, Waukesha, WI. These assays included Hexaplex (detection of several RNA respiratory viruses), Adenoplex (detection of adenoviruses), Pneumoplex (detection of several bacterial agents causing pneumonia) as well as other assays [50, 51]. The commercially available systems offer optimized hybridization conditions and reagent formulations. With in-house-developed assays, the user must optimize reagent formulations and assay conditions and verify performance.

An advantage of in-house-developed assays is the lower cost of reagents. All of the aforementioned commercial and in-house developed CMPS involved the capture of amplicons by a capture molecule immobilized in plate wells. There are additional commercially available nucleic acid amplification assays that utilize the microwell detection format, but without actual capture, or binding of amplicons. The ProbeTec *C. trachomatis* and *Neisseria gonorrhoeae* assays (Becton Dickinson, Sparks, MD) utilize microwell plates to perform strand displacement amplification, and detection of extended primers using fluorescein-labeled probes in solution. Another variation on the amplicon-microwell detection format is the hybrid capture chemistry employed by Digene (now Qiagen, Germantown, MD). During hybrid capture, target DNA–RNA probe hybrids are captured onto a solid microwell phase coated with antibodies specific for DNA:RNA hybrids. After capture, the hybrids are detected with multiple antibodies conjugated to alkaline phosphatase (signal amplification).

Clinical Application of CMPS for Amplicon Detection

There is substantial literature demonstrating the clinical utility of CMPS for PCR amplicon detection. The Hexaplex assay, which detects and quantitates RNA of influenza viruses A and B, respiratory syncytial viruses A and B, and human parainfluenza virus types 1, 2, and 3, was shown to be more sensitive than culture [51]. Of 109 total respiratory specimens tested, all 29 culture positive specimens were positive by Hexaplex. In addition, eight culture negative specimens were positive by Hexaplex. All eight specimens with presumptive false-positive Hexaplex results were from symptomatic patients with low RNA viral loads. The Argene Enterovirus Consensus assay was more sensitive than culture, and comparable to nucleic acid sequence-based amplification for detection of enteroviruses in a variety of clinical specimens [49]. The Amplicor *C. trachomatis* assay was significantly more sensitive than culture, and 100 % specific [46]. Stellrecht et al. developed a multiplex CMPS assay for detection of several genital mycoplasmas [45] and an assay for detection of enteroviruses [44]. Loeffelholz et al. developed a microwell detection assay for detection of *Bordetella pertussis* that was significantly more sensitive than culture or direct fluorescent assay [33]. Tang et al. devised comparison studies between microtiter detection assays and conventional standards of diagnoses finding that microtiter assays for viruses such as herpes simplex viruses, respiratory syncytial virus, influenza virus, cytomegalovirus, tuberculosis, hepatitis C virus, histoplasmas, *and Bartonella quintana*, to be more sensitive, specific, timely with a decreased risk of contamination that the previously held conventional methods used for detection provided [52–60]. Improving upon the benefit of rapid results of microassays was the ability to quickly assign a quantification, as Li et al. displayed the ability to measure the viral load of cytomegalovirus through the advent of a real-time PCR assay using a sequence detection system [58]. Some applications increased the degree of virus coverage in a CMPS assay with the testing of multiple viruses as in research by Tang et al. compiling an eight herpes simplex viruses

microassay allowing for an increase in the ability to screen and identify a specific virus [56]. Lastly, Rahimian et al. demonstrated the superiority of PCR-based technology to serology when differentiating *B. quintana* from *Coxiella burnetii,* wherein serological testing was unable to produce a specific positive between two bacteria; alluding to a false coinfections [60]. Further research and studies concerning the efficiency and specificity of PCR-ELISA assays were defined by developments in the tests for Alphavirus, chronic fatigue syndrome (CFS), *Neisseria meningtitidis*, avian influenza virus, *Listeria*, human rotavirus, parvovirus B19, RSV, enteroviruses, and noroviruses [61–72]. Further studies comparing the use of the aforementioned Prodesse EHA containing a panel of seven distinct viruses and subtypes demonstrates the range of specificity and sensitivity of these microtiter assays over previously used methods of detection [51].

Developments into the research of assays which allow for high throughput of samples such as with blood genotyping and detecting trace bacterial pathogens such as *Aeromonas salmonicida*, *Tenacibaculum maritimum*, *Lactococcus garvieae*, and *Yersinia ruckeri* were compared by the studies by St. Louis, Wilson, and Carson, respectively, to demonstrate the higher efficiency which RT-PCR ELISA hybridization assays afford one over lesser throughput serological methods [63, 72]. Other CMPS-based applications which include the Pneumoplex: a multiplex PCR-EHA that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, *Legionella micdadei*, and *B. pertussis*, and its real-time counterpart by Khanna et al.; development of a sensitive and specific multiplex RT-PCR assay for detection of 11 common viral and bacterial pathogens causing community-acquired pneumonia or sepsis using two detection strategies: enzyme hybridization and microarray and carriage study by Kumar et al.; rapid Multiplex RT-PCR typing of influenza A and B, and subtyping of influenza A into H1, 2, 3, 5, 7, 9, N1 (human), N1 (animal), N2, and N7 including typing of novel swine-origin influenza A (H1N1) virus during current 2009 outbreak in Milwaukee and simultaneous detection of CDC category "A" DNA and RNA bioterrorism agents by He et al. [50, 73–75] are all demonstrated high specificities and sensitivities.

How to Develop the CMPS for Amplicon Detection?

Among factors to consider when developing a CMPS assay are oligonucleotide labeling, microwell plate choices, immobilization of the probe in microwell plate wells, hybridization conditions, and detection of the hybridized amplicon. Microwell plate detection procedures are frequently based on capture and detection of biotinylated amplicons. This requires that the primer used in the PCR amplification to generate the amplicon strand complementary to the probe be tagged with biotin. The biotin substituent will allow the amplicon to be either captured or detected by avidin (or streptavidin), depending on the detection format. Most oligonucleotide vendors offer 5′-end biotin-labeled oligonucleotides.

Polystyrene microwell plates are typically used for amplicon detection because of their high DNA binding capabilities. Three common brands of commercially available microwell plates are Immulon (Thermo Fisher Scientific, Waltham, MA), Nunc (Thermo Fisher Scientific), and Costar (Corning, Corning, NY). Eight-well removable strips offer convenience and flexibility. Immobilization of DNA to the polystyrene surface is often accomplished by covalent binding (coating) of unmodified DNA probe. The probe is diluted to the appropriate concentration in coating buffer, added to microwells, and incubated under various conditions. Probe-coating conditions were described in several references [37, 48, 50, 64]. Using 1 M ammonium acetate as coating buffer, if the incubation condition is at 37 °C, overnight, the wash buffer is PBS, 0.1 % Tween-20; if the incubation condition is at 37 °C, 2 h, the wash buffer is 2× SSC, 1.0 % Tween-20. Using 25 mM KH_2PO_4, 25 mM $MgCl_2$ as coating buffer, the incubation condition is at room temperature, 2 h, the wash buffer is 25 mM KH_2PO_4, 100 mM $MgCl_2$. Using 1.5 M NaCl, 0.3 M Tris (pH 8.0), 0.3 M $MgCl_2$ as coating buffer, the incubation condition is at 37 °C, overnight, the wash buffer is 1 M NaCl; 0.1 M Tris (pH 9.3); 2 mM $MgCl_2$, 0.1 % Tween-20. Probe-coated microwell plates stored with desiccant at 4 °C are usually stable for weeks to months, although stability should be verified by the individual user. Also available from several vendors are microwell plates covalently bound with streptavidin. These plates are used to bind biotinylated amplicons. Once bound, these biotin–streptavidin complexes are stable at salt concentrations of 500 mM NaCl and detergent concentrations of 1 % SDS. In a second step, the captured amplicons bind a sequence-specific, labeled probe.

Conditions for hybridization of denatured amplicon to immobilized DNA probe vary considerably and have generally evolved into more simplified and rapid procedures with fewer steps and shorter incubation times. Table 21.1 lists hybridization conditions from several published procedures. The goal of the hybridization step is to allow efficient and stable binding of the complementary probe and amplicon sequence while preventing the binding of amplified DNA containing mismatches. The degree to which noncomplementary DNA molecules hybridize depends on the stringency of the hybridization conditions; as stringency increases, hybridization specificity increases. If conditions are too stringent, hybridization of molecules with 100 % complementarity will be prevented or reduced, resulting in lower sensitivity. The washing step immediately following hybridization is critical because it removes unhybridized, labeled molecules that would otherwise react with detection reagents. The stringency of the washing reagent must be such that bound amplicon is not removed. Table 21.1 lists wash buffer formulations. Typically, wash buffer is added to the microplate wells and removed immediately or allowed to soak for several seconds. This may be repeated several times. If using 4× SSC, 3.2× Denhardt's, 10 % dextran sulfate, 10 μg of salmon sperm DNA/mL as hybridization buffer, 2 h pre-hybridization at 65 °C is needed.

The final step of the CMPS assay is the colorimetric detection of hybridized (captured) amplicons. Biotin-labeled amplicons are usually detected using an avidin-enzyme conjugate followed by an enzyme substrate. Appropriate conjugates for solid-phase assays include alkaline phosphatase and horseradish peroxidase.

Table 21.1 Microwell plate hybridization conditions

Hybridization buffer	Time, temperature, other conditions	Wash buffer[a]	Reference
Amplicor (Roche)	60 min, 37 °C	Amplicor (Roche)	21
30 % formamide, 2× or 4× SSPE,[b] 1 % Triton X-100, 5 % dextran sulfate	30–90 min, room temperature	0.2× SSC,[c] 0.1 % Triton X-100	6
0.15 M NaCl, 0.12 M HEPES (pH 8.0), 25 % dextran sulfate, 33 % formamide	90 min, room temperature, shaking	2× SSC, 0.1 % Tween-20	24
4× SSC, 3.2× Denhardt's,[d] 10 % dextran sulfate, 10 μg of salmon sperm DNA/mL	Overnight, 65 °C	2× SSC (30 min at 65 °C)	22
50 % formamide, 5× SSC, 1× FPG,[e] 25 mM KH_2PO_4 (pH 7.0), 0.2 % SDS, 5 % dextran sulfate, 200 μg of salmon sperm DNA/mL	4 h, 42 °C	2× SSC, 0.1 % SDS	18
5× SSC, 5× Denhardt's, 0.2 % SDS, 200 μg of herring sperm DNA/mL	30 min, 50 °C	2× SSC	15
1 pmole of horseradish peroxidase-labeled probe, 7.5× Denhardt's, 3.5× SSPE, 200 μg of herring sperm DNA/mL	60 min, 42 °C	PBS,[f] 0.05 % Tween-20	26

[a]Unless otherwise stated, plate washing did not include prolonged incubation times
[b]1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate buffer (pH 7.0), and 1 mM EDTA
[c]1× SSC is 0.15 M NaCl and 15 mM sodium citrate
[d]1× Denhardt's is 0.02 % Ficoll 400, 0.02 % polyvinylpyrrolidone, and 0.02 % bovine serum albumin
[e]1× FPG is 0.2 % Ficoll 400, 0.02 % polyvinylpyrrolidone 360, and 0.02 % glycine
[f]*PBS* phosphate-buffered saline

The substrates for these enzymes are soluble in the aqueous buffers used in CMPS assays. Alkaline phosphatase substrates include *p*-nitrophenylphosphate and 5-bromo-4-chloro-3-indolyl phosphate. Peroxidase substrates include 3,3′,5,5′-tetramethylbenzidine and 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid). Kirkegaard & Perry Laboratories (Gaithersburg, MD) and Vector Laboratories (Burlingame, CA) are two commercial sources for enzyme conjugates and substrates. Some of the enzyme-substrate systems require the addition of a weak acid to stop color development. Plates employing colorimetric enzyme substrates are read in an ELISA plate reader at 405–450 nm, depending on the substrate used. Variations of this procedure include hybridization of a fluorescein isothiocyanate-labeled probe to capture amplicon, followed by detection of the complex with an anti-fluorescein isothiocyanate antibody conjugate (ChemFLASH; Millipore). A kit no longer commercially available used anti-double-stranded DNA antibody conjugate to detect amplicon-probe hybrids [76].

To achieve adequate PCR assay performance characteristics, including sensitivity, specificity, accuracy, and precision, the CMPS assay must be optimized. Among the components that require optimization are the probe-coating concentration and hybridization stringency. Probe concentrations that are too low will result in low or variable optical density readings. As discussed above, hybridization stringency must be optimized to achieve a desired balance between sensitivity and specificity. When PCR assays are used for diagnostic purposes and the results are reported as such, verification of the performance characteristics is required. For CMPS assays, an optical density value distinguishing positive and negative results (cutoff) must be established and verified using a panel of well-characterized clinical specimens. In addition, the stability of probe-coated microwell plates and other in-house-prepared reagents must be determined and expiration dates should be applied. It is the responsibility of the laboratory to develop and implement appropriate quality control testing of in-house-prepared reagents. The entire CMPS (plate, hybridization and wash buffers, enzyme conjugate, and substrate) may be QC tested as a complete system, using amplicon stocks. References for optimization, verification, and validation of PCR diagnostic tests are available [76, 77].

Summary of CMPS for Amplicon Detection

The CMPS have been widely used for over two decades, offering sensitivity and specificity. Sequence-specific DNA/DNA hybridization makes the PCR product detection more specific than gel detection. Enzyme detection of amplicon/probe hybrids is, in effect, a second amplification process in the CMPS detection format, providing additional sensitivity to that already provided by PCR gene amplification. The sensitivity of conventional PCR and CMPS detection is equivalent to that of real-time PCR or other nucleic acid amplification chemistries [18, 48, 49]. The CMPS detection format is familiar to most microbiologists, and can be adopted and performed using readily available equipment. The multi-well format allows for convenient detection of amplicons from multiplexed PCR. The CMPS detection format is also convenient and economical, particularly in a high-volume laboratory. There are also disadvantages to the CMPS detection format. There are multiple hands-on steps, generally requiring about 2–2.5 h for detection of amplified PCR product. The open system makes the assay susceptible to contamination. However, automation of the PCR and CMPS detection in a closed system, and software analysis of the data could mitigate these disadvantages. CMPS for amplicon detection are highly sensitive, highly specific, capable of high sample throughput of up to 96 samples per run, easy to use, reliable, safe, economic, and flexible multiple target detection. It has the potential to be one of the standard methods for virus and other microorganism detection.

References

1. Caliendo AM, Valsamakis A, Bremer JW et al (2011) Multi-laboratory evaluation of real-time PCR tests for hepatitis B virus DNA quantification. J Clin Microbiol 49(8):2854–2858
2. Yukl SA, Li P, Fujimoto K et al (2011) Modification of the Abbott RealTime assay for detection of HIV-1 plasma RNA viral loads less than one copy per milliliter. J Virol Methods 175:261–265
3. Sire JM, Vray M, Merzouk M et al (2011) Comparative RNA quantification of HIV-1 group M and non-M with the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 v2.0 and Abbott RealTime HIV-1 PCR assays. J Acquir Immune Defic Syndr 56:239–243
4. Mallory MA, Page SR, Hillyard DR (2011) Development and validation of a hepatitis B virus DNA sequencing assay for assessment of antiviral resistance, viral genotype and surface antigen mutation status. J Virol Methods 177(1):31–37
5. Cheval J, Sauvage V, Frangeul L et al (2011) Evaluation of high throughput sequencing for identifying known and unknown viruses in biological samples. J Clin Microbiol 49(9):3268–3275
6. Liu C, Zheng S, Wang Y et al (2011) Detection and molecular characterization of recombinant avian leukosis viruses in commercial egg-type chickens in China. Avian Pathol 40:269–275
7. Lung O, Fisher M, Beeston A et al (2011) Multiplex RT-PCR detection and microarray typing of vesicular disease viruses. J Virol Methods 175:236–245
8. Chen H, Mammel M, Kulka M, Patel I, Jackson S, Goswami BB (2011) Detection and identification of common food-borne viruses with a tiling microarray. Open Virol J 5:52–59
9. Moulton KR, Taylor AW, Rowlen KL, Dawson ED (2011) ampliPHOX colorimetric detection on a DNA microarray for influenza. J Vis Exp (52). pii: 2682. doi:10.3791/2682
10. Hoar DI (1987) Molecular diagnosis. Can Fam Physician 33:401–404
11. Stibenz D (1975) Introduction to polyacrylamide gel electrophoresis. Acta Histochem Suppl 15:57–71
12. Dunker AK, Rueckert RR (1969) Observations on molecular weight determinations on polyacrylamide gel. J Biol Chem 244:5074–5080
13. Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406–4412
14. Bickle TA, Traut RR (1971) Differences in size and number of 80 S and 70 S ribosomal proteins by dodecyl sulfate gel electrophoresis. J Biol Chem 246:6828–6834
15. Swanborg RH, Feldstein SR (1972) Molecular weight of encephalitogenic protein by electrophoresis in sodium dodecyl sulfate (SDS)-acrylamide gels. Proc Soc Exp Biol Med 139:238–241
16. Glickman RM, Phillips MA, Glickman BW (1988) Introduction to DNA-based genetic diagnostics. Can Fam Physician 34:883–889
17. Lab901 (2012) https://www.genomics.agilent.com/GenericB.aspx?pagetype=Custom&subpagetype=Custom&pageid=2617. 2012:6
18. Fortune Co., Ltd. (2011) FT-2020A auto agarose gel electrophoresis analyzer. http://fortunecd.en.made-in-china.com/product/EeinoWtvgbVP/China-FT-2020A-Auto-Agarose-Gel-Electrophoresis-Analyzer.html. 2011:1
19. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021
20. Berth M, Moser FM, Kolbe M, Bernhardt J (2007) The state of the art in the analysis of two-dimensional gel electrophoresis images. Appl Microbiol Biotechnol 76:1223–1243
21. Aebersold R (2003) Mann M Mass spectrometry-based proteomics Nature 422:198–207
22. Ye Y, Mar EC, Tong S et al (2010) Application of proteomics methods for pathogen discovery. J Virol Methods 163:87–95
23. Graham DRM, Mitsak MJ, Elliott ST et al (2008) Two-dimensional gel based approaches for the assessment of N-linked and O-GlcNAc glycosylation in human and simian immunodeficiency viruses. Proteomics 8:4919–4930

24. Kniemeyer O, Schmidt AD, Vodisch M, Wartenberg D, Brakhage AA (2011) Identification of virulence determinants of the human pathogenic fungi *Aspergillus fumigatus* and *Candida albicans* by proteomics. Int J Med Microbiol 301(5):368–377
25. Lu Y, Liu J, Lin C et al (2010) Peroxiredoxin 2: a potential biomarker for early diagnosis of hepatitis B virus related liver fibrosis identified by proteomic analysis of the plasma. BMC Gastroenterol 10:115
26. Rozek W, Ricardo-Dukelow M, Holloway S et al (2007) Cerebrospinal fluid proteomic profiling of HIV-1-infected patients with cognitive impairment. J Proteome Res 6:4189–4199
27. Maxwell KL, Frappier L (2007) Viral proteomics. Microbiol Mol Biol Rev 71:398–411
28. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517
29. Bobo L, Munoz B, Viscidi R, Quinn T, Mkocha H, West S (1991) Diagnosis of *Chlamydia trachomatis* eye infection in Tanzania by polymerase chain reaction/enzyme immunoassay. Lancet 338:847–850
30. Chee M, Yang R, Hubbell E et al (1996) Accessing genetic information with high-density DNA arrays. Science 274:610–614
31. DiDomenico N, Link H, Knobel R et al (1996) COBAS AMPLICOR: fully automated RNA and DNA amplification and detection system for routine diagnostic PCR. Clin Chem 42:1915–1923
32. Inouye S, Hondo R (1993) Type differentiation of herpes simplex virus by stringent hybridization of polymerase chain reaction products. Arch Virol 129:311–316
33. Loeffelholz MJ, Thompson CJ, Long KS, Gilchrist MJ (1999) Comparison of PCR, culture, and direct fluorescent-antibody testing for detection of *Bordetella pertussis*. J Clin Microbiol 37:2872–2876
34. Schachter J, Stamm WE, Quinn TC, Andrews WW, Burczak JD, Lee HH (1994) Ligase chain reaction to detect *Chlamydia trachomatis* infection of the cervix. J Clin Microbiol 32:2540–2543
35. White TJ, Madej R, Persing DH (1992) The polymerase chain reaction: clinical applications. Adv Clin Chem 29:161–196
36. Kawai S, Maekawajiri S, Yamane A (1993) A simple method of detecting amplified DNA with immobilized probes on microtiter wells. Anal Biochem 209:63–69
37. Keller GH, Huang DP, Manak MM (1989) A sensitive nonisotopic hybridization assay for HIV-1 DNA. Anal Biochem 177:27–32
38. Keller GH, Huang DP, Shih JW, Manak MM (1990) Detection of hepatitis B virus DNA in serum by polymerase chain reaction amplification and microtiter sandwich hybridization. J Clin Microbiol 28:1411–1416
39. Rapier JM, Villamarzo Y, Schochetman G et al (1993) Nonradioactive, colorimetric microplate hybridization assay for detecting amplified human immunodeficiency virus DNA. Clin Chem 39:244–247
40. Cook AF, Vuocolo E, Brakel CL (1988) Synthesis and hybridization of a series of biotinylated oligonucleotides. Nucleic Acids Res 16:4077–4095
41. Nagata Y, Yokota H, Kosuda O, Yokoo K, Takemura K, Kikuchi T (1985) Quantification of picogram levels of specific DNA immobilized in microtiter wells. FEBS Lett 183:379–382
42. Keller GH, Huang DP, Manak MM (1991) Detection of human immunodeficiency virus type 1 DNA by polymerase chain reaction amplification and capture hybridization in microtiter wells. J Clin Microbiol 29:638–641
43. Buck GE (1996) Detection of *Bordetella pertussis* by rapid-cycle PCR and colorimetric microwell hybridization. J Clin Microbiol 34:1355–1358
44. Stellrecht KA, Harding I, Hussain FM et al (2000) A one-step RT-PCR assay using an enzyme-linked detection system for the diagnosis of enterovirus meningitis. J Clin Virol 17:143–149
45. Stellrecht KA, Woron AM, Mishrik NG, Venezia RA (2004) Comparison of multiplex PCR assay with culture for detection of genital mycoplasmas. J Clin Microbiol 42:1528–1533

46. Bass CA, Jungkind DL, Silverman NS, Bondi JM (1993) Clinical evaluation of a new polymerase chain reaction assay for detection of *Chlamydia trachomatis* in endocervical specimens. J Clin Microbiol 31:2648–2653
47. Germer JJ, Gerads TM, Mandrekar JN, Mitchell PS, Yao JD (2006) Detection of HIV-1 proviral DNA with the AMPLICOR HIV-1 DNA Test, version 1.5, following sample processing by the MagNA Pure LC instrument. J Clin Virol 37:195–198
48. Hymas WC, Aldous WK, Taggart EW, Stevenson JB, Hillyard DR (2008) Description and validation of a novel real-time RT-PCR enterovirus assay. Clin Chem 54:406–413
49. Landry ML, Garner R, Ferguson D (2003) Comparison of the NucliSens Basic kit (Nucleic Acid Sequence-Based Amplification) and the Argene Biosoft Enterovirus Consensus Reverse Transcription-PCR assays for rapid detection of enterovirus RNA in clinical specimens. J Clin Microbiol 41:5006–5010
50. Khanna M, Fan J, Pehler-Harrington K et al (2005) The pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. J Clin Microbiol 43:565–571
51. Fan J, Henrickson KJ, Savatski LL (1998) Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). Clin Infect Dis 26:1397–1402
52. Tang YW, Heimgartner PJ, Tollefson SJ et al (1999) A colorimetric microtiter plate PCR system detects respiratory syncytial virus in nasal aspirates and discriminates subtypes A and B. Diagn Microbiol Infect Dis 34:333–337
53. Tang YW, Rys PN, Rutledge BJ, Mitchell PS, Smith TF, Persing DH (1998) Comparative evaluation of colorimetric microtiter plate systems for detection of herpes simplex virus in cerebrospinal fluid. J Clin Microbiol 36:2714–2717
54. Tang YW, Li H, Roberto A, Warner D, Yen-Lieberman B (2004) Detection of hepatitis C virus by a user-developed reverse transcriptase-PCR and use of amplification products for subsequent genotyping. J Clin Virol 31:148–152
55. Tang YW, Meng S, Li H, Stratton CW, Koyamatsu T, Zheng X (2004) PCR enhances acid-fast bacillus stain-based rapid detection of *Mycobacterium tuberculosis*. J Clin Microbiol 42:1849–1850
56. Tang YW, Johnson JE, Browning PJ et al (2003) Herpesvirus DNA is consistently detected in lungs of patients with idiopathic pulmonary fibrosis. J Clin Microbiol 41:2633–2640
57. Tang YW, Li H, Durkin MM et al (2006) Urine polymerase chain reaction is not as sensitive as urine antigen for the diagnosis of disseminated histoplasmosis. Diagn Microbiol Infect Dis 54:283–287
58. Li H, Dummer JS, Estes WR, Meng S, Wright PF, Tang YW (2003) Measurement of human cytomegalovirus loads by quantitative real-time PCR for monitoring clinical intervention in transplant recipients. J Clin Microbiol 41:187–191
59. Poehling KA, Griffin MR, Dittus RS et al (2002) Bedside diagnosis of influenza virus infections in hospitalized children. Pediatrics 110:83–88
60. Rahimian J, Raoult D, Tang YW, Hanna BA (2006) *Bartonella quintana* endocarditis with positive serology for *Coxiella burnetii*. J Infect 53:e151–e153
61. Tsang RS, Tsai CM, Henderson AM et al (2008) Immunochemical studies and genetic background of two *Neisseria meningitidis* isolates expressing unusual capsule polysaccharide antigens with specificities of both serogroup Y and W135. Can J Microbiol 54:229–234
62. Wang E, Paessler S, Aguilar PV et al (2006) Reverse transcription-PCR-enzyme-linked immunosorbent assay for rapid detection and differentiation of alphavirus infections. J Clin Microbiol 44:4000–4008
63. Wilson T, Carson J (2003) Development of sensitive, high-throughput one-tube RT-PCR-enzyme hybridisation assay to detect selected bacterial fish pathogens. Dis Aquat Organ 54:127–134

64. Chaharaein B, Omar AR, Aini I, Yusoff K, Hassan SS (2009) Detection of H5, H7 and H9 subtypes of avian influenza viruses by multiplex reverse transcription-polymerase chain reaction. Microbiol Res 164:174–179
65. Chia JK, Chia AY (2008) Chronic fatigue syndrome is associated with chronic enterovirus infection of the stomach. J Clin Pathol 61:43–48
66. Kim HJ, Cho JC (2008) Rapid and sensitive detection of *Listeria monocytogenes* using a PCR-enzymelinked immunosorbent assay. J Microbiol Biotechnol 18:1858–1861
67. Liolios L, Jenney A, Spelman D, Kotsimbos T, Catton M, Wesselingh S (2001) Comparison of a multiplex reverse transcription-PCR-enzyme hybridization assay with conventional viral culture and immunofluorescence techniques for the detection of seven viral respiratory pathogens. J Clin Microbiol 39:2779–2783
68. Milne SA, Gallacher S, Cash P, Lees DN, Henshilwood K, Porter AJ (2007) A sensitive and reliable reverse transcriptase PCR-enzyme-linked immunosorbent assay for the detection of human pathogenic viruses in bivalve molluscs. J Food Prot 70:1475–1482
69. Musiani M, Gallinella G, Venturoli S, Zerbini M (2007) Competitive PCR-ELISA protocols for the quantitative and the standardized detection of viral genomes. Nat Protoc 2:2511–2519
70. Santos N, Honma S, Timenetsky Mdo C et al (2008) Development of a microtiter plate hybridization-based PCR-enzyme-linked immunosorbent assay for identification of clinically relevant human group A rotavirus G and P genotypes. J Clin Microbiol 46:462–469
71. Schutzle H, Weigl J, Puppe W, Forster J, Berner R (2008) Diagnostic performance of a rapid antigen test for RSV in comparison with a 19-valent multiplex RT-PCR ELISA in children with acute respiratory tract infections. Eur J Pediatr 167:745–749
72. St-Louis M (2009) PCR-ELISA for high-throughput blood group genotyping. Methods Mol Biol 496:3–13
73. Kumar S, Wang L, Fan J et al (2008) Detection of 11 common viral and bacterial pathogens causing community-acquired pneumonia or sepsis in asymptomatic patients by using a multiplex reverse transcription-PCR assay with manual (enzyme hybridization) or automated (electronic microarray) detection. J Clin Microbiol 46:3063–3072
74. He J, Bose ME, Beck ET et al (2009) Rapid multiplex reverse transcription-PCR typing of influenza A and B virus, and subtyping of influenza A virus into H1, 2, 3, 5, 7, 9, N1 (human), N1 (animal), N2, and N7, including typing of novel swine origin influenza A (H1N1) virus, during the 2009 outbreak in Milwaukee, Wisconsin. J Clin Microbiol 47:2772–2778
75. He J, Kraft AJ, Fan J et al (2009) Simultaneous detection of CDC category "A" DNA and RNA bioterrorism agents by use of multiplex PCR & RT-PCR enzyme hybridization assays. Viruses 1:441–459
76. Elder BL, Hansen SA, Kellogg JA, Marsik FJ, Zabransky RJ (1997) Method selection, example.,Verification and validation of procedures in the clinical microbiology laboratory. In: McCurdy BW (ed) Cumitech 31, p. 14. Appendix A. American Society for Microbiology, Washington, DC
77. CLSI (2006) Molecular diagnostic methods for infectious diseases: approved guideline. In: CLSI document MM3-A2, 2nd edn. CLSI, Wayne

Chapter 22
Direct Nucleotide Sequencing for Amplification Product Identification

Tao Hong

Introduction

The advances of technology to determine the nucleotide sequence of DNA have fundamentally changed the field of biological research and medicine. For diagnostic molecular microbiology, the most precise method of identification of a PCR product (amplicon) is to determine its nucleotide sequence. Although it is not always necessary to sequence the entire amplicon for routine diagnostic procedures, DNA sequence has been used to analyze broad range PCR products for bacterial identification, for gene mutations related with antimicrobial resistance, and for bacterial strain typing and viral geno-typing, etc. Most of the amplicons of these applications are large (range approximately from 300 to 1,500 bp), and the exact nucleotide sequence of the amplicons are crucial for the results.

Two basic methods are created for DNA sequencing, the ddNTP-mediated chain termination method of Sanger et al. [33] and the chemical cleavage method of Maxam and Gilbert [24]. The Sanger method has been widely performed in most research laboratory, using radioisotope-labeled nucleotide (e.g., ^{32}P or ^{35}S) and standard manual method. The method relies on enzymatic DNA synthesis from a specific oligonucleotide primer. The primer is annealed to the complementary sequence adjacent to the DNA of interest on a genetic element [32]. The method of DNA sequencing developed by Maxam and Gilbert is based on the specific cleavage of DNA at specific nucleotide. A homogeneous sample of DNA radiolabeled at one end is treated with four separate chemical reactions, each of which modifies a particular type of base. Conditions of the subsequent cleavage reactions are set such that cleavage occurs an average of only once for each DNA molecule.

T. Hong (✉)
Department of Pathology, Clinical Microbiology Laboratory,
Hackensack University Medical Center, 30 Prospect Avenue, Hackensack, NJ 07601, USA
e-mail: thong@humed.com

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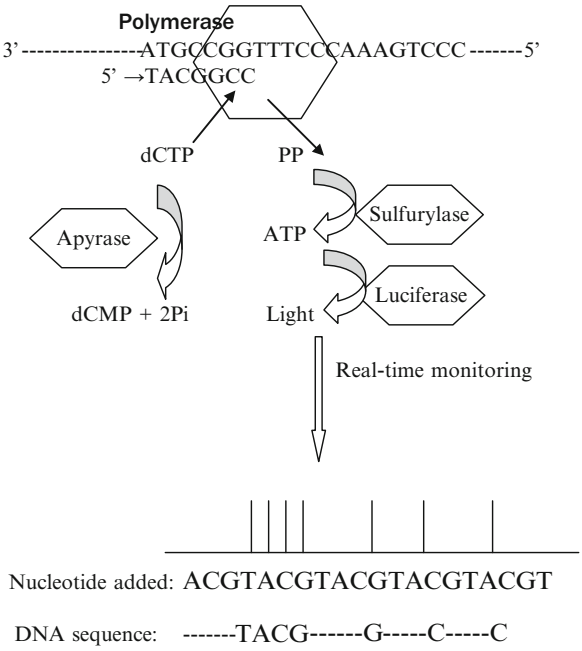

Fig. 22.1 Schematic diagram of pyrosequencing. The reaction mixture consists of single-stranded DNA with an annealed primer, DNA polymerase, ATP sulfurylase, luciferase, and apyrase. The four nucleotide bases are added to the reaction mixture in a particular order, e.g., A, C, G, and T. If the added nucleotide forms a base pair (in this case, two Cs base pair to the template), the DNA polymerase incorporates the nucleotide and a pyrophosphate (PPi) is released. The released pyrophosphate is converted to ATP by ATP sulfurylase, and luciferase uses this ATP to generate detectable light. This light is proportional to the number of nucleotides incorporated and is detected in real time. The pyrosequencing raw data are displayed simultaneously, and in this example the sequence generated reads TACGGCC. Excess quantities of the added nucleotide are degraded by apyrase. If the nucleotide does not form a base pair with the DNA template, it is not incorporated by the polymerase and no light is produced. Apyrase then rapidly degrades the nucleotide

Not long ago, DNA sequence-based analyses were laborious and time-consuming. These methods were available only for research setting. Recent advances in the use of fluorescent dye terminator chemistry and laser scanning in a polyacrylamide gel electrophoresis (PAGE) and the application of capillary electrophoresis technique combined with fluorescent dye terminator, combined with base-calling software, has made DNA sequencing much less labor intensive. Capillary electrophoresis allows for accurate size discrimination of fluorescently labeled nucleic acids from 50 to 1,000 bases with single base precision. Capillary electrophoresis base nucleic acid sequencing analysis has become a routing procedure in many molecular diagnostic laboratories.

Pyrosequencing is a non-gel-based DNA sequencing technique that is based on the detection of the pyrophosphate (PPi) released during DNA synthesis. In a cascade of enzymatic reactions, visible light is generated at a level that is proportional to the number of incorporated nucleotides (Fig. 22.1) This method generates 30- to 40-base sequences with each primer, and the throughput is 96 samples in approximately 10 min; i.e., the throughput is much higher than that which can be achieved

by conventional Sanger sequencing on gel or capillary-based automated sequencing machines. The limitation of pyrosequencing is that the sequence is only accurate within the first 30–40 bases, beyond that the data is unreliable.

Methodology

Four steps are required to obtain DNA sequence of PCR product: nucleic acid extraction (either RNA or DNA), PCR amplification (or RT-PCR for RNA target), nucleotide sequencing, and database homology search/analysis and reporting.

Nucleic Acid Extraction

Depends on the PCR primers, for broad range primers, pure culture of a bacterial/viral agent is generally required for its identification. If PCR primer is designed specifically for a particular microbial agent, clinical specimens may be used directly for nucleic acid extraction. Various DNA extraction methods can be used, such as traditional phenol chloroform method, commercial DNA extraction kits, etc. Pure culture and relatively large quantity of target DNA makes contamination by background DNA from reagents and other sources negligible. In our experience, for most bacterial target, no DNA purification is necessary. Two colonies or the pellet of 1 mL positive liquid medium are resuspended in 200 μL sterile saline, 2 μL of the suspension is used directly in the subsequent PCR reaction. Alternatively, the bacterial suspension can be boiled for 10 min and centrifuged for 5 min at 8,000×*g*, and the supernatant (2 μL) can be used for PCR.

Polymerase Chain Reaction

Depends on the target and primer set, the PCR condition varies. It is important to verify the purity of PCR product by visualizing the amplified DNA on an agarose gel before starting DNA sequencing, especially in the assay validation stage. Once the procedure is validated and a single PCR product is routinely obtained, the agarose gel step may not be necessary. Usually, PCR amplicon amplified from a pure target, produce large amount of DNA and sufficient for nucleotide sequencing. For PCR reaction that generates multiple products, a gel purification procedure is necessary to purify the amplicon of interest.

Nucleotide Sequencing

The PCR amplicon can be sequenced directly after the removal of unpolymerized primers and 4-NTPs that can be achieved by enzymatic digestion with exonuclease

and shrimp alkaline phosphatase. No further purification or concentration of the amplicon is generally necessary. Automated sequencing can be performed according to sequencing chemistry and sequencing instrument of your laboratory. Usually, one of the PCR primers is used as the primer for sequencing reaction. If both strands of the amplicon are to be sequenced, two separate reactions are needed. For clinical microbiology laboratories with no DNA sequencing equipment, this final step can usually be achieved by sending the purified amplicon with one of the PCR primers to an in-house core sequencing facility or a commercial laboratory providing DNA sequencing service.

Ruano and Kidd [31] have developed a method called coupled amplification and sequencing; it is a method for sequencing both strands of template as they are amplified. The procedure is biphasic, stage I selects and amplifies a single target from the genomic DNA, stage II accomplishes the sequencing as well as additional amplification of the target using aliquots from the stage I reaction mixed with end-labeled primer and dideoxynucleotides. A modified procedure (CLIP) has been developed using Clipper sequencer [44]. Two characteristics of the CLIP reaction as a modification of the original coupled amplification and sequencing method by Ruano and Kidd are: (1) An engineered mutant of thermostable DNA polymerase is used which lacks 5′–3′ exonuclease activity and therefore produces uniform band intensities. (2) Different far-red fluorescent dyes are linked to the two inward-facing CLIP primers, allowing a template to be sequenced in both directions in a single run.

Homology Search and Reporting

For sequence analysis, the sequence is compared with the data in nucleotide sequences database, whether in-house developed, commercial or public database (such as GenBank). The match (sometimes multiple matches) need to be interpreted cautiously; specifically, consensus of the matches and/or the match with type strain should be sought. Preferably, sequences from type strains with good quality (no unresolved nucleotides or artificial gaps) and from a reputable laboratory. One should be aware that the nucleotide sequence data in the public database have not been peer-reviewed. Early sequencing data generated by manual method may not be very accurate.

Application of DNA Sequencing in Molecular Diagnosis

Sequencing of hsp65 for Identification of Mycobacterial Species

Clinical microbiology laboratory usually uses a combined molecular/conventional approach for mycobacterium identification. Commercial probes are available for *Mycobacterium tuberculosis* complex, *Mycobacterium avian/intracellular* complex, *Mycobacterium kansasii*, and *Mycobacterium gornodae*. For other mycobacteria,

conventional/biochemical based (and time-consuming) methods are applied for identification. Atypical biochemical reactions have frequently causing problems for accurate species identification. Many molecular methods have been developed for identifying mycobacteria, the 16S rRNA gene sequencing is the most frequently used approach for sequence-based identification of mycobacteria (review in Chap. 30 of this book). The 65-kDa heat shock protein gene (*hsp65*), present in all mycobacteria, is more variable than the 16S rRNA gene sequence and is useful for the identification of genetically related species. Sequence variations in the *hsp65* gene have been exploited to identify both slowly growing mycobacteria and rapidly growing mycobacteria (RGM) to the species level. Hance et al. [20] reported amplifying a fragment of the *hsp65* to detect and, coupled with species-specific probes, identify mycobacteria from clinical samples. After this, Plikaytis et al. [28] and Telenti et al. [39] described, using separate gene regions, the successful identification of mycobacteria by using restriction digest analysis of amplified *hsp65* fragments (*hsp65* PRA). *Hsp65* PRA has been widely used for identification, and an algorithm based on this approach has recently been developed for differentiating 34 mycobacterial species, including members of the RGM group. A sequence-based strategy has several potential advantages. It generates direct, unambiguous data and can distinguish medically relevant subspecific phylogenetic lineages. Recent advances in automated DNA sequencing have also made this approach much easier. To overcome the limitations of *hsp65* PRA and the potential advantage of generating direct unambiguous data, Kapur et al. [22] developed a procedure for sequencing the *hsp65* amplicon generated by the Telenti primers as a means for identifying mycobacteria. This technique has since been used by many investigators to identify species, as well as characterize and define groups within a number of mycobacteria. A study by McNabb et al. [25] assessed the viability of using *hsp65* sequencing to identify all mycobacteria routinely isolated by a clinical mycobacteriology laboratory and the ability of an in-house database, consisting of 111 *hsp65* sequences from putative and valid mycobacterial species or described groups, to identify 689 mycobacterial clinical isolates from 35 species or groups. The overall agreement between *hsp65* sequencing and the other identification methods is 85.2 %. The study indicates that for *hsp65* sequencing to be an effective means for identifying mycobacteria a comprehensive database must be constructed. *Hsp65* sequencing has the advantage of being more rapid and less expensive than biochemical test panels, uses a single set of reagents to identify both rapid- and slow-growing mycobacteria, and can provide a more definitive identification. Due to limitations of appropriate database, the best approach for sequence identification of mycobacteria is to have both 16S rRNA gene (see Chap. 30 in this book) and the hsp65 gene methods available in the laboratory.

The recA Gene Sequence

The *rec*A gene sequence can be an alternative to the sequence analysis of the gene 16S rDNA for the differentiation of mycobacteria [3]. The *Rec*A protein encoded by

this gene exists in all bacteria. It plays an important role in homologous DNA recombination, DNA damage repair, and induction of the SOS response. Sequencing of the fragment A (915–970 bp) can distinguish *Mycobacterium leprae*, *Mycobacterium aurum*, and *Mycobacterium mucogenicum*. The *rec*A sequencing can differentiate the clinically important *M. kansasii* from the less clinically important *Mycobacterium gastri*. Sequencing of the fragment B (about 1 kbp) distinguished the species *Mycobacterium xenopi*, *Mycobacterium asiaticum*, *Mycobacterium shimoidei*, and *MTC*.

Internal Transcribed Spacer

The internal transcribed spacer (ITS) region, a stretch of DNA that lies between the 16S and 23S rRNA subunit genes, has proved to show a high degree of variability in both sequence and size at the genus and species level [2, 17]. Hence, this region may allow efficient identification of species due to its enhanced variability within a genus [12]. The diversity of the intergenic spacer regions is due in part to variations in the number and type of tRNA sequences found among these spacers. Sequence of ITS region has been used for the identification of mycobacterial species, for Staphylococcal species [8], Streptococcal species [7], and for rapid identification of medically important yeast [6].

For molecular identification of mycobacteria, the most frequently used DNA sequence-based method is the 16S rRNA gene. However, there are instances in which the sequences of 16S rDNA genes have been found to be very similar, if not identical, between different species in a genus, making it necessary to find alternative specific sequences. The intergenic 16S–23S ITS region is considered to be less prone to selective pressure and consequently can be expected to have accumulated a higher percentage of mutations than the corresponding rDNA. Sequencing of the ITS regions of diverse bacteria indicates that considerable length and primary sequence variation occurs, and this variability has been successfully used to distinguish between closely related mycobacteria, such as the *Mycobacterium avium–M. intracellulare* complex [9, 11], *M. gastri* and *M. kansasii,* both of them share an identical 16S rDNA sequence [29, 30]; *Mycobacterium farcinogenes* and *Mycobacterium senegalense* [19] and *Mycobacterium chelonae* complex.

Streptococci are a very diverse group of microorganism; many molecular techniques have been used for the identification of streptococci. Chen et al. [7] have evaluated the feasibility of sequence analysis of the 16S–23S ribosomal DNA (rDNA) intergenic spacer (ITS) for the identification of clinically relevant viridans group streptococci (VS). The ITS regions of 29 reference strains (11 species) of VS were amplified by PCR and sequenced. The ITS lengths (246–391 bp) and sequences were highly conserved among strains within a species. The intraspecies similarity scores for the ITS sequences ranged from 0.98 to 1.0, except for the score for *Streptococcus gordonii* strains. The interspecies similarity scores for the ITS sequences varied from 0.31 to 0.93. Phylogenetic analysis of the ITS regions revealed that evolution of the regions of some species of VS is not parallel to that of

the 16S rRNA genes. The accuracy of using ITS sequencing for the identification of VS was verified by 16S rDNA sequencing for all strains except strains of *Streptococcus oralis* and *Streptococcus mitis*, which were difficult to differentiate by their 16S rDNA sequences. It was concluded that the identification of species of VS by ITS sequencing is reliable and could be used as an alternative accurate method for the identification of VS [14].

In staphylococci, there are several copies of the *rrn* operon. Gürtler and Barrie [16] characterized the spacer sequences of *Staphylococcus aureus* strains, including methicillin-resistant *S. aureus* (MRSA) isolates, and identified nine *rrn* operons whose 16S–23S spacer region varied from 303 to 551 bp. Three of these spacers contain the tRNAIle gene and two contains both the tRNAIle and the tRNAAla genes, while the remaining four 16S–23S spacers have no tRNA gene. Forsman et al. [10] sequenced the 16S–23S spacer of five staphylococcal species (*S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, *Staphylococcus simulans*, and *Staphylococcus xylosus*) and found that in addition to *S. aureus*, *S. hyicus*, and *S. simulans* also had a tRNAIle gene in some of their *rrn* operons. The sequence conservation of the rrn operons argues for the use of the 16S–23S spacer region as a stable and direct indicator of the evolutionary divergence of *S. aureus* strains.

Fungi are an incredibly diverse and ubiquitous group of eukaryotes, traditional identification depends on morphological differences in their sexual or asexual reproductive structures. A PCR/sequencing-based approach may provide rapid and more accurate identification method. Coding regions of the 18S, 5.8S, and 28S nuclear rRNA genes evolve slowly, are relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationships [43]. Between coding regions are the internal transcribed spacer 1 (ITS1) and regions 2 (ITS2), respectively. The ITS region evolves more rapidly and vary among different species within a genus. The ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome (*Aspergillus* species). PCR amplification may facilitate the identification of ITS region DNA sequences with sufficient polymorphism to be useful for identifying medically important fungal species, the concordance rate between phonotypical and ITS2 is greater than 98 % [5, 6, 40]. The sequence variation of ITS regions has led to their use in phylogenetic studies of many different organisms [15].

HCV Genotyping by Nucleotide Sequencing

There are nearly four million persons infected with HCV in the United States and it is estimated that 30,000 acute new infections will occur annually. Progression to chronic disease occurs in approximately 85 % of individuals. Chronic HCV infection is known to progress to cirrhosis and hepatocellular carcinoma. Interferon alfa and ribavirin are used to treat this infection [26]. HCV genotyping is recommended after diagnosis of HCV. HCV is classified on the basis of the similarity of nucleotide

sequence into major genetic groups designated genotypes. Recent studies have shown that HCV viral genotyping may be able to help in selecting therapeutic regimen and outcome of therapy. The reference standard and most definitive method for HCV genotyping is sequencing of a specific PCR-amplified portion of the HCV genome obtained from the patient, followed by phylogenetic analysis. Although all these methods are able to identify correctly the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating among subtypes. [4, 37]

The TruGene™ HCV 5′NC Genotyping Kit (Bayer Diagnostics) has provided a method to obtain the sequence of the 5′ noncoding region of HCV viral RNA in plasma. RNA is extracted from plasma and a 244-base pair sequence in the 5′NC region is amplified by reverse transcription and polymerase chain reaction (RT-PCR) in a single-tube, one-step amplification technique. Sequencing reactions are generated from the amplified cDNA by CLIP sequencing. CLIP allows both directions of the cDNA to be sequenced simultaneously in the same tube using two different dye-labeled primers for each of the sequencing reactions. The CLIP sequencing ladders (each direction being labeled with one dye) are then detected on the OpenGene™ automated DNA sequencing system. The forward and reverse sequences are combined and compared to the sequences of several genotypes of HCV strains with the GeneLibrarian™ software in order to determine the genotype of HCV.

HIV-1 Genotyping

The ViroSeq HIV-1 genotyping assays (Celera Diagnostics, Alameda, CA; distributed by Abbott Molecular Diagnostics, Des Plaines, IL) also use dideoxy chain-terminating sequencing, but each dideoxynucleotide is labeled with a different fluorescent dye. Each reaction mixture contains one primer but all four uniquely labeled dideoxynucleotides. Separation of the terminated PCR products is done by capillary electrophoresis. The ViroSeq HIV-1 Genotyping System can be used for detecting HIV genomic mutations associated with resistance to specific types of antiretroviral drugs, facilitate monitoring, and treatment of HIV infection. Specifically, the ViroSeq HIV-1 Genotyping System can be used to: detect HIV-1 Subtype B viral resistance in plasma samples collected in EDTA with a viral load ranging from 2,000 to 750,000 copies/mL genotype the entire HIV-1 protease gene from codons 1-99 and two-thirds of the reverse transcriptase (RT) gene from codons 1-335.

Sequence-Based Bacterial Genome Typing

Many techniques are available to differentiate *S. aureus*, and specifically MRSA, isolates. Conventionally, isolates were distinguished by phenotypic methods, including antibiotic susceptibility testing and bacteriophage typing. Both methods have

limitations, as genetically unrelated isolates commonly have the same antibiogram, and many *S. aureus* isolates are nontypeable by phage typing. With the advancement of molecular biology, strain typing focused on DNA-based methods: restriction endonuclease patterns of chromosomal or plasmid DNA; Southern blot hybridization using gene-specific probes, ribotyping, PCR-based approaches, and pulsed-field gel electrophoresis (PFGE). These methods require subjective interpretation and comparison of patterns and fingerprint images.

Nucleotide sequence analysis is an objective genotyping method; sequencing data can be easily stored and analyzed in a relational database. Recent advances in DNA sequencing technology have made it possible for sequencing to be considered as a viable typing method. Two different strategies have been used to provide genotyping data: multi-locus sequence typing (MLST), which compares sequence variation in numerous housekeeping gene targets, and single-locus sequence typing, which compares sequence variation of a single target. MLST has been developed for *Neisseria gonorrhoeae*, *Streptococcus pneumoniae,* and *S. aureus* based on the classic multi-locus enzyme electrophoresis (MLEE) method used to study the genetic variability of a species. Sequence analysis of five to seven housekeeping genes provides a database from which to infer relationships in somewhat distantly related isolates that have had substantial time to diversify. The MLST approach is not practical to be used in a clinical laboratory because it is labor-intensive, time consuming and costly. A single-locus target, if discriminating, provides an inexpensive, rapid, objective, genotyping method to subspeciate bacteria. Two *S. aureus* genes conserved within the species, Shopsin et al. has developed the protein A (*spa*) [34] and coagulase (*coa*) [35] procedures for sequence-based staphylococci strain typing. DNA sequencing of the short sequence repeat (SSR) region of the protein A gene (*spa*) has been used as an alternative to current techniques for the typing of *S. aureus*. The SSR consists of a variable number of 24-bp repeats and is located immediately upstream of the region encoding the C-terminal cell wall attachment sequence. The sequencing of the *spa* SSR region combines many of the advantages of a sequencing-based system such as MLST but may be more rapid and convenient for outbreak investigation in the hospital setting since *spa* typing involves a single locus.

The coagulase gene (*coa*) variable region has been evaluated for use in conjunction with *spa* sequencing for the strain typing of MRSA. The coagulase protein is an important virulence factor of *S. aureus*. Like *spa*, *coa* has a polymorphic repeat region that can be used for differentiating *S. aureus* isolates. The variable region of *coa* comprises 81-bp tandem SSRs that are variable in both number and sequence, as determined by restriction fragment length polymorphism analysis of PCR products.

Coagulase gene (*coa*) SSR region sequencing was used to measure relatedness among a collection of temporally and geographically diverse methicillin-resistant *S. aureus* isolates. The results show that *coa* typing is a useful addition to *spa* typing for the analysis of *S. aureus*, including methicillin-resistant strains.

Pyrosequencing

Pyrosequencing, with its ability of rapidly sequence a short piece of DNA, has been evaluated for applications used many areas: GC strain typing (*porB* gene sequencing) [42]; linezolid resistance in Enterococci [38]; lamivudine resistance in HBV [23]; monitoring HIV protease inhibitor resistance [27]; rapid identification of bacteria from positive blood culture [21]; detection of HSV-1 and 2 [1].

For 16S rRNA gene-based bacterial identification, a minimum 200 bp or more is needed for any meaningful identification. However, many investigators have tried to use short representative regions for rapid identification, notably identification for mycobacteria and rapid ID for sepsis-related bacteria. Jordan et al. [21] have studied the possibility of using pyrosequencing to identify a15 base hyper variable region within the 16S rRNA gene for bacterial ID, as compared with 380 bp 16S rRNA fragment sequencing. The results were not very encouraging; the 380 bp sequencing can give species level identification while the 15 bp pyrosequencing can only give semi-genus level identification, such as staphylococcus, streptococcus, or enteric gram negative rods. The 15 bp pyrosequencing did not do much better than a simple gram stain smear reviewed by an experienced clinical microbiologist.

A 30-bp pyrosequencing method was evaluated mycobacterium identification. When blasted against GenBank, 179 of 189 sequences (94.7 %) assigned isolates to the correct molecular genus or group. Pyrosequencing of this hypervariable region afforded rapid and acceptable characterization of common, routinely isolated clinical Mycobacterium sp. However, additional sequencing primer or additional biochemical tests may be needed for more accurate identification [41].

Pyrosequencing did very well in identifying mutant genes associated with drug resistance. A pyrosequencing assay for the rapid characterization of resistance to HIV-1 protease inhibitors [27]. This sequencing approach allows parallel analysis of 96 reactions in 1 h, facilitating the monitoring of drug resistance in eight patients simultaneously. Twelve pyrosequencing primers were designed and were evaluated on the MN strain and on viral DNA from peripheral blood mononuclear cells from eight untreated HIV-1-infected individuals. The method had a limit of detection of 20–25 % for minor sequence variants. Pattern recognition (i.e., comparing actual sequence data with expected wild-type and mutant sequence patterns) simplified the identification of minor sequence variants. This real-time pyrosequencing method was applied in a longitudinal study monitoring the development of PI resistance in plasma samples obtained from four patients over a 2 1/2-year period. Pyrosequencing identified eight primary protease inhibitor resistance mutations as well as several secondary mutations.

A pyrosequencing method for detection and quantification of macrolide resistance mutations was developed and tested for *S. pneumoniae*, *Streptococcus pyogenes*, *Mycobacterium avium*, *Campylobacter jejuni*, and *Haemophilus influenzae*. The method detecting mutations at positions 2058 and 2059 (*Escherichia coli* numbering) of the 23S rRNA gene [18]. Pyrosequencing has also been used for fungal identification [13].

The Roche 454 platform, using pyrosequencing technology to carry out hundreds of thousands of sequencing reactions simultaneously on independent beads, this is one of the technologies referred as the "Next generation sequencing."

Next-Generation Sequencing

During the past decade, multiple new sequencing technology platforms have emerged. And they have surpassed conventional Sanger sequencing method in terms of increased total sequence production and significantly decreased cost, these new sequencing methods are referred to as next-generation sequencing, and they have considerable potential for clinical diagnostics. The five major next-generation sequencing platforms as of this writing are the Roche 454 GS-FLX (454, Branford, CT), the Illumina (San Diego, CA) Genome Analyzer, the ABI SOLiD (Applied Biosystems), HeliScope (Helicos), and SMRT by Pacific Bioscience. A detailed description of the mechanisms of each technology is beyond the scoop of this chapter. Briefly, these new generation sequencing technologies have a similar general approach: breaking DNA into multiple fragments, amplifying the fragments (in some technology, no amplification needed) and then simultaneously, rapidly sequencing multiple fragments, and then using a powerful bio-informatics software to align the sequences and generating final sequencing results. These new technologies are having a significant impact on human genome research, diagnosis of genetic disorders, cardiovascular disease, and cancer. In the field of clinical microbiology, the technologies will have tremendous impact on rapid whole genomic sequencing, identify new organisms, look into strain-to-strain variations and rare mutations. Ultradeep sequencing using the Roche 454 system can detect rare viral variants consisting of as little as 1 % of the population [36], significantly impact treatment outcomes in HIV-1 infections. This level of detection can never be achieved by traditional sequencing methods.

References

1. Adelson ME, Feola M, Trama J, Tilton RC, Mordechai E (2005) Simultaneous detection of herpes simplex virus types 1 and 2 by real-time PCR and Pyrosequencing. J Clin Virol 33(1):25–34
2. Barry T, Colleran G, Glennon M, Dunican LK, Gannon F (1991) The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods Appl 1:51–56
3. Blackwood KS, He C, Gunton J, Turenne CY, Wolfe J, Kabani AM (2000) Evaluation of *rec*A sequences for identification of *Mycobacterium* species. J Clin Microbiol 38:2846–2852
4. Bukh J, Miller RH, Purcell RH (1995) Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin Liver Dis 15:41–63
5. Chen YC, Eisner JD, Kattar MM, Rassoulian-Barrett SL, LaFe K, Yarfitz SL, Limaye AP, Cookson BT (2000) Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. J Clin Microbiol 38:2302–2310
6. Chen YC, Eisner JD, Kattar MM, Rassoulian-Barrett SL, Lafe K, Bui U, Limaye AP, Cookson BT (2001) Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. J Clin Microbiol 39:4042–4051

7. Chen CC, Teng LJ, Chang TC (2004) Identification of clinically relevant viridans group streptococci by sequence analysis of the 16S-23S ribosomal DNA spacer region. J Clin Microbiol 42:2651–2657
8. Couto I, Pereira S, Miragaia M, Sanches IS, de Lencastre H (2001) Identification of clinical staphylococcal isolates from humans by internal transcribed spacer PCR. J Clin Microbiol 39:3099–3103
9. De SmetA L, Brown IN, Yates M, Ivanyi J (1995) Ribosomal internal transcribed spacers are identical among *Mycobacterium avium-intracellulare* complex isolates from AIDS patients, but vary among isolates from elderly pulmonary disease patients. Microbiology 141:2739–2747
10. Forsman P, Tilsala-Timisjärvi A, Alatossava T (1997) Identification of staphylococcal and streptpcoccal causes of bovine mastitis using 16S–23S rRNA spacer regions. Microbiology 143:3491–3500
11. Frothingham R, Wilson KH (1993) Sequence-based differentiation of strains in the *Mycobacterium avium* complex. J Bacteriol 175:2818–2825
12. Garcia-Martinez J, Martinez-Murcia A, Anton AI, Rodriguez-Valera F (1996) Comparison of the small 16S to 23S intergenic spacer region (ISR) of the rRNA operons of some *Escherichia coli* strains of the ECOR collection and E. coli K-12. J Bacteriol 178:6374–6377
13. Gharizadeh B, Norberg E, Loffler J, Jalal S, Tollemar J, Einsele H, Klingspor L, Nyren P (2004) Identification of medically important fungi by the pyrosequencing technology. Mycoses 47:29–33
14. Gheldre YD, Vandamme P, Goossens H, Struelens MJ (1999) Identification of clinically relevant viridans streptococci by analysis of transfer DNA intergenic spacer length polymorphism. Int J Syst Bacteriol 49:1591–1598
15. Guarro J, Gene J, Stchigel AM (1999) Developments in fungal taxonomy. Clin Microbiol Rev 12:454–500
16. Gürtler V, Barrie HD (1995) Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S-23S rDNA spacer regions: characterization of spacer sequences. Microbiology 141:1255–1265
17. Gürtler V, Stanisich VA (1996) New approaches to typing and identification of bacteria using 16S-23S rDNA spacer region. Microbiology 142:3–16
18. Haanpera M, Huovinen P, Jalava J (2005) Detection and quantification of macrolide resistance mutations at positions 2058 and 2059 of the 23S rRNA gene by pyrosequencing. Antimicrob Agents Chemother 49:457–460
19. Hamid ME, Roth A, Landt O, Kroppenstedt RM, Goodfellow M, Mauch H (2002) Differentiation between *Mycobacterium farcinogenes* and *Mycobacterium senegalense* strains based on 16S-23S ribosomal DNA internal transcribed spacer sequences. J Clin Microbiol 40: 707–711
20. Hance AJ, Grandchamp B, Lévy-Frébault V, Lecossier D, Rauzier J, Bocart D, Gicquel B (1989) Detection and identification of mycobacteria by amplification of mycobacterial DNA. Mol Microbiol 3:843–849
21. Jordan JA, Butchko AR, Durso MB (2005) Use of pyrosequencing of 16S rRNA fragments to differentiate between bacteria responsible for neonatal sepsis. J Mol Diagn 7:105–110
22. Kapur V, Li LL, Hamrick MR, Plikaytis BB, Shinnick TM, Telenti A, Jacobs WR, Banerjee A, Cole S, Yuen YK, Clarridge JE, Kreiswirth BN, Musser JM (1995) Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. Arch Pathol Lab Med 119:131–138
23. Lindstrom A, Odeberg J, Albert J (2004) Pyrosequencing for detection of lamivudine-resistant hepatitis B virus. J Clin Microbiol 42:4788–4795
24. Maxam AM, Gilbert W (1977) A new method for sequencing DNA. Proc Natl Acad Sci U S A 74:560–564
25. McNabb A, Eisler D, Adie K, Amos M, Rodrigues M, Stephens G, Black WA, Isaac-Renton J (2004) Assessment of partial sequencing of the 65-kilodalton heat shock protein gene (*hsp65*) for routine identification of *Mycobacterium* species isolated from clinical sources. J Clin Microbiol 42:3000–3011

26. National Institutes of Health Consensus Development Conference Panel (1997) National Institutes of Health Consensus Development Conference Panel statement: management of hepatitis C. Hepatology 26(suppl 1):2S–10S
27. O'meara D, Wilbe K, Leitner T, Hejdeman B, Albert J, Lundeberg J (2001) Monitoring resistance to human immunodeficiency virus type 1 protease inhibitors by pyrosequencing. J Clin Microbiol 39:464–473
28. Plikaytis BB, Plikaytis BD, Yakrus A, Butler WR, Woodley WR, Silcox VA, Shinnick TM (1992) Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification and restriction fragment length polymorphism analysis. J Clin Microbiol 30:1815–1822
29. Rogall T, Wolters J, Floher T, Böttger EC (1990) Towards a phylogeny and definition of the species at the molecular level within the genus *Mycobacterium*. Int J Syst Bacteriol 40:323–330
30. Roth A, Fischer M, Hamid HE, Ludwig W, Michalke S, Mauch H (1998) Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. J Clin Microbiol 36:139–147
31. Ruano G, Kidd KK (1991) Coupled amplification and sequencing of genomic DNA. Proc Natl Acad Sci U S A 88:2815–2819
32. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harber Laboratory Press, Cold Spring Harbor
33. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74:5463–5467
34. Shopsin B, Kreiswirth BN (2001) Molecular epidemiology of methicillin-resistant *Staphylococcus aureus*. Emerg Infect Dis 7:323–326
35. Shopsin B, Gomez M, Waddington M, Riehman M, Kreiswirth BN (2000) Use of coagulase gene (*coa*) repeat region nucleotide sequences for typing of methicillin-resistant *Staphylococcus aureus* strains. J Clin Microbiol 38:3453–3456
36. Simen BB, Simons JF, Hullsiek KH, Novak RM, Macarther RD, Baxter JD, Hugan C, Lubeski C, Turenchalk GS, Braverman MS, Desany B, Rothberg JM, Egholm M, Kozai MJ, Beirn T, Community Programs for Clinical Research on AIDS (2009) Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naïve patients significantly impact treatment outcomes. J Infect Dis 199:610–612
37. Simmonds P (1995) Variability of hepatitis C virus. Hepatology 21:570–583
38. Sinclair A, Arnold C, Woodford N (2003) Rapid detection and estimation by pyrosequencing of 23S rRNA genes with a single nucleotide polymorphism conferring linezolid resistance in Enterococci. Antimicrob Agents Chemother 47:3620–3622
39. Telenti A, Marchesi F, Balz M, Bally F, Böttger EC, Bodmer T (1993) Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 31:175–178
40. Travis H, Iwen PC, Hinrichs SH (2000) Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. J Clin Microbiol 38:1510–1515
41. Tuohy MJ, Hall GS, Sholtis M, Procop GW (2005) Pyrosequencingtrade mark as a tool for the identification of common isolates of Mycobacterium sp. Diagn Microbiol Infect Dis 51:245–250
42. Unemo M, Olcén P, Jonasson J, Fredlund H (2004) Molecular typing of *Neisseria gonorrhoeae* isolates by pyrosequencing of highly polymorphic segments of the porB gene. J Clin Microbiol 42:2926–2934
43. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gefland DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, New York, pp 315–322
44. Yager TD, Baron L, Batra R, Bouevitch A, Chan D, Chan K, Darasch S, Gilchrist R, Izmailov A, Lacroix JM, Marchelleta K, Renfrew J, Rushlow D, Steinbach E, Ton C, Waterhouse P, Zaleski H, Dunn JM, Stevens J (1999) High performance DNA sequencing, and the detection of mutations and polymorphisms, on the Clipper sequencer. Electrophoresis 20:1280–1300

Chapter 23
Microarray-Based Amplification Product Detection and Identification

Joy D. Van Nostrand, Terry J. Gentry, and Jizhong Zhou

Introduction

Microorganisms play important roles in ecosystem functioning in both the environment and within host organisms. However, the diversity of microbial communities (an estimated 2,000–50,000 microbial species per gram of soil [1–4] and the human intestine is estimated to contain at least 500 phylotypes [5]) and their mostly unculturable status (>99 % [6–8]) make them difficult to study. As such, culture-independent approaches are essential to study even a fraction of microorganisms in the environment. Microarrays can examine tens of thousands of genes at one time in a simple, rapid, high-throughput, and parallel manner, making them ideal for the study of microbial communities. Microarrays can also provide quantitative information if amplification of target DNA is not required prior to analysis. Due to the large amount of data generated from each array, array-based analyses can be more cost-effective than other molecular methods. In addition, arrays are an ideal tool for comparing microbial communities from different sites, conditions, or times since samples are interrogated against a defined set of genes or microorganisms contained on the array. These features make microarrays excellent tools for assessing microbial community structure, functions, activities, and dynamics in natural settings. This chapter discusses various types of arrays and their applications to issues of clinical interest.

J.D. Van Nostrand • J. Zhou (✉)
Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, 101 David L Boren Blvd., Norman, OK 73019, USA
e-mail: Joy.vannostrand@ou.edu; jzhou@ou.edu

T.J. Gentry
Department of Soil and Crop Sciences, Texas A&M University, 550A Heep Center, College Station, TX 77843-2474, USA
e-mail: tgentry@ag.tamu.edu

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Principles and Types of Microarrays

Microarrays are comprised of probes for specific genes, sequences, or genomes on a solid surface. Labeled DNA or RNA is then hybridized to the probes. This is conceptually similar to traditional membrane-based Northern and Southern blots where a labeled probe molecule is hybridized to target nucleic acid attached to a membrane, only reversed. Microarrays can be manufactured on glass slides [9–11] or nylon membranes [12]; although, typically, glass slides are used since they produce less background fluorescence [13, 14] and allow higher probe density [15]. Several methods of array printing are available. Bubble Jet printing uses 0.02–1.6 mg/mL probe (10- to 300-mer) solution with no DNA loss or probe shearing and produces small spot sizes by using 24 pL droplets [16]. Laser-induced forward transfer (LIFT) uses lasers to transfer probes onto the array surface from a film support [17]. Variations in spot size are accomplished by adjusting the laser power with spots as small as 40 μm. Several companies produce custom microarrays. Roche NimbleGen uses Maskless Array Synthesizer technology to synthesize probes directly onto the glass array surface. With this technology 786,000–4.2 million micromirrors are used to deprotect the nascent oligonucleotide and allow addition of the next base (http://www.nimblegen.com/technology/manufacture.html). Agilent uses inkjet technology (http://www.genomics.agilent.com/GenericB.aspx?PageType=Custom&SubPageType=Custom&PageID=2011). Additionally, contact printing using printing pins is frequently used for in-house laboratory array printing.

Several types of arrays could be used in clinical and diagnostic applications or research. These include (1) phylogenetic oligonucleotide arrays (POAs), which contain probes for conserved genes such as the 16S rRNA gene and are used to detect specific organisms and can be used to compare microbial community composition; (2) functional gene arrays (FGAs), which contain probes for specific functional genes involved in various processes of interest, such as antibiotic resistance or virulence, and can provide information on the genes and populations within a community and provide direct linkages between gene functions and ecosystem processes; (3) community genome arrays (CGAs), which use whole genomic DNA of cultured microorganisms as probes and can be used to examine the relatedness of microbial isolates, characterize microbial communities, or detect the presence of specific microorganisms in a community; (4) metagenomic arrays (MGA), which contain probes created from clone libraries of environmental DNA and can be used as a high-throughput screening method, (5) whole-genome open reading frame (ORF) arrays (WGA), which are comprised of probes targeting all ORFs in one or more genomes and can be used to monitor gene expression under different conditions and (6) other miscellaneous arrays.

Phylogenetic Oligonucleotide Arrays

POA can be used to assess phylogenetic relatedness of microbial strains or to determine the presence of specific microorganisms using conserved genes such as the 16S rRNA [18–21]. The 23S rRNA gene [22] has also been used since it has been shown to provide a greater sequence variation than the 16S rRNA [23, 24]. In general, a shorter probe length (~20mers) is necessary to provide the needed level of specificity.

The PhyloChip is the most comprehensive POA reported to date. This array contains ~300,000 perfect-match (PM) and mismatch (MM) probes for the 16S rRNA gene and is capable of detecting 842 subfamilies or 8,741 taxa [18, 25, 26] at the family to subfamily levels [26].

Functional Gene Arrays

FGAs contain probes for genes for key proteins or enzymes involved in specific functional processes [27–30]. FGAs allow the examination of thousands of different functional genes at the same time [27–35]. The first published FGA used PCR-amplicons as probes and focused on N-cycling genes (*nirS*, *nirK*, *amoA*, and *pmoA*) [30]. Tests using soil and marine sediment microbial communities showed that the PCR-based probes worked well. However, the use of this type of probe greatly limits the number and type of genes that could be included on the array due to limits in primer availability and difficulty in obtaining large numbers of pure culture isolates. Oligonucleotide probes are now most commonly used for FGAs [10, 11] because it is easy to design custom probes based on sequences within public databases [27, 36, 37].

Since this first report, many FGAs have been described and most have been specific for individual functional groups, environments or microbial groups. For example, FGAs have been developed to study N-cycling (denitrification, N-fixation, ammonia oxidation, and nitrite reduction) [9], nitrogen fixation [38], methanotrophs (*pmoA/amoA* genes) [39, 40], nitrification, denitrification, nitrogen fixation, methane oxidation, and sulfate reduction [10], or organic contaminant degradation and metal resistance [11]. To date, the most comprehensive FGAs described are the GeoChip arrays [28, 29]. These arrays were developed to provide a truly comprehensive probe set covering many functional gene groups while providing the specificity necessary to distinguish nearly homologous sequences [29]. GeoChip 3.0, the most recently reported version, covers ~57,000 sequences from 292 gene families and includes antibiotic-resistance genes [28]. GeoChip 4.0 greatly expands sequence coverage and includes probes for virulence genes from human, animal, and plant pathogens; viral genes; and microbial stress response (Tu et al., unpublished data).

Due to the highly conserved nature of many functional genes and the functional diversity of microorganisms, FGA probes must be carefully designed to provide

specificity. The genes to be included on an FGA should be chosen with care. Selected genes should encode a protein critical to the process of interest, be fairly conserved but have enough sequence divergence to allow design of specific probes, and have a relatively large sequence set available in public databases [27]. To assure that all sequences covered by the GeoChip FGA are correct, a special protocol was developed. Public sequence databases (e.g., GenBank) are searched using broad keywords since gene annotations can vary or be more or less specific. Gene sequences are downloaded and then compared to experimentally confirmed seed sequences using HMMER software (http://hmmer.wustl.edu/). Seed sequence selection is a very important step and should be carefully performed. The sequences confirmed by HMMER are then verified by hand and used for probe design with CommOligo [41] using experimentally determined criteria: identity (sequence-specific probes, ≤90 %; group-specific probes, ≥96 %), continuous stretch (sequence-specific, ≤20 bases; group-specific, ≥35), and free energy (sequence specific, ≥−35 kJ/mol; group specific, ≤−60 kJ/mol) [29, 42]. Designed probes are then BLASTed against the GenBank database to assure specificity. The finalized probes can then be commercially synthesized.

Community Genome Arrays

CGA use whole genomic DNA as probes [43, 44]. The first CGA contained 67 bacterial strains and was able to distinguish isolates at the species level when hybridized at 55 °C and 50 % formamide and at the strain level at 75 °C and 50 % formamide [43]. Another CGA was developed which was specific to acid mine drainage and bioleaching systems and covered 51 prokaryotic strains specific to these environments [45]. This CGA had a detection limit of 5 ng for mixed communities at 55 °C and 50 % formamide [45].

Metagenomic Arrays

MGA have probes made from clone libraries created from environmental DNA and can be used as a high-throughput screening method [46]. The first reported WGA used ~1 kb PCR amplicon inserts from an enrichment culture-based cosmid library as probes [46]. The MGA was then tested with enrichment culture isolates or reference strains. Unhybridized probes were assumed to be from uncultured strains and were subsequently sequenced. Another WGA was later developed using marine microbial community BAC or fosmid clone libraries [47]. Probes were designed to cover all ORFs within each clone insert (20–160 kb) and the array was used to generate microbial community profiles.

Whole-Genome ORF Arrays

WGA contain probes for all ORFs in one or more genomes. WGA were originally developed to examine gene transcription in individual organisms [48], but they can also be used for comparative genomics [49]. The relatedness of ten metal-reducing *Shewanella* strains was evaluated using a WGA containing *Shewanella oneidensis* MR-1 ORFs and found that some genes were conserved across all strains while other genes were conserved only among halotolerant strains [49]. Several other studies have used this same approach to examine relatedness including *Klebsiella pneumoniae* an *E. coli* K-12 WGA [50] and *Pyrococcus* spp. using a *Pyrococcus furiosus* WGA [51]. Additional information on WGA can be found in other reviews [52, 53].

Other Arrays

Other novel arrays have been developed and could have application in clinical diagnosis and research. Kingsley et al. [54] developed a DNA fingerprinting array, which uses randomly generated nonamer probes designed from the genome of a selected microorganism. A "bar code" is generated based on the hybridization pattern. This method demonstrated a higher resolution than other methods such as gel electrophoresis and could even differentiate similar strains of microorganisms.

A relatively new type of microarray is the sequence capture array, which was designed to selectively enrich human nucleic acid samples for exons [55, 56]. These arrays cover an entire gene loci using oligonucleotide probes (>60mer) spaced approximately 1–10 bases apart across the entire sequence [55]. For this array, target DNA is fragmented to 250–1,000 base length using either sonication or nebulization. The fragmented DNA is then ligated with linkers for subsequent use in PCR amplification. The amplified DNA is hybridized to the array for 65 h and the "captured" DNA is then eluted using 95 °C water. The eluted DNA is amplified and then sequenced in order to detect gene variants. Most of the reported studies using this method have focused on human or mammalian DNA, looking for disease biomarkers. However, one study used an *E. coli*-specific capture array covering 68 genes involved in fatty acid biosynthesis, ß-oxidation, and glycolysis [57]. Sequencing results from the captured *E. coli* K12 DNA indicated an enrichment of ~50-fold for 750 ng of starting material and an error rate of <0.2 %.

Another array, the Symbiosis Chip, includes probes for both *Sinhorhizobium meliloti*, a symbiotic α-Proteobacterium, and the host plant, *Medicago truncatula*, allowing examination of concurrent gene expression in the symbiont and the host under the exact same hybridization conditions [58].

Pathogen Detection, Virulence Markers, Antibiotic Resistance, and Diagnostics

Microarray technology can be applied to field of pathogen detection either in clinical settings as a diagnostic tool or in food and water safety testing and studies have shown this to be a promising technology in these fields (Table 23.1). In addition, advances in hybridization technology will make microarray use more practical in clinical settings for high-speed, high-throughput diagnosis and testing. A microfluidic device has been developed which allows hybridization in 15 min and was able to discriminate between four *Staphylococcus* strains [59]. A similar device has also been developed for cell lysis [60]. The goal of these devices is to allow a clinical lab

Table 23.1 Application of diagnostic microarrays

Purpose of array	Type of array[a]	Organisms detected	Genes detected	Reference
Pathogen detection	FGA	*Salmonella*	Virulence factors (*invA, sopB*)	[62]
Bloodstream pathogen detection	FGA	*Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*	Virulence genes, antibiotic-resistance, metabolic- or structural genes	[63]
Waterborne pathogen detection	FGA	12 and 17 waterborne pathogens	Virulence markers	[64]
Pathogen detection	FGA	*Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Proteus* spp., *Klebsiella* spp., *Stenotrophomonas* spp., *Enterobacter* spp., *Acinetobacter* spp., *E. coli*, *P. aeruginosa*, *Candida albicans*	Antimicrobial-resistance genes	[65]
Detect antibiotic resistance	FGA	N/A	Tetracycline-resistance genes	[67]
Detect genes related to host specificity	WGA	*Pseudomonas syringae* strains	Virulence factors	[66]
Pathogen detection	POA	17 pathogens	23S rRNA gene	[22]
Biosurveillance	POA	>8,000 taxa	16S rRNA gene	[25]
Detect strains with specific virulence factors	CGA	*E. coli* reference collection	Whole genomic DNA	[44]
Identify novel viruses	Other	Viruses	Sequences specific to particular viral families	[69]

[a]FGA, functional gene array; WGA, whole-genome open reading frame array; POA, phylogenetic oligonucleotide array; CGA, community genome array

to take a patient specimen, dispense it into a cassette and have DNA extraction, labeling, hybridization, and scanning occur automatically [61]. The following are examples of microarrays used for pathogen monitoring or diagnosis and hold promise for use in clinical applications.

Several preliminary arrays have been developed for the purposes of pathogen detection either in patient samples or as part of water or food monitoring. Most of these are FGAs which utilize a range of virulence markers for probe design. A 70-mers oligonucleotide array was designed to target *Salmonella*-specific virulence factors (*invA* and *sopB*) and an *Enterobacteriaceae* 16S rRNA gene-specific probe as a positive control [62]. DNA from *Escherichia coli* DH5α and *Salmonella enterica* serovar Senftenberg were whole-genome amplified and hybridized at 42 °C and with 40 % formamide. The array had a detection limit of as little as 1 % of the original community. A bloodstream pathogen detection array using PCR amplicon probes (200–800 bp) was developed and covered 120 virulence genes, antibiotic-resistance genes, and metabolic or structural genes from *Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa* [63]. The array was tested with 45 clinical or reference strains of the target organisms and was found to be specific for each strain tested. Antibiotic-resistance genes detected in *S. aureus* strains were confirmed using traditional culture-based methods and agreed with microarray findings. Another array targeted virulence markers from waterborne pathogens and was comprised of two sets of probes: 791 targeting 35 virulence markers from 12 pathogens and 2,034 targeting 67 virulence markers from 17 pathogens [64]. This array had a sensitivity of 0.01 % of the original community for pathogens in tap water, wastewater treatment plant effluent, and river water.

A larger array covering eleven different pathogens (*Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Proteus* spp., *Klebsiella* spp., *Stenotrophomonas* spp., *Enterobacter* spp., *Acinetobacter* spp., *E. coli*, *P. aeruginosa*, and *Candida albicans*) was designed using 930 organism-specific genes in addition to antimicrobial-resistance genes, negative control probes from human, mouse, amoeba, and grass genes, and bacterial 16S rRNA gene probes as positive controls [65]. For this array, a multiplex PCR amplification strategy was used to increase sensitivity to 1 ng of DNA at 42 °C and 25 % formamide. Primers were specific for all genes on the array (800 primer pairs). Both the amplification method and the array were tested using an infected wound swab, and positive hybridization to multiple *Enterococcus faecium*- and *Staphylococcus epidermidis*-specific probes were observed and confirmed with culturing.

In addition to these FGA arrays, pathogen-specific arrays have been developed using other types of arrays. A WGA array contained probes for 353 virulence factors from 91 *Pseudomonas syringae* strains and was used to determine which genes were associated with host specificity [66]. A statistically significant association was found between several genes and specific hosts. For example, the *hopAA1-1* gene was significantly associated with *P. syringae* strains from rice while *shcA* was strongly associated with strains from soybean.

A POA using 23S rRNA gene-based probes incorporated an amplification step which increased the sensitivity from 1 μg of DNA to 100 fg [22]. This array was

able to detect *K. pneumoniae*, *P. aeruginosa*, and *Clostridium perfringens* in municipal wastewater. The PhyloChip has been used to monitor aerosols for microorganisms as part of a biosurveillance program to detect potential bioterrorism threats [25]. The PhyloChip was able to detect sequences similar to several potential pathogens including *Campylobacteraceae*, *Helicobacteraceae*, and *Francesella* and bacteria related to *Bacillus anthracis*, *Rickettsia*, and *Clostridium*.

A CGA of fragmented whole genomic DNA from an *E. coli* reference collection was used to test *E. coli* strains for *hly*, a hemolysin gene [44]. With 63 °C hybridizations, the array was able to discriminate between target strains with and without *hly* and was able to determine copy number as a twofold increase in signal intensity was observed for strains with two copies of the *hly* gene.

In addition to pathogen detection, microorganisms can also be tested for the presence of antibiotic-resistance genes using microarrays. A PCR amplicon (~550 bp) array of tetracycline genes was developed as a possible high-throughput screening tool for isolates [67]. Of 48 isolates tested, 38 showed positive hybridizations to one *tet* gene and one isolate hybridized to two probes, results which agreed with previous findings for these isolates.

Another unique pathogen array is a viral array that is able to identify novel viral pathogens [68, 69]. The array is comprised of 70-mer oligonucleotide probes specific for various sequenced viral families and covers hundreds of viruses [69]. Novel viruses can be detected based on hybridization patterns. This array was able to detect and diagnose viral infections in patients with acute exacerbation of idiopathic pulmonary fibrosis [70], detect viral pathogens in patients with respiratory infections [71], and identify a possible etiologic agent for proventricular dilation disease [72].

Nonpathogen specific arrays have also been used to gain a better understanding of what conditions trigger diseases in the environment. Microbial communities associated with the surface mucopolysaccharide layer and tissue of healthy and yellow band diseased coral, *Montastraea faveolata* was examined with GeoChip 2.0 and results suggested that increases of cellulose-degrading and nitrifying populations may provide a competitive advantage to coral pathogens [73].

The sequence capture arrays have been used to find disease biomarkers and detect mutations associated with certain cancers. For example, Ståhl et al. [74] selected potential biomarkers for breast cancer from data on protein expression patterns in healthy and breast cancer tissues from the Human Protein Atlas. The markers were then used to create a capture array and DNA from breast cancer tissue was hybridized and then the captured sequences were sequenced. Results confirmed that the original set of biomarkers were present in all samples.

Comparison of Microarrays and High-Throughput Sequencing

While the focus of this chapter is on microarrays, high-throughput sequencing is frequently used in microbial studies. Due to the unique advantages and disadvantages of microarrays and high-throughput sequencing and their ideal use as complementary

Table 23.2 Advantages and disadvantages of closed vs. open format detection

	Open format[a]	Closed format[a]
Sensitivity to random sampling errors	High	Low
Effects by dominant organisms	Yes	No
Finding new things	Yes	No
Sensitivity to contaminated DNA	Yes	No
Comparison across samples	?	Yes

[a]Open format methods (high-throughput sequencing, proteomic, or metabolomic analysis) can detect any substance (protein, nucleic acid sequence, etc.) in the sample being tested. Closed format methods (microarrays) are limited to detecting only those substances (nucleic acid sequences) that are within a predetermined probe set

approaches, sequencing methods will be briefly discussed (Table 23.2). Barcode-based 454 pyrosequencing can provide hundreds of thousands of sequences per sample [75–77]. An initial PCR step is required for sequencing, which introduces well-known biases and limits the quantitative ability of this technique [78–80]. While some microarrays require or use a PCR step, an alternative amplification method, whole-community genome amplification (WCGA), can be used for other microarrays (e.g., FGAs) and has been shown to have minimal bias [33]. However, a great advantage of high-throughput sequencing is the ability to find novel sequences. Microarrays, on the other hand, have a defined set of sequences with which to probe, so novel sequences cannot be detected, although new microarray applications are being developed which provide a way to find novel sequences [55, 56]. Primer limitations (conserved primers are difficult to design for all but a few functional genes, difficulty in amplifying some samples) greatly limit the number and type of genes that can be amplified.

Sequencing technologies also have advantages and disadvantages specific to the study of microbial communities. These include (1) Random sampling errors. In most studies, only a small portion of the microbial community is actually sampled, even though this provides a large amount of data. For example, only ~1,400 rRNA gene sequences were obtained from gut microbiome samples of Rhesus macaques when using 454 sequencing, an underestimate of the gut community based on Chao1 estimates [81]. Theoretically, with completely random sampling, there should be only a small probability of sampling the same community portion over multiple sampling events [37]; although dominant populations would more than likely be sampled multiple times. A recent comparison of 16S rDNA amplicon-based sequencing results indicated low reproducibility between technical replicates (17.2 ± 2.3 % for two replicates; 8.2 ± 2.3 % for three) [82]. In contrast, microarray-based approaches interrogate all samples against the same probe set (i.e., those contained on the array), ensuring that the same population is sampled across all samples. (2) Relative abundance. The abundance of species in a microbial community will vary greatly. While sequencing-based approaches are very sensitive to differences in species abundance, microarray-based approaches are not affected by abundance variation as long as it is above the detection limit. (3) Detecting new sequences. Since there are no limitations on what sequences are sequenced, sequencing-based technologies can find new or novel sequences. Microarrays, on the other hand, are

limited to detecting only those sequences covered by the probe set on the array, so detecting new sequences is impossible. However, as mentioned previously, a new type of array, the capture microarray, allows new sequences to be detected [55, 56] and a virus chip has been designed which can classify novel viruses based on the hybridization pattern [68, 69].

Challenges for Microarray Analysis

While there have been great advances in the development of microarray technology over the last decade, there are still challenges remaining. These include sensitivity and specificity of probes and hybridization conditions, and quantitative ability.

Sensitivity

Sensitivity is a major concern with all types of microarrays. Currently, a sensitivity of ~5 % of the microbial community has been observed with environmental samples [39, 83], which allows detection of only the most dominant community members.

There are several strategies available for increasing array sensitivity. First the target RNA or DNA can be amplified [18, 33, 65, 84, 85]. If only a few genes are covered on the array, a PCR-based amplification can easily be incorporated, although this does limit the quantitative information. PCR amplification is routinely used for PhyloChip analysis [18]. Multiplex PCR using primers for all genes on an array has been used for some FGAs [65], although this would be impractical if larger numbers of genes were present on an array. Another option is WCGA [33] or whole-community RNA amplification (WGRA) [84], both of which use random priming to amplify all nucleic acids in the sample, providing a representative, sensitive, and quantitative amplification using ng quantities of starting material.

Alterations to the probe or hybridization conditions can also increase sensitivity, although some of these methods lessen specificity. In general, longer probes are more sensitive (although less specific) [36, 86, 87]. PCR-based probes have a higher sensitivity than oligonucleotide probes although oligonucleotide probes are more specific [37]. Increasing the density of the probes can increase sensitivity [35, 87, 88], but too high a density can actually decrease sensitivity by reducing the overall signal intensity [36]. Reducing the hybridization volume increases sensitivity. Using a volume of 15 μL resulted in a sensitivity of 5 ng for pure culture DNA, while a volume of 3 μL increased sensitivity to 0.2 ng [43]. Mixing during hybridization will increase the signal-to-noise ratio by threefold [89]. Changing the target labeling method can also increase sensitivity. Cyanine dye-doped nanoparticles [35] and tyramide signal amplification labeling [36] both produce up to a tenfold increase in sensitivity over traditional cyanine dyes. Reducing the amount of ozone in rooms where array hybridization occurs may increase sensitivity since ozone causes a decrease in cyanine dye signal [90].

Specificity

Specificity is another concern for diagnostic microarrays, especially for use with samples containing many unknown sequences. Probe design greatly affects specificity. For FGAs, there are several design criteria that have been experimentally determined to modulate specificity: sequence identity, continuous sequence stretches, and free energy [29, 42, 91]. Perfect-match and mismatch probes have been used successfully with POAs to distinguish true signals from signals produced by cross-hybridization [18, 26]. A similar strategy has also been used for FGAs [92]. Hybridization conditions affect specificity; higher temperature and/or formamide concentration increases specificity.

Quantitation

The ability to provide quantitative information is a desirable quality for microarrays. As mentioned before, the use of PCR amplification limits the quantitative information available. A linear relationship ($r = 0.89–0.99$) has been shown between target DNA or RNA concentrations and hybridization signal intensities over a range of 0.5–100 ng DNA using PCR amplicon probes with the GeoChip FGA [30] over a concentration range of 8–1,000 ng DNA [10] or 50–100 ng for RNA [84].

Conclusion

There have been many advances in the development, technology, and applications of microarrays in the past decade. Some of the more exciting developments have been in the fields of clinical microbiology and pathogen detection. There are several types of arrays that have applications in diagnosis and detection of disease, and new uses and types of arrays are still being reported. However, challenges still exist in regard to technical, experimental, and analytical methods used for microarrays. Novel methods, technologies, and applications are necessary to improve array sensitivity, specificity, and quantitative accuracy.

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References

1. Torsvik V, Ovreas L, Thingstad TF (2002) Prokaryotic diversity-magnitude, dynamics, and controlling factors. Science 296(5570):1064–1066
2. Schloss PD, Handelsman J (2006) Toward a census of bacteria in soil. PLoS Comput Biol 2(7):e92
3. Hong S-H, Bunge J, Jeon S-O, Epstein SS (2006) Predicting microbial species richness. Proc Nat Acad Sci U S A 103(1):117–122
4. Roesch LF, Fulthorpe RR, Riva A et al (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J 1(4):283–290
5. Eckburg PB, Bik EM, Bernstein CN et al (2005) Diversity of the human intestinal microbial flora. Science 308(5728):1635–1638
6. Amann R, Ludwig W, Schleifer K (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59(1):143–169
7. Fuhrman JA, Steele JA (2008) Community structure of marine bacterioplankton: patterns, networks, and relationships to function. Aquat Microb Ecol 53(1):69–81
8. Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci USA 95(12):6578–6583
9. Taroncher-Oldenburg G, Griner EM, Francis CA, Ward BB (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl Environ Microbiol 69(2):1159–1171
10. Tiquia SM, Wu L, Chong SC et al (2004) Evaluation of 50-mer oligonucleotide arrays for detecting microbial populations in environmental samples. Biotechniques 36(4):1–8
11. Rhee SK, Liu X, Wu L et al (2004) Detection of genes involved in biodegradation and biotransformation in microbial communities by using 50-mer oligonucleotide microarrays. Appl Environ Microbiol 70(7):4303–4317
12. Steward GF, Jenkins BD, Ward BB, Zehr JP (2004) Development and testing of a DNA macroarray to assess nitrogenase (nifH) gene diversity. Appl Environ Microbiol 70(3):1455–1465
13. Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270(5235):467–470
14. Schena M, Shalon D, Heller R, Chai A, Brown PO (1996) Parallel human genome analysis: Microarray-based expression monitoring of 1000 genes. Proc Natl Acad Sci USA 93(20):10614–10619
15. Ehrenreich A (2006) DNA microarray technology for the microbiologist: an overview. Appl Microbiol Biotechnol 73(2):255–273
16. Okamoto T, Suzuki T, Yamamoto N (2000) Microarray fabrication with covalent attachment of DNA using Bubble Jet technology. Nat Biotechnol 18(4):438–441
17. Serra P, Colina M, Fernández JM, Sevilla L, Morenza JL (2004) Preparation of functional DNA microarrays through laser-induced forward transfer. Appl Phys Lett 85:1639–1641
18. Brodie EL, DeSantis TZ, Joyner DC et al (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol 72(9):6288–6298
19. Loy A, Lehner A, Lee N et al (2001) Oligonucleotide microarray for 16 S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl Environ Microbiol 68(10):5064–5081
20. Small J, Call DR, Brockman FJ, Straub TM, Chandler DP (2001) Direct detection of 16 S rRNA in soil extracts by using oligonucleotide microarrays. Appl Environ Microbiol 67(10):4708–4716
21. Wilson WJ, Strout CL, DeSantis TZ et al (2002) Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. Mol Cell Probes 16(2):119–127
22. Lee DY, Shannon K, Beaudette LA (2006) Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR. J Microbiol Methods 65(3):453–467

23. Anton AI, Martinez-Murcia AJ, Rodriguez-Valera F (1999) Intraspecific diversity of the 23 S rRNA gene and the spacer region downstream in *Escherichia coli*. J Bacteriol 181(9):2703–2709
24. Gurtler V, Stanisich VA (1996) New approaches to typing and identification of bacteria using the 16 S-23S rDNA spacer region. Microbiology 142(Pt 1):3–16
25. Brodie EL, DeSantis TZ, Moberg Parker JP et al (2007) Urban aerosols harbor diverse and dynamic bacterial populations. Proc Natl Acad Sci U S A 104(1):299–304
26. DeSantis TZ, Brodie EL, Moberg JP et al (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb Ecol 53(3):371–383
27. Gentry TJ, Wickham GS, Schadt CW, He Z, Zhou J (2006) Microarray application in microbial ecology research. Microb Ecol 52(2):159–175
28. He Z, Deng Y, Van Nostrand JD, Tu Q et al (2010) GeoChip 3.0 as a high-throughput tool for analyzing microbial community composition, structure, and functional activity. ISME J 4(9):1167–1179
29. He Z, Gentry TJ, Schadt CW et al (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. ISME J 1(1):67–77
30. Wu L, Thompson DK, Li G et al (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl Environ Microbiol 67(12):5780–5790
31. Wagner M, Smidt H, Loy A, Zhou Z (2007) Unraveling microbial communities with DNA-microarrays: challenges and future directions. Microb Ecol 53(3):498–506
32. Wang F, Zhou H, Meng J et al (2009) GeoChip-based analysis of metabolic diversity of microbial communities at the Juan de Fuca Ridge hydrothermal vent. Proc Natl Acad Sci USA 106(12):4840–4845
33. Wu L, Liu X, Schadt CW, Zhou J (2006) Microarray-based analysis of submicrogram quantities of microbial community DNAs by using whole-community genome amplification. Appl Environ Microbiol 72(7):4931–4941
34. Zhou J, Thompson DK (2002) Challenges in applying microarrays to environmental studies. Curr Opin Biotechnol 13(3):204–207
35. Zhou X, Zhou J (2004) Improving the signal sensitivity and photostability of DNA hybridizations on microarrays by using dye-doped core-shell silica nanoparticles. Anal Chem 76(18):5302–5312
36. Denef VJ, Park J, Rodrigues JLM et al (2003) Validation of a more sensitive method for using spotted oligonucleotide DNA microarrays for functional genomics studies on bacterial communities. Environ Microbiol 5(10):933–943
37. Zhou J, Kang S, Schadt CW, Garten CT Jr (2008) Spatial scaling of functional gene diversity across various microbial taxa. Proc Natl Acad Sci USA 105(22):7768–7773
38. Zhang L, Hurek T, Reihold-Hurek B (2007) A nifH-based oligonucleotide microarray for functional diagnostics of nitrogen-fixing microorganisms. Microb Ecol 53(3):456–470
39. Bodrossy L, Stralis-Pavese N, Murrell JC et al (2003) Development and validation of a diagnostic microbial microarray for methanotrophs. Environ Microbiol 5(7):566–582
40. Stralis-Pavese N, Sessitsch A, Weilharter A et al (2004) Optimization of diagnostic microarray for application in analysing landfill methanotroph communities under different plant covers. Environ Microbiol 6(4):347–363
41. Li X, He Z, Zhou J (2005) Selection of optimal oligonucleotide probes for microarrays using multiple criteria, global alignment and parameter estimation. Nucleic Acids Res 33(19):6114–6123
42. Liebich J, Schadt CW, Chong SC et al (2006) Improvement of oligonucleotide probe design criteria for functional gene microarrays in environmental applications. Appl Environ Microbiol 72(2):1688–1691
43. Wu L, Thompson DK, Liu X et al (2004) Development and evaluation of microarray-based whole genome hybridization for detection of microorganisms within the context of environmental applications. Environ Sci Technol 38(24):6775–6782
44. Zhang L, Srinivasan U, Marrs CF et al (2004) Library on a slide for bacterial comparative genomics. BMC Microbiol 4:12–18

45. Chen Q, Yin H, Luo H et al (2009) Micro-array based whole-genome hybridization for detection of microorganisms in acid mine drainage and bioleaching systems. Hydrometallurgy 95:96–103
46. Sebat JL, Colwell FS, Crawford RL (2003) Metagenomic profiling: Microarray analysis of an environmental genomic library. Appl Environ Microbiol 69(8):4927–4934
47. Rich VI, Konstantinidis K, DeLong EF (2008) Design and testing of 'genome-proxy' microarrays to profile marine microbial communities. Environ Microbiol 10(2):506–521
48. Wilson M, DeRisi J, Kristensen HH et al (1999) Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. Proc Natl Acad Sci USA 96(22):12833–12838
49. Murray AE, Lies D, Li G et al (2001) DNA–DNA hybridization to microarrays reveals gene-specific differences between closely related microbial genomes. Proc Natl Acad Sci USA 98(17):9853–9858
50. Dong Y, Glasner JD, Blattner FR, Triplett EW (2001) Genomic interspecies microarray hybridization: rapid discovery of three thousand genes in the maize Endophyte, *Klebsiella pneumoniae* 342, by microarray hybridization with *Escherichia coli* K-12 open reading frames. Appl Environ Microbiol 67(4):1911–1921
51. White JR, Escobar-Paramo P, Mongodin EF, Nelson KE, DiRuggiero J (2008) Extensive genome rearrangements and multiple horizontal gene transfers in a population of *Pyrococcus* isolates from Vulcano Island, Italy. Appl Environ Microbiol 74(20):6447–6451
52. Gresham D, Dunham MJ, Botstein D (2008) Comparing whole genomes using DNA microarrays. Nat Rev Genet 9(4):291–302
53. Mockler TC, Ecker JR (2005) Applications of DNA tiling arrays for whole-genome analysis. Genomics 85(1):1–15
54. Kingsley MT, Straub TM, Call DR et al (2002) Fingerprinting closely related *Xanthomonas pathovars* with random nonamer oligonucleotide microarrays. Appl Environ Microbiol 68(12):6361–6370
55. Albert TJ, Molla MN, Muzny DM et al (2007) Direct selection of human genomic loci by microarray hybridization. Nat Methods 4(11):903–905
56. Okou DT, Steinberg KM, Middle C et al (2007) Microarray-based genomic selection for high-throughput resequencing. Nature Meth 4(11):907–909
57. Schracke N, Kornmeyer T, Kränzle M et al (2009) Specific sequence selection and next generation resequencing of 68 E. coli genes using HybSelect™. N Biotechnol 26(5):229–233
58. Barnett MJ, Toman CJ, Fisher RF, Long SR (2004) A dual-genome Symbiosis Chip for coordinate study of signal exchange and development in a prokaryote-host interaction. Proc Nat Acad Sci U S A 101(47):16636–16641
59. Peytavi R, Raymond FR, Gagné D et al (2005) Microfluidic device for rapid (<15 min) automated microarray hybridization. Clin Chem 51(10):1836–1844
60. Kido H, Micic M, Smith D et al (2007) A novel, compact disk-like centrifugal microfluidics system for cell lysis and sample homogenization. Colloids Surf B Biointerfaces 58(1):44–51
61. Gorkin R, Park J, Siegris J et al (2010) Centrifugal microfluidics for biomedical applications. Lab Chip 10(14):1758–1773
62. Kostic T, Weilharter A, Sessitsch A, Bodrossy L (2005) High-sensitivity, polymerase chain reaction-free detection of microorganisms and their functional genes using 70-mer oligonucleotide diagnostic microarray. Anal Biochem 346(2):333–335
63. Cleven BEE, Palka-Santini M, Gielen J et al (2006) Identification and characterization of bacterial pathogens causing bloodstream infections by DNA microarray. J Clin Microbiol 44(7):2389–2397
64. Miller SM, Tourlousse DM, Stedtfeld RD et al (2008) In situ-synthesized virulence and marker gene biochip for detection of bacterial pathogens in water. Appl Environ Microbiol 74(7):2200–2209
65. Palka-Santini M, Cleven BE, Eichinger L, Krönke M, Krut O (2009) Large scale multiplex PCR improves pathogen detection by DNA microarrays. BMC Microbiol 9:1

66. Sarkar SF, Gordon JS, Martin GB, Guttman DS (2006) Comparative genomics of host-specific virulence in *Pseudomonas syringae*. Genetics 147(2):1041–1056
67. Call DR, Bakko MK, Krug MJ, Roberts MC (2003) Identifying antimicrobial resistance genes with DNA microarrays. Antimicrob Agents Chemother 47(10):3290–3295
68. Ksiazek TG, Erdman D, Goldsmith CS et al (2003) A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 348(20):1953–1966
69. Wang D, Coscoy L, Zylberberg M et al (2002) Microarray-based detection and genotyping of viral pathogens. Proc Natl Acad Sci USA 99(24):15687–15692
70. Wootton SC, Kim DS, Kondoh Y et al (2011) Viral infection in acute exacerbation of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 183(12):1698–1702
71. Kistler A, Avila PC, Rouskin S et al (2007) Pan-viral screening of respiratory tract infections in adults with and without asthma reveals unexpected human coronavirus and human rhinovirus diversity. J Infect Dis 196(6):817–825
72. Kistler AL, Gancz A, Clubb S et al (2008) Recovery of divergent avian bornaviruses from cases of proventricular dilatation disease: Identification of a candidate etiologic agent. Virol J 5:88
73. Kimes NE, Van Nostrand JD, Weil E et al (2010) Microbial functional structure of *Montastraea faveolata*, an important Caribbean reef-building coral, differs between healthy and yellow-band diseased colonies. Environ Microbiol 12(2):541–556
74. Ståhl PL, Bjursell MK, Mahdessian H et al (2011) Translational database selection and multiplexed sequence capture for up front filtering of reliable breast cancer biomarker candidates. PLoS One 6(6):e20794
75. Margulies M, Egholm M, Altman WE et al (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437(7057):376–380
76. Binladen J, Gilber MTP, Bollback JP et al (2007) The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. PLoS One 2(2):e197
77. Hamady M, Walker JJ, Harris JK, Gold NJ, Knight R (2008) Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nat Methods 5(3):235–237
78. Warnecke PM, Stirzaker C, Melki JR et al (1997) Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. Nucleic Acids Res 25(21):4422–4426
79. Lueders T, Friedrich MW (2003) Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Appl Envron Microbiol 69(1):320–326
80. Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16 S rRNA genes by PCR. Appl Environ Microbiol 62(2):625–630
81. McKenna P, Hoffmann C, Minkah N et al (2008) The Macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. PLoS Pathog 4(2):e20
82. Zhou J-Z, Wu L-Y, Deng Y et al (2011) Reproducibility and quantitation of amplicon sequencing-based detection. ISME J 5(8):1303–1313
83. Franke-Whittle IH, Klammer SH, Insam H (2005) Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. J Microb Methods 62(1):37–56
84. Gao H, Yang ZK, Gentry TJ et al (2007) Microarray-based analysis of microbial community RNAs by whole-community RNA amplification. Appl Environ Microbiol 73(2):563–571
85. Franke-Whittle IH, Goberna M, Pfister V, Insam H (2009) Design and development of the ANAEROCHIP microarray for investigation of methanogenic communities. J Microb Methods 79(3):279–288
86. He Z, Wu L, Fields MW, Zhou J (2005) Use of microarrays with different probe sizes for monitoring gene expression. Appl Environ Microbiol 71(9):5154–5162
87. Relógio A, Schwager C, Richter A, Ansorge W, Valcárcel J (2002) Optimization of oligonucleotide-based DNA microarrays. Nucleic Acids Res 30(11):e51

88. Cho JC, Tiedje JM (2002) Quantitative detection of microbial genes by using DNA microarrays. Appl Environ Microbiol 58(3):1425–1430
89. Schaupp CJ, Jiang G, Myers TG, Wilson MA (2005) Active mixing during hybridization improves the accuracy and reproducibility of microarray results. Biotechniques 38:117–119
90. Branham W, Melvin C, Han T et al (2007) Elimination of laboratory ozone leads to a dramatic improvement in the reproducibility of microarray gene expression measurements. BMC Biotechnol 7:8
91. He Z, Wu LY, Li XY, Fields MW, Zhou JZ (2005) Empirical establishment of oligonucleotide probe design criteria. Appl Environ Microbiol 71(7):3753–3760
92. Deng Y, He Z, Van Nostrand JD, Zhou J (2008) Design and analysis of mismatch probes for long oligonucleotide microarrays. BMC Genomics 9:491–503

Chapter 24
Real-Time Detection of Amplification Products Through Fluorescence Quenching or Energy Transfer

Shihai Huang

Introduction

Molecular diagnostics of microbiological agents involves detection of microbial nucleic acids (i.e., DNA and/or RNA) and often requires the amplification of targeted sequences due to low abundances of the microbes or relevant molecular targets. Target amplification technology became available with the advent of polymerase chain reaction (PCR) in the 1980s, which eventually revolutionized the field of clinical diagnostics. While PCR remains the mainstay ever since, several alternative isothermal target amplification technologies have also become available and been used in practice [1, 2], including nucleic acid sequence-based amplification (NASBA) [3, 4], self-sustained sequence replication (3SR) [5, 6], transcription-mediated amplification (TMA) [7], strand displacement amplification (SDA) [8, 9], loop-mediated isothermal amplification (LAMP) [10, 11], recombinase polymerase amplification [12], helicase-dependent amplification (HDA) [13, 14], single primer isothermal amplification (SPIA) [15], isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) [16], isothermal chain amplification (ICA) [17], and Ionian Technologies amplification [18].

When amplification products are detected, the signals generated are then used to derive the diagnostic results for clinical specimens. Target amplification, target detection, and signal generation can be achieved using methods in either a heterogeneous or homogeneous format. Heterogeneous detection is performed after target amplification (i.e., "end-point" reaction) and typically involves multiple steps such as target binding to a solid phase (i.e., beads or membranes), hybridization with excess amounts of labeled probes, washing of unbound probes, and signal generation. The types of signals that have been routinely detected include radioactive decay

S. Huang (✉)
Abbott Molecular Inc., 1300 E Touhy Ave, Des Plaines, IL 60018-3315, USA
e-mail: shihai.huang@abbott.com

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of radioisotopes, chemiluminescence, fluorescence emission, fluorescence polarization, light scattering, and others. The target specificity in signal generation is achieved by interrogating physical and chemical properties unique to the target sequence, such as size, electrostatic charge, and various bio-molecular interactions (e.g., biotin/avidin, antibody/antigen, and nucleic acid binding). Heterogeneous target amplification and detection methods have been developed and established in both research and clinical settings. There are, however, aspects intrinsic to the heterogeneous assay workflow that prevent its wider adoption in clinical microbiology in the era of expanded application of molecular testing. Association of targets to solid surfaces slows down reaction kinetics, which requires longer time for hybridization reactions. Separate reactions for target amplification, target hybridization, probe hybridization, separation of unbound molecules, and signal detection make it conceivably difficult to develop an automated assay procedure. Further, direct manipulation of amplification products will likely introduce contamination due to unintended carryover of amplicons. These limitations of heterogeneous assay format may lead to slower assay turnaround and elevated levels of unforced human errors or incorrect assay results. As a result, recent years have witnessed the increasing adoption of homogeneous methodologies.

In a homogeneous reaction, target amplification and detection are designed to take place in a closed reaction vessel. With proper technologies and instrumentations, targets can be detected as they are being amplified, thus a homogeneous reaction is often referred to as a "real-time" or "kinetic" reaction. The term "real-time" will be used to represent homogeneous methods throughout this chapter. In a real-time assay, it is the combination of two simultaneously occurring and mechanistically interdependent processes of target amplification and target detection/signal generation that enables sensitive and reproducible detection and/or quantification of input samples. Some close-tube assays detect amplified products as a separate step after amplification reaction is completed. Such assay technologies are not included in the discussion here.

Contrary to heterogeneous methods, real-time assays bypass the requirement of multi-step post-amplification sample processing, and therefore they can provide shorter result turnaround time and are amenable to full assay automation. In addition, because there is no need to open the reaction vessel containing amplification products throughout the assay procedure, amplicon contamination can be eliminated. In addition to the advantages in assay workflow, real-time amplification and detection methods provide more robustness in quantitative microbial measurements over wide dynamic ranges. Real-time assays can incorporate either PCR or non-PCR methods to achieve target amplification. For real-time PCR reactions, signal being generated during the course of the amplification is proportional to the amount of accumulated products at any given time. The continuous monitoring of signals allows the determination of the number of PCR cycles (threshold cycle) taken for the amplification reaction to accumulate products to a defined level at the early exponential phase. Threshold cycle is inversely proportional to the amount of the input target (i.e., higher target level leads to fewer cycles needed to achieve detection). The measurement of threshold cycles has been demonstrated to be highly

reliable and its relationship with input target level is well defined over a much wider dynamic range compared to the measurement of end-point PCR signals (as in the case for heterogeneous reactions). Therefore, real-time PCR has become the method of choice for the quantitative detection of microbial agents. Real-time assays using non-PCR amplification have also been developed to achieve quantitative measurements. It is to note that, even though the advantages of real-time assays in quantitative detection are obvious over the traditional end-point heterogeneous assays, real-time assays can be equally effective in providing accurate qualitative results. Further, an important advantage for real-time assays is that signals throughout the course of an amplification reaction can be recorded, thereby allowing the kinetics of the reaction to be analyzed. This information can then be used to detect abnormalities in the assay that could indicate potentially incorrect or unreliable results. The ability to provide assay validity criteria to ascertain reliable results with high confidence is a critical requirement in the highly regulated in vitro diagnostic field. As a result, most commercial real-time assays have been developed with sophisticated systems of validity checks around many of the kinetic characteristics of the reaction so that signals from abnormal reactions are not mistakenly used to determine patient results. This enables objective, automated data analysis and result reporting.

A real-time reaction contains components that support amplification reaction, target detection, and signal generation. Amplification reaction components typically include primers, enzymes, and nucleotides in a buffer, which are not different from heterogeneous amplification reactions. Target detection is usually achieved with components that bind to the amplified products. Signals for the amplified products are generated via target-binding components directly or are induced indirectly by target-binding components. These signals are then converted by data reduction processes to an output that can be accurately measured and recorded. Because detection of the amplification products needs to take place in a closed tube without post-amplification removal of the unbound target detection components, a signal generation mechanism that can spontaneously differentiate between bound and unbound status is necessary. To achieve this, a prominent technology in this application is the measurement of fluorescence signal intensity through the use of fluorophore-labeled oligomers (probes or primers). These oligomers consist of nucleotides or their analogs that can hybridize efficiently to amplification targets in a highly sequence-specific manner. Upon hybridization, the environment of the fluorophore is modified in such ways that the intensity of the fluorescence signals significantly increases or decreases. Several possible mechanisms can play a role in the fluorescence modification, among which the most prominent ones include radiation-less fluorescence quenching through direct contact by a quenching moiety [19, 20] and fluorescence resonance energy transfer (or Förster Resonance Energy Transfer, FRET) [19]. Fluorescence quenching or energy transfer-based real-time methods are among the most widely utilized molecular diagnostic methods in clinical microbiology [21], and will be the focus of this chapter. In addition to fluorophore-labeled oligomers, fluorescent dyes that can insert themselves in the amplified targets upon which fluorescence signal is modified have also been widely used in real-time assays

[21], and will be discussed in this chapter as well. Other fluorescence-related technologies besides signal intensity measurement, such as fluorescence polarization and fluorescent correlation analysis, will not be discussed in this chapter.

The scope of the discussions in this chapter covers the following topics related to fluorescence-based target detection and signal generation in real-time assays: (1) signal modification principles for fluorescence quenching and energy transfer, (2) target detection/signal generation technologies capable of real-time detection of amplification products, (3) instrument systems, and (4) data analysis and result reporting for real-time assays. Several other topics related to amplification and real-time assays are covered elsewhere in this book. PCR or non-PCR target amplification technologies are discussed in Chaps. 15 and 16. Real-time quantification and amplification product detection are discussed in Chaps. 19 and 20. It is to note that, besides target amplification, probe amplification (e.g., Qβ replicase [22], rolling cycle amplification (RCA) and ramification amplification method (RAM) [23–26]), and signal amplification (e.g., Invader chemistry [27]) have also been used to detect very small amount of analytes of interest which often involves enzymatic cleavage or replication. Signal amplification methods involve target detection and signal modification in which signals continue to accumulate and amplify. Signal amplification can be designed with or without concurrent target amplification (either homogeneous or heterogeneous). Some real-time methods involving both target amplification and signal amplification in one assay will be discussed in this chapter. The discussions on probe and signal amplification methods without target amplification or with heterogeneous target amplification may be found in Chaps. 17 and 18.

Fluorescence Signal Modification

In order to detect targets in a real-time assay, signals generated with the amplified targets need to be differentiated from signals when targets are absent or not amplified. Because fluorescence signals can be easily modified by the environment of the fluorescence moiety, detection of fluorescence signal modification has been the main technology to generate signals in real-time assays. The principle of fluorescence signal and three major mechanisms of fluorescence modifications will be discussed.

Principle of Fluorescence Signal

Fluorescence is a natural process by which certain substances can absorb light or other electromagnetic radiation at specific wavelengths and then emit light at another (usually higher) wavelength. Fluorescence emission is a dynamic process where energy conversion from the excited state to the ground state that generates

fluorescence signal competes with multiple other conversion processes, and the relevant spectroscopic rate constants of these processes are influenced by the environment for the fluorescent moiety. The types of conversions through which the excitation energy is lost spontaneously include the following: intrinsic fluorescence emission radiation decay, internal conversion from excited to ground state, intersystem crossing, collisional quenching, and resonance dipole energy transfer. In real-time assays, fluorescence technologies are used together with detection technologies in ways that these energy conversions will be directly or indirectly impacted by the presence of amplified products. As a result, fluorescence is modified and the modification can be detected and recorded as the testing data from which diagnostic results are derived. Refer to Clegg [28] and Morrison [19] for more detailed discussion on the principles of fluorescence signals. Refer to later sections of this chapter for further discussion on detection technologies.

Fluorescence Resonance Energy Transfer (FRET)

FRET is one of the mechanisms by which fluorescence modification can occur. FRET is a process involving nonradiative transfer of energy between two moieties, a "donor" and an "acceptor," over a long distance (10–100 Å) through dipole–dipole coupling. The acceptor may be a fluorescent or nonfluorescent moiety. When FRET occurs, the fluorescence of the donor will be reduced compared to when no acceptor is present; and the fluorescence of the acceptor will be increased. The prerequisites for FRET to occur are that the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor and that the quantum yield of the donor and absorption coefficient of the acceptor are sufficiently high. In addition, there is strong dependence of the efficiency of energy transfer on the sixth power of the distance between the donor and acceptor [29]. As a result, measurement of the fluorescence change due to FRET has been used effectively to study the static and dynamic molecular structures within the scale of 10–100 Å as well as the thermodynamics and kinetics of bio-molecular interactions. For this reason, FRET has often been referred to as a "spectroscopic" ruler [30].

To be used in a diagnostic method, the change in fluorescence signal due to FRET for a given donor–acceptor system needs to be measurable (i.e., significantly above the background noise) in response to the presence of the target. Considering the theory behind FRET as described above, the design of the donor–acceptor system is often focused on the distance between the two (or more) moieties when FRET is desired to occur as well as the selection of donor–acceptor pairs. It is most common that the donor and acceptor exist as labels on an oligonucleotide and thus the distance is often expressed as the nucleotide bases between the two labels. In this context, when estimating the distance between the donor and acceptor, it is important to consider the rigidity of the structures where labels are attached, spacing between labeled bases in the oligonucleotide (for example, the distance between adjacent base pairs in a DNA double helix is 3.4 Å), the length of linkage for the

labeled moieties, the position of attachment, and the interaction between the labeled moieties and the oligonucleotide. For DNA double helix, the distance also affects the dipole–dipole orientation which may in turn affect FRET efficiency. Use of nonfluorescent labels as acceptor or quencher would preclude the measurement option of monitoring the increase of signals from acceptor, but may simplify the design of a FRET system. In addition to the widely used fluorescent quenchers, several unique nonfluorescent quenchers (such as nucleotides [31] and gold particles [32, 33]) have been introduced for use and some are found to be significantly more efficient that conventional quenchers [32]. It is also important to point out that FRET occurs between moieties that do not contact each other. This is unlike the collisional quenching that requires direct contact. Refer to Tables 24.1 and 24.2 for lists of common dyes and quenchers used in oligonucleotide labeling and their fluorescence properties. To choose dyes for assay design, multiple factors have to be considered besides the fluorescence properties of the labels, such as compatibility with the optical design of existing instrument, oligonucleotide coupling chemistry where applicable, stability of the labels, non-FRET interference from other reaction components (e.g., fluorescence quenching by specific nucleotides bases or oligonucleotide sequences), and commercial availability of the labels.

Collisional Quenching

Besides FRET, collisional quenching is another mechanism by which fluorescence can be modified. Unlike FRET, collisional quenching requires direct contact between labels, and the energy conversion efficiency depends on the effective concentration of the quencher label and the bimolecular rate constant (that is usually controlled by diffusion) [28]. Even though there may be common outcome from collisional quenching and certain FRET system, i.e., the reduction of donor signals, collisional quenching has distinct spectroscopic mechanisms and conditions [20]. First, collisional quenching does not require overlapping emission spectrum and absorption spectrum between the labels and is not impacted by the extent of the overlap, whereas FRET efficiency is significantly impacted by the spectrum overlap. Second, when both labels are fluorescent, collisional quenching results in the reduction of emission of both labels whereas FRET leads to increase of emission of one label and reduction of the other. Third, the absorption spectrums of the labels in collisional quenching are altered compared with when the labels are separate, whereas the absorption spectrums stay unchanged in the case of FRET. Fourth, when same labels are involved, the efficiency of collisional quenching is higher than FRET-mediated quenching. Collisional quenching is often achieved by the blunt-end double-stranded oligonucleotide design where both strands are terminally labeled so that the two labels are in close contact. In such designs, hybridization stability of the oligonucleotides correlates positively with quenching efficiency. It is also to note that the interaction between the two labels can further increase the stability of double-stranded oligonucleotides, leading to higher quenching efficiency [20].

Table 24.1 Common dyes for oligonucleotide labeling

Dye name	Absorption peak wavelength (nm)	Emission peak wavelength (nm)
Dansyl-X	335	518
Alexa Fluor 350	343	441
Biosearch Blue	352	447
Cascade Blue	396	410
Alexa Fluor 430	434	539
Pulsar 650	460	650
Cy2	489	506
Oregon Green 488	494	517
Alexa Fluor 488	495	518
FAM	495	520
Rhodol Green	495	521
Orange Green 500	499	519
BODIPY FL	502	510
Rhodamine Green	503	528
Oregon Green 514	506	526
TET	521	536
CAL Fluor Gold 540	522	544
BODIPY R6G	528	547
JOE	529	555
Yakima Yellow	531	549
Alexa Fluor 532	532	554
BODIPY 530/550	534	554
HEX	535	556
VIC	538	554
CAL Fluor Orange 560	538	559
BODIPY TMR-X	544	570
NED	546	575
Quasar 570	547	570
Cy3	549	566
Alexa Fluor 555	555	565
Alexa Fluor 546	556	573
TAMRA	557	583
BODIPY 558/568	558	569
Rhodamine Red-X	560	580
BODIPY 564/570	563	569
CAL Fluor Red 590	569	591
BODIPY 576/589	575	588
ROX	575	602
Alexa Fluor 568	578	603
Redmond Red	579	595
BODIPY 581/591	581	591
Cy3.5	581	596
BODIPY TR-X	588	616
CAL Fluor Red 610	590	610
Alexa Fluor 594	590	617

(continued)

Table 24.1 (continued)

Dye name	Absorption peak wavelength (nm)	Emission peak wavelength (nm)
Texas Red	597	616
CAL Fluor Red 635	618	637
LC Red 640	625	640
BODIPY 630/650	625	640
Alexa Fluor 633	632	647
BODIPY 650/665	646	660
Cy5	646	669
Quasar 670	647	670
Alexa Fluor 647	650	665
Alexa Fluor 660	663	690
Cy5.5	675	694
IRDye 800RS	676	786
Alexa Fluor 680	679	702
LC Red 705	680	710
Quasar 705	690	705
Alexa Fluor 700	702	723
Cy7	743	767
Alexa Fluor 750	749	775

Table 24.2 Common quenchers for oligonucleotide labeling

Dye name	Absorption peak wavelength (nm)	Approximate quenching range (nm)
DDQ I	430	380–510
Dabcyl	453	403–507
QSY 35	475	434–509
QXL 490	490	410–540
BHQ-0	495	425–550
QXL 520	520	460–560
Eclipse	522	460–590
Iowa Black FQ	531	420–620
BHQ-1	534	460–595
QSY7	560	535–582
QSY9	562	535–587
QXL 570	570	520–600
BHQ-2	579	510–645
QXL 610	610	520–660
DDQ II	630	550–680
Blackberry Quencher 650	650	550–750
Iowa Black RQ	656	500–700
QSY 21	661	601–695
QXL 670	670	600–690
BHQ-3	672	575–730
QXL 680	680	610–710
IRDye QC-1	737	660–870

Collisional quenching, when achieved by oligonucleotide hybridization, does not necessarily require a quenching label. Instead, fluorescence quenching by specific nucleotides is well documented and has been used in methods based on collisional quenching [31].

Nucleic Acid Dyes

There are dyes that can bind to nucleic acids in a sequence-independent manner (without being conjugated to an oligonucleotide), and upon binding, their fluorescence signal increases significantly. These dyes are also called nucleic acid stains. Nucleic acid dyes can be grouped into three major classes based on the mechanism of signal generation: (1) intercalating dyes such as phenanthridine compound (also known ethidium bromide), cyanine dyes (including Sybr Green, Picogreen, etc.), and propidium iodide; (2) minor-groove binders such as DAPI and Hoechst dyes; and (3) other miscellaneous dyes that achieve external binding. Intercalating dyes are the most commonly used nucleic acid dyes. They contain planar structures that insert between stacked bases in the nucleic acids, thereby creating a large increase in fluorescence signal relative to the free dye in solution. The signal increase is a proportional response to the amount of nucleic acids. This property allows the dyes to be used in either direct quantification of nucleic acid samples or real-time amplification-based detection and quantification. SYBR Green I is one of the intercalating dyes that generate signals by inserting into double stranded DNA. It has been the most widely used nucleic acid dye in real-time PCR method due to high-level sensitivity for DNA detection and relatively cheap price. In addition to methods using only nucleic acid dyes to achieve non-sequence-specific detection, some methods have also been designed where nucleic acid dyes are covalently attached to an oligomer and provide increased fluorescent signal upon sequence-specific hybridization. Refer to Table 24.3 for a list of nucleic acid dyes that have been used in real-time assays [34–43].

Detection of Amplification Products

Real-time assay design starts with selection of target region and primer sequences as well as optimization of amplification efficiency (reagent composition, concentration, and cycling conditions). To monitor a real-time assay, signals have to be generated for the target being amplified. Target detection and signal generation can be achieved either in a sequence-specific manner by using oligomers consisting of nucleotides or their analogs, or in a sequence-nonspecific manner by using nucleic acid dyes. Multiple factors have to be considered when designing target detection/signal generation systems: (1) optimizing sensitivity for detection of

Table 24.3 Nucleic acid dyes used in real-time PCR

Dye name	Absorption peak wavelength (nm)	Emission peak wavelength (nm)
LC Green	440–470	470–520
ResoLight	450–500	487
BO-PRO-1	462	481
BEBO	467	492
SYTO 9	485	498
SYTO 13	488	509
SYTO 16	488	518
YO-PRO-1	491	509
SYBR Green I	497	520
EvaGreen	500	530
TO-PRO-1	515	531
BOXTO	515	552
SYTO 82	541	560
SYTOX Orange	546	570
SYTO 64	598	620
TO-PRO-3	649	655
SYTO 62	649	680
SYTO 60	652	678

low-level targets, (2) maximizing mismatch tolerance or discrimination depending on the goal of assay design, (3) optimizing specificity to achieve minimal signals when background or nonspecific targets are present, and (4) lowering pre-amplification background signals and improving signal-to-noise ratio.

Nucleic acid dyes have been used in highly sensitive real-time assays. Nucleic acid dyes detect double-stranded DNA targets and generate fluorescent signal while doing so. The limitation is that the specificity of the method solely depends on the specificity of the amplification reaction, i.e., primer design. A potential concern with the use of nucleic acid dyes is that high level of genomic background will increase the pre-amplification background signal. In addition, any unintended nonspecific amplification such as primer dimer formation can generate signals that lead to false-positive results. Therefore, care needs to be taken during primer design to eliminate potential nonspecific signals or reduce them to an acceptable level. One way to ascertain the specificity of the nucleic acid dye-based real-time signals is to perform melting experiment after amplification to determine the presence of intended targets and absence of unintended targets through characteristic melting temperatures.

The discussion here will focus instead on various sequence-specific detection technologies (namely primers and probes) that generate signals based on energy transfer, fluorescence quenching, or fluorescence enhancement (for certain probes labeled with intercalating dyes). Signals generation as a result of target detection are achieved either directly via signal components labeled on the primers or probes, or indirectly via mechanisms initiated by target detection (one example would be enzymatic cleavage as the result of Invader probe binding). Probes and primers consist of oligonucleotides or analogs; therefore, by nature they only bind to the target sequences with sufficient complementarity via specific biophysical interactions

between base pairs. Depending upon the purpose of a real-time assay, probe or primer design for the target of interest often needs to be specifically tailored either to tolerate sequence variations for detection of diverse DNA or RNA sequences or to differentiate between sequence polymorphisms. It is also important to point out that it is by using sequence-specific target detection mechanisms and optically distinct fluorescence dyes that real-time assays can be designed with multiplex capability, i.e., concurrent detection and differentiation between multiple analytes within one reaction.

There is a wide range of oligomer designs suitable for real-time target detection. They vary greatly in physical characteristics such as base type (DNA, RNA, or other analogs), length, and multiplicity of the components, direct or indirect signal generation (mentioned above), biochemical/biophysical mechanisms enabling signal generation by energy transfer or fluorescence quenching/enhancement (e.g., hybridization, conformational change, and enzymatic cleavage), and presence or absence of signal amplification in addition to target detection, etc. Needless to say, each design has its advantages and disadvantages, and though every design has its flexibility, certain designs may fit specific needs better than others. The section below will include discussions on some representative designs as found in published literatures, which is not intended to be a complete list. Only design principles and basic performance characteristics will be described. The discussion will not be focused on the findings or conclusions related to advantage or disadvantages of certain designs based on empirical experience from specific studies, with the notion in mind that each design can be optimized and each design may fit one utility better than others within specific contexts. To facilitate discussions, sequence-specific target detection technologies are roughly categorized in four groups depending upon the components enabling signal generation. The first group relies on design of oligonucleotide probes to recognize the amplified target sequences and generate signals; the second group relies on design of primers beyond their target amplification function to generate signals upon extension of the template; the third group requires both probe and primer in order to generate signals; and the fourth group requires fluorophore-labeled nucleobases. One important difference between groups one and three and groups two and four is that the former designs take advantage of both primer and probe sequences to ensure target specificity of the reaction, whereas the latter designs depend solely on target specificity provided by primer sequences. As a result, designs belonging to groups two and four are theoretically more prone to nonspecific signals due to nonspecific amplification such as primer dimers.

When multiple designs exist within each group, they are described sequentially without further subgrouping, due to the complexity and flexibility in design principles. Related designs viewed as derivatives or extensions of the same design principle are discussed together rather than being treated as separate designs. In addition, it is understood that hybridization between oligonucleotides and their target sequences can be enhanced by incorporating certain types of nucleotide analogs (e.g., LNA and PNA) in the oligonucleotides or by specific types of labeling (e.g., MGB). As a result, such design features alone are not treated as independent designs. Detailed discussions on these hybridization enhancements are not included in the discussion here and can be easily found in existing literature.

Probe Designs for Target Detection and Signal Generation

5′-to-3′ Exonuclease Assay

Many real-time assays are designed based on the 5′-to-3′ exonuclease activity of the polymerase that amplifies target sequences [44]. Probes used in such assays are often dual-labeled linear oligonucleotides containing one label at the 5′-end and the other label either at a location internal to the 5′-end or at the 3′-end. During the primer extension step, both the primers and the probe are hybridized to the target strand. When primer extension reaches the probe, the polymerase will cleave the 5′ label off of the probe via its 5′-to-3′ exonuclease activity. As a result, the fluorescence signal of one or both labels are modified due to the removal of the energy transfer or quenching effect between the labels. The fluorescence signal as measured during a real-time assay is proportional to the amount of cleaved probes as well as the accumulated amount of the amplification product (i.e., one cleaved probe per new copy of amplified product). In real-time PCR, the signal doubles after each cycle because the amounts of cleaved probes and amplified targets double as the result of primer extension. Since the 5′-to-3′ exonuclease activity has also been referred to as TaqMan activity, the probes are often called TaqMan probe. Similar to many other technologies in later discussions, assays can be designed to achieve multiplex detection by using multiple TaqMan probes labeled with spectrally distinct fluorescence labels. Due to its relative simplicity, TaqMan probe has been widely adopted. As a potential limitation, probe length needs to be sufficiently long to enable hybridization to the target at the relatively high polymerase extension temperature (approximately 65 °C). As a result, background signals in the absence of amplified target may be high, and the capability of relatively long TaqMan probe in detecting single nucleotide polymorphism (SNP) mutations may be limited. One new approach in 5′-to-3′ exonuclease assay [45, 46], called Snake, utilizes a primer with a 5′-flap containing an amplicon complementary sequence that results in the formation of a stem-loop structure of the PCR amplicon. A dual-labeled probe, together with this stem-loop structure, serves as an optimal substrate for the exonuclease activity of Taq polymerase. Unlike TaqMan probe, the Snake system prefers short probes, therefore may be more efficient in detecting SNPs. However, the design of the 5′-flap can be tricky, where the overstabilization of the secondary structure inhibits PCR efficiency.

Adjacent Probes

In this design, two probes bind to adjacent locations on the target sequence [47, 48]. The 3′-end of the upstream probe and the 5′-end of the downstream probe are labeled with two fluorescence moieties capable of energy transfer or quenching. When target sequences accumulate during amplification, these two probes will bind and the fluorescence signal modification (either signal increase for the fluorescence acceptor or signal decrease for the donor) can be monitored in real time. Real-time assays using adjacent probes often can be followed with melting

analysis for identification of mutations. It is to note, however, that the signal is generated with a tertiary structure involving two probes and the target, thus optimization in assay designs and conditions can be relatively complicated. Due to the requirement of two probe-binding regions and their proximity, a long contiguous sequence has to be evaluated for fitness as the probe-binding region regarding sequence context and mutations. In addition, when accumulated target level exceeds that of the probe concentration, probes will more likely bind to different target strands than to a single strand. This will lead to a decrease in real-time PCR signal complicating data analysis. A variation of the adjacent probe design has also been reported where probes bind to double-stranded DNA target (rather than single-stranded target) each forming a triplex structure [49].

A modification of adjacent probes is C-probe design [50]. C-probe is a single oligonucleotide probe dual-labeled with fluorophore/quencher at both ends containing two terminal regions that can bind to adjacent target sequences. As a result of the binding, the two labels are brought together enabling fluorescence modification. In another related C-probe design, the two target-binding regions are in two different probe sequences that associate with each other via a double-stranded stem [50].

Molecular Beacon Probe

Molecular beacon probe is a single-stranded oligonucleotide containing complementary sequences at or near its termini that can form an intramolecular helical stem as well as a loop sequence connecting the two stem sequences [51, 52]. The loop and sometimes part or all of the stems are designed to constitute the complementary sequence to the target. Molecular beacons are usually labeled with two fluorescent/quenching moieties each in the vicinity of either stem. Whether signal generation mechanism is based on energy transfer or collisional quenching depends on the stagger positions of the two labels [20]. It is conceivable that some designs may require only one label by using the natural quenching property of certain nucleotides. It is also conceivable that more than one fluorescent label can be used in order to generate more signals or to achieve wavelength shifting via energy transfer. In the absence of target, molecular beacon possesses the closed stem-loop structure due to the efficient intramolecular stem formation, resulting in energy transfer or collisional quenching. When target accumulates in a real-time assay, molecular beacon probes hybridize to the target via the target complementary sequence forming the double-stranded probe–target hybrid. As a result, the probe's stem-loop structure opens up thereby separating the two labels and causing the modification in fluorescence signals due to the loss of energy transfer or quenching effect. The thermodynamic balance between the open and closed states of the probe can be effectively modulated by adjusting the sequence and length of either the stem or the target binding/loop sequence. The opening and closing are synergistically affected by both the loop and stem sequences. Molecular beacon probes with a strong stem and relatively short loop have often been used effectively to detect SNP mutations. In fact, SNP detection is perceived to be a predominant advantage of molecular

beacon due to efficient closing of the stem-loop structure. However, assay sensitivity in such cases may be negatively impacted due to the stabilized closed state. A variation in the molecular beacon design [53], i.e., tentacle probe, was developed, which contains a capture probe in addition to the stem-loop structure. The capture probe can be designed to enhance the hybridization stability and rate while maintaining the sequence specificity of the typical molecular beacon. Molecular beacon can also be designed with long loop sequence and relatively short stem to detect heterogeneous sequences in a mutation tolerant manner, though the background signal will be higher. Another variation in molecular beacon is the "wavelength shifted" design, where a "harvester" dye is introduced at a position 5–18 bases away from the fluorophore [54]. The harvester dye has an emission spectrum that overlaps well with the excitation wavelengths of multiple fluorophore, which can presumably facilitate multiplex detection on instrument where only one excitation wavelength is available. Besides these above-mentioned designs, there are also other probe designs that use similar fundamental principles as the original molecular beacons, i.e., signal generation resulted from molecular conformational changes driven by target binding. These probe designs include bimolecular beacon [55], tripartite molecular beacon [56], molecular torches [57], dumbbell molecular beacon [58], and cyclicon probe [59].

Complementary Probes

Complementary probes, i.e., double-stranded probes, consist of two strands of oligonucleotides with complementary sequences [60–62]. The 5′-end of one strand and the 3′-end of the other strand are each labeled with a fluorescent or quenching moiety. Similar to molecular beacons, signal generation mechanism (energy transfer or quenching) depends on the positions of the labels; and in a preferred design there may be variations in the number of labels. In the absence of targets, due to the formation of double helix consisting of the two probe strands, the two labels are brought into the vicinity of each other. In the presence of targets, the probe strands hybridize to their corresponding complementary target sequence, thereby separating the two labels. To achieve desired assay performance (i.e., sensitivity, mismatch tolerance versus differentiation), the thermodynamic balance between probe binding and target binding can be modulated by multiple factors such as the lengths and sequences of both probe strands and their respective concentrations and molar ratio. The ability to modulate the molar ratio between the two strands is an advantage of the complementary probes over the single-strand probes (such as TaqMan probe and molecular beacon) for which sequence variation remains the only source of design flexibility. Multiple types of complementary probe designs (categorized by length symmetry) have been introduced, including partially double-stranded probes with differential lengths [60], and symmetric or near-symmetric probes with equal or near-equal lengths between the two strands [61, 62]. While symmetric or near-symmetric probes should work effectively to distinguish mutations, this is accomplished at the expense of slower binding kinetics and potentially lower sensitivity

due to competition of the equal length complementary probe with the target. The partially double-stranded probe can be designed either to achieve mismatch tolerance or mismatch discrimination. One significant advantage of partially double-stranded probe is that the design features (i.e., lengths of both probe strands, their molar ratio, and read temperature) can be thermodynamically modulated with considerable independence so that multiple performance requirements such as sensitivity and mismatch tolerance or differentiation can be achieved simultaneously. Partially double-stranded probes with two labeled oligonucleotides overlapping at the complementary region and containing noncomplementary single-stranded regions on both ends have also been described [63].

Single-Stranded Dual Label Hybridization Probes

Eclipse probes are singled-stranded probes labeled with a 5′ quencher and a 3′ fluorophore [64]. In addition, a minor groove binder (MGB) moiety that significantly stabilizes the double-stranded probe–target complex is also conjugated to the 5′ end. When not hybridized to the target, the fluorescence of the MGB Eclipse probe is efficiently quenched by the interaction between the fluorophore and quencher moieties. Upon hybridization, the probe is stabilized in the double-stranded structure, allowing fluorescence to be emitted. The mechanism of signal generation is independent from the 5′-to-3′ exonuclease activity because the 5′ MGB blocks such an activity.

Similar to the Eclipse probes, Pleiades probes are also hybridization probes labeled with a fluorophore, quencher, and a 5′ MGB [65]. The difference is that the fluorophore is labeled at the 5′ end and the quencher at the 3′ end. As a result, Pleiades probes may have lower background signal when probes are not hybridized and higher signal gain upon probe hybridization to the target.

Light-Up and FIT Probes

Light-up probe and FIT (forced intercalation) probe are short peptide nucleic acid (PNA) oligomer labeled with asymmetric cyanine dye thiazole orange (TO). Light-up probe is labeled with TO at the N-terminus of PNA [66], whereas FIT is labeled with a quinoline-linked TO as the substitution of an internal nucleobase [67]. TO is an intercalating dye that upon binding to DNA target exhibits large enhancement in fluorescence intensity relative to the free dye. Both Light-up and FIT probes take advantage of PNA to achieve sequence-specific hybridization. The extent of signal enhancement upon binding is much greater when complementary sequences are present compared to when noncomplementary sequences are present. The background signal in the absence of targets comes from the TO-labeled PNA probe and is highly dependent on the probe sequence. The use of PNA instead of nominal DNA bases eliminates the binding of TO to probe bases thus reducing the amount of background signal. Both Light-up and FIT probes can be designed very short (e.g., 10 base long) while maintaining sufficient hybridization stability due to the use of PNA. As a result of the short length, Light-up and FIT probes are highly sequence

specific with one mismatch causing clear differentiation between perfect-matched and mismatched targets. These short probes can be used to detect/quantify highly diverse microbial strains because it is relatively easy to find short conserved sequences within which to design probes [68]. The probes can also be designed to straddle the mismatches to detect/identify SNP mutations. It is interesting to note that the internally labeled TO in an FIT probe behaves as a universal base and can be designed at a position complementary to a mutation to achieve tolerance to such a mutation. Importantly, the similarities in both excitation and emission wavelengths between TO and fluorescein mean that these probe designs are compatible with the majority of the existing real-time PCR instruments. The utilities of Light-up and FIT probes have been demonstrated in qualitative or quantitative clinical diagnostic testing based on real-time PCR or isothermal amplification real-time assays [68, 69].

Hybridization-Induced Quenching or Dequenching of Fluorescein-Labeled Oligonucleotides

Fluorescence can be quenched by nucleobases due to photoinduced electron transfer, as well as possibly coupled electron/proton transfer and hydrophobic effects. Due to their electron-donating properties, guanine derivatives have been found to be strong quenchers of fluorescence. As a result, labeling of fluorophores close to a G base has been recommended against in probe designs. Interestingly, it has been found that a probe labeled with G-quenched fluorescence (HyBeacon) can be dequenched upon hybridization to the complementary target strand, which leads to an increase in fluorescence signal [70]. Besides guanine, the quenching effect of intercalating dye has also been utilized to design a probe for which the quenching of fluorescence signals is reduced upon dye's intercalation with probe–target double-stranded complex [71]. As a result, the fluorescence is generated when targets accumulate. In addition to the quenching effect based on primary sequence adjacency, the quenching effect from G bases brought in close vicinity of the fluorophore via stem-loop formation has also been explored for the design of "Smart" probe. Like molecular beacon, the stem-loop structure opens up upon binding to the target, thereby generating signals due to reduced quenching [72]. All these above-mentioned phenomena of hybridization-induced fluorescence dequenching have been or can be used to design probes for real-time assay-based nucleic acid detection and genotyping.

In addition to dequenching, the G-associated quenching phenomenon has been utilized to design assays based on hybridization-induced quenching [73, 74]. Specifically, a fluorophore-labeled probe is designed with such a sequence that upon binding the fluorophore will be in the vicinity of a G on the complementary target sequence. The extent of the quenching effect depends on the number and positions of the adjacent G bases on the complementary target strand. As target sequences accumulate in a real-time assay, the amount of quenched fluorescence can be measured that correlates with the amount of accumulated targets, much in the same way that an increased fluorescence signal is measured in a typical real-time assay.

Internally Cleavable Fluorescence Probe

BDProbeTecET is a single-stranded DNA probe consisting of a stem-loop structure where fluorescein and rhodamine labels are positioned on two opposite sides of the loop and a recognition sequence for the *Bso*BI enzyme is placed within the loop sequence [75]. This probe has been designed to achieve real-time detection of the nucleic acid targets generated with SDA, based on the FRET principle. Before target amplification by SDA, there is very little signal from fluorescein due to energy transfer to rhodamine. During SDA, the probes bind to the displaced single-stranded target sequences generated from the amplification reaction and are converted to double-stranded DNA as the result of extension by *Bst* DNA polymerase. This double-stranded product is then cleaved by the *Bso*BI restriction enzyme due to the presence of the restrictive site engineered in the probe sequence. This cleavage causes the physical separation of the fluorescein and rhodamine labels, and loss of energy transfer which in turn leads to increase in fluorescein signals. There have also been similar designs where the donor and acceptor are separated only by a restrictive enzyme site without a stem-loop structure.

Besides the above examples where each copy of amplified target supports one signal generation event, signals amplification can be achieved by using a cleavable fluorescence probe where one copy of amplified target supports multiple signal generation events. CataCleave probe (catalytically cleavable fluorescence probe) is one example [76]. CataCleave probe has a chimeric DNA-RNA-DNA sequence with four contiguous internal purine ribonucleotides. Upon binding to the DNA target, the RNA portion of the DNA:RNA hybrid is cleaved by RNase H. The cleaved products dissociate from the target DNA allowing further rounds of probe hybridization and cleavage. As such, each target DNA generates many cleaved probes that can accumulate over several orders of magnitude. Because the probe is labeled with fluorophore/quencher labels adjacent to both DNA/RNA sequence boundaries, probe cleavage results in fluorescence signal modification. Application of CataCleave probe has been demonstrated in real-time assays including both isothermal amplification and PCR.

Invader Chemistry

Invader chemistry detects target with probe-mediated sequence-specific hybridization and achieves signal amplification with probe cycling and structure-specific cleavage by Cleavase enzyme [27]. Invader chemistry involves two isothermal reactions. In the first reaction, an Invader oligonucleotide and a downstream probe bind to the single-stranded target and form a substrate structure for the Cleavase, which contains a one-base overlap at the 3′ end of the Invader oligonucleotide and 5′ end of the probe's target binding region. In addition to the target binding region, the probe is designed with an un-complementary arm (flap) 5′ to the target binding region. Cleavase enzyme cleaves the probe at the overlap position and generates the flap plus one nucleotide. The reaction temperature is set close to the melting

temperature of the probe therefore allowing probe to cycle on and off rapidly. As a result, cleaved probes are continuously being generated as reaction proceeds. In the second reaction, the flap released by the first reaction acts as an Invader oligo and binds to a synthetic hairpin structure that is labeled with a FRET dye–quencher pair (FRET cassette). The complex can again be cleaved by the Cleavase enzyme and as a result the FRET pair is separated allowing fluorescence signal to be detected. This second reaction involves rapid isothermal cycling of the flap on and off the FRET cassette, similar to the first reaction. As a result, Invader chemistry can generate significant signal amplification in a sequence-specific manner. Invader chemistry has been used in a wide range of application including genotyping, target quantification, and copy number variation. This signal amplification technology has also recently been combined with real-time PCR amplification to achieve multiplex quantification of viral genomes [77–79].

Primer Designs for Target Detection and Signal Generation

Stem-Loop Primers

Three types of primers containing a stem-loop structure have been described in real-time PCR assays: AmliFluor primer (also called Sunrise primer) [80], LUX primer (*L*ight *U*pon e*X*tension) [81], and Scorpion primer [82]. Besides the stem-loop structure, the common features between these primer types are that (1) they are labeled with at least one fluorophore and generate signals upon opening of the stem-loop structure (probes are not needed to generate signals), and (2) the 3′ end of the primer sequence can bind to the complementary target sequence and be extended by the DNA polymerase. AmpliFluor primers are labeled with fluorophore/quencher pair at the two ends of the stem-loop structure, so that no fluorescence is generated due to efficient quenching. When PCR amplification on the complementary strand extends through the AmpliFluor primer region, the primer adopts an extended conformation due to formation of double-stranded DNA separating the two labels. As a result, energy transfer or quenching effect reduces, resulting in increase in fluorescence signal. LUX primer is singly labeled with a fluorophore at the C-5 position of the thymidine close to the 3′-end. In addition, the primer needs to have G or C base around the 3′-end so that the fluorescence is efficiently quenched due to the nucleobase-dependent quenching. Similar to AmpliFluor, LUX primer will fluoresce when its complementary sequence is extended during PCR causing the stem-loop structure to open. It is to note that only those fluorophores that can be efficiently quenched by nearby dG-dC and dC-dG basepairs such as fluorescein, JOE, HEX, TET, ROX, and TAMRA can be used in the LUX design, whereas certain other dyes such as Texas Red, Cy3, and Cy5 cannot be used in LUX primers. The loop in both AmpliFluor and LUX primers simply serves as a connector between the two stem-forming sequences so that a stable stem-loop structure can be formed. In contrast, Scorpion primer contains a loop sequence that is complementary to the extended

target sequence downstream of the primer. When the new region is synthesized in the PCR, the loop will hybridize to the newly synthesized region on the same DNA strand and form a large stem-loop structure. As a result, the original stem-loop structure in the primer opens up leading to signal generation. It should be noted that a blocker moiety is inserted in the primer between the stem-loop sequence and target complementary sequence so that the extension of the reverse strand will not open up the primer. These above primer systems can be designed to tolerate or discriminate mutation dependent upon the 3′ end of the primer sequences.

Duplex Primer

Several duplex primer designs have been introduced. The common features between these duplex primer designs include the following: (1) two complementary strands are used including a primer strand that is extendable from the 3′ end, and (2) both strands are labeled, the primer strand with a fluorescence donor and the other with a fluorescence acceptor or quencher. In one such design, PCR extension of the region complementary to the primer strand displaces the other strand labeled with a quencher and thus allowing signal generation [83]. Another design is called duplex scorpion primer [84], which is a derivative to the original scorpion primer design described above. Instead of having a stem as part of the stem-loop structure, the stem for the duplex scorpion primer is formed by two separate oligonucleotides. The stem sequence in the primer strand is complementary to the PCR-extended target sequence downstream of the primer. Therefore, PCR amplification causes the stem on the primer strand to bind to the extended target region, displacing the other stem bearing the quencher, and thus resulting in signal generation.

DzyNA-PCR

DzyNA-PCR involves in vitro amplification of target sequences using a DzyNA primer that contains the complementary sequence of a DNAzyme [85, 86]. Upon amplification of the target, the reverse strand is generated that contains the active DNAzyme sequence. DNAzyme sequence consists of a catalytic domain of 15 nucleotides flanked by two substrate recognition domains and it can cleave nucleic acid substrate at specific RNA phosphodiester bonds (between an unpaired purine and a paired pyrimidine). A chimeric oligonucleotide that contains RNA substrate sequence flanked by DNA-binding sequences and is labeled with FRET donor/acceptor pair is included as the reporter substrate. During PCR, the reporter substrate will transiently associate with DNAzyme through Watson–Crick pairing yielding multiple cleavage product and signal amplification. This method obviously requires that the reaction conditions support both the target amplification and enzymatic reaction. In addition, the design of substrate recognition domains in the DNAzyme sequence needs to ensure sufficient hybridization at assay conditions and thus efficient cleavage.

Similar to DzyNA-PCR, methods have been described that utilize the hammerhead ribozyme sequence either existing in a target sequence or introduced as a primer tag to achieve real-time target detection and signal amplification in an isothermal amplification reaction such as NASBA or 3SR [87]. It is to note that, similar to any assay solely relying on primer for target recognition, nonspecific primer amplification such as false priming or primer dimer may generate nonspecific signals.

Primer Labeled with Unnatural DNA Bases

A real-time PCR method has been developed that uses a primer containing a 5-methylisocytosine nucleotide base (isoC) labeled with a fluorophore and an unlabeled primer in the reverse direction [88]. PCR amplification is performed in the presence of diGTP (isoG) labeled with a dabcyl quencher. Incorporation of the quencher through isoc:isoG paring leads to quenching of the fluorescence. Fluorescence signal decreases along the course of reaction, which is similar to a typical real-time PCR method, albeit with signal change in the opposite direction.

Energy Transfer Between Primers or Between Primer and Probe

Several methods have been described that involve energy transfer between primer and probe each labeled with a fluorescence donor or acceptor [89, 90]. The primer is labeled either at the 5′ end or at an internal position, while the probe is labeled at the 3′ end. After primer extension generates the probe-binding sequence, the probe will hybridize to the extended region bringing two labels within a short distance. As a result, energy transfer can occur and fluorescence signal change can be measured. One publication also described a method involving energy transfer between forward and reverse primers each labeled with donor and acceptor/quencher [91]. The labels are located at such positions in the primers that once the double-stranded amplicon is formed as the result of primer extension, energy can be efficiently transferred from the donor to acceptor or quencher.

FRET Mediated Through Acceptor-Labeled Nucleobases Incorporated in the Amplified Product

Several methods are designed based on acceptor fluorophore-labeled nucleobases. These nucleobases are incorporated in the amplified products via primer extension. Upon target binding of certain nucleic acid dyes or cationic conjugated polymers that can act as fluorescence donors, acceptor fluorescence will increase due to FRET [92, 93]. In an alternative design called template-directed dye-terminator incorporation, the primer is labeled with fluorescence donor [94]. As a result, incorporation of the next acceptor-labeled nucleobase leads to change in fluorescence signal.

Instruments for Real-Time Amplification and Detection

Instrument systems supporting automated real-time amplification and detection are becoming increasingly available in both research and clinical diagnostic spaces. Real-time PCR is the main target amplification and detection technology that underlies the core capability of the majority of systems, while real-time isothermal amplification and detection is also represented in a small number of systems. This discussion will not include instrumentation required for sample management or nucleic acid extraction/purification.

Each instrument supporting real-time assay and fluorescence detection has three basic technical functionalities: (1) supply excitation energy, (2) detect emission energy, (3) control and/or cycle temperature. There are currently three main types of excitation energy supply, namely, lamp, light-emitting diode (LED), and laser. Lamp provides relatively broad spectrum of lights while LED and laser provide narrow wavelengths. Detection of the emission light is achieved with three main types of devices, namely, charge-coupled device (CCD) camera, photomultiplier tube, and other types of photodetectors. It is to note that multiplex detection is achieved by using multiple discrete filters or channels at the emission detection as well as sometimes the excitation end of the instrument. It is more difficult to achieve multiplex performance when there is only one excitation spectrum with a narrow wavelength range. For multiplex detection, filter or channel-specific signals often have to be determined through mathematical decomposition to determine the analyte-specific signals due to the often overlapping spectrums of fluorescence dyes. Requirements of thermal control are different for real-time PCR and isothermal amplification. While any thermal device capable of maintaining specific temperatures suffices for the isothermal amplification, real-time PCR requires a device that can cycle temperature with speed, accuracy, and uniformity. Common types of thermal cycling devices include heating block (e.g., Peltier based) and heated air (for either tube or capillary-based reaction vessels). It is obvious that the performance of real-time assays depends on factors in assay chemistry (amplification and signal generation), instrument performance (optical and thermal), and the intricate interactions between assay and instrument factors. Critical assay performance characteristics include detection sensitivity, specificity, and reproducibility (e.g., sample-to-sample, run-to-run, reagent lot-to-lot, and instrument-to-instrument). For example, the sample-to-sample reproducibility is highly dependent upon the instrument's robustness against variables including sample positions and plate setup, which would be determined by the optical and thermal uniformity.

Design features, functionalities, and technical capabilities of an instrument system are complex and multifaceted. Institutional adoption of a system typically involves comparison and consideration in various areas including: instrument cost, physical dimension and other facility requirements, test menu/performance and regulatory status, open application capability, assay throughput and labor burden, service and support capability of the manufacturer, user interface, and laboratory information systems. The central consideration among all these factors has to be performance of the test and instrument. Interested readers are encouraged to review

a comprehensive review by Espy et al. [21] and a recent summary of automated molecular platforms for additional information [95]. The assessment of instrument systems for the purpose of assay or instrument development are typically focused on the technical design features and capabilities, such as excitation and detection systems, multiplex capability, reaction volume, throughput, thermal control, etc. It is understood that when designing an instrument, logistical, infrastructural, and other nontechnical aspects of the total system offering as stated previously have to be comprehended and optimized for the end-use laboratories and personnel. The technical features of the main instrument systems currently available in the commercial clinical diagnostic field are summarized in Table 24.4.

The future in molecular diagnostic instrumentation lies in areas such as higher degree of process integration, menu access flexibility (random access), sample processing flexibility (continuous access and STAT testing), testing throughput, quicker turnaround time, and less hands-on time. In addition, an area of increasing importance and growing needs is robust point-of-care (POC) systems, especially for the resource-limited areas. Such POC systems need to possess these following capabilities: full integration of assay process (sample in and result out), error-proof operation, environmental robustness, and minimal logistic requirements (e.g., power, water, reagent ambient storage, and waste containment).

Data Analysis and Result Reporting

Data analysis and result reporting are a critical and final process of the diagnostic testing. This process typically involves the following steps: (1) recording of optical measurements, (2) determining dye responses (analyte signals), (3) data normalization and amplification curve analysis, (4) baseline setting, (5) determining cycle number (for PCR) or time to positivity (for isothermal amplification), (6) determining qualitative and/or quantitative results based on outputs from step 5 as well as signal intensity, and (7) reporting results.

The automation in data analysis and the ease of use for reported results (i.e., objectivity without human intervention) are among the hallmarks of modern commercial clinical diagnostic systems. The objectivity of reported results is the outcome of both robust data analysis algorithms and sophisticated data validity criteria. Validity criteria include checks on multiple aspects of the assay data, including amplification curve, dye intensity, signal/noise abnormality, cycle number (or time to positivity) abnormality, as well as, depending on control and calibration strategies, various performance characteristics of controls and calibrators.

The output of data analysis is the generation of some sorts of actionable values from which to determine assay results. These actionable values may include cycle number (or time to positivity) or signal intensity. The algorithms in formulating assay results from these actionable values vary depending upon the diagnostic utilities that assay results are expected to fulfill. For a quantitative or semi-quantitative assay, analyte quantities may be calculated by comparing the cycle number (or time

Table 24.4 Real-time instruments used in clinical microbiology

Company	Model	Technology	Reaction format	Read system excitation	Read system detection	Multiplex capability	Thermal control
Abbott	m2000rt	Real-time PCR	96-Well plate	Halogen lamp; optical filters; full plate illumination	Optical filters; CCD camera	5 colors	96-Well block Peltier device
BD	Max	Real-time PCR	Microfluidic card	Individual well illumination	Discrete detector for each well	2 colors	Individual reaction heating circuit
bioMerieaux	EasyQ	Real-time NASBA	Reaction tube	Filters	Optical filter; PMT; individual tube	8 colors	Isothermal device
Cepheid	SmartCycler	Real-time PCR	Individual custom chamber	LED; individual well illumination	Optical filters; silicon detector for each reaction	4 colors	Individual reaction heating circuit; solid state heater and forced-air cooling
Focus	3M Integrated Cycler	Real-time PCR	Disc	LED; optical filters	Optical filters; PMT; individual well detection (one reader)	4 colors	Infrared heating; air cooling
Qiagen	Roter-Gene Q	Real-time PCR	Reaction tube	6 LED; individual well illumination	Optical filters; PMT; individual well detection (one reader)	6 colors	36, 72, 100 tube rotor with air heating/cooling
Roche	cobas TaqMan	Real-time PCR	Microtube	Halogen lamp; optical filters; individual well illumination	Fiber optic channels; optical filters; photo ASIC	4 colors	24-Well block Peltier device
Roche	LightCycler 480	Real-time PCR	96-Well plate	Xenon lamp; optical filters; full plate illumination	Optical filters; CCD camera	5 color excitation; 6 color emission	96-Well block Peltier device
Siemens	Versant kPCR	Real-time PCR	96-Well plate	Halogen lamp; optical filters; individual well illumination	Optical filters; PMT; individual well detection	5 colors	96-Well block Peltier device

to positivity) of the analyte against external calibrator(s) or internal quantitative standard(s). Similarly, relative quantification may be calculated by comparing the Δ cycle time (or Δ time to positivity) between the analyte and the endogenous control against calibrators/standards. For a qualitative assay, the positive or negative assay result may be determined by comparing the cycle number (or time to positivity) and/or signal intensity with respective cutoff values. For a genotyping assay, qualitative results from one or multiple analytes may be combined to determine the genotype profile of the sample.

Conclusions

Several main topics regarding fluorescence-based detection of real-time amplification and detection assays have been discussed in this chapter, including fluorescence principles, target detection/signal generation technologies, real-time instrument systems, and data analysis and result reporting. While existing real-time assays are playing an important role in clinical microbiology, new technology platforms and instrument systems for amplification and detection as well as sample management and preparation will continue to emerge. These new technologies hold great promises in further improving established clinical utilities as well as addressing emerging clinical needs or new microbiological agents. It is therefore imperative for the diagnostic community, including researchers, laboratories, clinicians, and device manufacturers, to make concerted and continued effort to develop, commercialize, and utilize more sophisticated and accurate diagnostic tools to fight against increasing burden of diseases and infections.

References

1. Ginocchio CC (2004) Life beyond PCR: alternative target amplification technologies for the diagnosis of infectious diseases, part I. Clin Microbiol Newslett 26:121–128
2. Ginocchio CC (2004) Life beyond PCR: alternative target amplification technologies for the diagnosis of infectious diseases, part II. Clin Microbiol Newslett 26:129–136
3. Deiman B, van Aarle P, Sillekens P (2002) Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol Biotechnol 20:163–179
4. Romano JW, van Gemen B, Kievits T (1996) NASBA: a novel, isothermal detection technology for qualitative and quantitative HIV-1 RNA measurements. Clin Lab Med 16:89–103
5. Fahy E, Kwoh DY, Gingeras TR (1991) Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. PCR Methods Appl 1:25–33
6. Mueller JD, Putz B, Hofler H (1997) Self-sustained sequence replication (3SR): an alternative to PCR. Histochem Cell Biol 108:431–437
7. Hill CS (2001) Molecular diagnostic testing for infectious diseases using TMA technology. Exp Rev Mol Diagn 1:445–455
8. Walker GT (1993) Empirical aspects of strand displacement amplification. PCR Methods Appl 3:1–6
9. Nycz CM, Dean CH, Haaland PD, Spargo CA, Walker GT (1998) Quantitative reverse transcription strand displacement amplification: quantitation of nucleic acids using an isothermal amplification technique. Anal Biochem 259:226–234

10. Notomi T, Okayama H, Masubuchi H et al (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:e63
11. Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K (2008) Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev Med Virol 18:407–421
12. Piepenburg O, Williams CH, Stemple DL, Armes NA (2006) DNA detection using recombination proteins. PLoS Biol 4:e204
13. Jeong YJ, Park K, Kim DE (2009) Isothermal DNA amplification in vitro: the helicase-dependent amplification system. Cell Mol Life Sci 66:3325–3336
14. Vincent M, Xu Y, Kong H (2004) Helicase-dependent isothermal DNA amplification. EMBO Rep 5:795–800
15. Kurn N, Chen P, Heath JD, Kopf-Sill A, Stephens KM, Wang S (2005) Novel isothermal, linear nucleic acid amplification systems for highly multiplexed applications. Clin Chem 51: 1973–1981
16. Mukai H, Uemori T, Takeda O et al (2007) Highly efficient isothermal DNA amplification system using three elements of 5′-DNA-RNA-3′ chimeric primers, RNaseH and strand-displacing DNA polymerase. J Biochem 142:273–281
17. Jung C, Chung JW, Kim UO, Kim MH, Park HG (2010) Isothermal target and signaling probe amplification method, based on a combination of an isothermal chain amplification technique and a fluorescence resonance energy transfer cycling probe technology. Anal Chem 82:5937–5943
18. Van Ness J, Van Ness LK, Galas DJ (2003) Isothermal reactions for the amplification of oligonucleotides. Proc Natl Acad Sci U S A 100:4504–4509
19. Morrison LE (2003) Fluorescence in nucleic acid hybridization assays. In: Lakowicz JR (ed) Topics in fluorescence spectroscopy: DNA technology. Springer, US, pp 69–103
20. Marras SA, Kramer FR, Tyagi S (2002) Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes. Nucleic Acids Res 30:e122
21. Espy MJ, Uhl JR, Sloan LM et al (2006) Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 19:165–256
22. Tyagi S, Landegren U, Tazi M, Lizardi PM, Kramer FR (1996) Extremely sensitive, background-free gene detection using binary probes and beta replicase. Proc Natl Acad Sci U S A 93:5395–5400
23. Baner J, Nilsson M, Mendel-Hartvig M, Landegren U (1998) Signal amplification of padlock probes by rolling circle replication. Nucleic Acids Res 26:5073–5078
24. Demidov VV (2002) Rolling-circle amplification in DNA diagnostics: the power of simplicity. Exp Rev Mol Diagn 2:542–548
25. Zhang DY, Brandwein M, Hsuih T, Li HB (2001) Ramification amplification: a novel isothermal DNA amplification method. Mol Diagn 6:141–150
26. Zhang DY, Brandwein M, Hsuih TC, Li H (1998) Amplification of target-specific, ligation-dependent circular probe. Gene 211:277–285
27. de Arruda M, Lyamichev VI, Eis PS et al (2002) Invader technology for DNA and RNA analysis: principles and applications. Exp Rev Mol Diagn 2:487–496
28. Clegg RM (1992) Fluorescence resonance energy transfer and nucleic acids. Methods Enzymol 211:353–388
29. Clegg RM (1995) Fluorescence resonance energy transfer. Curr Opin Biotechnol 6:103–110
30. Stryer L (1978) Fluorescence energy transfer as a spectroscopic ruler. Annu Rev Biochem 47:819–846
31. Nazarenko I, Pires R, Lowe B, Obaidy M, Rashtchian A (2002) Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. Nucleic Acids Res 30:2089–2195
32. Dubertret B, Calame M, Libchaber AJ (2001) Single-mismatch detection using gold-quenched fluorescent oligonucleotides. Nat Biotechnol 19:365–370
33. Dulkeith E, Morteani AC, Niedereichholz T et al (2002) Fluorescence quenching of dye molecules near gold nanoparticles: radiative and nonradiative effects. Phys Rev Lett 89:203002
34. Mao F, Leung WY, Xin X (2007) Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. BMC Biotechnol 7:76

35. Sutherland JW, Patterson DR, inventors; Johnson & Johnson Clinical Diagnostics, Inc., assignee (2004) Homogeneous method for assay of double-stranded nucleic acids using fluorescent dyes and kit useful therein. EP 0 684 316 B1
36. Gudnason H, Dufva M, Bang DD, Wolff A (2007) Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic Acids Res 35:e127
37. Horakova H, Polakovicova I, Shaik GM et al (2011) 1,2-Propanediol-trehalose mixture as a potent quantitative real-time PCR enhancer. BMC Biotechnol 11:41
38. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49:853–860
39. Bengtsson M, Karlsson HJ, Westman G, Kubista M (2003) A new minor groove binding asymmetric cyanine reporter dye for real-time PCR. Nucleic Acids Res 31:e45
40. Lind K, Stahlberg A, Zoric N, Kubista M (2006) Combining sequence-specific probes and DNA binding dyes in real-time PCR for specific nucleic acid quantification and melting curve analysis. Biotechniques 40:315–319
41. Takano EA, Mitchell G, Fox SB, Dobrovic A (2008) Rapid detection of carriers with BRCA1 and BRCA2 mutations using high resolution melting analysis. BMC Cancer 8:59
42. Monis PT, Giglio S, Saint CP (2005) Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal Biochem 340:24–34
43. Ishiguro T, Saitoh J, Yawata H, Yamagishi H, Iwasaki S, Mitoma Y (1995) Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Anal Biochem 229:207–213
44. Lee LG, Connell CR, Bloch W (1993) Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res 21:3761–3766
45. Kutyavin IV (2009) New approach to real-time nucleic acids detection: folding polymerase chain reaction amplicons into a secondary structure to improve cleavage of Forster resonance energy transfer probes in 5′-nuclease assays. Nucleic Acids Res 38:e29
46. Kutyavin IV (2011) Use of base modifications in primers and amplicons to improve nucleic acids detection in the real-time snake polymerase chain reaction. Assay Drug Dev Technol 9:58–68
47. Espy MJ, Uhl JR, Mitchell PS et al (2000) Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. J Clin Microbiol 38:795–799
48. Wittwer CT, Rasmussen RP, Lay MJ, inventors; University of Utah Research Foundation, assignee (2007) Monitoring amplification with FRET probes. US 7,160,998 B2
49. Mergny JL, Garestier T, Rougee M et al (1994) Fluorescence energy transfer between two triple helix-forming oligonucleotides bound to duplex DNA. Biochemistry 33:15321–15328
50. Rosmarin D, Pei Z, Blaser MJ, Tyagi S, inventors; C-shaped probe (2006) US 2006/0040275 A1
51. Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14:303–308
52. Bonnet G, Tyagi S, Libchaber A, Kramer FR (1999) Thermodynamic basis of the enhanced specificity of structured DNA probes. Proc Natl Acad Sci U S A 96:6171–6176
53. Satterfield BC, Kulesh DA, Norwood DA, Wasieloski LP Jr, Caplan MR, West JA (2007) Tentacle probes: differentiation of difficult single-nucleotide polymorphisms and deletions by presence or absence of a signal in real-time PCR. Clin Chem 53:2042–2050
54. Tyagi S, Marras SA, Kramer FR (2000) Wavelength-shifting molecular beacons. Nat Biotechnol 18:1191–1196
55. Chen AK, Davydenko O, Behlke MA, Tsourkas A (2010) Ratiometric bimolecular beacons for the sensitive detection of RNA in single living cells. Nucleic Acids Res 38:e148
56. Nutiu R, Li Y (2002) Tripartite molecular beacons. Nucleic Acids Res 30:e94
57. Becker MM, Schroth GP, inventors; Gen-Probe Incorporated, assignee (2003) Molecular Torches. US 6,534,274 B2
58. Lv C, Yu L, Wang J, Tang X (2010) A dumbbell molecular beacon for the specific recognition of nucleic acids. Bioorg Med Chem Lett 20:6547–6550

59. Kandimalla ER, Agrawal S (2000) 'Cyclicons' as hybridization-based fluorescent primer-probes: synthesis, properties and application in real-time PCR. Bioorg Med Chem 8:1911–1916
60. Huang S, Salituro J, Tang N et al (2007) Thermodynamically modulated partially double-stranded linear DNA probe design for homogeneous real-time PCR. Nucleic Acids Res 35:e101
61. Li Q, Luan G, Guo Q, Liang J (2002) A new class of homogeneous nucleic acid probes based on specific displacement hybridization. Nucleic Acids Res 30:e5
62. Yu G, Niu J, Shen M, Shao H, Chen L (2006) Detection of *Escherichia coli* O157 using equal-length double-stranded fluorescence probe in a real-time polymerase chain reaction assay. Clin Chim Acta 366:281–286
63. Keys DA, Farooqui F, Reddy P, inventors; Beckman Coulter, Inc., assignee (2003) Oligonucleotide probes for detecting nucleic acids through changes in fluorescence resonance energy transfer. US 6,593,091 B2
64. Afonina IA, Reed MW, Lusby E, Shishkina IG, Belousov YS (2002) Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. Biotechniques 32(940–944):6–9
65. Lukhtanov EA, Lokhov SG, Gorn VV, Podyminogin MA, Mahoney W (2007) Novel DNA probes with low background and high hybridization-triggered fluorescence. Nucleic Acids Res 35:e30
66. Svanvik N, Westman G, Wang D, Kubista M (2000) Light-up probes: thiazole orange-conjugated peptide nucleic acid for detection of target nucleic acid in homogeneous solution. Anal Biochem 281:26–35
67. Bethge L, Jarikote DV, Seitz O (2008) New cyanine dyes as base surrogates in PNA: forced intercalation probes (FIT-probes) for homogeneous SNP detection. Bioorg Med Chem 16: 114–125
68. Tonelli A, Tedeschi T, Germini A et al (2011) Real time RNA transcription monitoring by Thiazole Orange (TO)-conjugated Peptide Nucleic Acid (PNA) probes: norovirus detection. Mol Biosyst 7:1684–1692
69. Wirgart BZ, Andersson P, Grillner L (2005) Evaluation of the ReSSQ assay in relation to the COBAS AMPLICOR CMV MONITOR test and an in-house nested PCR method for detection of cytomegalovirus DNA. J Clin Microbiol 43:4057–4063
70. French DJ, Archard CL, Brown T, McDowell DG (2001) HyBeacon probes: a new tool for DNA sequence detection and allele discrimination. Mol Cell Probes 15:363–374
71. Yamane A (2000) Smart probe: a novel fluorescence quenching-based oligonucleotide probe carrying a fluorophore and an intercalator. Nucleic Acids Symp Ser 44:297–298
72. Knemeyer JP, Marme N, Sauer M (2000) Probes for detection of specific DNA sequences at the single-molecule level. Anal Chem 72:3717–3724
73. Crockett AO, Wittwer CT (2001) Fluorescein-labeled oligonucleotides for real-time PCR: using the inherent quenching of deoxyguanosine nucleotides. Anal Biochem 290:89–97
74. Kurata S, Kanagawa T, Yamada K et al (2001) Fluorescent quenching-based quantitative detection of specific DNA/RNA using a BODIPY((R)) FL-labeled probe or primer. Nucleic Acids Res 29:e34
75. Little MC, Andrews J, Moore R et al (1999) Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. Clin Chem 45:777–784
76. Harvey JJ, Lee SP, Chan EK et al (2004) Characterization and applications of CataCleave probe in real-time detection assays. Anal Biochem 333:246–255
77. Hosono N, Kubo M, Tsuchiya Y et al (2008) Multiplex PCR-based real-time invader assay (mPCR-RETINA): a novel SNP-based method for detecting allelic asymmetries within copy number variation regions. Hum Mutat 29:182–189
78. Tadokoro K, Akutsu Y, Tanaka K et al (2010) Comparative quantitative analysis of 14 types of human papillomavirus by real-time polymerase chain reaction monitoring Invader reaction (Q-Invader assay). Diagn Microbiol Infect Dis 66:58–64
79. Tadokoro K, Yamaguchi T, Egashira T, Hara T (2009) Quantitation of viral load by real-time PCR-monitoring Invader reaction. J Virol Methods 155:182–186

80. Nazarenko IA, Bhatnagar SK, Hohman RJ (1997) A closed tube format for amplification and detection of DNA based on energy transfer. Nucleic Acids Res 25:2516–2521
81. Nazarenko I, Lowe B, Darfler M, Ikonomi P, Schuster D, Rashtchian A (2002) Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Res 30:e37
82. Whitcombe D, Theaker J, Guy SP, Brown T, Little S (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol 17:804–807
83. Li J, Wang F, Mamon H et al (2006) Antiprimer quenching-based real-time PCR and its application to the analysis of clinical cancer samples. Clin Chem 52:624–633
84. Solinas A, Brown LJ, McKeen C et al (2001) Duplex Scorpion primers in SNP analysis and FRET applications. Nucleic Acids Res 29:e96
85. Santoro SW, Joyce GF (1997) A general purpose RNA-cleaving DNA enzyme. Proc Natl Acad Sci U S A 94:4262–4266
86. Todd AV, Fuery CJ, Impey HL, Applegate TL, Haughton MA (2000) DzyNA-PCR: use of DNAzymes to detect and quantify nucleic acid sequences in a real-time fluorescent format. Clin Chem 46:625–630
87. Singh KK, Hanne A, Krupp G (2004) Real-time monitoring of RNA and DNA reactions by fluorescence detection. Methods Mol Biol 252:33–48
88. Sherrill CB, Marshall DJ, Moser MJ et al (2004) Nucleic acid analysis using an expanded genetic alphabet to quench fluorescence. J Am Chem Soc 126:4550–4556
89. Lay MJ, Wittwer CT (1997) Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. Clin Chem 43:2262–2267
90. Rasmussen TB, Uttenthal A, de Stricker K, Belak S, Storgaard T (2003) Development of a novel quantitative real-time RT-PCR assay for the simultaneous detection of all serotypes of foot-and-mouth disease virus. Arch Virol 148:2005–2021
91. Ahmad AI, Ghasemi JB (2007) New FRET primers for quantitative real-time PCR. Anal Bioanal Chem 387:2737–2743
92. Takatsu K, Yokomaku T, Kurata S, Kanagawa T (2004) A FRET-based analysis of SNPs without fluorescent probes. Nucleic Acids Res 32:e156
93. Duan X, Yue W, Liu L et al (2009) Single-nucleotide polymorphism (SNP) genotyping using cationic conjugated polymers in homogeneous solution. Nat Protoc 4:984–991
94. Chen X, Zehnbauer B, Gnirke A, Kwok PY (1997) Fluorescence energy transfer detection as a homogeneous DNA diagnostic method. Proc Natl Acad Sci U S A 94:10756–10761
95. College of American Pathologists (2010) Automated molecular platforms. CAP Today 27–42 http://www.cap.org/apps/docs/cap_today/1110/1110_CAPTODAY_AutomatedMolecularPlatformsGuide.pdf

Chapter 25
Microbial Identification by PCR/Electrospray Ionization-Mass Spectrometry

David Metzgar, Rangarajan Sampath, Lawrence B. Blyn, Christian Massire, and David J. Ecker

Introduction

Pathogen detection and characterization have traditionally been accomplished through time-consuming, complex and expensive culture-based methods. These methods are reasonably inclusive within the limits of their design, but can only detect inherently cultureable agents that have not been rendered nonviable through preemptive antibiotic treatment, immune system challenge, or other processes. Although culture methods can be used retrospectively to provide an explanation for disease, these assays are often too slow to effectively inform treatment decisions or public health responses. For these reasons, many clinical laboratories have begun to rely on rapid molecular tests such as PCR and rapid antigen strip tests.

Most rapid molecular detection methods are designed to detect specific agents—that is, to answer the question "Is a certain pathogen in my sample?" [1] The technology discussed in this chapter, which couples PCR amplification to mass spectroscopy detection (PCR/ESI-MS (electrospray ionization-mass spectrometry)), is designed to answer a far more universal question—"What infectious organism(s) are in my sample?" [1] The PCR/ESI-MS technology offers the broad capture potential of culture methods enhanced by the detection of unculturable or nonviable organisms and returns high-resolution identifications with the speed offered by modern molecular genetic methods and process automation.

The motivation to design biological detection technologies based on PCR/ESI-MS derived from the recognition that bacterial and viral infections are caused by extremely diverse agents. More than 1,400 species of microorganism have been associated with human disease [2], and others are being identified at a rapid rate, in part thanks to broad-spectrum molecular methods such as 16S ribosomal sequencing [3]. It is clear

D. Metzgar (✉) • R. Sampath • L.B. Blyn • C. Massire • D.J. Ecker
Ibis Biosciences, Inc., an Abbott Company, 2251 Faraday Avenue, Suite 150, Carlsbad, CA 92008, USA
e-mail: DMetzgar@ibisbio.com

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that many infections and contaminations go undiagnosed if frontline testing methods are limited to specific targets. This can result in delays in appropriate treatment, misuse (and overuse) of antibiotics and antivirals, and other suboptimal but unavoidable medical responses. Furthermore, from a public health and biosecurity perspective, opportunities to detect and respond to cases involving emerging, rare, or variant agents are lost in the current system. The vast majority of samples that do not immediately yield a diagnosis using available specific molecular tests are discarded. Those that are not discarded are stored, shipped, and only much later tested broadly by public health agencies for agents that, while rare, actually represent the greatest health risk to the general public. These include emerging pandemic viruses and potential agents of biowarfare attacks. Recent efforts to model events resulting from the appearance of pandemic agents suggest that prophylaxis and quarantine efforts are only effective if the initial emergence of transmissible agents is detected, and the response initiated, within 1 or 2 days [4, 5]. Similarly, the literature suggests that delays in effective diagnosis of critical infections such as sepsis, which may be caused by an extremely diverse groups of pathogens, represent very significant risk factors for patients. Those risk factors increase on a scale of hours, not days [6].

All of these issues can (and will) eventually be addressed through the routine clinical and environmental use of technologies that can detect and identify bacteria and viruses in an inclusive manner, and do so in a timeframe consistent with the application of clinical treatment, biosecurity, and public health measures. Although the PCR/ESI-MS system described here has not yet been certified for diagnostic use, the technology was designed around these basic goals and has demonstrated significant potential. Detailed explanations of the principles of PCR/ESI-MS operation have been described elsewhere [7, 8]. In this chapter, we primarily discuss the realized and potential uses, benefits, and limitations of PCR/ESI-MS.

The PCR/ESI-MS system is built on the principle of universal detection and specific identification. The technology detects a wide variety of organisms with broad-spectrum PCR primers designed to amplify regions that vary in sequence (and therefore base pair) composition among the targeted organisms. Mass measurements achieved by ESI-MS analysis of the resulting amplicons provide data that is computationally deconvoluted to generate base pair composition signatures (Fig. 25.1) [9]. Identification is achieved by automated digital matching of species- (or strain-) specific base composition signatures to signatures contained in an integrated database. These comparison signatures are derived from either existing sequence databases or from previous empirical testing of well-characterized type strains using PCR/ESI-MS [9]. Each specific PCR/ESI-MS assay is designed to detect and identify all organisms within a particular interrelated phylogenetic group. Multiple primer pairs are used to amplify multiple genetic loci from most targets, increasing the resolution of identifications to the desired level [1] and precluding loss of sensitivity due to sequence variations in the primer target sequences [10]. Any group of related organisms that share conserved regions of sequence with sufficient homology to support broad-spectrum priming can be targeted by a universal PCR/ESI-MS detection and identification assay. This includes groups as large as the entire kingdom of bacteria, for which 16 primer pairs can provide enough organism-specific data to provide genus-specific, and often species-specific, identification [11], and certain families of

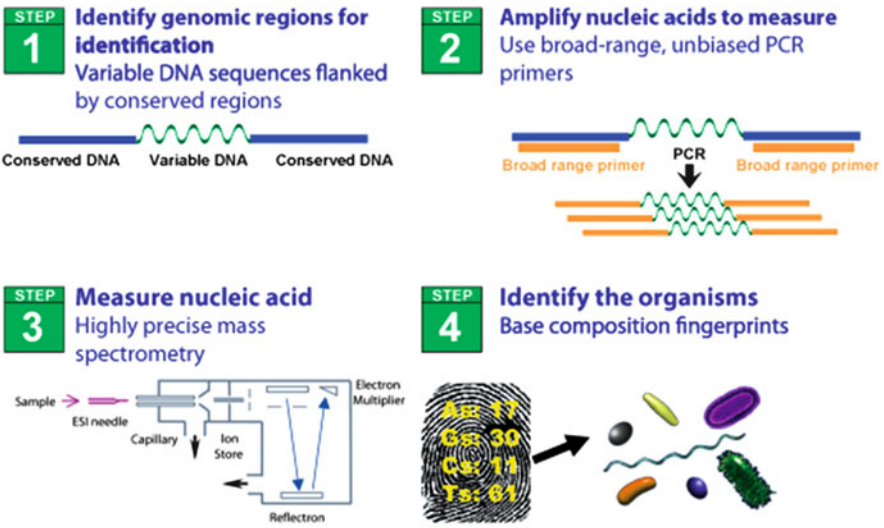

Fig. 25.1 Identification and genotyping of microbes by PCR/Electrospray Ionization-Mass Spectrometry (PCR/ESI-MS). (Reproduced with permission from Ecker, et al. [9])

viruses such as influenza, for which nine primer pairs are able to provide type, subtype, and lineage-specific identification for all recognized strains [12]. The following sections offer two examples of such tests and provide evidence for the functionality of the PCR/ESI-MS technology for both identification of common agents (as might be pursued in the future in a clinical setting) and detection and partial characterization of unexpected or emergent agents of significant medical and public health interest.

Universal Influenza Detection and Characterization

The PCR/ESI-MS universal influenza assay uses nine primer pairs to detect and discriminate among all previously characterized influenza A, B, and C subtypes. This test targets highly conserved regions of the influenza RNA genome and uses base composition signatures derived from these segments, in tandem with a database that includes all complete influenza genome sequences currently available, to identify detected viral strains. This assay can identify and characterize strains from both cultured isolates and uncultured patient specimens. The targets of the primers, as well as a diverse representation of the influenzas captured by these primers and their subtype-specific base composition signatures, are shown in Fig. 25.2. Notably, H and N subtype are inferred from the other segments targeted by the assay primers, as the hemagglutinin (HA) and neuraminidase (NA) segments are both significantly more variable than other segments and are hence not appropriate targets for universal amplification with conserved-site primers (this is discussed later in the section on limitations).

		Host	Serotype	All Influenza-PB1	Influenza A-NP	Influenza A-M1	Influenza A-PA	Influenza A-NS1	Influenza A-NS2
Influenza A virus	Mammalian species	Human	H1N1	36 30 26 36	33 23 20 25	21 32 22 30	36 27 27 22	37 32 22 28	33 28 18 26
			H1N1	36 30 26 36	31 25 20 25	22 31 25 27	36 26 28 22	37 32 22 28	35 26 18 26
			H1N1	36 30 26 36	33 23 20 25	22 31 23 29	36 26 29 21	38 31 22 28	35 26 18 26
			H1N1	33 32 27 36	34 22 21 24	24 27 26 28	36 26 27 23	36 33 22 28	33 28 18 26
			H1N1	33 32 27 36	34 22 21 24	24 27 27 27	36 26 27 23	36 33 22 28	33 28 18 26
			H1N1	33 32 27 36	34 22 21 24	24 27 27 27	36 26 27 23	35 34 22 28	32 29 18 26
			H3N2	39 32 24 33	32 24 20 25	24 30 23 28	36 24 28 24	38 32 21 28	35 27 17 26
			H3N2	39 32 23 34	32 24 20 25	24 30 23 28	36 24 28 24	38 32 21 28	35 27 17 26
			H3N2	39 32 23 34	32 23 20 26	24 30 23 28	36 24 28 24	38 32 21 28	35 27 17 26
			H3N2	40 31 24 33	31 25 21 24	25 29 22 29	36 24 27 25	39 31 21 28	36 26 17 26
			H3N2	40 31 24 33	32 24 21 24	25 29 22 29	36 24 27 25	39 31 21 28	36 26 17 26
			H3N2	39 32 24 33	31 25 21 24	25 29 22 29	36 24 27 25	39 31 21 28	36 26 17 26
			H3N2	40 31 24 33	32 24 21 24	25 29 23 28	36 24 27 25	39 31 21 28	36 26 17 26
		Swine	H1N1	39 32 26 31	33 24 20 24	25 27 25 28	34 28 28 22	35 35 21 28	33 29 17 26
			Unknown	39 32 25 32	35 22 21 23	22 28 26 29	35 27 27 23	35 36 21 27	33 30 17 25
		Equine	H7N7	39 32 23 34	38 20 21 22	25 27 21 32	35 27 27 23	40 29 21 29	35 26 17 27
	Avian Species	American Black Duck	H3N2	37 34 23 34	34 24 21 22	21 32 24 28	35 26 29 22	37 32 22 28	34 27 18 26
		American Green-winged Teal	H8N4	39 32 23 34	36 22 21 22	24 28 25 28	34 27 28 23	37 32 22 28	34 27 18 26
		Blue-winged Teal	H1N3	37 33 24 34	35 22 22 22	23 30 25 27	35 27 28 22	40 30 20 29	35 27 16 27
			H1N4	40 31 24 33	35 22 22 22	24 28 25 28	35 26 28 23	40 30 20 29	35 27 16 27
		CITE	H8N4	40 31 24 33	35 22 22 22	22 30 27 26	34 27 29 22	40 30 20 29	35 27 16 27
		Mallard	H10N7	40 31 24 33	35 22 21 23	23 30 26 26	34 27 29 22	37 32 22 28	34 27 18 26
			H4N6	39 32 23 34	35 22 23 21	22 31 27 25	34 27 28 23	37 32 22 28	34 27 18 26
			H4N8	40 31 22 35	34 24 21 22	25 28 25 27	34 27 28 23	37 32 22 28	34 27 18 26
			H5N2	39 32 23 34	36 21 22 22	23 31 26 25	35 26 27 24	37 32 22 28	34 27 18 26
			H5N3	39 32 24 33	34 24 21 22	21 32 25 27	34 27 28 23	37 32 22 28	34 27 18 26
			H6N5	39 32 22 35	36 21 22 22	21 32 25 27	34 27 28 23	40 30 20 29	35 27 16 27
			H7N7	39 32 23 34	36 22 22 21	20 32 27 26	34 27 29 22	37 32 22 28	34 27 18 26
		Ruddy Turnstone	H10N7	40 31 24 33	33 25 21 22	23 30 25 27	35 27 27 23	38 31 22 28	34 27 18 26
			H11N6	38 33 22 35	35 22 22 22	23 30 24 28	34 27 28 23	40 30 20 29	35 27 16 27
			H12N4	40 31 23 34	35 23 22 21	23 30 26 26	35 26 29 22	37 32 21 29	34 27 17 27
			H12N5	39 32 24 33	36 21 22 22	21 32 24 28	35 26 29 22	37 32 22 28	34 27 18 26
			H2N4	39 32 24 33	35 22 22 22	23 30 24 28	35 26 29 22	37 32 22 28	34 27 18 26
			H2N9	39 32 24 33	36 21 22 22	24 29 25 27	35 26 29 22	37 32 22 28	34 27 18 26
			H3N8	37 34 23 34	34 24 20 23	23 30 24 28	34 27 28 23	37 32 22 28	34 27 18 26
			H7N9	39 32 24 33	36 21 22 22	21 32 24 28	34 27 29 22	37 32 22 28	34 27 18 26
			H9N5	38 33 23 34	37 21 20 23	24 28 23 30	35 26 28 23	37 32 22 28	34 27 18 26
			H9N7	38 33 24 33	37 21 20 23	22 30 26 27	35 26 28 23	37 32 22 28	34 27 18 26
		Teal	H5N1	37 32 25 34	34 23 21 23	23 29 24 29	35 25 29 23	37 32 21 29	34 27 18 26
		Chicken	H5N1	37 32 25 34	34 23 21 23	23 29 24 29	35 25 29 23	38 31 21 29	35 27 16 27
			H5N1	37 32 25 34	34 23 21 23	23 29 24 29	35 25 29 23	37 32 21 29	34 28 16 27
		Human	H5N1	37 32 26 33	34 23 21 23	23 29 24 29	35 25 29 23	37 32 21 29	34 28 16 27
		Goose/Chicken/Duck/Human	H5N1	37 32 26 33	34 23 21 23	23 29 24 29	35 25 29 23	38 31 21 29	35 27 16 27
		Egret/Chicken/Duck/Human	H5N1	37 32 26 33	34 23 21 23	23 29 25 28	36 24 29 23	37 32 21 29	34 28 16 27
		Pintail	H5N3	38 32 26 32	34 24 23 20	23 30 25 27	34 27 29 22	40 30 20 29	35 27 16 27
		Goose	H7	38 32 26 32	34 24 22 21	23 30 25 27	34 27 28 23	39 31 21 28	35 27 17 26
		Shoveler	Unknown	37 33 24 34	36 23 19 23	24 29 22 30	33 27 30 22	43 30 20 26	34 28 16 27
			H10	39 31 26 32	34 24 22 21	23 30 25 27	34 27 29 22	37 32 22 28	34 27 18 26
			H10	38 32 26 32	35 23 22 21	24 30 26 25	35 26 29 22	37 32 22 28	34 27 18 26
			H9N2	37 32 26 33	34 24 22 21	23 30 25 27	33 28 29 22	37 32 22 28	34 27 18 26
		Teal	H10	38 32 26 32	34 24 22 21	24 29 25 27	34 27 29 22	37 32 22 28	34 27 18 26
			H11	37 33 26 32	34 24 23 20	24 29 25 27	34 27 29 22	37 32 22 28	34 27 18 26
			H5N2	38 32 26 32	34 24 23 20	24 29 25 27	34 27 29 22	40 30 20 29	35 27 16 27
			H9	39 31 26 32	33 25 21 22	24 30 25 26	34 27 29 22	37 32 21 29	34 27 17 27

Fig. 25.2 Detection and characterization of important human and avian influenza virus subtypes. Base composition signatures are shown in A, G, C, T order. Identical base compositions within a column are the same color. Base compositions represented only once are shown in *white*. (Reproduced with permission from Sampath, et al. [12])

The automated analysis program integrated into the PCR/ESI-MS platform is designed to compare signatures from multiple amplicons to base composition signatures contained in the associated database, such as those shown in Fig. 25.2. These signatures may be derived from existing sequence database entries, such as those in GenBank, or they may be generated directly using PCR/ESI-MS through the analysis of well-characterized type strains acquired from surveillance agencies [12]. Identical or close matches of multiple base composition signatures to any specific strain in the database will result in a reported detection of the appropriate subtype (H1N1, H3N2, etc.). The sensitivity, specificity, and accuracy of identification of this assay for circulating human influenzas, including influenza

AH1N1, influenza AH3N2, and influenza B, in uncultured human specimens, including throat swabs, nasal swabs, and nasopharyngeal swabs, have been demonstrated in multiple studies. PCR/ESI-MS identifications consistently agree with accepted methods including both culture and PCR [12–15]. These studies suggest that the PCR/ESI-MS influenza assay has the potential to provide rapid, high-throughput, frontline clinical diagnosis, and identification of common influenza viruses given appropriate validation and certification of the platform.

Manual analysis of base composition signatures from well-characterized influenza strains, aided by automated matching of individual amplicon signatures to specific database entries, allows discrimination of distinct lineages of circulating influenza (Fig. 25.3). This level of resolution provides an indication of the relatedness of any given strain to the strains currently offered in vaccine formulations, offers a way to track the evolution of influenza in real time, and, given sufficient external data linking specific strains to phenotypic characteristics, prediction of antiviral resistance [14].

The influenza assay is also able to detect and identify all influenza A types known to circulate in avian species, swine, horses, and other nonhuman mammals, including the well-characterized high pathogenicity strains of H5N1, H7N7, and others known to present significant threats to human and farm animal health [12]. Thus, the assay provides a tool for surveillance of these viruses in animal populations, as well as a rapid method of identifying these agents in the rare (but critical) instances in which they infect humans. Because the assay provides a similar level of strain resolution for all targeted subtypes, it can be used to discriminate lineages or strains of rare agents for research or epidemiological purposes.

All of the aforementioned capabilities could be matched by highly multiplexed PCR platforms. Massively multiplexed assays could provide a large number of simultaneous yes/no answers related to the presence or absence of specific well-characterized influenza variants. Any such system would be extremely complex insofar as it would require individual primer pairs with a unique identifier to amplify each targeted variant. The use of universal priming strategies in the PCR/ESI-MS assay offers a powerful advantage unavailable with type-specific molecular methods. Because sequences are amplified from the full spectrum of recognized influenza viruses using primers for which the target sites are highly conserved, the PCR/ESI-MS assay is also theoretically capable of detecting and, to a significant degree, characterizing, previously unrecognized or novel influenzas such as those that might cause pandemics in human populations.

Emergent (pandemic) influenza viruses are often reassortants, with segments originating from avian, swine, and/or human influenzas [16, 17]. These viruses are of great public health importance, as they are immunologically distinct from previously circulating viruses and may be transmitted quickly through universally susceptible human populations with significantly higher mortality rates than those observed with seasonal epidemic influenzas. Modeling efforts suggest that public health responses to events involving the emergence of highly contagious pandemic agents require recognition of initial cases within 48 h of their occurrence in order to effectively contain the pathogen through quarantine or targeted prophylaxis [4, 5]. The only imaginable way to achieve such rapid recognition, especially in cases where infection with the emergent agent initially presents with common symptoms,

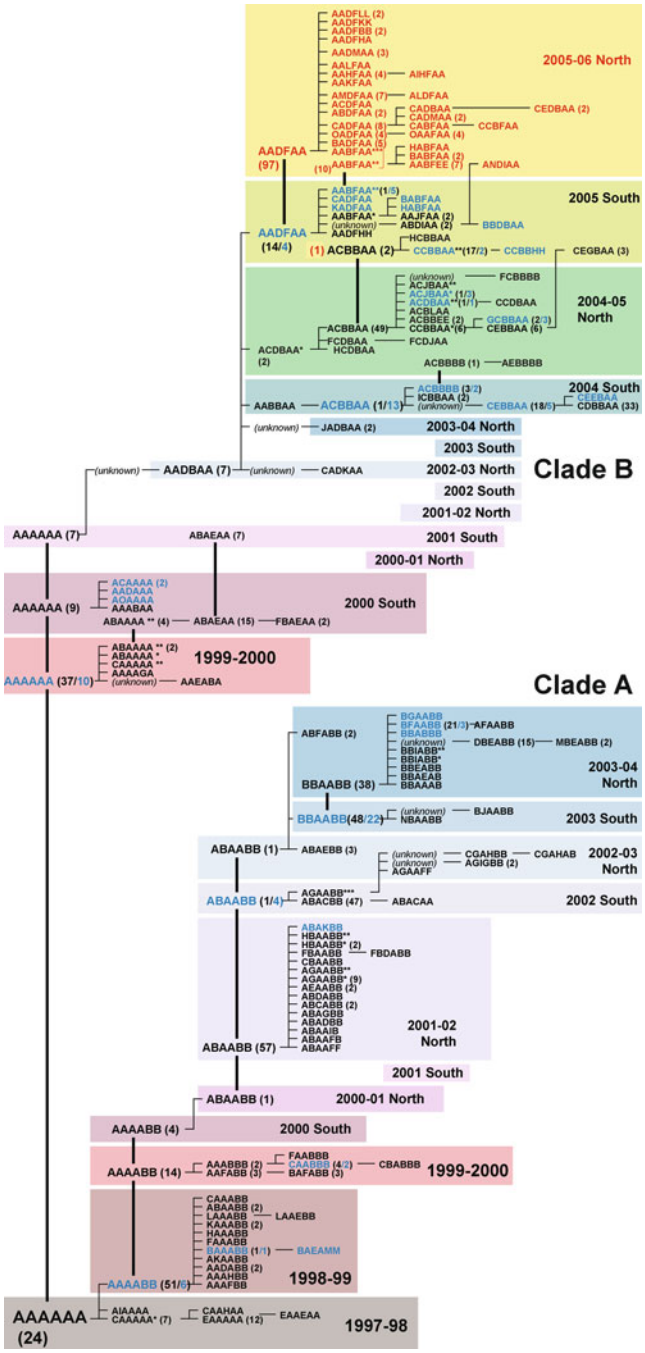

Fig. 25.3 (continued)

is to piggyback broad-spectrum surveillance capability on routine diagnostic procedures. This in turn can only be achieved with technologies that have universal detection and identification capability paired with sensitive, specific, high-throughput, and rapid capabilities to detect and identify common, treatable infections.

The theoretical ability of PCR/ESI-MS assays to detect and characterize novel influenzas was demonstrated during the emergence of the 2009 pandemic H1N1 (pH1N1) influenza. The first recognized case of this strain was detected using a PCR/ESI-MS influenza assay during screening of "untypeable" influenzas and PCR-negative specimens in the course of epidemiological research activities at the Naval Health Research Center in San Diego [13]. PCR/ESI-MS generated six amplicons from an uncultured patient specimen. As a novel reassortant, the signature of the pH1N1 strain was not in the database. However, the detection algorithm reported partial matches of multiple amplicons, in three combinations, to existing, previously characterized influenza strains (Table 25.1). Those strains included avian H3N2 and H7N1 viruses, and H1N2 swine viruses. Subsequent manual analysis of the spectral data revealed a single base composition signature derived from each primer pair, clearly indicating that the sample contained a single reassortant virus, rather than a coinfection of multiple avian and swine strains (Fig. 25.4). Coinfections result in multiple base composition signatures from individual primer pairs that amplify segments from multiple component viruses or from primer pairs designed to capture different influenza types (such as influenza A and influenza B) [12, 13]. The principle differences between signals obtained from reassortants and coinfections are illustrated in Fig. 25.4 and Table 25.2 using pH1N1 as an example of a novel reassortant and FluMist multivalent live-attenuated vaccine as an (artificial) example of a coinfection.

Following discovery of a previously uncharacterized agent such as pH1N1, the captured signatures from the novel strain need only be "named" in the PCR/ESI-MS database, and thereby associated with each other and with a specific characterized identity, to achieve accurate and unambiguous identification of further cases [13–15]. This principle was demonstrated through the analysis of pH1N1 strains following the initial emergence of the new reassortant, as shown in Fig. 25.5. With the existing broad primers, such a change does not require new reagent or assay development as would be required to produce a real-time PCR test specific to the newly emerging virus. Thus, the PCR/ESI-MS system can be rapidly adapted and deployed for use in

Fig. 25.3 Clade distribution of H3N2 influenza viruses. Unique base composition types are reported using a six-letter code (see text) and are chronologically sorted *bottom* to *top* (color boxes, seasons 1997 to 2006). From year 2000 onwards, seasons were labeled "*North*" and "*South*" to reflect the northern or southern hemispheric origin of the samples. *Thick vertical* bars represent the persistence of main types between consecutive seasons. Within each season, the number of isolates is reported between parentheses for types encountered more than once. *Thin horizontal* lines represent the spawning of new types through the accumulation of single mutations (*left* to *right*). *Black* font: types determined through sequence analysis; *blue* font: experimentally determined base composition types; *red* font: experimentally determined base composition types for season 2005–06. Ten rare sequence types (<1.5%) were not uniquely discernable by the base composition analysis of the eight amplicons used in this analysis, as more than one subtype produced the same BC-type. These BC-types are indicated by *asterisks*. (Reproduced with permission from Sampath, et al. [12])

Table 25.1 Best triangulated matches for all amplified products from the first characterized sample containing 2009 pandemic H1N1 influenza virus (novel flu) and FluMist trivalent influenza vaccine (Taken with permission from Metzgar et al. [13])[a]

Sample [apparent component strain][b]	Best matched strain (type/host/identifying site/unique sample No./year of isolation (subtype))	No. of amplified fragments matching/total No. of tested for this type	Contributing segment (No. of mismatches for that segment)[b]
Novel flu [1]	A/SWINE/ ILLINOIS/100085A/2001(H1N2)	3/6	PA [2] NS1 [0] NS2 [0]
Novel flu [1]	A/TURKEY/OHIO/313053/2004(H3N2)	3/6	PA [2] NS1 [0] NS2 [0]
Novel flu [1]	A/MALLARD/ALBERTA/34/2001(H7N1)	2/6	PB1 [0] M [4]
FluMist [1]	A/HUMAN/ANN ARBOR/6/1960 (H2N2)	6/6	PB1[0] NP [0] M [0] PA [0] NS1 [0] NS2 [0]
FluMist [2]	B/HUMAN/ANN ARBOR/1/1966 (flu B)	3/3	PB1 [0] PB2 [0] NP [0]

[a]Due to a lack of identically matched signatures in the database for the NP amplicon of Novel flu, and no triangulated set of signatures with which the NP signature clustered, the Novel flu NP amplicon was not initially matched to a target. Upon addition of the new signature to the database, this segment was identified as being closely related to recent H1N1 swine influenza isolates

[b]*M* matrix; *NP* nucleoprotein; *NS1* nonstructural protein 1; *NS2* nonstructural protein 2; *PA* polymerase; *PB1* polymerase basic protein 1; *PB2* polymerase basic protein 2. Includes only those segments that were recognized as having a common close match in the database of recognized signatures

an emerging situation. Continuing evolution of the virus, along with correlations between specific strains and clinically relevant phenotypes such as virulence and antiviral susceptibility, can be tracked immediately through signature-specific analysis of each amplicon, as discussed above for the case of common circulating influenzas.

Universal Bacterial Detection and Characterization

The broad bacterial test provides perhaps the best example of the potential power of PCR/ESI-MS [7, 9, 11]. This test is capable of detecting and identifying essentially all recognized genera of bacteria. The primers are designed such that multiple primer pairs amplify products from any given phylogenetic group of bacteria (Fig. 25.6). The resulting test is therefore closer to other universal capture/specific identification methods such as 16S ribosomal sequence analysis and multi-locus sequence typing than to tests such as real-time PCR, which typically detect a specific species of bacteria. The PCR/ESI-MS broad bacterial assay can identify virtually all common bacterial species without preliminary culture, detect and characterize bacteria which were not previously associated with specific diseases because of a lack of existing specific assays, detect emergent or previously

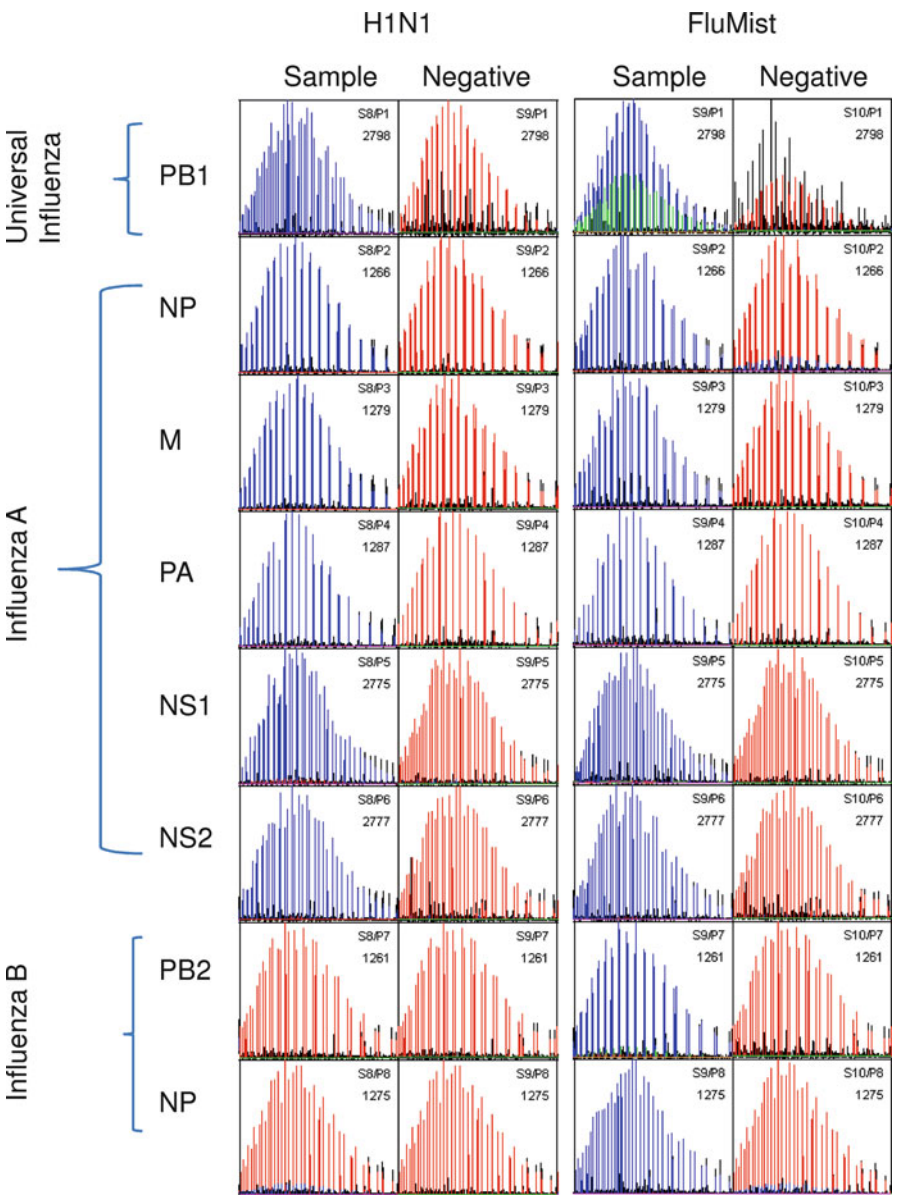

Fig. 25.4 Raw spectral data from the first characterized sample containing 2009 pandemic H1N1 influenza (H1N1) and FluMist trivalent vaccine. Individually colored peaks (series of *vertical* bars) represent multiple charge states of a single amplicon (allele). All peaks are normalized to their maxima. *Red* peaks represent internal positive controls (IPCs), *blue* peaks represent primary amplicons, and *green* peaks represent secondary detected amplicons of a different mass. *Red* peaks do not always show in tandem with positive amplifications, since the IPC signal may be obscuredby the higher titer of the target amplification. (Reproduced with permission from Metzgar, et al. [13])

Table 25.2 Primer pair detections (as base counts, A/G/C/T[b]) for 2009 pandemic H1N1 influenza virus and FluMist trivalent influenza vaccine, and base count proximity[c] to nearest match in database (Taken with permission from Metzgar et al. [13])[a]

Primer pair target (type/segment)	All Flu/PB1	Flu A/NP	Flu A/M	Flu A/PA	Flu A/NS1	Flu A/NS2	Flu B/PB2	Flu B/NP
H1N1 primary	39/32/24/33[b]	35/21/20/25	24/28/24/29	35/26/27/24	35/36/21/27	33/30/17/25	ND	ND
Proximity	[0000][c]	[+1-1-1+1]	[-2+2+2-2]	[-1+1+1-1]	[0000]	[0000]	NA	NA
FluMist primary	41/29/26/32	31/25/21/24	22/31/24/28	36/26/29/21	38/31/22/28	35/26/18/26	24/25/21/21	35/19/36/10
Proximity	[0000]	[0000]	[0000]	[0000]	[0000]	[0000]	[0000]	[0000]
FluMist secondary	37/31/21/33	ND	ND	ND	ND	ND	ND	ND
Proximity	[0000]	NA	NA	NA	NA	NA	NA	NA

[a]*ND* no detection; *NA* not applicable

[b]A/G/C/T = No. of adenines/No. of guanines/No. of cytosines/No. of thymines as derived from the mass of the amplicon. Actual base compositions may differ by up to one base from actual base compositions under some circumstances (specifically, when pairs of substitutions result in single Dalton differences in mass). This ambiguity is accommodated by the software used to match base compositions with signatures in the associated database. Primary and secondary refer to the relative apparent titer of multiple detections from the same primer pair

[c]Base count proximities represent the differences in base composition relative to the nearest match in the database of known strains for each of the four nucleotides

Influenza Virus	Sample	Closest Match Identified	PB1	NP	M1	PA	NS1	NS2
Seasonal Human Influenza A Virus	Previously detected human influenza samples (run at Ibis)	H3N2	A39 G32 C24 T33	A32 G24 C20 T25	A24 G30 C23 T28	A36 G24 C28 T24	A38 G32 C21 T28	A35 G27 C17 T26
			A39 G32 C23 T34	A32 G24 C20 T25	A24 G30 C23 T28	A36 G24 C28 T24	A38 G32 C21 T28	A35 G27 C17 T26
			A39 G32 C23 T34	A32 G23 C20 T26	A24 G30 C23 T28	A36 G24 C28 T24	A38 G32 C21 T28	A35 G27 C17 T26
			A40 G31 C24 T33	A31 G25 C21 T24	A25 G29 C22 T29	A36 G24 C27 T25	A39 G31 C21 T28	A36 G26 C17 T26
			A40 G31 C24 T33	A32 G24 C21 T24	A25 G29 C22 T29	A36 G24 C27 T25	A39 G31 C21 T28	A36 G26 C17 T26
			A39 G32 C24 T33	A31 G25 C21 T24	A25 G29 C22 T29	A36 G24 C27 T25	A39 G31 C21 T28	A36 G26 C17 T26
			A40 G31 C24 T33	A32 G24 C21 T24	A25 G29 C23 T28	A36 G24 C27 T25	A39 G31 C21 T28	A36 G26 C17 T26
		H1N1	A36 G30 C26 T36	A33 G23 C20 T25	A21 G32 C22 T30	A36 G27 C27 T22	A37 G32 C22 T28	A33 G28 C18 T26
			A36 G30 C26 T36	A31 G25 C20 T25	A22 G31 C25 T27	A36 G26 C28 T22	A37 G32 C22 T28	A35 G26 C18 T26
			A36 G30 C26 T36	A33 G23 C20 T25	A22 G31 C23 T29	A36 G26 C29 T21	A38 G31 C22 T28	A35 G26 C18 T26
			A33 G32 C27 T36	A34 G22 C21 T24	A24 G27 C26 T28	A36 G26 C27 T23	A36 G33 C22 T28	A33 G28 C18 T26
			A33 G32 C27 T36	A34 G22 C21 T24	A24 G27 C27 T27	A36 G26 C27 T23	A36 G33 C22 T28	A33 G28 C18 T26
			A33 G32 C27 T36	A34 G22 C21 T24	A24 G27 C27 T27	A36 G26 C27 T23	A35 G34 C22 T28	A32 G29 C18 T26
2009 pandemic Isolates	NHRC 1	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	NHRC 2	A/California/04/2009-PA SNP	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A35 G26 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 1	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 2	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 3	A/California/04/2009-PA SNP	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A35 G26 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 4	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 5	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 6	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 7	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 8	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 9	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
	CDC 10	A/California/04/2009	A39 G32 C24 T33	A35 G21 C20 T25	A24 G28 C24 T29	A36 G25 C27 T24	A36 G35 C20 T28	A34 G29 C16 T26
CDC Swine Control	CDC11	Classical Swine H1N1	A38 G32 C25 T33	A35 G22 C22 T22	A22 G30 C26 T27	A33 G28 C29 T22	A35 G36 C21 T27	A33 G30 C17 T25

Fig. 25.5 The genomic print of 2009 pandemic H1N1 (H1N1 pdm) viruses is highly conserved and distinct from other influenza viruses. Thirteen (13) seasonal H1N1 and H3N2 viruses from various seasons (*top* half); 12 samples from the H1N1pdm viruses (including the two viruses of the first two cases of the 2009 pandemic, referred to as NHRC1 and 2) (*bottom* half in *yellow* highlight); and a North American swine of H1N1 subtype were analyzed. All 2009 H1N1 pdm viruses have the same genomic print. BC signatures of five of the six targets in the H1N1pdm virus (NP, M1, PA, NS1, and NS2) were different from the other subtypes. The BC signature determined for the PB1 (A39, G32, C24, T33) was identical to some human H3N2 viruses, as expected. The H1N1pdm viruses were also distinguishable from the triple reassortant swine H1N1 at all six targets (compare *yellow* and *purple* signature). Differences in color patterns within the same subtype either reflects different genetic groups (H3N2 and H1N1), or a single nucleotide polymorphism (2009 H1N1 pdm). The numbers preceded by letters in each box correspond to the base counts (number A, G, C, and T) determined from the amplicons of the respective target genes. (Reproduced with permission from Deyde, et al [14])

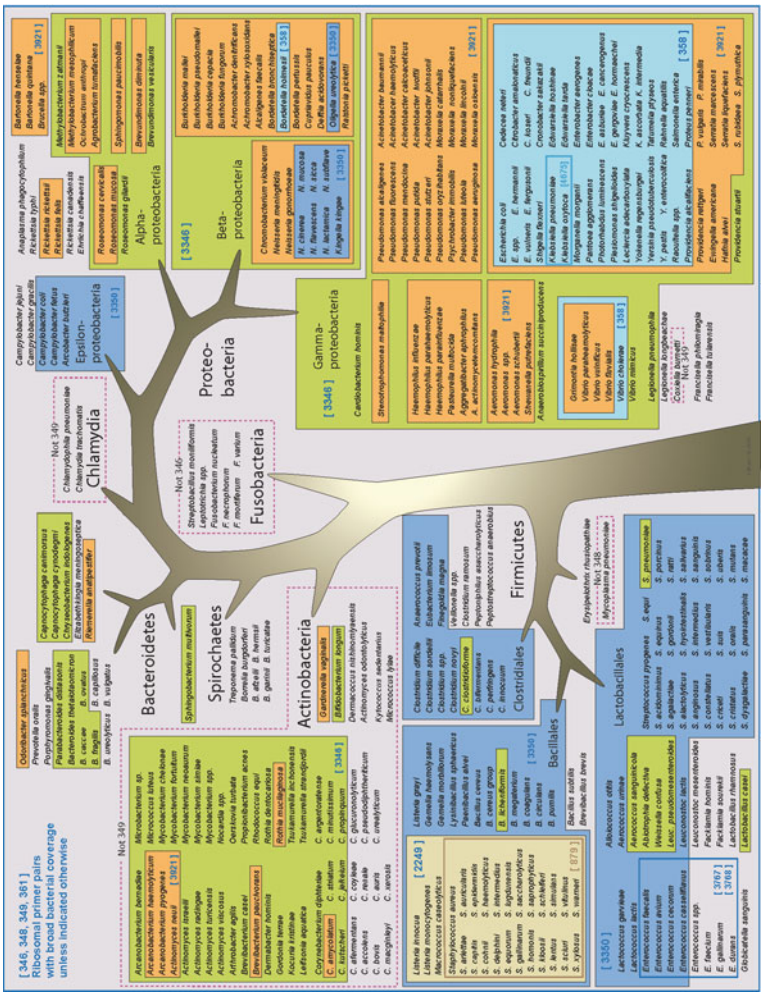

Fig. 25.6 Bacterial phylogenetic tree showing the primer coverage of the PCR/electrospray ionization-mass spectrometry assay. The primer coverage of the rDNA is represented by the *gray* background. Exceptions are indicated by *red* boxes. (Reproduced with permission from Ecker, et al. [9])

unrecognized species, and identify biowarfare agents that might initially present as an ordinary infection [18].

The current PCR/ESI-MS broad bacterial assay utilizes 16 primer pairs, including many of those whose range is depicted in Fig. 25.6, to identify bacteria to the species level in otherwise sterile samples such as whole blood, cerebrospinal fluid (CSF), or tissue specimens. *Candida* species (fungi commonly associated with bloodstream, CSF, and other sterile-site infections, particularly in immunocompromised hosts) are also targeted and amplified in the current assay, as are four of the most common bacterial antibiotic resistance elements. Two clade-specific primer pairs are also included to verify identification of critical infectious organisms including *Staphylococcus aureus*. Discussion here is limited to the broad-spectrum bacterial identification capabilities of the assay.

The bacterial assay's broad-spectrum primers target highly conserved loci such as those encoding ribosomal RNA and housekeeping genes (Fig. 25.6). These primers were chosen to insure priming of multiple loci in every phylogenetic clade of bacteria (Fig. 25.7). Automated results reporting for the broad bacterial assay is limited to those bacterial species for which direct demonstrations of sensitivity and accuracy of detection could be made. These are species for which sequence data was available to serve as a reference signature, or for which cultured specimens could be obtained for direct verification by PCR/ESI-MS. The broad bacterial assay has been validated experimentally with over 100 species of bacteria and is theoretically capable of identifying over 500. Other bacteria will also yield PCR amplicons and associated base count signatures; these species can be characterized by detailed manual analysis of individual primer pair amplicon data (or subsets of these data points) to yield near neighbor information as discussed previously in relation to the PCR/ESI-MS influenza assay.

The breadth of capture and identification afforded by the PCR/ESI-MS broad bacterial assay's design allows the identification of common causes of infection, such as *S. aureus*, *Klebsiella pneumonia*, and *Enterococcus faecium*. It can also detect and identify generally rare and unexpected agents capable of causing epidemics or locally high rates of endemic disease in specific regions, including *Yersinia pestis* (the agent of the plague) and *Bacillus anthracis* (anthrax—both an occasional environmental health hazard and a potential biowarfare agent), as demonstrated using a previous prototypical version of the assay [11].

High-Resolution Strain Analysis for Epidemiological Tracking

Bacteria generally evolve slowly, and truly threatening bacterial epidemics or outbreaks are likely to result from either gene transfer (resulting in the acquisition by one strain of new virulence elements or antibiotic resistance factors) or deliberate release of bacteria in biowarfare attacks. In such circumstances, the initial need is to identify the species causing illness, and the secondary need is to develop and deploy strain-specific markers to track emergent strains and define the sources and the epidemiology of outbreak. The PCR/ESI-MS broad bacterial assay, due the highly conserved nature of the loci targeted, can provide universal identification of bacteria at the genus, and often

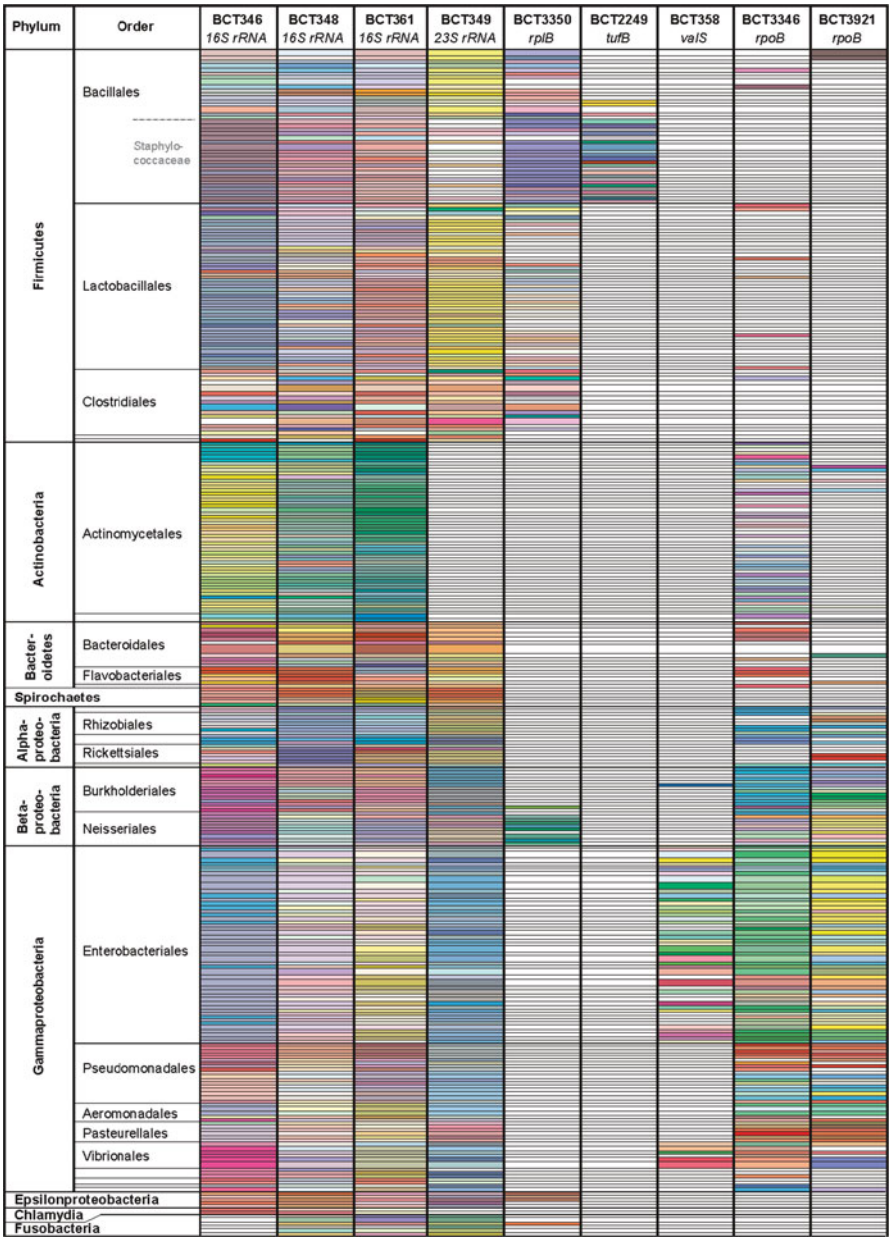

Fig. 25.7 Visualization of the diversity of base composition signatures for diverse bacteria. Colored cells represent individual base-composition signatures found for the 332 organisms tested (*rows*) in the nine loci (*columns*) that were used for broad bacterial identification. Row height occasionally varies as two or three distinct base counts may be found within the same locus for some organisms. Cells are left blank when no signature was found for the corresponding organism and locus. (Reproduced with permission from Ecker, et al. [9])

species, level, but it cannot provide strain-specific resolution for most bacteria. For these purposes, more focused PCR/ESI-MS assays have been developed for bacterial species of specific interest, including emerging nosocomial species such as *S. aureus* [19, 20] and *Acinetobacter baumanii* [7, 21–23, 27], agents of occasional but significant outbreaks such as *Streptococcus pyogenes* [24–26], and biowarfare agents such as *B. anthracis* [28, 29]. These assays use primer pairs designed to amplify loci that are conserved within species, often including "housekeeping" loci used in multilocus sequence typing (MLST) and strain-marker loci containing SNP variants and length-variable tandem repeats (VNTRs), methods which have been generally validated through sequencing or SNP-specific real-time PCR methods. Essentially, the PCR/ESI-MS platform offers a multilocus strain-typing methodology capable of returning high information content through a rapid, high-throughput, and automated process.

Research studies using a PCR/ESI-MS *A. baumanii* strain typing assay on samples collected from infected US troops returning from the conflict in Iraq, often via hospitals in Europe, demonstrated the capability of the PCR/ESI-MS genotyping assay through discrimination and identification of three species and 16 clonal lineages among 75 isolates (Table 25.3) [21]. The majority of isolates were members of well-recognized European clades associated with hospital transmission, suggesting that the hospitals at which the patients were initially treated in Europe were the proximal source of infection (Fig. 25.8) [21]. A follow-up study in similar populations of wounded service members conducted 3 years later, using the same PCR/ESI-MS method, identified many of the same clonal lineages, clearly discriminated those lineages from nosocomial strains circulating in nonmilitary US hospitals, and was able to track evolving changes in the *Acinetobacter* populations circulating in military hospitals (Fig. 25.8) [27]. Furthermore, direct analysis of antibiotic resistance phenotypes and genetic analysis of resistance determinants revealed a very close correlation between PCR/ESI-MS genotype and resistance phenotype in this sample set (Fig. 25.9) [21, 27]. This indicates that, given appropriate and contemporary association data based on wet-lab assays, the PCR/ESI-MS assay can effectively be used to predict variance in resistance phenotypes more quickly and efficiently than direct culture and resistance testing. These correlations were maintained over the period of 5 years of analysis.

A PCR/ESI-MS assay designed to detect and genotype group A streptococci (GAS, *S. pyogenes*) was used to conduct high-resolution epidemiology studies of GAS among the US military recruits, who suffer a high rate of GAS outbreaks and are the subject of active prophylactic antibiotic programs [24–26]. Analysis of 387 samples by PCR/ESI-MS, traditional sequencing methods targeting the primary antigen (emm typing), and culture-based antibiotic resistance testing demonstrated that PCR/ESI-MS could discriminate 27 GAS genotypes, and that these correlated very closely with emm typing methods. Both PCR-ESI/MS and emm typing methods offered very significant predictive power with respect to the most common and clinically relevant form of GAS antibiotic resistance (resistance to macrolides such as erythromycin) (Tables 25.4 and 25.5) [26]. Analysis of 415 additional samples allowed the construction of a very informative epidemiological comparison between multiple military sites and indicated that strain turnovers result from geographically widespread changes in strain dominance (Figs. 25.10 and 25.11) [25]. In turn, this

Table 25.3 Distribution of *Acinetobacter* sp. PCR/ESI-MS clone types isolated at the Walter Reed Army Medical Center (Taken with permission from Hujer et al. [21])[a]

European type	PCR ESI-MS type	No. of isolates	Hospital location(s)	Time course
I	15[b]	4	SICU, MICU, ICU step-down	May 2003–August 2004
I	16	1	ONP	July 2004
I	46	2	MICU, ONP	April 2003–May 2003
II	1	1	MICU	October 2003
II	10	9	SICU, ONP, surgery, ICU step-down, internal medicine, ER	August 2003–February 2005
II	11	15	SICU, MICU, ONP	May 2003–November 2004
II	12[b]	4	SICU, MICU, internal medicine	August 2004–December 2004
III	14[b]	16	SICU, MICU, ONP, surgery, ICU step-down	May 2003–December 2004
§	3	6	SICU, MICU, ONP	July 2003–February 2005
§	9	1	MICU	October 2004
§	24	11	SICU, MICU, ONP	June 2004–February 2005
§	39	1	MICU	September 2003
§	47	1	SICU	January 2004
§	48	1	SICU	April 2004
§	*Acinetobacter* genome sp. 3	1	ER	August 2003
§	*A. johnsonii*	1	ER	January 2004

[a]All isolates are *A. baumannii* except *Acinetobacter* genome sp. 3 and *A. johnsonii*. §, Isolates not related to European strains. *SICU* surgical intensive care unit; *MICU* medical intensive care unit; *ONP* orthopedics/neurology/physical medicine and rehabilitation; *ER* emergency room
[b]Exact matches to European reference strains

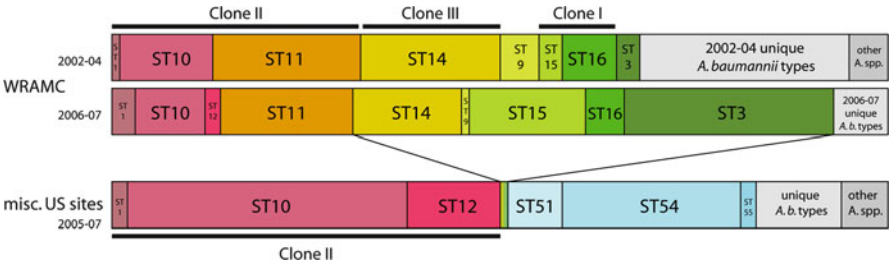

Fig. 25.8 Comparison of the typical distribution of *A. baumannii* types found in 4 American civilian hospitals in 2005–2007 with that of Walter Reed Army Medical Center (WRAMC) in 2002–2004 and 2006–2007. The width of each segment corresponds to the prevalence of the strain indicated. (Reproduced with permission from Wortmann, et al. [27])

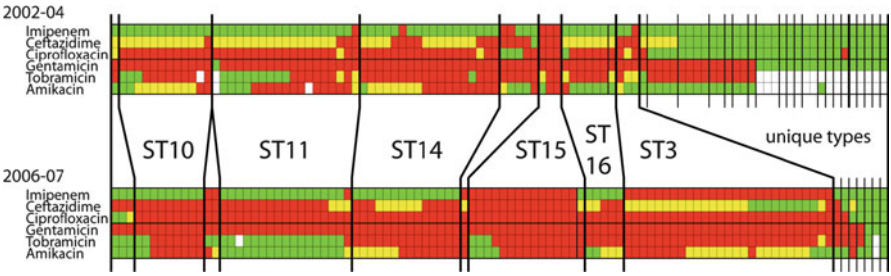

Fig. 25.9 Drug-resistance profiles for the *A. baumannii* isolates identified at Walter Reed Army Medical Center in 2002–2004 and in 2006 –2007. Each column corresponds to a single isolate, and cells are colored in *dark gray* (resistant), *medium gray* (intermediately resistant), or *light gray* (susceptible), in accordance with the level of resistance. *White* cells indicate a missing drug resistance characterization. Isolatecolumns are grouped by strain type (*heavy black* lines). (Reproduced with permission from Wortmann, et al. [27])

Table 25.4 Correlations between *emm* groups and *emm* types (Taken with permission from Metzgar et al. [26])

emm group		*emm* type (N)[a]	
1	1 (23)	**81 (1)**	
2	2 (10)		
3	3 (66)		
4	4 (16)		
6	6 (1)		
11	11 (13)		
78	78 (1)		
87	87 (4)		
94	94 (2)	**81 (1)**	
5/58	5 (58)	58 (6)	
12/77	12 (15)		
1/4/9/15/18/39/103/110/119	4 (1)	9 (2)	119 (1)
12/77/118	77 (10)	118 (16)	
17/22/47/84	22 (6)		
18/81/109	18 (13)		
22/25/28/75	75 (1)		
22/28	28 (16)		
22/61/81/104	**6 (1)**		
25/75	75 (33)		
29/53/91/101	29 (3)		
36/77/82/85/96/103	85 (1)		
52/93/101/104/116	101 (4)		
6/22/50/62/81/104	6 (22)		
68/75	68 (5)	75 (6)	
73/81/90	73 (6)		
75/78/89	89 (7)		
9/44/61/82	44 (15)	82 (1)	

[a]*emm* types (identified by *emm* gene sequence analysis) associated with each *emm* group (set of equally likely *emm* types, as identified by T-5000 PCR/ESI-MS analysis); contradictory identifications are shown in bold. All identities are shown as "identity (number identified)" for that specific associative category

Table 25.5 Isolate properties by PCR/ESI-MS emm group (Taken with permission from Metzgar et al. [26])[a]

PCR/ESI-MS *emm* group	*speA*	*speC*	ERY	CLI	TET	LEV	CHL	OFX
	N+(*N*)	*N*+(*N*)	*N*+(*N*)	*N*+(*N*)	*N*+(*N*)	*N*+(*N*)	*N*+(*N*)	*N*+(*N*)
1	14(15)	0(15)	0(38)[b]	0(38)	0(38)	0(38)	2(27)	2(27)
1/4/9/15/18/39/103/110/119	0(4)	3(4)	0(6)	0(6)	2(6)[b]	0(6)	0(2)	0(2)
11	0(9)	9(9)	2(16)	3(16)[c]	2(16)	0(16)	0(14)	0(14)
12/77	0(9)	6(9)	5(25)	3(24)[b]	0(25)	0(25)	1(12)	0(12)
12/77/118	0(24)	22(24)	0(64)[c]	0(64)	8(64)[c]	0(64)	0(23)	0(23)
17/22/47/84	0(3)	1(3)	1(10)	0(10)	0(10)	0(10)	0(10)	0(10)
18/81/109	1(10)	9(10)	0(56)[c]	0(56)	0(56)	1(56)	0(2)	0(2)
2	0(6)	5(6)	0(14)	0(14)	0(14)	0(14)	0(7)	0(7)
22/25/28/75	0(1)	1(1)	**1(1)**	0(1)	0(1)	0(1)	**1(1)**	0(1)
22/28	0(11)	10(11)	1(26)	0(26)	0(26)	0(26)	1(17)	1(17)
22/61/81/104	0(1)	1(1)	0(1)	0(1)	0(1)	0(1)	–	–
25/75	0(7)	6(7)	**66(73)**[c]	2(72)	0(73)	0(73)	2(70)[b]	1(70)[b]
29/53/91/101	0(3)	0(3)	0/(6)	0(6)	0(6)	0(6)	–	–
3	12(12)	5(12)	0(132)[c]	0(132)	0(132)[a]	3(132)	16(107)[b]	3(106)[b]
36/77/82/85/96/103	0(1)	0(1)	0(1)	0(1)	0(1)	0(1)	–	–
4	0(12)	9(12)	4(27)	1(27)	0(27)	0(27)	0(9)	0(9)
5/58	0(39)	34(39)	11(188)[b]	4(188)	8(188)	0(188)	13(81)[b]	0(81)[c]
52/93/101/104/116	0(1)	1(1)	0(4)	0/(4)	0(4)	0(4)	–	–
6	0(1)	1(1)	**1(1)**	0(1)	0(1)	0(1)	0(1)	**1(1)**
6/22/50/62/81/104	0(11)	8(11)	0(36)[b]	0(36)	0(36)	3(36)[c]	1(34)	**33(34)**[c]
68/75	0(11)	7(11)	0(13)	0(13)	6(13)[c]	0(13)	0(10)	1(10)

73/81/90	0(6)	4(6)	0(8)	1(8)	0(8)	0(8)	0(3)	0(3)
75/78/89	0(6)	6(6)	0(14)	0(14)	0(14)	0(14)	0(4)	0(4)
78	0(1)	1(1)	0/(2)	0(2)	0(2)	0(2)	0(2)	0(2)
87	0(4)	3(4)	0(5)	0(5)	0(5)	0(5)	0(2)	0(2)
9/44/61/82	0(8)	1(8)	1(28)	0(28)	1(28)	0(28)	6(21)[b]	0(21)
94	0(3)	0(3)	1(3)	0(3)	**2(3)**[b]	0(3)	**1(2)**	0(2)

[a]Observed rates of antibiotic resistance and scarlet fever exotoxin gene carriage for all identified PCR-ESI/MS emm groups. *N*+(*N*)=number of positives (number tested). Bold indicates 50 % or greater rates of resistance. Positives either carry a particular *spe* gene or show resistance to a particular antibiotic (inclusive of both intermediate and full resistance). *ERY* erythromycin; *CLI* clindamycin; *TET* tetracycline; *LEV* levofloxacin; *CHL* chloramphenicol; *OFX* ofloxacin. *speA* scarlet fever exotoxin gene A; *speC* scarlet fever exotoxin gene C. The following results are not shown in the table: 100 % of tested isolates (in all cases N.300) were susceptible to penicillin (PEN), vancomycin (VAN), cefepime (CPM), cefotaxime (CTX), ceftriaxone (CTR), linizolid (LNZ), meropenem (MEM), and gatifloxacin (GAT). Three hundred and eleven isolates were tested with quinupristindalfopristim (SYN) and one was resistant, six isolates were tested with trovafloxacin (TVA) and three were resistant

[b]Independently significant-specific association, positive or negative

[c]Significant after alpha (Bonferroni) adjustment for multiple measures (highly significant)

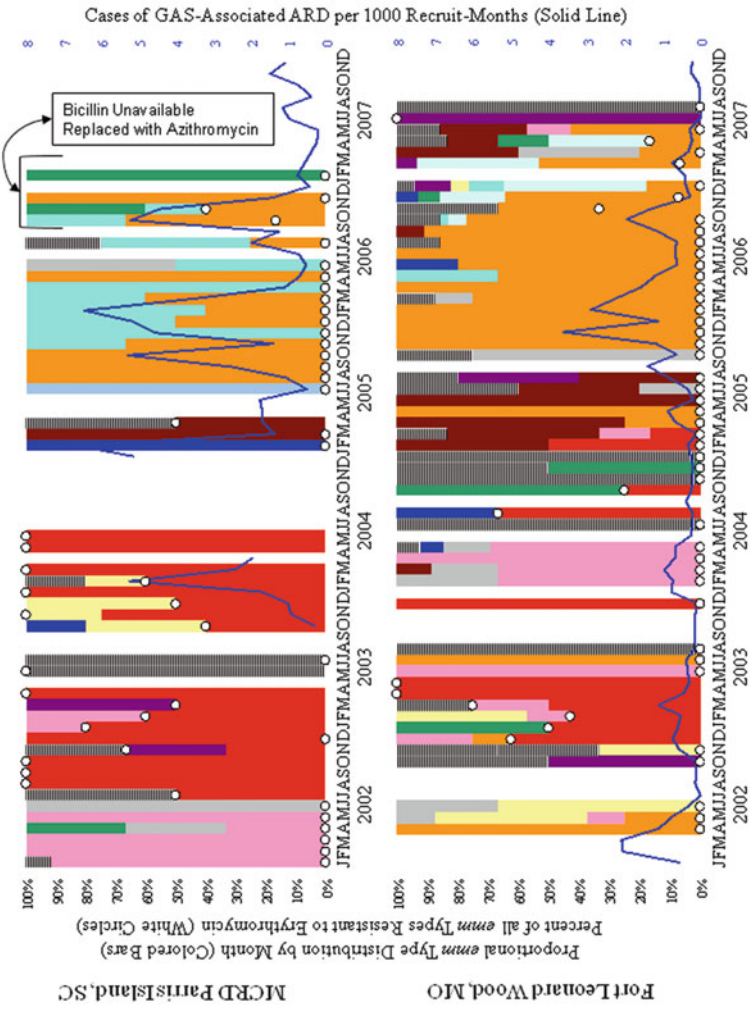

Fig. 25.10 *emm* type distribution, GAS disease rate and erythromycin resistance rate over 6 years at two sites. Temporally aligned *emm* type distributions for two sites that consistently contributed samples. Full names and locations of all sites are given in Acknowledgments. *emm* type color key appears in Fig. 25.11. (Reproduced with permission from Metzgar, et al. [25])

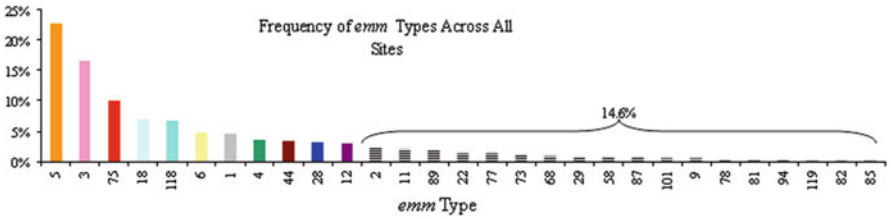

Fig. 25.11 Overall *emm* type distribution at ten sites and key to Fig. 25.10. (Reproduced with permission from Metzgar, et al. [25])

suggested that sudden changes in rates of antibiotic resistance observed at some sites were not, as previously thought, the result of specific use of erythromycin for prophylaxis, but rather the indirect result of temporary increases in dominance of a single strain which has long been associated with macrolide resistance [25]. These results show that properly constructed PCR/ESI-MS assays can be used to identify and characterize diverse members of any chosen group of related organisms. As with sequence comparison methodologies, the more narrowly targeted the assay, the greater the phylogenetic resolution of the resulting data.

Limitations of Universal PCR-Based Methods

Unlike single-target tests, in which individual primer pairs or probes are designed to recognize a component of an individual organism, the primers and PCR reactions used in universal assays such as PCR/ESI-MS or direct 16S ribosomal sequencing are subject to competition between closely related targets. This competition occurs at two stages: first, during the PCR reaction and associated amplicon characterization, in which multiple-related analytes will compete directly for amplification and signal acquisition; and second when information processing algorithms are used to transform raw signal into specific identifications. Unlike direct sequencing methods, signals from multiple amplicons generated by the same primers can be independently detected and identified by ESI-MS analysis, as shown in Fig. 25.4. However, if related organisms targeted by the same primer pairs are present in very different concentrations, competition will likely obviate detection of the less concentrated component. Furthermore, if many related organisms are present together in the same sample then the resulting collections of multiple mass identities for each amplified locus, combined with signature overlaps between components, may exceed the computational ability of the analysis platform to properly deconvolute the data into individual sets of signatures matched to specific targets. These issues limit the use of assays like the universal bacterial assay to sterile or semi-sterile samples (such as biopsy samples, blood, CSF, or sputum) or cultured isolates, where analysis is not compromised by the presence of complex background microbial

ecologies. Throat swabs, nasal swabs, and other nonsterile samples are less likely to afford clear, unambiguous detections of pathogens due to the presence of high titers of multiple carrier-state bacteria. For the latter purposes, more specific assays targeted towards specific pathogenic groups of bacteria are better suited.

Universal tests cannot be effectively targeted to hypervariable sequences and maintain the breadth of coverage that gives them their unique advantages. For these reasons, the highly variable markers that have long been used to discriminate organisms at high resolution using species-specific methods (like immunochemistry and antibody-mediated neutralization methods for serotyping) and sequencing methods targeting hypervariable loci (like emm-typing) are not readily targeted by universal methods. Existing PCR/ESI-MS methods, for example, do not directly target the HA and NA segments of all influenza H/N subtypes, because the HA and NA segments do not offer regions of sufficient conservation to support universal priming and amplification. The current assay includes HA-specific primers to capture drift and potential shift in the newly emerged pH1N1 and many other H1 strains, and some iterations of the assay have included NA primers capable of capturing multiple subtypes. For most influenza subtypes, the remaining (more highly conserved) six segments are analyzed, and associative database matching of signatures is used to infer HA and NA subtype. Similarly, the GAS genotyping assay targets several conserved housekeeping loci rather than directly targeting the primary antigenic determinant generally used to identify serotypes (the emm gene). Translation of PCR/ESI-MS data into commonly used identification schemes therefore requires associative data relating the sequence characteristics at targeted loci to the direct determinants of previously characterized and named strains, much as inference of phenotypic characteristics such as virulence must be determined by secondary (culture- or epidemiology-based) methods. The breadth and limits of the inferential method used to name detected organisms must be determined and considered in analysis of results. This is illustrated by the case of the first detection of the novel reassortant 2009 pandemic H1N1 influenza (Table 25.1), when various segments were identified as closely related to strains of varying H/N type, including avian H3N2 and H7N1 and swine H1N2 viruses.

Notes on Characterization of PCR/ESI-MS Tests

Like any assay, the sensitivity, specificity, and accuracy of PCR/ESI-MS tests must be characterized prior to use. PCR/ESI-MS primers are designed such that multiple primer pairs amplify products from a given group of organisms, and thus have an essentially infinite breadth of detection and identification within that group. The resulting assays are therefore closer to other universal capture/specific identification methods such as 16S ribosomal sequence analysis and multi-locus sequence typing than to tests such as real-time PCR. Due to the breadth of detection within the targeted group of organisms, targets representing every possible reportable signature set cannot possibly be tested in the process of characterization. We therefore

suggest that PCR/ESI-MS tests be characterized in a general fashion using a representative subset of the assay's possible targets to demonstrate the overall performance characteristics.

Characterization should begin with a demonstration of basic functionality and robustness and then proceed to full analytical testing of a small set of targets ("core organisms") which together exercise all of the primer pairs and biochemical reactions of an assay. This should be done using live target organisms spiked in the same matrix for which the test is intended to be used, and should otherwise follow the standard protocols for evaluation of molecular biological detection methods. Sensitivity should be determined for each of the core organisms, followed by traditional testing of parameters including reproducibility, potential interference by substances likely to be found in samples, cross-reactivity to organisms outside the phylogenetic range targeted by the assay, and any others deemed important in the context in which testing will be performed. These characterizations should be performed using the core organisms at concentrations near the limit of detection or at least in the lower range of concentrations expected in the context of the intended studies. These tests will fully characterize the biochemical properties of all test reagents and the performance of the device.

The second level of characterization involves demonstration of the degree of universality through the limit of detection testing using a larger set of diverse organisms representing the phylogenetic range of detection of the assay. This stage of analysis will demonstrate the variance in sensitivity of the assay to diverse targets and the accuracy of identification of those targets. Observed variance in these parameters can then be used to estimate the range and variance in sensitivity of detection and accuracy of identification with respect to all possible targets through statistical extrapolation. This characterization is used to test the level of "noise" in the system caused by variable matching quality between universally targeted primer pairs and the highly conserved (but still heterogeneous) target sequences presented by different phylogenetic target groups. These tests should be done in natural matrix, thus subjecting them to the same degree of interference from background nucleic acids (such as the DNA and RNA of animals from samples are intended to be collected) and other potential interfering substances found in the intended sample types.

Characterization of probable performance on untested targets should include in silico validation of the remaining target organisms for which the assay's database includes signatures. This involves computational analysis of the primer matches and melting temperatures for all targets for which sequences are available in public databases, and analysis of the masses of the expected amplicons to ensure that resulting signatures will uniquely identify the target organism. For those organisms for which the target sequences are unavailable, signatures can be obtained through direct physical analysis (using the assay) of representative characterized samples of the organisms obtained from existing strain collections. These tiered characterizations address the breadth and specificity of an assay's universal primers and demonstrate the robustness of the DNA mass determination (detection) and computational (identification) properties of the system.

Summary

This chapter describes a high-throughput technology for detection and characterization of bacteria and viruses using PCR amplification and mass spectrometry. Broadly targeted PCR reactions are used to generate amplicons from diverse groups of phylogenetically related organisms. PCR primers are chosen to amplify internally variable regions of sequence, as is done with 16S ribosomal sequencing and MLST. Amplicon masses are analyzed using ESI-MS, and the results are translated into amplicon base composition signatures. These signatures are matched with known signatures from existing sequencing data, providing specific identification of detected organisms. The combined process is called PCR/ESI-MS. The process is automated, allowing for rapid characterization of diverse microbes from more than 200 cultured or uncultured specimens in less than one day. The broad-spectrum capture and information-rich output of the technology render it capable of detecting and characterizing any organism within the designed phylogenetic breadth of a specific assay, including both previously recognized common agents and unique, rare, or emerging variants.

References

1. Ecker DJ, Drader JJ, Gutierrez J et al (2006) The Ibis T5000 Universal Biosensor: an automated platform for pathogen identification and strain typing. J Lab Autom 11:341–351
2. Taylor LH, Latham SM, Woolhouse ME (2001) Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci 356:983–989
3. Woo PC, Lau SK, Teng JL, Tse H, Yuen KY (2008) Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin Microbiol Infect 14:908–934
4. Ferguson NM, Cummings DA, Cauchemez S et al (2005) Strategies for containing an emerging influenza pandemic in Southeast Asia. Nature 437:209–214
5. Longini IM Jr, Nizam A, Xu S et al (2005) Containing pandemic influenza at the source. Science 309:1083–1087
6. Kumar A, Roberts D, Wood KE et al (2006) Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med 34:1589–1596
7. Ecker DJ, Massire C, Blyn LB et al (2009) Molecular genotyping of microbes by multilocus PCR and mass spectrometry: a new tool for hospital infection control and public health surveillance. Methods Mol Biol 551:71–87
8. Hofstadler SA, Sampath R, Blyn LB et al (2005) TIGER: the universal biosensor. Int J Mass Spectrom 242:23–41
9. Ecker DJ, Sampath R, Li H et al (2010) New technology for rapid molecular diagnosis of bloodstream infections. Expert Rev Mol Diagn 10:399–415
10. Metzgar D (2011) Adaptive evolution of diagnostic resistance. J Clin Microbiol 49(7):2774–2775
11. Baldwin CD, Howe GB, Sampath R et al (2009) Usefulness of multilocus polymerase chain reaction followed by electrospray ionization mass spectrometry to identify a diverse panel of bacterial isolates. Diagn Microbiol Infect Dis 63:403–408

12. Sampath R, Russell KL, Massire C et al (2007) Global surveillance of emerging Influenza virus genotypes by mass spectrometry. PLoS One 2:e489
13. Metzgar D, Baynes D, Myers CA et al (2010) Initial identification and characterization of an emerging zoonotic influenza virus prior to pandemic spread. J Clin Microbiol 48:4228–4234
14. Deyde VM, Sampath R, Garten RJ et al (2010) Genomic signature-based identification of influenza A viruses using RT-PCR/electro-spray ionization mass spectrometry (ESI-MS) technology. PLoS One 5:e13293
15. Sampath R, Hofstadler SA, Blyn LB et al (2005) Rapid identification of emerging pathogens: coronavirus. Emerg Infect Dis 11:373–379
16. Smith GJ, Vijaykrishna D, Bahl J et al (2009) Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122–1125
17. Garten RJ, Davis CT, Russell CA et al (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325:197–201
18. Ecker DJ, Sampath R, Massire C et al (2008) Ibis T5000: a universal biosensor approach for microbiology. Nat Rev Microbiol 6:553–558
19. Hall TA, Sampath R, Blyn LB et al (2009) Rapid molecular genotyping and clonal complex assignment of *Staphylococcus aureus* isolates by PCR coupled to electrospray ionization-mass spectrometry. J Clin Microbiol 47:1733–1741
20. Wolk DM, Blyn LB, Hall TA et al (2009) Pathogen profiling: rapid molecular characterization of *Staphylococcus aureus* by PCR/electrospray ionization-mass spectrometry and correlation with phenotype. J Clin Microbiol 47(10):3129–3137
21. Hujer KM, Hujer AM, Hulten EA et al (2006) Analysis of antibiotic resistance genes in multi-drug-resistant *Acinetobacter* sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. Antimicrob Agents Chemother 50:4114–4123
22. Whitman TJ, Qasba SS, Timpone JG et al (2008) Occupational transmission of *Acinetobacter baumannii* from a United States serviceman wounded in Iraq to a health care worker. Clin Infect Dis 47:439–443
23. Ecker JA, Massire C, Hall TA et al (2006) Identification of Acinetobacter species and genotyping of *Acinetobacter baumannii* by multilocus PCR and mass spectrometry. J Clin Microbiol 44:2921–2932
24. Ecker DJ, Sampath R, Blyn LB et al (2005) Rapid identification and strain-typing of respiratory pathogens for epidemic surveillance. Proc Natl Acad Sci U S A 102:8012–8017
25. Metzgar D, McDonough EM, Hansen CJ et al (2010) Local changes in rates of group A *Streptococcus* disease and antibiotic resistance are associated with geographically widespread strain turnover events. Virulence 1:247–253
26. Metzgar D, Baynes D, Hansen CJ et al (2009) Inference of antibiotic resistance and virulence among diverse group A *Streptococcus* strains using emm sequencing and multilocus genotyping methods. PLoS One 4:e6897
27. Wortmann G, Weintrob A, Barber M et al (2008) Genotypic evolution of *Acinetobacter baumannii* strains in an outbreak associated with war trauma. Infect Control Hosp Epidemiol 29:553–555
28. Van Ert MN, Hofstadler SA, Jiang Y, et al (2004) Mass spectrometry provides accurate characterization of two genetic marker types in *Bacillus anthracis*. Biotechniques 37:642–644, 646, 648
29. Okinaka RT, Henrie M, Hill KK et al (2008) Single nucleotide polymorphism typing of *Bacillus anthracis* from Sverdlovsk tissue. Emerg Infect Dis 14:653–656

Chapter 26
Amplification Product Inactivation

Susan Sefers, Charles W. Stratton, and Yi-Wei Tang

Introduction

"With great power comes great responsibility." This popular Spider Man movie quote infers the relationship between molecular nucleic acid amplification methods and amplification product inactivation procedures. The "power" is the ability of molecular amplification methods to produce billions of copies of nucleic acid from one template strand. This technique has been modified and adapted to serve as a means to detect viral and bacterial nucleic acids in clinical settings. The use of molecular methods is constantly evolving and expanding in its utility in this arena. With these methods, it is possible to detect small amounts of herpes simplex virus (HSV) in an infant's cerebrospinal fluid (CSF) with suspected meningitis [1]. With the detection of HSV in the CSF, antiviral therapy can be continued with confidence that the therapy is preventing brain damage that can be caused by HSV [2]. Before the advent of nucleic acid amplification techniques, there was little to no chance of recovering the virus from this sample type with traditional viral laboratory methods [3]. Empiric antiviral therapy was either continued or stopped without objective data upon which to base the decision.

The "responsibility" that comes with this "power" is making sure the amplification method is free of contaminating nucleic acids from previous amplification reactions. If one copy of the carryover nucleic acid contaminates the HSV reaction described above, this could cause a false positive test result. Antiviral therapy would be continued unnecessarily, and the infant thus would remain as an inpatient in a hospital for several additional days or longer. The therapy regimen can include IV

S. Sefers (✉) • C.W. Stratton
Departments of Pathology and Medicine, Vanderbilt University Medical Center, Nashville, TN 37212, USA
e-mail: Susan.sefers@vanderbilt.edu

Y.-W. Tang
Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 37212, USA

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Acyclovir for up to 3–4 weeks in some cases. This not only causes stress and worry for the infant's parents, but also wastes thousands of dollars on inpatient and home nursing care. In this chapter, most common causes of false positive amplification methods are discussed as well as methods laboratorians can take to prevent these from occurring in their molecular microbiology laboratories [4–6].

Causes of False Positive Amplification Reactions

The most common cause of false positive amplification reactions is carryover of previously amplified product (amplicon) to a new amplification reaction. The amplification reaction creates billions of copies of nucleic acids. Whenever the microcentrifuge tubes that contain these amplicons are opened post-amplification, there is a chance that these amplicons can be spread to future reactions. Even small aerosols that are produced when opening the tubes can be enough to eventually cause contamination problems in the laboratory [7].

Another common source of false positive amplification reactions is carryover of nucleic acid from a high titer sample to another sample in close proximity. This could occur, for example, if a genital sample that contained high titers of HSV was processed next to the infant's CSF sample described in the introduction example. The contamination happens when a small drop of the genital sample finds its way on a technologist's glove. The technologist then opens the CSF sample next and the small drop is transferred to this sample causing a false positive result. HSV positive genital or oral samples generally have very high titers of virus as compared to other sample types [8]. Unfortunately, the contaminating nucleic acid can also be transferred on a pipette tip, pipette, pen, vortex, or any other equipment that is used when processing and extracting samples. In most cases, the drop is not visible to the naked eye which makes this even more difficult to prevent. Besides a high titer patient sample, a high titer positive control or real-time PCR standard that utilizes plasmid DNA can also serve as a potential carryover sample [9]. When making positive controls for assays, it is important to make them lower titers but not so low that they flirt with the assay's limit of detection. If the positive control is of low titer but still consistently detectable, it can also serve a dual purpose to indicate reagent degradation (if sudden negative reactions occur).

Other possible sources of contamination leading to false positive amplification test results have also been described [4–6]. Many times it is difficult to determine the source of the contamination [10]. There has been contamination of a collection device that is repeatedly used from sample to sample or patient to patient and has not been cleansed adequately. The repeated use of an instrument to punch dried blood spots for HIV PCR showed that thorough cleaning between uses reduced the possibility of carryover contamination between samples [7]. Commercially sold reagents have been contaminated with the target for a particular amplification test. *Coxiella burnetti* was found to contaminate a commercially prepared PCR master mix [11]. 16S rRNA PCR was complicated by bacterial DNA that was found to

contaminate amplification reagents [12, 13]. Commercially manufactured extraction kits were contaminated with *Legionella* DNA [14]. There was an incident where RT-PCR enzymes used in a molecular HIV genotyping assay were contaminated with an HIV-based vector manufactured by the same vendor [15].

Principle Methods for Preventing Carryover Contamination

There are two principles that a laboratory can take to help prevent contamination events. The first principle is to physically separate post-amplification areas from the rest of the laboratory. Proper lab design is one of the most important methods for preventing false positive amplification reactions. The post-amplification area should be in a designated room that is separate from the sample processing area. The room should be separated by a door and most ideally down a short hallway from the sample preparation area. The sample preparation (pre-amplification) area should also be in a separate room (with a door). To keep reagents from being contaminated from both high titer samples and amplicons, the reagent preparation area should also be in a dedicated room (with a door). Each room should have separate set of refrigerators, freezers, centrifuges, computer workstations, lab coats, pens, pipettes, etc. These items must never migrate from room to room as they can carry nucleic acids.

The laboratory technologists must follow a "one-way traffic" flow throughout the work day. The sample processing and extraction should be preformed in the pre-amplification area, then the technologist should move to the post-amplification area to detect the amplicons. The technologist should not return to the pre-amplification area after working in the post-amplification area in the same shift. Figure 26.1 shows the ideal configuration for a molecular laboratory.

When performing a molecular test method, cleaning the work area is of utmost importance. Ten percent bleach is the most common cleaning solution used in molecular labs [16]. The bleach oxidizes any nucleic acid present and subsequently renders it unsuitable as a template for future amplification reactions. The technologist should wipe down the work area including pipettes, pens, and any equipment used with 10 % bleach before and after manipulating samples. Bleach can be corrosive to equipment over time so rinsing with 70 % ethanol can remove bleach to minimize the damage. Another cleaning agent that is used in molecular labs is DNA Away (Molecular BioProducts, San Diego, CA). This is a strong alkaline agent that degrades nucleic acids. DNA Away can be used to wipe down work areas. It is important to rinse with water after using DNA Away because residues can be inhibitory and cause false-negative reactions if they are not completely removed. Other areas of caution when cleaning is to wipe work areas in the extraction box with water prior to wiping with 10 % bleach as bleach can combine with guanidinium isothiocyanate (reagent commonly used in extraction) to create toxic gas. The use of DNA Away in a spray bottle is discouraged. If sprayed, the droplets can remain in an aerosolized form to contaminate future amplification reactions and cause a false negative result.

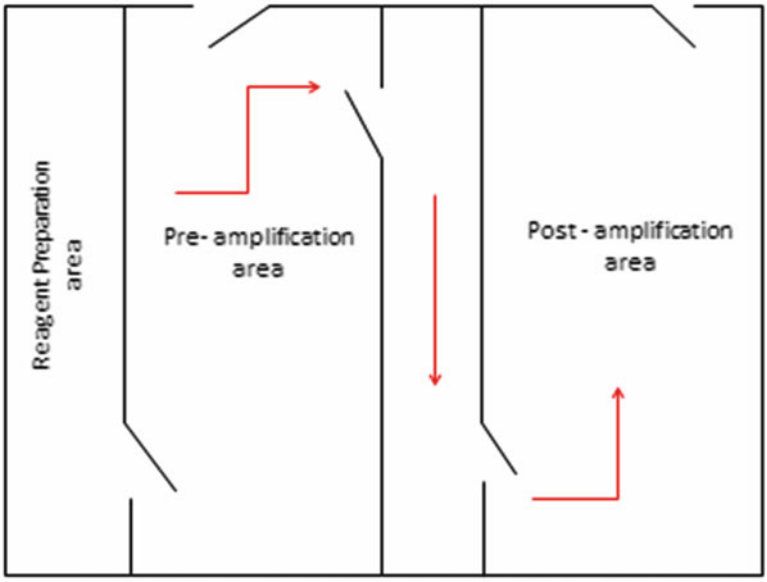

Fig. 26.1 Ideal design of a molecular laboratory. The pre-amplification area is a separated room from the post-amplification area. The reagent preparation area is separated from the remainder of the lab. There is a unidirectional workflow moving from pre-amplification to post-amplification with no opposite flow

Some labs employ the use of "dead air boxes" within the separate rooms which further separate the work areas thus providing even more protection (Fig. 26.2). The "dead air box" provides just what it is describing, an area of "dead air." Any aerosols that are created are contained inside the unit. Most facilities have heating or cooling systems with vents that can blow nucleic acids around the room and increase the chances of carryover. The "dead air box" creates an enclosed area that prevents these occurrences. Furthermore, they come equipped with an ultraviolet (UV) light that can be used to destroy any nucleic acids remaining after cleaning the work area. The use of UV light as a method for controlling contamination is discussed in the next section.

Molecular laboratories should use special pipette tips called "aerosol resistant tips" (ART). These pipette tips include a type of filter just below the point where the tip fits on the pipette. The filter blocks aerosols, nucleic acids, etc., from collecting on the end of the pipette thus greatly reducing the carryover from one sample to the next.

There are a variety of automated extraction instruments available that may assist the laboratory by providing automation as well as decreasing the chance for carryover contamination. These instruments are computer programmed and pipette very

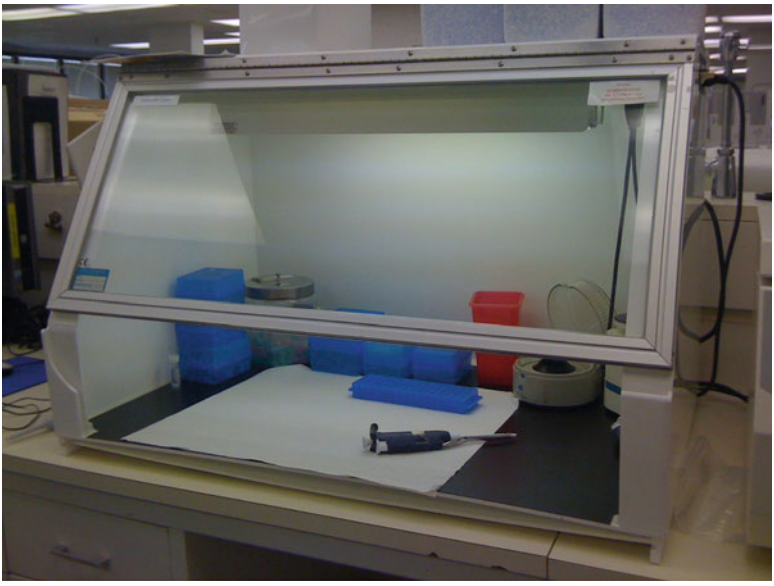

Fig. 26.2 Molecular laboratory "Dead Air Box." The dead air box creates an atmosphere that will lower chances of contamination in molecular samples. Aerosols are blocked from entering the unit from the surrounding environment. Before and after usage, the unit can be wiped down and cleaned. Then, the unit can be closed and the UV light can be turned on to further decontaminate the area

deliberately and precisely. The tubes and pipette tips are designed to be manipulated by a machine and are shaped to minimize contamination. Tubes have screw caps and are slowly opened to create fewer aerosols. The instruments are enclosed to cut down on aerosols entering or escaping the unit. They are usually equipped with a UV light that can be turned on at the end of the workday.

Recently, several "closed-tube" systems, especially those used in real-time PCR formats have diluted the essential need of using the facilities described above. Real-time amplification tests detect amplicons while amplification is performed; therefore, tubes are not required to be opened post-amplification. Methods that use gels (agarose gels or gels for southern or western blotting) or EIA methods for detection necessitate opening tubes post-amplification. Any time a tube is opened post-amplification it increases the risk of contamination [17].

Commonly Used Methods of Amplification Product Inactivation

Another principle for controlling carryover contamination is to implement chemical modifications. The following are common methods being used as routine laboratory practices.

Ultraviolet Light

UV light has been used pre-amplification to cut down on amplicon carryover [18, 19]. UV light irradiation produces pyrimidine dimer adducts between adjacent pyrimidine bases. These adducts prohibit *Taq* polymerase from processing along the amplicons. This renders nucleic acids unsuitable as templates for future reactions.

UV lights are included in "dead air" boxes and automated extraction instruments as a way to limit contamination. After being cleaned the units can be completely closed and the UV light can be turned on to aid in degrading any remaining nucleic acid. The units come with transparent shields or windows to protect the technologist's eyes from the harmful effects of UV light.

A UV light treatment at 254 nm for 10 min seems to be suitable to eliminate contaminating amplicons. Obviously, the action of UV light may be effected by the percentage of pyrimidines in the amplicons produced and the distance from the UV light source. Contaminating DNA was found to be eliminated at a distance of 5 cm [20]. As the distance was increased, UV light was less effective at controlling contamination. It has been found that there is better success if the amplicon is greater than 500 bp [21].

Enzymatic Methods

Uracil-*N*-Glycosylase

An enzymatic method that has proven to be an effective means for amplicon inactivation is uracil-*N*-glycosylase (UNG). This enzyme removes uracil bases from DNA by cleaving the uracil glycosidic bond between the base and the sugar phosphate backbone. Amplicons containing uracil are cleaved at these sites by UNG yielding them unsuitable as a template for amplification. UNG has no action on RNA and is only functional on single- or double-stranded DNA. Figure 26.3 shows the biochemistry of the UNG inactivation method.

Using UNG requires some changes in master mix recipe and in thermal cycling conditions. Master mix must contain UNG and there needs to be at least some, if not all, substitution of dTTP with dUTP. The optimal temperature of UNG activity is 55 °C. The enzyme is inactivated at 95 °C for 10 min. The thermal cycling profile needs to include two temperature holds before amplification, the first hold is 55 °C 2 for 10 min and the second is 95 °C for 10 min. After these two holds, thermal cycling can proceed although it is best if annealing temperatures remain above 55 °C. [22]. After cycling, it is advantageous to hold reactions at 72 °C (above 55 °C) or 4 °C to limit any residual UNG activity [23] (Fig. 26.4).

The success of UNG activity depends on the amount of uracil incorporated in the product, so products that are A+T rich will give better results with this method. The UNG protocol was not found to work well with small amplicons (<100 bp) [24]. Also, if it is desired to add UNG to an existing protocol, it may take some time

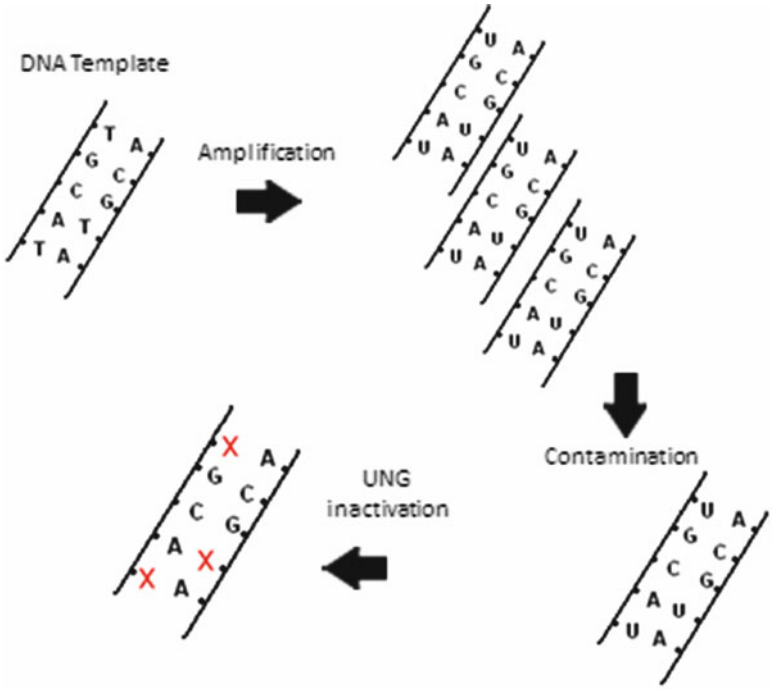

Fig. 26.3 Biochemistry of UNG. UNG and dUTP are included in the polymerase chain reaction (PCR) master mix. During amplification, dUTP is included in the amplicon. If contamination of a new PCR reaction occurs with this amplicon, UNG will cleave the apyrimidinic sites and the amplicon is not suitable as a template for further amplification

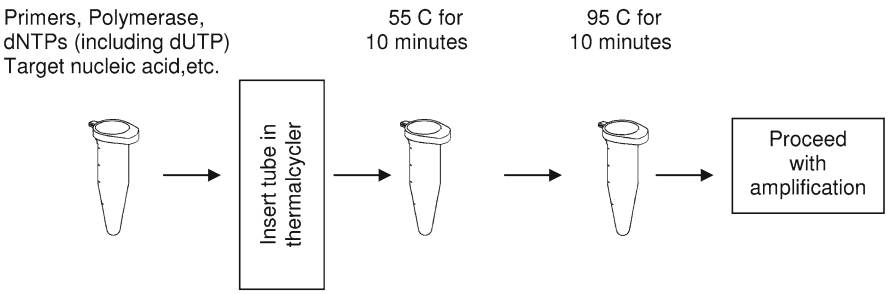

Fig. 26.4 Steps used in UNG amplification product inactivation protocol. The master mix has been formulated to contain UNG and dUTP (as opposed to dTTP). The master mix is allowed to incubate for 10 min to allow for UNG activity. Then, the mixture is warmed to 95 °C for 10 min to inactivate UNG. The amplification and detection are allowed to proceed as normal

to adjust master mix and thermal cycling programs to come to an optimal mean between good product yield and good UNG activity. The UNG method has been shown to inhibit up to 3×10^9 copies of contaminating DNA [25].

In most experiments, a 1 μL of 1 U/μL UNG is included in a 100 μL reaction volume [25]. A heat-labile UNG is also available that has proven to be more effective in some experiments when RNA is targeted. The heat-labile forms of UNG operate at lower temperatures than heat-stable versions. They are degraded at temperatures lower than 50 °C that is commonly used for the reverse transcriptase step [26, 27] in RT-PCR protocols. Sodium hydroxide used to denature DNA also has a dual purpose in helping to halt UNG activity more completely than just heat alone [28]. Concentrations of UNG higher than 0.1 U per reaction have been shown to inhibit amplification with some targets [29].

PCR protocols using UNG have been developed for a variety of targets including enterovirus [26, 29, 30], tuberculosis [31], *Toxoplasma gondii* [28], human herpesvirus 6 (HHV-6) [32], *Histoplasma* [33], *Bartonella henselae*, *Bartonella quintana*, and *C. burnetti* [34], HSV [1], CMV [35], and many others. The UNG procedure has been modified slightly to be compatible with DNA methylation experiments [36]. In fact, most commercial manufacturers have included UNG if they provide reagents in a kit format [35, 37].

UNG protocols have no effect on gel mobility, ethidium bromide staining, and hybridization reactions used in PCR product detection [23]. Although there have been reports of UNG changing melting temperatures for assays using them as part of their endpoint analysis [38]. RNA degradation has been reported when using heat-labile UNG [25] (Table 26.1).

Other Enzymatic Methods

Double-stranded DNase (dsDNase) is another enzymatic method of contamination control. These enzymes specifically target double-stranded DNA and have no activity with single-stranded DNA or RNA. Approximately 0.1–0.5 U of dsDNase can be added to the PCR master mix pre-amplification. This reaction mixture should be incubated at 42 °C for 15 min for DNA decontamination (and reverse transcription if RNA is the target). Then, the mixture is heated to 95 °C for 5 min to inactivate the enzyme. PCR thermal cycling can occur as normal after inactivation. Heat-labile forms of this enzyme are available that can be inactivated at 55 °C. The heat-labile enzyme is added to PCR master mix before template addition and is allowed to incubate at 37 °C for 10 min. Then, the enzyme is inactivated at 55 °C for 15 min. The template can be added after this step and thermal cycling proceeds as normal. An algorithm was created by Champlot et al. where Taq polymerase, dNTPs and primers were treated pre-amplification with heat-labile dsDNAse to rid these reagents of possible contaminants prior to PCR master mix formulation. Chemical means of decontaminating other components of PCR master mix (water, buffers, glycerol, etc.) were shown to work well. UV irradiation was not consistent at decontaminating or had negative effects on PCR efficiency when used on polymerase, dNTPs or primers thus heat-labile dsDNase was used [39].

Table 26.1 Comparison of inactivation protocols used in nucleic acid tests

Method	Application	Conditions	Advantages	Disadvantages	References
UV light irradiation	Pre-amplification	Lower G+C content, >500 bp amplicon	Inexpensive Simple procedure No changes to protocol	Efficacy varies	[18, 20, 21]
UNG	Pre-amplification	Lower G+C content, >100 bp amplicon	Simple procedure	Changes necessary in amplification cycling and master mix More expensive than other methods	[23, 24]
Photochemical cross-linkers	Post-amplification	Lower G+C content, >100 bp amplicon	Simple procedure	Additional instruments required Can change molecular mass of products which effects electrophoresis	[23, 24, 42]
Primer hydrolysis	Post-amplification	No specific requirements	No effect on amplicon analysis	Necessary for reagent addition post-amplification Efficacy varies	[23]
Hydroxylamine treatment	Post-amplification	Higher G+C content, >100 bp amplicon	Effective on some amplicons	Necessary for reagent addition post-amplification Some amplicon changes may effect analysis Hydroxylamine is a mutagenic agent	[43]
Restriction endonuclease	Pre-amplification	No specific requirements	Effective on some amplicons	May lengthen processing time	[40]

One more enzymatic option for pre-amplification product inactivation is the treatment with restriction endonucleases (RE). RE are added to master mix prior to amplification to chew up any contaminating nucleic acid. The RE incubates with the reaction mixture for a specified amount of time. Then, primers, template, and polymerase are added and amplification can proceed. The problems with this method are that more than one RE may be needed to efficiently inactivate amplicons, incubation times for RE action may be lengthy and there may need to be some experimentation to find which RE(s) work best for inactivating the amplicon [40].

Photolinkers

A vast majority of PCR protocols use UNG as the means of contamination control. In the early days of amplified molecular methods, several other options were investigated but most of these are not used in recent protocols. They are mentioned briefly here as then can be used if UNG is not an option in a particular situation and contamination control is desired other than stringent cleaning.

The use of psoralens is a method that can be used post-amplification. Two psoralens, isopsoralen, and methoxypsoralen have been used with molecular amplification methods. Isopsoralens are added to the master mix along with DNA template. After amplification, the tube must be exposed to UV light for 15 min [23, 41]. The activated isopsoralen forms adducts between pyrimidine residues blocking *Taq* polymerase from extending [42]. This method was shown to function as well as UNG in inactivating up to 3×10^9 copies of contaminating DNA [23]. Drawbacks to this method include some alteration to migration on gels [24] and additional cost in the need for a UV box.

Other Methods

Addition of hydroxylamine hydrochloride post-amplification is another method of amplification product inactivation. Hydroxylamine reacts with cytosine residues and blocks it from pairing with guanine. The modified base can bind with adenine and causes replacement with thymine if amplification occurs after treatment. The flaw with this system of amplification product control is the requirement to open tubes to add reagent post-amplification. Aerosols produced upon opening can spread amplicon [43].

Primer hydrolysis is another option for amplicon inactivation. Primers are designed to have ribose residues at or near the 3′ end. NaOH is added to the tube post-amplification which results in the primers being cleaved. The resulting nucleic acids are not suitable for amplification in future reactions. This procedure requires manipulation of products post-amplification which can spread amplicons before they have been inactivated [23].

Root Cause Analysis

Whenever a false-positive result occurs in a setting in which molecular testing is being used, a root cause analysis should be performed. The root cause analysis is key for uncovering the source of contamination. First, there is a detailed examination of the false-positive occurrence. Details to review are the test method performed, sample(s) tested, instrumentation used and staff involved. An investigation to find the source contamination should be completed. It is helpful to retest the original sample from start to finish, re-amplify extracted nucleic acid from the original test run and repeat detection of PCR amplicons from the original test run. This analysis will reveal the step where contamination occurred. When the step is identified, all equipment should be decontaminated. Reagents should be discarded and replenished with new, fresh reagents. Also, it is best to observe staff for possible technique errors leading to contamination. When the investigation is completed, all investigations and procedure changes should be documented in a written format for all staff to review. This document should include sections entitled (1) Definition of Problem, (2) Investigation, (3) Analysis of Cause-and-Effect Relationships, (4) Potential Changes in Procedure and any other sections that seem appropriate from the investigation findings. The root cause analysis will effectively uncover the underlying problems causing the false-positive result and aid in preventing these occurrences in the future [44].

Concluding Remarks

This chapter summarizes many ways for a laboratory to take "responsibility" for eliminating contamination and false-positive results in their amplified assays. Strict adherence to cleaning procedures and physical separation of pre- and post-amplification areas provide a first line of defense. UV light and addition of UNG protocols will maintain the clean environment. Adoption of these procedures will make the molecular laboratory very "powerful" with providing high quality results.

References

1. Smalling TW, Sefers SE, Li H, Tang YW (2002) Molecular approaches to detecting herpes simplex virus and enteroviruses in the central nervous system. J Clin Microbiol 40(7): 2317–2322
2. Kimberlin D (2004) Herpes simplex virus, meningitis and encephalitis in neonates. Herpes 11(Suppl 2):65A–76A
3. Long SS, Pool TE, Vodzak J, Daskalaki I, Gould JM (2011) Herpes simplex virus infection in young infants during 2 decades of Empiric Acyclovir Therapy. Pediatr Infect Dis J 30(7): 556–561

4. Sefers SE, Pei Z, Tang YW (2005) False positives and false negatives encountered in diagnostic molecular microbiology. Rev Med Microbiol 16(2):59–67
5. Aslanzadeh J, Preventing PCR (2004) amplification carryover contamination in a clinical laboratory. Ann Clin Lab Sci 34(4):389–396
6. Borst A, Box AT, Fluit AC (2004) False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. Eur J Clin Microbiol Infect Dis 23(4):289–299
7. Mitchell C, Kraft K, Peterson D, Frenkel L (2010) Cross-contamination during processing of dried blood spots used for rapid diagnosis of HIV-1 infection of infants is rare and avoidable. J Virol Methods 163(2):489–491
8. Tang JW, Lin M, Chiu L, Koay ES (2010) Viral loads of herpes simplex virus in clinical samples–a 5-year retrospective analysis. J Med Virol 82(11):1911–1916
9. Stals A, Werbrouck H, Baert L et al (2009) Laboratory efforts to eliminate contamination problems in the real-time RT-PCR detection of noroviruses. J Microbiol Methods 77(1): 72–76
10. McDermott SS, McDermott PF, Skare J et al (2000) Positive CSF HSV PCR in patients with GBM: a note of caution. Neurology 54(3):746–749
11. Tilburg JJ, Nabuurs-Franssen MH, van Hannen EJ, Horrevorts AM, Melchers WJ, Klaassen CH (2010) Contamination of commercial PCR master mix with DNA from *Coxiella burnetii*. J Clin Microbiol 48(12):4634–4635
12. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Kaczmarski EB, Fox AJ (2000) Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J Clin Microbiol 38(5):1747–1752
13. Muhl H, Kochem AJ, Disque C, Sakka SG (2010) Activity and DNA contamination of commercial polymerase chain reaction reagents for the universal 16S rDNA real-time polymerase chain reaction detection of bacterial pathogens in blood. Diagn Microbiol Infect Dis 66(1):41–49
14. Evans GE, Murdoch DR, Anderson TP, Potter HC, George PM, Chambers ST (2003) Contamination of Qiagen DNA extraction kits with Legionella DNA. J Clin Microbiol 41(7):3452–3453
15. Monleau M, Plantier JC, Peeters M (2010) HIV contamination of commercial PCR enzymes raises the importance of quality control of low-cost in-house genotypic HIV drug resistance tests. Antivir Ther 15(1):121–126
16. Prince AM, Andrus L (1992) PCR: how to kill unwanted DNA. Biotechniques 12(3):358–360
17. Khlif M, Mary C, Sellami H et al (2009) Evaluation of nested and real-time PCR assays in the diagnosis of candidaemia. Clin Microbiol Infect 15(7):656–661
18. Sarkar G, Sommer S (1990) More light on PCR contamination. Nature 347(6291):340–341
19. Kwok S, Higuchi R (1989) Avoiding false positives with PCR. Nature 339(6221):237–238
20. Ou CY, Moore JL, Schochetman G (1991) Use of UV irradiation to reduce false positivity in polymerase chain reaction. Biotechniques 10(4):442, 444, 446
21. Belak S, Ballagi-Pordany A (1993) Experiences on the application of the polymerase chain reaction in a diagnostic laboratory. Mol Cell Probes 7(3):241–248
22. Longo MC, Berninger MS, Hartley JL (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene 93(1):125–128
23. Rys PN, Persing DH (1993) Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. J Clin Microbiol 31(9):2356–2360
24. Espy MJ, Smith TF, Persing DH (1993) Dependence of polymerase chain reaction product inactivation protocols on amplicon length and sequence composition. J Clin Microbiol 31(9):2361–2365
25. Kleiboeker SB (2005) Quantitative assessment of the effect of uracil-DNA glycosylase on amplicon DNA degradation and RNA amplification in reverse transcription-PCR. Virol J 2:29
26. Taggart EW, Carroll KC, Byington CL, Crist GA, Hillyard DR (2002) Use of heat labile UNG in an RT-PCR assay for enterovirus detection. J Virol Methods 105(1):57–65

27. Pierce KE, Wangh LJ (2004) Effectiveness and limitations of uracil-DNA glycosylases in sensitive real-time PCR assays. Biotechniques 36(1):44–46, 48
28. Martins TB, Hillyard DR, Litwin CM, Taggart EW, Jaskowski TD, Hill HR (2000) Evaluation of a PCR probe capture assay for the detection of *Toxoplasma gondii.* Incorporation of uracil N-glycosylase for contamination control. Am J Clin Pathol 113(5):714–721
29. Mohamed N, Elfaitouri A, Fohlman J, Friman G, Blomberg J (2004) A sensitive and quantitative single-tube real-time reverse transcriptase-PCR for detection of enteroviral RNA. J Clin Virol 30(2):150–156
30. Kao SY, Niemiec TM, Loeffelholz MJ, Dale B, Rotbart HA (1995) Direct and uninterrupted RNA amplification of enteroviruses with colorimetric microwell detection. Clin Diagn Virol 3(3):247–257
31. Sarmiento OL, Weigle KA, Alexander J, Weber DJ, Miller WC (2003) Assessment by meta-analysis of PCR for diagnosis of smear-negative pulmonary tuberculosis. J Clin Microbiol 41(7):3233–3240
32. Flamand L, Gravel A, Boutolleau D et al (2008) Multicenter comparison of PCR assays for detection of human herpesvirus 6 DNA in serum. J Clin Microbiol 46(8):2700–2706
33. Qualtieri J (2009) Stratton CW, Head DR. Tang YW PCR detection of Histoplasma capsulatum var capsulatum in whole blood of a renal transplant patient with disseminated histoplasmosis Ann Clin Lab Sci 39(4):409–412
34. Tang YW (2009) Duplex PCR assay simultaneously detecting and differentiating *Bartonella quintana, B. henselae*, and *Coxiella burnetii* in surgical heart valve specimens. J Clin Microbiol 47(8):2647–2650
35. Li H, Dummer JS, Estes WR, Meng S, Wright PF, Tang YW (2003) Measurement of human cytomegalovirus loads by quantitative real-time PCR for monitoring clinical intervention in transplant recipients. J Clin Microbiol 41(1):187–191
36. Tetzner R (2009) Prevention of PCR cross-contamination by UNG treatment of bisulfite-treated DNA. Methods Mol Biol 507:357–370
37. Schutten M, Peters D, Back NK et al (2007) Multicenter evaluation of the new Abbott RealTime assays for quantitative detection of human immunodeficiency virus type 1 and hepatitis C virus RNA. J Clin Microbiol 45(6):1712–1717
38. Leblanc JJ, Pettipas J, Campbell SJ, Davidson RJ, Hatchette TF (2008) Uracil-DNA glycosylase (UNG) influences the melting temperature (T(m)) of herpes simplex virus (HSV) hybridization probes. J Virol Methods 151(1):158–160
39. Champlot S, Berthelot C, Pruvost M, Bennett EA, Grange T, Geigl EM (2010) An efficient multistrategy DNA decontamination procedure of PCR reagents for hypersensitive PCR applications. PLoS One 5(9)pii:e13042
40. DeFilippes FM (1991) Decontaminating the polymerase chain reaction. Biotechniques 10(1):26, 8, 30
41. Isaacs ST, Tessman JW, Metchette KC, Hearst JE, Cimino GD (1991) Post-PCR sterilization: development and application to an HIV-1 diagnostic assay. Nucleic Acids Res 19(1):109–116
42. Cimino GD, Metchette KC, Tessman JW, Hearst JE, Isaacs ST (1991) Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. Nucleic Acids Res 19(1):99–107
43. Aslanzadeh J (1993) Application of hydroxylamine hydrochloride for post-PCR sterilization. Mol Cell Probes 7(2):145–150
44. Woloshynowych M, Rogers S, Taylor-Adams S, Vincent C (2005) The investigation and analysis of critical incidents and adverse events in healthcare. Health Technol Assess 9(19):1–143, iii

Part II
Applications

Chapter 27
Bacterial Identification Based on Universal Gene Amplification and Sequencing

Susanna K.P. Lau, Jade L.L. Teng, and Patrick C.Y. Woo

Introduction

Accurate identification of bacterial isolates is one of the fundamental tasks in clinical microbiology laboratories. This is critical in providing a microbiological diagnosis to an infectious disease and guiding appropriate antibiotic treatment as well as infection control measures. On the population scale, accurate bacterial identification is important for defining epidemiology of infectious diseases. Traditionally, identification of bacteria in clinical microbiology laboratories is performed using conventional phenotypic tests, including Gram smear, cultural requirements, growth characteristics, and biochemical tests. These tests are relatively inexpensive and accurate for most commonly encountered bacteria in clinical laboratories. However, in certain circumstances, these phenotypic tests may fail to work and more sophisticated methods may be required. For example, accurate identification of anaerobic bacteria and mycobacteria may require special equipment and expertise such as gas chromatography–mass spectrometry. Moreover, phenotypic methods often fail to identify rare bacteria or bacteria which exhibit variable expression of certain traits, and are associated with ambiguity in determining end point reactions. As phenotypic methods rely on the availability of pure culture for the study of growth characteristics and biochemical profiles, it also takes considerable time for slow-growing bacteria to be identified. Furthermore, these methods are not applicable for noncultivable bacteria and in culture-negative infections.

S.K.P. Lau • J.L.L. Teng • P.C.Y. Woo (✉)
Department of Microbiology, The University of Hong Kong,
Pokfulam, Hong Kong

State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology,
The University of Hong Kong, University Pathology Building, Queen Mary Hospital,
Pokfulam, Hong Kong
e-mail: pcywoo@hkucc.hk.hk

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As a result of the widespread use of PCR and DNA sequencing in the last 2 decades, amplification and sequencing of universal gene targets represents an advanced technology that theoretically provides solutions to these problems, yielding reproducible and unambiguous results even for rare or slow-growing bacteria within 1 or 2 days. Among the various studied gene targets, 16S rDNA gene has been the most widely used, having played a pivotal role in rapid identification of bacteria in clinical microbiology laboratories. PCR and sequencing of 16S rDNA gene has been shown to be especially useful for identification of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria, and culture-negative infections. Application of this advanced technique in diagnostic microbiology has not only provided etiological diagnosis to infectious diseases but also assisted the choice and duration of antibiotics and deployment of appropriate infection control procedures. In addition, it has also enabled better understanding of the epidemiology and pathogenicity of rarely encountered bacteria or those that are "unidentifiable" by conventional phenotypic tests, which has not been possible in the past.

Apart from bacterial identification, the use of 16S rDNA gene sequencing has also led to the discovery of a large diversity of previously undescribed, novel bacterial species. More than 200 novel bacterial species have been discovered from human specimens in the past decade [1]. The highest numbers of novel species discovered were of the genera *Mycobacterium* and *Nocardia*, whereas the oral cavity/dental-related specimens and the gastrointestinal tract were the most important sites for discovery and/or reservoirs of novel species [1]. Among the novel species, *Streptococcus sinensis*, *Laribacter hongkongensis*, *Clostridium hathewayi*, and *Borrelia spielmanii* have been more thoroughly characterized, with the reservoirs and routes of transmission documented, and *S. sinensis*, *L. hongkongensis*, and *C. hathewayi* have been found globally [2–20], although most *Nocardia* and *Mycobacterium* species were probably from the environment and most anaerobes were probably from the oral cavity and/or gastrointestinal tract. However, the focus of the current chapter is on bacterial identification, and the discovery of novel bacterial species by 16S rDNA gene sequencing is not discussed in detail.

One of the limiting factors in putting 16S rDNA gene sequencing to routine use in diagnostic microbiology is the lack of full automation of the technology. Moreover, analysis and interpretation of 16S rDNA gene sequences is by no means a straightforward task to general technicians working in routine clinical microbiology laboratories. To solve the latter obstacle, several software packages with individualized databases have been developed to achieve automation of 16S rDNA gene sequence analysis for bacterial identification, which may make interpretation of results easier to users. Despite the well-recognized utility of 16S rDNA gene sequence in bacterial identification, there are limitations in using 16S rDNA gene sequence analysis for identification of certain groups of bacteria. In these situations, additional phenotypic or genotypic tests may be required for more accurate species identification. In this regard, a number of alternative gene targets have been explored for bacterial identification, especially those that cannot be readily identified to species level by

16S rDNA gene sequence analysis. New high-throughput technologies and availability of more complete bacterial genome sequences may allow the invention of improved methods for bacterial identification in diagnostic microbiology.

16S rDNA Gene Sequencing for Bacterial Identification

The discovery of conserved small rDNA gene sequences in the 1970s by Carl Woese and others has marked the beginning of new era for the study of evolution and classification of living organisms [21, 22]. These rDNA gene sequences are, in general, highly conserved within living organisms of the same genus and species, but different in organisms of different genera and species. Phylogenetic studies using these rDNA gene sequences have also "rediscovered" three domains of life: *Archaea*, *Bacteria*, and *Eukarya*, in contrast to the traditional dogma of classifying living organisms into prokaryotes and eukaryotes only [23]. With the subsequent invention of PCR and automated DNA sequencing technology in the 1990s, 16S rDNA gene sequences have been widely used for phylogenetic studies and considered a new standard for bacterial classification and identification. As a result, a large amount of bacterial 16S rDNA gene sequence data has been generated. Numerous bacterial genera and species have been reclassified and renamed, and many novel bacterial genera and species have been discovered. Moreover, classification of uncultivable bacteria has been made possible. In the last decade, bacterial genome sequencing projects have allowed study of bacterial phylogeny using various genes and genomes, which confirmed the representativeness of 16S rDNA gene in comparison to complete genome [24]. With the increasing availability of PCR and DNA sequencing facilities, the use of 16S rDNA gene sequencing has not been limited to research purposes, but also exploited in clinical microbiology laboratories for bacterial identification as well as other purposes such as direct identification of bacteria from clinical specimens or positive blood cultures [25–27].

The most common situations where 16S rDNA gene sequencing is employed for bacterial identification in the clinical laboratories are when a bacterium is "unidentified" by conventional phenotypic tests or commercial bacterial identification systems or the confidence level of identification by these methods is considered low; when the expected phenotypic profiles of a bacterium does not match its species identity; or when a rare bacterial species was encountered and technicians are uncertain about the accuracy of phenotypic identification. In these situations, 16S rDNA gene sequencing can be performed with the usual techniques for PCR and DNA sequencing. A pure bacterial culture will be used for DNA extraction and PCR is usually performed using universal or degenerate primers which target the conserved regions of bacterial 16S rDNA genes. While single universal or degenerate primer sets for all bacterial species are available from automated systems such as the MicroSeq systems, "less" degenerate primers for specific groups of bacteria are often used, which may help minimize the risk of PCR contamination and false

positive reactions. Once a PCR product is obtained and purified, it can be subject to DNA sequencing and sequence analysis by comparison to other 16S rDNA sequences from various sequence databases. To achieve maximum accuracy in identification, such sequence analysis results are best interpreted in light of conventional phenotypic test results.

Routine Bacterial Identification in Clinical Laboratories

Since conventional phenotypic tests are usually capable of accurately identifying commonly encountered bacteria, "routine" use of 16S rDNA gene sequencing in clinical microbiology laboratories has been largely limited to situations where bacterial isolates are difficult to identify by phenotypic tests. Nevertheless, various studies have been conducted to evaluate the usefulness of 16S rDNA gene sequencing for identification of various medically important bacteria in comparison to conventional or commercial methods. Depending on the group of bacteria studied and the criteria used for species definition, the success rate of species identification by 16S rDNA gene sequencing ranged from 62–92 % [28–34].

16S rDNA gene sequencing has been found to be particularly useful for identifying certain groups of bacteria. One notable example is anaerobic gram-positive rods which are notoriously difficult to identify by conventional methods even to genus level. Using 16S rDNA gene sequencing, many previously undescribed or "rarely encountered" anaerobic gram-positive rods have been characterized and found to contribute to cases of bacteremia [35–39]. Thus, the prevalence and pathogenicity of these often ignored anaerobes can be better defined. For example, the genus *Eggerthella* was found to contribute to an unexpectedly high proportion of clinically significant bacteremia due to anaerobic, nonsporulating, gram-positive rod, suggesting that this genus may be of high pathogenicity among this group of bacteria [35, 36]. Two novel *Eggerthella* species, now reclassified under the genus *Paraeggerthella*, were also discovered and may contribute to half of the cases of *Eggerthella* bacteremia [35, 40]. Using 16S rDNA gene sequencing, it was also found that patients with clinically significant clostridium bacteremia were associated with diseases in the gastrointestinal or hepatobiliary tract, which are also predictors of mortality [38]. 16S rDNA gene sequencing has been particularly useful in differentiating between *Actinomyces* and non-*Actinomyces* anaerobic gram-positive bacilli, which may not be easily achieved by conventional phenotypic tests [41–43]. A definitive diagnosis or exclusion of actinomycosis is considered clinically important, because prolonged antibiotic treatment, in terms of weeks to months, is often recommended in actinomycosis to prevent relapse.

Another group of bacteria that are readily identified by 16S rDNA gene sequencing are the catalase-negative gram-positive cocci. Application of this advanced technique has contributed to knowledge on the epidemiology and pathogenicity of the different *Streptococcus* and related bacterial species. For example, in the past, little was known about the relative importance of the four species of

Lancefield group G beta-hemolytic streptococci in causing bacteremia. Using 16S rDNA gene sequencing, these cases were found to be almost exclusively caused by *S. dysgalactiae* subsp. *equisimilis* by 16S rDNA gene sequencing, except in dog owners where *S. canis* infections may be rarely reported [44, 45]. As for α-hemolytic streptococci, the relative importance of the 3 species of the "*Streptococcus milleri* group" in infective endocarditis was previously largely unknown. Using 16S rDNA gene sequencing, all six cases of *S. milleri* endocarditis in one study were found to caused by *S. anginosus*, suggesting that this species may have the highest propensity to cause infective endocarditis among the three species of the "*S. milleri* group" [46]. The use of 16S rDNA gene sequencing in these clinically "unidentifiable" bacteria could be of clinical significance and carry treatment implications. For example, differentiation of *Enterococcus cecorum* from other *Enterococcus* species has allowed continuation of cefotaxime as treatment, as the organism is known to be susceptible to cefotaxime and ceftriaxone, unlike other *Enterococcus* species which are known to be resistant to cephalosporins. It was also found that the cephalosporin susceptibility of *E. cecorum* could well be explained by its unique phylogenetic position by 16S rDNA gene sequence, as it appeared to be the ancestor of other *Enterococcus* species and more closely related to *Streptococcus* species [47].

16S rDNA gene sequencing was also useful for identification of various gram-negative bacteria. Although *Haemophilus* species are commonly isolated in the clinical laboratories, these organisms are often fastidious and may not be readily identified by conventional phenotypic tests. It has been shown that 16S rDNA gene sequencing can accurately identified the various *Haemophilus* species isolated from clinical specimens [48–51]. Using this technique, it was also found that *Haemophilus segnis* is an important cause of non-*Haemophilus influenzae* bacteremia [48–50]. Examples of other commonly encountered gram-negative bacteria which are sometimes "unidentifiable" by phenotypic tests but may benefit from 16S rDNA gene sequencing include the *Enterobacteriaceae*, *Acinetobacter*, and *Campylobacter* species [52–56]. Apart from establishing the correct microbiological diagnosis and guiding antibiotic treatment, accurate species identification could have important management and public health significance. For example, differentiating *Salmonella enterica* serotype Typhi from other members of the *Enterobacteriaceae* family is important to determine if cholecystectomy and eradication of carrier state is indicated [54–56].

Identification of Rare Bacteria and Bacteria with Unusual Phenotypic Profiles

While microbiologists are usually facing common medically important bacteria most of the time in clinical laboratories, bacterial isolates that are rare or phenotypically aberrant are also encountered from time to time. These are the times where mistakes in identification often occur. 16S rDNA gene sequencing is most useful when these bacteria are suspected in clinical microbiology laboratories.

The biochemical profiles of rarely encountered bacteria are often poorly studied or not included in the commercial biochemical identification system databases. There are times where a rare bacterium may be misidentified as a more commonly encountered bacterium. For example, *Francisella tularensis* subsp. *novicida* has been consistently misidentified twice by phenotypic methods as *Neisseria meningitidis* or *Actinobacillus actinomycetemcomitans* [57]. As for a bacterium with an unusual or atypical phenotypic profile, the conventional tests are bound to fail. Unlike phenotypic characteristics which can be affected by deletion or mutation of various genes especially those encoding enzymes, 16S rDNA gene sequencing provides unambiguous identification of bacteria with atypical phenotypic characteristics. Therefore, in these situations, 16S rDNA gene sequencing is often the ultimate solution to diagnosing infections caused by these bacteria and guiding appropriate treatment [48, 58–68].

16S rDNA gene sequencing has been found to provide genus identification in >90 % and species identification in 65–83 % of these circumstances [69, 70]. Using the MicroSeq 500 16S rDNA-based identification system, 81 % of clinically significant bacterial isolates with ambiguous biochemical profiles and 89.2 % of unusual aerobic gram-negative bacilli have been identified to the species level [52, 71]. The use of 16S rDNA gene sequencing on rare or unusual bacteria has led to better understand of the epidemiology and pathogenic role of these bacteria. For example, cases of invasive *Streptococcus iniae* infections in Asia have been diagnosed by 16S rDNA gene sequencing [72–75]. This rare aquatic bacterium has only been previously reported to cause human infection in North America [76]. Many other rarely encountered bacteria, which may have been unrecognized without using 16S rDNA gene sequencing, are now better defined in terms of their disease association and pathogenicity. Examples are *Bordetella*, *Arcobacter*, *Tsukamurella*, and the *Streptococcus*-related gram positive cocci such as *Helcococcus*, *Gemella*, and the nutritionally deficient streptococci, *Granulicatella adiacens* and *Abiotrophia defectiva* [52, 58, 59, 62–64, 77, 78]. Using 16S rDNA gene sequencing, novel or rare clinical syndromes such as *Tsukamurella*-associated conjunctivitis and keratitis, prosthetic valve endocarditis due to *Streptobacillus monoliformis* and psoas abscess due to group A streptococcus can be recognized [77–80].

Using the technique, bacterial isolates with unusual biochemical profiles can now be identified unambiguously. For example, thermo-tolerant *Campylobacter fetus* strains have been identified as important causes of bacteremia in immunocompromised patients [81]. Melioidosis due to *Burkholderia pseudomallei* with ambiguous biochemical profile has been diagnosed [57]. Unusual strains of various gram-positive and gram-negative bacteria are also recognized [54, 55, 82, 83]. In summary, applications of 16S rDNA gene sequencing on rare or unusual bacteria can make significant impact on the decision whether to prescribe antibiotic treatment [54, 83, 84] and on the choice of specific antibiotic regimen [55, 80, 85], which could lead to improved clinical outcomes [52].

Identification of Slow-Growing and Uncultivable Bacteria

16S rDNA gene sequencing and similar molecular identification methods have the additional advantage of shortening the time to identify slow-growing bacteria and being able to identify bacteria that are even not cultivable. It is well known that most *Mycobacterium* species, except the rapidly growing mycobacteria, usually take 6–8 weeks to grow in culture and it often takes another few weeks to perform phenotypic tests using subcultures. Even for the "rapid growers," some biochemical reactions may take up to 28 days to complete. Moreover, whole-cell fatty acid analysis by gas chromatography, which is often required for definitive species identification, is not available in most routine clinical laboratories. 16S rDNA gene sequencing has been used for identification of *Mycobacterium* species, thereby speeding up clinical diagnosis and guiding prompt antibiotic treatment [84, 86]. Using the technique, a novel clinical syndrome, acupuncture mycobacteriosis, caused by relatively alcohol-resistant mycobacteria in patients receiving acupuncture has also been described [87, 88]. However, identification of mycobacteria by 16S rDNA gene sequencing is limited by the high sequence similarity among certain species, in which case alternative gene targets may be indicated.

Although bacterial culture plays a fundamental role in diagnosing bacterial infections in microbiology laboratories, some bacteria are known to be uncultivable even using modern techniques, which may make diagnosis difficult. Although direct microscopy and immunology-based assays has been used for such diagnosis, the sensitivities and specificities of these methods are often suboptimal and variable. The introduction of molecular diagnostics, in particular 16S rDNA gene sequencing, has enhanced our ability to diagnose these culture-negative infections. One of the most well-known examples of noncultivable bacteria is *Mycobacterium leprae*, the causative agent of leprosy which can be difficult to diagnose. Application of molecular detection such as PCR and sequencing of 16S rDNA gene from skin biopsies of leprosy patients has provided an additional diagnostic tool [89, 90]. In addition, the first breakthrough in the understanding of Whipple's disease, after its initial description in 1907, was only made with the identification of *Tropheryma whippeli* as the causative agent by PCR and sequencing of its 16S rDNA gene [91]. This state-of-the-art technique has also enabled the subsequent development of molecular diagnostic tests for this disease, and accelerated research in to its pathophysiology [92–95]. Similar successes in applying 16S rDNA gene sequencing have also been seen in recognizing the etiological agents of bacillary angiomatosis (caused by *Bartonella henselae* and *Bartonella quintana*) [96, 97] and human ehrlichiosis (caused by bacteria in the genera *Ehrlichia* and *Anaplasma*) [98–100].

Using 16S rDNA gene sequencing, etiological agents can also be established in various culture-negative infections. For example, up to a third of cases of infective endocarditis can be culture-negative [101], which may be due to prior antibiotic therapy, inadequate microbiological techniques, or infection caused by fastidious or noncultivable organisms [102]. It has been shown that 16S rDNA gene PCR amplification and sequencing performed on DNA extracted from infected valves

may provide a clue to the culprit organism [103–113]. Similar technique has also been used for diagnosis of culture-negative infections including meningitis [114–118], brain abscess [119], keratitis [120], urinary tract infections [121], empyema [122, 123], septic arthritis [124, 125], and septicemia [102, 126, 127]. Although recent progress in these areas has been made through the use of broad-range real-time PCR design [117, 128, 129], the use of universal primer sets is sometimes associated with problems of false-positives due to PCR contamination and picking up sequences from colonizing bacteria. With continued improvements in technology design and performance, it is expected that 16S rDNA gene sequencing will play an increasing role in the diagnosis of culture-negative infections.

Guidelines for Interpretation of 16S rDNA Gene Sequence Results

Despite its increasing use for bacterial identification in clinical microbiology laboratories, there are no widely accepted guidelines on the indications of using 16S rDNA gene sequencing as well as the interpretation of sequence data. In view of the limitations in identifying certain bacterial taxa, increasing taxonomic complexity, and large number of unvalidated 16S rDNA gene sequences in some databases, there have been recommendations on the use of 16S rDNA gene sequencing for bacterial identification [69, 130]. For indications of 16S rDNA sequencing, since certain bacterial taxa are known to present difficulties for identification by 16S rDNA gene sequence analysis, other gene targets should be considered for these bacteria [32, 130–139]. As far as the sequence analysis is concerned, it depends on the length and quality of sequences, the choice of appropriate programs and databases for analysis, and correct interpretation of similarity search results. It has been suggested that a minimum of 500–525 bp which covers the more variable 5′-region of the 16S rDNA gene may be adequate for identification, thus giving rise to the development of the MicroSeq databases. However, some recommended that full 16S rDNA gene sequences should be used whenever possible [69, 130]. This is particularly important for certain groups of bacteria such as *Campylobacter* species, where the 5′-region may not be sufficient for species differentiation [130]. A major difficulty and controversy in interpreting 16S rDNA gene sequence data is the lack of a universal threshold value or cutoff for species assignment, as different levels of sequence diversities are observed among different bacterial taxa which evolve at different rate. While a >97 % similarity level has been proposed for bacterial speciation, a >0.5 % difference may be indicative of a new species [140, 141]. Therefore, it may be necessary to use different cutoffs for different groups of bacteria [69]. For practical purposes, different cutoffs have also been used in different studies. For example, >99 and >97 % sequence similarity has been used as the cutoffs for species and genus identification respectively [69, 130].

Another commonly encountered problem when interpreting 16S rDNA gene sequence data is that when two different bacterial species share highly similar 16S rDNA gene sequence with <0.5–1 % difference, it is not a straightforward job to decide whether the “first hit” or “closest match” is the real identity of a bacterial

isolate, or this may lead to misidentification if the user is unaware of this problem. In view of this problem, studies have been carried out to systematically evaluate the usefulness of full and 527-bp 16S rDNA gene sequencing and the existing MicroSeq databases for identification of all medically important bacterial species listed in *Manual of Clinical Microbiology* [142–144]. Under the proposed guidelines, each medically important bacterial species was classified as follows: (1) can be confidently identified by 16S rDNA gene sequencing, with >3 % difference to other medically important bacteria, (2) cannot be confidently identified by 16S rDNA gene sequencing, with <2 % difference to a closely related medically important bacterium, or (3) can only be doubtfully identified by 16S rDNA gene sequencing with 2–3 % difference to a closely related medically important bacterium. If a bacterium belongs to (2) or (3), the bacterial species with similar 16S rDNA gene sequences will also be known and additional/supplementary tests may be considered for differentiation among these closely related species. For MicroSeq database analysis, the reason for failure to identify the bacterium is also indicated [143]. Using this algorithm, it was found that full and 527-bp 16S rDNA gene sequencing are able to identify 52–63 % of 130 anaerobic gram-positive rods, 72–73 % of 86 anaerobic gram-negative rods, and 78 % of 23 anaerobic cocci. Surprisingly, the MicroSeq databases were only able to identify 19–25 % of 130 gram-positive anaerobic rods, 38 % of 86 gram-negative anaerobic rods, and 39 % of 23 anaerobic cocci. As for medically important aerobic gram-positive bacteria, full and 527 bp 16S rDNA gene sequencing can identify 24 and 40 % of gram-positive cocci, and 21 and 34 % of gram-positive rods, whereas the full-MicroSeq and 500-MicroSeq databases can identify 15 and 34 % GPC and 14 and 25 % of GPR confidently to the species level [145]. These methods and databases are least useful for identification of staphylococci and nocardia, but are most useful for identification of *Bacillus* and related taxa. A similar study performed on medically important aerobic gram-negative bacteria showed that full and 527-bp 16S rDNA gene sequencing can identify 26.1 and 32.6 % of these bacteria, whereas the full-MicroSeq and 500-MicroSeq databases can identify 15.2 and 26.1 % confidently to the species level [144]. In particular, these methods or databases are least useful for identification of *Aeromonas*, *Bordetella*, and *Bartonella* species, and are most useful for identification of members of *Pasteurellaceae* and *Legionellaceae* and *Campylobacter* species. Compared to results on anaerobic and gram-positive bacteria, full and 527-bp 16S rDNA gene sequencing are able to confidently identify significantly more anaerobic gram-positive and gram-negative bacteria than aerobic gram-positive and gram-negative bacteria. In all three studies, the poor performance of the MicroSeq databases observed was mainly due to the absence of the sequences from the unidentified bacterial in their databases, suggesting that the MicroSeq databases can be much improved if they include more comprehensive and updated datasets. As such guidelines are still associated with a number of limitations, it is preferable to interpret the results of 16S rDNA sequencing with preliminary phenotypic test results. Nevertheless, such guidelines and similar studies may help easier interpretation of sequence data by inexperienced users in the clinical microbiology laboratories, and provide clues on the potential usefulness of 16S rDNA gene sequencing for selected bacterial isolates before they are chosen for such analysis.

Automation of 16S rDNA Gene Sequencing

One of the major obstacles to put 16S rDNA gene sequencing into routine use in clinical microbiology laboratories is the lack of automation of the technology. At the moment, conventional phenotypic tests are still considered the routine and most user-friendly tests for bacterial identification in clinical laboratories. This is partly attributed to the availability of various automated commercial bacterial identification systems based on panels of biochemical tests. However, similar systems are currently not available for 16S rDNA gene sequencing, because most of the steps involved in DNA extraction, PCR and sequencing have to be performed separately and manually. With high-throughput technologies being made more applicable, these steps may be incorporated into a robotic system for 16S rDNA gene sequencing platforms, making automation a possibility in future.

Another point of difficulty faced by technicians in using 16S rDNA gene sequencing in routine clinical microbiological laboratories is interpretation of sequencing results, which is often not straightforward to those not familiar with sequence and bioinformatics analysis. As a result, much effort has been put on development of automated 16S rDNA sequence analysis software packages or databases by various groups of scientists. These software packages usually contain a database of 16S rDNA gene sequences from selected bacterial species, against which the input 16S rDNA gene sequence is matched to generate the output bacterial identity. The best-known software and databases include BLASTn against GenBank, the Ribosomal Database Project (RDP-II) [146–150], MicroSeq [33, 71, 151, 152], Ribosomal Differentiation of Medical Microorganisms (RIDOM) [153–155]. Newer software packages and databases have also been developed in recent years, including SmartGene Integrated Database Network System (SmartGene IDNS™) [156], SILVA ribosomal RNA database [157], and 16Spath DB database [158] (Table 27.1). Among the currently available databases, the GenBank contains the largest databases, with 3,180,020 16S rDNA gene sequences (searches were conducted using the keyword "16S rRNA" and "16S rDNA"). Although this comprehensive database is extremely useful to researchers in the field, it is also well known to contain unvalidated, inaccurate, and redundant sequences. As a result, it is often not easy for inexperienced technicians working in clinical microbiology laboratories to interpret results of BLASTn against the GenBank database. For example, the user may not be aware that the "first hit" may not represent the true identity of a bacterial isolate.

The other databases contain sequences from selected bacteria, with differences in their selection criteria of bacterial species, quality control of sequences, inclusion of partial or full 16S rDNA gene sequences and cost. The databases of RDP-II and SmartGene IDNS™ contain selected sequences downloaded from GenBank and that of SILVA from EMBL, thus also with relatively large database sizes and associated with similar problems as GenBank. The databases of RIDOM and MicroSeq contain 16S rDNA gene sequences of selected bacterial strains from culture collections, thus with smaller database sizes. Although the sequence quality of these databases is better, their usefulness is limited by the choice of bacterial species. Since

Table 27.1 Currently available 16S rDNA gene sequence databases

Software packages	Partial/full 16S rDNA gene sequence included	Source of sequences	Database size	Quality control	Company/ Organization	Year of first description	Website
The Ribosomal Database Project (RDP) II	Partial and full	GenBank	1,613,063 (release 10.26)	Partial	Michigan State University, USA	1992	http://rdp.cme.msu.edu/
MicroSeq ID 16S rDNA 500 Library v2.2	Partial	Sequence 16S rDNA gene of one strain from each species	1,834	All type strains from culture collections	Life Technologies Corporation	1998	NA
MicroSeq ID 16S rDNA Full Gene Library v2.0	Full	Sequence 16S rDNA gene of one strain from each species	1,261	All type strains from culture collections	Life Technologies Corporation	1998	NA
Ribosomal Differentiation of Medical Microorganisms (RIDOM)	Partial	Sequence 16S rDNA gene of medical relevant bacteria, mainly belonging to the *Neisseriaceae, Moraxellaceae* and *Myco-bacterium* genus	236[a]	All strains from culture collections	Ridom GmbH, Würzburg, Germany	1999	http://rdna4.ridom.de/
SmartGene IDNS™ — Bacteria software	Partial and full	GenBank	243,000	Partial	SmartGene Services SARL, Switzerland	2006	http://www.smartgene.com/mod_bacteria.html

(continued)

Table 27.1 (continued)

Software packages	Partial/full 16S rDNA gene sequence included	Source of sequences	Database size	Quality control	Company/ Organization	Year of first description	Website
The SILVA ribosomal RNA database	Full	EMBL database	476,195 (release 104)	Partial	Microbial Genomics Group, Max Planck Institute for Marine Microbiology	2004	http://www.arb-silva.de/
16SpathDB	Partial and full	GenBank (include all medically important bacteria listed in Manual of Clinical Microbiology)	1010	All sequences manually selected from GenBank	Department of Microbiology, The University of Hong Kong, Hong Kong	2010	http://147.8.74.24/16SpathDB

[a]Number counted from http://rdna2.ridom.de/ridom2/servlet/link?page=list (31 May 2011)

they do not possess a very comprehensive database of all medically important bacterial species, they are unable to identify those bacterial species that are not included in the database. The RIDOM database contains only partial sequences of 236 medically relevant bacteria, mainly belonging to *Neisseriaceae*, *Moraxellaceae*, and *Mycobacterium*. The MicroSeq databases do not include a significant number of medically important bacteria that 16S rDNA gene sequencing is able to identify. For example, 98–108 (53.3–67.1 %), 38–39 (22.7–37.3 %), and 23–39 (19.8–41.9 %) medically important anaerobic, aerobic gram-positive, and aerobic gram-negative bacteria, respectively, which can be confidently identified by 16S rDNA gene sequencing, are not included [143, 145]. Another problem when using these software packages, including BLASTn against GenBank, is that, when there is only minimal difference among the sequences of closely related bacterial species, the inexperienced user may not be aware that 16S rDNA gene sequence alone is unable to identify these bacterial species in such circumstances and may wrongly accept the "first hit" or "closest match" as the identity of the bacterium. To solve these problems, a database, 16SpathDB, was recently developed, which includes the most representative 16S rDNA gene sequences of all medically important bacteria listed in the most current edition of the *Manual of Clinical Microbiology* [159], for identification of medically important bacteria using 16S rDNA gene sequencing in clinical microbiology laboratories [158]. All sequences were manually selected from GenBank, to ensure the quality of the sequences, and accurate identity and representativeness of the bacterial strains included. In contrast to RDP-II and SmartGene IDNS software packages, 16SpathDB includes only 16S rDRNA gene sequences of medically important bacteria to minimize ambiguity during data interpretation, as the target users of 16SpathDB are technicians and clinical microbiologists who work on 16S rDNA gene sequencing for identification of clinical isolates. This database also uses an automated user-friendly platform that indicated the most likely identity of the 16S rDNA gene sequence of a medically important bacterium, as well as other medically important bacteria with similar 16S rDNA gene sequences that may be alternative identities, which the user should be aware of [158]. For example, the 16S rDNA gene sequences of *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus mitis*, and *Streptococcus oralis* are known to share more than 99 % identity. In 16SpathDB, in addition to the species that shows the highest nucleotide identity to the query sequence, those species with 16S rDNA gene sequences having less than 1 % difference from the species that showed the highest nucleotide identity to the query sequence will also be reported, and the user will be alerted that further tests may have to be carried out in order to distinguish between these species. While MicroSeq and SmartGene IDNS software packages are commercially available for purchase, RDP II, RIDOM, SILVA and 16SpathDB are available for free via designated websites.

Various studies have also evaluated the usefulness of the different software packages for different groups of bacteria [28, 33, 52, 71, 151, 152, 156, 160–166]. However, these studies differ in study design, inclusion criteria for study strains, and interpretative criteria for "correct" identification, thus making direct comparison difficult [28, 151, 156, 161–164, 166]. As the intrinsic problems of the software

packages may not be fully addressed, some of the stated accuracies of the software packages mentioned in the publications may be overestimated. The usefulness of 16SpathDB has also been evaluated using the 16S rDNA gene sequences of 91 nonduplicated medically important bacterial isolates, among which 71 (78 %) were reported as a single bacterial species having >98.0 % nucleotide identity with the query sequence, 19 (20.9 %) as more than one bacterial species having >98.0 % nucleotide identity with the query sequence, and 1 (1.1 %) was reported as no match (*Gordonibacter pamelaeae* which has not been reported to cause human infection). With the development of more user-friendly software packages with high quality and comprehensive databases, 16S rDNA gene sequencing can be more readily used for routine bacterial identification in clinical laboratories.

Other Gene Targets for Bacterial Identification

Although 16S rDNA gene sequencing can achieve high accuracies in identifying many groups of bacteria to species level, there are "blind spots" within some major genera. A well-known problem is that 16S rDNA gene sequences are sometimes not discriminative enough for differentiation between related species. In these circumstances, alternative targets, usually based on highly conserved proteins, have to be investigated (Table 27.2). For example, *groEL* (bacterial homolog of *hsp60* encoding housekeeping chaperon proteins that assist in proper protein folding) is useful for classification and identification of various bacteria. This gene has been found useful in differentiating *B. pseudomallei* from *B. thailandensis*, of which the 16S rDNA gene sequences are indistinguishable [137, 138]. *groEL* is also useful for differentiating among the *Bartonella* species and in subtyping of *Bartonella henselae* [167]. Delineation of species within the genus *Acinetobacter* is often found to be problematic by phenotypic tests due to their catabolic diversity, and 16S rDNA gene sequences have failed to distinguish closely related genomic species due to its extremely low polymorphism [168]. The gene *rpoB*, another commonly used target encoding the beta subunit of RNA polymerase, has been found to be able to separate different *Acinetobacter* species, with higher bootstrap support in phylogenetic trees than those obtained with 16S rDNA gene [168]. The family *Enterobacteriaceae* contains a large number of pathogenic and frequently encountered bacterial species, some of which may be difficult to identify by phenotypic methods. Since the phylogenetic relations among certain closely related species, e.g., *Salmonella* species, *Citrobacter freundii*, and *E. coli*, are not well defined by 16S rDNA sequences, other targets, such as *rpoB* and *dnaJ* (encoding HSP40) have been explored [169, 170]. For example, *dnaJ* has been found to be useful in phylogenetic study and identification at species level of the family, with more monophyletic groups obtained and greater degree of divergences than that obtained with 16S rDNA gene sequences [169]. The high sequence similarity observed between members of the *Campylobacter* genus has also made differentiation between species such as *Campylobacter jejuni* and *C. coli* difficult based on 16S rDNA genes. Other gene targets, such as *gyrB*

Table 27.2 Commonly used gene targets other than 16S rDNA gene for bacterial identification

Gene target	Gene/protein function	Bacterial group	References
groEL	Heat shock protein	*Bartonella* species, *Burkholderia* species, rapidly growing mycobacteria, *Staphylococcus* species	[132, 135–138, 167]
gyrB	Beta-subunit of DNA gyrase	*Campylobacter* species, slowly growing mycobacteria	[133, 171]
gltA	Citrate synthase	*Ehrlichia* species, *Rickettsia* species	[183, 184]
dnaJ	Heat shock protein	*Enterobacteriaceae*, *Mycobacterium* species	[169, 180]
ITS	16S–23S rDNA gene internal transcribed spacer	*Campylobacter* species, slowly growing mycobacteria	[131, 172]
recA	Recombinase A	*Geobacillus* species, *Streptococcus mitis* group	[177, 179]
rpoB	Beta-subunit of RNA polymerase	*Acinetobacter* species, *Enterobacteriaceae*, *Geobacillus* species, nontuberculous mycobacteria, *Staphylococcus* species, *Streptococcus* species	[134, 168, 170, 173, 191]
sodA	Superoxide dismutase	*Enterococcus* species, *Streptococcus* species	[176, 178]
tuf	Elongation factor Tu	Coagulase-negative staphylococci	[32]

(encoding B subunit DNA gyrase protein) and 16S–23S rDNA internal transcribed spacer (ITS), have been found useful in this respect [171, 172].

As for gram-positive bacteria, 16S rDNA gene sequencing has limited discriminatory power for closely related *Staphylococcus* species which are sometimes misidentified by phenotypic tests. Therefore, sequencing of the *groEL*, *tuf* (elongation factor Tu), and *rpoB* genes has been proposed as more reliable methods for identification of staphylococci [32, 135, 136, 139, 173]. 16S rDNA gene sequences are also known to lack discriminatory power in distinguishing other aerobic gram-positive cocci, e.g., among the *Streptococcus mitis* group and among *Enterococcus* species. Different gene targets, such as *rpoB*, *sodA* (manganese-dependent superoxide dismutase), and *recA* (recombinase subunit), have been found to constitute a more discriminative target [173–178]. *rpoB* and *recA* have also been found to be advantageous to 16S rDNA gene for identification of *Geobacillus* species [179].

Although 16S rDNA gene sequencing can be used for identification of certain *Mycobacterium* species, a number of mycobacterial species are also known to be not distinguishable from one another by 16S rDNA gene sequencing, e.g., between *M. avium intracellulare* and *M. paratuberculosis*, between *M. chelonae* and

M. abscessus, between *M. kansasii* and *M.* gastri, between *M. malmoense* and *M. szulgai*, between *M. marinum* and *M. ulcerans,* between *M. mucogenicum* and *M. phocaicum*, and among the *M. tuberculosis* complex [84, 87, 88, 131, 180–182]. Therefore, different gene targets, sometimes supplemented by phenotypic results, have to be used for differentiation of specific mycobacterial species, such as *hsp65* (the *hsp60* homologue in mycobacteria), *rpoB*, ITS, *gyrB*, and *dnaJ* [131–134, 180]. For example, in a study evaluating the use of *hsp65* sequencing for identification of rapidly growing mycobacterium, the technique unambiguously differentiated *M. chelonae* and *M. abscesses* [132]. On the other hand, slowly growing mycobacteria were found to display high sequence variation in their ITS, which can be used to distinguish between *M. kansasii* and *M. gastri* [131]. *dnaJ* is also found to constitute a higher discriminatory power with mean sequence similarity of 80.4 % among the studied species compared to 16S rDNA, *rpoB*, and *hsp65* genes with 96.6, 91.3, and 91.1 % mean sequence similarities respectively, and is particularly useful for identifying the nontuberculous *Mycobacterium* species [180].

As for the rare bacteria, *Ehrlichia*, although 16S rDNA and *groEL* were useful for taxonomic classification and differentiation of the various species, other gene targets such as the citrate synthase gene, *gltA*, have also been explored to improve identification and diagnosis of ehrlichial diseases [183]. *gltA* has also been used as a complementary approach to 16S rDNA gene sequencing for phylogeneitc studies of the *Rickettsiaceae* [184]. The various alternative targets being explored to supplement 16S rDNA gene sequencing for identification of different groups of bacteria suggested that there is no single target that is superior to others. The limitations of 16S rDNA gene sequencing and other currently available gene targets also emphasize the importance of a "polyphasic" approach for accurate bacterial identification. By this "polyphasic" approach, it refers to the use of different methods, e.g., phenotypic tests plus 16S rDNA gene sequencing plus sequencing of other gene targets, to identify bacteria belonging to species which are known to be difficult to identify. With more bacterial complete genome sequences available in the near future from high-throughput sequencing technology, comparative genomic studies will also enable more comprehensive study of different gene targets for study of phylogeny and identification of bacteria [185].

Future Developments and New Technologies for Bacterial Identification

Not only has 16S rDNA gene sequencing helped answer some of our most fundamental questions in biology, but this technology has now developed beyond the research realm and matured into clinical applications. As 16S rDNA gene sequencing is associated with limitations for particular groups of bacteria, the development of alternative gene targets will continue to be important for identification of these bacteria by sequencing technology. Moreover, despite the wide range of software packages and databases available, automation of 16S rDNA gene sequencing is still not available

and interpretation of results often difficult by inexperienced users. The development of more user-friendly guidelines and software packages with high-quality, comprehensive databases, as well as the integration of high-throughput technologies will make automation of universal gene amplification and sequencing a possibility in the near future, which may replace the use of conventional phenotypic methods for routine bacterial identification in clinical microbiological laboratories in one day.

Apart from universal gene amplification and sequencing, other advanced technologies coming up in the twenty-first century may also emerge as new methods for bacterial identification in clinical laboratories, among which matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) may be the most promising technology. Although the MS technology has been established for over a century, it is only until recently that simple-to-use MALDI-TOF MS device has become available for the identification of pathogens in clinical microbiology laboratories. MALDI-TOF MS enables the analysis of biological molecules with no theoretical upper mass limit and MS detects the mass-to-charge ratio of a biological molecule. Using combination of these technologies, proteins and peptides are separated by their mass, giving an individual molecular fingerprint to each bacterium. In practice, a single bacterial colony or a centrifuged portion of a liquid culture is sufficient for MALDI-TOF MS analysis. A protein mass spectra database of known bacteria is then used to match the spectra of the bacterium under investigation. Theoretically, there is no limit to the identification ability of MALDI-TOF MS, as long as a suitable spectrum is present in the database. Researchers can also create their own library of bacterial mass spectra to increase its applicability. Different prospective studies have been carried out to examine the performance of MALDI-TOF MS identification using clinical samples [186–189]. A recent international study carried out in eight different laboratories also reported that this approach could achieve high inter-laboratory reproducibility [190]. Overall, these studies showed that MALDI-TOF MS has emerged as an efficient and reliable alternative method for bacterial identification. To date, MALDI-TOF MS identification still requires a growth step in order to obtain sufficient material for acquisition of mass spectra, and it is not able to identify all pathogens in mixed cultures. Nevertheless, along with further technical improvement and the potential to create inter-laboratory databases, MALDI-TOF MS technology will undoubtedly play a significant role in diagnostic microbiology in the near future.

References

1. Woo PC, Lau SK, Teng JL, Tse H, Yuen KY (2008) Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin Microbiol Infect 14:908–934
2. Woo PC, Tam DM, Leung KW et al (2002) *Streptococcus sinensis* sp. nov., a novel species isolated from a patient with infective endocarditis. J Clin Microbiol 40:805–810
3. Woo PC, Teng JL, Leung KW et al (2004) *Streptococcus sinensis* may react with Lancefield group F antiserum. J Med Microbiol 53:1083–1088

4. Woo PC, Teng JL, Lau SK, Yuen KY (2006) Clinical, phenotypic, and genotypic evidence for *Streptococcus sinensis* as the common ancestor of anginosus and mitis groups of streptococci. Med Hypotheses 66:345–351
5. Uckay I, Rohner P, Bolivar I et al (2007) *Streptococcus sinensis* endocarditis outside Hong Kong. Emerg Infect Dis 13:1250–1252
6. Faibis F, Mihaila L, Perna S et al (2008) *Streptococcus sinensis*: an emerging agent of infective endocarditis. J Med Microbiol 57:528–531
7. Yuen KY, Woo PC, Teng JL, Leung KW, Wong MK, Lau SK (2001) *Laribacter hongkongensis* gen. nov., sp. nov., a novel gram-negative bacterium isolated from a cirrhotic patient with bacteremia and empyema. J Clin Microbiol 39:4227–4232
8. Woo PC, Kuhnert P, Burnens AP et al (2003) *Laribacter hongkongensis*: a potential cause of infectious diarrhea. Diagn Microbiol Infect Dis 47:551–556
9. Lau SK, Woo PC, Hui WT et al (2003) Use of cefoperazone MacConkey agar for selective isolation of *Laribacter hongkongensis*. J Clin Microbiol 41:4839–4841
10. Woo PC, Lau SK, Teng JL et al (2004) Association of *Laribacter hongkongensis* in community-acquired gastroenteritis with travel and eating fish: a multicentre case–control study. Lancet 363:1941–1947
11. Teng JL, Woo PC, Ma SS et al (2005) Ecoepidemiology of *Laribacter hongkongensis*, a novel bacterium associated with gastroenteritis. J Clin Microbiol 43:919–922
12. Woo PC, Lau SK, Teng JL, Yuen KY (2005) Current status and future directions for *Laribacter hongkongensis*, a novel bacterium associated with gastroenteritis and traveller's diarrhoea. Curr Opin Infect Dis 18:413–419
13. Lau SK, Woo PC, Fan RY, Lee RC, Teng JL, Yuen KY (2007) Seasonal and tissue distribution of *Laribacter hongkongensis*, a novel bacterium associated with gastroenteritis, in retail freshwater fish in Hong Kong. Int J Food Microbiol 113:62–66
14. Ni XP, Ren SH, Sun JR et al (2007) *Laribacter hongkongensis* isolated from a patient with community-acquired gastroenteritis in Hangzhou City. J Clin Microbiol 45:255–256
15. Lau SK, Woo PC, Fan RY et al (2007) Isolation of *Laribacter hongkongensis*, a novel bacterium associated with gastroenteritis, from drinking water reservoirs in Hong Kong. J Appl Microbiol 103:507–515
16. Steer T, Collins MD, Gibson GR, Hippe H, Lawson PA (2001) *Clostridium hathewayi* sp. nov., from human faeces. Syst Appl Microbiol 24:353–357
17. Elsayed S, Zhang K (2004) Human infection caused by *Clostridium hathewayi*. Emerg Infect Dis 10:1950–1952
18. Woo PC, Lau SK, Woo GK, Fung AM, Yiu VP, Yuen KY (2004) Bacteremia due to *Clostridium hathewayi* in a patient with acute appendicitis. J Clin Microbiol 42:5947–5949
19. Linscott AJ, Flamholtz RB, Shukla D, Song Y, Liu C, Finegold SM (2005) Fatal septicemia due to *Clostridium hathewayi* and *Campylobacter hominis*. Anaerobe 11:97–98
20. Richter D, Schlee DB, Allgower R, Matuschka FR (2004) Relationships of a novel Lyme disease spirochete, *Borrelia spielmani* sp. nov., with its hosts in Central Europe. Appl Environ Microbiol 70:6414–6419
21. Fox GE, Magrum LJ, Balch WE, Wolfe RS, Woese CR (1977) Classification of methanogenic bacteria by 16S ribosomal RNA characterization. Proc Natl Acad Sci U S A 74:4537–4541
22. Gupta R, Lanter JM, Woese CR (1983) Sequence of the 16S ribosomal RNA from *Halobacterium volcanii*, an Archaebacterium. Science 221:656–659
23. Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains *Archaea, Bacteria*, and *Eucarya*. Proc Natl Acad Sci USA 87:4576–4579
24. Snel B, Bork P, Huynen MA (1999) Genome phylogeny based on gene content. Nat Genet 21:108–110
25. Qian Q, Tang YW, Kolbert CP et al (2001) Direct identification of bacteria from positive blood cultures by amplification and sequencing of the 16S rRNA gene: evaluation of BACTEC 9240 instrument true-positive and false-positive results. J Clin Microbiol 39:3578–3582

26. Li H, Turhan V, Chokhani L, Stratton CW, Dunbar SA, Tang YW (2009) Identification and differentiation of clinically relevant mycobacterium species directly from acid-fast bacillus-positive culture broth. J Clin Microbiol 47:3814–3820
27. Tang YW, Duplex PCR (2009) assay simultaneously detecting and differentiating *Bartonella quintana*, *B. henselae*, and *Coxiella burnetii* in surgical heart valve specimens. J Clin Microbiol 47:2647–2650
28. Hall L, Doerr KA, Wohlfiel SL, Roberts GD (2003) Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J Clin Microbiol 41:1447–1453
29. Bosshard PP, Abels S, Zbinden R, Bottger EC, Altwegg M (2003) Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). J Clin Microbiol 41:4134–4140
30. Bosshard PP, Abels S, Altwegg M, Bottger EC, Zbinden R (2004) Comparison of conventional and molecular methods for identification of aerobic catalase-negative gram-positive cocci in the clinical laboratory. J Clin Microbiol 42:2065–2073
31. Song Y, Liu C, Bolanos M, Lee J, McTeague M, Finegold SM (2005) Evaluation of 16S rRNA sequencing and reevaluation of a short biochemical scheme for identification of clinically significant *Bacteroides* species. J Clin Microbiol 43:1531–1537
32. Heikens E, Fleer A, Paauw A, Florijn A, Fluit AC (2005) Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. J Clin Microbiol 43:2286–2290
33. Lau SK, Ng KH, Woo PC et al (2006) Usefulness of the MicroSeq 500 16S rDNA bacterial identification system for identification of anaerobic Gram positive bacilli isolated from blood cultures. J Clin Pathol 59:219–222
34. Bosshard PP, Zbinden R, Abels S, Boddinghaus B, Altwegg M, Bottger EC (2006) 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory. J Clin Microbiol 44:1359–1366
35. Lau SK, Woo PC, Woo GK et al (2004) *Eggerthella hongkongensis* sp. nov. and *Eggerthella sinensis* sp. nov., two novel *Eggerthella* species, account for half of the cases of *Eggerthella* bacteremia. Diagn Microbiol Infect Dis 49:255–263
36. Lau SK, Woo PC, Fung AM, Chan KM, Woo GK, Yuen KY (2004) Anaerobic, non-sporulating, Gram-positive bacilli bacteraemia characterized by 16S rRNA gene sequencing. J Med Microbiol 53:1247–1253
37. Bosshard PP, Zbinden R, Altwegg M (2002) *Turicibacter sanguinis* gen. nov., sp. nov., a novel anaerobic, Gram-positive bacterium. Int J Syst Evol Microbiol 52:1263–1266
38. Woo PC, Lau SK, Chan KM, Fung AM, Tang BS, Yuen KY (2005) *Clostridium* bacteraemia characterised by 16S ribosomal RNA gene sequencing. J Clin Pathol 58:301–307
39. Lau SK, McNabb A, Woo GK et al (2007) *Catabacter hongkongensis* gen. nov., sp. nov., isolated from blood cultures of patients from Hong Kong and Canada. J Clin Microbiol 45:395–401
40. Würdemann D, Tindall BJ, Pukall R et al (2009) *Gordonibacter pamelaeae* gen. nov., sp. nov., a new member of the *Coriobacteriaceae* isolated from a patient with Crohn's disease, and reclassification of *Eggerthella hongkongensis* Lau et al. 2006 as *Paraeggerthella hongkongensis* gen. nov., comb. nov. Int J Syst Evol Microbiol 59:1405–1415
41. Colmegna I, Rodriguez-Barradas M, Rauch R, Clarridge J, Young EJ (2003) Disseminated *Actinomyces meyeri* infection resembling lung cancer with brain metastases. Am J Med Sci 326:152–155
42. Woo PC, Fung AM, Lau SK, Yuen KY (2002) Identification by 16S rRNA gene sequencing of *Lactobacillus salivarius* bacteremic cholecystitis. J Clin Microbiol 40:265–267
43. Woo PC, Fung AM, Lau SK, Hon E, Yuen KY (2002) Diagnosis of pelvic actinomycosis by 16S ribosomal RNA gene sequencing and its clinical significance. Diagn Microbiol Infect Dis 43:113–118

44. Woo PC, Fung AM, Lau SK, Wong SS, Yuen KY (2001) Group G beta-hemolytic streptococcal bacteremia characterized by 16S ribosomal RNA gene sequencing. J Clin Microbiol 39:3147–3155
45. Lam MM, Clarridge JE 3rd, Young EJ, Mizuki S (2007) The other group G *Streptococcus*: increased detection of *Streptococcus canis* ulcer infections in dog owners. J Clin Microbiol 45:2327–2329
46. Woo PC, Tse H, Chan KM et al (2004) "*Streptococcus milleri*" endocarditis caused by *Streptococcus anginosus*. Diagn Microbiol Infect Dis 48:81–88
47. Woo PC, Tam DM, Lau SK, Fung AM, Yuen KY (2004) *Enterococcus cecorum* empyema thoracis successfully treated with cefotaxime. J Clin Microbiol 42:919–922
48. Somers CJ, Millar BC, Xu J et al (2003) *Haemophilus segnis*: a rare cause of endocarditis. Clin Microbiol Infect 9:1048–1050
49. Lau SK, Woo PC, Mok MY et al (2004) Characterization of *Haemophilus segnis*, an important cause of bacteremia, by 16S rRNA gene sequencing. J Clin Microbiol 42:877–880
50. Lau SK, Woo PC, Chan BY, Fung AM, Que TL, Yuen KY (2002) *Haemophilus segnis* polymicrobial and monomicrobial bacteraemia identified by 16S ribosomal RNA gene sequencing. J Med Microbiol 51:635–640
51. Mak GC, Ho PL, Tse CW, Lau SK, Wong SS (2005) Reduced levofloxacin susceptibility and tetracycline resistance in a clinical isolate of *Haemophilus quentini* identified by 16S rRNA sequencing. J Clin Microbiol 43:5391–5392
52. Tang YW, Ellis NM, Hopkins MK, Smith DH, Dodge DE, Persing DH (1998) Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli. J Clin Microbiol 36:3674–3679
53. Lam JY, Wu AK, Ngai DC et al (2011) Three cases of severe invasive infections caused by *Campylobacter rectus* and first report of fatal *C. rectus* infection. J Clin Microbiol 49:1687–1691
54. Woo PC, Leung PK, Leung KW, Yuen KY (2000) Identification by 16S ribosomal RNA gene sequencing of an *Enterobacteriaceae* species from a bone marrow transplant recipient. Mol Pathol 53:211–215
55. Woo PC, Cheung EY, Leung K, Yuen K (2001) Identification by 16S ribosomal RNA gene sequencing of an *Enterobacteriaceae* species with ambiguous biochemical profile from a renal transplant recipient. Diagn Microbiol Infect Dis 39:85–93
56. Woo PC, Fung AM, Wong SS, Tsoi HW, Yuen KY (2001) Isolation and characterization of a *Salmonella enterica* serotype Typhi variant and its clinical and public health implications. J Clin Microbiol 39:1190–1194
57. Clarridge JE 3rd, Raich TJ, Sjosted A et al (1996) Characterization of two unusual clinically significant *Francisella* strains. J Clin Microbiol 34:1995–2000
58. Woo PC, Chong KT, Leung K, Que T, Yuen K (2001) Identification of *Arcobacter cryaerophilus* isolated from a traffic accident victim with bacteremia by 16S ribosomal RNA gene sequencing. Diagn Microbiol Infect Dis 40:125–127
59. Lau SK, Woo PC, Teng JL, Leung KW, Yuen KY (2002) Identification by 16S ribosomal RNA gene sequencing of *Arcobacter butzleri* bacteraemia in a patient with acute gangrenous appendicitis. Mol Pathol 55:182–185
60. Lau SK, Woo PC, Woo GK, Yuen KY (2002) Catheter-related *Microbacterium* bacteremia identified by 16S rRNA gene sequencing. J Clin Microbiol 40:2681–2685
61. Elshibly S, Xu J, McClurg RB et al (2005) Central line-related bacteremia due to *Roseomonas mucosa* in a patient with diffuse large B-cell non-Hodgkin's lymphoma. Leuk Lymphoma 46:611–614
62. Woo PC, Fung AM, Lau SK et al (2003) *Granulicatella adiacens* and *Abiotrophia defectiva* bacteraemia characterized by 16S rRNA gene sequencing. J Med Microbiol 52:137–140
63. Woo PC, Lau SK, Fung AM, Chiu SK, Yung RW, Yuen KY (2003) *Gemella* bacteraemia characterised by 16S ribosomal RNA gene sequencing. J Clin Pathol 56:690–693

64. Woo PC, Tse H, Wong SS et al (2005) Life-threatening invasive *Helcococcus kunzii* infections in intravenous-drug users and *ermA*-mediated erythromycin resistance. J Clin Microbiol 43:6205–6208
65. Lau SK, Woo PC, Woo GK et al (2006) Bacteraemia caused by *Anaerotruncus colihominis* and emended description of the species. J Clin Pathol 59:748–752
66. Lau SK, Woo PC, Li NK et al (2006) *Globicatella* bacteraemia identified by 16S ribosomal RNA gene sequencing. J Clin Pathol 59:303–307
67. Lau SK, Teng JL, Leung KW et al (2006) Bacteremia caused by *Solobacterium moorei* in a patient with acute proctitis and carcinoma of the cervix. J Clin Microbiol 44:3031–3034
68. Woo PC, Lau SK, Lin AW, Curreem SO, Fung AM, Yuen KY (2007) Surgical site abscess caused by *Lactobacillus fermentum* identified by 16S ribosomal RNA gene sequencing. Diagn Microbiol Infect Dis 58:251–254
69. Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol 38:3623–3630
70. Mignard S, Flandrois JP (2006) 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. J Microbiol Methods 67:574–581
71. Woo PC, Ng KH, Lau SK et al (2003) Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. J Clin Microbiol 41:1996–2001
72. Lau SK, Woo PC, Tse H, Leung KW, Wong SS, Yuen KY (2003) Invasive *Streptococcus iniae* infections outside North America. J Clin Microbiol 41:1004–1009
73. Koh TH, Kurup A, Chen J (2004) *Streptococcus iniae* discitis in Singapore. Emerg Infect Dis 10:1694–1696
74. Lau SK, Woo PC, Luk WK et al (2006) Clinical isolates of *Streptococcus iniae* from Asia are more mucoid and beta-hemolytic than those from North America. Diagn Microbiol Infect Dis 54:177–181
75. Sun JR, Yan JC, Yeh CY, Lee SY, Lu JJ (2007) Invasive infection with *Streptococcus iniae* in Taiwan. J Med Microbiol 56:1246–1249
76. Weinstein MR, Litt M, Kertesz DA et al (1997) Invasive infections due to a fish pathogen, *Streptococcus iniae*. N Engl J Med 337:589–594
77. Woo PC, Ngan AH, Lau SK, Yuen KY (2003) *Tsukamurella* conjunctivitis: a novel clinical syndrome. J Clin Microbiol 41:3368–3371
78. Woo PC, Fong AH, Ngan AH et al (2009) First report of *Tsukamurella* keratitis: association between *T. tyrosinosolvens* and *T. pulmonis* and ophthalmologic infections. J Clin Microbiol 47:1953–1956
79. Lau SK, Woo PC, Yim TC, To AP, Yuen KY (2003) Molecular characterization of a strain of group a streptococcus isolated from a patient with a psoas abscess. J Clin Microbiol 41:4888–4891
80. Chen PL, Lee NY, Yan JJ et al (2007) Prosthetic valve endocarditis caused by *Streptobacillus moniliformis*: a case of rat bite fever. J Clin Microbiol 45:3125–3126
81. Woo PC, Leung KW, Tsoi HW, Wong SS, Teng JL, Yuen KY (2002) Thermo-tolerant *Campylobacter fetus* bacteraemia identified by 16S ribosomal RNA gene sequencing: an emerging pathogen in immunocompromised patients. J Med Microbiol 51:740–746
82. Woo PC, Teng JL, Lau SK et al (2003) Analysis of a viridans group strain reveals a case of bacteremia due to lancefield group G alpha-hemolytic *Streptococcus dysgalactiae* subsp *equisimilis* in a patient with pyomyositis and reactive arthritis. J Clin Microbiol 41:613–618
83. Woo PC, Leung AS, Leung KW, Yuen KY (2001) Identification of slide coagulase positive, tube coagulase negative *Staphylococcus aureus* by 16S ribosomal RNA gene sequencing. Mol Pathol 54:244–247
84. Woo PC, Tsoi HW, Leung KW et al (2000) Identification of *Mycobacterium neoaurum* isolated from a neutropenic patient with catheter-related bacteremia by 16S rRNA sequencing. J Clin Microbiol 38:3515–3517

85. Lefevre P, Gilot P, Godiscal H, Content J, Fauville-Dufaux M (2000) *Mycobacterium intracellulare* as a cause of a recurrent granulomatous tenosynovitis of the hand. Diagn Microbiol Infect Dis 38:127–129
86. Heller R, Jaulhac B, Charles P et al (1996) Identification of *Mycobacterium shimoidei* in a tuberculosis-like cavity by 16S ribosomal DNA direct sequencing. Eur J Clin Microbiol Infect Dis 15:172–175
87. Woo PC, Li JH, Tang W, Yuen K (2001) Acupuncture mycobacteriosis. N Engl J Med 345:842–843
88. Woo PC, Leung KW, Wong SS, Chong KT, Cheung EY, Yuen KY (2002) Relatively alcohol-resistant mycobacteria are emerging pathogens in patients receiving acupuncture treatment. J Clin Microbiol 40:1219–1224
89. Kurabachew M, Wondimu A, Ryon JJ (1998) Reverse transcription-PCR detection of *Mycobacterium leprae* in clinical specimens. J Clin Microbiol 36:1352–1356
90. Phetsuksiri B, Rudeeaneksin J, Supapkul P, Wachapong S, Mahotarn K, Brennan PJ (2006) A simplified reverse transcriptase PCR for rapid detection of *Mycobacterium leprae* in skin specimens. FEMS Immunol Med Microbiol 48:319–328
91. Wilson KH, Blitchington R, Frothingham R, Wilson JA (1991) Phylogeny of the Whipple's-disease-associated bacterium. Lancet 338:474–475
92. Relman DA, Schmidt TM, MacDermott RP, Falkow S (1992) Identification of the uncultured bacillus of Whipple's disease. N Engl J Med 327:293–301
93. Fenollar F, Raoult D (2001) Molecular techniques in Whipple's disease. Expert Rev Mol Diagn 1:299–309
94. Fenollar F, Fournier PE, Raoult D, Gerolami R, Lepidi H, Poyart C (2002) Quantitative detection of *Tropheryma whipplei* DNA by real-time PCR. J Clin Microbiol 40:1119–1120
95. Fenollar F, Fournier PE, Robert C, Raoult D (2004) Use of genome selected repeated sequences increases the sensitivity of PCR detection of *Tropheryma whipplei*. J Clin Microbiol 42:401–403
96. Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS (1990) The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N Engl J Med 323:1573–1580
97. Regnery RL, Anderson BE, Clarridge JE 3rd (1992) Rodriguez-Barradas MC, Jones DC, Carr JH. Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. J Clin Microbiol 30:265–274
98. Maeda K, Markowitz N, Hawley RC, Ristic M, Cox D, McDade JE (1987) Human infection with *Ehrlichia canis*, a leukocytic rickettsia. N Engl J Med 316:853–856
99. Anderson BE, Dawson JE, Jones DC, Wilson KH (1991) *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J Clin Microbiol 29:2838–2842
100. Chen SM, Dumler JS, Bakken JS, Walker DH (1994) Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J Clin Microbiol 32:589–595
101. Kupferwasser LI, Darius H, Muller AM et al (2001) Diagnosis of culture-negative endocarditis: the role of the Duke criteria and the impact of transesophageal echocardiography. Am Heart J 142:146–152
102. Cursons RT, Jeyerajah E, Sleigh JW (1999) The use of polymerase chain reaction to detect septicemia in critically ill patients. Crit Care Med 27:937–940
103. Goldenberger D, Kunzli A, Vogt P, Zbinden R, Altwegg M (1997) Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. J Clin Microbiol 35:2733–2739
104. Wilck MB, Wu Y, Howe JG, Crouch JY, Edberg SC (2001) Endocarditis caused by culture-negative organisms visible by Brown and Brenn staining: utility of PCR and DNA sequencing for diagnosis. J Clin Microbiol 39:2025–2027
105. Millar B, Moore J, Mallon P et al (2001) Molecular diagnosis of infective endocarditis—a new Duke's criterion. Scand J Infect Dis 33:673–680
106. Gauduchon V, Chalabreysse L, Etienne J et al (2003) Molecular diagnosis of infective endocarditis by PCR amplification and direct sequencing of DNA from valve tissue. J Clin Microbiol 41:763–766

107. Grijalva M, Horvath R, Dendis M, Erny J, Benedik J (2003) Molecular diagnosis of culture negative infective endocarditis: clinical validation in a group of surgically treated patients. Heart 89:263–268
108. Bosshard PP, Kronenberg A, Zbinden R, Ruef C, Bottger EC, Altwegg M (2003) Etiologic diagnosis of infective endocarditis by broad-range polymerase chain reaction: a 3-year experience. Clin Infect Dis 37:167–172
109. Lang S, Watkin RW, Lambert PA, Bonser RS, Littler WA, Elliott TS (2004) Evaluation of PCR in the molecular diagnosis of endocarditis. J Infect 48:269–275
110. Lang S, Watkin RW, Lambert PA, Littler WA, Elliott TS (2004) Detection of bacterial DNA in cardiac vegetations by PCR after the completion of antimicrobial treatment for endocarditis. Clin Microbiol Infect 10:579–581
111. Breitkopf C, Hammel D, Scheld HH, Peters G, Becker K (2005) Impact of a molecular approach to improve the microbiological diagnosis of infective heart valve endocarditis. Circulation 111:1415–1421
112. Houpikian P, Raoult D (2005) Blood culture-negative endocarditis in a reference center: etiologic diagnosis of 348 cases. Medicine (Baltimore) 84:162–173
113. Kotilainen P, Heiro M, Jalava J et al (2006) Aetiological diagnosis of infective endocarditis by direct amplification of rRNA genes from surgically removed valve tissue. An 11-year experience in a Finnish teaching hospital. Ann Med 38:263–273
114. Xu J, Millar BC, Moore JE et al (2003) Employment of broad-range 16S rRNA PCR to detect aetiological agents of infection from clinical specimens in patients with acute meningitis—rapid separation of 16S rRNA PCR amplicons without the need for cloning. J Appl Microbiol 94:197–206
115. Schuurman T, de Boer RF, Kooistra-Smid AM, van Zwet AA (2004) Prospective study of use of PCR amplification and sequencing of 16S ribosomal DNA from cerebrospinal fluid for diagnosis of bacterial meningitis in a clinical setting. J Clin Microbiol 42:734–740
116. Xu J, Moore JE, Millar BC, Webb H, Shields MD, Goldsmith CE (2005) Employment of broad range 16S rDNA PCR and sequencing in the detection of aetiological agents of meningitis. New Microbiol 28:135–143
117. Deutch S, Pedersen LN, Podenphant L et al (2006) Broad-range real time PCR and DNA sequencing for the diagnosis of bacterial meningitis. Scand J Infect Dis 38:27–35
118. Welinder-Olsson C, Dotevall L, Hogevik H et al (2007) Comparison of broad-range bacterial PCR and culture of cerebrospinal fluid for diagnosis of community-acquired bacterial meningitis. Clin Microbiol Infect 13:879–886
119. Tsai JC, Teng LJ, Hsueh PR (2004) Direct detection of bacterial pathogens in brain abscesses by polymerase chain reaction amplification and sequencing of partial 16S ribosomal deoxyribonucleic acid fragments. Neurosurgery 55:1154–1162
120. Knox CM, Cevellos V, Dean D (1998) 16S ribosomal DNA typing for identification of pathogens in patients with bacterial keratitis. J Clin Microbiol 36:3492–3496
121. Domann E, Hong G, Imirzalioglu C et al (2003) Culture-independent identification of pathogenic bacteria and polymicrobial infections in the genitourinary tract of renal transplant recipients. J Clin Microbiol 41:5500–5510
122. Saglani S, Harris KA, Wallis C, Hartley JC (2005) Empyema: the use of broad range 16S rDNA PCR for pathogen detection. Arch Dis Child 90:70–73
123. Le Monnier A, Carbonnelle E, Zahar JR et al (2006) Microbiological diagnosis of empyema in children: comparative evaluations by culture, polymerase chain reaction, and pneumococcal antigen detection in pleural fluids. Clin Infect Dis 42:1135–1140
124. Rosey AL, Abachin E, Quesnes G et al (2007) Development of a broad-range 16S rDNA real-time PCR for the diagnosis of septic arthritis in children. J Microbiol Methods 68: 88–93
125. Fihman V, Hannouche D, Bousson V et al (2007) Improved diagnosis specificity in bone and joint infections using molecular techniques. J Infect 55:510–517
126. Sleigh J, Cursons R, La Pine M (2001) Detection of bacteraemia in critically ill patients using 16S rDNA polymerase chain reaction and DNA sequencing. Intensive Care Med 27: 1269–1273

127. Xu J, Moore JE, Millar BC et al (2004) Improved laboratory diagnosis of bacterial and fungal infections in patients with hematological malignancies using PCR and ribosomal RNA sequence analysis. Leuk Lymphoma 45:1637–1641
128. Marin M, Munoz P, Sanchez M et al (2007) Molecular diagnosis of infective endocarditis by real-time broad-range polymerase chain reaction (PCR) and sequencing directly from heart valve tissue. Medicine (Baltimore) 86:195–202
129. Schabereiter-Gurtner C, Nehr M, Apfalter P, Makristathis A, Rotter ML, Hirschl AM (2008) Evaluation of a protocol for molecular broad-range diagnosis of culture-negative bacterial infections in clinical routine diagnosis. J Appl Microbiol 104:1228–1237
130. Janda JM, Abbott SL (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol 45:2761–2764
131. Roth A, Fischer M, Hamid ME, Michalke S, Ludwig W, Mauch H (1998) Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. J Clin Microbiol 36:139–147
132. Ringuet H, Akoua-Koffi C, Honore S et al (1999) *hsp65* sequencing for identification of rapidly growing mycobacteria. J Clin Microbiol 37:852–857
133. Kasai H, Ezaki T, Harayama S (2000) Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. J Clin Microbiol 38:301–308
134. Adekambi T, Berger P, Raoult D, Drancourt M (2006) *rpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. Int J Syst Evol Microbiol 56:133–143
135. Kwok AY, Chow AW (2003) Phylogenetic study of *Staphylococcus* and *Macrococcus* species based on partial *hsp60* gene sequences. Int J Syst Evol Microbiol 53:87–92
136. Kwok AY, Su SC, Reynolds RP et al (1999) Species identification and phylogenetic relationships based on partial HSP60 gene sequences within the genus *Staphylococcus*. Int J Syst Bacteriol 49:1181–1192
137. Woo PC, Woo GK, Lau SK, Wong SS, Yuen K (2002) Single gene target bacterial identification. *groEL* gene sequencing for discriminating clinical isolates of *Burkholderia pseudomallei* and *Burkholderia thailandensis*. Diagn Microbiol Infect Dis 44:143–149
138. Woo PC, Lau SK, Woo GK et al (2003) Seronegative bacteremic melioidosis caused by *Burkholderia pseudomallei* with ambiguous biochemical profile: clinical importance of accurate identification by 16S rRNA gene and *groEL* gene sequencing. J Clin Microbiol 41: 3973–3977
139. Goh SH, Potter S, Wood JO, Hemmingsen SM, Reynolds RP, Chow AW (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. J Clin Microbiol 34:818–823
140. Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849
141. Palys T, Nakamura LK, Cohan FM (1997) Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int J Syst Bacteriol 47:1145–1156
142. Murray PR, Baron EJ, Pfaller MA, Jorgensen JH, Yolken RH (eds) (2003) Manual of clinical microbiology, 8th edn. American Society for Microbiology, Washington, DC
143. Woo PC, Chung LM, Teng JL et al (2007) In silico analysis of 16S ribosomal RNA gene sequencing-based methods for identification of medically important anaerobic bacteria. J Clin Pathol 60:576–579
144. Teng JL, Yeung MY, Yue G et al (2011) In silico analysis of 16S ribosomal RNA gene sequencing based methods for identification of medically important aerobic Gram-negative bacteria. J Med Microbiol 60(Pt 9):1281–6
145. Woo PC, Teng JL, Wu JK et al (2009) Guidelines for interpretation of 16S rRNA gene sequence-based results for identification of medically important aerobic Gram-positive bacteria. J Med Microbiol 58:1030–1036

146. Maidak BL, Cole JR, Parker CT Jr et al (1999) A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res 27:171–173
147. Maidak BL, Cole JR, Lilburn TG et al (2000) The RDP (Ribosomal Database Project) continues. Nucleic Acids Res 28:173–174
148. Maidak BL, Cole JR, Lilburn TG et al (2001) The RDP-II (Ribosomal Database Project). Nucleic Acids Res 29:173–174
149. Cole JR, Chai B, Farris RJ et al (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33:D294–D296
150. Cole JR, Chai B, Farris RJ et al (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res 35:D169–D172
151. Patel JB, Leonard DG, Pan X, Musser JM, Berman RE, Nachamkin I (2000) Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 16S rDNA bacterial identification system. J Clin Microbiol 38:246–251
152. Tang YW, Von Graevenitz A, Waddington MG et al (2000) Identification of coryneform bacterial isolates by ribosomal DNA sequence analysis. J Clin Microbiol 38:1676–1678
153. Harmsen D, Rothganger J, Singer C, Albert J, Frosch M (1999) Intuitive hypertext-based molecular identification of micro-organisms. Lancet 353:291
154. Harmsen D, Rothganger J, Frosch M, Albert J (2002) RIDOM: Ribosomal Differentiation of Medical Micro-organisms Database. Nucleic Acids Res 30:416–417
155. Harmsen D, Dostal S, Roth A et al (2003) RIDOM: comprehensive and public sequence database for identification of *Mycobacterium* species. BMC Infect Dis 3:26
156. Simmon KE, Croft AC, Petti CA (2006) Application of SmartGene IDNS software to partial 16S rRNA gene sequences for a diverse group of bacteria in a clinical laboratory. J Clin Microbiol 44:4400–4406
157. Pruesse E, Quast C, Knittel K et al (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196
158. Woo PC, Teng JL, Yeung JM, Tse H, Lau SK, Yuen KY (2011) Automated identification of medically important bacteria by 16S rRNA gene sequencing using a novel comprehensive database, 16SpathDB. J Clin Microbiol 49:1799–1809
159. Murray PR, Baron EJ, Louise MR, Jorgensen JH, Pfaller MA (eds) (2007) Manual of clinical microbiology, 9th edn. American Society for Microbiology, Washington, DC
160. Turenne CY, Tschetter L, Wolfe J, Kabani A (2001) Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. J Clin Microbiol 39:3637–3648
161. Cloud JL, Neal H, Rosenberry R et al (2002) Identification of *Mycobacterium* spp. by using a commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries. J Clin Microbiol 40:400–406
162. Mellmann A, Cloud JL, Andrees S et al (2003) Evaluation of RIDOM, MicroSeq, and Genbank services in the molecular identification of *Nocardia* species. Int J Med Microbiol 293:359–370
163. Cloud JL, Conville PS, Croft A, Harmsen D, Witebsky FG, Carroll KC (2004) Evaluation of partial 16S ribosomal DNA sequencing for identification of nocardia species by using the MicroSeq 500 system with an expanded database. J Clin Microbiol 42:578–584
164. Becker K, Harmsen D, Mellmann A et al (2004) Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. J Clin Microbiol 42:4988–4995
165. Patel JB, Wallace RJ Jr, Brown-Elliott BA et al (2004) Sequence-based identification of aerobic actinomycetes. J Clin Microbiol 42:2530–2540
166. Fontana C, Favaro M, Pelliccioni M, Pistoia ES, Favalli C (2005) Use of the MicroSeq 500 16S rRNA gene-based sequencing for identification of bacterial isolates that commercial automated systems failed to identify correctly. J Clin Microbiol 43:615–619
167. Zeaiter Z, Fournier PE, Ogata H, Raoult D (2002) Phylogenetic classification of *Bartonella* species by comparing *groEL* sequences. Int J Syst Evol Microbiol 52:165–171

168. La Scola B, Gundi VA, Khamis A, Raoult D (2006) Sequencing of the *rpoB* gene and flanking spacers for molecular identification of *Acinetobacter* species. J Clin Microbiol 44:827–832
169. Pham HN, Ohkusu K, Mishima N et al (2007) Phylogeny and species identification of the family *Enterobacteriaceae* based on *dnaJ* sequences. Diagn Microbiol Infect Dis 58:153–161
170. Mollet C, Drancourt M, Raoult D (1997) *rpoB* sequence analysis as a novel basis for bacterial identification. Mol Microbiol 26:1005–1011
171. Kawasaki S, Fratamico PM, Wesley IV, Kawamoto S (2008) Species-specific identification of *Campylobacters* by PCR-restriction fragment length polymorphism and PCR targeting of the gyrase B gene. Appl Environ Microbiol 74:2529–2533
172. Man SM, Kaakoush NO, Octavia S, Mitchell H (2010) The internal transcribed spacer region, a new tool for use in species differentiation and delineation of systematic relationships within the *Campylobacter* genus. Appl Environ Microbiol 76:3071–3081
173. Drancourt M, Raoult D (2002) *rpoB* gene sequence-based identification of *Staphylococcus* species. J Clin Microbiol 40:1333–1338
174. Poyart C, Quesne G, Trieu-Cuot P (2002) Taxonomic dissection of the *Streptococcus bovis* group by analysis of manganese-dependent superoxide dismutase gene (sodA) sequences: reclassification of '*Streptococcus infantarius* subsp. *coli'* as *Streptococcus lutetiensis* sp. nov. and of *Streptococcus bovis* biotype 11.2 as *Streptococcus pasteurianus* sp. nov. Int J Syst Evol Microbiol 52:1247–1255
175. Arbique JC, Poyart C, Trieu-Cuot P et al (2004) Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. J Clin Microbiol 42:4686–4696
176. Poyart C, Quesnes G, Trieu-Cuot P (2000) Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. J Clin Microbiol 38:415–418
177. Zbinden A, Köhler N, Bloemberg GV (2011) *recA*-based PCR assay for accurate differentiation of *Streptococcus pneumoniae* from other viridans streptococci. J Clin Microbiol 49:523–527
178. Poyart C, Quesne G, Coulon S, Berche P, Trieu-Cuot P (1998) Identification of streptococci to species level by sequencing the gene encoding the manganese-dependent superoxide dismutase. J Clin Microbiol 36:41–47
179. Weng FY, Chiou CS, Lin PH, Yang SS (2009) Application of *recA* and *rpoB* sequence analysis on phylogeny and molecular identification of *Geobacillus* species. J Appl Microbiol 107:452–464
180. Yamada-Noda M, Ohkusu K, Hata H et al (2007) Mycobacterium species identification—a new approach via *dnaJ* gene sequencing. Syst Appl Microbiol 30:453–462
181. Hong T, Butler WR, Hollis F et al (2003) Characterization of a novel rapidly growing *Mycobacterium* species associated with sepsis. J Clin Microbiol 41:5650–5653
182. Gray LD, Ketring KL, Tang YW (2005) Clinical use of 16S rRNA gene sequencing to identify *Mycoplasma felis* and *M. gateae* associated with feline ulcerative keratitis. J Clin Microbiol 43:3431–3434
183. Inokuma H, Brouqui P, Drancourt M, Raoult D (2001) Citrate synthase gene sequence: a new tool for phylogenetic analysis and identification of *Ehrlichia*. J Clin Microbiol 39:3031–3039
184. Roux V, Rydkina E, Eremeeva M, Raoult D (1997) Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the rickettsiae. Int J Syst Bacteriol 47:252–261
185. Ho CC, Lau CC, Martelli P et al (2011) Novel pan-genomic analysis approach in target selection for multiplex PCR identification and detection of *Burkholderia pseudomallei, Burkholderia thailandensis*, and *Burkholderia cepacia* complex species: a proof-of-concept study. J Clin Microbiol 49:814–821
186. Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G (2010) Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial

strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol 48:1549–1554
187. Cherkaoui A, Hibbs J, Emonet S et al (2010) Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. J Clin Microbiol 48:1169–1175
188. Eigner U, Holfelder M, Oberdorfer K, Betz-Wild U, Bertsch D, Fahr AM (2009) Performance of a matrix-assisted laser desorption ionization-time-of-flight mass spectrometry system for the identification of bacterial isolates in the clinical routine laboratory. Clin Lab 55:289–296
189. Seng P, Drancourt M, Gouriet F et al (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49:543–551
190. Mellmann A, Bimet F, Bizet C et al (2009) High interlaboratory reproducibility of matrix-assisted laser desorption ionization-time of flight mass spectrometry-based species identification of nonfermenting bacteria. J Clin Microbiol 47:3732–3734
191. Drancourt M, Roux V, Fournier PE, Raoult D (2004) *rpoB* gene sequence-based identification of aerobic Gram-positive cocci of the genera *Streptococcus, Enterococcus, Gemella, Abiotrophia*, and *Granulicatella*. J Clin Microbiol 42:497–504

Chapter 28
Molecular Techniques for Blood and Blood Product Screening

Yuan Hu

Introduction

The Food and Drug Administration (FDA) is responsible for ensuring the safety of the more than 15 million units of blood and blood components donated each year in the United States. "Blood banking has become a manufacturing industry, an industry that must conform to high standards and quality control requirements comparable to those of pharmaceutical companies or other regulated industries," said David A. Kessler, MD, former FDA commissioner [1]. Screening donated blood for infectious diseases that can be transmitted through blood transfusion is a very important step in ensuring safety. The United States has the safest blood supply in the world [1] and the FDA is striving to keep it safe by decreasing the risk of infectious disease transmission. The regulatory agency is continuously updating its requirements and standards for collecting and processing blood. As mentioned earlier, an important step in ensuring safety is the screening of donated blood for infectious diseases. In the United States, tests for infectious diseases are routinely conducted on each unit of donated blood, and these tests are designed to comply with regulatory requirements (Table 28.1). The field of clinical microbiology and virology are now focusing on molecular technology. Currently, nucleic acid testing techniques have been developed to screen blood and plasma products for evidence of very recent viral infections that could be missed by conventional serologic tests. It is time for all blood safety procedures to include molecular detection techniques.

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Y. Hu (✉)
U.S. Food and Drug Administration, Northeast Regional Laboratory,
158-15 Liberty avenue, Jamaica, NY 11433, USA
e-mail: yuan.hu@fda.hhs.gov

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Table 28.1 Licensed/approved clinical assays for infectious agents (Source: Center for Biologics Evaluation and Research, US Food and Drug Administration [48])

Tradename(s)	Format	Sample	Use	Manufacturer	Approval date
Antibody to hepatitis B surface antigen (HBsAg assay)					
Auszyme Monoclonal	EIA	Serum/Plasma/ Cadaveric serum	Donor screen and conf kit	Abbott Laboratories, Abbott Park, IL; US License 0043	4/1/1985
GS HBsAg EIA 3.0	EIA	Serum/Plasma/ Cadaveric serum	Donor screen and conf kit	Bio-Rad Laboratories, Redmond, WA; US License 1109	1/23/2003
ORTHO ANTIbody to HBsAg ELISA Test System 3	EIA	Serum/Plasma	Donor screen/diagnosis and conf kit	Ortho-Clinical Diagnostics, Inc, Raritan, NJ; US License 1236	4/23/2003
ABBOTT PRISM HBsAg, ABBOTT PRISM HBsAg Confirmatory	Chemiluminescent immunoassay (ChLIA)	Serum/Plasma	Donor screen	Abbott Laboratories, Abbott Park, IL; US License 0043	7/18/2006
Anti-HIV-1 oral specimen collection device					
OraSure HIV-1 Oral Specimen Collection Device	Oral specimen collection device	Oral fluid	For use with HIV diagnostic assays that have been approved for use with this device.	OraSure Technologies, Bethlehem, PA	12/23/1994
Anti-HIV-1 testing service					
Home Access HIV-1 Test System	Dried blood spot collection device	Dried blood spot	Diagnostic	Home Access Health Corp, Hoffman Estates, IL	7/22/1996
Hepatitis B surface antigen (anti-HBs assay)					
Ausab EIA	EIA	Serum/Plasma	Anti-HBs	Abbott Laboratories, Abbott Park, IL; US License 0043	11/18/1982
Hepatitis B virus core antigen (anti-HBc assay)					
CORZYME	EIA	Serum/Plasma	Donor screen	Abbott Laboratories, Abbott Park, IL; US License 0043	3/19/1991

Ortho HBc ELISA Test System	EIA	Serum/Plasma	Donor screen	Ortho-Clinical Diagnostics, Inc, Raritan, NJ; US License 1236	4/18/1991
ABBOTT PRISM HBcore	Chemiluminescent Immunoassay (ChLIA)	Serum/Plasma	Donor screen	Abbott Laboratories, Abbott Park, IL; US License 0043	10/13/2005
Hepatitis C virus encoded antigen (anti-HCV assay)					
Abbott HCV EIA 2.0	EIA	Serum/Plasma/ Cadaveric Serum	Donor screen	Abbott Laboratories, Abbott Park, IL; US License 0043	5/6/1992
Ortho HCV Version 3.0 ELISA Test System	EIA	Serum/Plasma	Donor screen	Ortho-Clinical Diagnostics, Inc, Raritan, NJ; US License 1236	5/20/1996
Chiron RIBA HCV 3.0 Strip Immunoblot Assay	SIA	Serum/Plasma	Donor supplemental	Chiron Corp, Emeryville, CA; US License 1106	2/11/1999
Nucleic acid testing					
Roche Amplicor HIV-1 Monitor Test	PCR	Plasma	Prognosis/Patient management; HIV-1 viral load assay	Roche Molecular Systems, Inc, Pleasanton, CA	3/2/1999
NucliSens HIV-1 QT	NASBA	Plasma	Prognosis/Patient management; HIV-1 viral load assay	bioMerieux, Inc, Durham, NC	11/19/2001
COBAS Ampliscreen HIV-1 Test	PCR	Plasma	Donor screen: expanded indications for use; Source: plasma donors, other living donors, and organ donors	Roche Molecular Systems, Inc, Pleasanton, CA; US License 1636	12/20/2002
Procleix HIV-1/HCV Assay	HIV-1/HCV nucleic acid test (TMA)	Plasma	Donor screen: expanded indications for use; Source: plasma donors, living organ donors, and cadaveric samples	Gen-Probe, San Diego, CA; US License 1592	6/4/2004
Trugene HIV-1 Genotyping Kit and Open Gene DNA Sequencing System	HIV-1 genotyping	Plasma	Patient monitoring	Siemens Medical Solutions Diagnostics, Berkeley, CA	4/24/2002

(continued)

Table 28.1 (continued)

Tradename(s)	Format	Sample	Use	Manufacturer	Approval date
UltraQual HIV-1 RT-PCR Assay	PCR	Plasma	Donor screen	National Genetics Institute, Los Angeles, CA 92121	9/18/2001
UltraQual HCV RT-PCR Assay	PCR	Plasma	Donor screen	National Genetics Institute, Los Angeles, CA 92121	9/18/2001
ViroSeq HIV-1 Genotyping System with the 3700 Genetic Analyzer	HIV-1 genotyping	Plasma	Patient monitoring	Celera Diagnostics, Alameda, CA	6/11/2003
Versant HIV-1 RNA 3.0 (bDNA)	Signal amplification nucleic acid probe	Plasma	Patient monitoring	Siemens Medical Solutions Diagnostics, Berkeley, CA	9/11/2002
COBAS AmpliScreen HCV Test	PCR	Plasma	Donor screen: expanded indications for use; Source: plasma donors, other living donors, and organ donors	Roche Molecular Systems, Inc, Pleasanton, CA; US License 1636	12/3/2002
COBAS AmpliScreen HBV Test	PCR	Plasma	Donor screen: indications for use; Source: plasma donors, other living donors, and organ donors	Roche Molecular Systems, Inc, Pleasanton, CA; US License 1636	4/21/2005
Procleix West Nile Virus (WNV) Assay	TMA	Plasma	Qualitative detection of West Nile virus (WNV) RNA	Gen-Probe, San Diego, CA; US License 1592	12/1/2005
APTIMA HIV-1 RNA Qualitative Assay	HIV-1 and HCV/Nucleic acid pooled testing/ Synthetic	Plasma	For use as an aid in diagnosis of HIV-1 infection, including acute or primary infection	Gen-Probe, Inc; US License 1592	10/4/2006
Procleix Ultrio Assay	TMA	Plasma and Serum	Qualitative detection of human immunodeficiency virus type 1 (HIV-1) RNA and hepatitis C virus (HCV) RNA	Gen-Probe, San Diego, CA; US License 1592	10/3/2006
Hepatitis C Virus RT-PCR assay	PCR	Plasma	Qualitative detection of HCV RNA	BioLife Plasma Services, L.P., Deerfield, IL; US License 1640	2/9/2007

Human Immunodeficiency Virus, Type 1 (HIV-1) Reverse Transcription (RT) Polymerase Chain Reaction (PCR) Assay	PCR	Plasma	Qualitative detection of HIV-1 RNA	BioLife Plasma Services, L.P., Deerfield, IL; US License 1640	1/31/2007
Abbott RealTime HIV-1 Amplification Kit	PCR	Plasma	Quantitation of human immunodeficiency virus type 1 (HIV- 1)	ABBOTT Molecular, Inc, Des Plaines, IL	5/11/2007
COBAS AmpliPrep/COBAS TaqMan HIV-1 Test	PCR	Plasma	Quantitation of human immunodeficiency virus type 1 (HIV-1) nucleic acid	Roche Molecular Systems, Inc, Pleasanton, CA	5/11/2007
COBAS TaqScreen West Nile Virus Test	PCR	Plasma	For the qualitative detection of WNV	Roche Molecular Systems, Inc, Pleasanton, CA; US License 1636	8/28/2007
Human immunodeficiency virus type 1 (anti-HIV-1 assay)					
GS rLAV EIA	IA	Serum/Plasma	Donor screen	Bio-Rad Laboratories Redmond, WA; US License 1109	6/29/1998
Cambridge Biotech HIV-1 Western Blot Kit	WB	Serum/Plasma	Donor supplemental	Calypte Biomedical Corp, Berkeley, CA; US License 1207	1/3/1991
GS HIV-1 Western Blot	WB	Serum/Plasma	Donor supplemental	Bio-Rad Laboratories, Redmond, WA; US License 1109	11/13/1998
Fluorognost HIV-1 IFA	IFA	Serum/Plasma	Donor supplemental	Waldheim Pharmazeutika GmbH, Vienna, Austria; US License 1150	2/5/1992
HIVAB HIV-1 EIA	EIA	Dried Blood Spot	Diagnostic	Abbott Laboratories	4/22/1992
HIV-1 Urine EIA	EIA	Urine Screen	Diagnostic	Calypte Biomedical Corp	8/6/1996

(continued)

Table 28.1 (continued)

Tradename(s)	Format	Sample	Use	Manufacturer	Approval date
GS rLAV EIA	EIA	Dried blood spot	Diagnostic	Bio-Rad Laboratories, Redmond, WA	6/29/1998
Cambridge Biotech HIV-1 Western Blot Kit	WB	Urine	Diagnostic supplemental	Maxim Biomedical, Inc	5/28/1998
GS HIV-1 Western Blot	WB	Dried blood spot	Diagnostic supplemental	Bio-Rad Laboratories, Redmond, WA	11/13/1998
OraSure HIV-1 Western Blot Kit	WB	Oral fluid	Diagnostic supplemental	OraSure Technologies, Bethlehem, PA	6/3/1996
Fluorognost HIV-1 IFA	IFA	Dried blood spot	Diagnostic supplemental	Waldheim Pharmazeutika GmbH	5/14/1996
Reveal Rapid HIV-1 Antibody Test	Rapid immunoassay	Serum/Plasma	Diagnostic	MedMira Laboratories, Inc; Halifax, Nova Scotia, Canada B3S 1B3	4/16/2003
Uni-Gold Recombigen HIV	Rapid immunoassay	Serum/Plasma/ Whole blood	Diagnostic	Trinity Biotech, plc; Bray Co., Wicklow, Ireland	12/23/2003
Human immunodeficiency virus types 1 and 2 (anti-HIV-1/2 assay)					
Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA	EIA	Serum/Plasma/ Cadaveric serum	Donor screen	Abbott Laboratories, Abbott Park, IL; US License 0043	2/14/1992
GS HIV-1/HIV-2 Plus O EIA	EIA	Serum/Plasma/ Cadaveric serum	Donor screen	Bio-Rad Laboratories, Redmond, WA; US License 1109	8/5/2003
Multispot HIV-1/HIV-2 Rapid Test	Rapid immunoassay	Plasma/Serum	Diagnostic	Bio-Rad Laboratories, Redmond, WA	11/12/2004

OraQuick ADVANCE Rapid HIV-1/2 Antibody Test	Rapid immunoassay	Whole blood, plasma, oral fluid	Diagnostic	OraSure Technologies, Bethlehem, PA	6/22/2004
ABBOTT PRISM HIV O Plus	Chemiluminescent immunoassay (ChLIA)	Serum/Plasma/ Cadaveric Serum	Screen	Abbott Laboratories	9/18/2009
ADVIA Centaur HIV 1/O/2 Enhanced ReadyPack Reagents	Microparticle chemilumino-metric immunoassay	Plasma/Serum	Diagnostic for qualitative determination of antibodies to the human immunodeficiency virus type 1, including Group O, and/or type 2	Siemens Medical Solutions Diagnostics, Tarrytown, NY	5/18/2006
HIV 1/2 STAT-PAK ASSAY SURE CHECK HIV 1/2 ASSAY	Rapid immunoassay	Fingerstick and venous whole blood, serum, plasma	Diagnostic	Chembio Diagnostic Systems, Inc, Medford, NY	5/25/2006
Human immunodeficiency virus type 2 (anti-HIV-2 assay)					
GS HIV-2 EIA	EIA	Serum/Plasma	Donor screen	Bio-Rad Laboratories, Redmond, WA; US License 1109	4/25/1990
Human T-lymphotropic virus types I and II (anti-HTLV-I/II assay)					
Abbott HTLV-I/HTLV-II EIA	EIA	Serum/Plasma	Donor screen	Abbott Laboratories, Abbott Park, IL; US License 0043	8/15/1997
ABBOTT PRISM HTLV-I/ HTLV-II	Chemiluminescent Immunoassay (ChLIA)	Serum/Plasma	Donor screen	Abbott Laboratories, Abbott Park, IL; US License 0043	1/16/2008
Trypanosoma cruzi (T. cruzi) (anti-T. cruzi assay)					
ORTHO *T. cruzi* ELISA Test System	EIA	Serum/Plasma	Donor screen	Ortho-Clinical Diagnostics, Inc, Raritan, NJ; US License 1236	12/13/2006

This approach can significantly aid in blood safety to reduce the risk of transmission of serious disease by transfusion. This chapter reviews the current antigen/antibody-based technology, molecular biological technology, and published regulatory policy data for blood safety.

Limitations for Current Technologies Used in Blood Safety

Direct detection of viral antigens and virus specific antibodies has been a common tool for the diagnosis of virus infections in the past 40 years. There are some limitations. For direct detection of virus antigens, shortly after virus infection, only a few viruses release antigens in amounts sufficiently detectable in the body by an antibody-mediated assay. For indirect virus detection by virus specific antibodies (e.g., an immunofluorescence assay or enzyme immunoassay (EIA), etc.), there is a problem in that shortly after infection by a pathogenic virus, there is a window period in which antibody generation is insufficient for detection [2]. To reduce this window period of low detection, direct nucleic acid tests are needed.

Application of Advanced Molecular Techniques in Blood Safety Applications

Through the application of molecular biology, biological and biochemical analyses have been revolutionized, and nucleic acid, gene-based techniques have been developed to screen blood and plasma donations for evidence of very recent and earlier viral infections that might otherwise be missed by conventional serologic testing. The nucleic acid tests can also provide evidence for genetic variation in viruses. Molecular methods include the use of nucleic acid probes as well as amplification-based and DNA sequence-based techniques. An increasing number of molecular diagnostic methods are now available commercially. In comparison to classical methods, molecular biological methods are superior in terms of rapidness, specificity, and sensitivity. The current nucleic acid detection methods in the field may be grouped into two major classes: amplifying techniques such as PCR and nonamplifying techniques such as Southern blot hybridization. Amplifying techniques are more sensitive than nonamplifying techniques. There are two different types of amplifying methods [3], target amplification methods and signal amplification methods. Target amplifying techniques include PCR, nucleic acid sequence-based amplification (NASBA) [4,5], self-sustaining sequence amplification (3SR), transcription-based amplification (TAS), transcription-mediated amplification (TMA), strand displacement amplification (SDA), and ligase chain reaction (LCR). Signal amplification methods include branched DNA (bDNA) signal amplification [6], cleavage-based signal amplification (cycling probe technologies and invader assay), Qß replicase, hybrid capture, cycling probe technologies (CPT), and rolling-circle amplification (RCA) [7]. To further insure the safety of blood products, it is of importance to further improve these and other types of nucleic acid testing.

Major Different Generations of Nucleic Acid Detection Techniques

Southern Blot Hybridization (1970s)

Southern blotting [8] was named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s. This technique is used to detect specific sequences within mixtures of DNA, which is size-fractionated by gel electrophoresis and then transferred by capillary action to a suitable membrane. After blocking of nonspecific binding sites, the nitrocellulose replica of the original gel electrophoresis experiment is then allowed to hybridize with an oligonucleotide probe representing the specific DNA sequence of interest. Should specific DNA be present on the blot, it will combine with the labeled probe and be detectable. By coelectrophoresing DNA fragments of known molecular weight, the size(s) of the hybridizing band(s) can then be determined. Southern blotting hybridization technology is one of the major tools that have already helped clinical staffs worldwide interpret genomic information. Other competing methodologies include in situ hybridization and solution hybridization. Important clinical examples of the use of this technology are DNA fingerprinting and the ability to detect DNA gene rearrangements.

Polymerase Chain Reaction (1980s)

In 1983, Dr. Kary Mullis at Cetus Corporation conceived of Polymerase Chain Reaction [9]. There is not a single technique that has had a greater impact on the practice of molecular biology than PCR. With this technique, we can detect infectious diseases agents at an extremely low level. It is based on the ability of sense and antisense DNA primers to hybridize to a DNA of interest. Following extension from the primers on the DNA template by DNA polymerase, the reaction is heat-denatured and allowed to anneal with the primers once again. Another round of extension leads to a multiplicative increase in DNA products. Therefore, a minute amount of DNA can be efficiently amplified in an exponential fashion to result in larger amounts of DNA that are more easily manipulated. By including critical controls, the technique can be made quantitative. The current level of the sensitivity and detection limit is as low as 10–50 copies per ml blood in HIV testing [1, 10, 11]. Important clinical examples of the use of PCR are detection of HIV and HCV [12–14]. PCR techniques have evolved into different branches. Some of them are now widely in use for virus detection in clinical diagnostics. These are real-time PCR by Taqman (Roche), Light Cycler (Roche) and Smart Cycler (Cepheid), and in situ PCR, nested-PCR, nested-real time PCR [15], broad-range PCR, multiplex PCR, RT-PCR, arbitrarily primer PCR, long PCR, and quantitative PCR. Real-time sequence technology will be coming soon for more detailed detection. In the past, identification of viral serotypes

was restricted to investigative methods using antibody detection and restriction fragment length polymorphism (RFLP). With real-time sequences technology, we will be able to detect a virus early as well as to obtain the viral sequence.

Microarrays (1990s)

Microarrays were developed at Stanford University by Schena and coworkers in the early 1990s [16]. For medical applications, a microarray analysis offers a very accurate screening technology. It allows hundreds or thousands of nucleic acid hybridization reaction to be performed on a solid substrate. It promises to be a fast and accurate diagnostic tool in the field of clinical microbiology and virology. Applied to infection safety for blood and blood products, it will be able to screen for the presence of viral pathogens by matching genetic sequences. Compared with existing technologies, it allows for a wider variety of specific tests to be carried out simultaneously to determine the quality of the blood and will provide consumers with extra safety. With the use of molecular biology protocols, the microarray will permit the detection of lower concentrations of microorganisms in the blood and the accurate identification of many types of pathogenic contaminants. In the near future, progress can be expected in the application of microarray technology for screening of donated blood for infectious agents. It can provide vast information about the identity of bloodborne pathogens as well as their gene expression profiles [17].

Screening of Donor Blood for Infectious Agents

To ensure a safe blood supply for those who may need a transfusion, an important step in ensuring safety is the screening of donated blood for infectious agents. After donation, each unit of donated blood undergoes a series of tests for bloodborne agents such as human immunodeficiency virus (HIV)-1, HIV-2, hepatitis B virus (HBV), hepatitis C virus (HCV), human T-Cell lymphotropic virus (HTLV)-1 and HTLV-II, West Nile virus (WNV), and *Treponema pallidum*, the agent of syphilis.

Confirmatory Testing of Donor Blood for Infectious Agents

All of the above tests are referred to as screening tests, and are designed to detect as many infectious agents as possible. Because these tests are so sensitive, some donors may have a false-positive result, even when the donor has never been exposed to the particular infection. In order to sort out true infections from such false-positive test results, screening tests that are reactive may be followed up with more specific tests

called confirmatory tests. Thus, confirmatory tests help determine whether a donor is truly infected. If any one of these tests fails, affected blood products are considered unsuitable for transfusion [18].

Application of Nucleic acid Testing for Infectious Agents

Nucleic acid testing (NAT) employs testing technology that directly detects the genomes of viruses. Because NAT detects a virus's genetic material instead of waiting for the body's response, the formation of antibodies, as with many current tests, it offers the opportunity to reduce the window period during which an infecting agent is undetectable by traditional tests [19], thus further improving blood safety. NAT will become the gold standard because of greater sensitivity compared to antibody tests.

Since 1999, NAT has been approved by the FDA and used to detect HIV-1 and HCV; this technology currently is under investigation for detecting other infectious disease agents. We know that for many viral infections, viral RNA appears very early in the infection, in 1–2 weeks, but the antibody does not appear until 10–12 weeks, e.g., HIV and HCV [20]. In order to virtually prevent infection by all the transfusion associated viruses, we need to detect the viruses in their window period, and a NAT or gene-based testing method is needed. NAT also provides an opportunity for the viral, e.g., HIV or HCV, infected donor to seek early treatment. On the other hand, NAT is not only a sensitive method, but also a rapid method which is suitable for a blood bank laboratory because the turnaround time for maintaining blood donations is extremely critical.

Hepatitis B Virus

The HBV is a highly infectious and often nonsymptomatic virus that is transmitted primarily through blood and blood-derived fluids and is a leading cause of liver infection worldwide. The World Health Organization (WHO) estimates that two billion people worldwide have been infected with HBV and 350,000,000 people are chronically infected. Chronic infection results in a high risk for liver cancer and cirrhosis of the liver, which cause about 1,000,000 deaths each year. Each year up to 200,000 people become newly infected in the United States alone. Since the beginning of screening for HBV in 1969, the rate of infection through blood transfusions has greatly decreased. However, as of 2000, HBV is still transmitted through blood transfusions in 1 out of 137,000 units of blood. One reason for this is that currently available blood screening technologies detect core antibodies or surface antigens, which appear up to 8 weeks after infection. Serologic tests for HBV include hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (HBcAb).

Hepatitis B Surface Antigen

HBV, which mainly infects the liver, has an inner core and an outer envelope (the surface). The HBsAg test detects the outer envelope, identifying an individual infected with the HBV. This virus can cause inflammation of the liver, and in the earliest stage of the disease, infected people may feel ill or even have yellow discoloration of the skin or eyes, a condition known as jaundice. Fortunately, most patients recover completely and test negative for HBsAg within a few months after the illness. A small percentage of people become chronic carriers of the virus, and in these cases, the test may remain positive for years. Chronically infected people can develop severe liver disease as time passes, and need to be followed carefully by an experienced physician. To reduce the occurrence of posttransfusion hepatitis, it is essential to screen all blood donations for HBsAg by the most sensitive and specific assays. Blood donations that are found to be reactive in the HBsAg test are automatically confirmed by the HBsAg confirmatory assay. If the specimen is neutralizable in the confirmatory test, the specimen is considered positive for HBsAg. HBsAg testing of donated blood has begun in 1975 (Table 28.1).

Currently, all blood donors are screened for HBsAg, but occasional transmission of HBV still occurs due to the inclusion of window period donations (i.e., blood from recently infected donors who are antibody negative but still viremic). Detection of early HBV infection of blood donors is still a major problem of blood transfusion. The current third-generation licensed HBsAg tests (mostly radioimmunoassay and enzyme immunoassays) can not detect HBV in the window period for HBV infection. This is a strong motivation for introducing molecular detection techniques to the field. There are some commercially available test methods for detecting HBV DNA in the market now, such as Chiron's Quantiplex HBV DNA [21], Digene's Hybrid Capture, Abbott's HBV DNA assay, and Roche's Amplicor HBV Monitor. Using these commercial hybridization or PCR-based assays, HBV DNA can be detected 1–3 weeks before the appearance of HBsAg [22]. Some chronically infected patients who have lost their HBsAg remain HBV DNA positive, but are disqualified as potential blood donors. Molecular detection of HBV DNA is more sensitive than current methods employed for HBsAg screening.

Antibodies to the Hepatitis B Core Antigen

Determination of antibodies to the hepatitis B core antigen (anti-HBc) (total) is also used to monitor the progress of the hepatitis B viral infection. Determination of anti-HBc (IgM) is employed to distinguish an acute hepatitis B infection from a chronic infection. The anti-HBc test developed in 1987 detects an antibody to the HBV that is produced during and after infection. If an individual has a positive anti-HBc test, but the HBsAg test is negative, it may mean that the person once had hepatitis B, but has recovered from the infection. Of the individuals with a positive test for anti-HBc, many have not been exposed to the HBV; thus, there is a frequent problem of false

positives. Although the individual may be permanently deferred from donating blood, it is unlikely that the person's health will be negatively affected. (Note: This antibody is not produced following vaccination against hepatitis B).

Hepatitis C Virus

The HCV is a member of the Flaviviridae family of viruses, which are associated with both human and animal diseases. Hepatitis caused by HCV is the most common chronic bloodborne infection in the United States. Over four million Americans are believed to be infected. HCV can also be transmitted through blood transfusion. HCV causes inflammation of the liver, and up to 80% of those exposed to the virus develop a chronic infection, which can lead to liver inflammation, cirrhosis, cancer, and death. Eventually, up to 20% of people with HCV may develop cirrhosis of the liver or other severe liver diseases. As in other forms of hepatitis, individuals may be infected with the virus, but may not realize they are carriers since they do not have any symptoms. Because of the risk of serious illness, people with HCV need to be followed closely by a physician with experience evaluating this infection. Since the first cloning of full-length HCV cDNA in 1989, significant progress has been made in characterizing its molecular biology [11]. But, the natural history of HCV infection is still largely unclear and current treatment options for patients are limited. There is no vaccine for HCV, and the only available treatment, a combination of alpha interferon and ribavirin, is efficacious in only a minority of patients [23]. The life cycle of the HCV is poorly understood due to the lack of an efficient cell culture system [24]. There is an urgent need to develop a highly sensitive detection method for studying possible extrahepatic sites for the replication of HCV. We have recently established a cell culture system for the replication of HCV by using human T and B leukemia cell lines [25]. This model should represent a valuable tool for the detailed study of the initial steps of the HCV replication cycle and for the evaluation of antiviral molecules. Currently, appropriate vaccine strategies for HCV have not been developed. Early detection and prevention of HCV infection are most important for blood safety.

It is a formidable task to design primers and probes for sensitive nucleic acid level diagnostic assays throughout the open reading frame of the HCV genome because of a high mutation rate in this genomic region. However, the untranslated region of about 341 nucleotides contains highly conserved domains which allows for stable primer design for qualitative and quantitative diagnostic tests which have equivalent sensitivity against the known six various genotypes of HCV.

Antibodies to the Hepatitis C Virus

In 1990, the first specific test for HCV, the major cause of "non-A, non-B" hepatitis was introduced. Now, a third generation ELISA kit is available to detect antibodies to HCV and screening blood for HCV antibodies is recommended. These assays are

based on detection of serum antibody to various HCV antigens because these antibodies are nearly universally present in patients who are chronically infected with HCV [26]. The HCV screening tests are known to have significant limitations and positive samples should be further tested by HCV confirmatory tests.

HCV Confirmatory Tests

Guidelines provided by the CDC recommend that HCV antibody screening test positive samples should be confirmed with serologic or nucleic acid supplemental testing. HCV confirmatory tests include the recombinant immunoblot assay in which several recombinant peptide antigens are applied on a strip that is then probed with the patient's serum. In this way, the response to individual antigens can be recognized, and some false-positive ELISA results can be eliminated (e.g., RIBA, Chiron HCV 3.0, and PCR assay) (e.g., Roche COBAS AMPLICOR HCV Test, version 2.0). Laboratories can choose to perform this testing on all positive specimens or based on screening test positive (signal to cutoff) ratios. The positive predictive values (s/co) can vary depending on the prevalence of infection in the population being screened.

HCV antibodies are not generally detectable for at least 6 weeks and may not appear for several months. Acute HCV infections are relatively rare among blood donors, but the antibody tests often fail to detect these patients in the window period between the time of infection and the time of appearance of antibody detectable by the above assays. High sensitivity detection of HCV during the window period is a long-term technical challenge in the field. Tests for HCV RNA genome detection based on the PCR or other highly sensitive RNA detection systems have been used for the diagnosis of acute hepatitis [26]. Sensitive detection of HCV RNA based on RT-PCR or other nucleic acid amplification techniques can be readily accomplished with kits that are now available commercially. For example, in 1999 the FDA approved Roche's Amplicor HIV-1 Monitor Ultra Sensitive quantitative assay. It can measure HIV levels at as few as 50 copies/mL and another commercial kit, the LCx HIV RNA Quantitative Assay from Abbott Laboratories, also has a detection limit at 50 copies/mL. Some studies even showed a sensitivity limit at 1 copy [27]. In fact, a qualitative assay should be much more sensitive than a quantitative assay for HIV/HCV screening. A sensitive qualitative HCV molecular detection assay will possibly interdict and virtually prevent all transfusion-associated HIV/HCV. The current sensitivity standard for clinical diagnostics is 100 copies/mL, but since there has been an improvement in technology, this would be the time to change sensitivity standard to 50 copies/mL.

Human Retroviruses

Antibodies to Human Immunodeficiency Virus, Types 1 and 2

HIV-1 and/or HIV-2 virus cause acquired immunodeficiency syndrome, or AIDS. The test is designed to detect antibodies directed against antigens of the HIV-1 or

HIV-2 viruses. HIV-1 is much more common in the United States, whereas HIV-2 is prevalent in Western Africa. Donors are tested for both viruses because both are transmitted by infected blood, and a few cases of HIV-2 have been identified in US residents. In1985, the first blood-screening EIA test to detect HIV was licensed and quickly implemented by blood banks to protect the blood supply. In 1992, testing of donor blood for both HIV-1 and HIV-2 antibodies (anti-HIV-1 and anti-HIV-2) was implemented. In 1996, HIV p24 antigen testing of donated blood was mandated. Now, the p24 antigen testing is going to be compared with a PCR-based test for their ability to detect HIV in the window period.

Antibodies to Human T-Lymphotropic Virus, Types I and II

HTLV retroviruses are endemic in Japan and the Caribbean but relatively uncommon in the United States. They cause adult T-cell leukemia/lymphoma and a neurological disorder similar to multiple sclerosis. The infection can persist for a lifetime but rarely causes major illnesses in most people who are infected. In rare instances, the virus may, after many years of infection, cause nervous system disease or an unusual type of leukemia. HTLV-II infections are usually associated with intravenous drug usage, especially among people who share needles or syringes. Disease associations with HTLV-II have been hard to confirm, but the virus may cause subtle abnormalities of immunity that lead to frequent infections, or rare cases of neurological disease.

In 1989, human-T-lymphotropic-virus-antibody testing of donated blood was begun. Blood is now routinely screened for antibodies to HTLV-I/II. These test screens for antibodies directed against epitopes of the HTLV-I/II viruses. Several commercial assays based on the enzyme-linked immunosorbent assay (ELISA) or particle agglutination formats are used for screening of HTLV antibodies, followed by confirmatory assays using western blotting. In some infected individuals, the serologic response to HTLV infection is very low. These problems have been solved by the application of PCR amplification of specific sequences in the virus genome. PCR can be used to detect HTLV-I/II proviruses and is now the method of choice for detection of HTLV DNA directly from blood and many other tissues. Commercial PCR kits for HTLV are available [28].

West Nile Virus

The WNV is a single-stranded RNA virus of the Flaviviridae family and is the most recent emerging infectious disease threat to public health and, potentially, to the safety of our blood supply. In 2002, WNV was identified as transfusion transmissible. It is transmitted by mosquitoes to birds and other animals through a mosquito bite. The virus can infect people, horses, many types of birds, and some other animals. WNV was shown in 2002 to be transmissible by blood [29], with an estimated mean risk of 2/10,000–5/10,000 in outbreak regions in the United States. The most common symptoms of transfusion-transmitted cases of WNV were fever and headache.

Detection of WNV includes either a measurement of WNV antibodies or of WNV nucleic acid (detecting genetic material from the virus itself). There are two types of WNV antibody testing: IgM and IgG. In most individuals, IgM antibodies will be present within 8 days after the initial exposure to WNV, followed by IgG production several weeks later. But, the antibodies tested to detect WNV are not expedient for donor blood screening. NAT involves amplifying and measuring the WNV's genetic material to detect the presence of the virus in blood or tissue. WNV NAT will be negative in the blood once clinical illness has occurred. In this situation, both NAT and IgM antibody testing may be needed. Nucleic acid tests to screen blood for WNV are commercially available and in current use. But, the viral yield for WNV infection is much lower than other viruses. Consequently, a more sensitive WNV NAT system for donor blood screening will be required, which could further reduce the risks of transfusion transmitted WNV.

Syphilis

Serum samples from all blood units should be subjected to either the Venereal Disease Research Laboratory (VDRL) test or a treponemal test, such as the *T. pallidum haemagglutination* (TPHA) test before transfusion. Any unit found positive should be discarded as per standard safety procedures. This test is done to detect evidence of infection with the spirochete that causes syphilis. Blood centers began testing for this shortly after World War II, when syphilis rates in the general population were much higher. The risk of transmitting syphilis through a blood transfusion is exceedingly small (no cases have been recognized in this country for many years) because the infection is very rare in blood donors, and because the spirochete is fragile and unlikely to survive blood storage conditions. Sensitivity and specificity of serologic tests vary depending on the type of test performed and the stage of the disease. If the donor has spirochetemia, their serologic tests are usually negative, and if the donors are antibody positive, their blood is not infectious. Syphilis serological tests for donors have less clinical significance. A nucleic acid test for accurately detecting syphilis is needed. It can be used to determine whether a blood donor is currently or has recently been infected with the spirochete.

Other Concerns

Hepatitis Viruses

In recent years, numerous infectious agents found worldwide have been identified as potential threats to the blood supply and among these are several newly discovered hepatitis viruses that present unique challenges in assessing possible risks. Even if the hepatitis virus test is negative for all known A–E hepatitis agents, there

are some unidentified hepatitis viruses, called non A–E hepatitis viruses that can still be transmitted by blood transfusion. In the future, advances in NAT may allow rapid discovery of the unknown hepatitis viruses.

Hepatitis Delta Virus

Hepatitis delta virus (HDV) is a small RNA virus that can infect only individuals who have HBV; worldwide more than 15 million people are coinfected [30]. HDV is clinically important because it generally makes HBV infections more damaging to the liver. Increased understanding of the molecular virology of HDV will identify novel therapeutic targets for this most severe form of chronic viral hepatitis. PCR and real-time PCR methods are available for HDV RNA detection [31].

TT Virus

TT virus (TTV) [32], named for the patient from whom it was first isolated with non-A–E and G posttransfusion hepatitis in Japan in 1997, is a newly discovered transfusion transmitted, single-stranded and circular DNA virus [33]. TTV is non-enveloped and its entire sequence of ~3.9 kb has been determined. It is also often interpreted as a transfusion-transmitted virus [32]. At least 16 genotypes have been identified, and TTV is now found all over the world. TTV infection was sought by detection of TTV DNA in serum by polymerase chain reaction using primers generated from a conserved region of the TTV genome, e.g., the UTR region [34]. Donor blood and blood product can be screened for TTV DNA by using PCR or real-time PCR. The significance of positive findings is still unclear, because high level TTV carriers in healthy populations are currently found [35, 36]. Whether TTV actually causes hepatitis remains to be determined.

Cytomegalovirus

Cytomegalovirus (CMV) is a virus belonging to the herpes group that is rarely transmitted by blood transfusion. Donor blood is not routinely tested for CMV, and the prevalence of CMV antibody ranges from 50 to 80 % of the population. But, blood contaminated with CMV can cause problems in neonates or immunocompromised patients. It also remains a major pathogen for solid-organ transplant recipients causing febrile syndromes, hepatitis, pneumonitis, retinitis and colitis. Potential problems in selected patient populations can be prevented by transfusing CMV negative blood or frozen, deglycerolized red blood cells. Serologic tests for antibody to CMV are useful for determining whether a patient had CMV infection in the past, a determination of great clinical importance for organ and blood donors, and in the pretransplant evaluation of prospective transplant recipients [37]. Commercial NAT kits are available for CMV [3], and these include the Amplicor PCR CMV Monitor test and Hybrid capture system CMV DNA test.

Malaria

Sensitive screening tests for malaria are neither commercially available nor officially approved yet. The most effective way of screening donors is to take a proper history of malaria or of fever that could be due to malaria. Donor selection criteria should be designed to exclude potentially infectious individuals from donating red blood cells for transfusion. Because there are no practical laboratory tests available to test donor blood, donors traveling to high risk malaria areas are excluded from donating blood for 6 months. However, there is a need to develop suitable screening tests, especially for use in an endemic area. A number of clinical research approaches have been developed for the extraction, amplification and detection of malaria parasite DNA from blood products [37].

Variant Creutzfeldt–Jakob Disease

Variant Creutzfeldt–Jakob disease (vCJD—a rare but fatal brain infection) [38] was first described in 1996 in the United Kingdom. vCJD is strongly linked with exposure to the bovine spongiform encephalopathy (BSE) agent. BSE is a transmissible spongiform encephalopathy (TSE) affecting cattle and was first reported in the UK in 1986. It has different clinical and pathologic characteristics from classic vCJD. Each disease also has a particular genetic profile of the prion protein gene. In recent years, questions have been raised concerning the potential risk of vCJD disease for recipients of plasma-derived clotting factors, including United States licensed Factor Eight (pdFVIII), Factor Nine (pdFIX), and other plasma-derived products such as immune globulins and albumin. In the past 10 years, there have been some reported cases of probable vCJD transmission by red blood cell transfusions in the United Kingdom. Prion infections are associated with long and clinically silent incubations. The number of asymptomatic individuals with vCJD prion infection is unknown, posing risk to others through blood transfusion, blood products, organ or tissue grafts, and contaminated medical instruments. In order to decrease the risk, there is a need to establish a blood-based molecular assay for detection of vCJD prion infection. Recently research papers have shown that sensitivity detection methods are available for vCJD prion [39]. However, commercial detection kits are not yet available.

Dengue Viruses

The Dengue virus (DENV) is a member of the virus family Flaviviridae and is transmitted to people through the bite of an infected mosquito. The Dengue virus has been shown to have four subtypes. These subtypes are different strains of dengue virus that have 60–80 % homology between each other. Dengue has emerged as a worldwide problem only since the 1950s. With more than one-third of the world's population living in areas at risk for transmission, dengue infection is a leading

cause of illness and death in the tropics and subtropics. According to CDC, as many as 100 million people are infected yearly. Dengue is caused by any one of four related viruses transmitted by mosquitoes. There are not yet any vaccines to prevent DENV infection, and the most effective protective measure is to avoid mosquito bites. There have been healthcare-related transmissions, including transmission by blood products [40]. Dengue infection has a viremic phase that lasts 4–8 days, and blood collected during this phase may be infective when transfused into susceptible hosts [40]. There are currently no tests for direct detection of dengue virus, but there are however, commercial ELISA tests to detect antibodies of the dengue virus in blood samples from patients. Recently, research papers have shown that PCR detection methods are available for any dengue virus strain [41].

Babesia Species

Babesia is a protozoan parasite of the blood that causes a hemolytic disease known as Babesiosis. Babesiosis is a malaria-like parasitic disease, and there are over 100 species of *Babesia* identified. In the United States, *Babesia microti* is the agent most commonly reported to cause human infection. Clinical confusion between human babesiosis and malaria is often reported in literature [42]. *Babesia* infection can also be acquired by blood transfusion. In fact, there have been many cases of transfusion-induced babesiosis documented [43]. Risk of developing this clinical infection is increased for elderly, asplenic, or immunosuppressed patients. Current standards issued by the American Association of Blood Banks (AABB) require the indefinite deferral of a blood donor with a history of babesiosis. [44] There is a need to develop methods for identification *B. microti* in order to reduce the risk of transmission of babesiosis by transfusion. Diagnosis depends upon finding parasites on blood film examination which can be detected 2–4 weeks after a tick bite. Hamster inoculation and serology have also been used for diagnosis. The Indirect Fluorescent Antibody Test (IFAT) is available for *B. microti* and is the most useful serological test for early diagnosis. Also, the PCR screen tests for Babesiosis are technically available in the field [45].

Chagas' Disease

Chagas disease is named after the Brazilian physician Carlos Chagas, who discovered the disease in 1909. Chagas disease is spread mainly by blood-sucking insects infected with *Trypanosoma cruzi*. Chagas disease can also be spread through blood transfusion, organ transplants, and from a mother to an unborn child. National screening of the blood supply was instituted in early 2007 by FDA, and more than 1,000 donors with *T. cruzi* infection have been identified within the past 3 years of testing. "Screening for *T. cruzi* is an important safety measure to help protect our blood supply and to help prevent the spread of Chagas disease," says Karen Midthun, M.D., acting director of the FDA's Center for Biologics Evaluation and Research.

Currently, serological ELISA tests are available for diagnose chronic Chagas disease. PCR test is not a tool for diagnosis of chronic Chagas disease in clinical practice yet, although some research results have showed that PCR is a very sensitive parasitological test for Chagas' disease in active transmission regions [46]. More studies are needed for the development of this molecular method.

Severe Acute Respiratory Syndrome

Coronavirus is an RNA virus known to be associated with respiratory disease. Severe acute respiratory syndrome (SARS) is a newly recognized coronavirus whose genome sequence does not belong to any of the known coronavirus groups and which quickly spread all over the world from Asia in 2003. There has been no evidence that this infection is transmitted from blood donors to transfusion recipients, but the virus associated with SARS is present in the blood of people who are sick, and it is possible that the virus could be present in blood immediately before a person gets sick, so that an individual with infection but no symptoms possibly could transmit SARS through a blood donation. To help determine whether or not an individual might be infected with SARS, a blood collection facility will ask a potential donor orally or in writing about any travel to a SARS-affected country or a history of SARS or possible exposure to SARS. Enzyme-linked immunoassays for detection of specific IgG and IgM antibodies and RT-PCR for detection of SARS coronavirus specific RNA in the SARS patients has been developed. Rapid, sensitive, and specific identification of SARS and other novel coronaviruses by molecular methods will be very important in the future.

Discovery of Unrecognized and Uncharacterized Viral Agents

Based on past history, it is not just a hypothetical risk that many people have been infected with unrecognized viruses, for example, many patients with symptoms of non A–E, G, and TTV posttransfusion hepatitis. It is still possible that unexplained cases of posttransfusion hepatitis may be caused by a new, undiscovered pathogen. In recent years, numerous new infectious agents found worldwide have been identified through time-consuming procedures. By the time a new virus, such as HCV, HIV and SARS, is found, many people are infected and there could be a large number of fatalities. There is an urgent need to develop methods for rapid identification and characterization of previously unknown pathogenic viruses. The most recent technologies for detecting and identifying previously unrecognized pathogens are expression library screening, representational difference analysis (RDA), and broad-range polymerase chain reaction (BR-PCR). But they are all time-consuming approaches. The new unrecognized and uncharacterized viral agents can be rapid identified by some of the new molecular approaches, e.g., subtraction hybridization [47] and DNA microarray.

Conclusion

Ensuring the safety and efficacy of blood and blood products is a critical regulatory challenge. The high safety level of the blood supply is the result of continued improvements in blood donor screening and testing. It will be achieved by introducing more updated nucleic acid tests to the field of blood safety. NAT is a method of testing blood that is more sensitive and specific than conventional tests that require the presence of antibodies to trigger a positive test result. Also, NAT works by detecting the low levels of viral genetic material present when an infection occurs but before the body develops an immune response to a virus. This improved sensitivity should enable us to significantly decrease the infection window period, allowing for earlier detection of the infection and diminishing the chances for transmission of the agent via transfusion. We are to protect the blood supply from not only known pathogens but also the emergence of new and unrecognized and uncharacterized infectious agents. The NAT methods are more sensitive and specific compared with non-NAT. In the future, NAT technology, such as PCR, may allow routine screening of donors for all the known and unknown pathogens of concern to blood safety.

References

1. Revelle M (1995) Progress in blood supply safety. FDA Cons 29:21–24
2. Chamberland ME (2001) Emerging infectious disease issues in blood safety. Emerg Infect Dis 7:552–553
3. Hayden RT (2004) (2004). In vitro nucleic acid amplification techniques. In: Persing DH, Tenover FC, Versalovic J, Tang YW, Unger ER, Relman DA, White TJ (eds) Molecular microbiology: diagnostic principles and practice. ASM, Washington, DC, pp 43–69
4. Guichon A, Chiparelli H, Martinez A, Rodriguez C, Trento A, Russi JC, Carballal G (2004) Evaluation of a new NASBA assay for the qualitative detection of hepatitis C virus based on the NucliSens Basic Kit reagents. J Clin Virol 29:84–91
5. Starkey WG, Millar RM, Jenkins ME, Ireland JH, Muir KF, Richards RH (2004) Detection of piscine nodaviruses by real-time nucleic acid sequence based amplification (NASBA). Dis Aquat Organ 5:93–100
6. Peter JB, Sevall JS (2004) Molecular-based methods for quantifying HIV viral load. AIDS Patient Care STDS 18:75–79
7. Tang YW, Procop GW, Persing DH (1997) Molecular diagnostics of infectious diseases. Clin Chem 1997(43):2021–2038
8. Mornet E (2004) DNA, 50 years of the double helix: from the concept of molecular hybridization to microarrays. Gynecol Obstet Fertil 3:895–899
9. Persing DH (1993) In vitro nucleic acid amplification techniques. In: Persing DH, Smith TF, Tenover FC, White TJ (eds) Diagnostic molecular microbiology principles and applications. ASM, Washington, DC, pp 51–87
10. Ginocchio CC, Kemper M, Stellrecht KA, Witt DJ (2003) Multicenter evaluation of the performance characteristics of the nucliSens HIV-1 QT assay used for quantitation of human immunodeficiency virus type 1 RNA. J Clin Microbiol 41:164–173
11. Lamballerie XD (1996) Serological and molecular biology screening techniques for HCV infection. Nephrol Dial Transplant 4:9–11

12. Katsoulidou AS, Moschidis ZM, Gialeraki RE, Paraskevis DN, Sypsa VA, Lazanas MC, Tassopoulos NC, Psichogiou MA, Boletis JN, Karafoulidou AS, Hatzakis AE (2004) Clinical evaluation of an HIV-1 and HCV assay and demonstration of significant reduction of the HCV detection window before seroconversion. Transfusion 44:59–66
13. Roche Amplicor (1999) HIV-1 Monitor UltraSensitive quantitative assay. Roche, Nutley
14. LCx HIV RNA quantitative assay from Abbott Laboratories. Abbott Laboratories, North Chicago
15. Hu Y, Arsov I (2009) Nested real-time PCR for hepatitis A detection. Lett Appl Microbiol 49:615–619
16. Schena M (ed) (2002) Introduction to microarray analysis. In: Microarray analysis. Wiley, Hoboken, pp. 1–26
17. Yu X, Susa M, Knabbe C, Schmid RD, Bachmann TT (2004) Development and validation of a diagnostic DNA microarray to detect quinolone-resistant Escherichia coli among clinical isolates. J Clin Microbiol 42:4083–4091
18. U.S. Food & Drug Administration (2004) Testing requirements for communicable disease agents. Federal Register (21CFR 610:40), pp. 75–80.
19. Stramer SL, Glynn SA, Kleinman SH, Strong DM, Sally C, Wright DJ, Dodd RY, Busch MP (2004) Detection of HIV-1 and HCV infections among antibody-negative blood donors by nucleic acid-amplification testing. N Engl J Med 19:819–822
20. CDC (1997) Immunization of health-care workers: recommendations of the advisory committee on immunization practices (ACIP) and the hospital infection control practices advisory committee (HICPAC). MMWR Morb Mortal Wkly Rep 46:1–42
21. Krajden M, Comanor L, Rifkin O, Grigoriew A, Minor JM, Kapke GF (1998) Assessment of hepatitis B virus DNA stability in serum by the Chiron quantiplex branched-DNA assay. J Clin Microbiol 36:382–386
22. Hollinger FB, Jake T (2001) Hepatitis B virus (Chap. 87). Section two: specific virus families. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields virology, 4th edn. Lippincott Williams & Wilkins, Philadelphia, pp 2971–3036
23. Wang QM, Heinz BA (2001) Recent advances in prevention and treatment of hepatitis C virus infections. Prog Drug Res Spec No. 79–110
24. Cohen J (1999) The scientific challenge of hepatitis C. Science 285:26–30
25. Hu Y, Shahidi A, Park S, Guilfoyle D, Hirshfield I (2003) Detection of extrahepatic HCV replication by a novel highly sensitive single tube nested-PCR. Am J Clin Pathol 119:95–100
26. Major ME, Rehermann B, Feinstone SM (2001) Hepatitis C viruses (Chap. 34). Section two: specific virus families. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields virology, 4th edn. Lippincott Williams & Wilkins, Philadelphia, pp 1127–1161
27. Palmer S, Wiegand AP, Maldarelli F, Bazmi H, Mican JM, Polis M, Dewar RL, Planta A, Liu S, Metcalf JA, Mellors JW, Coffin JM (2003) New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 41:4531–4536
28. Green PL, Chen ISY (2001) Human T-cell leukemia virus types 1 and 2 (Chap. 58). Section two: specific virus families. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields virology, 4th edn. Lippincott Williams & Wilkins, Philadelphia, pp 1941–1969
29. Biggerstaff BJ, Peterson LR (2003) Estimated risk of transmission of the West Nile Virus through blood transfusion in the US, 2002. Transfusion 43:1007–1017
30. Hughes SA, Wedemeyer H, Harrison PM (2011) Hepatitis delta virus. Lancet 378(9785):73–85
31. Gal FL, Gordien E, Affolabi D, Hanslik T, Alloui C, Deny P, Gault E (2005) Quantification of hepatitis delta virus RNA in serum by consensus real-time PCR indicates different patterns of virological response to interferon therapy in chronically infected patients. J Clin Microbiol 43(5):2363–2369
32. Cossart Y (2000) TTV-a virus searching for a disease. J Clin Virol 17:1–3

33. Okamoto H, Nishizawa T, Ukita M (1999) A novel unenveloped DNA virus (TT virus) associated with acute and chronic non-A to G hepatitis. Intervirology 42:192–204
34. Das K, Kar P, Gupta R, Das B (2004) Role of transfusion-transmitted virus in acute viral hepatitis and fulminant hepatic failure of unknown etiology. J Gastroenterol Hepatol 19:406–412
35. Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M (1997) A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun 241:92–97
36. Blut A (2000) TT virus. Infus Ther Transfus Med 27:114–116
37. Pass RF (2001) Cytomegalovirus (Chapter 77). Section two: specific virus families. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields virology, 4th edn. Lippincott Williams & Wilkins, Philadelphia, pp 2676–2705
38. Stramer SL, Hollinger FB, Katz LM, Kleinman S, Metzel PS, Gregory KR, Dodd RY (2009) Emerging infectious disease agents and their potential threat to transfusion safety. Transfusion 49:1S–29S
39. Julie Ann Edgeworth JA, Farmer M, Sicilia A, Tavares P, Beck J, Campbell T, Lowe J, Mead S, Rudge P, Collinge J, Jackson GS (2011) Detection of prion infection in variant Creutzfeldt-Jakob disease: a blood-based assay. Lancet 377(9764):487–493
40. Wilder-Smith A, Chen LH, Massad E, Wilson ME (2009) Threat of dengue to blood safety in dengue-endemic countries. Emerg Infect Dis 15(1):8–11
41. Gijavanekar C, Añez-Lingerfelt M, Feng C, Putonti C, Fox GE, Sabo A, Fofanov Y, Willson RC (2011) PCR detection of nearly any dengue virus strain using a highly sensitive primer cocktail. FEBS J 278:1676–1687
42. Clark IA, Jacobson LS (1998) Do babesiosis and malaria share a common disease process? Ann Trop Med Parasitol 92(4):483–488
43. Gubernot DM, Lucey CT, Lee KC, Conley GB, Holness LG, Wise RP (2009) Babesia infection through blood transfusions: reports received by the US Food and Drug Administration, 1997–2007. Clin Infect Dis 48:25–30
44. Leiby DA (2011) Transfusion-transmitted Babesia spp.: bull's-eye on Babesia microti. Clin Microbiol Rev 24(1):14–28
45. Ano H, Makimura S, Harasawa R (2001) Detection of Babesia species from infected dog blood by polymerase chain reaction. J Vet Med Sci 63(1):111–113
46. Wincker P, Telleria J, Bosseno MF, Cardoso MA, Marques P, Yaksic N, Aznar C, Liegeard P, Hontebeyrie M, Noireau F, Morel CM, Breniere SF (1997) PCR-based diagnosis for Chagas' disease in Bolivian children living in an active transmission area: comparison with conventional serological and parasitological diagnosis. Parasitology 114:367–373
47. Hu Y, Hirshfield I (2005) Rapid approach to identify an unrecognized viral agent. J Virol Methods 127:80–86
48. Center for Biologics Evaluation and Research, U.S. Food & Drug Administration. (2010). Complete list of donor screening assays for infectious agents and HIV diagnostic assays

Chapter 29
Molecular Diagnostics of Sexually Transmitted Diseases

Angus C.T. Lo and Kai Man Kam

Introduction

Molecular techniques for identifying and detecting microorganisms have been proven readily adaptable for use in the clinical diagnostic laboratory. Sexually transmitted diseases (STDs) constitute the commonest infectious diseases globally and bear significant consequences for the individual as well as public health of the community. The Centers for Disease Control and Prevention (CDC) estimates there are 19 million new STD cases each year. Nearly half of the cases occur in individuals between the ages of 15 and 24 years. In particular, cases of chlamydia and gonorrhea exceeded 1.2 million reported in 2009 and some estimates suggest that over half of the new chlamydia and gonorrhea infections remain undiagnosed [1]. Globally, an estimated one million new cases of bacterial sexually transmitted infections (STIs) occur each day, and half a million babies die in sub-Saharan Africa alone each year due to congenital syphilis [1, 2]. In the last decade, rapid development of molecular techniques have gradually shifted the paradigm of laboratory diagnosis from traditional biological to molecular detection of major agents of STIs.

A milestone in biotechnology heralded the beginning of molecular diagnostics was the development of the polymerase chain reaction (PCR) by Mullis and colleagues [1, 3]. Since then, numerous molecular detection techniques have been designed to detect specific nucleic acids without relying on the ability to culture or directly observe intact organisms. As a result, stringency in transport of clinical samples is less strict. With automation, a faster turnaround time of molecular tests

A.C.T. Lo
Department of Health, Centre for Health Protection, Kowloon, Hong Kong

K.M. Kam (✉)
Public Health Laboratory Services Branch, Department of Health,
Centre for Health Protection, Kowloon, Hong Kong

Public Health Laboratory Centre, 382 Nam Cheong Street, Room 731, Kowloon, Hong Kong
e-mail: kmkam@dh.gov.hk

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became a significant advantage that enhances this paradigm shift. Silent pathogens, such as human papillomavirus (HPV) that cannot be cultivated in vitro, can now be detected and typed by using molecular techniques that can also determine oncogenic potential and prognostic outcome of different infections [4, 5]. These powerful molecular techniques have a significant impact on strategies and public health programs designed for the control and prevention of STDs worldwide.

An estimated 50% of STDs occur asymptomatically, and this forms a major reservoir of infectious source that persists in the community. More sensitive detection techniques are often required for detecting asymptomatic individuals with low microbial load [6]. Currently available molecular techniques using nucleic acid amplification and hybridization can now offer high sensitivity in screening for these infections and disrupt the transmission chains within the community leading to decrease in case burden and ultimately eliminate the reservoir of infections.

This paper intends to review the currently developed and available molecular diagnostics of common STDs including (1) *Chlamydia trachomatis* and *Neisseria gonorrhoeae*; (2) *Treponema pallidum*; (3) *Haemophilus ducreyi*; (4) *Mycoplasma* and *Ureaplasma*; (5) *Trichomonas vaginalis*; and (6) Herpes simplex virus (HSV).

Chlamydia trachomatis and Neisseria gonorrhoeae

C. trachomatis and *N. gonorrhoeae* infections are the two most prevalent STDs worldwide. These two infections accounted for over 1.5 million cases of STIs reported by CDC [1] *C. trachomatis* is the causative agent of nongonococcal urethritis, epididymitis, proctitis, cervicitis, and pelvic inflammatory disease. Asymptomatic infections are common in men and women. Routine screening for chlamydia is commended by CDC on sexually active teenagers and adults of ≤24 years [1]. *N. gonorrhoeae* is the etiological agent of gonorrhea. In the United States, gonorrhea is the second most commonly reported notifiable disease leading to serious outcomes in women, such as tubal infertility, ectopic pregnancy, and chronic pelvic pain. There is an estimated 700,000 new *N. gonorrhoeae* infections every year [7]. Since coinfections of *C. trachomatis* and *N. gonorrhoeae* are common, many diagnostic test platforms or systems were designed for simultaneously detecting both microorganisms.

Several FDA-approved commercial detection kits currently available for *C. trachomatis* and *N. gonorrhoeae* include nucleic acid hybridization, signal amplification, PCR, strand displacement amplification (SDA), and transcription-mediated amplification (TMA).

Nucleic Acid Hybridization

Nucleic acid hybridization is a molecular technique based on annealing of complementary nucleic acid strands on a stable double-strand nucleic acid without amplification.

The Gen-Probe PACE 2NG and PACE 2C assays (Gen-Probe, San Diego, CA) are two commercially available systems using hybridization [8, 9]. Both assays have been approved by the Food and Drug Administration (FDA) in the United States for detecting *N. gonorrhoeae*. The PACE 2NG assay uses a chemiluminescent acridinium ester-labeled probe targeting the 16S ribosomal RNA of *N. gonorrhoeae*, while the PACE 2C assay uses combination probes to screen for presence of both 16S ribosomal RNAs of *C. trachomatis* and *N. gonorrhoeae* in a single-tube assay. The performance of the PACE 2C was found to be similar to PACE 2NG for detecting *N. gonorrhoeae* in endocervical specimens [10]. Sensitivity and specificity of these assays for both *C. trachomatis* and *N. gonorrhoeae* were found to be 96.3–100 and 98.8–99.6% [10, 11].

Signal Amplification

The Hybrid Capture II (HC2) (Digene Corporation, Gaithersburg, MD) is a signal amplification test targeting both genomic DNA and cryptic plasmid DNA sequences of *C. trachomatis* and *N. gonorrhoeae* [12, 13]. The RNA–DNA hybrids are captured by hybrid-specific antibodies in microtiter plates and detected in luminometer by adding chemiluminescent substrate with initial positive results. The sensitivity of the HC2 CT/NG assay was 95% for detection of *C. trachomatis* and *N. gonorrhoeae* in endocervical specimens, while the specificity was found to be greater than 98% compared with *N. gonorrhoeae* culture [12, 13].

Polymerase Chain Reaction

Several in-house PCR assays targeting different regions of *C. trachomatis* and *Neisseria gonorrhoeae* (NG) were developed (Table 29.1). The target regions for *C. trachomatis* detection include the cryptic plasmid [14], major outer membrane protein (MOMP) [15, 16], cysteine-rich protein [17], a protein from the phospholipase D endonuclease superfamily (CT157) [18], and 16S rRNA genes [19, 20]. A new variant of *C. trachomatis* with a 377-bp deletion in the cryptic plasmid has been reported in Halland County, Sweden [21]. A new real-time PCR assay was developed to detect this new variant [22]. The gene encoding outer membrane protein III (*ompIII*) [23, 24], the *cppB* gene [25, 26], the *opa* gene [25, 27], and the *porA* pseudogene [28] of *N. gonorrhoeae* are the target regions used in several PCR assays. Specificity and sensitivity of the *ompIII* assay were 96.4 and 78.6% [24]. No false-positive or false-negative results have been described in *ompIII* PCR assays [25]. Using a coded panel of 500 DNA samples, the *cppB* gene missed in 5.8% of NG strains, and therefore the *cppB* gene, to be an unsuitable target [25]. The *opa* and *porA* pseudogene have been validated as suitable confirmatory test for positive nucleic acid amplification tests [28, 29].

The Cobas Amplicor CT/NG assay (Roche Molecular Systems, Branchburg, NJ) is an FDA-approved nucleic acid amplification test for detecting *C. trachomatis* and

Table 29.1 In-house PCR assays of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

Organism	Target regions	Primer and probe sequences	References
C. trachomatis	Cryptic plasmid	CT2A: 5′-CGCATGCAAGATATCGAGTATGCGTTGTTAGG-3′ CT2B: 5′-GACCGGCCTCTAGCGCTGCG-3′	[14]
	Cryptic plasmid (377-bp deletion)	Probe: swCT: FAM-GGATCCGTTTGTTCTGG-MGB ForwardswCT:5′-TCCGGATAGTGAATTATAGAGACTATTTAATC-3′ Reverse swCT: 5′-GGTGTTTTACTAGAGGACTTACCTCTTC-3′	[22]
	Cysteine-rich protein	Forward: 5′-CAAACTCATCAGACGAG-3′ Reverse: 5′-CCTTCTTTAAGAGGTTTTACCC-3′	[17]
	Major outer membrane protein (MOMP)	Forward: 5′-GACTTTGTTTTCGACCGTGTT-3′ Reverse: 5′-ACATAATACATCAAATCGATCCCA-3′	[15, 16]
	Phospholipase D endonuclease superfamily (CT157)	P1: 5′-TCTTTTTAAACCTCCGGAACCCACTT P2: 5′-GGATGGCATCGCATAGCATTCTTTG	[18]
	16s RNA	Forward: 5′-AGCAATTGTTTCGGCAATTG-3′ Reverse: 5′-CACATAGACTCTCCCTTAAC-3′	[19, 20]
N. gonorrhoeae	Outer membrane protein III (*ompIII*)	Forward: 5′-CGTCGGCATCGCTTTTG-3′ Reverse: 5′-CAGGCTGTTCATGCGGTAGTC-3′	[23, 24]
	cppB gene	Forward: 5′-GCTASCGCATACCCGCGTTGC-3′ Reverse: 5′-CGAAGACCTTCGAGCAGACA-3′	[25, 26]
	opa gene	papTM-P: FAM-CGCCTATACGCCTGCTACTTTCACGC-BHQ1 papTM-F: 5′-CAGCATTCAATTTGTTCCGAGTC-3′ papTM-R: 5′-GAACTGGTTTCATCTGATTACTTTCCA-3′	[25]
	porA pseudogene	GcopaF: FAM-CCGATATAATCGCTCCTTCAACATCAG-TAMRA GcopaF: 5′-TTGAAACACCGCCCGGAA-3′ GcopaR: 5′-TTTCGGCTCCTTATTCGGTTTAA-3′	[25]

N. gonorrhoeae [18]. The targets for assay are a 207 bp sequence within a cryptic plasmid in *C. trachomatis*, and a 210 bp sequence in the cytosine methyltransferase gene of *N. gonorrhoeae*. Internal control target DNA is available for coamplification in each reaction to detect the presence of inhibitors in each specimen. Sensitivity and specificity of this assay were ranged from 94.2 to 98.1% and 98.4 to 100% respectively [30, 31]. However, it has been reported that the Cobas Amplicor CT/NG assay for *N. gonorrhoeae* cross-reacts with certain strains of nonpathogenic *Neisseria* species, such as *N. subflava*, *N. lactamica*, and *N. cinerea* [32]. Consequently, supplementary confirmatory testing or *N. gonorrhoeae* nucleic acid amplification test is now widely used [33, 34]. In 2006, a new variant of *C. trachomatis* (nvCT) with 377 bp deletion in the cryptic plasmid was discovered in Sweden that was not detectable with Cobas Amplicor [21, 35]. It becomes a strong drawback on this assay because the proportion of nvCT was found to be 20–64% of the detected Chlamydia cases in the Swedish counties [36].

The Cobas 4800 CT/NG assay (Roche Molecular Systems, Branchburg, NJ) is a fully automated real-time PCR system for detection of *C. trachomatis* and *N. gonorrhoeae*, while the Cobas TaqMan CT assay is for detection of *C. trachomatis* only. This multiplex real-time PCR assay has incorporated a dual-target strategy for detecting *C. trachomatis*, including a 206 bp conserved sequence in cryptic plasmid of *C. trachomatis* and 182 bp sequence of the MOMP gene, to ensure accurate and reliable detection of Chlamydia, including the variant strain found in Sweden. The *N. gonorrhoeae* assay was newly designed to target a direct repeat region called DR-9. This target region has two highly conserved sequence variations and is repeated three times on the genome. The sensitivity and specificity of this assay on urine and swab samples were ranged from 92.0 to 94.5% and 99.5 to 100%, respectively, for *C. trachomatis*, and 92.9–100% and 99.4–100%, respectively, for *N. gonorrhoeae* [37].

Another commercially available real-time PCR assay for detection of *C. trachomatis* and *N. gonorrhoeae* is the Abbott RealTime CT/NG assay (Abbott Molecular, Des Plaines, IL) [38, 39]. The assay contains two sets of primers targeting a 122 bp conserved sequence in the *C. trachomatis* cryptic plasmid and a 140 bp sequence located outside the deleted region of nvCT. The target region for *N. gonorrhoeae* is a 122 bp conserved sequence in *N. gonorrhoeae* opacity (Opa) gene. The sensitivity and specificity of this assay were ranged from 92.4 to 99.6% and 99.2 to 99.7% for *C. trachomatis* and from 96.9 to 100% and 99.7 to 99.8% for *N. gonorrhoeae*, respectively [38, 40].

Strand Displacement Amplification

The SDA is a multiplex isothermal DNA target amplification method. The BD ProbeTec ET *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (CT/NG) Amplification DNA Assays and Probe Tec GC Q^{x} assay (Becton Dickinson, Franklin Lakes, NJ) utilize SDA technology and fluorescent resonance energy transfer probes targeting DNA sequence homologous to the cryptic plasmid in CT and different

regions within the multicopy pilin gene-inverting protein homologue. The assays can be performed using either a semi-automated BD ProbeTec ET System or an automated BD VIPER System. The sensitivity and specificity of the BD ProbeTec assays were ranged from 95.2 to 100% and 92.6 to 100% for *C. trachomatis* and from 84.9 to 98.5% and 92.5 to 98.6% for *N. gonorrhoeae*, respectively [23, 41–43].

Transcription-Mediated Amplification

The APTIMA Combo 2 Assay (Gen-Probe, San Diego, CA) is a target amplification nucleic acid probe test combining target capture, TMA, and dual kinetic assay (DKA) technologies. This assay replicates a specific region of the 23S rRNA from CT and a specific region of the 16S rRNA from GC using unique sets of primers. The target rRNA molecules are isolated by the use of capture oligomers and separated with magnetic particles. The rRNA amplification products are detected by hybridization with chemiluminescent single-strand DNA probes and an enzyme-labeled DNA–RNA duplex antibody. Sensitivity and specificity for CT were ranged from 94.2 to 100% and 97.6 to 100% in swab and urine, while the corresponding values for GC were ranged from 91.3 to 99.2% and 98.7 to 99.3% [44, 45].

Confirmation Tests for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

As false-positives and decreased positive predictive values may be a problem in low-prevalence populations, the Centers for Disease Control (CDC) has recommended that the confirmatory tests should be performed when the positive predictive value is less than 90% [46]. According to CDC guidelines, less-sensitive diagnostic tests should not be used for confirmatory tests for more-sensitive nucleic acid amplification tests. There are four approaches to additional molecular confirmatory tests: (1) testing a second specimen with a different test using different target; (2) testing the original specimen with a different test that uses a different target or format; (3) repeating the original test on the original specimen with a blocking antibody or competitive probe; and (4) repeating the original test on the original specimen [46]. The APTIMA CT assay (ACT), which detects *C. trachomatis*, the APTIMA GC assay (AGC), which detects *N. gonorrhoeae*, and the APTIMA Combo 2 assay (AC2), which detects both pathogens, are three assays from Gen-Probe, Inc. The ACT and AGC oligonucleotide probes target rRNA sequences different from those for AC2. Therefore, these three assays can be used as confirmatory tests for each other assay [47, 48]. Besides, two previously published in-house real-time assays targeting *opa* and *porA* pseudogene were also used as independent real-time PCR confirmatory assays in some laboratories [27, 47, 49].

Treponema pallidum

Syphilis is a chronic and multistage STI caused by the spirochaete *Treponema pallidum* subspecies *pallidum*, which continues to be a worldwide public health problem with estimated 12 million new cases per year [50]. Syphilis has three main stages: primary, secondary, and tertiary. Owing to the slow generation time and the inability to survive and multiply outside the mammalian body, despite repeated attempts over the years, *T. pallidum* cannot be cultured in vitro or stained using simple techniques. Dark-field microscopic examination in lesion exudate or tissue and serology are the definitive methods for diagnosing early syphilis. The presumptive diagnosis of syphilis is possible by using nontreponemal serological tests, such as rapid plasma reagin (RPR) and Venereal Disease Research Laboratory (VDRL), and treponemal tests, such as fluorescent treponemal antibody absorption (FTA) and *T. pallidum* hemagglutination assay (TPHA). However, these tests may be problematic in the early stages of primary syphilis, as both serological tests and microscopic examinations are limited by low degrees of sensitivity and specificity [51].

Polymerase Chain Reaction

PCR provides a fast and reliable alternative for the diagnosis and rapid identification of the disease (primary, secondary, and tertiary syphilis), specifically in settings in which dark-field microscopy cannot be performed [52–55]. Although no commercial *T. pallidum* detection test is available, some laboratories provide locally developed PCR tests for the detection of *T. pallidum*. Several PCR-based tests of *T. pallidum* were developed on the basis of membrane lipoproteins [56], *tmpA* and *4D* genes [57], 16S rRNA [58], *tp47* gene [59], and DNA polymerase I (*polA*) gene [52, 60] (Table 29.2). The levels of detection of these assays ranged between 10^{-3} organisms by reverse-transcriptase PCR (RT-PCR) [58] to 10–50 organisms by amplifying the gene fragment encoding the 47 kDa membrane lipoprotein [56]. A multiplex PCR assay for simultaneous detection of *H. ducreyi*, *T. pallidum*, and HSV type 1 and 2 was developed to give three diagnoses from one assay in a single genital ulcer swab specimen [56]. A real-time PCR assay was also developed, which was based on this multiplex PCR assay to detect *H. ducreyi* and *T. pallidum* [61].

Two real-time PCR assays using TaqMan probes targeting *polA* gene of *T. pallidum* were developed [62, 63] (Table 29.2). The assays provide a robust, sensitive, and specific assay to directly detect the presence of *T. pallidum* with the short turnaround time and the ease of performance. Compared directly with serology, one real-time PCR assay showed 95% agreement with a sensitivity of 80.4% and a specificity of 98.4% [62], while the other real-time PCR assay reported high sensitivities and specificities of 94–100% using two real-time PCR platforms, the Rotor-Gene (QIAGEN Inc., Valencia, CA) and iCycler (Bio-Rad Laboratories, Hercules, CA) [64]. However, the real-time PCR assay had a disappointingly low sensitivity (43%) for the detection of secondary syphilis that it has no added value for the clinical diagnosis of secondary syphilis, even though the specificity was high (98%) [63].

Table 29.2 PCR assays of *Treponema pallidum*

Target regions	Primer and probe sequences	References
Membrane lipoproteins	Forward: 5′-GACAATGCTCACTGAGGATAGT-3′ Reverse: 5′-ACGCACAGAACCGAATTCCTTG-3′	[56]
tmpA and 4D genes	Forward: 5′-CAGGTAACGGATGCTGAAGT-3′ Reverse: 5′-AACGCCTCCATCGTCAGACC-3′	[57]
16S rRNA	Forward: 5′-CTCTTTTGGACGTAGGTCTTTGAG-3′ Reverse: 5′-TTACGTGTTACCGCGGCGTGG-3′	[58]
tp47 gene	Forward: 5′-CGTGTGGTATCAACTATGG-3′ Reverse: 5′-TCAACCTGTACTCAGTGC-3′	[59]
DNA polymerase I (*polA*) gene	Forward: 5′-AGACGGCTGCACATCTTCTCCA-3′ Reverse: 5′-AGCAGACGTTACATCGAGCGGA-3′	[60]
	SyphTP: 6-Carboxyfluorescein-ATGCACCAGCTTCGA-MGB SyphTF: 5′-AGGATCGCCCATATGTCCAA-3′ SyphTR: 5′-GTGAGCGTCTCATCATTCCAAA-3′	[62]

Haemophilus ducreyi

Chancroid is a genital ulcer caused by *H. ducreyi* and is prevalent mainly in developing countries, such as the countries in Africa, Asia, and Latin America [65]. As genital ulceration has been shown strong associations with increased risk of transmission of human immunodeficiency virus (HIV) infection, effective diagnosis and treatment of genital ulcer disease have become increasingly important [1, 66, 67]. Several studies have shown that the accuracy of clinical diagnosis for chancroid ranged from 33 to 80% [68, 69]. *H. ducreyi* is a fastidious organism requiring complex media and growth conditions for culture, and is difficult to detect using traditional culture method. The optimal sensitivity of culture can only be 50–90% even in experienced and well-equipped laboratories, while the sensitivity can be less than 50% in inexperienced laboratories [70, 71]. The "gold standards" for the diagnosis of chancroid were clinical diagnosis and laboratory culture of *H. ducreyi* in the past. The advent of DNA amplification methods using the PCR offer a simple and more sensitive and specific approach to diagnosis.

Several DNA amplification-based techniques that detect *H. ducreyi* directly in patient samples were developed and significantly improved the sensitivity of laboratory diagnostic tests for chancroid. Specific DNA primers have been designed to target and identify the bacteria in these techniques, which include nucleic acid hybridization and PCR assays.

Nucleic Acid Hybridization

Two probe hybridization assays for detection of *H. ducreyi* were developed [72, 73]. In one assay, three ^{32}P-labeled DNA probes designed on the basis of encoding *H. ducreyi*-specific proteins demonstrated to react strongly with *H. ducreyi* DNA in both bacterial suspensions as well as in infected rabbit lesion material blotted onto

nitrocellulose membranes [72]. The sensitivity of this probe hybridization assay was around 10^3–10^4 CFU of *H. ducreyi* in both pure and mixed cultures. The other probe hybridization assay was based on the development of specific rRNA-derived oligonucleotide probes for *H. ducreyi* [73]. Hybridization probes were chemically synthesized on eight oligonucleotide sequences complementary to different regions in the 16S and 23S rRNA molecules. This DNA–RNA hybridization assay demonstrated high specificity on culture grown isolates, but the sensitivity of the technique has not been provided. No complete evaluation was reported on the usefulness of these DNA or RNA probe hybridization techniques in the diagnosis of chancroid by *H. ducreyi* detection in clinical specimens.

Polymerase Chain Reaction

Several PCR assays have been developed to improve the sensitivity of laboratory diagnosis of chancroid [56, 74–77]. Target regions of the primers of these assays include 16S rRNA gene [56, 74], the *rrs* (16S)-*rrl*(23S) ribosomal intergenic spacer region [77], an anonymous fragment of cloned DNA [75], and the *groEL* gene encoding the GroEL heat shock protein [76]. As mentioned in previous *T. pallidum* section, a multiplex PCR assay with colorimetric detection and a real-time PCR assay were developed for simultaneous detection of *H. ducreyi*, *T. pallidum* and HSV type 1 and 2 [56, 61]. The sensitivity and specificity of the multiplex PCR detection of *H. ducreyi* were 98.4% and 99.6% respectively, as compared to 74.2 and 100% for culture. Expectedly, the sensitivity of culture is relatively low in comparison with PCR. Provided adequate clinical correlation studies can be carried out, the PCR assays have the potential to become an accurate and easily available reference method for laboratory diagnosis of chancroid.

Mycoplasma and Ureaplasma

Urethritis is one of the most common STDs among heterosexual men and categorized etiologically as gonococcal or non-gonococcal urethritis (NGU). *C. trachomatis* is a cause of acute NGU and accounts for 30–50% NGU cases in men [78, 79]. For non-chlamydial NGU, there are some evidences that mycoplasma and ureaplasmas might be pathogens of NGU and are associated with persistent and recurrent NGU cases [80, 81]. *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma parvum*, and *Ureaplasma urealyticum* are important etiological agents of postpartum fever, infertility, and pelvic inflammatory diseases [82, 83]. Genital mycoplasma and ureaplasma infections are commonly diagnosed by culture. However, the time-consuming culture requires 2–5 days for *Ureaplasms* spp. and *M. hominis*, and up to 8 weeks for *M. genitalium*, while they can be detected in less than 8 h by nucleic acid amplification methods. No commercial diagnostic system is available for the detection of mycoplasmas and ureaplasmas.

Introduction of molecular detection techniques provides a new horizon in the identification and detection of *Mycoplasma* and *Ureaplasma* species. These techniques

provide improved specificity and sensitivity compared with the time-consuming traditional culture methods. The molecular detection techniques on mycoplasmas and ureaplasmas include nucleic acid hybridization and PCR methods.

Nucleic Acid Hybridization

Sequences based on the variable species-specific regions of 16S rRNA genes have been widely utilized for synthesis of specific probes for hybridization. Several rRNA probe hybridization assays were developed for detecting mycoplasmas, even though many of rRNA probes were designed for the purpose of contamination detection in tissue cell cultures [84, 85]. Specific probes designed from genomic libraries of *Mycoplasma pneumonia* and *M. genitalium* have also been reported [86]. Dot blot hybridization methods with ^{32}P-labeled, digoxigenin or biotin-labeled probe for detection of mycoplasmas were also developed [87]. However, the detection limit of these assays is approximately 1 ng of specific mycoplasma DNA or 10^4–10^5 CFU, which is often not sufficiently sensitive for use in clinical laboratory [84, 88]. A rapid PCR-microtiter plate hybridization assay has been developed to detect *M. genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum* in genitourinary samples [81]. In this assay, four species-specific capture probes were used to detect the targets by PCR amplification of a part of the 16S rRNA gene followed by 96-well microtiter plate hybridization. Sensitivity of this assay was ten copies of the 16S rRNA gene of each of the four species without cross-reactions with other human mycoplasmas or ureaplasmas [81].

Polymerase Chain Reaction

Several PCR assays have been developed for detection of clinically relevant mycoplasmas and ureaplasmas [89–92]. Specific primers were designed for different target regions including 16S rRNA genes and other repetitive sequences, such as MgPa adhesion gene of *M. genitalium* and urease genes of *Ureaplasma* species [93–95]. As PCR assay is highly sensitive compared with traditional culture method, it is a potentially useful tool for detection of slow-growing and fastidious organisms, such as *M. genitalium*, where only small amount of bacterial DNA is required. With many difficulties in cultivating *M. genitalium*, PCR assay has become an important tool to detect this highly fastidious organism in the absence of direct isolations from genital specimens. Several different real-time PCR assays for quantifying *M. genitalium* were developed and have been validated in detection of a fragment of MgPa adhesion gene and 16S rRNA gene [91, 96–98]. As an alternative to PCR, a TMA-based test for detection of *M. genitalium* was developed by Gen-Probe Incorporated (San Diego, CA) [99]. As The TMA assay targets multiple copies of rRNA instead of single copy genes in PCR assay, it potentially increases the sensitivity of detection relative to the sensitivity of PCR assay.

Apparently, PCR assay seems to be less valuable in other more rapidly growing and easily cultivable mycoplasmas and ureaplasmas, such as *M. hominis* and *U. urealyticum*.

Difficulties to culture are also encountered in certain specimens, such as amniotic fluids, and endotracheal aspirates of newborns [95]. The use of PCR technology in these situations, in contrast to culture, can enhance the detection of the mycoplasmas and ureaplasmas. A multiplex PCR assay has been developed to simultaneously detect six sexually transmitted pathogens, *C. trachomatis*, *N. gonorrhoeae*, *M. hominis*, *M. genitalium*, *T. vaginalis*, and genital *Ureaplasma* (*U. urealyticum* and *U. parvum*) [100]. The development of the multiplex PCR tests is useful in improving our understanding of the epidemiology of these important STDs in areas where these are endemic. A denaturing gradient gel electrophoresis (DGGE) fingerprinting of 16S rRNA of 32 mycoplasma species has also been developed for rapid identification of mycoplasma species of human origin [101]. It represents a significant improvement on current diagnosis of Mycoplasma infection directly from clinical samples in less than 24 h.

Trichomonas vaginalis

Trichomoniasis is one of the most common STIs worldwide and is caused by a parasitic protozoan, *T. vaginalis*. The infection in women has been linked to increased risk of HIV transmission and complications including premature labor, low infant birth-weight, and post-abortion or post-hysterectomy infection [102, 103]. Over 180 million cases of trichomoniasis are reported annually worldwide, while estimated five million women and one million men in United States are infected annually [104]. However, the actual figures are expected to be higher than these estimates because (1) the infection can be asymptomatic, particular in men; (2) trichomoniasis is not a reportable disease in United Sates and other countries; and (3) the sensitivities of different diagnostic tests varied between different laboratories, which often have little quality control on these methods. Although direct microscopy examination of vaginal secretion and urine samples remains the most common diagnostic test for this infection, detection of *T. vaginalis* by culture remains the "gold standard" for the diagnosis of this infection. The sensitivities of both direct microscopy examination and culture are low, only 40–70% [105]. As nucleic acid amplification technique becomes more widely used for diagnosis of STI, this technique for trichomoniasis would be highly desirable.

Several molecular detection methods are available for detection of *T. vaginalis*, which include nucleic acid hybridization, PCR assays, and TMA. These assays have been devised to target *T. vaginalis*-specific 16S rRNA, and various regions or genes of genome including 2.3 kb *T. vaginalis* fragment [106], the ferredoxin gene [107], beta tubulin gene [108], highly repeated DNA sequences [109] and 18S rRNA genes [110].

Nucleic Acid Hybridization

BD Affirm VPIII assay (Becton Dickson, Sparks, MD) is a commercially available kit that is the only FDA-cleared RNA probe-based diagnostic test to directly detect

Gardnerella vaginalis, *T. vaginalis*, and *Candida* species [111, 112]. The sensitivity and specificity of the Affirm VPIII assay for detection of *T. vaginalis* were 100% and 80%, respectively, compared with wet mount microscopy and culture. There were no false-positives and three false-negatives for the Affirm VP test compared with culture and/or wet mount for *T. vaginalis* [111, 112]. A dot-blot hybridization assay has also been developed by utilizing a 2.3 kb *T. vaginalis* DNA fragment as a probe to detect *T. vaginalis* DNA from vaginal exudates [106]. However, the drawbacks of this assay are the instability of the probe, and the necessity to handle and dispose of radioactive materials. To overcome these limitations, fluorescence-labeled DNA probe can be used for identification of *T. vaginalis* by DNA in situ hybridization technique.

Polymerase Chain Reaction

Several PCR assays have been developed to detect *T. vaginalis* from clinical samples. A PCR assay with eight specific primer pairs targeting the unique sequences of geome of *T. vaginalis* has been designed. In one primer pair, a 102 bp genomic fragment was amplified and termed as A6p sequence, which appears highly selective for a broad range of *T. vaginalis* isolates [107]. Beta-tubulin gene of *T. vaginalis* is a well-conserved region that has been used to develop a PCR assay [113]. The sensitivity and specificity of the beta-tubulin gene PCR assay were 97 and 98%, while the sensitivities of culture and wet preparation were only 70 and 36% [113]. Another target region of *T. vaginalis* that has been used for PCR amplification was 2,000 bp repeated fragment of *T. vaginalis*, in which two sets of highly *T. vaginalis*-specific primers were used [109]. A PCR assay targeting a specific region of 18S rRNA gene of *T. vaginalis* has also been developed [110]. The PCR amplification product was subsequently confirmed by enzyme digestion with *HaeIII*. Overall sensitivity and specificity of the 18S rRNA PCR assay on vaginal swab samples were 100% and 98% respectively [110]. A multiplex PCR for direct simultaneous detection of six sexually transmitted pathogens, including *T. vaginalis*, in clinical specimens has been developed [100].

Recently, real-time PCR assays for detection of *T. vaginalis* were developed using TaqMan-based or fluoresence resonance energy transfer (FRET)-based probes [108, 114, 115]. High levels of agreement between these real-time PCR assays and culture have been obtained for detection of *T. vaginalis*. By saving hands-on time and labor, a real-time PCR method has the potential for rapid, large-scale screening of patients at risk for *T. vaginalis*.

Transcription-Mediated Amplification

The Gen-Probe Aptima *Trichomonas vaginalis* (ATV) assay (Gen-Probe, San Diego, CA) is a TMA assay for detection of *T. vaginalis*-specific 16S rRNA to aid in the diagnosis of trichomoniasis using the TIGRIS DTS System. This assay combines

the technologies of target capture, TMA, and hybridization protection assay (HPA). Compared the ATV assay with another commercially available molecular assay, BD Affirm VPIII assay (Becton Dickson, Sparks, MD), the ATV assay was statistically more sensitive than the Affirm assay (100% vs. 63.4%, $P < 0.0001$), identifying 36.6% more positive patients [116].

Herpes Simplex Virus

HSV, type 1 (HSV1) and type 2 (HSV2), causes a wide range of clinical manifestations including genital, dermal, and central nervous system diseases. The primary transmission modes for HSV1 are via oral secretions and nongenital contact, while infection with HSV2 is usually via sexual transmission. In United States, up to 80% of children are infected with HSV1 by adulthood, 20–25% of the population has HSV2 antibodies by the age of 40, and overall there are at least 50 million people with genital herpes [117]. Historically, the preferred method for diagnosis of herpes infection was viral isolation in tissue culture followed by type-specific immunofluorescence detection. However, the nucleic acid amplification methods for detection of HSV are increasingly adopted due to their enhanced sensitivity, robustness, and short turnaround time [118–120].

Nucleic acid amplification techniques, including PCR and SDA, have been well developed for detection of HSV. PCR technique is now widely recognized as the reference standard laboratory assay method for the sensitive and specific diagnosis of CNS infections caused by HSV [118, 119, 121].

Polymerase Chain Reaction

PCR has become the mainstay laboratory diagnostic method for HSV encephalitis over the past decade and has been considered as the most sensitive method for direct detection of HSV [118, 122]. Several multiplex PCR assays have been developed that enable simultaneous screening of three to five viruses or bacteria in one assay. These multiplex PCR assays include PCR assay of *T. pallidum*, *H. ducreyi*, HSV1, and HSV2 [56], PCR assay of HSV-1, varicella-zoster (VZV), and enteroviruses [123], PCR assay of HSV-1, HSV-2, VZV, human cytomegalovirus (CMV), and Epstein-Barr virus (EBV) [124], and multiplex herpesvirus PCR assay on CMV, EBV, VZV, HSV, and human herpesvirus 6 (HHV-6) [121]. In recent years, real-time PCR assay has been widely adopted for molecular infectious disease testing and replaced the conventional PCR. Real-time PCR is more amenable to automation with high throughputs, short turnaround time, and reduction of likelihood of contamination. Several gene targets have been selected for the detection of HSV by real-time PCR, including gene coding for the DNA polymerase, glycoproteins B, C, D, and G, thymidine kinase, DNA-binding protein [120, 125, 126]. A real-time PCR assay of simultaneous detection of five human herpesviruses (CMV, EBV,

HSV-1, HSV-2, and VZV) in a single LightCycler assay has been developed [127]. There are three commercial real-time PCR HSV assays available, including artus HSV-1/2 PCR kit (QIAGEN Inc., Valencia, CA), SmartCycler Non-typing and SmartCycler Typing ASR kits (Cepheid, Sunnyvale, CA). The artus HSV-1/2 PCR kit, SmartCycler Non-typing, and SmartCycler Typing ASR kits showed improved sensitivity (100%, 98%, and 99%, respectively) compared to culture (37%), and all real-time PCR assays were highly specific (100%) [128].

Strand Displacement Amplification

The BD ProbeTec Herpes Simplex Virus (1 and 2) Q^x Amplified DNA Assay (Becton Dickson, Sparks, MD) is the first fully automated, FDA-cleared molecular assay for detection of HSV1 and HSV2. This assay uses SDA technology to qualitatively detect and differentiate HSV1 and HSV2 DNA in clinical-collected external anogenital lesion specimens as an aid in the diagnosis of herpes infection. The advantages of this fully automated assay are that it greatly reduces the turnaround time by reading up to 98 results in 2.5 h and can also run tests for chlamydia and gonorrhea, along HSV1 and 2 on a single run with the BD Viper System.

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

Identification and classification of microorganisms can now be achieved using protein "fingerprints" from the whole bacterial cells measured by the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MADLI-TOF MS) [129, 130]. By comparing these fingerprints to a database of reference spectra by the use of various algorithms, bacteria can be rapidly identified [130]. The matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS technique has been used of rapid identification and speciation of *Haemophilus* bacteria [131]. This technique can identify *H. ducreyi* and at the same time determine strain differences between different *H. ducreyi* isolates. The acquisition time for this technique is only about 10 min for identification of *Haemophilus* spp., which is far shorter than all other traditional biological methods and nucleic acid amplification tests. Atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry (AP-MALDI MS) was also applied as a proteomics-based method to detect and identify *Neisseria* species. Heat-inactivated clinical isolate cell suspensions of *N. gonorrhoeae* were subjected to on-probe protein/peptide extraction and tryptic digestion followed by AP-MALDI tandem MS (MS/MS)-based proteomic analysis. Amino acid sequences derived from three protonated peptides, neisserial acyl carrier protein, neisserial-conserved hypothetical protein, and neisserial putative DNA-binding protein, were identified by AP-MALDI MS/MS and MASCOT proteome database search analysis. These three peptide masses can thus be potential biomarkers for *Neisseria* species identification by AP-MALDI MS [132]. Another similar study was also demonstrated

in which significant interspecies differences between *N. gonorrhoeae*, *N. meningitidis*, and several nonpathogenic *Neisseria* species but little intraspecies diversity was revealed by visual inspection and bioinformatics examination using the MALDI BioTyper software. Cluster analysis successfully separated mass spectra collected from three groups that corresponded to *N. gonorrhoeae*, *N. meningitidis*, and nonpathogenic *Neisseria* isolates. This approach, requiring only one bacterial colony for testing and using a fast and easy measuring protocol, provides a powerful tool for the rapid identification of pathogenic *Neisseria* and can be adopted for other sexually transmitted pathogens [133].

Conclusion

The diagnosis of the STI has dramatically changed in the past 10 years. Molecular detection methods have now been demonstrated to be a powerful tool for research and diagnosis of STDs and become increasingly important for disease control and prevention [134, 135]. From an epidemiological perspective, accurate delineation of sexual networks and disease transmission patterns within populations can be constructed and understood by molecular typing methods. With the experiences gained in the different molecular techniques and approaches in several STDs including *N. gonorrhoeae*, *C. trachomatis*, *T. pallidum*, *Haemophilus duceryi*, *Mycoplasma and Ureaplasma*, *T. vaginalis*, and HSV, the advantages of molecular detection of STDs are readily apparent compared with traditional methodologies, such as direct examination, culture, and serology.

The major advantages of molecular approaches to STD diagnosis have usually included increased sensitivity, improved specificity, and reduced turnaround time for test results. Development of real-time PCR method can be used for both qualitative and quantitative analysis, as well as for genotyping, with shorter amplification time. It can also minimize some of the cross-contamination problems by using a closed-tube format without need for post-PCR processing. The ability to simultaneously identify the most prevalent STI pathogens (viral and bacterial) by multiplex real-time PCR assays will provide a rapid and more cost-effective diagnostic tool than current traditional tests that look for single pathogens, benefiting the patient by reducing the time from presentation to treatment and minimizing sample requirements.

Automation and miniaturization will be the main future directions in the development of molecular diagnostics, as many of the available molecular tests are still labor-intensive. For most automated systems, the biggest challenge will remain at the initial sample preparation and processing procedures, even though a few recent developments of molecular detection workstations may be able to provide solutions for this longstanding issue. Highly automated extraction and detection systems are in the development pipeline that will allow small laboratories to perform high-throughput molecular detection. In recent years, the extensive development of microarray technology and MALDI-TOF MS has provided new impetus and horizons on the molecular diagnostic front. It will not be surprising that in the not-too-distant

future, a small chip or plate would be used for the automated screening of several infectious pathogens, including STD pathogens.

References

1. CDC (2010) Sexually transmitted disease surveillance 2009. US Department of Health and Human Services, Atlanta, GA
2. Schmid G (2004) Economic and programmatic aspects of congenital syphilis prevention. Bull World Health Organ 82(6):402–409
3. Saiki RK, Gelfand DH, Stoffel S et al (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239(4839):487–491
4. Zaravinos A, Mammas IN, Sourvinos G, Spandidos DA (2009) Molecular detection methods of human papillomavirus (HPV). Int J Biol Markers 24(4):215–222
5. Dinc B, Bozdayi G, Biri A et al (2010) Molecular detection of cytomegalovirus, herpes simplex virus 2, human papillomavirus 16–18 in Turkish pregnants. Braz J Infect Dis 14(6):569–574
6. Deguchi T, Yoshida T, Yokoi S et al (2002) Longitudinal quantitative detection by real-time PCR of *Mycoplasma genitalium* in first-pass urine of men with recurrent nongonococcal urethritis. J Clin Microbiol 40(10):3854–3856
7. Workowski KA, Berman S (2010) Sexually transmitted diseases treatment guidelines, 2010. MMWR Recomm Rep 59(RR-12):1–110
8. Harkins AL, Munson E (2011) Molecular diagnosis of sexually transmitted *Chlamydia trachomatis* in the United States. ISRN Obstet Gynecol 2011:279149
9. Levett PN, Brandt K, Olenius K, Brown C, Montgomery K, Horsman GB (2008) Evaluation of three automated nucleic acid amplification systems for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in first-void urine specimens. J Clin Microbiol 46(6):2109–2111
10. Iwen P, Walker R, Warren K, Kelly D, Hinrichs S, Linder J (1995) Evaluation of nucleic acid-based test (PACE 2C) for simultaneous detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in endocervical specimens. J Clin Microbiol 33(10):2587–2591
11. Hanks JW, Scott CT, Butler CE, Wells DW (1994) Evaluation of a DNA probe assay (Gen-Probe PACE 2) as the test of cure for *Neisseria gonorrhoeae* genital infections. J Pediatr 125(1):161–162
12. Schachter J, Hook EW III, McCormack WM et al (1999) Ability of the digene hybrid capture II test to identify *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in cervical specimens. J Clin Microbiol 37(11):3668–3671
13. Darwin LH, Cullen AP, Arthur PM et al (2002) Comparison of digene hybrid capture 2 and conventional culture for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in cervical specimens. J Clin Microbiol 40(2):641–644
14. Wong K, Ho B, Egglestone S, Lewis W (1995) Duplex PCR system for simultaneous detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in clinical specimens. J Clin Pathol 48(2):101–104
15. Jalal H, Verlander NQ, Kumar N, Bentley N, Carne C, Sonnex C (2011) Genital chlamydial infection: association between clinical features, organism genotype and load. J Med Microbiol 60(pt 7):881–888
16. Fredlund HANS, Falk LARS, Jurstrand MARG, Unemo MAGN (2004) Molecular genetic methods for diagnosis and characterisation of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*: impact on epidemiological surveillance and interventions. APMIS 112(11–12): 771–784

17. Watson M, Lambden P, Clarke I (1991) Genetic diversity and identification of human infection by amplification of the chlamydial 60-kilodalton cysteine-rich outer membrane protein gene. J Clin Microbiol 29(6):1188–1193
18. Sachdeva P, Patel AL, Sachdev D, Ali M, Mittal A, Saluja D (2009) Comparison of an in-house PCR assay, direct fluorescence assay and the Roche AMPLICOR *Chlamydia trachomatis* kit for detection of *C. trachomatis*. J Med Microbiol 58(pt 7):867–873
19. Mahony JB, Song X, Chong S, Faught M, Salonga T, Kapala J (2001) Evaluation of the NucliSens Basic Kit for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genital tract specimens using nucleic acid sequence-based amplification of 16S rRNA. J Clin Microbiol 39(4):1429–1435
20. Madico G, Quinn TC, Boman J, Gaydos CA (2000) Touchdown enzyme time release-PCR for detection and identification of *Chlamydia trachomatis*, *C. pneumoniae*, and *C. psittaci* using the 16S and 16S-23S spacer rRNA genes. J Clin Microbiol 38(3):1085–1093
21. Soderblom T, Blaxhult A, Fredlund H, Herrmann B (2006) Impact of a genetic variant of *Chlamydia trachomatis* on national detection rates in Sweden. Euro Surveill 11(12):E061207
22. Catsburg A, van Dommelen L, Smelov V et al (2007) TaqMan assay for Swedish *Chlamydia trachomatis* variant. Emerg Infect Dis 13(9):1432–1434
23. Palmer HM, Mallinson H, Wood RL, Herring AJ (2003) Evaluation of the specificities of five DNA amplification methods for the detection of *Neisseria gonorrhoeae*. J Clin Microbiol 41(2):835–837
24. Liebling MR, Arkfeld DG, Michelini GA et al (1994) Identification of Neisseria gonorrhoeae in synovial fluid using the polymerase chain reaction. Arthritis Rheum 37(5):702–709
25. Bruisten SM, Noordhoek GT, van den Brule AJC et al (2004) Multicenter validation of the cppB gene as a PCR target for detection of *Neisseria gonorrhoeae*. J Clin Microbiol 42(9):4332–4334
26. Ho BS, Feng WG, Wong BK, Egglestone SI (1992) Polymerase chain reaction for the detection of *Neisseria gonorrhoeae* in clinical samples. J Clin Pathol 45(5):439–442
27. Tabrizi SN, Chen S, Tapsall J, Garland SM (2005) Evaluation of opa-based real-time PCR for detection of *Neisseria gonorrhoeae*. Sex Transm Dis 32(3):199–202
28. Whiley DM, Buda PJ, Bayliss J, Cover L, Bates J, Sloots TP (2004) A new confirmatory *Neisseria gonorrhoeae* real-time PCR assay targeting the porA pseudogene. Eur J Clin Microbiol Infect Dis 23(9):705–710
29. Mangold KA, Regner M, Tajuddin M et al (2007) Neisseria species identification assay for the confirmation of *Neisseria gonorrhoeae*-positive results of the COBAS amplicor PCR. J Clin Microbiol 45(5):1403–1409
30. Martin DH, Cammarata C, Van Der PB et al (2000) Multicenter evaluation of AMPLICOR and automated COBAS AMPLICOR CT/NG tests for *Neisseria gonorrhoeae*. J Clin Microbiol 38(10):3544–3549
31. Van Der Pol B, Martin DH, Schachter J et al (2001) Enhancing the specificity of the COBAS AMPLICOR CT/NG test for *Neisseria gonorrhoeae* by retesting specimens with equivocal results. J Clin Microbiol 39(9):3092–3098
32. Farrell DJ (1999) Evaluation of AMPLICOR *Neisseria gonorrhoeae* PCR using cppB nested PCR and 16S rRNA PCR. J Clin Microbiol 37(2):386–390
33. Whiley DM, Tapsall JW, Sloots TP (2006) Nucleic acid amplification testing for *Neisseria gonorrhoeae*: an ongoing challenge. J Mol Diagn 8(1):3–15
34. Smith DW, Tapsall JW, Lum G (2005) Guidelines for the use and interpretation of nucleic acid detection tests for *Neisseria gonorrhoeae* in Australia: a position paper on behalf of the Public Health Laboratory Network. Commun Dis Intell 29(4):358–365
35. Herrmann B (2007) A new genetic variant of *Chlamydia trachomatis*. Sex Transm Infect 83(4):253–254
36. Klint M, Hadad R, Christerson L et al (2011) Prevalence trends in Sweden for the new variant of *Chlamydia trachomatis*. Clin Microbiol Infect 17(5):683–689
37. Rockett R, Goire N, Limnios A et al (2010) Evaluation of the cobas 4800 CT/NG test for detecting *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. Sex Transm Infect 86(6): 470–473

38. Cheng A, Qian Q, Kirby JE (2011) Evaluation of the Abbott RealTime CT/NG assay in comparison to the Roche Cobas Amplicor CT/NG assay. J Clin Microbiol 49(4):1294–1300
39. Maze MJ, Young S, Creighton J, Anderson T, Werno A (2011) Nucleic acid amplification of the opa gene for detection of *Neisseria gonorrhoeae*: experience from a diagnostic laboratory. J Clin Microbiol 49(3):1128–1129
40. Gaydos CA, Cartwright CP, Colaninno P et al (2010) Performance of the Abbott RealTime CT/NG for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. J Clin Microbiol 48(9):3236–3243
41. Little MC, Andrews J, Moore R et al (1999) Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. Clin Chem 45(6):777–784
42. Van Der Pol B, Ferrero DV, Buck-Barrington L et al (2001) Multicenter evaluation of the BDProbeTec ET system for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urine specimens, female endocervical swabs, and male urethral swabs. J Clin Microbiol 39(3):1008–1016
43. Pope CF, Hay P, Alexander S et al (2010) Positive predictive value of the Becton Dickinson VIPER system and the ProbeTec GC Q x assay, in extracted mode, for detection of *Neisseria gonorrhoeae*. Sex Transm Infect 86(6):465–469
44. Gaydos CA, Quinn TC, Willis D et al (2003) Performance of the APTIMA Combo 2 assay for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in female urine and endocervical swab specimens. J Clin Microbiol 41(1):304–309
45. Verkooyen RP, Noordhoek GT, Klapper PE et al (2003) Reliability of nucleic acid amplification methods for detection of *Chlamydia trachomatis* in urine: results of the First International Collaborative Quality Control Study among 96 laboratories. J Clin Microbiol 41(7):3013–3016
46. Johnson RE, Newhall WJ, Papp JR et al (2002) Screening tests to detect *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections—2002. MMWR Recomm Rep 51(RR-15):1–38
47. Alexander S, Coelho da Silva F, Manuel R, Varma R, Ison C (2011) Evaluation of strategies for confirming *Neisseria gonorrhoeae* nucleic acid amplification tests. J Med Microbiol 60(7):909–912
48. Schachter J, Hook EW, Martin DH et al (2005) Confirming positive results of nucleic acid amplification tests (NAATs) for *Chlamydia trachomatis*: all NAATs are not created equal. J Clin Microbiol 43(3):1372–1373
49. Whiley DM, Sloots TP (2005) Comparison of three in-house multiplex PCR assays for the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* using real-time and conventional detection methodologies. Pathology 37(5):364–370
50. Gerbase AC, Rowley JT, Heymann DH, Berkley SF, Piot P (1998) Global prevalence and incidence estimates of selected curable STDs. Sex Transm Infect 74(suppl 1):S12–S16
51. Jethwa HS, Schmitz JL, Dallabetta G et al (1995) Comparison of molecular and microscopic techniques for detection of *Treponema pallidum* in genital ulcers. J Clin Microbiol 33(1):180–183
52. Liu H, Rodes B, Chen CY, Steiner B (2001) New tests for syphilis: rational design of a PCR method for detection of *Treponema pallidum* in clinical specimens using unique regions of the DNA polymerase I gene. J Clin Microbiol 39(5):1941–1946
53. Marfin AA, Liu H, Sutton MY, Steiner B, Pillay A, Markowitz LE (2001) Amplification of the DNA polymerase I gene of *Treponema pallidum* from whole blood of persons with syphilis. Diagn Microbiol Infect Dis 40(4):163–166
54. Pope V, Fox K, Liu H et al (2005) Molecular subtyping of *Treponema pallidum* from North and South Carolina. J Clin Microbiol 43(8):3743–3746
55. Bruisten SM, Cairo I, Fennema H et al (2001) Diagnosing genital ulcer disease in a clinic for sexually transmitted diseases in Amsterdam, The Netherlands. J Clin Microbiol 39(2):601–605
56. Orle K, Gates C, Martin D, Body B, Weiss J (1996) Simultaneous PCR detection of *Haemophilus ducreyi*, *Treponema pallidum*, and herpes simplex virus types 1 and 2 from genital ulcers. J Clin Microbiol 34(1):49–54

57. Hay PE, Clarke JR, Strugnell RA, Taylor-Robinson D, Goldmeier D (1990) Use of the polymerase chain reaction to detect DNA sequences specific to pathogenic treponemes in cerebrospinal fluid. FEMS Microbiol Lett 56(3):233–238
58. Centurion-Lara A, Castro C, Shaffer J, Van Voorhis W, Marra C, Lukehart S (1997) Detection of *Treponema pallidum* by a sensitive reverse transcriptase PCR. J Clin Microbiol 35(6):1348–1352
59. Buffet M, Grange PA, Gerhardt P et al (2007) Diagnosing *Treponema pallidum* in secondary syphilis by PCR and immunohistochemistry. J Invest Dermatol 127(10):2345–2350
60. Casal CA, Silva MO, Costa IB, Araüjo Eda C, Corvelo TC (2011) Molecular detection of *Treponema pallidum* sp. pallidum in blood samples of VDRL-seroreactive women with lethal pregnancy outcomes: a retrospective observational study in northern Brazil. Rev Soc Bras Med Trop 44:451–456
61. Suntoke TR, Hardick A, Tobian AAR et al (2009) Evaluation of multiplex real-time PCR for detection of *Haemophilus ducreyi*, *Treponema pallidum*, herpes simplex virus type 1 and 2 in the diagnosis of genital ulcer disease in the Rakai District, Uganda. Sex Transm Infect 85(2):97–101
62. Leslie DE, Azzato F, Karapanagiotidis T, Leydon J, Fyfe J (2007) Development of a real-time PCR assay to detect *Treponema pallidum* in clinical specimens and assessment of the assay's performance by comparison with serological testing. J Clin Microbiol 45(1):93–96
63. Heymans R, van der Helm JJ, de Vries HJC, Fennema HSA, Coutinho RA, Bruisten SM (2010) Clinical value of *Treponema pallidum* real-time PCR for diagnosis of syphilis. J Clin Microbiol 48(2):497–502
64. Koek AG, Bruisten SM, Dierdorp M, van Dam AP, Templeton K (2006) Specific and sensitive diagnosis of syphilis using a real-time PCR for *Treponema pallidum*. Clin Microbiol Infect 12(12):1233–1236
65. Trees DL, Morse SA (1995) Chancroid and *Haemophilus ducreyi*: an update. Clin Microbiol Rev 8(3):357–375
66. Fleming DT, Wasserheit JN (1999) From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. Sex Transm Infect 75(1):3–17
67. Pepin J, Quigley M, Todd J et al (1992) Assoication between HIV2 infection and genital ulcer diseases among male sexually transmitted disease patients in The Gambia. AIDS 6:489–493
68. Dangor Y, Ballard R, da L Exposto F, Fehler G, Miller S, Koornhof H (1990) Accuracy of clinical diagnosis of genital ulcer disease. Sex Transm Dis 17(4):184–189
69. Chapel T, Brown W, Jeffres C, Stewart J (1977) How reliable is the morphological diagnosis of penile ulcerations? Sex Transm Dis 4(4):150–152
70. Dylewski J, Hsanze H, Maitha G, Ronald A (1986) Laboratory diagnostics of chancroid: sensitivity of culture medium. Diagn Mircrobiol Infect Dis 4:241–245
71. Nsanze H, Plummer FA, Maggwa AB et al (1984) Comparison of media for the primary isolation of *Haemophilus ducreyi*. Sex Transm Dis 11(1):6–9
72. Parsons LM, Shayegani M, Waring AL, Bopp LH (1989) DNA probes for the identification of *Haemophilus ducreyi*. J Clin Microbiol 27(7):1441–1445
73. Rossau R, Duhamel M, Jannes G, Decourt JL, Van HH (1991) The development of specific rRNA-derived oligonucleotide probes for *Haemophilus ducreyi*, the causative agent of chancroid. J Gen Microbiol 137(2):277–285
74. Chui L, Albritton W, Paster B, Maclean I, Marusyk R (1993) Development of the polymerase chain reaction for diagnosis of chancroid. J Clin Microbiol 31(3):659–664
75. Johnson SR, Martin DH, Cammarata C, Morse SA (1994) Development of a polymerase chain reaction assay for the detection of *Haemophilus ducreyi*. Sex Transm Dis 21(1):13–23
76. Parsons L, Waring A, Otido J, Shayegani M (1995) Laboratory diagnosis of chancroid using species-specific primers from *Haemophilus ducreyi* groEL and the polymerase chain reaction. Diagn Mircrobiol Infect Dis 23(3):89–98
77. Gu X, Rossau R, Jannes G, Ballard R, Laga M, Van Dyck E (1998) The rrs (16S)-rrl (23S) ribosomal intergenic spacer region as a target for the detection of *Haemophilus ducreyi* by a heminested-PCR assay. Microbiology 144(4):1013–1019

78. Taylor-Robinson D (1996) The history of nongonococcal urethritis. Thomas Parran Award Lecture. Sex Transm Dis 23(1):86–91
79. Bowie WR, Wang SP, Alexander ER et al (1977) Etiology of nongonococcal urethritis. Evidence for *Chlamydia trachomatis* and *Ureaplasma urealyticum*. J Clin Invest 59(5):735–742
80. Maeda S, Deguchi T, Ishiko H et al (2004) Detection of *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma parvum* (biovar 1) and *Ureaplasma urealyticum* (biovar 2) in patients with non-gonococcal urethritis using polymerase chain reaction-microtiter plate hybridization. Int J Urol 11(9):750–754
81. Yoshida T, Maeda SI, Deguchi T, Miyazawa T, Ishiko H (2003) Rapid detection of *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma parvum*, and *Ureaplasma urealyticum* organisms in genitourinary samples by PCR-microtiter plate hybridization assay. J Clin Microbiol 41(5):1850–1855
82. Debattista J, Gazzard CM, Wood RN et al (2004) Interaction of microbiology and pathology in women undergoing investigations for infertility. Infect Dis Obstet Gynecol 12(3–4): 135–145
83. Simms I, Eastick K, Mallinson H et al (2003) Associations between *Mycoplasma genitalium*, *Chlamydia trachomatis* and pelvic inflammatory disease. J Clin Pathol 56(8):616–618
84. Razin S (1994) DNA probes and PCR in diagnosis of mycoplasma infections. Mol Cell Probes 8(6):497–511
85. Kessler HH, Dodge DE, Pierer K et al (1997) Rapid detection of *Mycoplasma pneumoniae* by an assay based on PCR and probe hybridization in a nonradioactive microwell plate format. J Clin Microbiol 35(6):1592–1594
86. Hyman H, Yogev D, Razin S (1987) DNA probes for detection and identification of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. J Clin Microbiol 25(4):726–728
87. Razin S, Hyman HC, Nur I, Yogev D (1987) DNA probes for detection and identification of mycoplasmas (Mollicutes). Isr J Med Sci 23(6):735–741
88. Marmion BP, Williamson J, Worswick DA, Kok TW, Harris RJ (1993) Experience with newer techniques for the laboratory detection of *Mycoplasma pneumoniae* infection: Adelaide, 1978–1992. Clin Infect Dis 17(suppl 1):S90–S99
89. Blanchard A, Hamrick W, Duffy L, Baldus K, Cassell G (1993) Use of the polymerase chain reaction for detection of *Mycoplasma fermentans* and *Mycoplasma genitalium* in the urogenital tract and amniotic fluid. Clin Infect Dis 17(suppl 1):272–279
90. Bebear C, de Barbeyrac B, Bebear C, Renaudin H, Allery A (1997) New developments in diagnostic and treatment of mycoplasma infections in humans. Wien Klin Wochenschr 109(14–15):594–599
91. Yoshida T, Deguchi T, Ito M, Maeda S-I, Tamaki M, Ishiko H (2002) Quantitative detection of *Mycoplasma genitalium* from first-pass urine of men with urethritis and asymptomatic men by real-time PCR. J Clin Microbiol 40(4):1451–1455
92. Mirnejad R, Amirmozafari N, Kazemi B (2011) Simultaneous and rapid differential diagnosis of *Mycoplasma genitalium* and *Ureaplasma urealyticum* based on a polymerase chain reaction-restriction fragment length polymorphism. Indian J Med Microbiol 29(1):33–36
93. Jensen JS, Borre MB, Dohn B (2003) Detection of *Mycoplasma genitalium* by PCR amplification of the 16S rRNA gene. J Clin Microbiol 41(1):261–266
94. Dutro SM, Hebb JK, Garin CA, Hughes JP, Kenny GE, Totten PA (2003) Development and performance of a microwell-plate-based polymerase chain reaction assay for *Mycoplasma genitalium*. Sex Transm Dis 30(10):756–763
95. Blanchard A, Hentschel J, Duffy L, Baldus K, Cassell GH (1993) Detection of *Ureaplasma urealyticum* by polymerase chain reaction in the urogenital tract of adults, in amniotic fluid, and in the respiratory tract of newborns. Clin Infect Dis 17(suppl 1):S148–S153
96. Jensen JS, Bjornelius E, Dohn B, Lidbrink P (2004) Use of TaqMan 5¢ nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. J Clin Microbiol 42(2):683–692

97. Hardick J, Giles J, Hardick A, Hsieh YH, Quinn T, Gaydos C (2006) Performance of the Gen-Probe transmission-mediated amplification research assay compared to that of a multitarget real-time PCR for *Mycoplasma genitalium* detection. J Clin Microbiol 44(4):1236–1240
98. Lillis RA, Nsuami MJ, Myers L, Martin DH (2011) Utility of urine, vaginal, cervical, and rectal specimens for detection of *Mycoplasma genitalium* in women. J Clin Microbiol 49(5): 1990–1992
99. Wroblewski JK, Manhart LE, Dickey KA, Hudspeth MK, Totten PA (2006) Comparison of transcription-mediated amplification and PCR assay results for various genital specimen types for detection of *Mycoplasma genitalium*. J Clin Microbiol 44(9):3306–3312
100. Samra Z, Rosenberg S, Madar-Shapiro L (2011) Direct simultaneous detection of 6 sexually transmitted pathogens from clinical specimens by multiplex polymerase chain reaction and auto-capillary electrophoresis. Diagn Microbiol Infect Dis 70(1):17–21
101. McAuliffe L, Ellis RJ, Lawes JR, Ayling RD, Nicholas RA (2005) 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species. J Med Microbiol 54(8):731–739
102. Cotch MF, Pastorek JG, Nugent RP et al (1997) *Trichomonas vaginalis* associated with low birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group. Sex Transm Dis 24(6):353–360
103. Soper DE, Bump RC, Hurt WG (1990) Bacterial vaginosis and trichomoniasis vaginitis are risk factors for cuff cellulitis after abdominal hysterectomy. Am J Obstet Gynecol 163(3): 1016–1021
104. Sutton M, Sternberg M, Koumans EH, McQuillan G, Berman S, Markowitz L (2007) The prevalence of *Trichomonas vaginalis* infection among reproductive-age women in the United States, 2001–2004. Clin Infect Dis 45(10):1319–1326
105. McCann JS (1974) Comparison of direct microscopy and culture in the diagnosis of trichomoniasis. Br J Vener Dis 50(6):450–452
106. Rubino S, Muresu R, Rappelli P et al (1991) Molecular probe for identification of *Trichomonas vaginalis* DNA. J Clin Microbiol 29(4):702–706
107. Riley D, Roberts M, Takayama T, Krieger J (1992) Development of a polymerase chain reaction-based diagnosis of *Trichomonas vaginalis*. J Clin Microbiol 30(2):465–472
108. Simpson P, Higgins G, Qiao M, Waddell R, Kok T (2007) Real-time PCRs for detection of *Trichomonas vaginalis* beta-tubulin and 18S rRNA genes in female genital specimens. J Med Microbiol 56(pt 6):772–777
109. Kengne P, Veas F, Vidal N, Rey JL, Cuny G (1994) *Trichomonas vaginalis*: repeated DNA target for highly sensitive and specific polymerase chain reaction diagnosis. Cell Mol Biol (Noisy-le-Grand) 40(6):819–831
110. Mayta H, Gilman RH, Calderon MM et al (2000) 18S ribosomal DNA-based PCR for diagnosis of *Trichomonas vaginalis*. J Clin Microbiol 38(7):2683–2687
111. Briselden AM, Hillier SL (1994) Evaluation of affirm VP microbial identification test for *Gardnerella vaginalis* and *Trichomonas vaginalis*. J Clin Microbiol 32(1):148–152
112. Brown HL, Fuller DD, Jasper LT, Davis TE, Wright JD (2004) Clinical evaluation of affirm VPIII in the detection and identification of *Trichomonas vaginalis*, *Gardnerella vaginalis*, and *Candida* species in vaginitis/vaginosis. Infect Dis Obstet Gynecol 12(1):17–21
113. Madico G, Quinn TC, Rompalo A, McKee KT Jr, Gaydos CA (1998) Diagnosis of *Trichomonas vaginalis* infection by PCR using vaginal swab samples. J Clin Microbiol 36(11):3205–3210
114. Jordan JA, Lowery D, Trucco M (2001) TaqMan-based detection of *Trichomonas vaginalis* DNA from female genital specimens. J Clin Microbiol 39(11):3819–3822
115. Hardick J, Yang S, Lin S, Duncan D, Gaydos C (2003) Use of the Roche LightCycler instrument in a real-time PCR for *Trichomonas vaginalis* in urine samples from females and males. J Clin Microbiol 41(12):5619–5622
116. Andrea SB, Chapin KC (2011) Comparison of Aptima *Trichomonas vaginalis* transcription-mediated amplification assay and BD Affirm VPIII for detection of *T. vaginalis* in symptomatic women: performance parameters and epidemiological implications. J Clin Microbiol 49(3): 866–869

117. Fatahzadeh M, Schwartz RA (2007) Human herpes simplex virus infections: epidemiology, pathogenesis, symptomatology, diagnosis, and management. J Am Acad Dermatol 57(5): 737–763
118. Tang YW, Mitchell PS, Espy MJ, Smith TF, Persing DH (1999) Molecular diagnosis of herpes simplex virus infections in the central nervous system. J Clin Microbiol 37(7): 2127–2136
119. Smalling TW, Sefers SE, Li H, Tang YW (2002) Molecular approaches to detecting herpes simplex virus and enteroviruses in the central nervous system. J Clin Microbiol 40(7): 2317–2322
120. Espy MJ, Uhl JR, Mitchell PS et al (2000) Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. J Clin Microbiol 38(2):795–799
121. Quereda C, Corral I, Laguna F et al (2000) Diagnostic utility of a multiplex herpesvirus PCR assay performed with cerebrospinal fluid from human immunodeficiency virus-infected patients with neurological disorders. J Clin Microbiol 38(8):3061–3067
122. Espy MJ, Rys PN, Wold AD et al (2001) Detection of herpes simplex virus DNA in genital and dermal specimens by LightCycler PCR after extraction using the IsoQuick, MagNA Pure, and BioRobot 9604 methods. J Clin Microbiol 39(6):2233–2236
123. Read SJ, Kurtz JB (1999) Laboratory diagnosis of common viral infections of the central nervous system by using a single multiplex PCR screening assay. J Clin Microbiol 37(5): 1352–1355
124. Shin CH, Park GS, Hong KM, Paik MK (2003) Detection and typing of HSV-1, HSV-2, CMV and EBV by quadruplex PCR. Yonsei Med J 44(6):1001–1007
125. Stevenson J, Hymas W, Hillyard D (2005) Effect of sequence polymorphisms on performance of two real-time PCR assays for detection of herpes simplex virus. J Clin Microbiol 43(5):2391–2398
126. Aldea C, Alvarez CP, Folgueira L, Delgado R, Otero JR (2002) Rapid detection of herpes simplex virus DNA in genital ulcers by real-time PCR using SYBR green I dye as the detection signal. J Clin Microbiol 40(3):1060–1062
127. Stocher M, Holzl G, Stekel H, Berg J (2004) Automated detection of five human herpes virus DNAs by a set of LightCycler PCRs complemented with a single multiple internal control. J Clin Virol 29(3):171–178
128. Rose L, Herra CM, Crowley B (2008) Evaluation of real-time polymerase chain reaction assays for the detection of herpes simplex virus in swab specimens. Eur J Clin Microbiol Infect Dis 27(9):857–861
129. Carbonnelle E, Mesquita C, Bille E et al (2011) MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin Biochem 44(1):104–109
130. Bizzini A, Greub G (2010) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin Microbiol Infect 16(11):1614–1619
131. Haag AM, Taylor SN, Johnston KH, Cole RB (2000) Rapid identification of *Haemophilus* and other bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Am Clin Lab 19(3):20–21
132. Gudlavalleti SK, Sundaram AK, Razumovski J, Doroshenko V (2008) Application of atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry for rapid identification of *Neisseria* species. J Biomol Tech 19(3):200–204
133. Ilina EN, Borovskaya AD, Malakhova MM et al (2009) Direct bacterial profiling by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry for identification of pathogenic *Neisseria*. J Mol Diagn 11(1):75–86
134. Peeling RW, Holmes KK, Mabey D, Ronald A (2006) Rapid tests for sexually transmitted infections (STIs): the way forward. Sex Transm Infect 82(suppl 5):v1–v6
135. Ronald A, Kuypers J, Lukehart SA, Peeling RW, Pope V (2006) Excellence in sexually transmitted infection (STI) diagnostics: recognition of past successes and strategies for the future. Sex Transm Infect 82(suppl 5):v47–v52

Chapter 30
Advances in the Diagnosis of *Mycobacterium tuberculosis* and Detection of Drug Resistance

Abdullah Kilic

Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB) and is an airborne infectious disease that primarily affects the lungs. More than 125 years after the discovery of the tubercle bacillus, by Dr. Robert Koch in 1882, TB, while both preventable and curable, remains one of the leading infectious causes of death worldwide. TB continues to be a disease associated with crowded living conditions, depressed immunity, and poverty. MTB infects one-third of the world's population; the World Health Organization (WHO) declared TB to be a global health emergency in 1993. There are approximately nine million new tuberculosis cases and two million deaths reported each year [1, 2]. The directly observed treatment, short-course (DOTS) is the internationally recommended strategy developed by the WHO in the 1990s in order to control MTB by decreasing TB-related morbidity, preventing TB deaths, and decreasing TB transmission. The DOTS achieved cure rates of nearly 80% and was subsequently expanded as an internationally recommended approach for TB control [3].

Mycobacterial Cell Wall

MTB is an aerobic, nonspore forming, nonmotile, and pleomorphic bacilli that is typically 1–5 μm long and 0.2–5 μm wide [4]. Its generation time is 15–20 h, so visible growth takes 3–6 weeks on solid media [5]. The mycobacterial cell wall is

A. Kilic (✉)
Department of Microbiology, Gulhane Military Medical Academy,
Ankara 06018, Turkey
e-mail: abkilic@gata.edu.tr

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composed of superficial lipids (e.g., cord factor), mycolic acids, arabinogalactan, peptidoglycan, and lipoarabinomannan. The cell wall confers shape, size, and protection against osmotic pressure, and it probably protects the plasma membrane from deleterious molecules in the cellular environment [6]. The cell wall components of mycobacteria determine their most prominent feature: staining of the cell wall by carbol fuchsin is resistance to decolorization by acid alcohol (i.e., "acid-fast"). Despite the use of decolorizing agents containing ethyl alcohol-hydrochloric acid, carbol fuchsin cannot be readily removed from the cell wall. Mycobacteria are not classified as either gram-positive or gram-negative. They are commonly referred as acid-fast bacilli (AFB) [7]. This important feature of MTB allows differential staining in contaminated specimens like sputum. Polysaccharides in the MTB and gram-positive bacteria cell walls are similar; however, mycobacterium cell wall contains lipid while gram-positive bacteria have proteins in their cell wall [6, 8].

Mycosides including mycolic acids (long chain fatty acids C78–90), waxes, and phosphatides are present in the exterior surface of the MTB cell envelope. In the cell wall, the mycolic acids are largely bound to peptidoglycan by phosphodiester bridges and to arabinogalactan by esterified glycolipid linkages. The agglutination serotype of strain and colony morphology is related to the mycosides [6, 9]. Another important cell component of mycobacteria is cord factor (trehalose 6,6′-dimycolate) that is thought to correlate with virulence. Cord factor may cause chronic granulomas and inhibits migration of leukocytes [7].

Epidemiology of Tuberculosis Infections

After acquired immunodeficiency syndrome (AIDS), tuberculosis is the most common infectious disease resulting in death [10]. Tuberculosis cases occur predominantly in individuals 15–59 years old [4]. According to WHO, in 2008 an estimated 11.1 million people were living with TB, including 9.4 million new cases. It is estimated that 1.8 million MTB deaths in the world, including 500,000 deaths that were HIV-positive. Newly acquired tuberculosis infections occur at a frequency of every second in the world. In addition, one-third of the world population is currently infected with MTB. Due to malnutrition, crowded living conditions, and poor access to health care, the highest incidence rate of TB cases is found in developing countries. Twenty two countries are contemplated "high-burden countries (HBCs)" accounting for approximately 80% of new TB cases each year. In 2008, the global TB incidence rate was reported with 139/100,000 populations, decreasing from a peak of 143/100,000 in 2004. Africa with three million new TB cases has the highest incidence and prevalence rates of tuberculosis, accounting for almost a third of the global total. The Americas have the lowest TB incidence and prevalence rates in the world, with only Brazil being a HBC. In Europe, 425,000 new TB cases have been reported in 2008. The only HBC in Europe is the Russian Federation where the treatment rate was lower than all other HBCs in 2007. South-East Asia is the second

most affected region in the world with 3.2 million new TB cases. There are five HBCs in this region, four (Bangladesh, India, Indonesia, and Myanmar) of which have met global treatment targets [2].

Sub-Saharan Africa has the highest incidence rate of tuberculosis (350 cases/100,000 population). In England and Wales, due to immigration and HIV infection, the incidence of new TB cases increased from 8.4/100,000 in 1993 to 9.2/100,000 in 1998. The greatest increase was in London at 11% [11]. Five to ten percent of immunocompetent persons infected with MTB will develop active disease sometime during their lifetime. Each year, approximately 10% of people who are co-infected with MTB and HIV develop active tuberculosis [10]. Most cases are in South-East Asia and India. Co-infection with human immunodeficiency virus (HIV) has led to an even greater increase in both developed and developing countries [12]. In 2008, 1.4 million of the 9.4 million new TB cases were also estimated HIV-positive. Africa was the hardest hit region by HIV in the world with 78% of co-infections. In 2008, of the 1.8 million people who died from the TB, an estimated 500,000 were HIV-positive [2].

Other risk factors for the development of TB include being foreign-born, traveling to a high-prevalence country, intravenous drug use, imprisonments, homelessness, health care workers who care for high-risk patients, and children exposed to adults in high-risk groups (except health care workers) [4].

Epidemiology of Multidrug-Resistant TB

Drug resistance in TB is a global problem. Over the past 5 years, resistant in TB strains have increased at an alarming rate. There are two forms of drug-resistant TB: Multi drug-resistant TB (MDR-TB) is defined as bacilli resistant to at least two first-line agents, isoniazid (INH) and rifampin (RIF). Extensively drug resistant TB (XDR-TB) is defined as bacilli resistant to both first- and second-line drugs. Drug-resistance has increased especially in regions where TB control programs are poorly enforced. According to WHO, of 114 countries that provided information between 1994 and 2009 on resistance to first-line anti-TB drugs, 109 countries reported data on resistance occurring among new TB cases. Overall, there were an estimated 390,000–510,000 cases of MDR-TB (primary and acquired) in 2008, with the best estimate at 440,000 cases. Among all incident TB cases globally, 3.6% are estimated to have MDR-TB. The highest numbers of MDR-TB were found in India, China, and the Russian Federation. China and India account for almost 50% of the estimated global number of incident MDR-TB cases. In some areas of the former Soviet Union, more than 20% of new cases were MDR-TB. An estimated 150,000 deaths caused by MDR-TB occurred globally in 2008, including those with HIV infection (range: 53,000–270,000). The 46 countries have been reported continuous surveillance or representative surveys of second-line drug resistance among MDR-TB cases. “XDR-TB was first described in March 2006, generally evolves

from MDR strains [3]. By March 2010, a cumulative total of 58 countries have confirmed at least one case of XDR-TB. Combining the data from these countries, 5.4% of MDR-TB cases were found to have XDR-TB. Eight countries reported XDR-TB in more than 10% of MDR-TB cases; six of these countries were located in Eastern Europe and Central Asia. In an outbreak in South Africa, 52 out of 53 people infected with XDR-TB died within an average of 3 weeks of being diagnosed [13].

MDR-TB development not only hinders treatment but also increases the costs of treatment by 100-fold and lengthens the treatment time [14]. The cost of drugs alone for treating the average MDR-TB patient is 50–200 times higher than for treating a drug-susceptible TB patient, and the overall costs for care have been found to be ten times higher or more. Among new cases, treatment success averaged to 64%, and 8% die; whereas, treatment success for previously treated cases was 58%, and 13% died. In the 27 high MDR-TB burden countries alone, approximately 1.3 million M/XDR-TB cases will need to be treated between 2010 and 2015. The associated cost of care for these patients has been estimated to amount to US$ 16.2 billion over the 6 years, rising from US$ 1.3 billion in 2010 to US$ 4.4 billion in 2015 [15].

The basic treatment of mycobacterial infection is chemotherapy. First-line anti-TB antibiotics target actively replicating MTB cells in the lung and reduce transmission rates of MTB to other persons within the first 2 months of treatment [16]. Second-line antibiotics are introduced into treatment regimens when resistance to primary antibiotics emerges [17]. In TB treatment, since single drug therapy can cause an increase in drug resistance strains, a combined treatment should be applied. With a combined treatment, cure can be >95% [7]. INH, RIF, pyrazinamide (PZA), ethambutol (EMB), and streptomycin (SM) are the first-line agents. These drugs are used for drug-susceptible TB infections. The most important risk factor for MDR-TB is failure to complete treatment for tuberculosis. Six months short-course therapy, starting with treatment with INH, RIF, EMB, and PZA for 2 months, following with 4 months treatment with INH and RIF, have still been used. The American Thoracic Society, the Center for Diseases Control and Prevention (CDC), the Infectious Diseases Society of America, WHO, and IUATLD advise slight modifications to this treatment protocol [18].

In case of MDR-TB, second-line drugs are the choice for treatment of infection [7]. There are nine second-line drugs: kanamycin, capreomycin, ethionamide, cycloserine, ofloxacin, clofazimine, levofloxacin, *para*-aminosalicylic acid, and ciprofloxacin [4]. Unfortunately, XDR-TB is often considered very difficult to treat, or is even untreatable, with existing chemotherapeutic [19]. A TB cavity usually contains 10^7–10^9 bacilli. Between 1 in 10^6 and 1 in 10^8 replications of tubercle bacilli may result in spontaneous mutation which confers resistance to antituberculosis therapy. When INH and RIF are used together, spontaneous mutations resulting in resistance would be extremely rare (1 in 10^{14}). If the single- or multidrug therapy is used episodically, resistant tubercle bacilli multiply under selective pressure and emerge rapidly [7, 20].

The genetic mutations responsible for resistance to tuberculosis therapy are myriad. INH is the most frequently used first-line antituberculosis drug. It is only active against growing tubercle bacilli, and it is not active against non-replicating bacilli or under anaerobic conditions [21]. The genes indan enoyl acp reductase (*inhA*), catalase-peroxidase (*katG*), alkyl hydroperoxide (*ahpC*), and oxidative stress regulator (*oxyR*) are responsible for INH resistance [20]. RIF is an important first-line drug for the treatment of TB with MICs ranging from 0.05 to 1 μg/mL in liquid or agar medium [22]. RIF interferes with RNA synthesis by binding to the β subunit of the RNA polymerase. RIF resistance is caused by mutations in RNA polymerase subunit 12 (*rpsl*) genes [23]. PZA, a nicotinamide analog, is an important first-line drug and was first discovered to have anti-TB activity in 1952. PZA targets an enzyme involved in fatty acid synthesis and is responsible for killing persistent tubercle bacilli in the initial intensive phase of chemotherapy. PZA is a prodrug converting to its active form, pyrazinoic acid (POA) by the pyrazinamidase enzyme encoded by *pncA* gene of MTB. Mutations in *pncA* gene lead to resistance against PZA [24]. SM is an alternative first-line anti-TB drug recommended by the WHO. The effect of SM has been demonstrated to take place at the ribosomal level. SM interacts with the 16S rRNA and S12 ribosomal protein (*rrs* and *rpsL*) [25]. SM resistance is associated with mutations in ribosomal protein subunit 12 (*rpsL*), 16S ribosomal RNA (*rrs*), and aminoglycoside phosphotransferase gene (*strA*) [20]. EMB is a first-line drug that is used with INH, RIF, and PZA as combination to prevent the emergence of drug resistance. EMB interfered with the biosynthesis of cell wall arabinogalactan. Strains resistant to EMB have MICs >7.5 μg/mL [26]. Resistance to EMB occurs secondary to mutations in arabinosyl transferase (*emb A*, *B*, and *C*) genes. However, about 35% of EMB-resistant strains do not have *emb* gene, suggesting that there may be other mechanisms of EMB resistance [27]. Second-line drugs are generally used in the treatment of MDR-TB cases and as a result prolong the total treatment time from 6 to 9 months [25]. Aminoglycosides (kanamycin and enviomycin), polypeptides (capreomycin, viomycin, and enviomycin), fluoroquinolones (ofloxacin, ciprofloxacin, and gatifloxacin), D-cycloserine, and thionamides (ethionamide and prothionamide) have been classified as second-line drugs according to the WHO. Unfortunately, second-line drugs have inherently known more toxic and less effective than first-line drugs [25]. Fluoroquinolones inactivate DNA gyrase, a type II DNA topoisomerase and resistance to fluoroquinolones occurs secondarily to mutations in DNA gyrase (*gyr A* and *B*) gene [20]. Aminoglycosides inhibit protein synthesis and cannot be used against dormant MTB. Mutation in the *rrs* gene encoding for 16S rRNA are associated with resistance to aminoglycosides [25]. Ethionamide is an important drug in the treatment of the MDR-TB and is bactericidal only against MTB, *Mycobacterium avium-intracellulare* and *Mycobacterium leprae*. Like INH, ethionamide is also a prodrug that is activated by bacterial metabolism. *EthA* catalyses two-step activation of ethionamide. Mutations in these activator enzymes cause resistance to ethionamide. In addition, mutations in the promoter of the *inhA* gene confer resistance to both ethionamide and INH [21].

Optimal Specimen Collection of MTB

Early diagnosis of TB is important for infection control, as well as to introduce the treatment in a timely manner and determine the resistance profile. During the last 2 decades, dedicated efforts have been resulted in enhancing TB diagnosis techniques. In this review we discuss the application of currently available methods and their impact on the diagnosis of TB and detection of drug resistance. Samples such as sputum, bronchial or gastric washings, pleural fluid, urine, or cerebrospinal fluid (CSF) are collected from patients suspected of having TB [9]. Collection of good quality specimens containing the highest number of mycobacteria is important for diagnosis. Specimen must be sent to the laboratory within 30 min or at most within 24 h after collection [28].

Sputum. The properly collected sputum is the best specimen for diagnosis of pulmonary TB. Expectorated sputum specimen should be collected early in the morning on three occasions and sent to laboratory in a wide-mouthed, sterile, plastic container (wax free) with a tight fitting cap at 0–4 °C. The US CDC recommends that TB be identified and first-line drug susceptibility testing be completed within 30 days of specimen collection. However, by traditional culture methods, TB identification and drug susceptibility testing can take 4–6 weeks [28].

Gastric lavage. Other specimens which may yield a diagnosis include the CSF and gastric lavage [29]. Gastric lavage for swallowed sputum is generally collected from young children who do not produce suitable expectorated sputum. Since *Mycobacterium* cannot survive for long periods in an acidic gastric washing environment, gastric lavage should be sent to laboratory promptly in 10% sodium carbonate. Gastric lavage specimen should be collected before breakfast on three separate occasions [6].

Cerebral spinal fluid (*CSF*). CSF require high volume aspirates to successful stain and culture MTB. Typically, clinical microbiology labs require at least 5 mL for a TB culture [6].

Urine. Midstream urine specimens should be collected in a sterile plastic container (wax free) with a leak-proof cap at three early mornings. Blood specimens should be collected in a sodium polyanethol sulfonate (SPS) tube and must be treated with lytic agent such as deoxycholate and should be concentrated by centrifugation before inoculating the media. Fluids should be processed promptly and inoculated into a liquid growth medium as well as a solid media. Biopsy specimens should be immediately sent to the lab [6].

It can be expected that specimens collected from nonsterile sites be contaminated by normal microflora. Therefore, in order to reduce contamination by normal flora, decontamination processes are necessary. The most commonly used mucolytic agents for sputum specimens are freshly prepared *N*-acetyl-L-cysteine (NALC) and dithiothreitol (DTT or sputolysin). NALC and sodium hydroxide are generally used for digesting and decontaminating because they kill most bacteria and fungi. Following decontamination, specimens are neutralized with buffer and concentrated by centrifugation. Since swab specimens do not generally contain sufficient materials for culture, 1 g of tissue or 10 mL of fluid are preferred [6].

Conventional Methods for Identifying MTB

Microscopic Techniques

Acid-fast staining is a fast, cheap, and convenient method for direct detection of mycobacteria from clinical specimens [6]. Although microscopy provides preliminary information, it is not an adequate method for differentiating MTB from other *Mycobacterium* species. Also microscopic techniques are not suitable for examination of specimens, such as urine, which are contaminated with non-pathogenic bacteria [29].

In microscopy, Ziehl-Neelsen (ZN), kinyoun's stain, and fluorochrome stain methods are used. It is necessary that reference laboratory report results of acid-fast stain within 24 h of receiving the specimens. In this method, due to mycolic acid-rich cell wall, carbol fuchsin dye is retained after washing with acid alcohol. This method is advised by WHO and IUATLD [10]. An alternative method is a fluorochrome stain made of auramine–rhodamine, which stains mycolic acids in the AFB cell wall. To visualize one AFB, approximately 5×10^3 bacilli per mL sputum should be present. In smear examination it is necessary to assess 300 fields before reporting a specimen as AFB negative [28]. With respect to culture, microscopy specificity is 99% and sensitivity is 25–75%. In some patients who receive antituberculosis therapy, it is possible to have positive smears and negative cultures, which reflect nonviable bacilli [9]. A recent advance in light-emitting diode (LED) technology-based fluorescence microscopy provides an overview of the major commercial LED technologies for microscopy. It has shown that this technique increases the sensitivity of sputum smears by nearly 10% over conventional staining [30].

Traditional Culture Techniques

Mycobacterial culture remains the gold standard for detection and drug susceptibility testing. There are nonselective and selective mediums for culture of mycobacteria. Selective media contains antibiotics that inhibit growth of normal flora [31]. In order to culture mycobacteria from clinical specimens, there are various kinds of solid and liquid media such as Lowenstein-Jensen, Kirchner, and the various Middlebrook formulations (7H9, 7H10, and 7H11) [32]. Specimens contaminated with normal bacterial flora such as sputum are inoculated in a selective medium containing antimicrobial agents; sterile body fluids are inoculated with solid and a broth media [9]. The growth of solid culture media is 6 weeks or longer whereas that of liquid culture media is usually 7–21 days. Therefore, specimens should be cultured on solid and liquid media at 35–37 °C, with 5–10% CO_2. All cultures should be examined weekly for 8 weeks. The major advantages of solid cultures are that they make it possible to examine the morphology of colonies and visualize the pigmentation. These advantages are useful to differentiate the MTB from other nontuberculous mycobacteria (NTM) [9, 10].

Biochemical Tests and Morphological Features

There are various kinds of biochemical tests and morphological features for identification of mycobacteria. Based on pigment production, mycobacteria are classified into three groups: photochoromogens, scotochromogens, and nonchromogens. Photochoromogens produce pigmented colonies in the light. Scotochromogens produce pigmented colonies when grown in the dark. Nonchromogens are nonpigmented in both light and dark, but only have light tan or buff-colored colonies [7, 33]. Pigmented mycobacteria are classified as NTM because *M. tuberculosis* does not produce pigments [33].

Growth rate, colony morphology, and biochemical tests are other important differentiating factors among mycobacteria. Mycobacteria that grow less than 14 days are called rapid growers, and those that grow after 14 days are called slow growers. Examination of the morphology of colonies is important especially in mixed cultures. After 15 days growth, MTB produces thin, nonpigmented, rough colonies on 7H11 agar. Biochemical tests also aid in the identification of MTB from NTM. TB has the ability to reduce nitrate to nitrite. There are also other tests such as production of catalase or urease, arylsulfatase test, iron uptake, tween hydrolysis, tellurite reduction, and positive niacin test, which aid in the diagnosis of tuberculosis [33].

Modern Methods for Identifying MTB

BACTEC 460TB (Becton Dickinson, USA)

Automated or semi-automated liquid culture systems that detect growth of mycobacteria species earlier than direct visualization have been developed. BACTEC 460TB system was commercially developed in the early 1980s by Becton Dickinson Company [6, 32]. This method is based on radiometric analysis of liquid growth medium containing palmitic acid, labeled with radioactive carbon (^{14}C-palmitic acid) as the substrate [28]. As the mycobacteria metabolize these fatty acids, radioactive carbon dioxide ($^{14}CO_2$) is released and measured by the system [20]. This system has evolved to become the "gold standard" [34]. The advantage of this method is reduced detection time of both smear-positive and smear-negative samples by nearly 50%; also the bacterial growth can be detected in 5–10 days [35, 36]. Although this system considerably reduces the mycobacteria detection time, it is labor intensive and has other limitations, including cumbersome manual loading and unloading, potential risk of cross contamination, lack of computerized data management, use of radioactive material, and accumulation of low-level radioactive waste [37].

BACTEC 9000 MB (Becton Dickinson, USA)

BACTEC 9000 MB, a fully automated, nonradiometric method developed by Becton Dickinson Company. This fluorescence-based system uses an oxygen specific sensor to detect the mycobacterial growth [38, 39]. In every vial, there is a silicon rubber disk impregnated a ruthenium metal complex as oxygen specific sensor [40]. Although this system is more rapid and less labor-intensive than solid media, it requires a solid media, MYCO/F medium, a modified Middlebrook 7H9 broth to suppress the growth of contaminating microorganisms that can be supplemented with antibiotics. The lower risk of cross contamination and data management has greatly facilitated its use [41].

BACTEC MGIT 960 (Becton Dickinson, USA)

The mycobacteria growth indicator tube (MGIT) system is a completely automated, nonradiometric, continuously monitoring, noninvasive instrument that requires neither needles nor other sharp instrument culture system [42]. Similar to Bactec 9000 MB, it uses the same oxygen-quenching fluorescence technology [39]. It has greater capacity (960 bottles vs. 240 bottles) than other automated culture systems such as Bactec 460TB. This method detects MTB in 2 days [42]. Every 60 min culture vials are monitored by instrument, and based on specific growth algorithms, it is tagged as positive [34]. This system is accepted as an alternative to radiometric culture system [43]. Also, it is preferred by laboratory personnel as it is a safe and easy method [44]. However, although it has significant safety advantages and is less labor intensive, the detection time for MTB, especially in smear-negative specimens, is longer than Bactec 460TB [45]. Moreover, some studies have shown that this system has a higher contamination rate than radiometric Bactec 460TB system [42, 46].

MB/BacT (Biomerieux, France)

MB/BacT is a continuously monitored nonradiometric system, developed by Organon Teknika Company, which contains a computerized database management system. It uses a gas-permeable sensor embedded in a colorimetric indicator at the bottom of culture vials. Carbon dioxide released by growing mycobacteria is detected by sensor [47]. Every sensor in the bottom of culture vial changes from dark green to yellow depending on the CO_2 concentration. Every incubating drawer in instrument is monitored by reflometric detection unit [48]. Calculated values are transmitted into computer in every 10 min and mycobacterial growth is indicated based on a predetermined algorithm [49]. In previous studies, it was shown that this radiometric system is an automated, sensitive, rapid method, and requires less labor for detection of TB in clinical specimens. It serves as a good alternative to radiometric systems and solid media [47, 50–52].

Septi-Chek AFB

Septi-Chek AFB (Roche, Basel, Switzerland) contains biphasic culture medium and a self-contained CO_2 (20%) environment. This system uses 20.0 mL of modified Middlebrook 7H9 broth, a paddle with agar media enclosed in a plastic tube, and enrichment broth. One side of the paddle is covered with nonselective Middlebrook 7H11 agar. The reverse side is separated into two sections: one section contains chocolate agar for the detection of contaminants, other side contains modified L-J medium. Vials were inoculated with 0.5 mL of the processed sediment and incubated at 35–37 °C for 10 weeks. Vials are inspected four times per week during the first 2 weeks for growth, then inspected weekly for an additional 6–8 weeks. It does not require specialized equipment or use of radioactive material [53–55]. Also this system is quite useful and has potential advantages for detection and isolation of mycobacteria. However, it requires more incubator space and is more labor intensive than the manual method [56].

Trek Diagnostic ESP Culture System II (AccuMed, Chicago)

The ESP Culture System II developed from AccuMed is a fully automated, continuously monitoring method based on the detection of pressure changes, which are related to either gas production or gas consumption as a result of mycobacterial growth [57]. A special detection algorithm has been developed for very slowly growing mycobacteria [58, 59]. Bottles contain a modified Middlebrook 7H9 medium, which is enriched prior to use with OADC (oleic acid, albumin, dextrose, and catalase) growth supplement (Myco GS) and with Myco PVNA (polymyxin B, vancomycin, nalidixic acid, and amphotericin B). The bottles also contain a compressed sponge submerged in the broth, which provides a growth support platform, and they should be incubated at 35 °C for 42 days or until a positive signal is achieved. If this system is used with solid media, it has the same advantages as BACTEC in terms of being fully automated and does not use radioisotopes [57]. However, when compared with BACTEC MGIT, time to detect MTB is significantly longer in this system [60].

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is based on variation of *Mycobacterium* species cell wall mycolic acid profiles. This method can detect mycobacteria in smear-positive clinical specimens and positive liquid cultures [6]. It was demonstrated that HPLC proved to be a more reliable, easy to perform, cost effective, and rapid analysis technique than other identification methods after 18 months of routine use [61]. However, its routine use has not implemented in large hospitals, reference and public health laboratories due to the equipment cost and the expertise required to perform the assays [6].

Molecular Methods

Nucleic Acid Amplification Tests

The extremely slow growing nature of *M. tuberculosis* in culture method delays the diagnosis of TB disease. Nucleic acid amplification tests (NAATs) are developed for rapid diagnosis of TB disease (Table 30.1). These methods are directly detected to mycobacterial DNA and RNA in clinical specimens [62]. There are many commercial NAATs for direct detection of TB in clinical samples. However, the Food and Drug Administration (FDA) has approved only two of them for direct detection of TB in AFB smear-positive respiratory specimens: Enhanced Amplified Mycobacterium Tuberculosis Direct Test (MTD) (Gen-Probe, San Diego, CA, USA) and COBAS Amplicor MTB test (Amplicor, Roche Diagnostic Systems, Indianapolis, IN, USA) [63]. No assay has been licensed for use in nonrespiratory specimens [64]. Therefore, several rapid and accurate molecular methods have recently been developed and now available for the microbiological diagnosis of mycobacterial infection.

Enhanced Amplified Mycobacterium Tuberculosis Direct Test

On September 30, 1999, the FDA approved the MTD for the detection of TB in AFB smear-positive and smear-negative respiratory samples (Gen-probe Inc., San Diego, CA, USA). It is a rapid isothermal (42 °C) method based on the amplification of 16S rRNA. This test is based on detection of RNA by isothermal transcription-mediated amplification system developed by Kwoh. In this test, the mycobacterial rRNA is released from target cells by sonication, followed by promoter-primer binding to the target rRNA. Reverse transcriptase is used to copy rRNA to a cDNA-RNA hybrid. After degrading the initial RNA strand, a second primer binds to the cDNA and it is extended, resulting in the formation of double-stranded cDNA, which is then transcribed by DNA-dependent RNA polymerase to produce more rRNA molecules. The new transcripts then serve as targets for amplification and reverse transcription. The RNA amplicons are detected with an acridinium ester labeled DNA probe in a hybridization assay solution. The esterified acridinium on the hybridized probe is hydrolyzed by addition of alkaline hydrogen peroxide, resulting in the production of visible light, which is measured in a luminometer. The test is performed at 42 °C in a single tube to reduce contamination [62, 63]. The system works well with both AFB smear-positive and smear-negative specimens. The overall sensitivity for respiratory specimens compared to culture is 85.7–97.8%. Sensitivity of smear-positive specimens (91.7–100.0%) is higher than smear-negative specimens (40.0–92.9%) [63]. Test specificity of test was 100.0%. The major disadvantage of this test is lack of internal control for accessing amplification inhibitors [62].

Table 30.1 Comparison of nucleic acid amplification tests for direct detection of *Mycobacterium tuberculosis* complex from clinical samples

Assays	Amplification Method	Target	Detection	Sensitivity (%)	Specificity (%)	References
COBAS Amplicor MTB test	PCR	16S rRNA	Colorimetric	79.4–1.9	85.7–100.0	[63]
Enhanced amplified *Mycobacterium tuberculosis* Direct (MTD) test	TMA	16S rRNA	Chemiluminescent	85.7–97.8	100.0	[62, 63]
BD ProbeTec ET direct TB system (DTB)	SDA	IS*6110*-16S rRNA	Fluorimetric	90–100	92	[71, 73]
Xpert *Mycobacterium tuberculosis*/rifampicin assay	Real-time PCR	*rpo*B gene	Fluorimetric	98.2	98	[113]
The GenoType MTBDR *plus* assay	PCR-hybridization	*rpo*B, *kat*G and *inh*A genes	Colorimetric	94–99	99–100	[115]
INNO LiPA Rif.TB	Nested PCR	*rpo*B gene	Colorimetric	82.2	66.7	[100]
Real-time PCR	Real-time PCR	16S rRNA	Fluorimetric	71.6–98.1	100.0	[63]

NASBA nucleic acid sequence-based amplification; *SDA* strand displacement amplification; *TMA* transcription-mediated amplification; *PCR* polymerase chain reaction

Polymerase Chain Reaction-Based Amplicor and COBAS Amplicor MTB Test

The Amplicor test is based on the polymerase chain reaction (PCR) amplification of a 584-bp segment of the gene encoding the 16S rRNA. After amplification, amplicons are denaturated and added to a microtiter plate containing a bound, MTB complex-specific oligonucleotide probe. Detection is achieved with the help of avidin-horseradish peroxidase conjugate and 3,3′, 5,5′-tetramethyl benzidine in dimethylformamide. The results are measured with spectrophotometer. The test has an internal amplification control for the detection of PCR inhibition. Uracil-*N*-glycosylase (UNG) carries over prevention system is used in order to detect contamination by DNA remaining from previous runs. The results are available in 6–7 h. An automated version of this test, Cobas Amplicor, is available in Europe [62, 63]. The overall sensitivity and specificity of this test after resolving discordant results for respiratory specimens is 79.4–91.9% and 85.7–100.0%, and for non-respiratory specimens is 27.3–98.6% and 85.7–100.0%, respectively. Because the sensitivity of this test is lower (40.0–73.1%) for smear-negative specimens, FDA has approved this test only for smear-positive respiratory specimens [63].

Classical Polymerase Chain Reaction

PCR technique has been used to detect MTB in clinical specimens since 1989, a year after it was first described. This method has been developed for the detection of mycobacteria because of the slow growth rate of most pathogenic mycobacteria [32]. PCR not only allows rapid diagnosis, but also it can detect theoretically one DNA copy [65]. PCR offers better accuracy than AFB smears and has greater speed than culture [66]. Different PCR methods used for the detection of MTB have been developed. In these PCR methods, targets can be either DNA or rRNA [36]. IS*6110*, MPB64, 16S rRNA genes have been used as a target in PCR assays. Overall sensitivity and specificity are 84.2–100% and 83–100%, respectively, using IS*6110* as target [63].

Real-Time PCR

Real-time PCR has been developed for rapid and specific detection of MTB in the clinical specimens [36]. In the past few years, real-time PCR assays have been implemented in clinical microbiological laboratories [67]. Real-time PCR is based on hybridization of amplified nucleic acids with fluorescent-labeled probes spanning DNA regions of interest and monitored by inclusion of optical devices or CCD cameras in thermocyclers [63]. This technique has been used for identification of a variety of the bacteria and viruses [68]. Different format of probes are used for the detection of MTB. The most frequently used ones are TagMan probe, molecular beacons, and fluorescence resonance energy transfer (FRET) probes [69]. Real-time PCR sensitivity is 71.6–98.1% and the specificity is 100.0% [63].

Nucleic Acid Probe Methods

The AccuProbe (GenProbe, San Diego, CA, USA) is based on the use of species-specific DNA probes that hybridize with rRNA. Briefly, the rRNA is released from mycobacteria by sonication and heat. The labeled DNA probe is combined with the rRNA-containing lysate to form a DNA-rRNA hybrid. The labeled product is detected in a luminometer [33]. The results can be available in 2 h for culture-positive specimens [62]. The test is used only for culture identification. It cannot be used for direct detection of mycobacteria in clinical specimens [6]. This method is rapid and very easy to perform and special instrumentation is not required. Overall sensitivity and specificity are both 100% when MTB colonies are tested [33]. Another commercial probe test is SNAP (Syngene, San Diego, CA, USA). This assay is used, for MTB and MAC culture identification, using covalently labeled to alkaline phosphatase oligonucleotide probes directed against ribosomal RNA. Sensitivity and specificity of this test are 100% and 99%, respectively. This test is also highly versatile and is useful in many clinical and public health laboratories [70].

DNA Microarrays

High-density oligonucleotide arrays (DNA microarrays) provide an opportunity for rapid examination of large amounts of DNA sequence in a single hybridization step. This method is based on hybridization of fluorochrome-labeled PCR amplicons to DNA produced from bacterial colonies to DNA arrays. Oligonucleotide probes based on the 16S rRNA, DNA gyrase subunit B (*gyrB*), or the *rpoB* genes are used. The bound amplicons emit a fluorescent signal that is detected by a scanner [63]. This method has been used for identification of simultaneous species and detection of RIF resistance mutants of mycobacteria. This test contains 82 unique 16S rRNA sequence probes, and allows the differentiation of 54 mycobacterial species and 51 sequences that contain unique *rpoB* gene mutations. It is easy to perform and permits identification of a large number of mycobacteria in one reaction. This method is used in research and clinical laboratories for identification of mycobacteria. The results of the test are reliable, reproducible and obtained only 4 h for culture-positive specimens [62].

GenoType Mycobacteria Direct Assay

GenoType Mycobacteria Direct (GTMD) test (Hain Lifescience GmbH, Germany) is a commercial assay based on nucleic acid sequence-based amplification (NASBA) and amplifies single-stranded nucleic acids from the 23S rRNA gene in an isothermal reaction. The assay detects member of the MTB complex, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, and *Mycobacterium malmoense* directly from decontaminated respiratory samples and the results are

available in 1 day [71]. This assay is divided into three parts and was performed according to manufacturer's instructions. In the first part, RNA is performed RNA isolation and stabilization from contaminated samples using a capture method. The second part of the procedure comprised an isothermic amplification of RNA by the NASBA method, in which there is a mixture of primers/nucleotides labeled with biotin and a mixture of enzymes. In the third part of the test, reverse hybridization of the amplified products was performed using an automated system. Sensitivity and specificity were found to be 92% and 100%, respectively. This assay is an easy, relatively fast when used during the normal routine of clinical laboratory [72].

BD ProbeTec ET Direct TB System

BD ProbeTec ET Direct TB System (DTB) (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) was introduced in 1998 as a semi-automated technique for rapid detection of MTB in respiratory samples. The test based on strand displacement amplification (SDA) of target sequences in IS*6110* and the 16S rRNA genes. It is an isothermal (52 °C) enzymatic amplification process for generating multiple copies target sequences of the IS*6110* and the 16S rRNA genes. In this assay, amplification product is detected by the fluorescent method with a sensitivity level of 90–100% and specificity level of 92% in smear-positive sputum samples [71, 73].

Immunodiagnostic Methods

Antigen Detection Tests

A number of immunodiagnostic tests have been described. None of these tests has found widespread clinical use. Serological methods test the 38-kDa antigen, lipoarabinomannan, antigen 60, the antigen 85 complex, glycolipids including phenolic glycolipid, Tb1, 2,3-diacyltrehalose, and lipooligosaccharide as antigens [33]. Because of low sensitivity and specificity of the antigen tests, it cannot be recommended at this time [29]. However, some emerging data suggest that lipoarabinomannan may perform better in HIV-positive individuals with advanced immunosuppression [74].

Interferon-Gamma Release Assays

The tuberculin skin test (TST) has been widely used to detect latent infection of MTB. However, TST gives the positive reaction due to recent BCG immunization or exposure to nontuberculosis *Mycobacterium* that is present in the environment with major drawback. Interferon-gamma release assay have been developed as a test to replace TST. It is a T-cell-based assay, which is explored in many laboratories

using MTB-specific antigens such as ESAT-6, CFP-10, and TB7.7. Release of interferon-gamma from MTB specific T-cell is used as a marker of infection [75]. There are some commercial kits available in the market that claim to provide efficient and specific detection of latent MTB infection. The Quantiferon In-Tube and T-SPOT.TB has been approved from FDA. The T-SPOT.TB assay uses purified peripheral blood mononuclear cells with an enzyme-linked immunospot technique, whereas the Quantiferon In-Tube is a whole blood format that uses an enzyme-linked immunosorbent assay to detect IFN-gamma. Interferon assays have generally higher sensitivity and specificity compared with TST [76].

Typing Methods

IS*6110*-Based Restriction Fragment Length Polymorphism

The restriction fragment length polymorphism (RFLP) technique using the IS*6110* repetitive as a probe was published in 1990 and was widely used for typing strains of mycobacteria. This method is considered "the gold standard" for typing strains of mycobacteria. IS*6110* is a 1,355 bp insertion sequence in the MTB genome; zero to 25 copies of IS*6110* is found in the most strains of MTB. It is not known to be in other organisms [77]. Genomic DNA is extracted and then digested with specific restriction enzyme. Then, the restricted fragments are separated with electrophoresis [6]. This method has several disadvantages. First, it requires large amount of high quality DNA, extracted from a large number of bacteria grown from clinical material. Secondly, this method doesn't have good discrimination of isolates which contain less than six copies of IS*6110* [77].

Spoligotyping

Spoligotyping is a PCR-based typing method. A small direct repeat (DR) with 36 bp in the genomic DR region of MTB DNA is used to PCR amplifications with using appropriate primers [6]. The resulting PCR products are hybridized to 43 different oligonucleotides fixed to a membrane. The presence and absence of spacers in a given biotinylated strain are determined by hybridization with a set of 43 oligonucleotides derived from spacer sequences in *M. tuberculosis* H37Rv. Although this method is weaker than IS*6110* typing for differentiation of MTB strains, it has higher sensitivity for strains with low copy numbers of IS*6110* [77].

Variable Number of Tandem DNA Repeats

Variable number of tandem DNA repeats (VNTR) method is based on 12 loci of a type of VNTR sequences called mycobacterial interspersed repetitive units

(MIRUs) [77]. MIRUs are located in the intergenic region separating two genes, SenX3-RegX3, which encode a mycobacterial two-component system; they are present as tandem repeats. The number of the repeats is different among MTB strains. The PCR amplicons are detected in agarose gel or by a DNA sequences, allowing numbers of the repeats to be detected. Compared with culture, this method was found to be 100% sensitive and 100% specific [63].

Susceptibility Tests for MTB

Mycobacterial susceptibility testing is important in determining the appropriate treatment of mycobacterial infections [78]. CDC advises that susceptibility results should be available an average 28–30 days after receipt of a specimen from microbiology laboratory [48]. Different types of susceptibility tests are performed in clinical laboratories. It is recommended that the Clinical and Laboratory Standards Institute (CLSI) be used as a standardization method. CLSI document M24-A2 has a tentative standard method for using only MTB. Among the methods used for susceptibility testing of MTB, the agar proportion method (MOP) is universally accepted as the "gold standard." The result of this method takes generally 3–4 weeks; new methods such as radiometric or nonradiometric are being developed [79].

Conventional Methods

Three methods are used for susceptibility testing of MTB: MOP, absolute concentration method, and resistance ratio method. MOP is most frequently used in USA and Western Europe [6]. MOP for mycobacterial susceptibility testing was developed in the 1960s by G. Canetti [80]. This method was modified with a standard method published by NCCLS in 2003 [18]. The preferred medium for this test is Middlebrook 7H10 agar plates because it has a simple composition, is easy to prepare, and allows the early detection and quantitation of colonies. The Lowenstein-Jensen medium is recommended by WHO and IUATLD as an alternative medium [80]. The inoculum can be prepared as either a direct or an indirect test. A smear-positive specimen is used as the source of inoculum in the direct test. In the indirect test, the pure culture is used as the inoculum source. Several dilutions of a standardized suspension are inoculated onto suitable agar plates. The numbers of colony forming unit (CFU) on the drug-containing plates are compared with the numbers of CFU on a drug-free plate. Strains of tubercle bacilli that exceed 1% growth on drug-containing media, compared to growth on drug-free media are considered resistant to that agent [6].

In the absolute concentration method, the minimum inhibition concentration (MIC) of the agent is detected. The inoculating control media and drug-containing media are used for serial twofold dilutions of each agent. The lowest concentration of antibiotic that inhibits growth of the agent indicates resistance [20].

In resistance ratio method, MIC of the isolate is shown as a multiple of the MIC of a standard susceptible strain to avoid intra- and inter-laboratory variations. Inoculum size should be strictly controlled in both tests. These tests are not suitable for direct sensitivity testing of concentrated clinical specimens [20].

BACTEC 460TB (Becton Dickinson, USA)

In 1975, it was reported that MTB metabolic end-products could be detected by using a ^{14}C-labeled substrate [18]. This system has been widely performed in the clinical laboratories in the last 2 decades and is considered the gold standard in many ways [81]. Radioactive BACTEC 460TB system has been developed based on detection of $^{14}CO_2$ produced by consumption of a ^{14}C-labeled substrate. This system is used to test the metabolism of the organism in the presence of all first-line drugs (SM, INH, RIF, EMB, and PZA) [82]. This system is reliable and rapid for the detection of susceptibility of MTB to these drugs [83]. Results of susceptibility test can be available in 1 week compared to 3–4 weeks when solid media is used [18]. BACTEC 460TB is the proposed reference method for PZA susceptibility testing by CLSI [83], but this system uses radioactive substrates [84].

MB/BacT Alert 3D (Biomerieux, France)

MB/BacT Alert 3D is a fully automated, nonradiometric system, which allows susceptibility testing of MTB to EMB, INH, PZA, RIF, and SM [6]. The system is based on the detection of CO_2 released into the medium by actively growing mycobacteria. The CO_2 generated decreases the pH and color change occurs in a sensor in the bottom of the vial. Color changes are detected by a reflectometric detection unit in the instrument [85]. This system is likely to increase biosafety profiles, and it does not contain radioactive waste [82].

BACTEC MGIT 960 (Becton Dickinson, USA)

BACTEC MGIT 960 system is a nonradiometric, fully automated, continuous monitoring system that allows susceptibility testing for INH, RIF, SM, and EMB. Recently the automated BACTEC MGIT 960 system was developed to make the susceptibility testing of MTB to PZA possible [86]. This system consists of a modified Middlebrook 7H9 broth in a test tube in conjunction with a fluorescence-quenching oxygen sensor. The fluorescent compound is sensitive to the presence of oxygen in the test tube. Fluorescence light emitted from growing mycobacteria is detected using a 365-nm UV transilluminator [87]. This system has been reported as a rapid, reliable, and automated method for susceptibility testing of MTB to first-line drugs in many studies [84, 88–90].

ESP Culture System II (Trek Diagnostic, Westlake, OH, USA)

ESP Culture System II is a fully automated, continuous monitoring system for testing the susceptibility of MTB to INH, RIF, EMB, and SM [80]. This system is based on detection of produced or consumed gas due to microbial growth within the headspace above the broth culture medium in a sealed bottle [91]. It was reported as a reliable, rapid, and automated method for performing susceptibility testing of MTB [92].

Microscopic-Observation Drug-Susceptibility Assay

The microscopic-observation drug-susceptibility (MODS) assay is a novel liquid culture-based test that can detect MTB members and their drug resistances directly from sputum samples. MTB grows faster in liquid medium than in solid medium, and characteristic cord formation can be visualized microscopically in liquid medium at an early stage. Cultures containing supplemented Middlebrook 7H9 medium are microscopically examined for microcolonies, which can be detected in a median of 7 days. To detect drug resistance in MTB, INH and RIF can be incorporated in the testing process. Patient specimens or MTB isolates are inoculated to drug-free and drug-containing media, and cultures are microscopically examined. If the MTB grows in drug-free media, it indicates a positive culture. If the MTB grows in both drug-free and drug-containing media, it indicates resistance [93]. Despite the short timescale and the low cost of reagents, the WHO only endorses its implementation at reference laboratory level [94].

Nitrate Reductase Assay

The nitrate reductase assay (NRA), also known as the Griess method, is a simple technique based on the capacity of MTB to reduce nitrate to nitrite, which is detected by adding the Griess reagent to the medium. The biggest advantage of the NRA is that it is performed in the classical LJ medium that TB laboratories use routinely for the diagnosis of TB. Results are simple to interpret by a change of color. By incorporating 1 mg/mL potassium nitrate (KNO_3) in the Lowenstein-Jensen medium, the reduction of nitrate can be detected using the Griess reagent, which produces a colored reaction [95]. In the presence of antibiotic such as rifampicin or isoniazid at the critical concentration, the appearance of a red–pink color represents resistance to the drug. Susceptible strains will lose the capacity to reduce nitrate in the presence of the antibiotics, thus producing a non-colored reaction, as they are inhibited by the antibiotic. Because of the use of nitrate reduction as an indicator of growth, results can be obtained faster than by visual detection of colonies. It has been shown that the NRA is 97% sensitive and 100% specific for the detection of RIF resistance and 97% sensitive and 99% specific for the detection of INH resistance. The NRA has been shown to be highly sensitive and specific in the detection of RIF and INH resistance when used on clinical isolates [2].

Thin-Layer Agar

The thin-layer agar (TLA) method has been described as a simple, rapid, and inexpensive method, which is able to detect growth within 10 days while also allowing the initial identification of MTB based on colony morphology, microscopic visualization, and incorporation of *para*-nitrobenzoic acid (PNB) in the medium [96]. Preparing TLA plates, 100 μL of decontaminated sample is inoculated on a biplate Petri dish containing 20 mL of 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase plus piperacillin 0.05 μg/mL, trimethoprim 0.02 μg/mL, and amphotericin B 0.02 μg/mL. PNB at 500 μg/mL is incorporated in one compartment. After inoculation and sealed with sterile parafilm, the plates are incubated at 37 °C in 5% CO_2. After 24 h, the plates are checked for contamination and examined twice weekly for up to 6 weeks using a standard microscope. Whereas a positive culture is identified by the characteristic cords of *M. tuberculosis* growth, nontuberculous mycobacteria (NTM) are recognized by their lack of cording and growth on PNB. The TLA detects RMP and INH resistance directly from sputum samples using solid media with 100% sensitivity and specificity [97].

Molecular Methods

PCR-DNA Sequencing

PCR-DNA sequencing is the main method and gold standard for the detection of the genetic mechanisms of drug resistance in *MTB* [63]. This method includes PCR amplification of the DNA region of interest in clinical isolates or samples. Each isolate needs several sequencing reactions to be performed. Unfortunately this method is not used for routine identification of drug resistance in mycobacteria because the mutations responsible for antibiotic resistance involve many different genes. However, *rpoB* resistance is contained in a very short segment of the gene and this method is useful to accurately identify mutations which are associated with RIF resistance [62]. It is labor intensive and identifies previously recognized mutations. This system does not require expensive equipment but needs a high level of expertise. Although automatic sequencers are increasingly present in health care institutions, they are not available in numerous number microbiology laboratories [69].

INNO LiPA Rif.TB

RIF is an important drug in the treatment of tuberculosis and a marker for MDR-TB. The *rpoB* gene is target for amplification and detection point mutations, small deletions or insertions in *rpoB* gene, which lead to RIF resistance. LiPA has been developed for the detection of RIF resistance. It is based on the reverse hybridization method and consists of PCR amplification of a segment of the *rpoB* gene,

denaturation, and hybridization of the biotinylated PCR amplicons to capture probes bound to a membrane strip. The formed hybrids are colorimetrically detected [98]. LiPA test strip includes four probes for specific *rpoB* mutations and five probes for wild-type *rpoB* sequences. It also contains conjugated control and MTB control probes. MTB or the *rpoB* mutation is detected in the interpretation of the banding pattern on the strip. In this test, MTB culture or clinical specimens are used directly. Results are available in less than 48 h [62]. This method is simple, rapid, and does not require expertise in molecular biology [99]. The sensitivity when compared to conventional identification/culture methods was 82.2% and the specificity was 66.7%; the sensitivity and specificity were 100.0% and 96.9%, respectively, for the detection of RIF resistance [100]. However, this method is expensive and impractical for routine use [101] and also cannot detect rare mutations [62].

Rifampicin Oligonucleotide Typing (Rifoligotyping)

Rifoligotyping is analyzed by hybridization by the reverse line blotting technique. The 437-bp fragment of the *rpoB* gene is amplified with primer, one of which is labeled with biotin. The amplicons are hybridized to a set of wild-type and mutant oligonucleotides covalently bound to a membrane by reverse line blotting and are detected by enzyme chemiluminescence. The results of this method are available in a 1 day and performance is easy. In each run, a total of 43 samples can be studied. Also, if more than one mutation is known, the number of the probes should increase [63].

PCR-Based Single Strand Conformational Polymorphism

Single-strand conformation polymorphism (SSCP) technique is one of the methods based on PCR-electrophoresis. The principal of SSCP is conformational distortion resulting from a nucleotide substitution in a single-strand DNA fragment. A difference in sequence, even a change in a single base, leads to an electrophoretic mobility different to that of the wild-type single-strand fragment [102]. DNA fragment is denaturated to single strand and amplified by PCR. It is run on a polyacrylamide gel together with the denatured wild-type reference sample. When a mutation occurs, mobility shifts would occur in the clinical sample [99]. This system has been frequently used for the detection of mutation in RIF and INH and may be the most cost-effective method to detect point mutations within the 69-nucleotide region of RIF-resistant *MTB* [103]. This system is cheap, easy, rapid, and suitable for analysis of a large number of samples [99].

PCR-Heteroduplex Formation Assay

This assay was developed by Williams and coworkers for detecting RIF resistance. Assay is based on the amplification and detection of mutation in the *rpoB* gene

[104]. The amplicon of a 305-bp fragment of the *rpoB* gene is denatured and then mixed with an equivalent amount of a denatured amplicon from a reference wild-type strain. Then the mixture of amplicons is heated to denature the DNA, after which, the DNA is allowed to reconform on ice and then the aliquot of the amplified DNA is analyzed by electrophoresis. If there is a difference in electrophoretic mobility between the susceptible strain and clinical isolate, it indicates that clinical isolate has a mutation [63, 99]. This assay is rapid and sensitive for the detection of RIF genotype of *MTB* directly from sputum specimens [104].

Real-Time PCR

In many studies, real-time PCR was used for the detection of drug resistance in *MTB* [69, 105, 106]. Van Doorn et al. designed a real-time PCR assay that can be performed directly on clinical samples to detect INH resistance. The sensitivities and specificities of the probes were 82% and 100% for the mutant probe and 70% and 94% for the wild-type probe. They reported that this assay allows rapid identification of a mutant *katG* allele and could be easily implemented in clinical microbiology laboratories [107]. Torres et al. developed a single-tube method for detecting mutations associated with resistance to RIF and INH. They demonstrated that this assay is the fastest available method for the detection of RIF and INH resistance-associated mutations in *MTB* [106]. In another study, Sajduda et al. reported that real-time PCR method is fast and reliable for the detection of RIF and INH resistance-associated mutations in *MTB* [105]. Viedma et al. designed a new genotypic approach that can be simultaneously used for detecting resistance to RIF and INH in a single reaction tube. They reported that their design could be a model for new, rapid genotypic methods and is able to simultaneously detect a wide variety of antibiotic resistance mutations [69].

DNA Microarrays

DNA microarray technology is used for rapid detection of mutation associated with TB drugs [108, 109]. Seventy mycobacterial isolates from 27 different species and 15 RIF-resistant *MTB* strains were tested. A total of 26 of 27 species were correctly identified, including all of the *rpoB* mutants [109]. Yue et al. demonstrated that this method is an efficient, specialized technique to implement and can be used as a rapid method for detecting RIF resistance to complete standard culture-based method [110].

Xpert *Mycobacterium tuberculosis*/Rifampicin Assay

Xpert MTB/RIF assay was developed by the GeneXpert (Cepheid, Sunnyvale, CA, USA) as one of the most promising new point-of-care diagnostics technologies. The Xpert MTB/RIF assay consists of two main components: (1) the Xpert MTB/RIF

plastic cartridge, which contains liquid sample-processing and PCR buffers and lyophilized real-time PCR reagents; and (2) the GeneXpert instrument, which controls intracartridge fluidics and performs real-time PCR analysis [111, 112]. This system is a fully automated closed system that performs both sample preparation and real-time PCR and results are typically available within 1 h 55 min. Each module within the GeneXpert instrument operates independently, which enables the user to test each sputum sample as it arrives in the laboratory instead of saving samples for batch processing. Xpert MTB/RIF assay system rapidly detect the presence of MTB and identify the mutations most frequently associated with RIF resistance (targeting the RRDR of the *rpoB* gene) directly from smear-negative and smear-positive clinical sputum samples. The sensitivity of a single Xpert MTB/RIF assay (performed at reference facilities and compared with culture) was 98.2% among 561 patients with smear-positive MTB and 72.5% among 171 patients with culture-positive smear-negative TB in a multicountry study (Peru, Azerbaijan, South Africa, and India) of 1,730 individuals with suspected MTB. Sensitivity for detecting smear-negative TB increased to 85.1% with two assays and 90.2% when three Xpert MTB/RIF assays were performed. The specificity of the assay among 609 individuals without TB was at least 98% [113].

The GenoType MTBDR*plus* Assay

The GenoType MTBDR*plus* assay is a molecular test that was developed by Hain Lifesciences Technologies (Hain Lifesciences GmbH, Nehren, Germany). This assay for MDR TB is highly sensitive and specific, and significantly more rapid than conventional indirect susceptibility testing. The assay detects mutations in the *rpoB* gene for RIF resistance, the *katG* gene for high-level INH resistance, and the *inhA* gene for low-level INH resistance directly from smear-positive sputum. Results are available within 1 day [114]. The test involves DNA extraction, multiplex PCR, solid-phase reverse hybridization, and detection of the resistance mutations. Each strip consists of 27 reaction zones (bands), including six controls (conjugate, amplification, *M. tuberculosis* complex, *rpoB*, *katG*, and *inhA* controls), eight *rpoB* wild-type (WT) and four mutant (MUT) probes, one *katG* wild-type and two mutant probes, and two *inhA* wildtype and four mutant probes. The sensitivity for the detection of RIF resistance, INH resistance, and multidrug resistance was notified as 99, 94, and 99%, respectively. The specificity for the detection of RIF, INH, and multidrug resistance was notified as 99, 100, and 100%, respectively. The results of the present study have shown that the MTBDRplus assay is easy to perform and has the capability for the rapid detection of RMP- and INH-resistant MTB [115].

Conclusion and Outlook

Although in most developed countries, TB prevalence is at a low level, MDR-TB is increasing due to immigration of the foreign born. Drug-resistant TB prevalence has alarmingly increased in some of these developed countries [116]. So, the

mycobacteriology laboratories have important role in the diagnosis and control of TB [66]. The ideal TB diagnostic would be a simple, low-technology, rapid and could simultaneously identify drug resistance. The turnaround time for microscopy is ~24 h in most laboratories. Every laboratory should have experienced, well-trained technicians established in the mycobacteriology labs [117]. Molecular techniques are being used in mycobacteriology laboratories in developed nations for the detection of *MTB*, and drug susceptibility testing is being performed directly from clinical specimens. These tests allow rapid identification of *MTB* and detection of drug resistance. However, these techniques are complicated and costly. Also, they require experienced personal; separate rooms to prevent cross contamination; and expensive tools including thermocyclers, electrophoresis equipment, sequencing, and other automated systems [63].

Current opinions support molecular methods in combination with smear results, culture data, and clinical suspicion to diagnose tuberculosis [63]. Molecular techniques are currently unable to replace the traditional smears and culture [10].

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References

1. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC (1999) Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA 282:677–686
2. WHO (2010) Non-commercial culture and drug-susceptibility testing methods for screening of patients at risk of multi-drug resistant tuberculosis. World Health Organisation, Geneva
3. Haydel SE (2010) Extensively drug-resistant tuberculosis: a sign of the times and an impetus for antimicrobial discovery. Pharmaceuticals (Basel) 3:2268–2290
4. Starke JR (2004) Tuberculosis. In: Anne Gershon PH, Katz S (eds) Gershon: Krugman's infectious diseases of children, 11th edn. Mosby, St. Louis, pp 731–767
5. Haas DW (2000) *Mycobacterium tuberculosis*. In: Mandell G, Bennett JE, Dolin R (eds) Mandell: principles and practice of infectious diseases (chapter 240), 5th edn. Churchill Livingstone, New York, pp 2576–2611
6. Inderlied CB (2004) Mycobacteria. In: Jonathan Cohen WP (ed) Cohen & Powderly: infectious diseases (chapter 233), 2nd edn. Mosby, New York, pp 2285–2308
7. Brooks GF, Butel JS, Morse SA (2001) Mycobacteria. In: Geo JSB, Brooks F, Morse SA (eds) Jawetz, Melnick, & Adelberg's medical microbiology (chapter 24), 22nd edn. McGraw-Hill, New York, pp 275–284
8. Portaels F (1995) Epidemiology of mycobacterial diseases. Clin Dermatol 13:207–222
9. McMurray DN (2000) Mycobacteria and Nocardia. In: Baron S (ed) Medical microbiology, 4th edn. Churchill Livingstone, New York
10. Frieden TR, Sterling TR, Munsiff SS, Watt CJ, Dye C (2003) Tuberculosis. Lancet 362:887–899
11. Kumar D, Watson JM, Charlett A, Nicholas S, Darbyshire JH (1997) Tuberculosis in England and Wales in 1993: results of a national survey. Public Health Laboratory Service/British Thoracic Society/Department of Health Collaborative Group. Thorax 52:1060–1067
12. Huggett JF, McHugh TD, Zumla A (2003) Tuberculosis: amplification-based clinical diagnostic techniques. Int J Biochem Cell Biol 35:1407–1412
13. http://www.kff.org/globalhealth/upload/7883-02.pdf

14. WHO/74, P. R. (WHO: 22 Oct 22 1997). New report confirms global spread of drug resistant tuberculosis, http://www.who.int/archives/inf-pr-1997/en/pr97-74.html
15. http://whqlibdoc.who.int/publications/2010/9789241599191_eng.pdf
16. Hafner R, Cohn JA, Wright DJ et al (1997) Early bactericidal activity of isoniazid in pulmonary tuberculosis. Am J Respir Crit Care Med 156:918–923
17. Dorman SE, Chaisson RE (2007) From magic bullets back to the Magic Mountain: the rise of extensively drug-resistant tuberculosis. Nat Med 13:295–298
18. Parsons LM, Somoskovi A, Urbanczik R, Salfinger M (2004) Laboratory diagnostic aspects of drug resistant tuberculosis. Front Biosci 9:2086–2105
19. Matteelli A, Migliori GB, Cirillo D, Centis R, Girardi E, Raviglione M (2007) Multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis*: epidemiology and control. Expert Rev Anti Infect Ther 5:857–871
20. Sharma SK, Mohan A (2004) Multidrug-resistant tuberculosis. Indian J Med Res 120:354–376
21. Zhang Y, Yew WW (2009) Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 13:1320–1330
22. Mitchison DA (1985) The action of anti-tuberculosis drugs in short course chemotherapy. Tubercle 66:219–225
23. Bodmer T, Zurcher G, Imboden P, Telenti A (1995) Mutation position and type of substitution in the beta-subunit of the RNA polymerase influence in-vitro activity of rifamycins in rifampicin-resistant *Mycobacterium tuberculosis*. J Antimicrob Chemother 35:345–348
24. Scorpio A, Zhang Y (1996) Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med 2:662–667
25. Johnson R, Streicher EM, Louw GE, Warren RM, van Helden PD, Victor TC (2006) Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol 8:97–111
26. Heifets L, Desmond E (2005) Clinical mycobacteriology laboratory. In: Cole S, Eisenach K, McMurray D, Jacobs W Jr (eds) Tuberculosis and the tubercle bacillus. ASM Press, Washington, DC, pp 49–70
27. Safi H, Sayers B, Hazbon MH, Alland D (2008) Transfer of embB codon 306 mutations into clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol, isoniazid, and rifampin. Antimicrob Agents Chemother 52:2027–2034
28. Tenover FC, Crawford JT, Huebner RE, Geiter LJ, Horsburgh CR Jr, Good RC (1993) The resurgence of tuberculosis: is your laboratory ready? J Clin Microbiol 31:767–770
29. Gray JW (2004) Childhood tuberculosis and its early diagnosis. Clin Biochem 37: 450–455
30. Steingart KR, Henry M, Ng V et al (2006) Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis 6:570–581
31. Adjers-Koskela K, Katila ML (2003) Susceptibility testing with the manual mycobacteria growth indicator tube (MGIT) and the MGIT 960 system provides rapid and reliable verification of multidrug-resistant tuberculosis. J Clin Microbiol 41:1235–1239
32. Watterson SA, Drobniewski FA (2000) Modern laboratory diagnosis of mycobacterial infections. J Clin Pathol 53:727–732
33. Vincent V, Brown-Elliott BA, Jost KC Jr, Wallace RJ (2003) *Mycobacterium*: phenotypic and genotypic identification. In: Patrick EJB, Murray R, Pfaller MA, Tenover FC, Yolken RH (eds) Manual of clinical microbiology, 7th edn. ASM, Washington, pp 560–584
34. Scarparo C, Piccoli P, Rigon A, Ruggiero G, Ricordi P, Piersimoni C (2002) Evaluation of the BACTEC MGIT 960 in comparison with BACTEC 460 TB for detection and recovery of mycobacteria from clinical specimens. Diagn Microbiol Infect Dis 44:157–161
35. Brunello F, Favari F, Fontana R (1999) Comparison of the MB/BacT and BACTEC 460 TB systems for recovery of mycobacteria from various clinical specimens. J Clin Microbiol 37:1206–1209
36. Katoch VM (2004) Newer diagnostic techniques for tuberculosis. Indian J Med Res 120:418–428

37. Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G, Mengoli C (2004) Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria. J Clin Microbiol 42:2321–2325
38. Caws M, Drobniewski FA (2001) Molecular techniques in the diagnosis of *Mycobacterium tuberculosis* and the detection of drug resistance. Ann N Y Acad Sci 953:138–145
39. Flanagan PG, Williams R, Paull A (1999) Comparison of two automated systems for the isolation of mycobacteria from clinical specimens. Eur J Clin Microbiol Infect Dis 18: 912–914
40. van Griethuysen AJ, Jansz AR, Buiting AG (1996) Comparison of fluorescent BACTEC 9000 MB system, Septi-Chek AFB system, and Lowenstein-Jensen medium for detection of mycobacteria. J Clin Microbiol 34:2391–2394
41. Pfyffer GE, Cieslak C, Welscher HM, Kissling P, Rusch-Gerdes S (1997) Rapid detection of mycobacteria in clinical specimens by using the automated BACTEC 9000 MB system and comparison with radiometric and solid-culture systems. J Clin Microbiol 35:2229–2234
42. Hanna BA, Ebrahimzadeh A, Elliott LB et al (1999) Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. J Clin Microbiol 37:748–752
43. Pfyffer GE, Welscher HM, Kissling P et al (1997) Comparison of the Mycobacteria Growth Indicator Tube (MGIT) with radiometric and solid culture for recovery of acid-fast bacilli. J Clin Microbiol 35(2):364–368
44. Cornfield DB, Beavis KG, Greene JA, Bojak M, Bondi J (1997) Mycobacterial growth and bacterial contamination in the mycobacteria growth indicator tube and BACTEC 460 culture systems. J Clin Microbiol 35:2068–2071
45. Chew WK, Lasaitis RM, Schio FA, Gilbert GL (1998) Clinical evaluation of the Mycobacteria Growth Indicator Tube (MGIT) compared with radiometric (Bactec) and solid media for isolation of *Mycobacterium* species. J Med Microbiol 47:821–827
46. Tortoli E, Cichero P, Piersimoni C, Simonetti MT, Gesu G, Nista D (1999) Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: multicenter study. J Clin Microbiol 37:3578–3582
47. Piersimoni C, Scarparo C, Callegaro A et al (2001) Comparison of MB/Bact alert 3D system with radiometric BACTEC system and Lowenstein-Jensen medium for recovery and identification of mycobacteria from clinical specimens: a multicenter study. J Clin Microbiol 39:651–657
48. Brunello F, Fontana R (2000) Reliability of the MB/BacT system for testing susceptibility of *Mycobacterium tuberculosis* complex isolates to antituberculous drugs. J Clin Microbiol 38:872–873
49. Rohner P, Ninet B, Metral C, Emler S, Auckenthaler R (1997) Evaluation of the MB/BacT system and comparison to the BACTEC 460 system and solid media for isolation of mycobacteria from clinical specimens. J Clin Microbiol 35:3127–3131
50. Alcaide F, Benitez MA, Escriba JM, Martin R (2000) Evaluation of the BACTEC MGIT 960 and the MB/BacT systems for recovery of mycobacteria from clinical specimens and for species identification by DNA AccuProbe. J Clin Microbiol 38:398–401
51. Benjamin WH Jr, Waites KB, Beverly A et al (1998) Comparison of the MB/BacT system with a revised antibiotic supplement kit to the BACTEC 460 system for detection of mycobacteria in clinical specimens. J Clin Microbiol 36:3234–3238
52. Mirovic V, Lepsanovic Z (2002) Evaluation of the MB/BacT system for recovery of mycobacteria from clinical specimens in comparison to Lowenstein-Jensen medium. Clin Microbiol Infect 8:709–714
53. D'Amato RF, Isenberg HD, Hochstein L, Mastellone AJ, Alperstein P (1991) Evaluation of the Roche Septi-Chek AFB system for recovery of mycobacteria. J Clin Microbiol 29:2906–2908
54. Sewell DL, Rashad AL, Rourke WJ Jr, Poor SL, McCarthy JA, Pfaller MA (1993) Comparison of the Septi-Chek AFB and BACTEC systems and conventional culture for recovery of mycobacteria. J Clin Microbiol 31:2689–2691

55. Sharp SE, Lemes M, Erlich SS, Poppiti RJ Jr (1997) A comparison of the Bactec 9000 MB system and the Septi-Chek AFB system for the detection of mycobacteria. Diagn Microbiol Infect Dis 28:69–74
56. Sharp SE, Suarez CA, Lemes M, Poppiti RJ Jr (1996) Evaluation of the mycobacteria growth indicator tube compared to Septi-Chek AFB for the detection of mycobacteria. Diagn Microbiol Infect Dis 25:71–75
57. Tortoli E, Cichero P, Chirillo MG et al (1998) Multicenter comparison of ESP Culture System II with BACTEC 460 TB and with Lowenstein-Jensen medium for recovery of mycobacteria from different clinical specimens, including blood. J Clin Microbiol 36:1378–1381
58. Tholcken CA, Huang S, Woods GL (1997) Evaluation of the ESP Culture System II for recovery of mycobacteria from blood specimens collected in isolator tubes. J Clin Microbiol 35:2681–2682
59. Woods GL, Fish G, Plaunt M, Murphy T (1997) Clinical evaluation of difco ESP culture system II for growth and detection of mycobacteria. J Clin Microbiol 35:121–124
60. Williams-Bouyer N, Yorke R, Lee HI, Woods GL (2000) Comparison of the BACTEC MGIT 960 and ESP culture system II for growth and detection of mycobacteria. J Clin Microbiol 38:4167–4170
61. Thibert L, Lapierre S (1993) Routine application of high-performance liquid chromatography for identification of mycobacteria. J Clin Microbiol 31:1759–1763
62. Soini H, Musser JM (2001) Molecular diagnosis of mycobacteria. Clin Chem 47:809–814
63. Shamputa IC, Rigouts L, Portaels F (2004) Molecular genetic methods for diagnosis and antibiotic resistance detection of mycobacteria from clinical specimens. APMIS 112:728–752
64. Pai M, Flores LL, Hubbard A, Riley LW, Colford JM Jr (2004) Nucleic acid amplification tests in the diagnosis of tuberculous pleuritis: a systematic review and meta-analysis. BMC Infect Dis 4:6
65. Rodrigues MA, Serafini AB, Pereira Mde S et al (2004) Standardization of in-house polymerase chain reaction for the identification of *Mycobacterium tuberculosis* at the reference Tropical Disease Hospital in the State of Goias, Brazil. Mem Inst Oswaldo Cruz 99:415–419
66. Su WJ (2002) Recent advances in the molecular diagnosis of tuberculosis. J Microbiol Immunol Infect 35:209–214
67. Bruijnesteijn Van Coppenraet ES, Lindeboom JA, Prins JM, Peeters MF, Claas EC, Kuijper EJ (2004) Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadenitis in children. J Clin Microbiol 42:2644–2650
68. Miller N, Cleary T, Kraus G, Young AK, Spruill G, Hnatyszyn HJ (2002) Rapid and specific detection of *Mycobacterium tuberculosis* from acid-fast bacillus smear-positive respiratory specimens and BacT/ALERT MP culture bottles by using fluorogenic probes and real-time PCR. J Clin Microbiol 40:4143–4147
69. Garcia de Viedma D, del Sol Diaz Infantes M, Lasala F, Chave F, Alcala L, Bouza E (2002) New real-time PCR able to detect in a single tube multiple rifampin resistance mutations and high-level isoniazid resistance mutations in *Mycobacterium tuberculosis*. J Clin Microbiol 40:988–995
70. Lim SD, Todd J, Lopez J, Ford E, Janda JM (1991) Genotypic identification of pathogenic *Mycobacterium* species by using a nonradioactive oligonucleotide probe. J Clin Microbiol 29:1276–1278
71. Syre H, Myneedu VP, Arora VK, Grewal HM (2009) Direct detection of mycobacterial species in pulmonary specimens by two rapid amplification tests, the gen-probe amplified *Mycobacterium tuberculosis* direct test and the genotype mycobacteria direct test. J Clin Microbiol 47:3635–3639
72. Franco-Alvarez de Luna F, Ruiz P, Gutiérrez J, Casal M (2006) Evaluation of the GenoType Mycobacteria Direct assay for detection of *Mycobacterium tuberculosis* complex and four atypical mycobacterial species in clinical samples. J Clin Microbiol 44:3025–3027

73. Alcaide F, Coll P (2011) Advances in rapid diagnosis of tuberculosis disease and anti-tuberculous drug resistance. Enferm Infecc Microbiol Clin 29:34–40
74. Lawn SD, Edwards DJ, Kranzer K, Vogt M, Bekker LG, Wood R (2009) Urine lipoarabinomannan assay for tuberculosis screening before antiretroviral therapy diagnostic yield and association with immune reconstitution disease. AIDS 23(14):1875–1880
75. Cho SN (2007) Current issues on molecular and immunological diagnosis of tuberculosis. Yonsei Med J 48:347–359
76. Nyendak MR, Lewinsohn DA, Lewinsohn DM (2009) New diagnostic methods for tuberculosis. Curr Opin Infect Dis 22:174–182
77. Narayanan S (2004) Molecular epidemiology of tuberculosis. Indian J Med Res 120: 233–247
78. Woods GL (2000) Susceptibility testing for mycobacteria. Clin Infect Dis 31:1209–1215
79. Jassal M, Bishai WR (2009) Extensively drug-resistant tuberculosis. Lancet Infect Dis 9: 19–30
80. Pfyffer CBI (2003) Susceptibility test methods: fastidious bacteria. In: Patrick EJB, Murray R, Pfaller MA, Tenover FC, Yolken RH (eds) Manual of clinical microbiology, 7th edn. ASM, Washington, pp 1149–1177
81. Tortoli E, Benedetti M, Fontanelli A, Simonetti MT (2002) Evaluation of automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to four major antituberculous drugs: comparison with the radiometric BACTEC 460 TB method and the agar plate method of proportion. J Clin Microbiol 40:607–610
82. Tortoli E, Mattei R, Savarino A, Bartolini L, Beer J (2000) Comparison of *Mycobacterium tuberculosis* susceptibility testing performed with BACTEC 460 TB (Becton Dickinson) and MB/BacT (Organon Teknika) systems. Diagn Microbiol Infect Dis 38:83–86
83. Scarparo C, Ricordi P, Ruggiero G, Piccoli P (2004) Evaluation of the fully automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide, streptomycin, isoniazid, rifampin, and ethambutol and comparison with the radiometric BACTEC 460 TB method. J Clin Microbiol 42:1109–1114
84. Johansen IS, Thomsen VO, Marjamaki M, Sosnovskaja A, Lundgren B (2004) Rapid, automated, nonradiometric susceptibility testing of *Mycobacterium tuberculosis* complex to four first-line antituberculous drugs used in standard short-course chemotherapy. Diagn Microbiol Infect Dis 50:103–107
85. Angeby KA, Werngren J, Toro JC, Hedstrom G, Petrini B, Hoffner SE (2003) Evaluation of the BacT/ALERT 3D system for recovery and drug susceptibility testing of *Mycobacterium tuberculosis*. Clin Microbiol Infect 9:1148–1152
86. Kontos F, Nicolaou S, Kostopoulos C et al (2003) Multicenter evaluation of the fully automated Bactec MGIT 960 system for susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide: comparison with the radiometric Bactec 460 TB system. J Microbiol Methods 55:331–333
87. Ardito F, Posteraro B, Sanguinetti M, Zanetti S, Fadda G (2001) Evaluation of BACTEC Mycobacteria Growth Indicator Tube (MGIT 960) automated system for drug susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol 39:4440–4444
88. Bemer P, Palicova F, Rusch-Gerdes S, Drugeon HB, Pfyffer GE (2002) Multicenter evaluation of fully automated BACTEC Mycobacteria Growth Indicator Tube 960 system for susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol 40:150–154
89. Kontos F, Maniati M, Costopoulos C et al (2004) Evaluation of the fully automated Bactec MGIT 960 system for the susceptibility testing of *Mycobacterium tuberculosis* to first-line drugs: a multicenter study. J Microbiol Methods 56:291–294
90. Marttila HJ, Marjamaki M, Viljanen MK, Soini H (2003) Performance of BACTEC 960 Mycobacteria growth indicator tube in the susceptibility testing of genetically characterized *Mycobacterium tuberculosis* isolates. Eur J Clin Microbiol Infect Dis 22:757–759
91. Bergmann JS, Woods GL (1998) Evaluation of the ESP culture system II for testing susceptibilities of *Mycobacterium tuberculosis* isolates to four primary antituberculous drugs. J Clin Microbiol 36:2940–2943

92. Ruiz P, Zerolo FJ, Casal MJ (2000) Comparison of susceptibility testing of *Mycobacterium tuberculosis* using the ESP culture system II with that using the BACTEC method. J Clin Microbiol 38:4663–4664
93. Mendoza A, Castillo E, Gamarra N et al (2011) Reliability of the MODS assay decentralisation process in three health regions in Peru. Int J Tuberc Lung Dis 15:217–222
94. O'Grady J, Maeurer M, Mwaba P et al (2011) New and improved diagnostics for detection of drug-resistant pulmonary tuberculosis. Curr Opin Pulm Med 17:134–141
95. Rosales S, Pineda-García L, Andino N, Almendarez N, Membreño H, Hoffner SE (2009) Evaluation of the nitrate reductase assay for rapid detection of extensively drug-resistant tuberculosis. Int J Tuberc Lung Dis 13:1542–1549
96. Martin A, Paasch F, Von Groll A et al (2009) Thin-layer agar for detection of resistance to rifampicin, ofloxacin and kanamycin in *Mycobacterium tuberculosis* isolates. Int J Tuberc Lung Dis 13:1301–1304
97. Robledo J, Mejia GI, Paniagua L, Martin A, Guzmán A (2008) Rapid detection of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* by the direct thin-layer agar method. Int J Tuberc Lung Dis 12:1482–1484
98. Rossau R, Traore H, De Beenhouwer H et al (1997) Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob Agents Chemother 41:2093–2098
99. Garcia de Viedma D (2003) Rapid detection of resistance in *Mycobacterium tuberculosis*: a review discussing molecular approaches. Clin Microbiol Infect 9:349–359
100. Viveiros M, Leandro C, Rodrigues L et al (2005) Direct application of the INNO-LiPA Rif. TB line-probe assay for rapid identification of *Mycobacterium tuberculosis* complex strains and detection of rifampin resistance in 360 smear-positive respiratory specimens from an area of high incidence of multidrug-resistant tuberculosis. J Clin Microbiol 43:4880–4884
101. Lemus D, Martin A, Montoro E, Portaels F, Palomino JC (2004) Rapid alternative methods for detection of rifampicin resistance in *Mycobacterium tuberculosis*. J Antimicrob Chemother 54:130–133
102. Gong G, Lee H, Kang GH, Shim YH, Huh J, Khang SK (2002) Nested PCR for diagnosis of tuberculous lymphadenitis and PCR-SSCP for identification of rifampicin resistance in fine-needle aspirates. Diagn Cytopathol 26:228–231
103. Kim BJ, Lee KH, Park BN et al (2001) Detection of rifampin-resistant *Mycobacterium tuberculosis* in sputa by nested PCR-linked single-strand conformation polymorphism and DNA sequencing. J Clin Microbiol 39:2610–2617
104. Mayta H, Gilman RH, Arenas F et al (2003) Evaluation of a PCR-based universal heteroduplex generator assay as a tool for rapid detection of multidrug-resistant *Mycobacterium tuberculosis* in Peru. J Clin Microbiol 41:5774–5777
105. Sajduda A, Brzostek A, Poplawska M et al (2004) Molecular characterization of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Poland. J Clin Microbiol 42:2425–2431
106. Torres MJ, Criado A, Palomares JC, Aznar J (2000) Use of real-time PCR and fluorimetry for rapid detection of rifampin and isoniazid resistance-associated mutations in *Mycobacterium tuberculosis*. J Clin Microbiol 38:3194–3199
107. van Doorn HR, Claas EC, Templeton KE et al (2003) Detection of a point mutation associated with high-level isoniazid resistance in *Mycobacterium tuberculosis* by using real-time PCR technology with 3′-minor groove binder-DNA probes. J Clin Microbiol 41:4630–4635
108. Gingeras TR, Ghandour G, Wang E et al (1998) Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic *Mycobacterium* DNA arrays. Genome Res 8:435–448
109. Troesch A, Nguyen H, Miyada CG et al (1999) *Mycobacterium* species identification and rifampin resistance testing with high-density DNA probe arrays. J Clin Microbiol 37:49–55
110. Yue J, Shi W, Xie J et al (2004) Detection of rifampin-resistant *Mycobacterium tuberculosis* strains by using a specialized oligonucleotide microarray. Diagn Microbiol Infect Dis 48: 47–54

111. Raja S, Ching J, Xi L, Hughes SJ et al (2005) Technology for automated, rapid, and quantitative PCR or reverse transcription-PCR clinical testing. Clin Chem 51:882–890
112. Van Rie A, Page-Shipp L, Scott L, Sanne I, Stevens W (2010) Xpert(®) MTB/RIF for point-of-care diagnosis of TB in high-HIV burden, resource-limited countries: hype or hope? Expert Rev Mol Diagn 10:937–946
113. Boehme CC, Nabeta P, Hillemann D et al (2010) Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med 363:1005–1015
114. Hillemann D, Rüsch-Gerdes S, Richter E (2007) Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol 45:2635–2640
115. Barnard M, Albert H, Coetzee G, O'Brien R, Bomsan ME (2008) Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. Am J Respir Crit Care Med 177:787–792
116. Raviglione MC, Gupta R, Dye CM, Espinal MA (2001) The burden of drug-resistant tuberculosis and mechanisms for its control. Ann N Y Acad Sci 953:88–97
117. Espinal MA, Laszlo A, Simonsen L et al (2001) Global trends in resistance to antituberculosis drugs. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. N Engl J Med 344:1294–1303

Chapter 31
Rapid Screening and Identification of Methicillin-Resistant *Staphylococcus aureus*

Patrice Francois and Jacques Schrenzel

Clinical Relevance

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community-acquired infections. While the first *S. aureus* isolates displaying resistance to methicillin (MRSA) were reported in the early 1960s [1], endemic strains of MRSA carrying multiple resistance determinants have become a worldwide nosocomial problem only in the early 1980s [2]. The presence of MRSA in an institution is paralleled by an increased rate of bacteremia, or other severe MRSA infections [3]. MRSA-related bacteremia carries a threefold attributable cost and a threefold excess length of hospital stay when compared with methicillin-susceptible *S. aureus* (MSSA) bacteremia [4].

During the early 2000s, the emergence of endemic community-acquired MRSA (CA-MRSA) has been reported [5, 6]. In contrast to hospital-acquired MRSA (HA-MRSA), CA-MRSA are frequently isolated from healthy people and strains are generally susceptible to several older but clinically important antibiotics [6–8]. Population dynamic analysis of CA-MRSA revealed that this part of the MRSA population is in constant expansion [9] and was only recently detected as an emerging but worldwide infectious concern [7, 10].

P. Francois
Department of Specialties in Medicine, Genomic Research Laboratory, Rue Gabrielle-Perret-Gentil 4, CH-1211, Geneva 14, Switzerland
e-mail: patrice.francois@genomic.ch

J. Schrenzel (✉)
Department of Specialties in Medicine, Genomic Research Laboratory, Rue Gabrielle-Perret-Gentil 4, CH-1211, Geneva 14, Switzerland

Clinical Microbiology Laboratory, Rue Gabrielle-Perret-Gentil 4, CH-1211, Geneva 14, Switzerland
e-mail: jacques.schrenzel@genomic.ch

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The spread of MRSA in health care centers is difficult to control and requires elaborated infection control guidelines [11–13]. The difficulty to eradicate nosocomial MRSA infections may be explained by (1) the presence of an unknown hidden reservoir of MRSA carriers, (2) the emergence of novel highly epidemic *S. aureus* clonotypes such as EMRSA-15, EMRSA-16, or EMRSA-17 [14–16], with unspecified selective advantages, and/or (3) failure in enforcement of infection control prescriptions. Some authors reported that the screening of high-risk patients for MRSA colonization was a cost-effective measure for limiting the spread of the organism in hospitals [17]. As said before, early detection of MRSA carriers is also crucial for therapeutic decisions with last-line antibiotics against MRSA, e.g., glycopeptides and oxazolidinones [18]. This situation was recently the object of a CLSI report [19].

An extensive screening of MRSA carriers at hospital admission, despite its important cost, appears to have a major impact in reducing MRSA nosocomial infection rates [20], as recently shown by Wernitz et al. Indeed, MRSA carriage or colonization is a major risk factor for becoming infected. The preferred colonization sites are the nose, the throat, and the skin surface [21]. The spread of MRSA occurs generally after contact with carriers [22] or "MRSA reservoirs" (parts of which are probably unknown). The spread of MRSA in health care centers is difficult to control and requires elaborated infection control guidelines [3, 12, 17, 23–26] including (1) large-scale screening of suspected carriers, (2) automated computerized alerts, (3) specific recommendations for at-risk patients, such as contact isolation [12, 27, 28], and (4) significant improvement of hand hygiene compliance [13]. These data, together with successful containment effort programs [3, 12, 17, 23–26], prompt for screening high-risk patients even in a highly endemic setting [29]. However, discrepancies persist between various authors on the cost/benefit ratios of large-scale screening strategies [30–33]. The performance of the assay is obviously a crucial parameter bearing a major impact on this estimation and the cost-effectiveness appears also significantly affected by the MRSA prevalence of the studied wards [33, 34]. Several international guidelines now recommend the screening of potential MRSA-positive patients at hospital admission [35–37]. However, despite intensive efforts in the application of such guidelines, MRSA spread remains difficult to control.

Molecular Epidemiology

Molecular techniques dedicated to bacterial detection and identification have been recently reviewed [38, 39]. In the case of MRSA, the *mecA* gene encoding for the low-affinity penicillin-binding protein PBP2′ is the genetic basis of methicillin resistance in MRSA isolates. This gene, originating from a mobile genetic element designated staphylococcal cassette chromosome *mec* (SCC*mec* [40]), flanked by terminal inverted and direct repeats [41], is invariably inserted into the *orfX* gene of *S. aureus* chromosome (Fig. 31.1). This element contains two site-specific cassette chromosome recombinases, *ccrA* and *ccrB*, responsible for the precise excision and integration of SCC*mec* within the bacterial chromosome [40].

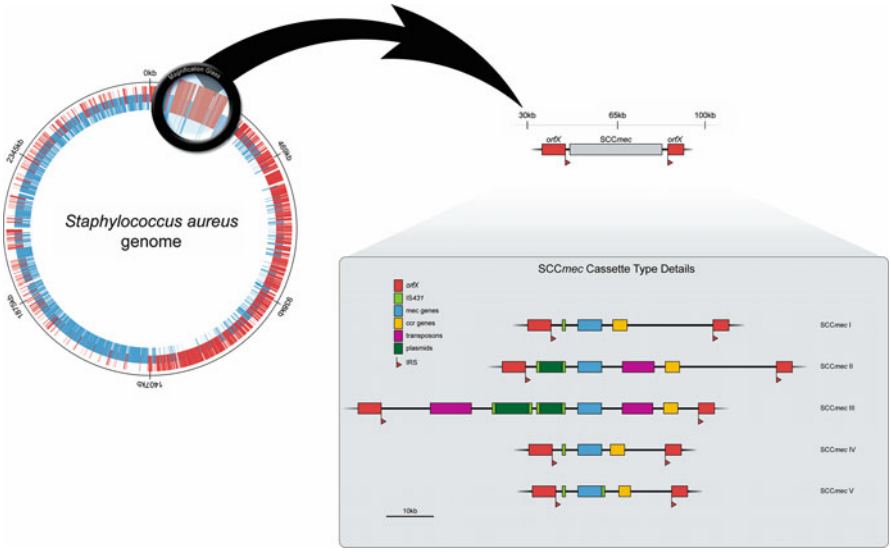

Fig. 31.1 General molecular organization of the main *Staphylococcus aureus* genomic regions containing the determinant of methicillin resistance. The circular chromosomes of several *S. aureus* strains are fully sequenced (**a**) allowing prediction of all ORFs and their respective coding sequences (*red* for ORFs encoded on the plus strand; *blue* on the minus strand). The SCC*mec* element encoding the methicillin-resistance determinant *mecA* is located in the *orfX* region at the very beginning of the chromosome (**b**). The five characterized variants of the SCC*mec* element show variable lengths; they are all flanked by inverted repeat regions (*red arrows*), contain recombinase(s) (*yellow blocks*) and the methicillin-resistance determinant (*cyan blocks*) (**c**). Variability in sequence contents concerns the presence of mobile elements harboring other antibiotic resistance determinants (*green blocks*) (**d**)

To date, eleven differently organized SCC*mec* elements have been characterized [42] and (http://www.sccmec.org/Pages/SCC_TypesEN.html). Three types of SCC*mec* elements are typically found in HA-MRSA strains: (1) type I, a 34 kb element that was prevalent in MRSA isolates in the 60s; (2) type II, a 53 kb element that was identified in 1982 and is ubiquitous in Japan, Korea, and the USA; and (3) type III, the largest 67 kb element identified in 1985, currently prevalent in Germany, Austria, India, and other South Asian and Pacific areas [40, 43]. In contrast to HA-MRSA, CA-MRSA isolates generally carry the SCC*mec* type IV element, whose size is much smaller than those of SCC*mec* types I, II, and III [42, 44]. At least four subtypes of the type IV SCCmec element, whose size is varying from 20 to 24 kb, have been reported so far (IVa to IVc) [42] and IVd (accession number AB097677)). The molecular structure of the recently described type V cassette [45] encoding a new *ccrC* recombinase presents some analogy with the type IV allele: both are more frequently associated to CA-MRSA, do not contain additional antibiotic resistance determinants, and are the smallest SCC*mec* cassettes with a size <30 kb (Fig. 31.1).

Molecular composition of the four SCC*mec* elements reveals key components useful for SCCmec typing [41, 46]. Variations in this gene set have allowed identifying five classes of *mecA* gene complexes [42, 43, 45], as discussed before.

A second essential region is the *ccr* gene complex. Types I and III harbor *ccrA1-B1* and *ccrA3-B3* recombinases, respectively, whereas types II and IV contain *ccrA2-B2* recombinases, showing some difference in the amino acid sequences [41]. Distinguishably to these four types, SCCmec V strains harbor a new *ccrC* recombinase type [45] (Fig. 31.1). This figure displays the organization of the prevalent SCC*mec* cassettes; extensive details and references describing all presently known cassettes are now summarized at http://www.SCCmec.org/Pages/SCC_TypesEN.html.

Recent efforts in the field of high-throughput sequencing yielded to the release of numerous bacterial genome sequences. To date, 14 fully sequenced strains of *S. aureus* are publicly available [47–56] providing opportunities for searching conserved or variable targets within the genome of MRSA, thus allowing improved molecular identification and characterization [57, 58]. For example, the markers described above (e.g., *mecA* and SCC*mec* organization) are important epidemiological indicators of strain origin [5, 59]. Note that the improvement of the molecular methods contributed to the possibility to rapidly document punctual changes in the epidemiology of MRSA such as emergence or discovery of new cassettes [60–63].

Culture-Based Methods Dedicated to MRSA Identification

Liquid cultures have been instrumental to the nascent field of microbiology and are still used in routine laboratories to recover minute amounts of bacteria or are employed as backup media. For example, Mueller-Hinton supplemented by oxacillin is still currently used to detect or confirm the presence of MRSA from swabs sampled for surveillance programs. This medium appears in the guidelines for the prevention and control of antibiotic-resistant organism from the CLSI recommendations [64]. However, solid media are now commonly used for organism isolation and identification, allowing MRSA identification in approximately 24 h. Agar-plates provide numerous advantages, such as the possibility for microbiologists to detect the presence of relevant colony morphologies, isolate them by sub-plating, and assess their purity on isolation plates. Pure isolates are essential for further phenotypic testing, including speciation (when required), antimicrobial susceptibility testing, and typing. Agar-plates developed for MRSA isolation or detection represent a particularly active field for microbiology companies. To date, numerous selective media containing β-lactam antibiotics and chromogenic substances are commercially available. The general principles are simple and consist in providing selective medium supplemented in (1) Gram-negative growth inhibitor (required for samples containing mixed flora), (2) antibiotic (allowing the selection of methicillin-resistant organisms only), and (3) a chromogenic substrate allowing the specific detection of growing *S. aureus* colonies. ORSAB plates (Oxoid), a solid variant of the liquid mannitol-salt medium containing oxacillin and aniline blue, allows detection of mannitol-fermenting organisms as blue colonies due to medium acidification. This medium appears adapted to high-risk population [65], but presents limitation for surveillance applications [66] as some coagulase-negative staphylococci (CNS)

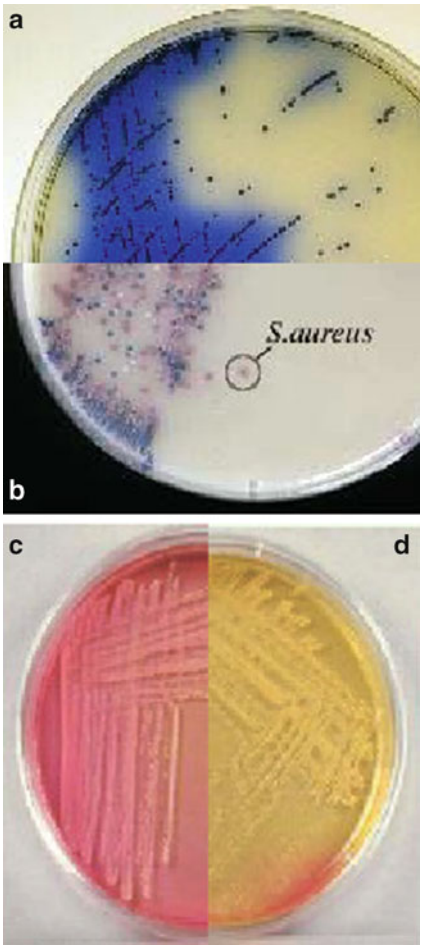

Fig. 31.2 Illustrative examples of chromogenic media. (**a**) ORSAB plates (Oxoid) contain oxacillin and a tracer of mannitol fermentation yielding blue *S. aureus* colonies. (**b**) Selective ChromAgar plate (ChromAgar), yielding *pink S. aureus* colonies (pictures are visible on the respective Web sites of the companies). Standard mannitol-salt agar plate showing colony aspects resulting from negative (**c**) or positive (**d**) MRSA cultures

(mainly *S. haemolyticus*, a frequent skin colonizer) appear also blue [66]. Thus, the utilization of this plate requires additional tests for robust identification. To date, ChromAgar/MRSA (ChromaAgar, Paris, France) stands among the most popular agar-plates dedicated to the detection of MRSA. This medium where MRSA colonies appear mauve, while other bacteria display different colors (Fig. 31.2), has been extensively tested and shows appreciable sensitivity and specificity [67, 68]. Other chromogenic agar media showing interesting performance are MRSA ID (bioMérieux, France), containing cefoxitin and a chromogenic substrate of α-glucosidase. Recent evaluation of this medium has shown improved performance compared to ChromAgar/MRSA [69]. Comparable results were reported with MRSA Select (Bio-Rad). Important efforts are still underway by these manufacturers to develop the fourth generation of chromogenic medium of this "gold standard culture method." However, performances—quite appreciable—tend to stagnate both in terms of TAT

and of sensitivity/specificity. Several recent studies published by different groups described similar performances of these different commercial products [34, 70–74]. All these media represent appreciable improvement of the current situation in the field of MRSA screening and identification, albeit these culture-based methods require at least 20–24 h to yield identification results. This timing remains theoretical as in real conditions, TAT appears significantly more important. During this period of time, infection control measures cannot be optimally applied. And, in case of empirical treatment, options include usually glycopeptide prescription leading to important costs and suboptimal use of last barrier drugs. A nice summary of the main properties of these chromogenic agar-plates appears in a recent review by Harbarth et al. [75].

Molecular Methods for MRSA Identification

Rapid detection of MRSA by standard clinical microbiological procedures appears then tedious and time consuming, since it first requires identification of isolated *S. aureus* colonies from mixed flora samples before assessing their antibiotic susceptibility profile. Direct or indirect particle agglutination assays using antibody-coated beads offer a rapid alternative to oxacillin susceptibility testing. The commercial test MRSA-Screen, manufactured by Denka Sieken (Tokyo, Japan), provides sensitive and specific immuno-detection of MRSA in a pure culture by using anti-PBP2′ antibody-coated latex beads, reveals similar to standard oxacillin disk diffusion or oxacillin salt agar screening [76, 77]. However, the specific immuno-detection of MRSA based on PBP2′ cannot be performed in the presence of other methicillin-resistant staphylococcal species, organisms that are frequently recovered as commensal contaminant of mixed flora samples [76]. Indeed, the high level of sequence homology of the *mecA* gene present in *S. aureus*, *S. epidermidis*, and potentially other CNS species [78, 79] precludes discrimination of methicillin-resistant strains of *S. aureus* from CNS. Furthermore, the sensitivity of the latex agglutination based on detection of PBP2′ antigenic motif requires the induction of its expression to reliably obtain detectable levels of the protein [80]. The fourth generation of agglutination test has been recently evaluated but the performances appear similar to the previous version [81]. Since the *mecA* gene, encoding the low-affinity penicillin-binding protein (PBP2′) [82], represents the “gold standard” for detecting methicillin resistance [83], several assays based on the direct detection of the *mecA* gene have been described, using chemiluminescent probes [84], cycling probe technology [85], or a two-step immuno-PCR assay based on the amplification and immuno-detection of *mecA* and *femB* amplicons, and performed after overnight culture enrichment of clinical samples [86]. These techniques appear promising in terms of sensitivity but also of turnaround time as they generally require a few hours before obtaining results. However, a common drawback is the requirement for induction of protein expression and the fact that they are not designed to identify MRSA among other MR-CNS, rather they appear much more adapted to isolated pathogens or sterile samples containing a single bacterial species.

Improved knowledge about genome sequences has allowed the design of numerous PCR-based methods. Rohrer and colleagues reported the utilization of a duplex PCR recognizing the genes *mecA* and *femA* (a gene specific for and recovered in all *S. aureus* strains [87, 88]) on a collection of isolated MRSA [80]. Other similar multiplexed PCR assays, using different targets (coagulase and *femA* genes) [89, 90] or *S. aureus* toxins [91], yielded promising results with good specificity. More recently, other promising results were also obtained by using triplex PCR assays based on the detection of *S. aureus* rRNA, *mecA*, and *nuc* [92, 93] or clfA genes [94]. Very recently, a smart approach was reported by Cuny and colleagues who used a simplex PCR amplification using primers complementary to a conserved region of *orfX* and a *S. aureus*-specific region selected by the authors (personal communication). Overall, PCR-based methods perform extremely well when applied to isolated strains or samples containing a pure culture, not exactly what is required for direct MRSA screening.

Multiplexed real-time qPCR techniques using fluorescently labeled detection of PCR products [95] combine accurate identification with limits of detection close to a single gene copy/sample. They have provided a significant technological advantage for the rapid and large-scale identification of various microorganisms [96–103]. These techniques outperform conventional detection methods by providing rapid and sensitive detection, and avoiding the use of acrylamide gel. Using the *nuc* gene as target, Fang and Hedin reported a fast screening and identification assay applicable to isolated bacteria [104]. More recently, using five specific primer pairs in the SCCmec and *orfX* regions, Huletsky et al. reported a very fast assay allowing direct identification of MRSA in 2 h, even in the presence of other staphylococcal species [105]. Note, however, that the emergence of new SCCmec elements warrants further modifications of the design and revalidation of the whole assay as new molecular variants continuously emerge [61, 62, 106]. Donnio et al. nicely described that the partial excision of the SCCmec cassette occurs not infrequently in MSSA strains, and might compromise the efficiency of this assay [107] whereas following geographical specificities, prevalent clones failed to be detected by tests based on SCC*mec* junction [19, 108, 109].

In this field, our group reported recently the use of a novel immuno-qPCR procedure allowing rapid detection of MRSA from mixed flora samples [110]. The procedure consists in a direct one-step enrichment of MRSA present in either nasal or inguinal swabs, followed by DNA extraction of immune-captured bacteria and their identification by a triplex qPCR. The specificity of MRSA identification is based on the quantitative correlation of the *mecA* gene and that of the *S. aureus*-specific *femA* signal, a probe that does not cross-react with other bacterial species, including *S. epidermidis*. This assay allows detection and identification of MRSA in less than 6 h after sample collection, thus allowing same-day identification (Fig. 31.3). We have successfully applied this method to the rapid screening of patients admitted to the ICU and shown that infection control measures could be implemented earlier than with optimized culture-based methods (22.2 h vs. 93.1 h, respectively). Finally, this molecular screening strategy resulted in decreased MRSA infections in the MICU after coupled implementation of strict control measures [111]. More recently, in a

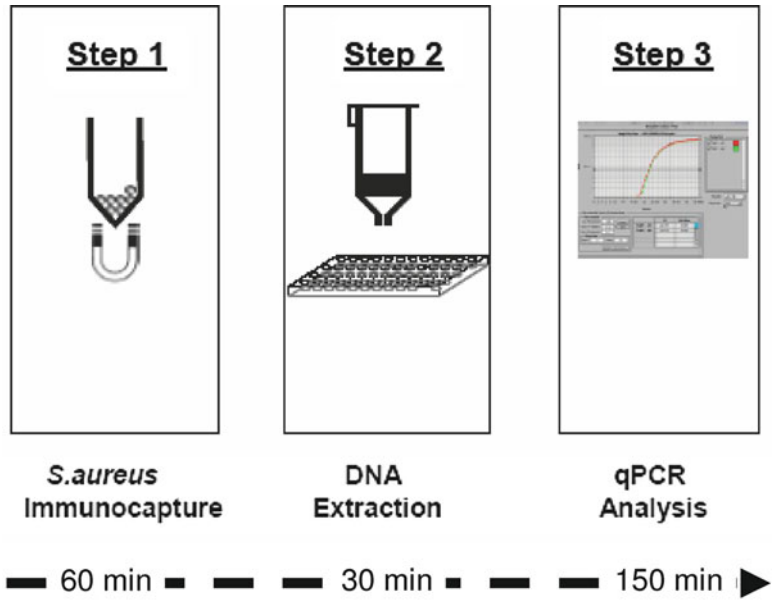

Fig. 31.3 Flowchart of an immuno-qPCR assay for rapid MRSA identification. After sampling and conditioning, mixed flora-containing samples are adsorbed using *S. aureus*-specific antibodies coupled to metallic particles (step 1). After washes, antibody-coated bacteria are disrupted by bead beater, and then purified on columns (step 2). The lysis medium is subjected to qPCR analysis (step 3). Note that turnaround time required for the complete procedure requires only a few hours whereas culture-based methods imply a minimum of 24 h

low prevalence area (around 5 %), large-scale screening in our hospital setting revealed of questionable value according to the results obtained by Harbarth et al. [30]. The main properties and performances of these commercially available molecular assays relying on the detection of the SCC*mec* junction are summarized in a recent review by Harbarth et al. [75].

Impact of Recent Hybridization Technologies on the Identification of MRSA

Hybridization technologies benefit from the efforts in high-throughput sequencing and allow the deposition onto solid surfaces of thousands of capture oligonucleotides able to hybridize with complementary target sequences. PCR-amplified products are detectable with low-density oligoarrays, enabling the parallel detection of several targets during the same experiment. The basic principle of DNA probe technology is hybridization, relying on denaturation of double-stranded DNA and

detection of hybridized labeled DNA. Recent reports relying on ribosomal or other specific markers demonstrated the identification of *Staphylococcus* at the species level [85, 112] and even provided an overview of the virulence factors harbored by clinical isolates during the same experiment [58, 113].

Despite parallelism and robustness, these analyses based on PCR product hybridization require several time-consuming steps (e.g., enzymatic amplification, product isolation, and labeling), thus impairing real-time screening demand. Sensitivity issue is another major challenge that has to be addressed before transferring direct hybridization technologies into routine laboratories. Efforts have been recently realized in this field, using direct labeling of target nucleic acids and/or optimized optic for the detection of hybridized products [114].

Note that commercially available low-density microarrays manufactured by ClonDiag and evaluating the presence of important accessory elements (virulence factors, regulators, and mobile genetic elements) allow the rapid characterization and genotyping of epidemiological strains [115, 116].

Impact of Mass Spectrometry on the Rapid Identification of MRSA

The rapid identification of *S. aureus* in various biological samples by using mass spectrometry has been documented some years ago [117] and predicted the possibility to identify directly MRSA on specific peptide traces in adapted database. The method used has been recently optimized and appears robust [118]. Various mass spectrometry methods require a minimum amount of material to prove reliable but show a particularly interesting discriminative power that allows clustering of isolates within their respective clonal complexes [119, 120]. Future developments of these methods in terms of analytical sensitivity as well as for the identification of specific signatures could rapidly contribute to revolutionize the field in terms of bacterial identification but also for the rapid determination of antibiotic susceptibilities, at least those requiring translation of specific target genes.

Conclusions

Nosocomial infections due to MRSA are frequent and represent an economical burden, requiring prompt isolation measures and utilization of last barrier drugs. Rapid detection and identification of MRSA is an absolute prerequisite. Tight collaboration with clinicians involved in prevention or infection control can contribute to the reduction of transmission and control of costs. Until recently, microbiological methods dedicated to MRSA identification relied on the utilization of selective growth media, which are time-consuming and preclude same-day diagnosis. This remains true nowadays with the fourth generation of chromogenic media. Nucleic acid-based

assays have raised the promise to revolutionize the diagnosis of infectious diseases during the 1990s. For more than 1 decade, nucleic acid-based assays have demonstrated their usefulness for the detection of hardly cultivable, non-cultivable, and even killed microorganisms, as well as for the identification of specific pathogens against the background of a mixed microflora. The current view is still that molecular methods are used to supplement, but not to replace, cultures. MRSA molecular detection nicely illustrates this paradigm: it provides early warning but cultures are still required for further antimicrobial susceptibility testing or epidemiological typing. Molecular assays based on target nucleic acid amplification, and especially real-time PCR, have proven rapid, affordable, and successful in terms of sensitivity and specificity even when performed in complex medium containing a mixture of organisms. Detection of specific amplifications is still improving and future development coupled to the parallelism of hybridization techniques might provide more broadly usable tools allowing not only to identify but also to obtain genotyping characterization providing genetically encoded antibiotic resistance and virulence determinants in a single experiment. Protein-based methods are currently evolving rapidly and will likely allow a rapid and reliable identification at a moderate unit cost.

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References

1. Barber M (1961) Methicillin-resistant staphylococci. J Clin Pathol 14:385–393
2. Hryniewicz W (1999) Epidemiology of MRSA. Infection 27(suppl 2):S13–S16
3. Harbarth S, Martin Y, Rohner P, Henry N, Auckenthaler R, Pittet D (2000) Effect of delayed infection control measures on a hospital outbreak of methicillin-resistant *Staphylococcus aureus*. J Hosp Infect 46(1):43–49
4. Abramson MA, Sexton DJ (1999) Nosocomial methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* primary bacteremia: at what costs? Infect Control Hosp Epidemiol 20(6):408–411
5. Naimi TS, LeDell KH, Como-Sabetti K et al (2003) Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. JAMA 290(22): 2976–2984
6. Saiman L, O'Keefe M, Graham PL III et al (2003) Hospital transmission of community-acquired methicillin-resistant *Staphylococcus aureus* among postpartum women. Clin Infect Dis 37(10):1313–1319
7. Vandenesch F, Naimi T, Enright MC et al (2003) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 9(8):978–984
8. Harbarth S, Francois P, Schrenzel J et al (2005) Community-associated methicillin-resistant *Staphylococcus aureus*, Geneva, Switzerland. Emerg Infect Dis 11(6):962–965
9. Carleton HA, Diep BA, Charlebois ED, Sensabaugh GF, Perdreau-Remington F (2004) Community-adapted methicillin-resistant *Staphylococcus aureus* (MRSA): population dynamics of an expanding community reservoir of MRSA. J Infect Dis 190(10):1730–1738

10. Said-Salim B, Mathema B, Kreiswirth BN (2003) Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging pathogen. Infect Control Hosp Epidemiol 24(6): 451–455
11. Herwaldt LA (1999) Control of methicillin-resistant *Staphylococcus aureus* in the hospital setting. Am J Med 106(5A):11S–18S
12. Pittet D, Safran E, Harbarth S et al (1996) Automatic alerts for methicillin-resistant *Staphylococcus aureus* surveillance and control: role of a hospital information system. Infect Control Hosp Epidemiol 17(8):496–502
13. Pittet D, Hugonnet S, Harbarth S et al (2000) Effectiveness of a hospital-wide programme to improve compliance with hand hygiene. Infection Control Programme. Lancet 356(9238):1307–1312
14. Aucken HM, Ganner M, Murchan S, Cookson BD, Johnson AP (2002) A new UK strain of epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA-17) resistant to multiple antibiotics. J Antimicrob Chemother 50(2):171–175
15. Cox RA, Conquest C, Mallaghan C, Marples RR (1995) A major outbreak of methicillin-resistant *Staphylococcus aureus* caused by a new phage-type (EMRSA-16). J Hosp Infect 29(2):87–106
16. Richardson JF, Reith S (1993) Characterization of a strain of methicillin-resistant *Staphylococcus aureus* (EMRSA-15) by conventional and molecular methods. J Hosp Infect 25(1):45–52
17. Papia G, Louie M, Tralla A, Johnson C, Collins V, Simor AE (1999) Screening high-risk patients for methicillin-resistant *Staphylococcus aureus* on admission to the hospital: is it cost effective? Infect Control Hosp Epidemiol 20(7):473–477
18. Sakoulas G, Eliopoulos GM, Moellering RC Jr et al (2002) Accessory gene regulator (agr) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. Antimicrob Agents Chemother 46(5):1492–1502
19. Salgado C, Tenover FC, Arbeit RD et al (2010) Surveillance for methicillin-resistant *Staphylococcus aureus*: principles, practices, and challenges; a report. X07-R. A CLSI report for global application. clinical and laboratory standards institute. Advancing Quality in Health Care Testing (30):1–55
20. Wernitz MH, Swidsinski S, Weist K et al (2005) Effectiveness of a hospital-wide selective screening programme for methicillin-resistant *Staphylococcus aureus* (MRSA) carriers at hospital admission to prevent hospital-acquired MRSA infections. Clin Microbiol Infect 11(6):457–465
21. Kluytmans J, Van Belkum A, Verbrugh H (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev 10(3): 505–520
22. Grundmann H, Barwolff S, Tami A et al (2005) How many infections are caused by patient-to-patient transmission in intensive care units? Crit Care Med 33(5):946–951
23. Chaix C, Durand-Zaleski I, Alberti C, Brun-Buisson C (1999) Control of endemic methicillin-resistant *Staphylococcus aureus*: a cost-benefit analysis in an intensive care unit. JAMA 282(18):1745–1751
24. Cohen SH, Morita MM, Bradford M (1991) A seven-year experience with methicillin-resistant *Staphylococcus aureus*. Am J Med 91(3B):233S–237S
25. Cosseron-Zerbib M, Roque Afonso AM, Naas T et al (1998) A control programme for MRSA (methicillin-resistant *Staphylococcus aureus*) containment in a paediatric intensive care unit: evaluation and impact on infections caused by other micro-organisms. J Hosp Infect 40(3):225–235
26. Nettleman MD, Trilla A, Fredrickson M, Pfaller M (1991) Assigning responsibility: using feedback to achieve sustained control of methicillin-resistant *Staphylococcus aureus*. Am J Med 91(3B):228S–232S
27. Pittet D, Waldvogel FA (1997) To control or not control colonization with MRSA … that's the question! Q J Med 90:239–241
28. Harbarth S, Pittet D (1998) MRSA—a European currency of infection control. Q J Med 91:519–521

29. Rubinovitch B, Pittet D (2001) Screening for methicillin-resistant *Staphylococcus aureus* in the endemic hospital: what have we learned? J Hosp Infect 47(1):9–18
30. Harbarth S, Fankhauser C, Schrenzel J et al (2008) Universal screening for methicillin-resistant *Staphylococcus aureus* at hospital admission and nosocomial infection in surgical patients. JAMA 299(10):1149–1157
31. Struelens MJ (2006) Rapid identification of methicillin-resistant *Staphylococcus aureus* (MRSA) and patient management. Clin Microbiol Infect 12(s9):23–26
32. Hallin M, Maes N, Byl B, Jacobs F, De Gheldre Y, Struelens MJ (2003) Clinical impact of a PCR assay for identification of *Staphylococcus aureus* and determination of methicillin resistance directly from blood cultures. J Clin Microbiol 41(8):3942–3944
33. Jeyaratnam D, Whitty CJ, Phillips K et al (2008) Impact of rapid screening tests on acquisition of meticillin resistant *Staphylococcus aureus*: cluster randomised crossover trial. BMJ 336(7650):927–930
34. Nulens E, Descheemaeker P, Deurenberg RH, Stobberingh EE, Gordts B (2010) Contribution of two molecular assays as compared to selective culture for MRSA screening in a low MRSA prevalence population. Infection 38(2):98–101
35. Coia JE, Duckworth GJ, Edwards DI et al (2007) Guidelines for the control and prevention of meticillin-resistant *Staphylococcus aureus* (MRSA) in healthcare facilities. J Hosp Infect 63(suppl 1):S1–S44
36. Ayliffe GA, Buckles A, Casewell MW et al (1999) Revised guidelines for control of MRSA: applying appropriately-based recommendations. J Hosp Infect 43(4):315–316
37. Muto CA, Jernigan JA, Ostrowsky BE et al (2003) SHEA guideline for preventing nosocomial transmission of multidrug-resistant strains of *Staphylococcus aureus* and *enterococcus*. Infect Control Hosp Epidemiol 24(5):362–386
38. Nolte FS, Calidendo AM (2003) Molecular detection and identification of microorganisms. In: Murray PR, Barron EJ, Jorgensen JH, Pfaller MA, Yolken RH (eds) Manual of clinical microbiology. ASM Press, Washington, DC, pp 234–256
39. Diekema DJ, Dodgson KJ, Sigurdardottir B, Pfaller MA (2004) Rapid detection of antimicrobial-resistant organism carriage: an unmet clinical need. J Clin Microbiol 42(7):2879–2883
40. Katayama Y, Takeuchi F, Ito T et al (2003) Identification in methicillin-susceptible *Staphylococcus hominis* of an active primordial mobile genetic element for the staphylococcal cassette chromosome mec of methicillin-resistant *Staphylococcus aureus*. J Bacteriol 185(9):2711–2722
41. Ito T, Katayama Y, Asada K et al (2001) Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 45(5):1323–1336
42. Ito T, Okuma K, Ma XX, Yuzawa H, Hiramatsu K (2003) Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. Drug Resist Updat 6(1):41–52
43. Hiramatsu K, Cui L, Kuroda M, Ito T (2001) The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends Microbiol 9(10):486–493
44. Ma XX, Ito T, Tiensasitorn C et al (2002) Novel type of staphylococcal cassette chromosome mec identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. Antimicrob Agents Chemother 46(4):1147–1152
45. Ito T, Ma XX, Takeuchi F, Okuma K, Yuzawa H, Hiramatsu K (2004) Novel type V staphylococcal cassette chromosome mec driven by a novel cassette chromosome recombinase, *ccrC*. Antimicrob Agents Chemother 48(7):2637–2651
46. Francois P, Renzi G, Pittet D et al (2004) A novel multiplex real-time PCR assay for rapid typing of major staphylococcal cassette chromosome *mec* elements. J Clin Microbiol 42(7):3309–3312
47. Baba T, Takeuchi F, Kuroda M et al (2002) Genome and virulence determinants of high virulence community-acquired MRSA. Lancet 359(9320):1819–1827
48. Kuroda M, Ohta T, Uchiyama I et al (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet 357(9264):1225–1240

49. Holden MT, Feil EJ, Lindsay JA et al (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci U S A 101(26):9786–9791
50. Gill SR, Fouts DE, Archer GL et al (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J Bacteriol 187(7):2426–2438
51. Howden BP, Seemann T, Harrison PF et al (2010) Complete genome sequence of *Staphylococcus aureus* strain JKD6008, an ST239 clone of methicillin-resistant *Staphylococcus aureus* with intermediate-level vancomycin resistance. J Bacteriol 192(21):5848–5849
52. Tenover FC, McDougal LK, Goering RV et al (2006) Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J Clin Microbiol 44(1):108–118
53. Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K (2008) Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. J Bacteriol 190(1): 300–310
54. Nair D, Memmi G, Hernandez D et al (2011) Whole genome sequencing of *Staphylococcus aureus* strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. J Bacteriol 193(9): 2332–2335
55. Le Marechal C, Hernandez D, Schrenzel J et al (2011) Genome sequence of two *Staphylococcus aureus* ovine strains that induce severe (strain O11) and mild (strain O46) mastitis. J Bacteriol 193(19):2353–2354
56. Mwangi MM, Wu SW, Zhou Y et al (2007) Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. Proc Natl Acad Sci U S A 104(22):9451–9456
57. Charbonnier Y, Gettler BM, Francois P et al (2005) A generic approach for the design of whole-genome oligoarrays, validated for genomotyping, deletion mapping and gene expression analysis on *Staphylococcus aureus*. BMC Genomics 6:95
58. Couzinet S, Yugueros J, Barras C et al (2005) Evaluation of a high-density oligonucleotide array for characterization of *grlA*, *grlB*, *gyrA* and *gyrB* mutations in fluoroquinolone resistant *Staphylococcus aureus* isolates. J Microbiol Methods 60(2):275–279
59. Dufour P, Gillet Y, Bes M et al (2002) Community-acquired methicillin-resistant *Staphylococcus aureus* infections in France: emergence of a single clone that produces Panton-Valentine leukocidin. Clin Infect Dis 35(7):819–824
60. Berglund C, Ito T, Ikeda M, Ma XX, Soderquist B, Hiramatsu K (2008) Novel type of staphylococcal cassette chromosome mec in a methicillin-resistant *Staphylococcus aureus* strain isolated in Sweden. Antimicrob Agents Chemother 52(10):3512–3516
61. McClure JA, Conly JM, Elsayed S, Zhang K (2010) Multiplex PCR assay to facilitate identification of the recently described staphylococcal cassette chromosome mec type VIII. Mol Cell Probes 24(4):229–232
62. Zhang K, McClure JA, Elsayed S, Conly JM (2009) Novel staphylococcal cassette chromosome mec type, tentatively designated type VIII, harboring class A mec and type 4 ccr gene complexes in a Canadian epidemic strain of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 53(2):531–540
63. Garcia-Alvarez L, Holden MT, Lindsay H et al (2011) Meticillin-resistant *Staphylococcus aureus* with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect Dis 11(8):595–603
64. Brown DF, Edwards DI, Hawkey PM et al (2005) Guidelines for the laboratory diagnosis and susceptibility testing of methicillin-resistant *Staphylococcus aureus* (MRSA). J Antimicrob Chemother 56(6):1000–1018

65. Simor AE, Goodfellow J, Louie L, Louie M (2001) Evaluation of new medium, oxacillin resistance screening agar base, for the detection of methicillin-resistant *Staphylococcus aureus* from clinical specimens. J Clin Microbiol 39(9):3422
66. Becker A, Forster DH, Kniehl E (2002) Oxacillin resistance screening agar base for detection of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 40(11):4400–4401
67. Diederen B, van Duijn I, van Belkam A, Willemse P, van Keulen P, Kluytmans J (2005) Performance of CHROMagar MRSA medium for detection of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 43(4):1925–1927
68. Kluytmans J, van Griethuysen A, Willemse P, van Keulen P (2002) Performance of CHROMagar selective medium and oxacillin resistance screening agar base for identifying *Staphylococcus aureus* and detecting methicillin resistance. J Clin Microbiol 40(7): 2480–2482
69. Perry JD, Davies A, Butterworth LA, Hopley AL, Nicholson A, Gould FK (2004) Development and evaluation of a chromogenic agar medium for methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 42(10):4519–4523
70. Cherkaoui A, Renzi G, Francois P, Schrenzel J (2007) Comparison of four chromogenic media for culture-based screening of meticillin-resistant *Staphylococcus aureus*. J Med Microbiol 56(pt 4):500–503
71. Compernolle V, Verschraegen G, Claeys G (2007) Combined use of Pastorex Staph-Plus and either of two new chromogenic agars, MRSA ID and CHROMagar MRSA, for detection of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 45(1):154–158
72. Carson J, Lui B, Rosmus L, Rennick H, Fuller J (2009) Interpretation of MRSASelect screening agar at 24 hours of incubation. J Clin Microbiol 47(3):566–568
73. Danial J, Noel M, Templeton KE et al (2011) Real-time evaluation of an optimized real-time PCR assay versus Brilliance chromogenic MRSA agar for the detection of meticillin-resistant *Staphylococcus aureus* from clinical specimens. J Med Microbiol 60(pt 3): 323–328
74. Van HF, Deloof N, Claeys G (2011) Performance evaluation of a modified chromogenic medium, ChromID MRSA New, for the detection of methicillin-resistant *Staphylococcus aureus* from clinical specimens. Eur J Clin Microbiol Infect Dis 30(12):1595–1598
75. Harbarth S, Hawkey PM, Tenover F, Stefani S, Pantosti A, Struelens MJ (2011) Update on screening and clinical diagnosis of meticillin-resistant *Staphylococcus aureus* (MRSA). Int J Antimicrob Agents 37(2):110–117
76. Cavassini M, Wenger A, Jaton K, Blanc DS, Bille J (1999) Evaluation of MRSA-screen, a simple anti-PBP 2a slide latex agglutination kit, for rapid detection of methicillin resistance in *Staphylococcus aureus*. J Clin Microbiol 37(5):1591–1594
77. Hussain Z, Stoakes L, Garrow S, Longo S, Fitzgerald V, Lannigan R (2000) Rapid detection of mecA-positive and mecA-negative coagulase-negative staphylococci by an anti-penicillin binding protein 2a slide latex agglutination test. J Clin Microbiol 38(6):2051–2054
78. Wielders CL, Vriens MR, Brisse S et al (2001) Evidence for in-vivo transfer of *mecA* DNA between strains of *Staphylococcus aureus*. Lancet 357(9269):1674–1675
79. Ryffel C, Tesch W, Birch-Machin I et al (1990) Sequence comparison of *mecA* genes isolated from methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Gene 94(1):137–138
80. Rohrer S, Tschierske M, Zbinden R, Berger-Bachi B (2001) Improved methods for detection of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis 20(4):267–270
81. Andriesse GI, Elberts S, Vrolijk A, Verhulst C, Kluytmans JA (2011) Evaluation of a fourth-generation latex agglutination test for the identification of *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis 30(2):259–264
82. Ryffel C, Kayser FH, Berger-Bachi B (1992) Correlation between regulation of *mecA* transcription and expression of methicillin resistance in staphylococci. Antimicrob Agents Chemother 36(1):25–31

83. Murakami K, Minamide W, Wada K, Nakamura E, Teraoka H, Watanabe S (1991) Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. J Clin Microbiol 29(10):2240–2244
84. Youmans GR, Davis TE, Fuller DD (1993) Use of chemiluminescent DNA probes in the rapid detection of oxacillin resistance in clinically isolated strains of *Staphylococcus aureus*. Diagn Microbiol Infect Dis 16(2):99–104
85. Hamels S, Gala JL, Dufour S, Vannuffel P, Zammatteo N, Remacle J (2001) Consensus PCR and microarray for diagnosis of the genus *Staphylococcus*, species, and methicillin resistance. Biotechniques 31(6):1364–1366
86. Towner KJ, Talbot DC, Curran R, Webster CA, Humphreys H (1998) Development and evaluation of a PCR-based immunoassay for the rapid detection of methicillin-resistant *Staphylococcus aureus*. J Med Microbiol 47(7):607–613
87. Berger-Bachi B, Barberis-Maino L, Strassle A, Kayser FH (1989) FemA, a host-mediated factor essential for methicillin resistance in *Staphylococcus aureus*: molecular cloning and characterization. Mol Gen Genet 219(1–2):263–269
88. Hurlimann-Dalel RL, Ryffel C, Kayser FH, Berger-Bachi B (1992) Survey of the methicillin resistance-associated genes *mecA*, *mecR1-mecI*, and *femA-femB* in clinical isolates of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 36(12):2617–2621
89. Kearns AM, Seiders PR, Wheeler J, Freeman R, Steward M (1999) Rapid detection of methicillin-resistant staphylococci by multiplex PCR. J Hosp Infect 43(1):33–37
90. Vannuffel P, Laterre PF, Bouyer M et al (1998) Rapid and specific molecular identification of methicillin-resistant *Staphylococcus aureus* in endotracheal aspirates from mechanically ventilated patients. J Clin Microbiol 36(8):2366–2368
91. Schmitz FJ, Steiert M, Hofmann B et al (1998) Development of a multiplex-PCR for direct detection of the genes for enterotoxin B and C, and toxic shock syndrome toxin-1 in *Staphylococcus aureus* isolates. J Med Microbiol 47(4):335–340
92. Louie L, Goodfellow J, Mathieu P, Glatt A, Louie M, Simor AE (2002) Rapid detection of methicillin-resistant staphylococci from blood culture bottles by using a multiplex PCR assay. J Clin Microbiol 40(8):2786–2790
93. Maes N, Magdalena J, Rottiers S, De Gheldre Y, Struelens MJ (2002) Evaluation of a triplex PCR assay to discriminate *Staphylococcus aureus* from coagulase-negative staphylococci and determine methicillin resistance from blood cultures. J Clin Microbiol 40(4):1514–1517
94. Mason WJ, Blevins JS, Beenken K, Wibowo N, Ojha N, Smeltzer MS (2001) Multiplex PCR protocol for the diagnosis of staphylococcal infection. J Clin Microbiol 39(9):3332–3338
95. Lee LG, Livak KJ, Mullah B, Graham RJ, Vinayak RS, Woudenberg TM (1999) Seven-color, homogeneous detection of six PCR products. Biotechniques 27(2):342–349
96. Mackay IM, Arden KE, Nitsche A (2002) Real-time PCR in virology. Nucleic Acids Res 30(6):1292–1305
97. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB (2001) Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol 39(4):1553–1558
98. Svenstrup HF, Jensen JS, Bjornelius E, Lidbrink P, Birkelund S, Christiansen G (2005) Development of a quantitative real-time PCR assay for detection of *Mycoplasma genitalium*. J Clin Microbiol 43(7):3121–3128
99. Hsu CF, Tsai TY, Pan TM (2005) Use of the duplex TaqMan PCR system for detection of Shiga-like toxin-producing *Escherichia coli* O157. J Clin Microbiol 43(6):2668–2673
100. Lee SO, Na DK, Kim CM et al (2005) Identification and prevalence of *Ehrlichia chaffeensis* infection in *Haemaphysalis longicornis* ticks from Korea by PCR, sequencing and phylogenetic analysis based on 16S rRNA gene. J Vet Sci 6(2):151–155
101. He JW, Jiang S (2005) Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. Appl Environ Microbiol 71(5):2250–2255

102. Gieseler S, Konig B, Konig W, Backert S (2005) Strain-specific expression profiles of virulence genes in *Helicobacter pylori* during infection of gastric epithelial cells and granulocytes. Microbes Infect 7(3):437–447
103. Brancart F, Rodriguez-Villalobos H, Fonteyne PA, Peres-Bota D, Liesnard C (2005) Quantitative TaqMan PCR for detection of *Pneumocystis jiroveci*. J Microbiol Methods 61(3):381–387
104. Fang H, Hedin G (2003) Rapid screening and identification of methicillin-resistant *Staphylococcus aureus* from clinical samples by selective-broth and real-time PCR assay. J Clin Microbiol 41(7):2894–2899
105. Huletsky A, Giroux R, Rossbach V et al (2004) New real-time PCR assay for rapid detection of methicillin-resistant *Staphylococcus aureus* directly from specimens containing a mixture of staphylococci. J Clin Microbiol 42(5):1875–1884
106. Berglund C, Molling P, Sjoberg L, Soderquist B (2005) Predominance of staphylococcal cassette chromosome mec (SCC*mec*) type IV among methicillin-resistant *Staphylococcus aureus* (MRSA) in a Swedish county and presence of unknown SCC*mec* types with Panton-Valentine leukocidin genes. Clin Microbiol Infect 11(6):447–456
107. Donnio PY, Oliveira DC, Faria NA, Wilhelm N, Le Coustumier A, de Lencastre H (2005) Partial excision of the chromosomal cassette containing the methicillin resistance determinant results in methicillin-susceptible *Staphylococcus aureus*. J Clin Microbiol 43(8): 4191–4193
108. Bartels MD, Boye K, Rohde SM et al (2009) A common variant of staphylococcal cassette chromosome mec type IVa in isolates from Copenhagen, Denmark, is not detected by the BD GeneOhm methicillin-resistant *Staphylococcus aureus* assay. J Clin Microbiol 47(5): 1524–1527
109. Thomas L, van Hal S, O'Sullivan M, Kyme P, Iredell J (2008) Failure of the BD GeneOhm StaphS/R assay for identification of Australian methicillin-resistant *Staphylococcus aureus* strains: duplex assays as the "gold standard" in settings of unknown SCC*mec* epidemiology. J Clin Microbiol 46(12):4116–4117
110. Francois P, Pittet D, Bento M et al (2003) Rapid detection of methicillin-resistant *Staphylococcus aureus* directly from sterile or nonsterile clinical samples by a new molecular assay. J Clin Microbiol 41(1):254–260
111. Harbarth S, Masuet-Aumatell C, Schrenzel J et al (2006) Evaluation of rapid screening and pre-emptive contact isolation for detecting and controlling methicillin-resistant *Staphylococcus aureus* in critical care: an interventional cohort study. Crit Care 10:-R25
112. Couzinet S, Jay C, Barras C et al (2005) High-density DNA probe arrays for identification of staphylococci to the species level. J Microbiol Methods 61(2):201–208
113. Saunders NA, Underwood A, Kearns AM, Hallas G (2004) A virulence-associated gene microarray: a tool for investigation of the evolution and pathogenic potential of *Staphylococcus aureus*. Microbiology 150(pt 11):3763–3771
114. Francois P, Bento M, Vaudaux P, Schrenzel J (2003) Comparison of fluorescence and resonance light scattering for highly sensitive microarray detection of bacterial pathogens. J Microbiol Methods 55(3):755–762
115. Monecke S, Slickers P, Ellington MJ, Kearns AM, Ehricht R (2007) High diversity of Panton-Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated methicillin-resistant *S. aureus*. Clin Microbiol Infect 13(12):1157–1164
116. Monecke S, Ehricht R (2005) Rapid genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates using miniaturised oligonucleotide arrays. Clin Microbiol Infect 11(10):825–833
117. Edwards-Jones V, Claydon MA, Evason DJ, Walker J, Fox AJ, Gordon DB (2000) Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. J Med Microbiol 49(3):295–300

118. Jackson KA, Edwards-Jones V, Sutton CW, Fox AJ (2005) Optimisation of intact cell MALDI method for fingerprinting of methicillin-resistant *Staphylococcus aureus*. J Microbiol Methods 62(3):273–284
119. Syrmis MW, Moser RJ, Whiley DM et al (2011) Comparison of a multiplexed MassARRAY system with real-time allele-specific PCR technology for genotyping of methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect 17(12):1804–1810
120. Wolters M, Rohde H, Maier T et al (2011) MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. Int J Med Microbiol 301(1):64–68

Chapter 32
Advanced Methods for Detection of Foodborne Pathogens

Heather Harbottle and Michael Pendrak

Traditional Methods

The detection of bacterial and viral foodborne pathogens has been in practice for many decades to assure the safety and cleanliness of human food. During this long and storied history of food safety, the most traditional methods for detection have been employed with great success, consisting of bacterial and viral culture of the food sample using microbiological media and biochemical identification of bacterial genera or cell culture techniques. The current method accepted by the Food and Drug Administration for detection of bacteria, viruses, yeast and mold is FDA's Bacterial Analytical Manual (BAM) method [1]. These fundamental microbiological assays remain the cornerstone of most pathogen detection schemes, in spite of the fact that traditional culture methods are slow and labor intensive. In a bacterial foodborne disease outbreak, a minimum of 5–7 days are required to obtain an identified isolated colony, which delays the proper diagnosis and treatment regime, resulting in longer hospital stays [2]. Therefore, a significant demand for the rapid detection of pathogens in minutes, rather than days, has arisen. Alternate molecular methods have been developed to attempt reduce or eliminate this rate limiting step and provide identification and characterization information to public health officials in the event of a foodborne disease outbreak. A partial list of some rapid methods and alternative molecular methods are listed in the FDA BAM in Appendix I, although these are not methods officially used or endorsed by the FDA [1].

H. Harbottle (✉)
Microbial Food Safety, Office of New Animal Drug Evaluation,
Center for Veterinary Medicine, U.S. FDA, 7500 Standish Place Rm. E411,
Derwood, MD 20855, USA
e-mail: heather.harbottle@fda.hhs.gov

M. Pendrak
Laboratory of Pathology, National Cancer Institute, NIH, Bethesda, MD 20892-1500, USA
e-mail: pendrakm@mail.nih.gov

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The top four foodborne pathogens, according to the CDC's latest estimation, are norovirus, nontyphoidal *Salmonella*, *Clostridium perfringes*, and *Campylobacter* spp. [2]. The leading cause of foodborne illness-related hospitalization and deaths was attributed to *Salmonella enterica* serotypes, and therefore, a major focus of this chapter is on detection and characterization of *S. enterica* serotypes [2].

Antibody Mediated

After identification of a bacterial foodborne pathogen utilizing selective media and biochemical testing, a common and successful method for further characterizing strains involves the use of antibodies. For microbes such as *S. enterica* strains, serotyping according to the Kauffman/White scheme is one of the oldest and most successful subtyping methods available [3]. Serotyping is based on antibody recognition of the O and the H antigens present on *S. enterica* flagella, and typing is achieved via agglutination testing using monoclonal antibodies specific for each variant. There are recognized to be over 2,500 serotypes of *S. enterica*, with varying degrees of affinity for particular hosts. For example, *Salmonella enterica* serotype Dublin (or *S.* Dublin) has shown to be isolated almost exclusively from cattle, and *S. choleraesuis* has been isolated mostly from swine. Whole genome sequence analysis of *S. enterica* serotypes has given some credence to host specificity in the case of serotypes with an affinity for a particular animal host [4]. Many zoonotic serotypes exist that do not display host-animal affinity, such as *S*. Typhimurium, which are the main focus of surveillance and outbreak identification. Although serotyping is a widely used and specific method to characterize *S. enterica* strains, it is laborious and time consuming. Molecular serotyping methods, such as multiplex polymerase chain reaction (PCR) or real-time PCR systems, have been established for some of the most common foodborne serotypes of *S. enterica* in the USA and Europe [5–7]. Leader et al. [6] tested over 700 strains of *Salmonella* using a multiplex PCR system capable of detecting the 50 most common serotypes in the USA with an accuracy of 89 %, when compared to traditional serotyping. Microarray methods to determine serotype of *S. enterica* strains have been developed, but do not have a 100 % correlation with the traditional Kauffman–White method to date [8]. Koyuncu et al. [8] tested feed samples for *S. enterica* strains using two microarray platforms and found that 56 and 81 % of serotypes were correctly identified. Molecular serotyping methods are much faster and orders of magnitude less labor intensive, but are not 100 % accurate and have not been established for all 2,500 serotypes of *S. enterica*, particularly veterinary strains. Additionally, very limited information can be gleaned from establishing a serotype, and this method of detection is considered a first step in the broad characterization of a *S. enterica* strain.

Additional antibody-mediated detection methods for foodborne pathogens include enzyme-linked immunosorbent assays or ELISA. An example of a commercially available automated system is the VIDAS system (bioMerieux SA, Marcy l'Etoile, France), which detects *S. enterica*, *Escherichia coli*, *Campylobacter*,

Vibrio, and *Listeria* strains from a mixed culture via an immunoassay strip-based method, whose inner surfaces are coated with specific antibodies. The VIDAS allows for automated rapid detection of *Salmonella* in 1–2 days, versus the longer process of identifying via traditional culture methods (5–7 days) and serotyping (5–7 days). Meyer et al. [9] conducted a study to compare detection of *Salmonella* using the VIDAS system and traditional microbiological methods from raw retail meats and meat byproducts in Germany. These investigators found the results to be comparable; however, the VIDAS detected twofold more *Salmonella* in the samples than the traditional methods, leading to the suggestion that VIDAS may be more sensitive at detecting low levels of bacterial strains which were outcompeted by other flora, or that the VIDAS detected unculturable bacteria. Although these investigators compared the VIDAS results with traditional microbiological culture identification, the possibility of false positives cannot be ruled out.

Although traditional ELISA methods can be sensitive and specific for the detection of foodborne pathogens, those conducted using a plate/well scheme are time consuming and can require large volumes of antibody or sample for accurate detection. Therefore, immunoassays which function similarly to ELISA have been developed which are faster, can detect more samples or further characterize the strains, and are more sensitive. Karoonuthaisiri et al. [10] developed an antibody-based microarray to detect the foodborne pathogens *E. coli* and *Salmonella* and compared its efficacy to the traditional ELISA method. These researchers tested the antibody-based microarray for simultaneous detection of each pathogen as well as cross-reactivity to other similar bacteria and common foodborne pathogens such as *Listeria*, *Staphylococcus*, *Klebsiella*, and *Corynebacterium* spp. The study showed that the antibody-based microarray was as specific as traditional ELISA, used about tenfold less antibody to detect the foodborne contaminants, did not cross-react with other foodborne pathogens and could be run in 1 h, versus a minimum of 4 h for a traditional ELISA. Other technologies based on immunoassays, such as microbead-based immunoassays (such as the Luminex technology) are replacing traditional ELISA methods. Microbead assays are capable of detecting a multiplex of 40–100 or more different targets including foodborne pathogens and associated virulence genes, are faster, more reproducible, and more sensitive [11]. In addition to Luminex technology, immunoassays to detect foodborne bacteria such as *Salmonella* are being developed whereby polyclonal antibodies fixed to gold nanoparticles capture the bacterial antigens and the signal is enhanced using a silver-based chemiluminescent detection system. This system detected *Salmonella* in food samples with a limit of 5 cfu/ml [12].

Bacteriophage Typing

Bacteriophage are naturally occurring viruses that target and infect specific strains of bacteria. Due to the specificity of bacteriophage, typing schemes have been developed to further classify very specific strains of bacteria. An example is the

definitive phage type (DT) 104, which identified a specific strain of *S.* Typhimurium which was resistant to five antimicrobials (ACSSuT—ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) and has been the cause of foodborne illness from the 1980s to today [13, 14]. This specific strain was differentiated by the phage type from all other *Salmonella* Typhimurium strains, demonstrating the ability of phage typing to characterize very similar strains within the same serotype. Therefore, phage typing systems have been employed to detect and characterize foodborne bacteria such as *Salmonella* serotypes, *E. coli*, *Campylobacter*, and *Listeria* strains with some success [15, 16]. Benefits to phage typing systems include great specificity, detection of only live cells (unlike DNA-based methods like PCR which detect both living and dead cell DNA), and in some cases, epidemiological relatedness [16, 17]. Phage typing systems can demonstrate positive detection of specific strains via simple plaque assays, or be coupled with fluorophores, antibodies, or other signal amplification schemes, or used as part of a biosensor system [17, 18].

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) is the gold standard for outbreak tracking and molecular characterization of zoonotic foodborne bacteria such as *S. enterica*, *Campylobacter species*, *E. coli*, *Shigella*, *Vibrio cholerae*, and *Listeria monocytogenes* [19, 20]. The PulseNet program, a system of state and public health laboratories and the CDC, share macrorestriction digest gel fingerprints of each strain of foodborne bacteria within a common database, and can identify indistinguishable patterns which may be linked in a foodborne outbreak. The National Antimicrobial Resistance Monitoring System (NARMS), CIPARS, and other international surveillance systems use PFGE for the primary method of molecular characterization of foodborne bacterial pathogens [21, 22]. The PFGE method is dependent on culture and isolation of the bacterial strain from the food product; however, it has been identified nationally and internationally as the gold standard for molecular characterization of foodborne outbreaks. The benefits of the PFGE method include national and international validation and standardization, a full genome "finger print" or banding pattern that is stored electronically, and a shared database between the state, local, and federal food safety agencies. Although single or two enzyme PFGE analysis provides a whole genomic snapshot of the bacterial strain, and does provide a high level of discrimination between very similar strains or serotypes, the actual sequence of these genomic differences are not identified. Additionally, plasmids, due to their small size, often are not visible on the PFGE "fingerprint," and often are not identified when only using the PFGE method. Finally, microbiological culture and isolation/identification of the bacterial pathogen must be conducted before PFGE can be conducted, resulting in a wait time of about 10 days before the results are realized.

Optical Mapping

Optical mapping is an analytical tool which operates on a similar level as PFGE: the genome of a pathogen is restricted and the fragments are measured. PFGE is accomplished by separating the restricted bacterial genomic DNA in an agarose gel that is subjected to alternating polarities, whereas an optical map is an aligned assembly of restricted DNA fragments into a complete genome map on a glass surface. Whole chromosomes are fixed, immobilized and digested with a restriction enzyme on the glass surface for each bacterial strain. Measurements of each contiguous restriction fragment across 20–100 long pieces of DNA are "read," and overlapping chromosomal pieces are assembled by alignment software [23]. In this manner, an entire bacterial genome is analyzed and differences as small as 2 kb can be detected between the analyzed strain and a reference strain, including insertions, deletions, prophage, or inverted sequences. However, and similar to the PFGE method, the exact DNA sequence of the identified change by optical mapping is unknown and must be investigated using sequencing technologies. Kotewicz et al. [23] used optical mapping to investigate the *E. coli* O157:H7 strains linked to the 2006 spinach outbreak in the USA. After identifying 14 informative sites using optical mapping, 454 sequencing was used to define the 14 markers specific to the outbreak strain. Saunders et al. [24] compared optical maps of 20 *Salmonella enterica* serotypes implicated in disease outbreaks to PFGE and microarray data, finding agreement between the methods but greater information regarding geographical lineages and novel insertions from the optical mapping data. Optical mapping was used to characterize the recent *E. coli* O104:H4 outbreak in Germany, suggesting that the outbreak strains were single-sourced and clonal, prior to next-generation sequencing of the strains [25]. Although optical mapping gives a genomic "snapshot" and elucidates novel insertions, deletions, inversions, and prophage, the level of resolution provided by this method is far outweighed by next-generation sequencing technologies of foodborne pathogens.

PCR

Polymerase chain reaction (PCR) is one of the gold standard methods for detecting and characterizing foodborne pathogens, be they bacterial or viral. Because PCR can be conducted on impure samples, mixed samples, and can be performed without the time consuming microbiological culture and isolation methods, it is one of the fastest, most robust, and most reliable methods to date. Methods to detect and characterize the major foodborne pathogens (*Salmonella*, *Campylobacter*, *E. coli*, *Listeria*, *Vibrio*, and Norovirus, to name a few) have been developed for contamination detection in a variety of food products [26–28]. Other methods of bacterial characterization and inter- and intrastrain discrimination based on PCR methods have been developed, such as MultiLocus Sequence Typing (MLST).

This method measures the single or multiple nucleotide changes in well-conserved housekeeping genes and uses this method to assess the evolutionary relatedness of the tested strains, which can provide information for outbreak tracking or virulence. Sangal et al. [29] used MLST and a database of thousands of sequence types contributed by researchers all over the world to study the relatedness and population structure of five major serotypes of *Salmonella*, with a particular focus on *Salmonella* Newport and its MDR-AmpC phenotype expressing resistance to nine antimicrobials. Schemes for multilocus virulence typing, plasmid typing, and other hybrid housekeeping/virulence gene schemes have been developed for many of the foodborne pathogens in order to better characterize these strains. However, primary identification systems such as bacteriological culture and isolation must be used prior to MLST characterization for strain detection. As useful as MLST or any variant multilocus scheme is to define strains, a combination of PFGE and other methods such as MLST have been shown to be the most discriminatory [30, 31].

Real-Time PCR

Real-time reverse transcriptase (RT) PCR is one of the most common and sensitive methods to detect foodborne viruses currently available. Methods to detect noroviruses and hepatitis A viruses, which are difficult to propagate using conventional cell culture methods, have been developed. A simultaneous real-time RT-PCR detection method was developed for noroviruses and hepatitis A virus in contaminated oyster and mussel tissue, exhibiting 100 % specificity when testing for each concurrently [32]. In addition to being one of the most common methods to detect foodborne viruses, real-time PCR methods are some of the most utilized methods to detect and quantify bacterial foodborne contamination events. *Vibrio* species *parahemolyticus*, *cholerae*, and *vulnificus* are common bacterial zoonotic contaminants of seafood and fish and can be detected simultaneously using a validated real-time PCR scheme with as little as 10 cfu/ml in food products [33]. Due to the popularity of real-time PCR methods, commercial kits have been developed and validated by the AOAC for diagnostic tests for food products. For example, two kits were developed by Roche and/or BIOTECHON Diagnostics to individually detect *L. monocytogenes* and *S. enterica* in a variety of food matrices using a real-time PCR scheme. The FoodProof kit allows for rapid isolation of the DNA from food matrices such as peanut butter, milk, vegetables, retail meats, and many other food products [34, 35]. These FoodProof real-time PCR detection kits have been shown to be validated to be equivalent in performance to the FDA-BAM reference method, however much more rapidly. The ability to test for more than one pathogen concurrently is a characteristic essential to the rapid diagnosis of a foodborne illness. Real-time PCR is easily manipulated to test for multiple targets, and was used by Fukushima et al. [36] to detect the causative agents of 21 foodborne outbreaks in 2 days. Therefore, the benefits of using real-time PCR to detect the foodborne pathogen contamination in food products or in an outbreak include increased

sensitivity and specificity, quantification of the pathogen, the ability to multiplex the reaction, and the detection of live bacteria or virus within the sample, versus the potential to detect dead cells for traditional PCR.

Biosensors

Biosensors convert a biological event or response to an electrical signal. These devices or molecular complexes are composed of a recognition element or bioreceptor and a transducer, which converts the biological recognition of the specific receptor element into a measurable electronic signal [18]. These receptors can consist of antibodies, nucleic acids, enzymes, or other organic tissues or microorganisms. The transducer can report the recognition event of the bioreceptor through an electrochemical, thermal, magnetic, or optical transmission. Depending on the system, combinations of transducers can be coupled to amplify detection. Therefore, the general function of a biosensor is the biological recognition of a specific analyte, the transduction of that interaction into a measurable electrical transmission, and the capture of that transmission for further analysis.

Because biosensors must be customized to a specific molecular process, the system must suit the biological process being measured. The most common types of bioreceptors include enzymes, nucleic acids, and antibodies. Biosensor systems that utilize the very specific antibody–antigen interaction to detect foodborne pathogens are some of the most common systems. Tokarskyy and Marshall [37] reviewed a number of *E. coli* O157:H7 immunosensor systems utilizing antibody-based detection systems of this common foodborne illness causative agent for use in the meat processing industry. Although immunosensors have been investigated to detect *E. coli* O157:H7 since the 1990s and can be much more rapid and sensitive than traditional cultural or ELISA methods, direct detection of the bacterial cells via antibody-based immunosensor methods have some drawbacks. Immnunosensors lack the ability to detect bacterial cells within the meat matrix, can result in false positive/false negative results due to cross-reactivity with other foodborne bacteria in the food matrix, and have displayed some variability within lots of immunosensors [37]. Commercially available immunosensor kits are available to detect *E. coli* O157:H7; however, performance validation according to AOAC standards has been a challenge.

PCR-based systems are another common type of biosensors. Chua et al. [38] used DNA biosensors consisting of oligonucleotides specific to *V. cholerae* labeled with biotin or fluorescein, detectors consisting of gold nanoparticles bioconjugated with antifluorescein antibodies, and capture molecules consisting of streptavidin-coated microspheres labeled with antidigoxigenin antibodies, with detection accomplished using lateral-flow. Chua et al. [38] tested this DNA biosensor system against a panel of 174 bacteria spiked into healthy stool specimens and found 100 % sensitivity and specificity with a limit of detection of 5 ng of target *V. cholerae* DNA. Geng et al. [39] used magnetic beads capped with specific DNA sequences to magnetically

separate and bind *E. coli* in an electrochemical biosensor system with a detection limit of 50 cells/ml. With whole genome sequencing of outbreak strains for particular markers, this method of sensitive and rapid detection has great potential.

Microarrays

Microarrays have been used with success to identify and characterize foodborne pathogens such as *E. coli*, *Salmonella*, and *Campylobacter* in purified or mixed samples since their first description in 1995 [40–43]. Microarrays are composed of substrate-coated glass slides or "chips," which have been printed with probes, or 25–75 bp pieces of DNA that are specific to particular genes or genetic elements. Labeled DNA (bacterial or viral samples) are hybridized these chips, the labeled sample binds to the probe immobilized on the chip, and detection is achieved by the visualization of the fluorescence of the probe–sample complex after scanning at a particular wavelength.

Microarrays have been used to detect antimicrobial resistance genes, mobile genetic elements, and virulence genes individually and concurrently [40, 44–47]. Microarrays are a high throughput and information dense tool that are particularly useful when screening multiple pathogen types with multidrug resistant phenotypes in a foodborne pathogen surveillance program such as NARMS. Given the threat of outbreaks due to enteric bacteria resistant to drugs of choice for human clinical treatment, Lascols et al. [48] used a microarray designed to detect, among other genes, extended spectrum beta-lactamase genes in 235 enteric bacteria from human patients including *E. coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter* strains. 28 % of isolates contained a carbapenemase gene, and only isolates from India carried the recently discovered bla_{NDM-1} gene. All but one isolate carrying the bla_{NDM-1} gene carried multiple carbapenemase genes concurrently. This study showcases the use of microarrays to characterize multidrug resistance in clinical isolates with a particular focus. Lindsey et al. [49] constructed a microarray which additionally identifies a number of common plasmid backbones which are associated with enteric foodborne bacteria, and used the microarray to screen isolates from food animals to investigate the prevalence and coincidence of antimicrobial resistance genes and particular plasmid types. Beutlich et al. [50] used DNA microarrays to perform an in-depth investigation of virulence and virulence associated genes in *Salmonella* strains of with serotypes of clinical importance, and identified differences in the *Salmonella* Genomic Island (SGI) variants that are associated with particular serotypes. Coupled with antimicrobial resistance phenotypes, these types of studies can provide data indicating host adaptation associated with particular serotypes. Traditional printed microarrays can accommodate up to 40,000 probes which are specific for DNA sequences. Photolithographic microarrays, such as Affymetrix arrays, are being designed for foodborne pathogens, which can accommodate millions of probes due to the photolithographic technology (Affymetrix Inc., Santa Clara, CA). These very information dense and high-throughput microarrays contain probes

for entire genomes of foodborne pathogens, and can define a single strain. Jackson et al. [51] used this technology to define and describe the genomic content of *E. coli* isolates from a reference collection and human illnesses. This type of microarray which can define entire genomes of particular strains will be very useful for outbreak source tracking.

Although bacterial foodborne pathogens are more commonly detected using microarray technology, platforms to detect foodborne viral pathogens are becoming more commonly available. With cell culture methods and PCR/RT-PCR methods representing the gold standards, the higher-throughput methods are an attractive alternative for rapid diagnostics. Tiling microarrays, or arrays with overlapping oligonucleotides capable of detecting single nucleotide polymorphisms, were developed to detect and characterize hepatitis A virus, human coxsackieviruses A and B (CVA and CVB), genogroups I and II of norovirus (NV), and human rotavirus (RV) [52, 53]. As the technology improves, microarrays will be very useful for use in outbreak investigation and source tracking, and thereby aid in food safety risk strategy development.

Multiplexed Microsphere-Based Flow Cytometry

Flow cytometry using fluorescent microspheres was pioneered commercially by the Luminex Corporation and now a number of companies offer this technology [54]. This platform is a high-throughput assay system that uses polystyrene microspheres that can be coupled to either nucleic acids or protein using straightforward chemistries. Up to 100 spectral addresses can be measured in each sample mixture providing the potential for rapid, large scale screening of complex sample mixtures. The feasibility of this approach was tested using nucleic acid targets for common bacterial pathogens in 2003 and further developed into a high sensitivity assay system for the simultaneous discrimination of *Salmonella* from other enteric microorganisms [55, 56]. A single-step RT-PCR multiplexed assay has been recently developed for norovirus GI and GII, rotavirus, astrovirus, sapovirus, and adenovirus [57]. A multiplex RT-PCR assay system is now commercially available from Luminex Corporation and combines bacterial, viral, and parasitic detection in a single assay tube (xTAG® Gastrointestinal Pathogen Panel).

The versatility of this assay system is suggested by an antibody-based approach to detect abrin, botulinum toxins, ricin, and *Staphylococcus* enterotoxins A, B, and C in food. This assay used paramagnetic fluorescent microspheres so a magnetic separation step prior to spectral analysis could be performed on samples containing large amounts of particulates [58]. The immunoassay format has been used for serotyping *E. coli* O157 and detecting Stx(1) and/or Stx(2) and for *Salmonella*, *Campylobacter*, *E. coli*, *Listeria*, and staphylococcal enterotoxin B [11]. Overall, the versatility and sensitivity of the system should make assay development for the detection of human pathogens in food more attractive.

Next-Generation Sequencing (NGS) Technologies

The advancements in the last decade from the first generation automated Sanger sequencing used for the human genome project to the current NGS platforms represent a remarkable advancement for the molecular epidemiologist (for a technology review see Metzker [59]). The ability to identify and subtype strains involved in a disease outbreak is now a reality. Within the next decade, the projected cost for de novo sequencing of a microbial genome will be well within the reach of most diagnostic laboratories, and its widespread use is inevitable [60, 61].

A number of retrospective studies have provided the "proof of principle" for this emerging technology in the study of food-related disease outbreaks. The likely long-term persistence of a *L. monocytogenes* strain in a food processing plant was confirmed by the NGS approach. Isolates from 1988 and 2000 were virtually identical at the chromosomal level and differed only in prophage recombination events [62]. In this case, all isolates were of a rare PFGE subtype, but it is also likely that different PFGE subtypes could be generated by such events. A related investigation of a listeriosis outbreak originating from ready-to-eat meat products in Canada in 2008 highlighted the ability of NGS to map microevolutionary changes during a disease outbreak [61]. Recently, a salmonellosis outbreak has been addressed using NGS and was able to identify the origin for the Montevideo strain associated with this event, even though the strain was judged identical to other tested isolates by conventional typing methods [63]. A different sort of investigation was conducted on the origin of the Haitian cholera outbreak. NGS provided investigators with evidence that the current outbreak was most likely caused by a strain introduced by "human activity" [64]. The speed with which investigators are able to sequence an entire genome in response to an outbreak is increasing as the technology improves. A team at the Beijing Genomic Institute and in Germany concomitantly sequenced and assembled the recent German *E. coli* O104:H4 outbreak strain in 2–3 days using a new integrated semiconductor nonoptical genomic sequencer (Ion Torrent from Life Technologies) [25, 65]. This allowed the outbreak investigators to analyze and identify the newly emergent and recombinant *E. coli* in a matter of a few short months, lightening fast for this type of whole genome analysis. Moreover, this genotyping approach allowed investigators to discover that the *E. coli* outbreak strain was a hybrid of enteroaggregative (EAEC) and enterohemorrhagic (EHEC) pathotypes, allowing for rapid symptom prediction and improved development of appropriate treatment regimes [25].

The NGS technology has spawned the field of "metagenomics," or the identification of genetic material isolated directly from environmental samples. This approach was tested for a case of human illness using DNA from a patient's feces during and after an idiopathic diarrhea. In this case, *Campylobacter jejuni* sequences were identified only from the illness sample [66]. This approach has also been used in the detection of viral diarrheal illness [67]. Metagenomics is suited not only for the investigations of food contamination but also for the identification of commensal and food spoilage organisms as well as assisting the development of metabolic models for commercial fermentation [68, 69].

Overall, full genome sequencing is coming to the forefront for follow-up investigations of epidemiological and source tracking studies, and will likely be used in a real-time format to identify contaminant point sources. The latter is especially important during high priority public health events, and will become more efficient and "user-friendly" as the data pool increases and the technology advances.

Conclusions

With food safety at the forefront of the mind due to a number of recent outbreaks involving retail meats, peanut butter, and fresh vegetables, it is imperative that the programs which protect our food supply from accidental or intentional contamination are strengthened. Traditional methods, while validated and internationally accepted, are often too laborious and time consuming to provide information that could curtail a foodborne pathogen outbreak or provide information useful for treatment strategies. New and advanced technologies, such as high density microarray and whole genome sequencing, are becoming more rapid and affordable for regular use for surveillance of our food supply. Multilaboratory validations must be conducted to put these exciting and advanced technologies into the hands of testing agencies to protect and preserve the quality of our food.

References

1. U.S. Food and Drug Administration (2011) Bacteriological analytical manual (BAM). Chapter 5.Salmonella(http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070149.htm)
2. Scallan E, Hoekstra R, Angulo F et al (2011) Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis 17:7–15
3. Bopp CA, Brenner FW, Fields PI, Wells JG, Stockbine NA (eds) (2003) Manual of clinical microbiology, vol 1. ASM Press, Washington, DC
4. Jacobsen A, Hendriksen RS, Aaresturp FM, Ussery DW, Friis C (2011) The *Salmonella enterica* Pan-genome. Microb Ecol 62(3):487–504
5. Kim S, Frye JG, Hu J, Fedorka-Cray PJ, Gautom R, Boyle DS (2006) Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp. enterica. J Clin Microbiol 44(10):3608–3615
6. Leader BT, Frye JG, Hu J, Fedorka-Cray PJ, Boyle DS (2009) High-throughput molecular determination of *Salmonella enterica* serovars by use of multiplex PCR and capillary electrophoresis analysis. J Clin Microbiol 47(5):1290–1299
7. Rajtak U, Leonard N, Bolton D, Fanning S (2011) A Real-Time Multiplex SYBR Green I Polymerase Chain Reaction Assay for rapid screening of *Salmonella* serotypes prevalent in the European Union. Foodborne Pathog Dis 8(7):769–780
8. Koyuncu S, Andersson G, Vos P, Häggblom P (2011) DNA microarray for tracing *Salmonella* in the feed chain. Int J Food Microbiol 145:S18–S22
9. Meyer C, Thiel S, Ullrich U, Stolle A (2011) *Salmonella* in raw meat and by-products from pork and beef. J Food Protect 73(10):1780–1784

10. Karoonuthaisiri N, Charlermroj R, Uawisetwathana U, Luxananil P, kirtikara K, Gajanandana O (2009) Development of antibody array for simultaneous detection of foodborne pathogens. Biosens Bioelectron 24:1641–1648
11. Clotilde LM, Bernard C 4th, Hartman GL, Lau DK, Carter JM (2011) Microbead-based immunoassay for simultaneous detection of Shiga toxins and isolation of *Escherichia coli* O157 in foods. J Food Protect 74(3):373–379
12. Wang Z, Duan N, Li J, Ye J, Ma S, Le G (2011) Ultrasensitive chemiluminescent immunoassay of *Salmonella* with silver enhancement of nanogold labels. Luminescence 26(2):136–141
13. Davies A, O'Neill P, Towers L, Cooke M (1996) An outbreak of *Salmonella typhimurium* DT104 food poisoning associated with eating beef. Commun Dis Rep CDR Rev 6(11):R159–R162
14. Majtánová L, Majtán T, Majtán V (2010) Detection of the class 1 integrons and SGI1 among *Salmonella enterica* Serovar Typhimurium DT104, U302, DT120, DT193, and nontypable human isolates. Jpn J Infect Dis 63(4):292–295
15. Pearce MC, Chase-Topping ME, McKendrick IJ, Mellor DJ, Locking ME, Allison L, Ternent HE, Matthews L, Knight HI, Smith AW, Synge BA, Reilly W, Low JC, Reid SW, Gunn GJ, Woolhouse ME (2009) Temporal and spatial patterns of bovine *Escherichia coli* O157 prevalence and comparison of temporal changes in the patterns of phage types associated with bovine shedding and human *E. coli* O157 cases in Scotland between 1998–2000 and 2002–2004. BMC Microbiol 29(9):276
16. Hagens S, Loessner MJ (2007) Application of bacteriophages for detection and control of foodborne pathogens. Appl Microbiol Biotechnol 76(3):513–519
17. Smartt AE, Ripp S (2011) Bacteriophage reporter technology for sensing and detecting microbial targets. Anal Bioanal Chem 400:991–1007
18. Velusamy V, Arshak K, Korostynska O, Oliwa K, Adley C (2010) An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnol Adv 28:232–254
19. Swaminathan B, Barrett TJ, Hunter SB, Tauxe RV, CDC PulseNet Task Force (2001) PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg Infect Dis 7(3):382–389
20. Boxrud D, Monson T, Stiles T, Besser J (2010) The role, challenges, and support of pulsenet laboratories in detecting foodborne disease outbreaks. Public Health Rep 125(Suppl 2):57–62
21. FDA (2011) National Antimicrobial Resistance Monitoring System—enteric bacteria (NARMS): 2009 retail meat report. U.S. Department of Health and Human Services, Food and Drug Administration, Rockville, MD
22. BC Integrated Surveillance Epidemiology Sub-Group. BC integrated *Salmonella* surveillance annual report 2010, May 2010
23. Kotewicz ML, Mammel MK, LeClerc JE, Cebula TA (2008) Optical mapping and 454 sequencing of *Escherichia coli* O157:H7 isolates linked to the US 2006 spinach-associated outbreak. Microbiology 154(Pt 11):3518–3528
24. Saunders MP, Wu G, Abuoun M, Pan Z, Anjum M, Woodward MJ (2010) Optical genetic mapping defines regions of chromosomal variation in serovars of *S. enterica* subsp. enterica of concern for human and animal health. Epidemiol Infect 22:1–10
25. Mellmann A, Harmsen D, Cummings CA, Zentz EB et al (2011) Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. PLoS One 6(7):e22751
26. Wang RF, Cao WW, Cerniglia CE (1997) A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J Appl Microbiol 83(6):727–736
27. Boxman IL, Tilburg JJ, te Loeke NA, Vennema H, de Boer E, Koopmans M (2007) An efficient and rapid method for recovery of norovirus from food associated with outbreaks of gastroenteritis. J Food Protect 70(2):504–508
28. Stals A, Baert L, De Keuckelaere A, Van Coillie E, Uyttendaele M (2011) Evaluation of a norovirus detection methodology for ready-to-eat foods. Int J Food Microbiol 145:420–425
29. Sangal V, Harbottle H, Mazzoni CJ, Helmuth R, Guerra B, Didelot X, Paglietti B, Rabsch W, Brisse S, Weill FX, Roumagnac P, Achtman M (2010) Evolution and population structure of *Salmonella* enterica serovar Newport. J Bacteriol 192(24):6465–6476

30. Harbottle H, McDermott PF, White DG, Walker RD, Zhao S (2006) Comparison of multi-locus sequence typing with pulsed-field gel electrophoresis and antimicrobial susceptibility profiling of *Salmonella* enterica Serotype Newport isolates. J Clin Microbiol 44(7): 2449–2457
31. Thakur S, White DG, Kroft B, McDermott PF et al (2009) Genotyping of *Campylobacter* coli isolated from humans and retail meats using multi locus sequence typing and pulsed field gel electrophoresis. J Appl Microbiol 106(5):1722–1733
32. Uhrbrand K, Myrmel M, Maunula L, Vainio K et al (2010) Evaluation of a rapid method for recovery of norovirus and hepatitis A virus from oysters and blue mussels. J Virol Methods 169:70–78
33. Messelhäusser U, Colditz J, Thärigen D, Kleih W et al (2010) Detection and differentiation of Vibrio spp. in seafood and fish samples with cultural and molecular methods. Int J Food Microbiol 142:360–364
34. Junge B, Berghof-Jager K (2006) Roche/BIOTECON Diagnostics LightCycler foodproof *L. monocytogenes* Detection Kit in combination with ShortPrep foodproof II Kit. J AOAC Int 89(2):374–398
35. Lindhardt C, Schonenbrucher H, Slaghuis J, Bubert A et al (2009) Foodproof® *Salmonella* Detection Kit. J AOAC Int 92(6):1876–1884
36. Fukushima H, Katsube K, Tsunomori Y, Kishi R, Atsuta J, Akiba Y (2009) Comprehensive and rapid real-time PCR analysis of 21 foodborne outbreaks. Int J Microbiol 2009:917623
37. Tokarskyy O, Marshall DL (2008) Immunosensors for rapid detection of *Escherichia coli* O157:H7—perspectives for use in the meat processing industry. Food Microbiol 25:1–12
38. Chua A, Yean C, Ravichandran M, Lima B, Lalitha P (2011) A rapid DNA biosensor for the molecular diagnosis of infectious disease. Biosens Bioelectron 26:3825–3831
39. Geng P, Zhang X, Teng Y, Fu Y, Xu L, Xu M, Jin L, Zhang W (2011) A DNA sequence-specific electrochemical biosensor based on alginic acid-coated cobalt magnetic beads for the detection of *E. coli*. Biosens Bioelectron 26(7):3325–3330
40. Bruant G, Maynard C, Bekal S et al (2006) Development and validation of an oligonucleotide microarray for detection of multiple virulence and antimicrobial resistance genes in *Escherichia coli*. Appl Environ Microbiol 72(5):3780–3784
41. Cooke FJ, Brown DJ, Fookes M et al (2008) Characterization of the genomes of a diverse collection of *Salmonella* enterica serovar Typhimurium definitive phage type 104. J Bacteriol 190(24):8155–8162
42. Dorrell N, Mangan JA, Laing KG et al (2001) Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. Genome Res 11(10):1706–1715
43. Kostic T, Weilharter A, Rubino S et al (2007) A microbial diagnostic microarray technique for the sensitive detection and identification of pathogenic bacteria in a background of nonpathogens. Anal Biochem 360(2):244–254
44. Batchelor M, Hopkins KL, Liebana E et al (2008) Development of a miniaturised microarray-based assay for the rapid identification of antimicrobial resistance genes in Gram-negative bacteria. Int J Antimicrob Agents 31(5):440–451
45. Chen S, Zhao S, McDermott PF et al (2005) A DNA microarray for identification of virulence and antimicrobial resistance genes in *Salmonella* serovars and *Escherichia coli*. Mol Cell Probes 19(3):195–201
46. Frye JG, Jesse T, Long F et al (2006) DNA microarray detection of antimicrobial resistance genes in diverse bacteria. Int J Antimicrob Agents 27(2):138–151
47. Porwollik S, Boyd EF, Choy C et al (2004) Characterization of *Salmonella* enterica subspecies I genovars by use of microarrays. J Bacteriol 186(17):5883–5898
48. Lascols C, Hackel M, Marshall SH, Hujer AM, Bouchillon S, Badal R, Hoban D, Bonomo RA (2011) Increasing prevalence and dissemination of NDM-1 metallo-blactamase in India: data from the SMART study (2009). J Antimicrob Chemother 66(9):1992–1997
49. Lindsey RL, Frye JG, Fedorka-Cray PJ, Welch TJ, Meinersmann RJ (2010) An oligonucleotide microarray to characterize multidrug resistant plasmids. J Microb Methods 81:96–100

50. Beutlich J, Jahn S, Malorny B, Hauser E, Hühn S et al (2011) Antimicrobial resistance and virulence determinants 1 in European "*Salmonella* Genomic Island 1 (SGI1)" positive *Salmonella enterica* isolates from different origins. Appl Environ Microbiol 77(16): 5655–5664
51. Jackson SA, Patel IR, Barnaba T, LeClerc JE, Cebula TA (2011) Investigating the global genomic diversity of *Escherichia coli* using a multi-genome DNA microarray platform with novel gene prediction strategies. BMC Genomics 12:349
52. Chen H, Mammel M, Kulka M, Patel I et al (2011) Detection and identification of common food-borne viruses with a tiling microarray. Open Virol J 5:52–59
53. Ayodeji M, Kulka M, Jackson SA, Patel I, Mammel M et al (2009) A microarray based approach for the identification of common foodborne viruses. Open Virol J 3:7–20
54. Keller KL, Oliver KG (2004) Multiplexed microsphere assays for protein and DNA binding reactions. Methods Cell Biol 75:409–429
55. Dunbar SA, Vander Zee CA, Oliver KG, Karem KL, Jacobson JW (2003) Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. J Microbiol Methods 53(2):245–252
56. Dunbar SA, Jacobson JW (2007) Quantitative, multiplexed detection of Salmonella and other pathogens by Luminex xMAP suspension array. Methods Mol Biol 394:1–19
57. Liu J, Kibiki G, Maro V, Maro A et al (2011) Multiplex reverse transcription PCR Luminex assay for detection and quantitation of viral agents of gastroenteritis. J Clin Virol 50(4): 308–313
58. Garber EAE, Venkateswaran KV, O'Brien TW (2010) Simultaneous multiplex detection and confirmation of the proteinaceous toxins abrin, ricin, botulinum toxins, and Staphylococcus enterotoxins A, B, and C in food. J Agric Food Chem 58(11):6600–6607
59. Metzker ML (2009) Sequencing technologies—the next generation. Nat Rev Genet 11(1):31–46
60. Pallen MJ, Loman NJ, Penn CW (2010) High-throughput sequencing and clinical microbiology: progress, opportunities and challenges. Curr Opin Microbiol 13(5):625–631
61. Gilmour MW, Graham M, Van Domselaar G, Tyler S et al (2010) High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. BMC Genomics 11(120):120
62. Orsi RH, Borowsky ML, Lauer P, Young SK et al (2008) Short-term genome evolution of *Listeria monocytogenes* in a non-controlled environment. BMC Genomics 9(539):539
63. Lienau EK, Strain E, Wang C, Zheng J et al (2011) Identification of a Salmonellosis outbreak by means of molecular sequencing. New Engl J Med 364(10):981–982
64. Chin CS, Sorenson J, Harris JB, Robins WP et al (2011) The origin of the Haitian cholera outbreak strain. N Engl J Med 364(1):33–42
65. Beijing Genome Institute (BGI) (2011) Sequencing data of *Escherichia coli* TY-2482 isolate based on Ion torrent technology and the assembly. Available: <ftp://ftp.genomics.org.cn/pub/Ecoli_TY-2482/>. Accessed 14 June 2011
66. Nakamura S, Maeda N, Miron IM, Yoh M et al (2008) Metagenomic diagnosis of bacterial infections. Emerg Infect Dis 14(11):1784–1786
67. Finkbeiner SR, Allred AF, Tarr PI, Klein EJ et al (2008) Metagenomic analysis of human diarrhea: viral detection and discovery. PLoS Pathog 4(2):e1000011
68. Liu GE (2009) Applications and case studies of the next-generation sequencing technologies in food, nutrition and agriculture. Recent Pat Food Nutr Agric 1(1):75–79
69. Henry CS, DeJongh M, Best AA, Frybarger PM et al (2010) High-throughput generation, optimization and analysis of genome-scale metabolic models. Nat Biotechnol 28(9):977–982

Chapter 33
Technical and Clinical Niches for Point of Care Molecular Devices

Ellen Jo Baron and Sheldon Campbell

Definition and Limitation of Point of Care Tests

A point of care (POC) device is one that is used outside of a central laboratory environment; generally near, or at the site of the patient/client. Point of care testing (POCT) varies from tests performed in physician's office labs, or "satellite" or "stat" labs, to tests performed on tabletop instruments in a clinic area, to testing performed with hand-held instruments at the bedside. In peripheral lab settings, POCT may be performed by trained laboratory staff, but clinic and bedside POCT is frequently performed by staff who lack specialized laboratory training and whose primary job is something other than doing lab tests.

In the industrialized world POCT is most commonly used to provide results within medical decision-making or infection control intervention actionable timeframes in order to accelerate and streamline care. That timeframe differs depending on the nature and seriousness of the infectious process, but for purposes of this discussion, a maximum of 4 h may be a reasonable upper limit from sample collection to results delivery within the scope of a POC test. In some clinical situations, this will be too long, and clinical decisions must be made without test results for guidance. POCT is also used (especially in resource-limited settings) to provide laboratory results unavailable in any other way. This may be provided by simple test systems that use stable reagents and provide rapid results. Novel systems for remote

E.J. Baron (✉)
Department of Pathology, Stanford University School of Medicine,
Palo Alto, CA 94304, USA

Research and Development, Cepheid, Sunnyvale, CA 94089, USA
e-mail: ejbaron@stanford.edu

S. Campbell
Department of Laboratory Medicine, Yale School of Medicine,
New Haven, CT 06520, USA

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testing (e.g., viewing of malaria smears via mobile phones) are also coming into use which might fit an operational definition of POCT [1].

There are several reasons to develop a POC test for an infectious disease. These include:

- The need to quickly provide highly targeted therapy. Current algorithms for seriously ill patients depend on empiric treatment based on the most likely pathogens for a given clinical presentation; however, this method involves broad-spectrum therapy to cover the likely contingencies. Knowing the exact identification of the pathogen will allow more focused therapeutic decisions. If a molecular method also detects important resistance factors in the pathogen, then a therapeutic decision can be made specifically to both treat the pathogen and limit development of resistant organisms.
- POCT infectious disease molecular assays may be developed to detect specific infections for which a rapid response is desirable. Examples include common outpatient infections such as group A streptococcal pharyngitis where immediate diagnosis saves follow-up effort; or *Chlamydia* and gonorrhea, where rapid results may allow immediate treatment of patients who might otherwise be lost to follow-up. There is the potential for both clinical and public health benefits from this class of test.
- Another potential objective of a POCT is to recognize quickly which patients require infection control precautions as they are admitted to the healthcare institution to prevent the spread of the agent to other patients or to caregivers. Some POC assays are meant for surveillance only and in such cases, interventions are taken to break transmission routes and prevent the development of infections. Increasingly, healthcare institutions are being asked to become more cost-effective, and rapid applications of infection control activities have been shown to be most effective. The potential for POCT to impact on infection-control is particularly significant for long-term care facilities and other health care settings without on-site laboratories.

Clinical Situations for Which POC Molecular Tests Are Currently Available

Platforms employing molecular technology which are simple enough for potential POC use are just beginning to come to market. No molecular test has yet achieved CLIA "waived" status (although some are being developed for FDA submission), so POC molecular tests are still physician's office lab or "satellite lab" tests rather than true bedside methods. Several comparatively simple, rapid molecular methods, though, are available and increasingly used.

The most widely used molecular test at patient POC sites is the real-time PCR (RT-PCR) assay for detection of nasal colonization of methicillin-resistant *Staphylococcus aureus* (MRSA) [2]. Colonized patients can be placed into contact isolation, decolonization protocols can be initiated, and appropriate surgical

prophylaxis can be used [3]. The use of this assay within the United States Veterans' Administration hospitals is one factor credited with lowering healthcare-associated MRSA infections 59 % since universal screening and additional infection control interventions were implemented. Selected nosocomial infections due to *Clostridium difficile* and vancomycin-resistant enterococci (VRE) also decreased [4]. Two further reports on the use of surveillance for MRSA illustrate the effectiveness of this intervention. With rapid results available within hours of patient admission, the Northshore Hospital System showed 69.6 % decrease in hospital-associated MRSA disease over the study period [5]. In contrast, another healthcare institution used a slower method for MRSA nasal surveillance with results available more than a day later and results were disappointing [6]. Molecular POC tools are virtually the only method possible to achieve the most effective infection control. Additional assays that detect both MRSA and methicillin-susceptible staphylococci in patients' skin and soft tissue wound sites and in nares are also available [7].

Testing feces for the presence of toxigenic *C. difficile* is another use of rapid molecular technology today [8]. An RT-PCR platform and a loop-mediated isothermal amplification (LAMP) platform are FDA-cleared. They each employ different targets. The LAMP assay seeks a genetic locus in the TcdA gene of *C. difficile* whereas the PCR assays either identify a portion of the toxin B gene (TcdB) [9] or a second FDA-cleared assay presumptively identifies the epidemic, hypervirulent 027 strain by detecting both a binary toxin sequence and a deletion in the toxin regulatory gene, in addition to the TcdB gene [10, 11].

The same RT-PCR platform is FDA-cleared for detection of influenza A, B, and influenza A H1N1 novel 2009 in respiratory secretions, which is a modification of a previous test available for a limited time during the 2009 H1N1 Influenza A outbreak [12]. In addition, a self-contained PCR technology using packets of reagents in plastic pouches has also been FDA-cleared for detection of respiratory viruses [13]. Another rapid molecular test has been FDA-cleared for multiplex detection of 15 respiratory viruses, including adenovirus, 2 coronavirus strains, 5 influenza strains, human metapneumovirus, parainfluenza virus types 1–4, RSV, and rhino-enterovirus, with a time-to-result of 1–1.5 h using a novel multiplex PCR and array detection format [14]. Although other PCR methods for virus detection and identification in respiratory secretions are available and show excellent sensitivities and specificities, they are not candidates for POC tests due to their complexity, long performance time, or format that leads to inefficiencies when performing non-batch (such as stat) testing [14].

An FDA-cleared RT-PCR assay can be used to detect gastrointestinal colonization with VRE using rectal swabs [15]. The US version of the test was developed to detect the vanA gene only because vanB VREs are uncommon in the United States today, and because there are more vanB-containing non-enterococci than enterococci in feces. A commonly used FDA-cleared PCR platform has another enterococcal assay that detects vanA and vanB, but specificity of the vanB marker is poor and the format is not optimized for POC [16].

The same platform as described for VRE and staphylococci is FDA-cleared for direct detection of group B streptococci (GBS) in vaginal/rectal swabs [17].

This assay has been used at the time of delivery to test women who never received antenatal surveillance cultures for GBS, or for women who tested negative in their surveillance cultures but whose colonization status may have changed between the time of the culture and presentation in labor [18].

In addition, this platform has an FDA-cleared test for the presence of enterovirus in cerebrospinal fluid [19]. Unlike all the other tests available on this platform, the CSF enterovirus assay is designated "high complexity" due to the need for the testing person to pipette a specific 200 μL volume of CSF into the cartridge.

Lightcycler® and other RT-PCR platforms have been used to develop tests for herpes simplex and varicella zoster virus in cerebrospinal fluid. They could be considered borderline POC tests because if ordered stat, a highly trained laboratory scientist could theoretically perform the test and report results within the 4 h timeframe [20]. It is unlikely, however, that in cases of severe disease, the clinicians treating the patient would be willing to wait that long before treating based on clinical presentation and CSF cell count separate from microbiology laboratory results.

A direct DNA hybridization assay for identification of *Gardnerella vaginalis* (as a marker for bacterial vaginosis), *Candida albicans*, and *Trichomonas vaginalis* has been in use in large physician offices and reference laboratories for many years [21]. Tests can be run in batches of 6 or fewer samples and results are available within 2 h.

In countries other than the United States, the RT-PCR method is used to detect *Mycobacterium tuberculosis* and rifampin-resistance in *M. tuberculosis* using a hemi-nested PCR protocol that uses five molecular beacons to bind to different regions of the ribosomal polymerase B gene in which most mutations conferring rifampin resistance are found [22]. If all five regions bind to their specifically colored fluorescent beacons, the organism is a wild-type *M. tuberculosis*. If one or more of the regions fails to bind its specific beacon, but at least two regions are present, the *M. tuberculosis* strain is reported as resistant [23]. This test can be used with unprocessed respiratory tract secretions at the patient location and results are available within 2 h. Studies have shown that even unskilled workers can achieve high levels of accuracy with this assay [24]. After achieving endorsement by the World Health Organization, it is being broadly disseminated throughout the resource-poor world.

It should not be overlooked that there are several POC diagnostics for detection of agents of bioterrorism. Anthrax, *Yersinia pestis*, and *Francisella tularensis* are all easily weaponized agents, and tests have been developed and field tested for their detection in both the environment (e.g., powders) and in or on patients [25].

Clinical Situations for Which POC Tests Should Be Developed in Future

Several attractive targets for POC infectious disease diagnostics exist. These include:

- Diagnosis of bacteremia and fungemia. Current culture-based technology requires incubation for at least 8 hours before the first indication of a positive result, after which some organisms can be rapidly identified using molecular

methods [26]. However, appropriate therapy within the first few hours often makes the difference between severe morbidity or death and recovery [27]. Because the numbers of circulating bacteria or yeast in the bloodstream of septicemic patients can be low, the volume of blood necessary to detect small numbers of organisms has limited the application of molecular methods. Once an effective front-end concentration system is developed, the diagnosis of these extremely severe infections can be approached as a POC test.

- Meningitis and encephalitis are potentially severe infections that benefit from early diagnosis so that patients can be appropriately managed. The limited number of common pathogens associated with CNS infections makes development of such tests feasible. Additional agents for which rapid, simple molecular tests are needed include *Neisseria meningitidis*, *Streptococcus pneumoniae*, GBS, *Listeria*, *Haemophilus influenzae*, herpes simplex and varicella zoster virus.
- Tests for diagnosis and management of diseases seen in outpatients, particularly those in hard-to-manage populations. POC tests for STDs, for respiratory viruses and group A streptococci, and for HIV and HCV viral load can potentially streamline care for these conditions, allowing same-visit management and decreasing both the effort of follow-up and the potential public health impact of patients who cannot be contacted to deliver their results.
- Many clinicians and infection preventionists are concerned about the rising incidence of multidrug-resistant gram-negative rods. Metallo-beta-lactamases, carbapenemases, cephalosporinases, etc., pose risks to patients and problems for infection control. A rapid molecular test to detect major determinants of resistance, regardless of the organism carrying them, would be a desirable rapid or POC test.

Limitations and Current POC Technologies

Several pathogens can be detected in patient samples using molecular tests within 4 h. However, there are formidable obstacles to moving molecular diagnostics into the POC setting. There include:

- The impracticality of performing some methods in a random access, non-batched mode.
- The need for highly trained individuals to perform the test; and if they must be located at the POC, the inefficiency of having such individuals waiting during the time between test requests.
- The need for space for instruments and other supplies and physical infrastructure that do not exist at most POC locations.
- The delay incurred when an additional sample is received for testing once a testing process has commenced that cannot be stopped in the middle.
- The need to test all necessary controls with individual samples rather than groups of patient samples.
- The need for additional instruments for sample preparation or pre-amplification.
- The possibility of contamination.

After these factors have been considered, the remaining current technologies include polymerase chain reaction, isothermal loop-mediated amplification, and direct DNA hybridization. Other methods are in earlier stages of development but may show potential in the future.

Molecular methods, when brought to routine POC use, have the potential to provide performance equivalent to that of laboratory-based methods. Methods must be chosen to have extreme sensitivity to detect small numbers of organisms in limited sample volumes, and further automation and miniaturization of platforms is desirable [28]. This is the situation in a number of infectious diseases; for example, tuberculous meningitis, where the paucibacillary nature of the cerebrospinal fluid has challenged the development of effective molecular assays [29].

Molecular methods at POC will bring new challenges to those who administer and perform POCT. In addition to the usual QA and QC associated with any POCT, molecular POCT will require procedures for controlling contamination with both amplified material and patient-derived materials. QC of each stage of the analytical procedure; extraction, amplification, and detection, may make trouble-shooting more challenging. The phenomenon of inhibited specimens may require operators to report more complex results than "positive" or "negative." POC molecular instruments are likely to be more complex than current systems such as glucose testing systems and may, initially, lack some of the sophisticated POC management tools associated with traditional POC platforms [30].

Molecular diagnostic technologies are transforming the diagnosis of infectious diseases. Current and emerging clinical needs; increased acuity of inpatient care, expanded outpatient care, and an increasingly mobile population; the need to control healthcare-acquired infections, and novel antibiotic resistance mechanisms, will all drive molecular microbiology to the POC [31].

References

1. Breslauer DN, Maamari RN, Switz NA, Lam WA, Fletcher DA (2009) Mobile phone based clinical microscopy for global health applications. PLoS One 4:e6320
2. Kim DH, Spencer M, Davidson SM et al (2009) Institutional prescreening for detection and eradication of methicillin-resistant *Staphylococcus aureus* in patients undergoing elective orthopaedic surgery. J Bone Joint Surg Am 92:1820–1826
3. Bode LG, Kluytmans JA, Wertheim HF et al (2010) Preventing surgical-site infections in nasal carriers of *Staphylococcus aureus*. N Engl J Med 362:9–17
4. Jain R, Kralovic SM, Evans ME et al (2011) Veterans Affairs initiative to prevent methicillin-resistant *Staphylococcus aureus* infections. N Engl J Med 364:1419–1430
5. Robicsek A, Beaumont JL, Paule SM et al (2008) Universal surveillance for methicillin-resistant *Staphylococcus aureus* in 3 affiliated hospitals. Ann Intern Med 148:409–418
6. Huskins WC, Huckabee CM, O'Grady NP et al (2011) Intervention to reduce transmission of resistant bacteria in intensive care. N Engl J Med 364:1407–1418
7. Wolk DM, Picton E, Johnson D et al (2009) Multicenter evaluation of the Cepheid Xpert methicillin-resistant *Staphylococcus aureus* (MRSA) test as a rapid screening method for detection of MRSA in nares. J Clin Microbiol 47:758–764

8. Voelker R (2010) Increased *Clostridium difficile* virulence demands new treatment approach. JAMA 303:2017–2019
9. Noren T, Alriksson I, Andersson J, Akerlund T, Unemo M (2011) Rapid and sensitive loop-mediated isothermal amplification (LAMP) test for *Clostridium difficile* diagnosis challenges cytotoxin b cell test and culture as gold standard. J Clin Microbiol 49(2):710–711
10. O'Connor JR, Johnson S, Gerding DN (2009) *Clostridium difficile* infection caused by the epidemic BI/NAP1/027 strain. Gastroenterology 136:1913–1924
11. Tenover FC, Akerlund T, Gerding DN et al (2011) Comparison of strain typing results for *Clostridium difficile* isolates from North America. J Clin Microbiol 49:1831–1837
12. Sambol AR, Iwen PC, Pieretti M et al (2010) Validation of the Cepheid Xpert Flu A real time RT-PCR detection panel for emergency use authorization. J Clin Virol 48:234–238
13. Rand KH, Rampersaud H, Houck HJ (2011) Comparison of two multiplex methods for detection of respiratory viruses: FilmArray RP and xTAG RVP. J Clin Microbiol 49(7):2449–2453
14. Ginocchio CC (2011) Strengths and weaknesses of FDA-approved/cleared diagnostic devices for the molecular detection of respiratory pathogens. Clin Infect Dis 52(Suppl 4):S312–S325
15. Gazin M, Lammens C, Goossens H, Malhotra-Kumar S (2012) Evaluation of GeneOhm VanR and Xpert vanA/vanB molecular assays for the rapid detection of vancomycin-resistant enterococci. Eur J Clin Microbiol Infect Dis 31(3):273–276
16. Stamper PD, Cai M, Lema C, Eskey K, Carroll KC (2007) Comparison of the BD GeneOhm VanR assay to culture for identification of vancomycin-resistant enterococci in rectal and stool specimens. J Clin Microbiol 45:3360–3365
17. Church DL, Baxter H, Lloyd T, Miller B, Gregson DB (2011) Evaluation of the Xpert(R) group B streptococcus real-time polymerase chain reaction assay compared to StrepB Carrot Broth for the rapid intrapartum detection of group B streptococcus colonization. Diagn Microbiol Infect Dis 69:460–462
18. El Helali N, Nguyen JC, Ly A, Giovangrandi Y, Trinquart L (2009) Diagnostic accuracy of a rapid real-time polymerase chain reaction assay for universal intrapartum group B streptococcus screening. Clin Infect Dis 49:417–423
19. Kost CB, Rogers B, Oberste MS et al (2007) Multicenter beta trial of the GeneXpert enterovirus assay. J Clin Microbiol 45:1081–1086
20. Petitjean J, Vabret A, Dina J, Gouarin S, Freymuth F (2006) Development and evaluation of a real-time RT-PCR assay on the LightCycler for the rapid detection of enterovirus in cerebrospinal fluid specimens. J Clin Virol 35:278–284
21. Brown HL, Fuller DD, Jasper LT, Davis TE, Wright JD (2004) Clinical evaluation of affirm VPIII in the detection and identification of *Trichomonas vaginalis*, *Gardnerella vaginalis*, and *Candida* species in vaginitis/vaginosis. Infect Dis Obstet Gynecol 12:17–21
22. Boehme CC, Nabeta P, Hillemann D et al (2010) Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med 363:1005–1015
23. Helb D, Jones M, Story E et al (2010) Rapid detection of *Mycobacterium tuberculosis* and rifampin resistance by use of on-demand, near-patient technology. J Clin Microbiol 48: 229–237
24. Boehme CC, Nicol MP, Nabeta P et al (2011) Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. Lancet 377:1495–1505
25. Lim DV, Simpson JM, Kearns EA, Kramer MF (2005) Current and developing technologies for monitoring agents of bioterrorism and biowarfare. Clin Microbiol Rev 18:583–607
26. Loiez C, Herwegh S, Courcol RJ (2011) Usefulness of real-time PCR for the diagnosis of sepsis in ICU-acquired infections. Infect Disord Drug Targets 11(4):348–353
27. Garnacho-Montero J, Aldabo-Pallas T, Garnacho-Montero C et al (2006) Timing of adequate antibiotic therapy is a greater determinant of outcome than are TNF and IL-10 polymorphisms in patients with sepsis. Crit Care 10:R111
28. Kiechle FL, Holland CA (2009) Point-of-care testing and molecular diagnostics: miniaturization required. Clin Lab Med 29:555–560

29. Baron EJ, Carroll K (2005) Rapid diagnosis of tuberculous meningitis: what is the optimal method? Infect Med 22:633–637
30. Kim JY, Lewandrowski K (2009) Point-of-care testing informatics. Clin Lab Med 29:449–461
31. American Academy of Microbiology Report (2008) Clinical microbiology in the 21st century: keeping the pace. American Academy of Microbiology, Washington, DC. Available at http://www.escmid.org/fileadmin/src/media/PDFs/4ESCMID_Library/5Directives_Reports/other_reports/Clinical_Microbiology_in_the_21st_Century.pdf

Chapter 34
Multiplex PCR in Molecular Differential Diagnosis of Microbial Infections: Methods, Utility, and Platforms

Jian Han

Introduction: The Needs, Wants, and Challenges

We are entering the age of personalized medicine where treatments are designed to target specific causes, rather than a group of patients with similar symptoms. However, personalized medicine is impossible without a personalized diagnosis that considers all the possible causes of a person's disease. Traditional molecular diagnostic methods, such as PCR and qPCR, cannot provide the necessary information to practice personalized medicine, because they cannot be multiplexed, allowing the detection of only one or a few (no more than 3) targets at a time in one sample. Practicing personalized medicine, therefore, requires multiplex PCR (mPCR), which can evaluate many molecular targets at once, in one reaction, from one sample.

For most infectious diseases, the clinical presentations are often not specific enough to allow for a definitive diagnosis of the causative pathogen. Coughing and fever, for example, are symptoms that may be caused by many different bacterial or viral infections. Thus, for better treatment and disease control, a molecular differential diagnostic (MDD) assay that can pinpoint the offending pathogen associated with a clinical syndrome is needed. MDDs are essential tools for effective infectious disease surveillance, biodefense, and personalized medicine.

MDDs are *needed* for emerging infectious disease surveillance and control. When outbreaks such as SARS occur, public health officials and laboratory scientists often struggle for weeks, if not longer, to identify the offending pathogen. With MDDs available, scientists involved in an outbreak investigation can quickly rule out many pathogens associated with similar clinical symptoms and focus on new, emerging infections. An MDD test can also aid in the management of a public health crisis by helping healthcare personnel in triaging patients and determining

J. Han (✉)
HudsonAlpha Institute for Biotechnology, 601 Genome Way, Huntsville, AL 35806, USA
e-mail: jhan@hudsonalpha.org

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which patients should be isolated, as well as identifying environmental sources of contamination within an intensive care unit (ICU) or patient hospital room.

MDDs are needed for homeland security and biodefense. With the current global political atmosphere, biodefense threats are a reality. A first-response technology could quickly identify a bioterrorism agent and control the spread of the pathogen. Without the availability of MDDs for rapid pathogen identification, the bioterrorism agent may go undetermined for days. Every hour wasted in determining the causative agent provides a greater opportunity for pathogen spread and panic to occur.

MDDs are needed for delivering true personalized medicine, which focuses on treating the patient, rather than the disease. It is genotype-based, rather than phenotype- or symptom-based medicine. An MDD test also makes it possible to practice *theranosis* (therapy guided by a diagnosis) by developing or reclassifying drugs that specifically target the molecular cause of the disease. If pharmacogenomics is the development of drugs based on individual genotypes, then theranosis is the administration of drugs based on individual (or infectious agent) genotypes.

It is clear that MDDs are *needed*, but in order to make the assays practical, we *want* them to have the following advanced features:

- *Multiplex capabilities*. The definition of multiplexing is "receiving multiple signals from the same source." For MDDs, multiplexing refers to the ability to conduct multiple genotyping tests at the same time and within the same sample. We *want* multiplexing because it requires only small amounts of precious patient sample; it allows the clinician to run fewer tests, while acquiring more relevant information; it reduces the amount of reagents, consumables, and time involved; and, most importantly, it can save lives. For infectious disease MDDs, we *want* a multiplex test that can identify all pathogens related to a clinical syndrome or detect all the genes and mutations responsible for the drug-resistance phenotype.
- *Specificity*. Even though multiple microorganisms are studied simultaneously, we want only the pathogens associated with the infection to be identified and with a high level of confidence.
- *Sensitivity*. We want an MDD to be able to identify a pathogen or drug resistance *directly* from a patient sample rather than from an enrichment culture. Using the patient sample directly eliminates the time required for bacterial or viral culture preparation and enzymatic testing. However, in order to bypass this propagation step, the assay must be sensitive enough to detect only a small amount of pathogen material in the patient sample.
- *Reliability*. For clinical application of MDDs, a consistent performance from assay to assay and from lot to lot is required.
- *Speed*. For an MDD to be practical for infectious disease diagnosis and treatment, it must be locally available and produce results within a few hours.
- *Automation*. An MDD should not require a PhD scientist: it should be user-friendly, and no special training should be required to perform the assay. The MDD system should also be easily integrated into standard molecular laboratory practice and should be automatable.

- *Closed system*. A challenge to widespread use of MDD is the risk of template or amplicon contamination of the work environment, which may lead to false positives. One advantage of qPCR is that the specific detection step is carried out inside the PCR tube, in real time, without the need to reopen the tube. To make MDD practical, it must be carried out in a closed system.
- *Affordability*. MDDs should be cost-effective.

The technology advances in this postgenomic era have made sequence information readily available for almost all known pathogenic microorganisms. Based on this information and armed with standard molecular tools, scientists have developed molecular assays, usually PCR-based, for almost every infectious pathogen. A simple internet keyword search for a pathogen name together with "PCR" will produce several pages referencing specific tests for that pathogen. From this exercise, it seems possible that the basic *needs* for molecular differential diagnosis can be met. However, to produce the MDD assay we really *want*, some unique technical challenges must be addressed.

The most difficult challenge is multiplexing. While PCR technology has been established for nearly 30 years, multiplex PCR is still very difficult to accomplish. There are many publications that claim multiplex PCR applications, but here we consider true multiplexing to be the detection of 5 or more targets in one reaction. The following is a list of common challenges associated with multiplexing.

- *Incompatible loci*. Each target in a multiplex PCR requires its own optimal conditions, and these may be incompatible between targets; therefore, increasing the number of multiplex targets makes finding satisfactory common conditions difficult and, in many instances, impossible.
- *Lack of specificity*. Multiple sets of high-concentration primers in a system often generate primer dimers or nonspecific background. Lack of specificity also adds operational burdens by requiring post-PCR cleanup and multiple posthybridization washes, which make automation very difficult.
- *Lack of sensitivity*. Crowded primers reduce amplification efficiency and waste resources by occupying enzymes and consuming substrates.
- *Uneven amplification*. Differences in amplification efficiency may lead to large discrepancies in amplicon yield. In a multiplex system, some loci may amplify very well, while others may amplify poorly or even fail to amplify. Uneven amplification makes it impossible to accurately perform end-point quantitative analysis.
- *Lot-to-lot variation*. Because large amounts of primers are consumed in each reaction, and manufacturers can generate only a limited number of assays per lot, quality control and quality assurance can be difficult. The resulting poor repeatability makes it very difficult to gain FDA approval and therefore restricts large-scale use of the technology.

In the following discussions of this chapter, we present several mPCR methods and describe their applications. We also introduce multiplex hardware platforms.

Novel Multiplex PCR Technologies

The difficulty of mPCR assays may be appreciated by an analogy to a three-legged race: each primer pair is like two people tied together at the ankle who must work together to reach the finish line (amplify their target). However, the goal of an mPCR race is not for one team to finish first, but for all teams (primer pairs) to reach the finish line at about the same time (obtain equal amplification of all targets).

This is why mPCR is so difficult. There are two major conflicts: for a particular amplification target, the forward and reverse primers need to be compatible and in sync, while for all the targets, the amplification efficiency also need to be in sync. The following table (Table 34.1) outlines four recently developed methods that have tried to resolve these conflicts to make mPCR work.

Target-Enriched Multiplex PCR or tem-PCR

The tem-PCR method was first reported in 2006 by Han and colleagues [1]. In 2010, the US patent (7,851,148) was awarded, and the technology has been commercialized by Qiagen (Products) and Diatherix (Services). Figure 34.1 illustrates the tem-PCR method.

For each target in the multiplex PCR reaction, nested gene-specific primers are designed and included in the reaction (*Fo* forward out, *Fi* forward in, *Ri* reverse in, and *Ro* reverse out). These primers are used at extremely low concentrations and are used only to enrich the targets during the first few cycles of PCR. Some of these gene-specific primers have tag sequences that can be recognized by a universal set of primers, called SuperPrimers™, which are included in the same reaction system together with the gene-specific nested primers. Only the SuperPrimers are included at a concentration necessary for exponential amplification, and only the reverse SuperPrimer is labeled. For detection, labeled PCR products are detected with a

Table 34.1 Current multiplex nucleic acid amplification techniques

Method	Reference	Patent#	Company
Target enriched multiplex PCR (tem-PCR)	Han et al. [1]	7,851,148	Qiagen/Diatherix
Dual-priming oligonucleotide PCR (DPO)	Chun et al. [2]	WO/2008/143367	Seegene
Nested Patch PCR	Varley and Mitra [3]	20100129874 (pending)	Kailos Genetics
Amplicon rescued multiplex PCR (arm-PCR)	Wang et al. [4]	7,999,092	HudsonAlpha Institute for Biotechnology

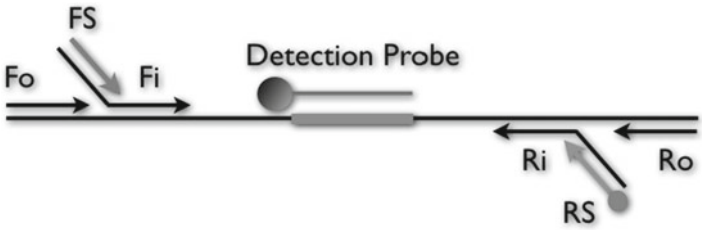

Fig. 34.1 In the tem-PCR method (target-enriched multiplex PCR), nested gene-specific primers are designed to enrich the targets during the initial cycles. Later, a SuperPrimer™ pair is used to amplify all targets

complementary capture probe that is covalently coupled to a color-coded bead (Luminex platform) or printed array, or are sequenced.

tem-PCR addresses two of the most difficult problems inherent in multiplex PCR: (1) incompatibility of amplification conditions between different primer sets and (2) background amplification associated with high concentrations of primers.

First, in a standard multiplex PCR reaction, if there are six targets to be amplified, each may require a different optimal annealing temperature or buffer formula. When the number of multiplex targets increases, it forces all primer sets to work under a single amplification profile; therefore, multiplex PCR is nearly impossible under standard conditions. With tem-PCR, there are two sets of nested primers for each target in the enrichment stage. This design gives rise to four possible forward and reverse primer combinations for amplification. Each combination may have its own optimal amplification profile, but given four amplification opportunities, a common condition that satisfies all targets can be attained.

Second, standard multiplex PCR utilizes multiple sets of labeled primers at high concentrations, which can associate with one another to form dimers or create nonspecific background amplification. Reduced amplification efficiency can also occur when primers occupy active sites on the polymerase. In addition, unused labeled primers produce background signal and use up reagents during the detection part of the assay. Because of these issues, post-PCR cleanup (such as spin column purification) is often required to remove these labeled primers before they can be used as probes. Yet, high-concentration primers are only required in the last cycles of a PCR reaction. With tem-PCR, the amounts of gene-specific primers used is only enough to enrich the targets and incorporate the SuperPrimer tag into the PCR products. After enrichment and tag incorporation, amplification is carried out with only one pair of primers. Because only one pair of primers is labeled, the background is low; therefore, no post-PCR cleanup is required. The PCR reaction is also very specific and sensitive, and no posthybridization washes are necessary. This feature makes it feasible to fully automate the laboratory procedures and perform high-throughput clinical studies.

The tem-PCR method also allows semiquantitative analysis of coinfections. With traditional multiplex PCR, each primer set, or each locus, has its own amplification efficiency. Hence, at the end of amplification cycling, the signal ratio of

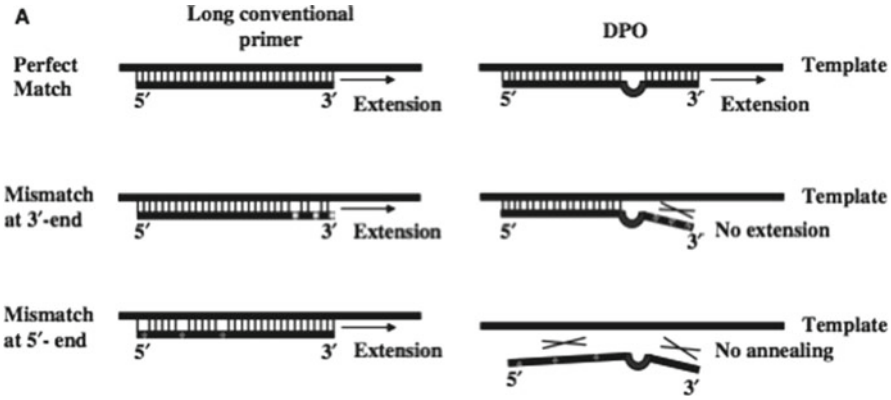

Fig. 34.2 Schematic diagram of long conventional primer-based and dual-primer oligonucleotide (DPO)-based PCR strategies

PCR products from different loci will not reflect the original ratio of the templates. With tem-PCR, the only primers used for exponential amplification are the pair of SuperPrimers. Consequently, all coamplified loci will have the same amplification efficiency. As a result, the end-point reading reflects the original copy number ratios between the coamplified targets.

tem-PCR is a flexible technology. Increased compatibility among multiple targets means that existing panels can be reorganized and remixed to build new panels. In addition, new amplification targets can be added without significantly reducing the sensitivity of the panel.

Continuing with the three-legged race analogy, the tem-PCR method does not rush all the paired runners to the finish line, but instead gives each time to run at their own pace at the beginning of the race. Once all the pairs (all the targets) have learned how to run in sync, the "SuperPrimer bus" will pick everyone up and carry them to the finish line together.

Dual-Priming Oligonucleotide (DPO) mPCR

The DPO mPCR method was first reported by Chun and colleagues [2]. The International patent (WO/2008/143367) describing the application of the method was awarded in 2008, and the technology has been commercialized by Seegene (Korea). Figure 34.2 illustrates the DPO method.

The DPO system has two separate primer segments, one of which is longer than the other, joined by a polydeoxyinosine linker. Deoxyinosine is known to have a relatively low melting temperature compared to the natural bases, due to weaker hydrogen bonding. Thus, the authors hypothesized that the poly(I) linker inserted between two stretches of natural bases would form a bubble-like structure and

separate a signal primer into two functional regions at a certain annealing temparature: a 5′-segment 18–25 nt in length and a 3′-segment 6–12 nt in length. This unequal distribution of nucleotides leads to a different annealing preference for each segment. The longer 5′-segment preferentially binds to the template DNA and initiates stable annealing, whereas the short 3′-segment selectively binds to its target site and blocks nonspecific annealing. Therefore, only target-specific extension will result from the successive priming of both the 5′ and 3′ segments of the DPO.

The DPO mPCR method successfully addresses the nonspecific priming issue. If we use the three-legged race analogy again here, the DPO method is successful by preselecting the athletes: only those fit to run are allowed in the race. There is no SuperPrimer bus to pick up teams in the middle of the race (no universal primers), so every pair has to run their own race from beginning to end.

Nested Patch PCR Method

The Nested Patch mPCR method was first reported by Varley and Mitra [3]. The patent (20100129874) is pending, and the technology is commercialized by Kailos Genetics (US). Figure 34.3 illustrates the Nested Patch method.

Like the DPO mPCR method, the Nested Patch PCR also intends to solve the problem of mispriming events that are typical of standard multiplex PCR. Like tem-PCR, the Nested Patch PCR method also requires four oligonucleotide hybridizations per locus, giving more specific amplification than standard multiplex PCR, which requires only two hybridizations per target locus.

Nested Patch PCR begins with a regular PCR reaction using primers with uracil substituted for thymine. PCR is first carried out for only a few cycles, which aims to define the ends of the targeted regions. The primers are then cleaved from the amplicons by the addition of an enzyme mix containing uracil DNA glycosylase. The ends of the target region are then internal to the PCR primer sequences. Next, Nested Patch oligonucleotides are annealed to the target amplicons and serve as a patch between the correct amplicons and universal primers. The universal primers are then ligated to the amplicons. The ligation reaction is highly specific, because thermostable ligases are used and will discriminate against mismatches at the junction. Signal is further enhanced by exonuclease digestion to remove mis-priming products or genomic DNA. The selected amplicons are protected from digestion by a 3′ modification of the universal primer. The selected amplicons are then amplified together by PCR with the universal primers. Even though there are many enzymatic steps involved in the protocol, it is an addition-only process; therefore, it is quite easy to perform and can be automated.

With Nested Patch PCR, the exponential phase of the amplification is carried out with the universal primers. Again, using the three-legged race analogy, all the paired runners are picked up by a universal primer bus after the initial enrichment of specific targets; in this case, the target enrichment is carried out with the aid of specially designed primers and a series of enzymes.

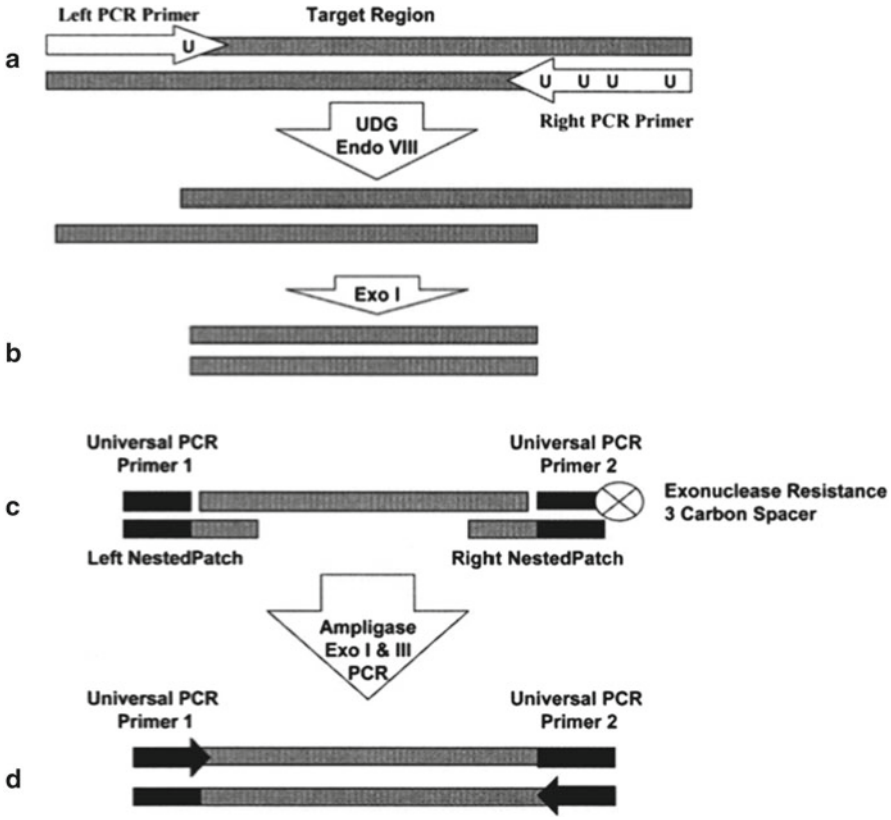

Fig. 34.3 Schematic diagram of Nested Patch PCR. (**a**) Using primers with uracil substituted for thymine, multiple primer pairs PCR amplify different targets from genomic DNA. The primers are then cleaved from the amplicons by the addition of heat-labile uracil DNA glycosylase, endonuclease VIII, and single-strand-specific exonuclease I. (**b**) The ends of the target regions are now internal to the PCR primers (nested). (**c**) Nested-patch oligonucleotides are annealed to the target amplicons and serve as a patch between the correct amplicons and universal primers. The universal primers are then ligated to the amplicons. The universal primer on the 3′ end of the amplicon is modified with a three-carbon spacer that protects the selected amplicon from the final exonuclease reaction that degrades nonspecific products. (**d**) The selected amplicons are then PCR amplified together using universal primers

Amplicon Rescued Multiplex PCR (arm-PCR)

The arm-PCR method was first reported in 2010 by Wang and colleagues [4]. The patent (7,999,092) describing the arm-PCR process was awarded in 2011, and the technology has been assigned to the HudsonAlpha Institute for Biotechnology (US) and licensed to iCubate and iRepertoire for commercialization in diagnostic and immune repertoire sequencing applications, respectively. Figure 34.4 illustrates the arm-PCR method.

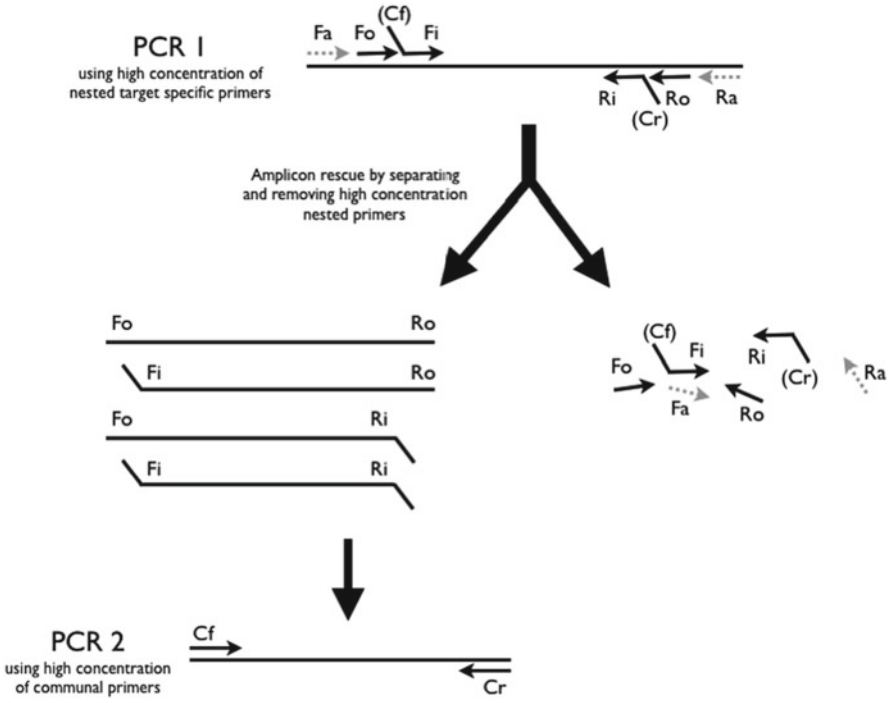

Fig. 34.4 Schematic diagram of amplicon-rescued multiplex PCR (arm-PCR). For each target in a mPCR reaction, a set of nested sequence-specific primers is designed (*Fo* forward-out, *Fi* forward-in, *Fa* additional forward primers, *Ro* reverse-out, *Ri* reverse-in, *Ra* additional reverse primers). A pair of common sequence tags is linked to all internal primers (Fi and Ri). Once these tags are incorporated into PCR products in the first few PCR cycles, an exponential phase of amplification can be carried out with a pair of communal primers, which can pair with the tag sequences. In the first round of amplification, only sequence-specific nested primers are used. The nested primers are then removed by exonuclease digestion and the first-round PCR products are used as templates for a second round of amplification by adding communal primers and a mixture of fresh enzyme and dNTP

Similar to tem-PCR, the arm-PCR method also uses nested gene-specific primers to solve the loci incompatibility problem. There are, however, two major differences between tem-PCR and arm-PCR: (1) with tem-PCR, the concentrations of gene-specific nested primers are very low, but with arm-PCR, the primer concentrations are high in order to increase amplification sensitivity and efficiency; (2) with tem-PCR, the universal SuperPrimers are included in the initial reaction together with the nested gene-specific primers, but with arm-PCR, the universal primers are not included in the initial reaction. The amplicons from the first round of PCR are rescued after removing the nested primers, and the universal, communal primers are then added for a second round of PCR.

If we refer to the three-legged race analogy again, like the tem-PCR and Nested Patched PCR methods, the arm-PCR method uses a SuperPrimer bus to carry all the

runners to the finish line once initial target enrichment is achieved. The first round of amplification is used to achieve specificity and introduce the universal tag into the PCR products. The second round of amplification is used to achieve sensitivity by efficient and semiquantitative amplification of all targets by using only one pair of primers.

We have described four mPCR methods, and there are many additional publications claiming multiplexing capabilities. When it is time to decide which method to use for infectious disease molecular diagnosis, however, we need to ask a few key questions: (1) Is the technology really a multiplex amplification method or it is only a multiplex detection method? (2) Is it easy to develop assays based on the method? (3) Is the method easy to use and can it be automated? (4) How is this multiplex amplification method incorporated with a downstream detection platform? (5) Finally, what about the assay specificity and sensitivity?

Vertical Integration to Provide a Complete Molecular Diagnostic Solution

MDD is a comprehensive process that includes three major steps: nucleic acid isolation, amplification, and detection (Fig. 34.5). There are many methods for completing each of the three steps in this process. Furthermore, a wide variety of instrument platforms are available to facilitate or automate each of these methods. To make MDD a routine clinical practice, these choices must be weighed against each other to obtain the best possible combination of methods and platforms to carry

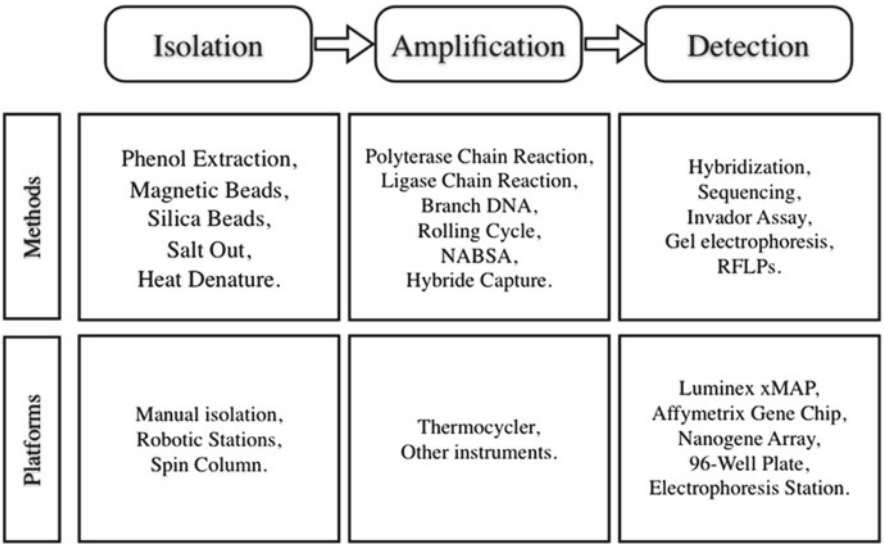

Fig. 34.5 System integration to achieve practical multiplex PCR solutions

Table 34.2 Comparison of information technology and biotechnology for multiplex PCR

	IT	Biotech
Hardware	PC or Apple	Luminex xMAP, Affymetrix chip, flow cytometry, next-gen sequencing, iCubate
Operation systems	Windows, Mac OS, Linux	PCR, qPCR, mPCR
Applications	Word, Excel, Powerpoint	HIV, bloodstream infection panels, HPV typing

Table 34.3 Comparison of different nucleic acid detection methods and platforms

Method	Platform	Throughput	Specificity	Sensitivity	Ease of use	Company	Price
Sequencing	HiSeq	+++++	+++++	++	++	Illumina	High
Sequencing	MiSeq	++++	+++++	++	++	Illumina	Medium
Sequencing	454	+++++	+++++	++	++	Roche	High
Sequencing	454 Jr	++++	+++++	++	++	Roche	Medium
Hybridization	Luminex	+++	+++	++++	+++	Luminex	Medium
Hybridization	eSensor	++	+++	+++	+++	GenMark Dx	Medium
Hybridization	Verigene	++	+++	+++	+++	Nanosphere	Medium
Hybridization	iCubate	++	+++	++++	++++	iCubate	Medium
Invader assay	Invader	++	+++	+++	++	Hologic	Medium
Electrophoresis	Gel box	+	+	+	+	Bio-Rad	Low

out the task. This process of vertical integration can produce multiple possibilities. Thus, if MDD is to be the next breakthrough in modern medicine, we must choose wisely which technology integration path to take.

The biotech industry is very much like the information technology industry (Table 34.2), where an application is developed by using a combination of hardware (the platform) and software (the basic methodology and reagent system). A research tools company may choose different combinations of methods and platforms, so that a particular amplification method can be followed by one of many different detection methods. For example, to build a molecular diagnostic system, PCR amplification may be paired with multiple detection methods, such as direct hybridization, gel analysis, or sequencing. One should also note that a particular method can be performed on multiple platforms. The most successful biotech companies and clinical laboratories are those that are able to develop such applications through technology integration and innovation.

Detection Methods and Platforms

We have described four novel mPCR technologies in this chapter. The next step after amplification is detection of the specific PCR products. Currently, there are two major detection methods: sequencing and hybridization. Table 34.3 lists several methods and representative platforms.

Before discussing detection platforms, it will be helpful to address a common misunderstanding regarding the use of next-generation sequencing in molecular diagnosis of infectious diseases. It is sometimes believed that, with the advance of high throughput sequencing technology and the accompanying rapid price drop, PCR will soon become an outdated technology and will no longer be needed. This is not true. Because sequencing is only a detection technology, it cannot increase the signal-to-noise ratio, which is critical in infectious disease diagnosis. For example, in bloodstream infections, peripheral blood samples from patients may have only 20 copies of the bacterial genome per milliliter of blood, but will have millions copies of the host genome. At that low signal-to-noise ratio, false positive rates will be extremely high and unacceptable for clinical applications. High-throughput sequencing will be useful in infectious disease diagnosis only if the signal-to-noise ratio can be increased significantly, and that requires mPCR.

mPCR products can be sequenced directly with relatively low cost thanks to technology advances in this field. To further reduce the cost, a molecular tag (barcode) system can be used during amplification; then, after amplification, hundreds of samples can be pooled together for one sequencing run, and software can be used to identify and differentiate the samples. The most promising sequencing platforms are Illumina MiSeq (www.ilumina.com) and Roche 454 Jr (www.roche.com), which both cost around $120,000 USD, while the reagents cost a few hundred dollars per run. If 10–20 samples are pooled in one run, the sequence analysis cost per sample will be under $50.

If abundant and specific DNA targets can be generated by an efficient amplification method, detection is more straightforward and rapid. The challenge then becomes providing an accurate measurement of the amplification products in a rapid, high-throughput, and low-cost format.

The simplest detection method is hybridization, which occurs without an enzymatic reaction. One strand of DNA binds to its complementary strand in solution via hydrogen bonding, and specificity is controlled by temperature and salt concentration. Typically, a detectable molecule (fluorescent dye or radioisotope) is attached to one strand of DNA, which can be recognized by a detection device. Because of its ease of use, hybridization is the method of choice for many detection platforms.

High-throughput DNA hybridization is performed with arrays. Currently, nucleic acids are arrayed on solid supports that are either glass slides or nylon membranes. Depending on the type of array, targets can be composed of oligonucleotides, PCR products, cDNA vectors, or purified inserts. The sequences on an array may represent entire genomes, including both known and unknown sequences, or they may be collections of sequences, such as apoptosis-related genes or cytokines. Many premade and custom arrays are available from commercial manufacturers, though many labs prepare their own arrays with the help of robotic arrayers. The methods of probe labeling, hybridization, and detection depend on the solid support to which the sequences are bound. Typically, fluorescent-labeled probes are used with glass arrays.

Luminex xMAP technology (www.luminexcorp.com) is also an array of sorts; however, unlike other arrays, the solid support for probe binding is provided by microspheres in suspension. Therefore, Luminex xMAP technology is also known

as a "liquid chip" or "suspension array." With xMAP technology, molecular reactions take place on the surface of color-coded beads (microspheres). For each pathogen, target-specific capture probes are covalently linked to a specific set of color-coded microspheres. Labeled PCR products are captured by the bead-bound capture probes in a hybridization suspension. A microfluidics system delivers the suspension hybridization reaction mixture to a dual-laser detection device. A red laser identifies each bead by its color-coding, while a green laser detects the hybridization signal associated with each bead. Software is used to collect the data and report the results in a matter of seconds.

This platform is specific, because only the probes that are captured by the beads are recognized by the green laser as signal. Any signal not associated with a specific set of color-coded beads is considered background. The platform is also very sensitive. Each bead has as many as 10^8 COOH groups on its surface for linking capture oligos. The green laser can detect the signal for as few as eight fluorescent-labeled probes that are captured by a bead. Another important feature of the xMAP platform is its repeatability. Because everything occurs in a homogeneous solution (from bead manufacture, color-code staining, and capture probe coupling to product hybridization and data collection), highly repeatable results are obtained with this platform. The xMAP method for collecting and reporting data also contributes to repeatability. Typically, there are 5,000 beads added per reaction for each color-coded bead set. Each bead set is specific for a particular disease marker, such as a mutation or a pathogen. The laser counts 100 microspheres from each bead set and reports the median fluorescent intensity (MFI). Thus, the data represents 100 microbead-associated data points, not just one data point produced by a standard array.

The eSensor XT-8 system developed and marketed by GenMark Dx (www.GenMarkdx.com) is also an array-based detection platform. Capture oligos are printed onto an electronic circuit board directly and eSensor detects electronic signals from the hybridized PCR products. The system is compact, very sensitive, and GenMark Dx has obtained FDA approval for several products for genetic mutation detection.

The Verigen® system developed by Nanosphere (www.nanosphere.us) is another very sensitive detection system, with a benchtop molecular diagnostic workstation that utilizes patented gold nanoparticle technology to detect nucleic acids. To use the Verigenii® system, target nucleic acid is simultaneously hybridized to capture oligonucleotides arrayed in replicate on a solid support (an array) and sequence-specific mediator oligonucleotides, with gold nanoparticle probes, that detect single-copy DNA regions in each target of interest. A washing step is carried out to remove unhybridized gold nanoparticle probes. Silver signal amplification is performed on the gold nanoparticle probes that are hybridized to captured DNA targets of interest. One more washing step is performed to remove unreacted signal amplification reagents. Qualitative analysis of results (reading the array) can then be performed on the Verigen® Reader.

The iCubate system (www.icubate.com) is a new molecular diagnostic platform that uses a disposable cassette, a processor, and a reader to carry out sample prep, mPCR, array hybridization, washing, and detection steps. The detection is carried

Table 34.4 Comparison of different integrated multiplex PCR solutions

Company	Amplification method	Detection platform	Multiplex capability	Sample-to-answer automation	Closed system
Luminex	PCR	Luminex xMAP	Yes	No	No
Qiagen/Diatherix	tem-PCR	Luminex xMAP	Yes	No	No
Seegene	DPO PCR	Gel electrophoresis	Yes	No	No
iCubate	arm-PCR	iCubate	Yes	Yes	Yes
Roche	Real-time PCR	Light Cycler™	Limited	No	Yes
Cepheid	Real-time PCR	GeneXpert™	Limited	Yes	Yes
Gentura Dx	Real-time PCR	IDbox™	Limited	Yes	Yes
Idaho Technology	Real-time PCR	FilmArray	Limited	Yes	Yes

out on a glass array, where capture probes are printed onto a 1-cm^2 piece of glass. mPCR products are captured by the array, and a gene-specific detection probe is then hybridized to the PCR products for detection.

Integrated Solutions

An integrated solution is one that incorporates different methodologies and instruments to allow sample-to-answer results. Table 34.4 lists some examples of companies providing integrated solutions for molecular diagnosis of infectious pathogens. These companies are compared in the following categories: amplification methods; detection platforms; multiplexing capability of more than five targets; fully integrating sample prep, amplification, and detection steps to allow a maximum hands-on time of <3 min; and a closed reaction system so that amplicon contamination can be eliminated.

The Luminex Corporation, for example, has incorporated PCR (also reverse transcription PCR) with their xMAP/xTag detection platform and is offering an FDA-approved respiratory viral panel (RVP) *FAST* that detects eight viruses and subtypes: influenza A, influenza A subtype H1, influenza A subtype H3, influenza B, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), rhinovirus, and adenovirus [5]. While Luminex is a highly efficient detection platform, if traditional PCR is used to amplify multiple targets, the poor amplification efficiency and low signal-to-noise ratio has forced users to perform post-PCR cleanup and posthybridization washes. These steps are difficult to automate and perform in an enclosed system, risking amplicon contamination that may lead to false-positive results or high background.

Qiagen (www.Qiagen.com) and Diatherix (www.Diatherix.com) are using tem-PCR and Luminex xMAP technologies to provide infectious disease detection products and services. The tem-PCR method was first reported for HPV typing,

where 25 types of HPV were amplified and detected with one assay [1]. Since then, this mPCR method has been used to develop many multiplexed assays.

In 2006, Brunstein and Thomas reported the use of a tem-PCR-based multiplex respiratory pathogen identification assay on clinical specimens [6]. The same group also conducted a large-scale clinical study. From 1,742 clinical samples, they found that rapid molecular multiplex assays significantly increased the detection rate when compared with rapid direct fluorescent-antibody (DFA) assays, with an overall detection rate of 68 %, compared to 35 %. More interestingly, they found that over 30 % of the patients had coinfections by detecting more than one pathogen in a sample [7]. Using the same tem-PCR technology, Li and coworkers evaluated a respiratory viral panel [8], Zou and coworkers reported the development of a human influenza typing panel that identified 15 human H5N1 infections in China [9], and Tang and coworkers reported an evaluation of the StaphPlex System with 360 GPCC (gram-positive cocci in clusters) specimens [10]. This system detected 18 molecular targets in a multiplex assay for Staph identification and drug resistance gene detection. The StaphPlex system demonstrated 100 % sensitivity and specificity ranging from 95.5 to 100 % when used for staphylococcal cassette chromosome *mec* typing and PVL detection [10]. Benson and coworkers at the CDC reported the development of a respiratory bacterial panel that detected six pathogens, including *S. pneumoniae*, *Neisseria meningitidis*, encapsulated or nonencapsulated *Haemophilus influenzae*, *L. pneumophila*, *Mycoplasma pneumoniae*, and *C. pneumoniae* [11], while Media Gegia and coworkers reported the evaluation of a tem-PCR based panel that detects 24 *Mycobacterium tuberculosis* gene mutations responsible for resistance to isoniazid, rifampin, streptomycin, and ethambutol [12].

Even though Qiagen/Diatherix has also used Luminex xMAP technology for detection, the incorporation of tem-PCR multiplex amplification technology has made their assay much more user friendly. The Luminex RVP product uses several enzymatic steps, but the Qiagen ResPlex uses only one enzymatic step and eliminates the washing steps after amplification and hybridization. However, the potential risk of amplicon contamination still exists, because the amplification and hybridization reactions are still set up in an open environment.

Seegene (www.Seegene.com) has also developed a mPCR method (DPO PCR) that has made them a powerhouse of multiplex assay development. However, their detection platform lags behind and still uses gel electrophoresis to separate PCR products. This low-cost solution, however, has made their products more acceptable in emerging markets. The lack of automation and potential amplicon contamination may limit the ability of their products to penetrate the western market.

iCubate (www.iCubate.com) has developed a novel mPCR method (arm-PCR) and also a fully automated system that can carry out magnetic bead-based DNA/RNA extraction, arm-PCR amplification, array hybridization, washing, and signal acquisition steps. At the core of the iCubate technology is a single-use cassette that comes preloaded with all the reagents necessary to perform extraction, amplification, and detection steps. The closed design of the cassette guarantees that the high-concentration amplicons contained inside have no chance of contaminating the lab. The iCubate iC-Processor allows for the automated processing of iCubate cassettes.

Computer-controlled robotics allow for automated sample prep, arm-PCR, hybridization, and washing procedures to be performed. Each processor can run from 1 to 4 cassettes in a random access fashion; if more throughput is needed, up to 12 units can be linked together to run up to 48 samples simultaneously. The iCubate iC-Reader allows for automated data collection from iCubate cassettes. A high-speed rotating platter, laser, and photomultiplier tube allow the acquisition of data from each cassette in just seconds. The iCubate iC-Report software performs automated data analysis and generates individual reports for each cassette. It also monitors and tracks cassette progress, as well as system performance.

iCubate is also an open platform. The company launched the iCubate 2.0 open platform (www.icubate2.com) recently, allowing researchers to develop their own assays with the aid of the free online software iC-Architect, which incorporates the patented arm-PCR technology and novel algorithm called PPI (Polymerase Preference Index, patent pending). The PPI can help improve primer design by identifying the priming sites that are preferred by thermostable polymerases.

Table 34.4 also lists several fully integrated platforms that are based on real-time PCR technology. Companies like Cepheid, Gentura Dx, and Idaho Technology have all developed sample-to-answer solutions that allow molecular assays to be performed in a contamination-free closed system. However, in these real-time PCR-based platforms, multiplexing to amplify more than five targets in one reaction is very difficult. Nevertheless, the ease of use of these platforms has revolutionized the molecular diagnostics industry and benefited millions of patients.

Delivering Value Through Reducing Cost and Saving Lives

The advances of genomic technology have changed the way we define diseases from a phenotypic, symptomatic description of clinical presentations to a genotypic, molecular classification of underlying causes. Molecular differential diagnosis has become the hallmark of 21st century medical practice.

Every infectious disease starts with an invasion by a microorganism's genetic material into the human body. The expression of pathogen genes inside human cells can interrupt normal cellular function and induce systemic responses or clinical syndromes. The goal for infectious disease MDD is to investigate all possible causes of a common clinical syndrome and identify the offending pathogen. To achieve this goal, we need a multiplex technology that uses one sample, one test, one technician, one machine, and a short period of time to obtain multiple answers.

MDD is necessary for controlling an outbreak, such as avian flu or SARS. Poorly controlled outbreaks that lead to public health crises are costly. During the SARS outbreak, for example, it is estimated that East Asian countries suffered a loss of almost 2 % GDP in the second quarter of 2003. A difficult cycle is often set into motion: a lack of rapid and accurate diagnostic tests combined with a lack of communication to the public and lack of scientific knowledge about the disease lead to panic and disruption of economic systems. With an early and accurate differential

diagnosis, infected patients can be identified, isolated, and treated. In addition, the general population can be informed and protected.

Following the recent swine flu pandemic, Koon and coworkers reported mPCR results on 10,624 clinical samples with respiratory symptoms [13]. Of those tested, about 71.5 % of the patients with respiratory symptoms were *not* sick from the pandemic strain of H1N1 [13]. Health-care practitioners therefore quickly identified and properly treated those with pandemic flu infection and those requiring regular care. Furthermore, these findings contradicted the conventional wisdom at the time, which was that anyone with flu-like symptoms probably had the H1N1 virus and should be treated accordingly.

The Koon study revealed a second critical point: among those with the H1N109 infection, 28 % were also infected with at least one other bacterial or viral pathogen [13]. These patients with coinfections require more medical care. A Center for Disease Control (CDC) study has shown that a large percentage of deaths associated with H1N109 were due to coinfection with pathogenic bacteria; thus, a multiplex diagnostic test could help physicians to better triage patients and better allocate treatment resources.

Antibiotic treatment depends even more on MDD. The genes and mutations that lead to resistance can be detected by studying bacterial DNA. Almost all genomes of human pathogens have been sequenced, and newly emerging resistant strain genomes are being sequenced as quickly as possible. This DNA sequence information is publicly available in the federally funded GenBank database, allowing scientists to develop specific assays to detect these genes and mutations. However, without mPCR, scientists usually study only one gene segment at a time. This is a problem because, for a particular bacterial strain, drug resistance capability may come from many different genes and mutations. Therefore, a multiplex PCR assay that detects multiple targets in one reaction, instead of just one molecular target like other PCR tests, allows scientists to immediately identify multiple pathogens and multiple segments of a specific viral or bacterial genome.

Healthcare-associated bloodstream infections (BSIs) are the tenth leading cause of death in the United States, with over 350,000 cases reported every year, resulting in an estimated 90,000 attributable deaths. Studies have estimated the cost of treating BSIs to be approximately $27,000 per patient for community-associated bloodstream infections and $58,000–101,000 per patient for healthcare-associated (hospital-acquired) bloodstream infections. Eiland and coworkers showed that for each BSI patient, when mPCR technology is used, drug costs can be reduced by $100 and ICU stay reduced by 3 days, which represents an additional savings of over $10,000 per patient after the cost of the test has been deducted [14]. Multiplex PCR technology allowed for the optimization antimicrobial therapy in 27 % of the patients and de-escalation therapy in 23 % of the patients evaluated [14].

mPCR-enabled MDDs are exciting methods that are bringing revolutionary changes to many aspects of medical practice, especially to infectious disease management. First, it changes the way a doctor treats a patient. Instead of waiting days for culture results, a doctor can now act immediately based on a comprehensive molecular diagnosis. Instead of guessing what may be the offending pathogen,

a doctor can identify the microorganism with confidence. Instead of ordering the blood cultures to gain knowledge for future empirical treatment, a doctor can prescribe the test to seek immediate solutions. Instead of offering antibiotics to put families or parents (and sometimes the doctor) at ease, a doctor can now provide accurate treatment to actually improve a patient's condition.

Second, MDDs will change the way hospitals operate. Hospitals can implement MDDs as an active surveillance measure to prevent hospital-acquired infections (HAIs). Many studies have shown that active surveillance, plus patient isolation, is one of the most effective methods to reduce HAIs [15]. Regularly scheduled surveys of critical environments (such as the ICU), instruments, and healthcare providers will raise the level of awareness and identify problems early. When an outbreak of HAI occurs, MDDs can quickly identify the source of an infection, helping healthcare providers determine which patients should be isolated to prevent the spread of the microorganisms. In an increasing number of states, hospitals are required to publish their rate of HAI, which is calculated based on discharge records. However, some patients may be misclassified as having an HAI because they were asymptomatic carriers before being admitted to the hospital. MDDs can help hospitals better identify, control, and report HAIs, thereby lessening their liability. As a result, MDDs can help reduce costs, shorten hospital stays, and improve the quality of care, while protecting profits.

Third, MDDs will lead to many changes in the healthcare industry. Healthcare spending in the United States has grown rapidly over the past few decades—from $27 billion in 1960 to $900 billion in 1993 to $1.8 trillion in 2004 [16]. Depending on how you measure it, the healthcare industry represents between 15 and 16 % of the gross domestic product. Traditionally, these financial activities occurred in three subcategories: providers (such as hospitals, nursing homes, and diagnostic laboratories), payers (such as insurance companies), and life sciences (such as biotechnology and pharmaceuticals). For example, the cost of developing a new drug can be as high as $800 million [17]. That cost is passed on from the life science sector to the payers and then to the providers. How could MDDs help in this situation? They can help by allowing the three healthcare sectors to work with each other instead of against each other.

In the life science sector, biotech companies with MDD technologies can work with pharmaceutical companies to develop pharmacogenomic or theranostic solutions. This kind of collaboration will improve treatment outcomes without significantly increasing development costs. Instead of developing blockbuster drugs that are one-size-fits-all, more effective treatment can be obtained by utilizing an MDD to tailor the treatment options to the patient's needs. MDDs will make drugs more effective by providing a genotype-based targeting system.

For payers in the healthcare industry, MDDs will change the risk calculation equations used by the insurance companies, such as health maintenance organizations (HMOs) and preferred provider organizations (PPOs). The healthcare payers make money by managing the "risk capital" associated with healthcare services. Reducing costs and risks will directly result in increased revenue. Hallin and coworkers studied the clinical impact of a PCR assay for identification of MRSA directly from blood cultures [18]. They found that, on average, results were available about 39 h earlier

than with the culture method, and about 25 % of the treatments were modified following diagnosis [18]. MDDs can provide faster, more accurate diagnosis that directly influences the clinical outcome and reduces the risks and costs associated with traditional diagnostic methods.

For healthcare providers, the benefit of MDDs is even more apparent. An MDD can help doctors make the right treatment decisions much sooner, thereby shortening the patients' hospital stay and improving the overall quality of care.

Fourth, MDDs will bring about societal changes. Society is threatened by emerging infectious diseases, including many drug-resistant super bugs. The global economy, with its traveling professionals, makes the spread of diseases much faster. Rising costs make quality healthcare more difficult to manage. Moreover, the cost of developing new antibiotics is too high and the process is too slow. We have been promised a better system, and have been awaiting the arrival of MDDs for a long time. Now that the technology has finally arrived, we must maximize its utility and benefit.

Finally, MDDs offer all of the benefits needed for patient care, at once. Using current bacterial or viral culture methods, patients and physicians often need to wait for days before a result is available. Conventional PCR-based molecular analyses are labor-intensive, expensive, and often inconclusive. Powerful mPCR methods can provide a faster answer, leading to a faster recovery. The ultimate value of MDDs is found in its ability to save lives.

References

1. Han J, Swan DC, Smith SJ, Lum SH, Sefers SE, Unger ER, Tang Y (2006) Simultaneous amplification and identification of 25 human papillomavirus types with templex technology. J Clin Microbiol 44(11):4157–4162
2. Chun J, Kim K, Hwang I, Kim Y, Lee D, Lee I, Kim J (2007) Dual priming oligonucleotide system for the multiplex detection of respiratory viruses and SNP genotyping of CYP2C19 gene. Nucleic Acids Res 35(6):e40
3. Varley KE, Mitra RD (2008) Nested Patch PCR enables highly multiplexed mutation discovery in candidate genes. Genome Res 18:1844–1850
4. Wang C, Sanders CM, Yang Q, Schroeder HW, Wang E, Babrzadeh F, Gharizadeh B et al (2010) High throughput sequencing reveals a complex pattern of dynamic interrelationships among human T cell subsets. PNAS 107:1518–1523
5. Gadsby NJ, Hardie A, Claas ECJ, Templeton KE (2010) Comparison of the Luminex respiratory virus panel fast assay with in-house real-time PCR for respiratory viral infection diagnosis. J Clin Microbiol 48(6):2213–2216
6. Brunstein J, Thomas E (2006) Direct screening of clinical specimens for multiple respiratory pathogens using the Genaco respiratory panels 1 and 2. Diagn Mol Pathol 15(3):169–173
7. Brunstein J, Cline CL, McKinney S, Thomas E (2008) Evidence from multiplex molecular assay for complex multi-pathogen interactions in acute respiratory infections. J Clin Microbiol 46:97–102
8. Li H, McCormac MA, Estes RW, Sefers SE, Dare RK, Chapell JD et al (2007) Simultaneous detection and high-throughput identification of a panel of RNA viruses causing respiratory tract infections. J Clin Microbiol 45(7):2105–2109

9. Zou S, Han J, Wen L, Liu Y, Cronin K, Lum SH et al (2007) Human Influenza A Virus (H5N1) detection by a novel multiplex PCR typing method. J Clin Microbiol 45(6):1889–1892
10. Tang Y, Killic A, Yang Q, McAllister SK, Li H, Miller RS et al (2007) StaphPlex System for rapid and simultaneous identification of antibiotic resistance determinants and Panton-Valentine leukocidin detection of Staphylococci from positive blood cultures. J Clin Microbiol 45(6):1867–1873
11. Benson R, Tondella ML, Bhatnagar J, Carvalho MGS, Sampson JS, Talkington DF et al (2008) Development and evaluation of a novel multiplex PCR technology for molecular differential detection of bacterial respiratory disease pathogens. J Clin Microbiol 46:2074–2077
12. Gegia M, Mdivani N, Mendes RE, Li H, Akhalaia M, Salakaia A et al (2008) Prevalence of and molecular basis for tuberculosis drug resistance in the Republic of Georgia: validation of a QIAplex System for detection of drug resistance-related mutations. Antimicrob Agents Chemother 52:725–729
13. Koon K, Sanders CM, Green J, Malone L, White H, Zayas D et al (2006) Co-detection of pandemic (H1N1) 2009 virus and other respiratory pathogens. EID J 16(12):1976–1978
14. Eiland EH III, Beyda N, Han J, Lindgren W, Ward R, English TM et al (2010) The utility of rapid microbiological and molecular technique in optimizing antimicrobial therapy. SRX Pharmacol. 10.3814/2010/395215
15. Farr BM, Bellingan G (2004) Pro/con clinical debate: isolation precautions for all intensive care unit patients with methicillin-resistant *Staphylococcus aureus* colonization are essential. Crit Care 8(3):153–156
16. Heffler S, Smith S, Keehan S, et al (2004 Feb) Health spending projections through 2013. Health Affairs Web Exclusive W4-79–W4-93
17. Adams C, Brantner Van V (2004 Dec) Estimating the costs of new drug development: is it really $802m? Social Science Research Network
18. Hallin M, Maes N, Byl B, Jacobes F, De Gheldre Y, Struelens MJ (2003) Clinical impact of a PCR assay for identification of *Staphylococcus aureus* and determination of methicillin resistance directly from blood cultures. J Clin Microbiol 41:3942–3944

Chapter 35
Technical Advances in Veterinary Diagnostic Microbiology

Dongyou Liu

Introduction

Forming a significant part of biomass on earth, microorganisms are renowned for their abundance and diversity. From submicroscopic infectious particles (viruses), small unicellular cells (bacteria and yeasts) to multinucleate and multicellular organisms (filamentous fungi, protozoa, and helminths), microorganisms have found their way into virtually every environmental niche, and show little restrain in making their presence felt. While a majority of microorganisms are free-living and involved in the degradation of plant debris and other organic materials, others lead a symbiotic, mutually beneficial life within their hosts. In addition, some microorganisms have the capacity to take advantage of temporary weaknesses in animal and human hosts, causing notable morbidity and mortality. Because clinical manifestations in animals and humans resulting from infections with various microorganisms are often nonspecific (e.g., general malaise and fever), it is necessary to apply laboratory diagnostic means to identify the culprit organisms for treatment and prevention purposes.

Veterinary diagnostic microbiology is devoted to the identification and detection of microorganisms that cause diseases in animals. Considering the close similarity among microorganisms causing diseases in humans and animals, many laboratory techniques that have been developed for the identification and detection, subtyping and phylogenetic analysis, virulence determination, and drug resistance assessment of human pathogens, have been thus readily adopted for the investigation of animal pathogens, or vice versa. Furthermore, apart from zoonotic pathogens that occur in both human and animals, animals of different classes and categories often have unique pathogens of their own. Therefore, veterinary diagnostic microbiology faces

D. Liu (✉)
Royal College of Pathologists of Australasia Biosecurity Quality Assurance Program, Virology Research Laboratory, Prince of Wales Hospital, Randwick, NSW 2031, Australia
e-mail: liu@cvm.msstate.edu

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even greater challenges than its medical counterpart in achieving accurate, sensitive, and rapid identification and detection of pathogenic microorganisms in animals.

In view of the fact that many human pathogens have originated/evolved from microorganisms commonly occurring in animals, accurate identification and tracking of animal pathogens are crucial for the control and prevention of zoonotic infections in human populations. The threat of zoonotic pathogens (e.g., *Bacillus anthracis*) being used in bioterrorism attacks, and the emergence of rapidly evolving antibiotic-resistant microorganisms and animal pathogens causing severe diseases in humans (e.g., SARS coronaviruses, and avian influenza viruses), have made the development and application of improved diagnostic methods for animal pathogens increasingly important.

Identification and Detection

Accurate identification and detection of pathogenic microorganisms in animals have been and will remain the primary objective for veterinary diagnostic microbiology.

Similar to its medical counterpart, veterinary diagnostic microbiology has traditionally relied on various phenotypic procedures for microbial characterization. These procedures assess the morphological, biological, biochemical, serological, in vitro and in vivo characteristics as well as other phenotypic properties of microorganisms, and have played an essential role in the identification and detection of microbial pathogens affecting humans and animals. More recently, molecular techniques have been increasingly applied for identification and detection of microbial pathogens (Table 35.1) [1].

Morphological characterization is based on the premise that various classes of microorganisms often show distinct morphological features (e.g., size, shape, internal and external components, colony morphology) which allow their initial identification upon macroscopic and microscopic examination. Application of light microscopy or transmission and scanning electron microscopy (EM) together with stains/dyes helps reveal additional morphological details. Hematoxylin and eosin (H&E), Gram, Giemsa, crystal violet stains are common stains used to enhance the contrast of microbes to their background. Additionally, Gimenez' and Pinkerton's stains are useful for detection of rickettsial organisms in tissue sections; Ziehl-Nielsen, Kinyoun, or auramine O stains for initial detection of mycobacteria; KOH, lactophenol cotton blue, India ink, and Southgate's mucicarmine stains for detection of fungi; periodic acid-Schiff (PAS), Grocott's methenamine silver (GMS), Fontana-Masson, Gridley's, and H&E stains for detection of mycotic elements in tissue biopsies. In general, morphological characterization is rapid and inexpensive, but has relatively low sensitivity and specificity, and its result interpretation is somewhat subjective. To improve the sensitivity and specificity of microscopic detection of pathogenic microorganisms (especially viruses), fluorescently labeled antibodies may be utilized. Application of highly sensitive and specific fluorescent sensor molecules in electron microscopy, fluorescence microscopy, or time-lapse microscopy

Table 35.1 Common laboratory techniques for identification and detection of microbial pathogens

Technique	Key features
Morphological	Macroscopic and microscopic examination of morphological features (e.g., size, shape, internal and external components, colony morphology) allows for rapid, inexpensive identification of microorganisms. Use of general or specialized stains/dyes further enhances the contrast of microbes to their background. Nonetheless, morphological characterization often lacks desired sensitivity and specificity
Biochemical	Examination of metabolic or enzymatic products of microorganisms (e.g., carbohydrate, protein, amino acid, fat, and enzyme) using biochemical techniques permits their discrimination at genus- and species-levels. However, the performance of biochemical tests is impacted by factors that affect microbial growth and metabolism
Serological	Detection of specific interactions between host antibodies and microbial antigens (e.g., protein or carbohydrate) by serological techniques provides indirect evidence for causal relationships between diseases and microbial pathogens. Serological tests have a relatively high sensitivity, specificity and quick turn-around time, but may show cross-reactivity with closely related microbial species
Biological, in vitro and in vivo	Assessment of biological features (e.g., host range, transmission pattern, pathological effects, geographical origin) of microorganisms helps diagnose microbial infections in cases where other relevant data are scarce
	In vitro culture techniques using laboratory media and cell lines facilitate isolation and propagation of target microorganisms for subsequent morphological, biochemical, serological, and molecular characterization
	In vivo assays using laboratory animals and chicken embryos allow for recovery of microorganisms that fail to grow on culture media or cell lines, and help determine host susceptibility and immune response to, and pathogenic effects of microorganisms
Molecular	Detection of nucleic acids using molecular techniques offers direct evidence on the presence of microorganisms. Application of nucleic acid amplification technologies further improves the speed, sensitivity, and specificity of microbial identification and detection

has further enhanced morphological characterization of microorganisms. Besides unraveling paradigms of pathogen entry and pinpointing the exact intracellular location, these new techniques permit direct monitoring of the intracellular lifestyle of microbial pathogens and yield insights into the underlying mechanisms of their pathogenicity [2, 3]. Furthermore, atomic force microscopy (AFM) techniques offer a powerful platform for analyzing the structure, properties and functions of microbial pathogens as well as the localization, mechanics, and interactions of the individual cell wall constituents, contributing to the elucidation of the molecular bases of cell adhesion (nanoadhesome) and mechanosensing (nanosensosome) [4, 5].

Biochemical characterization focuses on the metabolic or enzymatic products of microorganisms, including distinct patterns of carbohydrate, protein, amino acid, fat metabolisms and production of particular enzymes. Biochemical tests are often conducted to distinguish between aerobic and anaerobic breakdown of carbohydrates, to show carbohydrates that can be attacked, to detect specific breakdown products of

carbohydrates (e.g., the formation of acids, alcohols and gases when grown in selective liquid or solid media), to determine the ability of carbohydrates to utilize substrates such as citrate and malonate, to examine the metabolism of protein and amino acids (e.g., gelatine liquefaction, indole production, amino acid decarboxylase test, and phenylamine deaminase test) as well as of fats (e.g., hydrolysis of tribtyrin), and detect production of enzymes (e.g., catalase test, oxidase test, urease test, ONPG test, and nitrate reduction). Assessment of fungal primary metabolites such as ubiquinones (coenzyme Q) is useful for the taxonomy of black yeasts and filamentous fungi, whereas examination of fungal secondary metabolites (e.g., steroids, terpenes, alkaloids, cyclopeptides, and coumarins) by chromatographic techniques provides another means for fungal identification. A recent approach for biochemical characterization of microorganisms centers on the characteristic outer surface charges of microbes that contribute to their distinct migration under a direct-current electric field such as capillary electrophoresis (CE), leading to rapid and efficient separation, identification, quantitation, and characterization of intact microorganisms (i.e., bacteria, viruses, and fungi) [6]. Another useful technique for biochemical characterization of microbes is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), which has been shown to be useful for specific identification of *Francisella* and other microbial pathogens [7].

Serological characterization on the basis of specific reactions between host antibodies and microbial antigens (usually protein or carbohydrate) provides highly sensitive, specific, and rapid identification of microorganisms. Detection of rising levels of specific IgA, IgM, and IgG antibody titers or seroconversion in blood, urine, and fecal materials offers indirect evidence for causal relationships between diseases and microbial pathogens [8]. An interesting development in the serological characterization of disease-causing microorganisms is the use of chemically synthesized peptides. Generated by chemical approaches, these peptides are composed of two or more amino acids linked together by peptide bonds. By mimicking naturally occurring peptides or segments of proteins, these peptides serve as synthetic antigens in peptide microarrays as potential diagnostic tools in high-throughput immunoassays [9]. Other new developments in serological characterization of microorganisms include biosensors and nanotechnology (nanoarrays and nanochips). Biosensors involve the use of a microbe-specific antibody and a transducer (e.g., electrochemistry, reflectometry, interferometry, resonance, and fluorimetry) to convert a biological interaction into a measurable signal. In the particle concentration fluorescence immunoassay (PCFIA) for brucellosis, submicron polystyrene particles are coated with antigen and placed in a 96-well vacuum plate. After addition of fluorescent conjugate followed by vacuum filtration to remove unbound conjugate, the total particle-bound fluorescence is measured by front surface fluorimetry. Nanotechnology (nanoarrays and nanochips) offers small scale platforms to identify an array of infectious agents or serotypes on a single chip.

Biological characterization focuses on the issues related to the host susceptibility, transmission patterns, pathological effect(s), and geographical origin of microbial pathogens, which are critical in helping achieve correct diagnosis of microbial infections in cases where other relevant data are scarce [10]. In vitro isolation and

propagation on laboratory media and cell lines offers a valuable tool for identification and diagnosis of microbial infections. The size, color, shape and form of colonies formed by microorganisms on nutritional agar and other selective media are diagnostically informative. In case of viral pathogens, the formation of a region of dead cells resulting from viral growth (called a "plaque") suggests their cytopathogenic effects (CPE). Parasitic protozoa may also be cultivated as a means of identification [11]. However, because not all microorganisms will grow in laboratory media and cell lines, embryonated eggs, insect vector, and laboratory animals (e.g., rodents) may be utilized. For example, *Trypanosoma cruzi*, the causal agent for Chagas disease, is grown in the guts of its vector triatomine bug for confirmation and diagnosis. The availability of cultured isolate/strain permits further antigenic studies, antibiotic susceptibility testing, and genetic studies. Despite their relatively high expense and length of time required, in vitro and in vivo techniques have contributed to the studies of microbial taxonomy, biology, epidemiology, pathogenesis, and treatment response. A recent development in the use of in vivo techniques for microbial characterization relates to the in vivo bioluminescence imaging or biophotonic imaging (BPI). Based on genetically engineered bioluminescent/fluorescent microorganisms, this technique enhances the study of microbial infections and host immune responses [12]. Application of genetically engineered mice with luciferase reporters for specific microbial or host genes helps overcome the limitations of in vivo bioluminescence imaging for assessment of microbial replication, activation of key genes in host immunity, and response to tissue damage in vivo [13].

Because of their time-consuming, occasionally variable nature, and/or their limited sensitivity and specificity, phenotypic approaches (e.g., morphological, biochemical, serological, and biological characterization) to the identification of microorganisms are increasingly supplemented with molecular techniques. Progresses in the areas of genetic target selection, template preparation, transition from nonamplified to amplified approaches, and product detection over the past two decades have made molecular methods an indispensable tool in the laboratory diagnosis of microbial pathogens in veterinary medicine [1].

With regard to the selection of genetic targets, the following three types may be considered: nonspecific, shared, and specific genetic targets. Nonspecific genetic targets include the guanine and cytosine composition (or G+C content), short random primer sites, randomly dispersed repetitive extragenic palindromes (REP), enterobacterial repetitive intergenic consensus sequences (ERIC), variable-number tandem repeats (VNTR) (also known as simple sequence repeats (SSRs), or microsatellites), restriction enzyme sites, and so on. Shared genetic targets include ribosomal RNA (rRNA) genes (e.g., 16/18S rRNA, 23/25/28S rRNA), internal transcribed spacer (ITS) regions, mitochondrial DNA (mtDNA), and housekeeping genes, etc. Specific genes are uniquely present and represent ideal targets for the identification of pathogenic bacteria, fungi and parasites.

Preparation of nucleic acid templates from cultured isolates and clinical specimens represents an important initial step for molecular identification and detection of microorganisms. This often involves (1) disruption of cell walls, (2) denaturation of nucleoprotein complexes, (3) inactivation of endogenous DNase/RNAse, and

(4) removal of contaminating proteins, polysaccharides, polyphenolic pigments, and other compounds [14]. While enzymatic digestion (e.g., using lyticase, zymolase, chitinase, gluculase, and/or proteinase K) and occasionally acid and alkali treatments may be effective for breaking up bacterial and yeast cells, mechanical grinding, sonication or bead-beating is often necessary to disrupt the mycelial and helminth cell walls. Following extraction with organic solvents (e.g., phenol/chloroform) and detergents (e.g., sodium dodecyl sulfate, SDS; hexadecyltrimethylammonium bromide, CTAB; and *N*-lauroylsarcosine), which denatures cytosolic proteins and lipid membranes and inactivates endogenous DNase/RNAse, nucleic acids of high purity are obtained after precipitation with ethanol or isopropanol. The recent development of various easy-to-use commercial kits has negated the need to use hazardous organic solvents in the isolation of microbial DNA/RNA. Furthermore, automated nucleic acid extraction systems have become increasingly sophisticated and affordable, contributing to the streamlining of template preparation and reduction of potential cross-contamination during manual handling.

The early generation molecular procedures rely on nonamplified, hybridization approaches, such as DNA–DNA hybridization (for estimation of guanine–cytosine ratio or G-C content), and use of gene probes in dot blot, Southern blot, and fluorescence in situ hybridization (FISH), etc. [15]. A more recent development in the DNA hybridization-based approach is DNA microarray, in which high-density oligonucleotide probes (or segments of DNA) are immobilized on a solid surface, and used to hybridize (catch) any complementary sequences (labeled with fluorescent nucleotides) in a test sample. Subsequent detection and quantification of fluorescence signal permits identification and determination of the relative abundance of nucleic acid sequences in a sample [16]. Although these nonamplified procedures have adequate specificity, they are relatively insensitive, often requiring large quantity of starting materials for reliable detection. Nonetheless, some of these nonamplified techniques remain valuable for comparison of microbial genomes and for identification of species- and virulence-specific gene regions. For example, dot blot hybridization was employed for screening genomic DNA libraries of *Dichelobacter nodosus* strains causing virulent and benign footrot, and several virulent- and benign-specific gene regions for subsequent differentiation of virulent and avirulent *D. nodosus* strains were identified as a result [17, 18]. This approach was also applied for identification of novel virulence-specific gene regions in zoonotic bacterial pathogen *Listeria monocytogenes* and novel species-specific gene in animal bacterial pathogen *Listeria ivanovii* [19, 20].

The mid-1980s witnessed the advent of a novel, highly efficient in vitro nucleic acid amplification technique known as polymerase chain reaction (PCR). This technique has the capacity to synthesize billions of copies from a single nucleic acid template within 3–4 h, and demonstrates superior sensitivity, exquisite specificity, rapid turnover time and amenableness to automation for high throughput testing. Since then, PCR and its variants (e.g., nested PCR, multiplex PCR, real-time PCR, quantitative PCR, reverse transcription PCR (RT-PCR), arbitrarily primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD), degenerate oligonucleotide primed PCR (DOP-PCR), sequence-independent single primer amplification (SISPA), and rolling circle amplification (RCA)) have been widely applied in research and clinical laboratories for identification and phylogenetic analysis of microorganisms

[21–24]. Apart from PCR, other nucleic acid amplification procedures include nucleic-acid-sequence-based amplification (NASBA), ligase chain reaction (LCR), strand displacement amplification, Q-β replicase-mediated amplification, linear-linked amplification, and loop-mediated isothermal amplification (LAMP), etc.

Conventional methods for detection of nucleic acid products are based on electrophoretic separation followed by staining with ethidium bromide, gelstar, or SYBR Green. Whereas agarose gel electrophoresis provides a convenient, inexpensive way for separation and semiquantitation of DNA and RNA, polyacrylamide gel electrophoresis (PAGE) is useful for separating small nucleic acid fragments (<500 bp). Among the various PAGE-based procedures, single strand conformational polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are widely applied. SSCP is capable of detecting single nucleotide variations, and in combination with capillary electrophoresis (CE), SSCP-CE provides an automated system for rapid separation of nucleic acid products. Recent advances in instrument automation and fluorescent dye chemistry permit real-time monitoring of PCR amplicons (so-called real-time PCR). Besides the use of double-stranded DNA intercalating dye (e.g., SYBR Green), specifically designed probes such as hydrolysis dual-labeled probes (TaqMan®), hybridization probes (LightCycler), molecular beacons, peptide nucleic acid (PNA) probes, TaqMan minor groove binding (MGB™) probes, locked nucleic acid (LNA®) primers and probes, and scorpions™ may be utilized [25].

Other nucleic acid detection approaches include DNA microarray (also known as DNA chip, gene or genome chip, or gene array), biochips (biosensors), line probe assay (LiPA), enzymatic signal amplification (e.g., ELISA and flow cytometry), and DNA sequencing. Biochips (biosensors) are small analytical devices designed for nucleic acid-based electrical/optic detection (fluorescence or chemiluminescence) [26]. DNA sequencing analysis provides a most accurate way to determine the identity of microbial organisms. While the classic Sanger method (also known as "chain termination method" or "dideoxy sequencing") can read up to 900 bp per and produce 100 kb of sequence data per run, the "next generation sequencing" technologies (e.g., the 454 pyrosequencing-based instrument (Roche Applied Sciences), genome analysers (Illumina), and the SOLiD system (Applied Biosystems)) show improved efficiency for DNA sequencing analysis. Although Illumina and 454 sequencing technologies read 76–106 bp and 250–400 bp, they have the capacity to generate 20 Gb and 400 Mb of sequence data per run, respectively.

Subtyping and Phylogenetic Analysis

Microbial pathogens are noted for the diversity and their ability to adapt and survive in challenging environments. The ability to identify and track microbial strains and varieties involved in disease outbreaks is crucial for their control and prevention. For this reason, a number of phenotypic and molecular procedures have been developed and applied for subtyping and phylogenetic analysis of microbial strains and varieties causing animal diseases (Table 35.2) [27].

Table 35.2 Common laboratory techniques for typing and phylogenetic analysis of microbial pathogens

Technique	Key features
Biotyping	Biotyping separates microbial strains into "biotypes" on the basis of their metabolic and enzymatic activities (e.g., sugar fermentation, amino acid decarboxylation/deamination, urease activity, hydrolysis of compounds, hemagglutination, and hemolysis), colonial morphology, and environmental tolerances (e.g., tolerance to pH, chemicals, dyes, and heavy metals). Biotyping is generally reproducible and easy to perform and interpret. However, it has poor discriminatory power due possibly to variation in gene expression and point mutation
Phage typing	Phage typing distinguishes microbial strains into "phage types" by their patterns of resistance or susceptibility to a standard set of bacteriophages, depending on the presence or absence of particular receptors on the bacterial surface for phage (virus) binding. Phage typing shows good reproducibility, discriminatory power and ease of interpretation, but requires maintenance of biologically active phages and demands technical skills. In addition, many strains are nontypeable
Serotyping	Serotyping differentiates microbial strains into serotypes (serovars) according to the antigenic variations present on the surface structures (e.g., lipopolysaccharides, membrane proteins, capsular polysaccharides, flagella and fimbriae). Agglutination, latex agglutination, coagglutination, or fluorescent and enzyme labeled assays may be used for serotyping. Serotyping has good reproducibility, and ease of interpretation and performance. However, serotyping depends on the availability of good quality reagents, and some autoagglutinable (rough) strains are nontypeable. Additionally, the technique tends to have poor discriminatory power due to cross-reactive antigens
Bacteriocine typing	Bacteriocine typing assesses microbial strains for their susceptibility to a set of bacterial peptides (bacteriocine), and has been employed to type stains of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Yersinia pestis*, etc. The technique has good reproducibility, discriminatory power, and ease of interpretation, but it is technically demanding and many strains are nontypeable
Multilocus enzyme electrophoresis (MLEE) typing	MLEE typing separates strains into "electromorphs" (typically reflecting amino acid substitution that alters the charge of the protein) in accordance with their distinct electrophoretic mobilities of a set of metabolic enzymes. The technique has excellent reproducibility and ease of interpretation, but shows moderate discriminatory power, and requires expensive equipments
Antibiogram typing	Antibiogram typing compares different microbial isolates in their susceptibility to a set of antibiotics. The technique has ease of performance and interpretation and reasonable reproducibility. However, it has poor discriminating power
Restriction endonuclease analysis (REA) or restriction fragment length polymorphism (RFLP)	Digestion of chromosomal DNA with certain restriction endonuclease produces various fragments whose number and sizes (from 0.5 to 50 kb) are distinct among microbial strains and varieties. This technique has good reproducibility, but generates complex profile of hundreds of bands that may be difficult to interpret

(continued)

Table 35.2 (continued)

Technique	Key features
Pulse-field gel electrophoresis (PFGE)	Based on restriction fragment length polymorphism (RFLP), PFGE uses selected restriction enzymes to yield between 8 and 25 large DNA bands of 40–600 kb in size, and alternating currents to cause DNA fragments to move back and forth, resulting in a higher level of resolution of large fragments. This technique has good reproducibility, and ease of interpretation. However, it requires costly reagents and equipment
Ribotyping	Ribotyping uses a ribosomal RNA (rRNA) probe derived from the *Escherichia coli* to detect the restriction fragment patterns of 16S rRNA, 23S rRNA, and tRNAs after digestion of chromosomal DNA with appropriate restriction enzymes. Microorganisms are classified as separate species if their sequences show <98 % homology and are classified as different genera if their sequences show <93 % identity. As a derivative of RFLP, this technique is reproducible and is easy to interpret. However, it requires costly reagents and equipment
Amplified fragment length polymorphism (AFLP)	AFLP is a modification of RFLP through the addition of adaptors to restriction enzyme-digested DNA followed by PCR amplification and electrophoretic separation of PCR products, generating highly informative, polymorphic patterns of 40–200 bands for individual microbial strains. An obvious shortcoming of AFLP is its requirement for the ligation of linkers and indexers to enzyme-digested DNA from individual strains
PCR-restriction fragment length polymorphism (PCR-RFLP)	PCR-RFLP involves PCR amplification of one or more microbial housekeeping or virulence-associated genes followed by digestion with selected restriction enzymes and separation by agarose gel electrophoresis. The resultant band patterns allow differentiation of microbial subtypes. The technique obviates the need to ligate linkers and indexers before PCR amplification (as in AFLP), and represents a sensitive, discriminatory, and reproducible method for tracking and epidemiological investigation of microbial strains and varieties
Multilocus sequence typing (MLST)	In MLST, multiple DNA segments are amplified by PCR and examined by DNA sequencing analysis, leading to phylogenetic comparison of multiple isolates, and definitive identification of microbial strains and subtypes. MLST is reliable and easy to interpret
Mobile genetic element-PCR (MGE-PCR)	MGE-PCR uses a single primer in PCR to amplify particular MGEs followed by electrophoresis to discriminate amplicon profiles. This technique has been utilized to characterize different isolates of *Trypanosoma brucei* by targeting RIME which has a relatively high copy number in the genome

The common phenotypic subtyping procedures include biotyping, phage typing, serotyping, bacteriocine typing, multilocus enzyme electrophoresis (MLEE) typing, and antibiogram typing (Table 35.2). The genotypic subtyping (fingerprinting) approach targets the microbial chromosome and plasmid DNA such as their composition, homology, and presence or absence of specific genes. The genotypic subtyping techniques consist of two categories: nonamplified techniques (e.g., restriction endonuclease analysis (REA) or restriction fragment length polymorphism

(RFLP), pulse-field gel electrophoresis (PFGE), and ribotyping) and amplified techniques (e.g., amplified fragment length polymorphism (AFLP), PCR-restriction fragment length polymorphism (PCR-RFLP), and multilocus sequence typing (MLST), and mobile genetic element-PCR (MGE-PCR)) (Table 35.2) [28].

Virulence Determination

Many microbial species encompass a diversity strains with varied virulence potential. The availability of laboratory techniques to accurately assess the pathogenic potential of these microorganisms is vitally important to their control and prevention. For example, gram-negative bacterium *Dichelobacter nodosus* harbors strains that cause virulent, intermediate or benign footrot in sheep. As virulent and some intermediate footrot induces lameness and severe pain in affected sheep, leading to ill-thrift and reduced weight gain, it is necessary to apply control measures to stem the economic losses. On the other hand, benign footrot causes minimal harm to affected sheep and has the tendency to self-cure, it is unnecessary and indeed wasteful to treat benign footrot. Traditionally, the virulence of *D. nodosus* strains is determined by elastase test and gelatin gel test, which may take up to 4 weeks to complete, and often demonstrate notable variability. After comparative analysis of recombinant DNA libraries from *D. nodosus* virulent and benign strains, a panel of virulent- and benign-specific genes was identified. Use of gene probes and primers derived from these genes facilitate rapid and sensitive determination of *D. nodosus* virulence [17, 18].

Gram-positive bacterium *L. monocytogenes* is a zoonotic pathogen that encompasses a spectrum of strains with various pathogenic inclination. While some *L. monocytogenes* strains are highly pathogenic and sometimes deadly, others are relatively avirulent and cause little harm in the host. The current laboratory techniques for assessing the virulence of *L. monocytogenes* strains include the mouse virulence assay and in vitro cell assays. While the mouse virulence assay is capable of providing an in vivo measurement of all virulent determinants, its high expense limits its application. Representing a low-cost alternative to the mouse virulence assay for assessing *L. monocytogenes* virulence, in vitro cell culture techniques measure the ability of *L. monocytogenes* to cause cytopathogenic effects in the enterocyte-like cell line Caco-2, to form plaques in the human adenocarcinoma cell line HT-29, or to cause death in chicken embryos. Several other cell lines (e.g., hepatocyte Hep-G2, macrophage-like J774, epithelial Henle 407 and L2) are also useful for studies on *L. monocytogenes* ability to adhere, invade, escape from vacuoles, grow intracellularly and spread to neighboring cells. However, these techniques are time-consuming, and occasionally variable. Following recent identification of novel virulence-specific genes (e.g., *inlJ*), the virulence of *L. monocytogenes* strains can be rapidly and specifically determined by PCR [19, 29].

Drug Resistance Assessment

Microorganisms have the ability to acquire resistance to drugs that are used for their treatment. As drug are often used in animals (e.g., such as cows, pigs, chickens, fish, etc.) that provide an important source of human food, microorganisms exposed to these drugs can develop antibiotic resistance through horizontal gene transfer events (e.g., conjugation, transduction, or transformation) and point mutations [30]. The resistant bacteria in animals due to antibiotic exposure can be transmitted to humans through the consumption of meat, from close or direct contact with animals, or through the environment. For example, use of fluoroquinolone in poultry production has been linked to the emergence of fluoroquinolone resistant campylobacter infections in humans. Some bacteria (e.g., *Staphylococcus aureus*, enterococci, gonococci, streptococci, salmonella, and *Mycobacterium tuberculosis*) have developed multidrug resistance [31]. For example, methicillin-resistant *S. aureus* (MRSA) are resistant to non-beta-lactam antimicrobial drugs as well. Identification of MRSA has implications not only for treatment of infected animals, but for potential zoonotic transmission [32, 33]. While application of in vitro culture technique facilitates determination of MIC (medium inhibition concentration) of the strains, detection of specific gene mutations provides an alternative approach for assessment of antimicrobial drug resistance in microorganisms such as *B. hyodysenteriae* [34].

Conclusion

Given the diversity of animal hosts that are susceptible to a wide variety of microbial infections, veterinary diagnostic microbiology faces a greater challenge than its medical counterpart in achieving a correct and timely identification of culprit microorganisms causing significant economic losses in agricultural production. Although phenotypic procedures are useful for microbial identification, their time-consuming nature and occasional variability have provided the impetus for the development of nucleic acid detection methodology. With a high sensitivity, exquisite specificity and speed, molecular procedures especially those involving nucleic acid amplification (e.g., PCR) have been widely adopted in clinical and research laboratories for identification, typing, virulence determination, and drug resistance assessment of microorganisms of veterinary and medical importance. Further improvement through miniature and multiplexing will help reduce the cost of conducting molecular testing in diagnostic microbiology.

References

1. Rao SS, Mohan KV, Atreya CD (2010) Detection technologies for Bacillus anthracis: prospects and challenges. J Microbiol Methods 82:1–10

2. Ray K, Bobard A, Danckaert A et al (2010) Tracking the dynamic interplay between bacterial and host factors during pathogen-induced vacuole rupture in real time. Cell Microbiol 12:545–556
3. Bobard A, Mellouk N, Enninga J (2011) Spotting the right location-imaging approaches to resolve the intracellular localization of invasive pathogens. Biochim Biophys Acta 1810: 297–307
4. Dupres V, Alsteens D, Andre G, Dufrêne YF (2010) Microbial nanoscopy: a closer look at microbial cell surfaces. Trends Microbiol 18:397–405
5. Alsteens D, Dupres V, Andre G, Dufrêne YF (2011) Frontiers in microbial nanoscopy. Nanomedicine (Lond) 6:395–403
6. Simonet BM, Ríos A, Valcárcel M (2008) Capillary electrophoresis separation of microorganisms. Methods Mol Biol 384:569–590
7. Seibold E, Maier T, Kostrzewa M, Zeman E, Splettstoesser W (2010) Identification of Francisella tularensis by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry: fast, reliable, robust, and cost-effective differentiation on species and subspecies levels. J Clin Microbiol 48:1061–1069
8. Prickett JR, Zimmerman JJ (2010) The development of oral fluid-based diagnostics and applications in veterinary medicine. Anim Health Res Rev 11:207–216
9. Saravanan P, Kumar S (2009) Diagnostic and immunoprophylactic applications of synthetic peptides in veterinary microbiology. Microbiol Res 1:1e
10. Houpikian P, Raoult D (2002) Traditional and molecular techniques for the study of emerging bacterial diseases: one laboratory's perspective. Emerg Infect Dis 8:122–131
11. Wilkins W, Raji A, Parker S et al (2010) Examining heterogeneity in the diagnostic accuracy of culture and PCR for Salmonella spp. in swine: a systematic review/meta-regression approach. Zoonoses Public Health 57:S121–S134
12. Andreu N, Zelmer A, Wiles S (2011) Noninvasive biophotonic imaging for studies of infectious disease. FEMS Microbiol Rev 35:360–394
13. Luker KE, Luker GD (2010) Bioluminescence imaging of reporter mice for studies of infection and inflammation. Antiviral Res 86:93–100
14. Liu D (2008) Preparation of Listeria monocytogenes specimens for molecular detection and identification. Int J Food Microbiol 122:229–242
15. Gibson W (2009) Species-specific probes for the identification of the African tsetse-transmitted trypanosomes. Parasitology 136:1501–1507
16. Banér J, Gyarmati P, Yacoub A et al (2007) Microarray-based molecular detection of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses, using padlock probes. J Virol Methods 143:200–206
17. Liu D (1994) Development of gene probes of Dichelobacter nodosus for differentiating strains causing virulent, intermediate or benign ovine footrot. Br Vet J 150:451–462
18. Liu D, Webber J (1995) A polymerase chain reaction assay for improved determination of virulence of Dichelobacter nodosus, the specific causative pathogen for ovine footrot. Vet Microbiol 43:197–207
19. Liu D, Ainsworth AJ, Austin FW, Lawrence ML (2003) Characterization of virulent and avirulent Listeria monocytogenes strains by PCR amplification of putative transcriptional regulator and internalin genes. J Med Microbiol 52:1065–1070
20. Liu D, Ainsworth AJ, Austin FW, Lawrence ML (2004) PCR detection of a putative N-acetylmuramidase gene from Listeria ivanovii facilitates its rapid identification. Vet Microbiol 101:83–89
21. Drozd M, Kassem II, Gebreyes W, Rajashekara G (2010) A quantitative polymerase chain reaction assay for detection and quantification of Lawsonia intracellularis. J Vet Diagn Invest 22:265–269
22. Tarlinton RE, Dunham SP (2011) Pushing the envelope: advances in molecular techniques for the detection of novel viruses. Vet J 190:185–186

23. Tasker S (2010) The polymerase chain reaction in the diagnosis of infectious diseases. Vet Clin Pathol 39:261–262
24. Bexfield N, Kellam P (2011) Metagenomics and the molecular identification of novel viruses. Vet J 190:191–198
25. Clothier KA, Jordan DM, Thompson CJ, Kinyon JM, Frana TS, Strait EL (2010) Mycoplasma bovis real-time polymerase chain reaction assay validation and diagnostic performance. J Vet Diagn Invest 22:956–960
26. Gabig-Ciminska M (2006) Developing nucleic acid-based electrical detection systems. Microb Cell Fact 5:9
27. Belák S, Thorén P, LeBlanc N, Viljoen G (2009) Advances in viral disease diagnostic and molecular epidemiological technologies. Exp Rev Mol Diagn 9:367–381
28. Johansson A, Petersen JM (2010) Genotyping of Francisella tularensis, the causative agent of tularemia. J AOAC Int 93:1930–1943
29. Liu D, Lawrence ML, Austin FW, Ainsworth AJ (2007) A multiplex PCR for species- and virulence-specific determination of Listeria monocytogenes. J Microbiol Methods 71:33–40
30. Weese JS (2010) Methicillin-resistant Staphylococcus aureus in animals. ILAR J 51:233–244
31. Morgan M (2008) Methicillin-resistant Staphylococcus aureus and animals: zoonosis or humanosis? J Antimicrob Chemother 62:1181–1187
32. Cohn LA, Middleton JR (2010) A veterinary perspective on methicillin-resistant staphylococci. J Vet Emerg Crit Care (San Antonio) 20:31–45
33. Smout MJ, Kotze AC, McCarthy JS, Loukas A (2010) A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. PLoS Negl Trop Dis 4:e885
34. Hidalgo A, Carvajal A, Vester B, Pringle M, Naharro G, Rubio P (2011) Trends towards lower antimicrobial susceptibility and characterization of acquired resistance among clinical isolates of Brachyspira hyodysenteriae in Spain. Antimicrob Agents Chemother 55:3330–3337

Chapter 36
Recent Advances in Veterinary Diagnostic Virology: Report from a Collaborating Centre of the World Organization for Animal Health (OIE)

Sándor Belák and Lihong Liu

Introduction

Infectious diseases have a very high impact on animal and human health and welfare today, despite of strong efforts and good results in diagnostics, vaccine developments and control measures, including the early warning systems. There are many reasons, which contribute to the spread of infectious diseases, such as the open borders of the European Union, allowing rather free movement of animals over a whole continent, the globalization, the released and accelerated international and national trade and animal transfer. Simultaneously, the emergence and re-emergence of new or already known pathogens is a serious issue in veterinary and in human medicine. This scenario is clearly illustrated by the regular occurrence of transboundary animal diseases (TADs), such as foot-and-mouth disease (FMD), classical swine fever (CSF), African swine fever (ASF), among others. The recent occurrence of African swine fever in the Caucasus region and the spread afterwards to large territories of Russia clearly illustrates that our health authorities require a

S. Belák (✉)
OIE Collaborating Centre for Biotechnology-Based Diagnosis of Infectious Diseases in Veterinary Medicine, Joint R&D Division in Virology, The National Veterinary Institute, The Swedish University of Agricultural Sciences (SLU), Uppsala 751 89, Sweden

Departments of Virology, Immunobiology, and Parasitology (VIP), The National Veterinary Institute (SVA) & The Swedish University of Agricultural Sciences (SLU), Ulls väg 2B, Uppsala 751 89, Sweden
e-mail: Sandor.Belak@slu.se

L. Liu
OIE Collaborating Centre for Biotechnology-Based Diagnosis of Infectious Diseases in Veterinary Medicine, Joint R&D Division in Virology, The National Veterinary Institute, The Swedish University of Agricultural Sciences (SLU), Uppsala 751 89, Sweden

Departments of Virology, Immunobiology, and Parasitology (VIP), The National Veterinary Institute (SVA), Ulls väg 2B, Uppsala 751 89, Sweden
e-mail: Lihong.Liu@sva.se

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very strong preparedness, including prompt and powerful diagnosis, for the successful fight against the novel scenarios.

Considering these requirements the European Union (EU) is strongly supporting the development of powerful novel diagnostic methods, which are assured by international comparison, standardization and validation. Thus, the EU has launched and supported a large range of various projects for these purposes recently.

At the *World Organization for Animal Health (OIE) Collaborating Centre for the Biotechnology-Based Diagnosis of Infectious Diseases in Veterinary Medicine*, at the National Veterinary Institute (SVA) and the Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden, we have participated/participate in a high number of these projects, such as LAB-ON-SITE, EPIZONE, FLUTRAIN, FLUTEST, CSFV_goDIVA, ASFRISK, ConFluTech, AsemDialog, among others. As an example, the LAB-ON-SITE project (coordinated by SB) strongly contributed to the development of veterinary diagnostic virology. Ten infectious TADs, listed as notifiable to the OIE—FMD, swine vesicular disease (SVD), vesicular stomatitis (VS), CSF, ASF, bluetongue (BT), African horse sickness (AHS), Newcastle disease (ND), highly pathogenic avian influenza (HPAI) and swine influenza (SI)—were the subjects of this international research project of the EU. A range of new diagnostic assays were developed to to improve the current detection of the targeted ten viruses, such as novel real-time PCR assays, isothermal amplification methods, padlock probes, and novel ELISA systems. In order to improve the front-line, on-site diagnosis in the field, simple methods and equipment were adapted, such as portable PCR machines, simple thermo-platforms for isothermal amplification, dipsticks and lateral flow devices. Many of the developed methods were compared in the large international consortium, validation was performed and standard operating procedures (SOPs) of the new methods were disseminated via international organizations, such as the OIE with its 178 member countries and the International Atomic Energy Agency (IAEA) with its large networks in Asia, Africa and South America. Honouring the strong output, this project was considered as one of the Success Stories in FP6 of the European Union (http://ec.europa.eu/research/agriculture/success_labonsite_en.htm).

Considering the above-listed scenarios and requirements, *the prompt detection and very rapid and exact identification of various pathogens* is a very important and essential task in veterinary virology. While classical diagnostic methods, such as virus isolation, remain technically unaltered or show rather little steps of changes and development, the molecular diagnostic methods have advanced dramatically in the last decades. These new techniques provide powerful novel tools for the rapid detection and identification of a wide range of causative agents, as well as for supporting disease control and surveillance. For example, real-time quantitative PCR assays are highly sensitive and specific methods, which are widely used for detection of various pathogens in diagnostic units, including the Reference Laboratories and the Collaborating Centres of the World Health Organization (WHO; http://www.who.int) and of the World Organization for Animal Health (OIE; www.oie.int).

At the *OIE Collaborating Centre for the Biotechnology-Based Diagnosis of Infectious Diseases in Veterinary Medicine*, Uppsala, Sweden, great efforts have been made for the development, standardization and dissemination of new diagnostic

assays within the frame of several large EU and other projects in the recent years. Some achievements have been summarized in previously published review articles [1, 2]. Herewith, an update and selected examples are given in order to illustrate the trends of recent advances in veterinary diagnostic virology at our OIE Collaborating Centre, in collaboration with a wide network of partner laboratories in Europe and on other continents.

Sample Collection, Transportation, Storage, Enrichment and Nucleic Acid Preparation

Sample Collection, Preparation and Transportation

Proper sample collection, storage, transportation and enrichment are crucial for the reliable diagnosis of infectious diseases. To avoid potential degradation of the targeted nucleic acids, the samples are normally transported at low temperature using dry ice, cooling batteries and/or are stored in −20/70 °C freezers before further processing. Heat- and/or chemical inactivation of samples is required to avoid the transmission and spread of infectious agents. In order to diagnose the various known and "unknown" diseases in a safe and reliable way, proper methodologies are needed, which simplify the handling, processing of the samples, and the transport to the diagnostic laboratories. Currently, several simple tools, such as different filter papers or cards are commercially available for such purposes. For example, FTA™ Cards, produced by Whatman plc, now a part of GE Healthcare, are suitable for the collection, transportation and storage of samples at room temperature. The company claimed that such FTA Cards contain chemicals that lyse cells, denature proteins and protect nucleic acids from nucleases, as well as from the damages caused by heat, oxidative and UV effects. At the Collaborating Centre, our colleagues have used these types of cards for transportation of samples from other countries and continents to Sweden. For example, ASF serum samples were collected in Uganda, placed on the filter papers, and transported to Sweden at room temperature. The same type of filter papers was used for transportation of Peste des Petits Ruminants (PPR) samples from Pakistan. Bat coronavirus samples were sent from Brazil to Sweden in an envelope, by post. Viral nucleic acids, eluted from the cards, are successfully amplified by various PCR assays or detected by other means of molecular diagnostic virology. We perform real-time PCR assays, using small pieces of cards containing samples as templates and various viruses are detected in a convincing way after this simple way of sample transportation.

Sample Enrichment

The diagnosis in veterinary virology is frequently complicated by the fact that the amount of targeted viruses in the certain types of clinical samples, in food and feed products or in water samples, as well as in other diagnostic specimens, is often very

low. The low target content may lead to false negative results in the various PCR assays and in other molecular diagnostic procedures. In order to avoid or to reduce this very important bottleneck effect, diagnostic laboratories develop and apply a range of sample enrichment methods, in order to "fish out" the targeted pathogens or their components, such as nucleic acids or proteins, from the analyzed specimens. For example, DNA enrichment is achieved by functionalized magnetic nanoparticles that are coupled with probes to which the target DNA could be hybridized [3].

Nucleic Acid Preparation Processes

The proper preparation of viral nucleic acids as targets of PCR and other molecular diagnostic assays is also a very important task in the diagnostic laboratories. Simplicity and high-throughput capacity are major concerns in case of outbreaks where a huge number of samples are processed within a relatively short period of time. It is almost impossible to complete such a task by manual extraction methods such as TRIzol reagent or spin columns. The manual extraction processes are labour- and time-consuming and expensive. In addition, personnel costs and working processes have to be considered. For those reasons, various kinds of automated equipment have been developed and commercialized for nucleic acid preparation and/or handling of samples. At our OIE Collaborating Centre the automated nucleic acid extraction steps are performed with robotic extraction machines.

International Comparison and Standardization

In an exercise to compare the performance of nucleic acid extraction robots (12 separate instruments, comprising eight different models) in five European veterinary laboratories, similar results were observed from best performing robots when dilutions of a cell culture supernatant were tested, whereas up to 1,000-fold difference was obtained from less optimized robots when dilutions of a serum sample were tested [4]. It was observed that the same instrument performed differently when tested with different types of samples. Therefore, a proficiency test or "ring trial" of different clinical samples would be of great help to validate in-house assays, identify critical steps and improve performance of downstream PCR tests.

Real-Time Quantitative PCR Assays

Since the publication of quantitative real-time PCR in 1996 [5], it has become a very reliable, powerful, high-throughput and robust molecular tool for early, rapid and sensitive detection of pathogens in both human and veterinary medicine. Compared to the conventional gel-based PCR, the real-time PCR has several advantages, including high-throughput capacity, less hands-on time, lower risk of contamination,

and the potential to be fully automated [6]. By using a gene-specific probe, the specificity and analytic sensitivity of the real-time PCR may be further improved. The research activities at our Collaborating Centre aimed to develop real-time PCR assays for diagnostics to differentiate infected from vaccinated animals (DIVA), for specific detection of emerging viruses, and for detection of zoonotic agents, in particular water- and food borne pathogens.

Herewith, several examples are presented regarding specific problems, illustrating the diagnostic application of various real-time quantitative PCR assays developed and used at our OIE Collaborating Centre, in collaboration with other European veterinary institutes in various research projects of the European Union. Further important achievements are also listed here, for example the development of real-time RT-PCR assay using a minor-groove binder probe for the pan-serotypic detection of *foot and mouth disease virus* (FMDV) [7] and the application of loop-mediated isothermal amplification (LAMP) for detection of wild type *classical swine fever virus* (CSFV) [8] and vaccine strain [9], and *swine vesicular disease virus* (SVDV) [10].

Real-Time PCR Assays for the Detection of Various Variants of Classical Swine Fever Virus, Including Vaccine Strains

CSFV is a pestivirus within the family of *Flaviviridae*. It is the causative agent of classical swine fever (CSF), a highly contagious disease affecting both wild boars and domestic pigs. In Europe, the virus is largely maintained in the wild boar populations that serve as a reservoir for reintroduction to domestic pigs. Recently, a chimeric vaccine candidate, CP7_E2alf has been developed and has the potential to be used as a safe and efficient marker vaccine in wild boars, which enables differentiation of infected from vaccinated animals [11, 12]. A vaccine-specific TaqMan real-time RT-PCR assay was developed and evaluated, and a second, wild type-specific assay was modified from an established one in such a way that both can be performed in two wells side-by-side in a microplate in a single run [13]. Both assays could be applied in CSFV vaccination and control programs in the wild boar population.

DIVA Approaches

CSF remains endemic in domestic pigs in some countries, for example, China, where vaccination with the traditional lapinized live virus is still in practice. DIVA diagnostics is a potentially powerful tool to discriminate whether pigs are naturally infected with wild type strains or vaccinated with the lapinized live virus. The Chinese Hog Cholera Lapinized virus (HCLV) has been through extensive serial passages in rabbits in China in 1950s and is completely attenuated, but retains its efficacy as vaccine. Due to its safety and efficacy, this vaccine was introduced into European countries and named as “Chinese” strain (C-strain). “Riems” C-strain is one of the commercial vaccines derived from the Chinese strain. Development of a vaccine-specific real-time RT-PCR would be of great value, which could be used in

parallel with a wild type-specific or generic real-time RT-PCR for detection and differentiation of wild type viruses from vaccine strains. Leifer et al. studied differentiation of Riems C-strain-vaccinated pigs from animals infected by CSFV field strains using real-time TaqMan RT-PCR [14]. Leifer et al. also reported escape of CSF C-strain vaccine virus from detection by C-strain specific real-time RT-PCR caused by a point mutation in the primer-binding site [15]. At the Collaborating Centre, we have developed a real-time PCR based on primer-probe energy transfer technology (PriProET) for the improved detection of CSFV [16]. The PriProET technology was developed initially as a novel quantitative real-time PCR assay for the simultaneous detection of all serotypes of FMDV [17], and subsequently was used for detection of other pathogens, including SVDV [18], and *bluetongue virus* (BTV) [19]. Following PCR amplification, the melting curve analysis allows confirmation of specific amplicons, and differentiation between wild type CSFV and the HCLV vaccine strain (Fig. 36.1). Further evaluation of the assay demonstrated that in an RNA mixture of both wild type CSFV and C-strain vaccine, the melting curves displayed only one curve either a wild type-like or a vaccine-like depending on the dominating RNA [20]. Therefore, the PriProET melting curve analysis could identify the presence of CSFV field strain in equivocal samples or in animals vaccinated with C-strain, but would not reliably detect infections with wild-type virus in a population vaccinated with the Riems strain. In a recent study, a generic real-time TaqMan RT-PCR was developed for the specific detection of three lapinized vaccine strains, namely, the Taiwanese Lapinized Philippines Coronel (LPC), the Chinese HCLV, and the European Riems C-strain [21]. The new assay was able to detect the Riemser C-strain vaccine viral RNA in experimental samples, indicating that the assay could be a useful tool to facilitate outbreak control if a strategy using these lapinized vaccines is deployed.

Real-Time PCR Assay for the Detection of the Recently Detected Atypical Bovine Pestiviruses

Over the past few years, new bovine pestiviruses have been detected in biological products, e.g., foetal bovine serum (FBS) batches, and in naturally infected cattle. Due to nucleotide sequence variations, the identification of these viruses might fail, since primers and probes had mismatches to their target, leading to a false negative result. For example, the well-known "pan-pestivirus" primer pair 324/326 failed to react with D32/00_'HoBi' virus that was found in a batch of contaminated foetal calf serum [22]. To overcome the limitations of current molecular methods, a new one-step real-time TaqMan RT-PCR assay was developed for the specific detection of atypical bovine pestiviruses in clinical samples and in biological products [23]. This new assay provides a useful tool for highly sensitive and specific detection of atypical bovine pestiviruses and can be applied in combination with other diagnostic methods to ensure that biological products, including FBS and vaccines, are free from pestivirus contamination. Recently, we have tested 33 batches of commercial

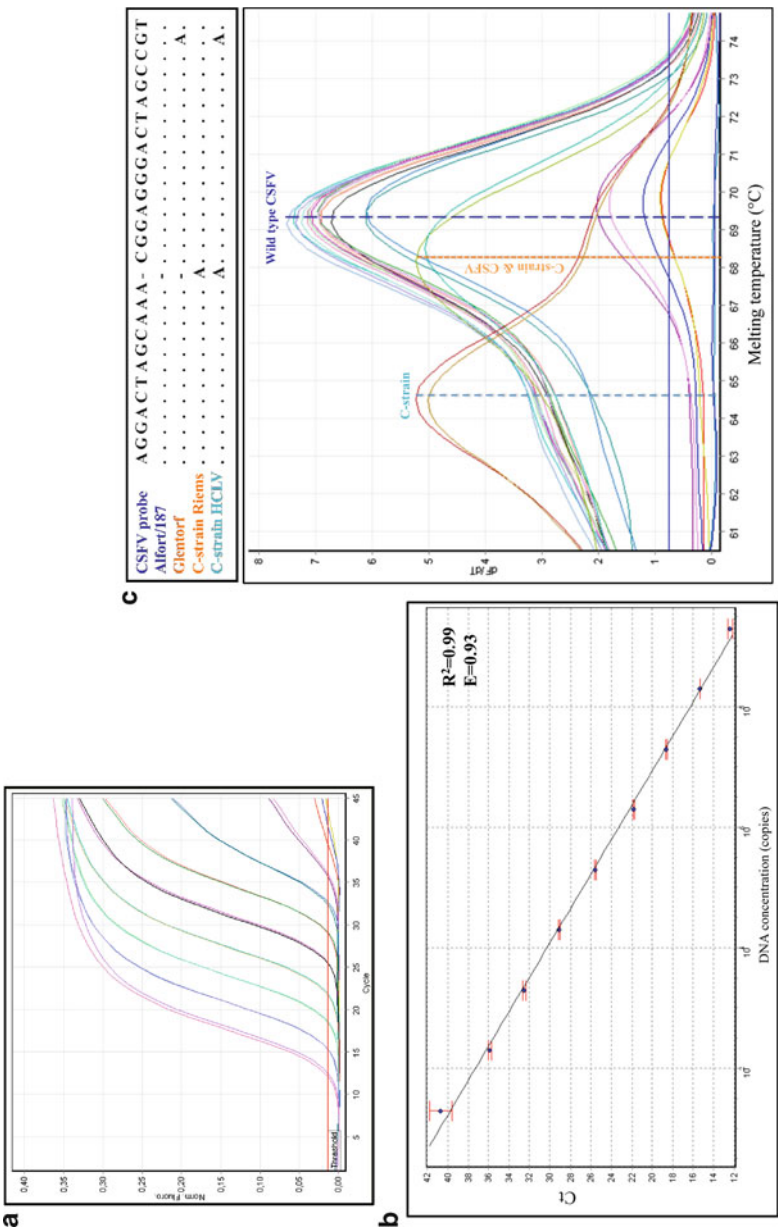


Fig. 36.1 A real-time RT-PCR assay based on primer-probe energy transfer technology for the improved detection classical swine fever virus. RNA standards containing the amplicon were ten-fold diluted in water and tested in duplicate. The results are demonstrated in amplification curve (**a**), linearity (**b**), of the PCR step for detection and melting curve analysis (**c**) to differentiate wild type viruses from the Chinese strain (C-strain) vaccine. Reprint from ref [16], with permission. Copyright © 2009, Elsevier

FBS by this approach and found all batches were positive for at least one species of bovine pestiviruses. The consequence of using contaminated FBS in diagnostic laboratories is detrimental. Isolation of bovine viral diarrhea virus (BVDV) in bovine turbinate cells is a routine method, which requires FBS during preparation of the cell cultures. A batch of contaminated FBS of South American origin had disturbed virus isolation. Tests showed presence of both the recognized species and the newly described atypical bovine pestivirus in the FBS batch. The contaminating atypical pestivirus was further characterized, as it is described by our group [24].

Broadly Targeting Real-Time RT-PCR Assays for the Generic Detection of Viruses

Apart from pathogens only affecting animals, a high number of pathogens can cross the species barrier(s) and cause diseases in humans, which is referred as zoonosis. Of more than 1,400 human pathogens, approximately 60 % are zoonotic, of which 25 % are estimated being able to be transmitted from human to human [25]. Therefore, it is of great importance to develop broadly targeting real-time RT-PCR for the generic detection of zoonotic viruses at our Collaborating Centre. Herewith, several examples are described.

Coronaviruses are the causative agents of a broad spectrum of animal and human diseases, including the Severe Acute Respiratory Syndrome (SARS) in humans. Considering this scenario, it is very important to develop very "general coronavirus" PCR assays, which are able to detect a wide range of viruses in various hosts simultaneously. In order to fulfil these requirements, we have developed a pan-coronavirus real-time RT-PCR, which is able to detect 36 strains of animal and human coronaviruses simultaneously, including HCoV-NL63 and SARS-CoV [26]. In this PCR panel the degenerated primers are targeting a conserved region of the ORF1b of the viral and have a low annealing temperature. We have compared this real-time RT-PCR system with two published TaqMan assays for detection of four animal coronaviruses in 75 clinical samples, and obtained the same results.

It has to be mentioned that eventhough this SYBR Green-based PCR assay is very promising in the general detection of coronaviruses, it also has weakness with regards to its specificity. Apart from the coronavirus positive samples, primer dimers and non-specific amplicons may also produce amplification curves and this can lead to false positive results. In order to avoid false diagnosis, melting curve analysis is needed in this assay. The melting curve analysis is a practical tool to verify the truly positive results. In summary, it was concluded that this SYBR Green-based real-time pan-coronavirus PCR assay, along with melting curve analysis, provides a powerful novel tool for the laboratory diagnosis of coronavirus infections and for detecting the emerging, yet to be uncharacterized new coronavirus variants. Considering the strong emergence rate of many RNA viruses, including the coronaviruses, the use of such pan-virus family PCR assays is very important. The new assay provides a novel tool for our diagnostic laboratories in veterinary and in human virology.

Concerning other real-time PCR approaches, such as the TaqMan principle, we have found that it is challenging and difficult to develop a pan-coronavirus detecting system using a single probe. Even though the genomic stretches are relatively conserved, it is almost impossible to design a TaqMan probe for efficient general detection of coronaviruses. To overcome this limitation, three overlapping TaqMan probes were designed and used together with the above-mentioned primers for sensitive and specific detection of coronaviruses of all three groups [27]. We have found this three-probe approach reduced the degree of probe degeneration and maintained a high sensitivity and specificity. The assay detected coronaviruses in human nasopharyngeal aspirate samples and in duck faecal samples. Furthermore, this method was able to detect SARS-CoV in a reference material, and detect the synthetic sequences of bat coronaviruses (HKU3-CoV, HKU5-Cov and HKU9-CoV), indicating it is a useful tool for screening samples of animal origin for zoonotic or potentially zoonotic pathogens. The assay was used as a tool for epidemiological studies on coronaviruses in wild birds from the Bering Strait area [28].

Molecular Tests for the Improved Detection of Food- and Waterborne Zoonotic Pathogens

Food- and waterborne zoonotic pathogens frequently cause large outbreaks in regions where sanitation is poor. *Hepatitis E virus* (HEV) is an important food- and waterborne zoonotic pathogen, which is an emerging virus of global importance. This single-stranded, positive-sense RNA virus belongs to the genus *Hepevirus* in the family of *Hepeviridae* that includes at least four recognized genotypes. While genotypes 1 and 2 are associated with large outbreaks and epidemics in humans in developing countries with poor sanitary conditions, genotypes 3 and 4 HEV strains are able to infect both humans and various animal species, in particular, domestic pigs and wild boars, and are responsible for sporadic cases of acute hepatitis E not only in the developing, but also in the industrialized countries. It has been recognized that hepatitis E is a zoonotic disease. At our OIE Collaborating Centre, Gyarmati et al. developed two real-time PCR assays for universal detection of all four genotypes of HEV [29]. Both the TaqMan and PriProET assays were able to detect 20 viral genome equivalents per reaction. The results obtained from both real-time PCR assays were comparable to those from a nested gel-based PCR. However, the TaqMan assay had higher reaction efficiency and performed slightly better than the PriProET assay. Such tools are important not only for veterinary diagnostics but also for molecular epidemiology of HEV in humans, pigs and wild boars [30].

Proximity Ligation Assays

It is not widely known yet that real-time PCR assays can be used not only for the detection of viral nucleic acids, but *also for the detection of protein components or*

antigens of pathogens. This technique is referred to proximity ligation assay (PLA) [31]. In a proximity ligation assay, two oligonucleotides are coupled to antibodies. Binding of the antibodies to the same target will bring the two oligonucleotides sufficiently close to each other (or in proximity) such that the two ends could be ligated with the help of a third connector oligonucleotide. The ligation products are then used as target DNA for subsequent amplification and detection by real-time PCR. At the OIE Collaborating Centre in Uppsala, Schlingemann et al. used this technique to detect antigens of avian influenza viruses in biological samples [32]. We have found that the method is four orders of magnitude more sensitive than a sandwich ELISA, which utilized the same antibody. This study opens a new window for diagnostic virology, by using PLA for the highly effective detection of viral proteins in clinical specimens.

Suspension Microarray Technologies

Although real-time RT-PCR offers multiplex detection of pathogens, its capacity is limited to no more than approximately five targets in a single diagnostic block. By contrast, suspension microarray technology, such as Luminex xMAP technology (Luminex Corporation, Austin, TX) has the capacity of *detecting up to 100 targets in a single platform, or even more*. At our OIE Collaborating Centre a range of Luminex assays have been developed for the multiplex detection of pathogens important in human and veterinary medicine. A suspension microarray assay was constructed for detection and differentiation of pestiviruses including CSFV, BVDV-1, BVDV-2, *border disease virus* (BDV) and the atypical bovine pestivirus [33]. Following PCR amplification of target genetic region, one strand of the amplicons was removed by Lambda exonuclease to generate single-stranded PCR products for efficient hybridization to the microsphere-coupled oligo probes. After incubation with a reporter dye streptavidin-R-phycoerythrin, the median fluorescent intensity (MFI) of each reaction was measured in a Luminex instrument. The system was able to provide positive/negative results and to identify each species by comparing MFI values. Such an assay is a useful tool for the genetic detection of various pestiviruses in cattle.

Another Luminex suspension microarray assay was developed at our laboratories for the serological diagnosis of bovine viral diarrhoea (BVD) [34]. In this blocking system, microspheres were coupled with a monoclonal antibody (MAb) and were incubated with viral antigen and serum samples. After addition of another MAb that is biotinylated, R-phycoerythrin-conjugated streptavidin was added and the MFI values were measured on a Luminex 200 analyzer. This blocking microsphere-based immunoassay had intra- and inter-assay variability of 4.9 % and <7 %, respectively, and variability of bead conjugations of <6.6 %. By testing a total of 509 serum samples, the assay had a sensitivity of 99.4 % and a specificity of 98.3 % relative to a commercial blocking ELISA. The new microsphere immunoassay provides an alternative to conventional ELISA systems and can be used for high-throughput screening in the BVD control and eradication program.

As BVDV is one of the pathogens causing bovine respiratory disease (BRD) complex, our ongoing work at the Collaborating Centre is to develop a multiplex Luminex assay for the high-throughput, simultaneous detection of antibodies against four major viruses that cause BRD, namely, BVDV, *bovine respiratory syncytial virus* (BRSV), *parainfluenza type-3 virus* (PIV3) and *bovine coronavirus* (BCoV). This novel multiplex assay not only allows a higher sample throughput but also reduces the time and labour required. The Luminex platform has a strong potential to replace ELISA as a powerful novel system for the complex diagnosis of pathogens.

Genome Sequencing and Viral Metagenomics, Detection of "Unknown" or Emerging New Viruses in Veterinary Medicine

A pathogen's genome can be determined by various methods, such as the traditional cycle sequencing and by the advanced "next-generation sequencing" technologies. While the cycle sequencing is still the method of choice in most laboratories, next-generation sequencing is becoming available in some laboratories. Both methods are a powerful tool in veterinary diagnostic virology. Furthermore, genome sequencing will contribute substantially to a better understanding of pathogens, which would be helpful in veterinary diagnostic virology. Particularly, viral metagenomics is a generic technology using large-scale sequencing to identify viral genome sequences without prior knowledge. Viral metagenomics has helped researchers with the *investigation of complex diseases, diseases of unknown aetiology, and identification of emerging novel viruses in samples.* In the followings, several examples are given on the use of viral metagenomics at our OIE Collaborating Centre to detect new and/or emerging and re-emerging viruses in animals.

Detection of Novel Viruses in the Postweaning Multisystemic Wasting Syndrome (PMWS) of Pigs

PMWS is a viral disease worldwide affecting postweaning pigs of about 2–4 months of age. *Porcine circovirus type 2* (PCV-2) has been found to play an important role in the complex infection biology of PMWS. PCV-2 is a small circular DNA virus belonging to the family *Circoviridae.* The virus is ubiquitously distributed in almost all pig populations, not only in PMWS pigs but also in clinically healthy ones. Therefore, it is hypothesized that other known or unknown factors may have a contribution to the development of PMWS. Aiming to find possible factors contributed to PMWS in piglets, our group tested lymph node samples that were collected from Swedish pigs with confirmed PMWS, by using GS-FLX 454 technology for large-scale sequencing of randomly amplified products [35]. Analysis of about 9,000 unique sequences obtained from the 454 run showed presence of PCV-2, *Torque Teno virus* genotypes 1 and 2 (TTV-1 and TTV-2), as well as a novel

parvovirus-like agent, termed as porcine boca-like virus in the lymph nodes from the diseased pigs. Based on this study, Blomström et al. screened 34 PMWS pigs and 24 animals without PMWS by PCR for the presence of PCV-2, TTV-1, TTV-2 and porcine boca-like virus [36]. PCV-2 was found in all PMWS pigs and in 80 % of pigs without PMWS. Presence of all three viruses TTV-1, TTV-2 and porcine boca-like virus was found in 71 % of the PMWS pigs and in 33 % pigs without PMWS. The exact role of these viruses, particularly the porcine boca-like virus in the development of PMWS requires further investigations.

Detection of Novel Astroviruses in a Neurological Disease of Minks, Termed Shaking Mink Syndrome

At our OIE Collaborating Centre viral metagenomic approaches, including the GS-FLX 454 technology was also used to investigate the cause of a neurological disease of minks termed *shaking mink syndrome*. The disease was first observed in farmed mink kits in Denmark in 2000 and subsequently in Sweden, Denmark and Finland in 2001, and in Denmark again in 2002 [37]. General virological investigations, including virus culture, negative-staining electron microscopy, immunoelectron microscopy, broad targeting PCR for herpesviruses, adenoviruses, pestiviruses and coronaviruses, and specific tests for six viral diseases, for three protozoa, for bacteria and intracerebral inoculation of neonatal mice were conducted but with negative results. It was postulated that it is likely that the disease was caused by a yet unidentified virus [37]. To investigate the cause, brain samples from experimental infection were prepared for nucleic acid extraction and random amplification and large-scale sequencing using GS-FLX 454 technology [38]. Analysis of the 454 sequencing data revealed eight sequence fragments similar to mink astrovirus. Based on the result, new primers were designed in order to determine the nucleotide sequences of the complete viral genome. The comparative analysis of complete genome sequences showed a similarity of 80.4 % to that of a mink astrovirus causing pre-weaning diarrhoea in mink. As the virus was not detected in healthy mink kits, we suppose an association between the astrovirus and the neurological disease of mink.

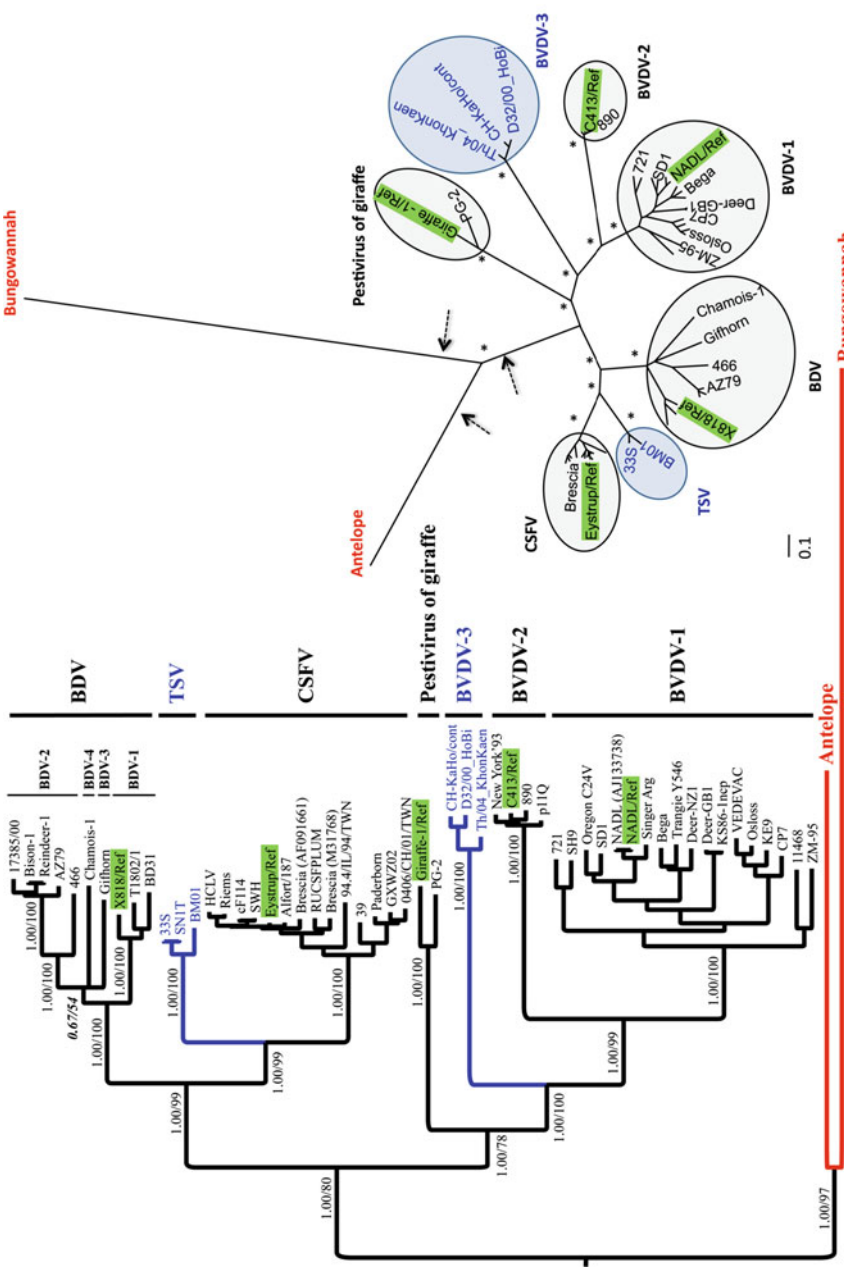
Genetic Characterization of Novel Bovine Pestiviruses in Biological Products, Such as Foetal Bovine Serum

Genome sequencing and subsequent phylogenetic analysis have been considered as important tools for the exact identification of the “unknown” or emerging new pathogens. From the initial discovery of the pestivirus D32/00_‘HoBi’ in a batch of contaminated FBS, it was unclear how this virus is related to the two recognized bovine pestivirus species: *bovine viral diarrhoea virus* 1 (BVDV-1) and *bovine viral diarrhoea virus* 2 (BVDV-2). The answer to this question has implication in veterinary diagnostic virology as well as in the BVD control and eradication program in various countries. Phylogenetic analysis of three genetic regions showed three different

relationships to both BVDV-1 and BVDV-2 [22]. To unequivocally solve the relationship, the pestivirus strain Th/04_KhonKaen was recovered from a serum sample of a naturally infected calf and the complete genome sequence was determined [39]. Phylogenetic analysis of complete genome sequences by Neighbor-joining, maximum likelihood, and the Bayesian approach, unanimously placed Th/04_KhonKaen in a single lineage, distinct from the established pestivirus species, and close to BVDV-1 and BVDV-2. A further evolutionary study proposed to term these newly described bovine pestiviruses as BVDV-3 (Fig. 36.2) [40].

Summary

During the last decades substantial progress has been made in veterinary diagnostic virology at our laboratories at the National Veterinary Institute (SVA) and the Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden. As the *World Organization for Animal Health (OIE) Collaborating Centre for the Biotechnology-Based Diagnosis of Infectious Diseases in Veterinary Medicine*, we have developed and tested a wide range of novel molecular diagnostic methods. Many of these novel assays provided powerful novel tools for the improved detection of viruses in veterinary and human medical virology. A wide range of the novel molecular diagnostic methods has been internationally compared in ring tests and validated. In order to illustrate this trend of development, several examples are summarized in this chapter. For molecular methods, upstream *nucleic acid extraction* is crucial for the success of the downstream diagnostic tests. We came to the conclusion that the *real-time PCR* principle, using different chemistries, such as TaqMan and PriProET, provides very reliable, highly sensitive and specific novel diagnostic tools for the direct detection of a wide range of pathogens, as it is demonstrated here with several examples. Simultaneously, the real-time PCR technology allows the development of *novel DIVA tests*, which are highly required for the improved control of infectious diseases, using marker vaccines and accompanied diagnostic packages. In parallel, high-throughput *suspension microarray technologies* enable the simultaneous detection and identification of multiple pathogens in single test platforms. The liquid-phase microarray platforms, such as Luminex panels, are accelerating the detection of emerging animal viruses and zoonotic, in particular, the water- and foodborne pathogens. *Proximity ligation* assay has emerged as a novel method for the highly sensitive and specific detection of the viral proteins. *Viral metagenomics and large-scale genome sequencing* establish powerful tools for the detection of "unknown" viruses, as well for the identification of emerging and re-emerging pathogens. These novel approaches strongly support the investigation of disease complexes and/or emerging novel disease scenarios in veterinary diagnostic virology, with regard to diseases in domestic animals and in wildlife, with special regard to zoonotic infections, by following the principles of "One World One Health."


Bungowannah
Pestivirus of giraffe
Giraffe -1/Ref
PG-2
TN/04_Khonkaen
CH-KaHo/cont
D32/00_HoBi
BVDV-3
C413/Ref
890
BVDV-2
721
SD1
NADL/Ref
Bega
Deer-GB1
CP7
Osloss77
ZM-95
BVDV-1
Chamois-1
Gifhorn
466
AZ79
X818/Ref
BM01
33S
Brescia
Eystrup/Ref
CSFV
TSV
BDV
Antelope
0.1
BDV
TSV
CSFV
Pestivirus of giraffe
BVDV-3
BVDV-2
BVDV-1
BDV-2
BDV-4
BDV-3
BDV-1
17385/00
Bison-1
Reindeer-1
AZ79
466
Chamois-1
Gifhorn
X818/Ref
T1802/1
BD31
33S
SN1T
BM01
HCLV
Riems
cF114
SWH
Eystrup/Ref
Alfort/187
Brescia (AF091661)
RUCSFPLUM
Brescia (M31768)
94.4/IL/94/TWN
39
Paderborn
GXWZ02
0406/CH01/TWN
Giraffe-1/Ref
PG-2
CH-KaHo/cont
D32/00_HoBi
Th/04_KhonKaen
New York'93
C413/Ref
890
p11Q
721
SH9
Oregon C24V
SD1
NADL (AJ133738)
NADL/Ref
Singer Arg
Bega
Trangie Y546
Deer-NZ1
Deer-GB1
KS86-1ncp
VEDEVAC
Osloss
KE9
CP7
11468
ZM-95
Antelope
Bungowannah
0.3
1.00/100
1.00/100
0.67/54
1.00/100
1.00/100
1.00/100
1.00/99
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1.00/100
1.00/78
1.00/100
1.00/100
1.00/99
1.00/100
1.00/80
1.00/97

Fig. 36.2 Phylogenetic analysis of pestiviruses by maximum likelihood and Bayesian approach. The molecular dataset contains 56 sampled pestiviruses and 2,089 characters, comprising the 5′UTR, N^{pro} and E2 gene regions. PHYML (v2.4.4) was used for phylogeny inference according to maximum likelihood criterion. MrBayes 3.1 was used for Bayesian analysis. This is a representative consensus tree: mid-point rooted (*left*) showing all sampled pestiviruses and their relationships, and unrooted (*right*). The reference sequences are highlighted in *green*. The new species proposed in this study are in *blue* (BVDV-3 and TSV) and in *red* (Antelope and Bungowannah). The numbers at a node are posterior probability (*left*) and percentage of 1,000 bootstrapping replicates (*right*). A "*" indicates strong statistical support for a node by a posterior probability value of 0.99–1.00 and by a bootstrap value of 78–100 %. The scale bar represents changes per site. The *arrows* show the probable placements of the root for the given unrooted network. Reprint from ref [40], with permission. Copyright © 2009, Elsevier

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References

1. Belák S, Thorén P (2008) Validation and quality control of polymerase chain methods used for the diagnosis of infectious diseases. Office International desEpizooties (OIE), manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), 6th edn. pp 46–55 (Chapter I.1.5). http://www.oie.int/eng/normes/mmanual/2008/pdf/1.1.05._VALID_PCR.pdf
2. Belák S, Thorén P, LeBlanc N, Viljoen G (2009) Advances in viral disease diagnostic and molecular epidemiological techniques. Exp Rev Mol Diagn 9:367–381
3. Tuan MA, Hai NH (2009) DNA enrichment by functionalized magnetic nanoparticles for on-site and fast detection of virus in biomedical application. J Phys: Conf Ser 187:012059
4. Rasmussen TB, Uttenthal A, Hakhverdyan M et al (2009) Evaluation of automated nucleic acid extraction methods for virus detection in a multiCentre comparative trial. J Virol Methods 155:87–90
5. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994
6. Belák S (2007) Molecular diagnosis of viral diseases, present trends and future aspects: a view from the OIE Collaborating Centre for the application of polymerase chain reaction methods for diagnosis of viral diseases in veterinary medicine. Vaccine 25:5444–5452
7. McKillen J, McMenamy M, Reid SM et al (2011) Pan-serotypic detection of foot and mouth disease virus using a minor-groove binder probe reverse transcription polymerase chain reaction assay. J Virol Methods 174:117–119
8. Zhang XJ, Sun Y, Liu L, Belák S, Qiu HJ (2010) Validation of a loop-mediated isothermal amplification assay for visualised detection of wild-type classical swine fever virus. J Virol Methods 167:74–78
9. Zhang XJ, Han QY, Sun Y, Belák S, Liu L, Qiu HJ (2011) Development of a loop-mediated isothermal amplification for visual detection of the HCLV vaccine against classical swine fever in China. J Virol Methods 171:200–205
10. Blomström AL, Hakhverdyan M, Reid SM, Dukes JP, King DP, Belák S, Berg M (2008) A one-step reverse transcriptase loop-mediated isothermal amplification assay for simple and rapid detection of swine vesicular disease virus. J Virol Methods 147:188–193
11. Reimann I, Depner K, Trapp S, Beer M (2004) An avirulent chimeric pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus. Virology 322:143–157
12. Koenig P, Lange E, Reimann I, Beer M (2007) CP7_E2alf: a safe and efficient marker vaccine strain for oral immunisation of wild boar against classical swine fever virus (CSFV). Vaccine 25:3391–3399
13. Liu L, Hoffmann B, Baule C, Beer M, Belák S, Widén F (2009) Two real-time RT-PCR assays of classical swine fever virus, developed for the genetic differentiation of naturally infected from vaccinated wild boars. J Virol Methods 159:131–133
14. Leifer I, Depner K, Blome S et al (2009) Differentiation of C-strain "Riems" or CP7_E2alf vaccinated animals from animals infected by classical swine fever virus field strains using real-time RT-PCR. J Virol Methods 158:114–122

15. Leifer I, Everett H, Hoffmann B et al (2010) Escape of classical swine fever C-strain vaccine virus from detection by C-strain specific real-time RT-PCR caused by a point mutation in the primer-binding site. J Virol Methods 166:98–100
16. Liu L, Xia H, Belák S, Widén F (2009) Development of a primer-probe energy transfer real-time PCR assay for improved detection of classical swine fever virus. J Virol Methods 160:69–73
17. Rasmussen T, Uttenthal Å, de Stricker K, Belák S, Storgaard T (2003) Development of a novel quantitative real-time PCR assay for the simultaneous detection of all serotypes of foot-and-mouth disease virus. Arch Virol 148:2005–2021
18. Hakhverdyan M, Rasmussen T, Thorén T, Uttenthal Å, Belák S (2006) Development of a real-time PCR assay based on primer-probe energy transfer for the detection of swine vesicular disease virus. Arch Virol 151:2365–2376
19. Leblanc N, Rasmussen TB, Fernández J et al (2010) Development of a real-time RT-PCR assay based on primer-probe energy transfer for the detection of all serotypes of bluetongue virus. J Virol Methods 167:165–171
20. Zhang XJ, Xia H, Everett H et al (2010) Evaluation of a primer-probe energy transfer real-time PCR assay for detection of classical swine fever virus. J Virol Methods 168:259–261
21. Liu L, Xia H, Everett H et al (2011) A generic real-time TaqMan assay for specific detection of lapinized Chinese vaccines against classical swine fever. J Virol Methods 175:170–174
22. Schirrmeier H, Strebelow G, Depner K, Hoffmann B, Beer M (2004) Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. J Gen Virol 85:3647–3652
23. Liu L, Xia H, Belák S, Baule C (2008) A TaqMan real-time RT-PCR assay for selective detection of atypical bovine pestiviruses in clinical samples and biological products. J Virol Methods 154:82–85
24. Liu L, Xia H, Baule C, Belák S (2009) Maximum likelihood and Bayesian analyses of a combined nucleotide sequence dataset for genetic characterization of a novel pestivirus, SVA/cont-08. Arch Virol 154:1111–1116
25. Taylor LH, Latham SM, Woolhouse ME (2001) Risk factors for human disease emergence. Philos Trans R Soc Lond B: Biol Sci 356:983–989
26. Escutenaire S, Mohamed N, Isaksson M et al (2007) SYBR Green real-time reverse transcription-polymerase chain reaction assay for the generic detection of coronaviruses. Arch Virol 152:41–58
27. Muradrasoli S, Mohamed N, Hornyák A et al (2009) Broadly targeted multiprobe QPCR for detection of coronaviruses: coronavirus is common among mallard ducks (Anas platyrhynchos). J Virol Methods 159:277–287
28. Muradrasoli S, Bálint A, Wahlgren J et al (2010) Prevalence and phylogenetic relationship of coronaviruses in wild birds from the Bering Strait Area (Beringia). PLoS One 5:e13640
29. Gyarmati P, Mohammed N, Norder H, Blomberg J, Belák S, Widén F (2007) Universal detection of hepatitis E virus by two real-time PCR assays: TaqMan and Primer-Probe Energy Transfer. J Virol Methods 146:226–235
30. Widén F, Sundqvist L, Matyi-Toth A et al (2011) Molecular epidemiology of hepatitis E virus in humans, pigs and wild boars in Sweden. Epidemiol Infect 139:361–371
31. Gullberg M, Gústafsdóttir SM, Schallmeiner E et al (2004) Cytokine detection by antibody-based proximity ligation. Proc Natl Acad Sci U S A 101:8420–8424
32. Schlingemann J, Leijon M, Yacoub A et al (2010) Novel means of viral antigen identification: improved detection of avian influenza viruses by proximity ligation. J Virol Methods 163:116–122
33. LeBlanc N, Leijon M, Jobs M, Blomberg J, Belák S (2010) A novel combination of TaqMan RT-PCR and a suspension microarray assay for the detection and species identification of pestiviruses. Vet Microbiol 142:81–86
34. Xia H, Liu L, Nordengrahn A et al (2010) A microsphere-based immunoassay for rapid and sensitive detection of bovine viral diarrhoea virus antibodies. J Virol Methods 168:18–21
35. Blomström AL, Belák S, Fossum C et al (2009) Detection of a novel porcine boca-like virus in the background of porcine circovirus type 2 induced postweaning multisystemic wasting syndrome. Virus Res 146:125–129

36. Blomström AL, Belák S, Fossum C, Fuxler L, Wallgren P, Berg M (2010) Studies of porcine circovirus type 2, porcine boca-like virus and torque teno virus indicate the presence of multiple viral infections in postweaning multisystemic wasting syndrome pigs. Virus Res 152:59–64
37. Gavier-Widén D, Bröjer C, Dietz HH et al (2004) Investigations into shaking mink syndrome: an encephalomyelitis of unknown cause in farmed mink (Mustela vison) kits in Scandinavia. J Vet Diagn Invest 16:305–312
38. Blomström AL, Widén F, Hammer AS, Belák S, Berg M (2010) Detection of a novel astrovirus in brain tissue of mink suffering from shaking mink syndrome by use of viral metagenomics. J Clin Microbiol 48:4392–4396
39. Liu L, Kampa J, Belák S, Baule C (2009) Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen. Vet Microbiol 138:62–68
40. Liu L, Xia H, Wahlberg N, Belák S, Baule C (2009) Phylogeny, classification and evolutionary insights into pestiviruses. Virology 385:351–357

Chapter 37
Deep Sequencing: Technical Advances and Clinical Microbiology Applications

Chunlin Wang

Introduction

The ability to generate sequence information for millions of DNA fragments in a short time cheaply by next-generation sequencing (NGS) technologies has enabled numerous projects covering diverse application areas that include de novo sequencing, genome resequencing, transcriptome sequencing, chip-sequencing, and exome sequencing. Several sequencing platforms are available in the market and many more are being developed at various stages [1]. Among them, the Roche/454 Genome Sequencer (GS) has the ability to sequence 400–600 million base pairs per run with 400–500 bp read lengths, and the Illumina HiSeq 2000 (HiSeq in brief) has the capacity to generate up to 200 billion base pair per run with 100 bp read lengths. The long reads by the GS and the high yield by the HiSeq make these two platforms stand out among various NGS techniques in the current NGS market.

The Roche/454 GS is the first commercially available NGS instrument, which integrates the emulsion PCR (emPCR) for template preparation and array-based pyrosequencing for sequence detection [2]. A library of DNA fragments of 300–800 bp prepared from genomic DNA are ligated to adaptor which contains universal priming sites. After denaturation, single-strand template DNA (sstDNA) fragments, beads with adaptor complementary to one adaptor on sstDNA on their surface, amplification reagents, and oil are mixed together under conditions that favor one DNA molecule per bead and emulsified through vigorous shaking to create zillions of water-in-oil vesicles. The water-in-oil vesicles serve as micro-bioreactors for the PCR reaction. After the successful amplification and enrichment of emPCR beads, clonally amplified DNA fragments are immobilized onto the surface of each bead. Single-stranded template DNA library beads are deposited into individual

C. Wang (✉)
Stanford Genome Technology Center, Department of Biochemistry,
Stanford University, 855 S. California Ave, Palo Alto, CA 94304, USA
e-mail: wangcl@stanford.edu

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PicoTiterPlate (PTP) wells and the loaded PTP device is placed into the GS instrument for sequencing. The four nucleotides (A, C, T, G) are added sequentially in a fixed order across the PTP device during a sequencing run. When a nucleotide complementary to the template strand is added into a well, the polymerase extends the existing DNA strand by adding nucleotide(s) and releases pyrophosphate molecule(s). Through a series of enzymatic reactions converting pyrophosphate into light where light intensity is proportional to the amount of released pyrophosphate, addition of one (or more) nucleotide(s) generates a light signal that is recorded by the CCD camera in the instrument and interpreted by a software to generate sequence information.

The Illumina technology uses solid-phase amplification to achieve clonal amplification of sequencing templates on the surface of a glass slide where high-density forward and reverse primers are covalently attached [3]. Adaptor-modified single-stranded template DNA is hybridized to the anchored oligonucleotides. After the successful amplification, a single DNA molecule is converted into a clonally amplified cluster consisting of approximately 1,000–2,000 molecules, followed by chemical reaction to release free ends to which a universal sequencing primer can be hybridized to initiate the sequencing reaction. The Illumina HiSeq uses the cyclic reversible termination method, which comprises nucleotide incorporation, fluorescence imaging, and cleavage steps. The Illumina sequencing chemistry uses four different fluorescent dye-labeled nucleotides and the 3′-OH group of those nucleotides is modified to ensure that only a single nucleotide is incorporated in each sequencing cycle. In each sequencing cycle, all four different modified nucleotides are loaded. An imaging step follows each nucleotide incorporation step to capture the incorporated nucleotide at each cluster. The fluorescent group and the 3′-OH blocking group are removed after imaging for the next base incorporation cycle.

The large volume of sequencing data from NGS instruments demands customized approaches to extract biologically meaningful information. This includes filtering noise, analyzing data, and extracting knowledge. Here I introduce our practices analyzing NGS data on the context of detecting low-frequency viral drug-resistant mutants and profiling sequence composition of samples.

Prolonged antiviral drug treatment of infections with HIV, HBV, or HCV usually leads to the emergence of resistant viruses and consequent treatment failure because those viral RNA-dependent DNA or RNA polymerases have no 3′–5′ proofreading activity and are error-prone and virus replication cycle is much shorter. The availability of genotypic information on the viral drug targets allows doctors to adjust treatment regiment and to select a new potent drug combination after failure of antiviral therapy. PCR-Sanger sequencing is the conventional approach to gather genotypic information on the viral drug targets, where the viral DNA fragments encoding drug-targeted enzymes such as polymerase or proteinase are amplified by PCR and sequenced by Sanger sequencing. Due to an associated replication or competitive disadvantage compared to the wild-type virus, newly emerged drug-resistant clone only represents a small proportion of the total viral load. Traditional Sanger sequencing is insensitive for minor alleles in a heterogeneous mixture of mutant and wild-type sequences with detection limit about 10 %. To detect those

minor mutants under 10 % frequencies, traditionally it requires cloning PCR product and sequencing hundreds of bacterial clones. According to Poisson distribution, it needs to sequence about 300 clones to detect mutants at 1 % frequency with 95 % confidence. The labor-intensive feature of this approach limits its usage in academic research settings. The high-throughput in vitro cloning and sequencing techniques offered by NGS techniques such as GS and HiSeq offer a significant advantage in time, cost, and labor over the conventional PCR-Sanger sequencing approach for detecting low-frequency mutants.

NGS techniques could generate a large amount of sequence data in a week or two. However, those sequencing reads are noisier with errors than those generated by Sanger sequencing. For instance, pyrosequencing—the underlying sequence detection technique in Roche/454 GS—cannot detect the length of long(>3)-stretch homopolymers accurately and results in higher error rates in homopolymeric and its surrounding regions than other regions [4]. The Illumina sequencers have more substitution-type miscalls than indel-type miscalls, while the Roche/454 sequencers have more indel-type miscalls than substitution-type miscalls. For Illumina sequencers, miscalls are more frequently found in the GC-rich regions and A to C and C to G miscalls are observed more often than other substitution errors [5]. The insertion/deletion of one or two bases change the frame of coding region, which is lethal to viruses. Therefore, it is much easier to distinguish indel-type miscalls from actual indel mutations selected under the drug pressure than substitution miscalls from actual substitution mutations. Substitution miscalls resemble with actual mutations in many aspects and more sophisticated statistical procedures are needed to identify them. From those points, it appears that Roche/454 sequencer is more suitable for rare mutation detection than the Illumina one in the meantime. The following sections describe the data analysis procedures for detecting low-level viral drug-resistant mutants with the Roche/454 technique.

Analyze Pyrosequencing Data for Detecting Low-Level Variants

Map Pyrosequencing Reads onto Reference Sequences

The output from the Roche/454 sequencing platform includes a quality score for every position in a read. Quality score in the Roche/454 sequencing platform is originally designed to measure the confidence that the homopolymer length at that position is correct [2]; however, we found that the quality score of a position is also a good measurement of confidence that the correct base is called at any position, as with a traditional Phred score [6]. The Phred-equivalent quality value (q) is given by the log-transformed probability p of the base call being incorrect according to the equation $q = -10 \times \log_{10} p$. Thus a base call with a quality value of q will have a probability of $10^{-q/10}$ of being incorrect. To apply this algorithm, we transformed the Phred scores into reliability weights $W = 1.0 - 10^{-q/10}$, and applied these weights in the selectively weighted Smith–Waterman (SW2) algorithm (see Fig. 37.1).

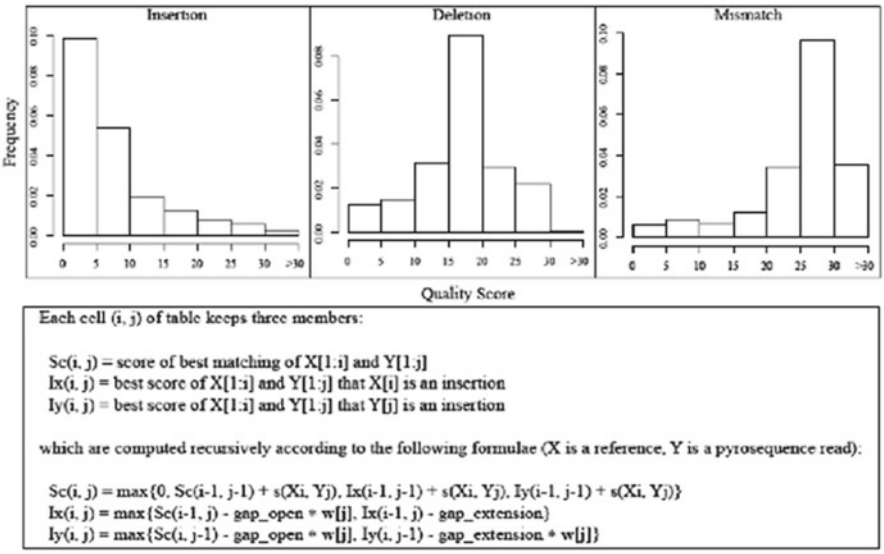

Fig. 37.1 The *upper panel* shows score distributions for sequencing errors in three categories: insertion, deletion (the score of the first upstream base), and mismatch. These distributions are based on pyrosequencing data from two DNA sources: PCR product of 1,548 bp HBV genome sequence amplified from a plasmid and an entire (3,221 bp) HBV genome sequence excised from the same plasmid. The PCR reaction was performed with the Expand High Fidelity PCR plus system (Roche Diagnostics Corporation). The pyrosequencing reactions were carried out using the GS FLX system in the 454 Life Sciences Company. Sequencing errors are determined as difference between mapped pyrosequencing reads and corresponding reference sequence using the program PyroMap described in this manuscript (note that we observed similar distributions for the corresponding categories for pyrosequencing reads generated with the GS 20 system). The lower panel describes the SW2 algorithm. *X* represents a reference sequence and *Y* denotes a pyrosequencing read. To help placing gaps in right positions, gap_open in the pyrosequencing read is weighted using score from its preceding base (Ix), and both gap_open and gap_extension in the reference sequence are weighted using score from their corresponding bases in the pyrosequencing read (Iy)

In our previous study, we developed an asymmetric Smith–Waterman (ASW) algorithm, which, basically, weights everything except gaps occurring in pyrosequencing reads, using quality score to map pyrosequencing reads onto reference sequences [4]. Although the ASW algorithm outperforms both BLAST [7] and the conventional Smith–Waterman algorithm [8], our further studies found that quality scores for bases preceding to deletion errors are much lower than average while quality scores for substitution errors are close to the average (see Fig. 37.1). To take in account the information, we designed the SW2 algorithm which weights the penalty for opening a gap in reads using the score from the preceding base and weights the penalty for both opening a gap and extending a gap in reference sequences (see Fig. 37.1). As weight ($W = 1.0 - 10^{-q/10}$) is always <1, weighting generally lowers the penalty and encourages opening or extending gaps, which are largely caused by sequencing errors. As scores for bases in other regions are not lower than average, weighting will not introduce artificial gaps in those regions. We found that the

sizes of deletion (and insertion) errors follow the geometric distribution (data not shown) and the chance of missing more than one nucleotide due to sequencing errors is extremely low, so we did not weight gap-extension at reads (Ix in Fig. 37.1).

We did not weight mismatches to avoid mis-pairing due to low quality score of a particular base. We compared the performance of four algorithms: SW2, ASW, SW, and BLAST by aligning Roche/454 GS20 reads for HIV-1 subtype B clone sequences to a more distantly related (HIV-1 subtype C) sequence as we did in an earlier study [4]. The SW2 algorithm has a slightly lower error rate than the three algorithms.

The computational complexity of the Smith–Waterman algorithm is $O(n\,m)$ where **n** is the length of a reference sequence and **m** is the length of a pyrosequencing read. The average read length from the current Roche/454 sequencing platform (GS FLX) is about 250, while the length of reference sequences could be several orders of magnitude longer. We implemented a seeding strategy to speed up the program. Briefly, the reference sequences are preprocessed to build a large lookup table of *k*-mers called "seeds" in the main memory for determining the positions of seeds in the long sequences efficiently. The query sequence (pyrosequencing read) is then scanned to associate individual seeds in the query with their positions in the reference, thereby listing candidate regions in the reference where the query and reference are likely similar. Finally, we attempt to compute the precise alignments with the SW2 algorithm between the query and each of candidate regions in the reference sequence. Moreover, we implemented a parallel version of the PyroMap program using the library pyMPI (http://pympi.sourceforge.net/), which could speed up the overall aligning procedure linearly in proportion to the number of processors.

Filter Sequencing Artifacts

Due to imperfection of sequencing technique, variants (mismatches between reads and consensus) of varying frequencies were observed at almost every position. It is critical to filter out those variants to remove sequencing miscalls and lower the number of false positives to make the NGS technique really useful for clinical applications.

The Poisson distribution is commonly encountered in biology in describing random occurrences of rare events in time when the events are independent. In other words, the occurrence of an event is assumed to have no effect on the probability of a second occurrence of the same event. The sequencing miscalls observed in pyrosequencing reads are rare in comparison to the correct base-calling and approximately independent. Although miscalls within a few bases in the same reads might be related, miscalls far away and those in separate reads are absolutely independent. With this consideration, we chose the Poisson distribution to model the distribution of sequencing miscalls.

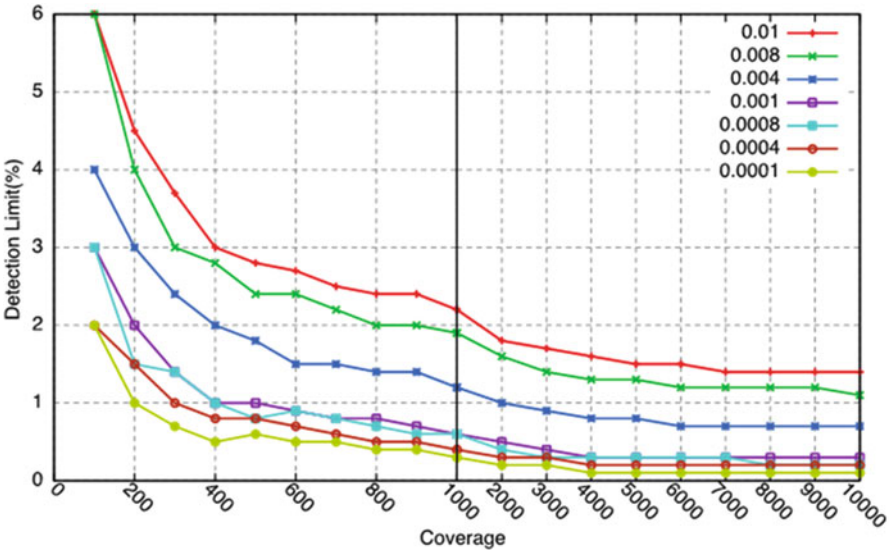

Fig. 37.2 Relationship between the number of required ultra-deep pyrosequencing reads per position (coverage) and the detection limit of the frequency of a minority sequence variant (detection limit). *Different lines* represent different error rates. The excess number of reads required to detect minor variants is a result of the Poisson model for handling potential sequencing errors. It is clear that more reads are needed to detect the variants of the same frequency in regions with higher error rate

In order to use the Poisson distribution to model miscall, we first need to estimate pyrosequencing error per base. We sequenced some plasmid DNA fragments with known sequences and counted mismatches between mapped bases and their references. As we described earlier, the pyrosequencing technique tends to make more miscalls in homopolymeric regions than non-homopolymeric regions. We classified miscalls into two categories, those in homopolymeric regions and those in non-homopolymeric regions, and computed the error rate for each category.

With the availability of pyrosequencing error rates, we can compute the probability of miscalls observed n times for each position with the formula $p = ((e^{\lambda} \cdot \lambda^{k}) / k!)$, where λ is the expected number of errors given N reads and is computed by $\lambda = N \cdot \mu$ and μ is the error rate per site estimated from the sequences of control plasmid clones. To classify whether a minor variant with n occurrences in N reads is sequencing miscall or actual mutations in a viral genome, we calculated the probability that such a variant would occur n or more times if it were a sequencing miscall, using the following formula: $p = 1 - \sum_{k=0}^{n-1} ((e^{\lambda} \cdot \lambda^{k}) / k!)$. Variants that yielded $P < 0.001$ were considered highly unlikely to be sequencing miscalls and reported as real mutants.

Using the Poisson model, we can also estimate the minimum coverage needed for the low-level variants of a particular frequency. Figure 37.2 displays relationship between the number of required ultra-deep pyrosequencing reads per position

(coverage) and the detection limit of the frequency of a minority sequence variant (detection limit) at various levels of error rates. The higher the error rate of a region, the higher the coverage needed to detect the variants of the same level of frequency. For detecting extremely rare variants of the frequency lower than the error rate, excess number of sequencing reads are needed to obtain confidence.

Analyze NGS Data for Profiling Sequence Content of Unknown Samples

There is always great interest in detecting new viral pathogens. Conventional diagnostic techniques include detection of virus-specific antigens and viral-specific sequences. With the advent of more powerful and economical DNA sequencing technologies, gene discovery and characterization is transitioning from single-organism studies to revealing the potential biotechnology applications embedded in communities of microbial genomes, or metagenomes. Metagenomics has given us a powerful new tool for finding virus sequences from a specimen. However, data management and sequence analysis of millions, even up to billions, of sequences generated by each instrument run pose a great challenge to field experts who used to deal with a few sequences. To address this challenge, we have developed a viral *m*etagenomic *s*equence *m*anagement *s*ystem (MS2). MS2 includes sequence analysis pipeline to compare reads to all known sequences from public database and to classify reads based on their best-matching hits. MS2 also includes an effective data management system to ensure field experts to use, preserve, and access the information.

Filter/Preprocess Module

Regions with low-complexity sequence have an unusual composition that can create problems in sequence similarity searching as it can cause artificial hits. In the filter/preprocess module, we have implemented a simple low-complex sequence detecting procedure using the Bio.SeqUtils.lcc module from the Biopython package. The Bio.SeqUtils.lcc module calculates the Shannon entropy [9] for a sequence. The filtering procedure in this module puts aside all those low-complex sequences before moving to the de-multiplexing procedure.

Although the NGS techniques have brought down the per-base cost dramatically, the reagent cost of each instrument run is very high and around $10,000 for Roche/454 FLX system and $25,000 for Illumina HiSeq2000 system. Multiplexing is a widely adopted strategy to lower the cost where many samples are pooled and sequenced together. To do that, each sample was amplified with PCR primers, which are tagged a short stretch of nucleotides (barcode). After amplification, the PCR product is tagged with barcode unique to each sample. After sequencing, reads are

needed to separate according to the barcode sequence. To de-multiplex reads, we identify regions in each reads matching to input barcode sequences and keep sequences between two nearest barcodes. To make reads cleaner, we trimmed eight nucleotides from each end of each remaining fragment to remove the random fraction of PCR primer.

Sequence Exclusion Module

In comparison to prokaryotic or eukaryotic genome, viral genomes are much smaller. Although their numbers present in a sample could be high, their overall biomass is small. Even in a metagenomic project setting where millions or even billions of sequences in a complex genetic material pool are randomly amplified and sequenced in a high-throughput experiment, only a very small portion of overall sequences are similar to viral sequences. Depending on the sample, the majority of the remaining sequences are either from eukaryotic genomes or prokaryotic genomes. Those off-target sequences pose challenge to both assembly module and sequence comparison module. This module is designed to filter out off-target eukaryotic and prokaryotic sequences. To speed up the filtering procedure, we run the blastn with large word size. After sequence comparison, we eliminate sequences where alignment region is more than 80 % of reads and identity is more than 90 %.

Assembly Module

Sequences passed through the sequence exclusion module are subjected to assembly. For Roche/454 sequencing data, de novo assembly is performed with the program mira (http://chevreux.org/projects_mira.html). For the Illumina sequencing data, de novo assembly is carried out using the program SOAPdenovo (http://soap.genomics.org.cn/soapdenovo.html). After assembly, we compute the coverage for each contig. Any contig or singlet shorter than 100 bp is eliminated from the following analysis.

Sequence Comparison Module

Sequences from the assembly module are compared with NCBI nonredundant nucleotide (nt) or protein (nr) using the program BLASTN and BLASTP, respectively. In addition, sequences are compared to the curated viral-specific version of nonredundant nucleotide (vnt) or protein (vnr) with more sensitive parameters using the program BLASTN, BLASTP, and TBLASTX. Finally, sequences are compared with the internal accumulated sequences database using the program BLASTN.

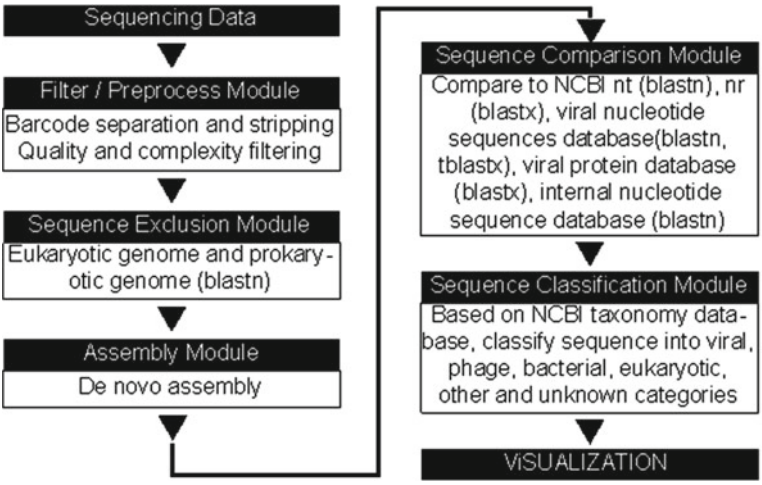

Fig. 37.3 The flowchart of MS2

Due to the large number of query sequences and the large size of database, it takes months or even years to complete sequence comparison in a single-CPU desktop. The current nt database is around 40 GB and the nr database is around 6 GB. The BLAST program runs faster when the entire database is loaded into physical memory. We normally divided the nt database into 20 smaller ones of equal size and divided the nr database into ten smaller ones of equal size. We then compare each query sequences to every small sub-database and merge all the results together. To speed up the entire procedure, the sequence comparison module is normally carried out in the computer-cluster with several hundred CPUs.

The BLAST *E*-value is a function of the size of the effective search space, which is dependent on three factors: the number of sequences in the database, the total combined length of all sequences in the database, and the length of the query sequence [10]. In the BLAST search procedure, we specify the effective length of the database to a fixed larger number with the option of $-z$ to maintain the *E*-value comparable.

Sequence Classification Module

Each query sequence is classified into domain based on its best hit according to the flowchart in Fig. 37.3. If the *E*-value of the best hit is greater than 1e−5, the query sequence is labeled as unknown. Otherwise, the query sequence is labeled eukaryotic, prokaryotic, virus, phage, or other.

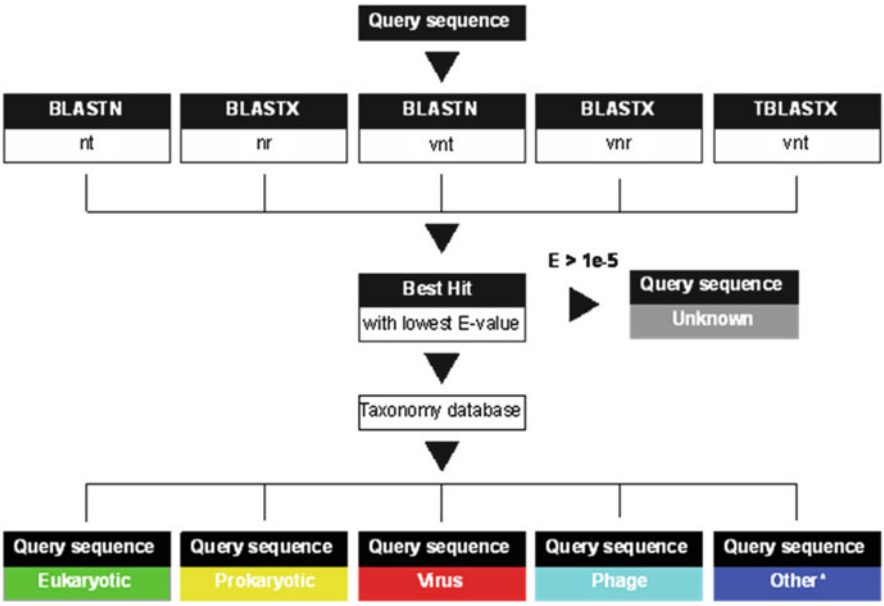

Fig. 37.4 The classification flowchart for query sequences. *Asterisk* indicates that the best-hit sequence is outside the four categories: eukaryotic, prokaryotic, virus, and phage according to the taxonomy database. It likely belongs to either synthetic sequence or Achaean sequence

Visualization

One key component of MS2 is to disseminate sequences and analysis results through a Web portal. In the Web portal, all sequences, and intermediate results such as sequence annotation and alignment, are stored in a centralized database. We also allow users to adjust parameter and some intensive computing on the fly, which needs some special tools and demands hardware. Both database and Web server are hosted in high-end machines and experimental biologists can access the results from regular desktop or laptop using popular browsers. After we started to develop this system 4 years ago, we have incorporated many suggestions from experimental biologists into both sequence analysis part and visualization part [11–16]. Their feedbacks keep improving this system.

Figure 37.5 displays a screen-shot of a Web page. Data are organized in a hierarchical fashion: study, sample, and sequence. In Fig. 37.5, pane A lists some studies we have done recently. Click one of them to get a list of samples (not shown). Click the sample name to get the list of sequences (Fig. 37.5, pane B). Each sequence name is highlighted with the color schema in Fig. 37.4. Click a sequence name to

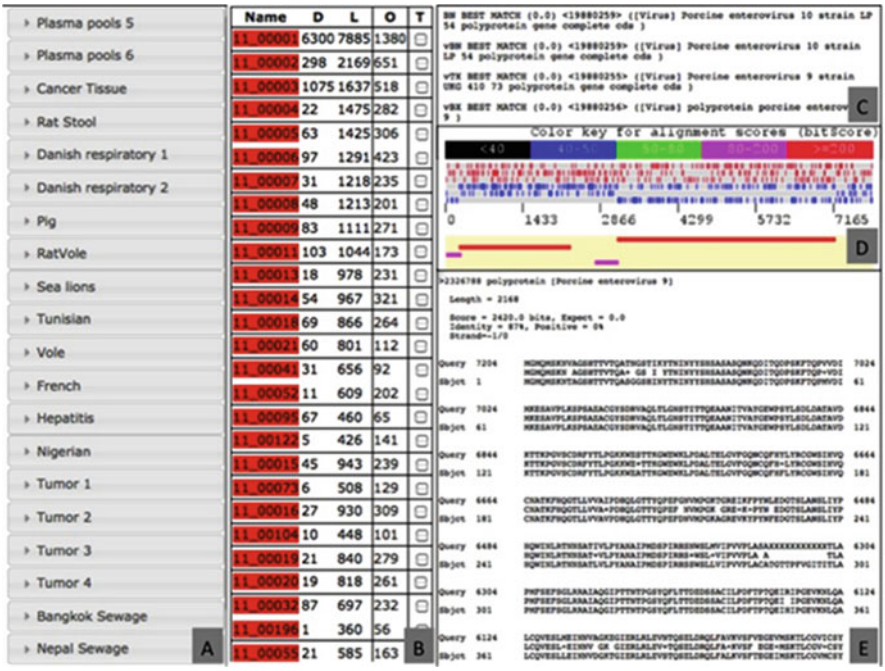

Fig. 37.5 Screen-shot of a Web page. **Pane A** lists studies; **pane B** lists sequences from one study where ID, coverage, and length of the longest open reading frame are listed from left to right; **pane C** lists classification reason for one sequence; **pane D** displays the alignment schematics and **pane E** shows the alignment of a sequence and its hit

get the reasoning for classification (Fig. 37.5, pane C) and a list of alignments for that particular sequence (Fig. 37.5, pane D, E).

In the Web portal, users can collaborate through commenting interesting sequences. Those comments will be shared with all users who are allowed to access those sequences.

To facilitate the effort to hunting new viruses, we organize all potential viral sequences from either one sample or one study in a hierarchical order according to their best hits' taxonomy information (Fig. 37.6). Most components in the hierarchical tree (Fig. 37.6) are clickable to reach more detail information.

Challenge of Data Analysis

Figure 37.7 displays the read and contig distribution based on 28 studies we conducted recently using the Roche/454 FLX system. It is interesting to look at the read/contig ratio for each category: virus 5.6, unknown 1.5, phage 6.5, other 3.6, eukaryotic 1.9, and bacteria 3.2. The ratios indicate the relative abundances of the species in samples. Phage and virus read/contig ratios top in the group suggest their

Fig. 37.6 Screen-shot of a vhit page displays the hierarchical organization of viral sequences according to their taxonomy order. *Inside brackets* list the number of contigs and *inside parentheses* list the number of raw reads

high abundance in a sample. This might be due to the special viral enrichment procedure where samples are passed through a 0.45-μm filter to remove bacterium-sized particles. The viral particles containing filtrate were digested with a mixture of DNases and RNase to remove unprotected nucleic acids (i.e., those not in viral capsids). Viral nucleic acids were then extracted. Nevertheless, due to their small biomass, only a small fraction of overall sequence reads and contigs are from viruses and phages.

Figure 37.7 shows that 37 % of contigs (from 23 % of reads) do not share statistically significant similarity with any sequences in the public database (*E*-value > 1e−5). The prevalence of unknown sequence reflects the fact that although

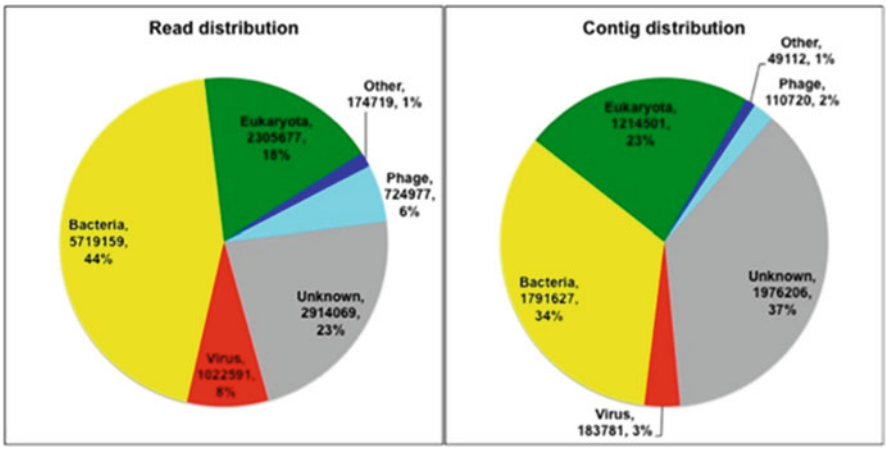

Fig. 37.7 Read and contig distribution from 28 studies done with Roche/454 FLX system without passing through the sequence exclusion module

the public NCBI database is large with millions, even billions of sequences, these databases are very redundant and extremely sparse in the sequence space. A lot of more sequences have not been recorded. In addition, the current sequence comparison method may not be able to detect remote similarity. Viruses typically evolve rapidly and gene sequence conservation is typically much less than that in cellular organisms. Therefore, there are opportunities to find novel viruses from those unknown sequences using methods beyond sequence similarity search.

Closing Remarks

The advance in NGS techniques in the past few years has impacted on many fields of biology science. However, most researchers are experimental experts and are likely to be overwhelmed by the amount of sequence data generated by the NGS platforms. Therefore, data analysis and management is essential for projects using the high-throughput sequencing approach. Here I just briefly touch our experience on sequence analysis and management for detecting rare mutants and profiling viral sequences. However, improvement in data analysis and management is needed to keep pace with the continuing development of the NGS technology. Understanding the underlying technique and the need of experimental experts is essential to the success of the computational tools.

References

1. Metzker ML (2010) Sequencing technologies—the next generation. Nat Rev Genet 11:31–46
2. Margulies M, Egholm M, Altman WE et al (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376–380
3. Bentley DR, Balasubramanian S, Swerdlow HP et al (2008) Accurate whole human genome sequencing using reversible terminator chemistry. Nature 456:53–59
4. Wang C, Mitsuya Y, Gharizadeh B, Ronaghi M, Shafer RW (2007) Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. Genome Res 17:1195–1201
5. Nakamura K, Oshima T, Morimoto T et al (2011) Sequence-specific error profile of Illumina sequencers. Nucleic Acids Res 39(13):e.90
6. Ewing B, Green P (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res 8:186–194
7. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
8. Smith TF, Waterman MS (1981) Identification of common molecular subsequences. J Mol Biol 147:195–197
9. Shannon CE (1951) Prediction and entropy of printed English. Bell Syst Tech J 30:14
10. Karlin S, Altschul SF (1990) Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. Proc Natl Acad Sci U S A 87:2264–2268
11. Li L, Victoria JG, Wang C et al (2010) Bat guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. J Virol 84:6955–6965
12. Victoria JG, Kapoor A, Li L et al (2009) Metagenomic analyses of viruses in stool samples from children with acute flaccid paralysis. J Virol 83:4642–4651
13. Victoria JG, Wang C, Jones MS et al (2010) Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol 84:6033–6040
14. Li L, Kapoor A, Slikas B et al (2010) Multiple diverse circoviruses infect farm animals and are commonly found in human and chimpanzee feces. J Virol 84:1674–1682
15. Li L, Victoria J, Kapoor A et al (2009) A novel picornavirus associated with gastroenteritis. J Virol 83:12002–12006
16. Shan T, Lan D, Li L et al (2011) Genomic characterization and high prevalence of bocaviruses in swine. PLoS One 6:e17292

Chapter 38
Detection of Viral RNA Splicing in Diagnostic Virology

Vladimir Majerciak and Zhi-Ming Zheng

Introduction

Diagnostic virology is to identify the etiologic cause of infection from patient's samples. In the past the diagnostic virology relied on three classical techniques to make a diagnosis of viral infection: (a) virus isolation by direct virus cultivation, (b) viral antigen detection, (c) indirect detection of virus-specific antibodies. While being important tools in the diagnostic virology today, these techniques are time-consuming and require specific tools such as cultivation media, cell or tissue cultures, antibodies, purified antigens. In the past decade the number of new molecular-based methods grew rapidly and gained more popularity in diagnostic labs. The core of these techniques constitutes of techniques based on nucleic acid detection by specific amplification, hybridization, and/or sequencing (reviewed in ref. [1]). The most nucleic acid-based diagnostic methods are simple, speed, sensitive and specific and thus meet the gold four-S-standard for their application in any diagnostic laboratories. The methods are simple and speed because only a specific primer pair and a PCR machine are needed in a lab setting and identification of a viral pathogen takes within few hours. They are sensitive and specific and require only a small amount of patients' materials to detect a specific nucleotide sequence region. In general, these techniques can be used to detect almost all types of viral pathogens and even to identify multiple viral pathogens or their variants at the same time. In this chapter we focus on detection of viral RNA splicing as a new tool for diagnostic virology.

V. Majerciak • Z.-M. Zheng (✉)
Tumor Virus RNA Biology Section, HIV and AIDS Malignancy Branch,
Center for Cancer Research, National Cancer Institute, National Institutes of Health,
10 Center Dr., Rm. 10/6 N106, Bethesda, MD 20892-1868, USA
e-mail: zhengt@exchange.nih.gov

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Principle of RNA Splicing

Definition of RNA Splicing

RNA splicing was discovered more than 30 years ago by Berget [2] and Chow [3] by mapping adenovirus transcription and identification of intervening sequences (introns) in type 2 adenovirus primary transcripts. Subsequently, RNA splicing is recognized as an essential nuclear event for mammalian gene expression and for virus multiplication of almost all DNA viruses and some RNA viruses. The majority of mammalian genes consist of multiple segments called exons which separated by noncoding or intervening sequences named introns. Genes which are composed of exons and introns are "split" genes. After transcription a nascent or primary transcript (pre-mRNA) contains both exons and introns. The introns are removed from the pre-mRNA by a molecular process called "RNA splicing" resulting in production of spliced mature mRNA. RNA splicing takes place both in coding as well as in non-coding primary transcripts. RNA splicing is a posttranscriptional event; however, recent studies showed that it often occurs cotranscriptionally [4, 5]. Only those transcripts which are fully processed are eventually exportable from the nucleus to the cytoplasm for protein synthesis.

Molecular Mechanism of RNA Splicing

All introns are defined by three *cis*-elements: a 5′ splice site (donor site), a branch point, and a 3′ splice site (acceptor site) with a polypyrimidine track immediately upstream (Fig. 38.1a). These *cis*-elements allow cellular splicing machinery to recognize and remove the intron from pre-mRNA. Most of mammalian introns start with GU dinucleotide on its 5′ end and a AG dinucleotide on its 3′ end ("GU-AG" introns). The GU-AG pairs are conserved sequences defining exon–intron boundaries. Introns with an AU on its 5′ end and an AC on its 3′ end are rare and this set of the introns are AU-AC introns [6, 7]. The presence of splice sites is not sufficient for intron definition. All introns must contain an additional element called "branch point" located 20–50 nts upstream from 3′ splice site with consensus sequence CU(A/C)A(C/U) where A is a most conserved base. Sequence between the branch point and acceptor site is a run of 15–40 pyrimidines (mostly U) and is referred as a "polypyrimidine track."

Fig. 38.1 (continued) two exons (*black*) is removed by two transesterification reactions. First the intron is recognized by cellular splicing machinery via splicing factors binding to intron specific sequences as described in (**a**). Splicing factors carry out the 1st transesterification reaction between the branch point and the 5′ donor site, resulting in an RNA cleavage at the 5′ donor site and releasing exon 1 and formation of a lariat intermediate. Subsequently, the free 3′ end OH group of exon 1 attacks the 5′ end phosphate of exon 2 and joints with the 5′ end of exon 2 via the 2nd transesterification reaction to form a mature mRNA. Intron is removed in the form of lariat structure and quickly degraded

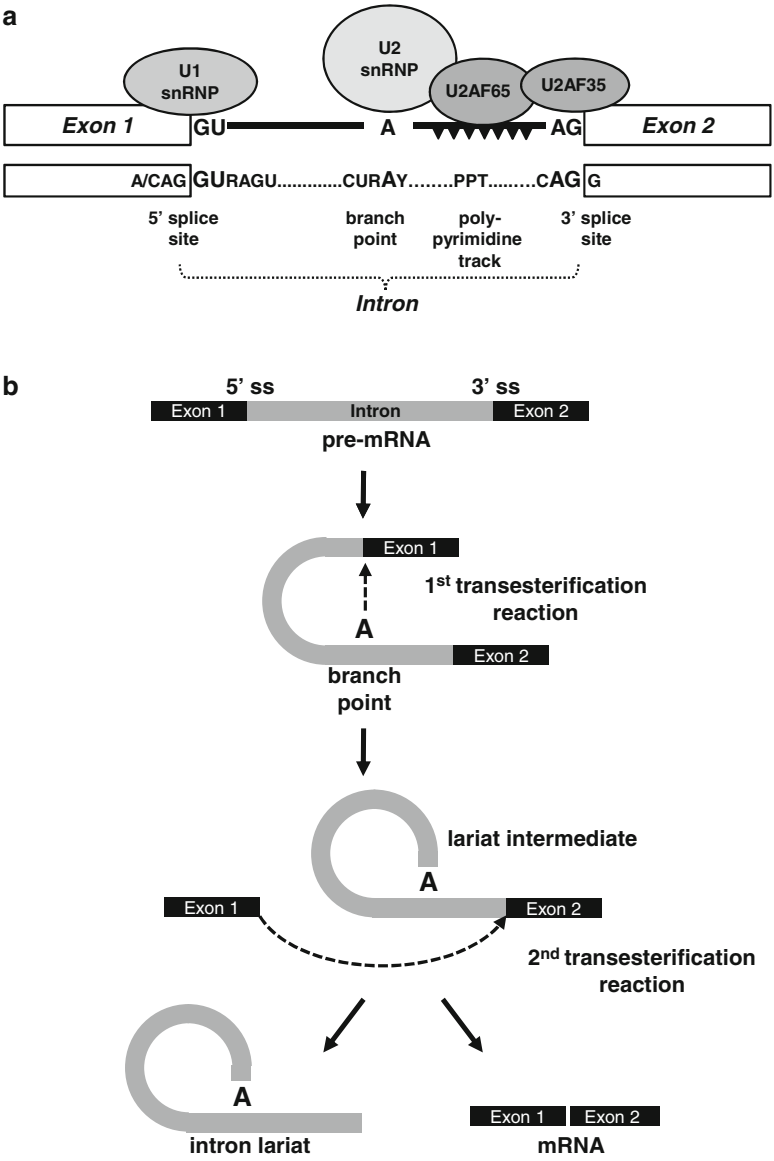


Fig. 38.1 Pre-mRNA structure and splicing reactions. (**a**) Structure of a pre-mRNA containing an intron (*solid line*) and two exons (*empty boxes*). An intron is defined by several specific sequence motifs which allow intron recognition by cellular splicing machinery. A 5′ splice site or donor site GU at the intron 5′ end is recognized by U1 small nuclear ribonucleoprotein (snRNP), a major component of the cellular splicing machinery. A 3′ splice site or acceptor site at the intron 3′ end consists of an AG dinucleotide, an upstream polypyrimidine track (PPT) and a further upstream branch point, which are recognized correspondently by U2AF35, U2AF65, and U2 snRNP. These initial recognitions of the intron elements by the components of RNA splicing machinery are essential for spliceosome formation on a pre-mRNA, leading to intron removal. (**b**) RNA splicing is catalyzed by two transesterification reactions. During RNA splicing the intron (*grey*) between

RNA splicing is catalyzed by cellular splicing machinery consisting of several components: small nuclear ribonucleoproteins (snRNPs, U1, U2, U4, U5, and U6) and splicing factors. During initial step snRNP U1 and U2 recognized intron sequences at 5′ splice site and branch point via complementary base-pairing, along with U2 accessory proteins U2AF65 and U2AF35 association with polypyrimidine track and 3′ splice site, respectively (Fig. 38.1a). Intron recognition is a signal for formation of a large protein complex called "spliceosome" where intron removal takes place [8, 9] by two transesterification reactions (Fig. 38.1b). First, the primary transcript is cleaved at the intron 5′ end (5′ splice site) to leave the upstream exon free and followed by branching of the cleaved intron 5′ end to the branch point (usually A) to create a looped structure named "lariat intermediate." In the second step, the hydroxyl group of the free exon attacks the intron 3′ splice site leading to the 3′ splice site cleavage and lariat formation. Simultaneously, a covalent bound is created between two exons to create a mature mRNA. In general, the lariats are quickly released from the spliceosome and degraded in the nucleus.

The efficiency of RNA splicing is regulated at multiple levels and both RNA *cis*-elements and cellular splicing factors play major roles in regulation of RNA splicing. As we described above, the level of conservation of the sequences at splice sites and branch point will affect the strength of binding of core splicing factors and determine the splicing efficiency. RNA splicing is also modulated by a large family of cellular splicing factors containing serine-arginine-rich proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs). Most of the splicing factors differentially expressed in a specified tissues and/or a development stage are the RNA-binding proteins which bind to specific RNA *cis*-element (splicing enhancers or silencers) located within introns and exons [10]. It is now well documented that splicing factors binding to the *cis*-elements either increase or decrease RNA splicing efficiency depending on type of splicing factors, positions of the binding sites and overall spliceosome composition [11, 12]. Current studies showed that beside splicing factors, other processes such as RNA polymerase rate and chromatin structure also affect RNA splicing [13, 14].

Alternative RNA Splicing

Although all introns in a pre-mRNA could be constitutively spliced out and all exons are supposedly included in a mature mRNA, there are many cases showing that an RNA splice site may be not selected constitutively, but rather skipped, during RNA splicing. Consequently, this alternative RNA splicing leads to production of RNA isoforms with different exon composition and sizes often resulting in production of different protein isoforms. In general, there are four major classes of alternative RNA splicing (Fig. 38.2), including exon skipping, intron retention, usage of alternative exons and usage of alternative 5′ and alternative 3′ splice site [15]. In addition, alternative promoter or polyadenylation usage can further complicate the alternative RNA splicing. The reason why some of the exons or introns in a pre-mRNA are alternatively spliced is because of the presence of weak or suboptimal splice signals in the RNA or lack of a particular splicing factor in that cell or tissue. It has been noticed that usage of weak

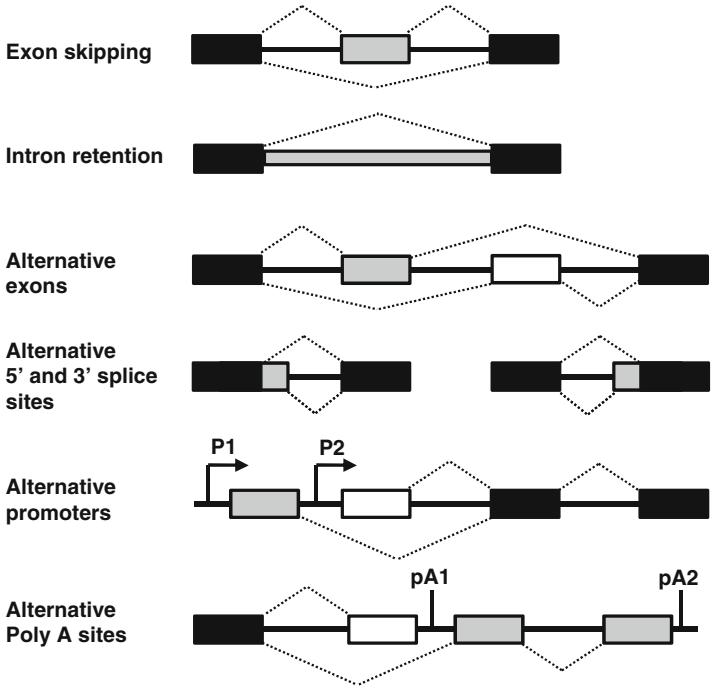


Fig. 38.2 Alternative RNA splicing. Alternative RNA splicing allows production of multiple splicing isoforms from a single pre-mRNA species. Constitutive exons (*black boxes*) are included in all splicing products while alternative exons (*grey* and *white boxes*) are either included or excluded in various isoforms of mature mRNAs. Major forms of alternative RNA splicing includes: exon skipping, intron retention, usage of alterative exons, and usage of alternative splice sites. In addition, usage of alternative promoters (P1 or P2) or polyadenylation sites (pA1 or pA2) may affect exon composition in a final mature transcript

splice sites is highly dependent on auxiliary splicing factors binding to the regulatory *cis*-elements. Since the expression of these factors is variable from cells to cells and from tissues to tissues, alternative RNA splicing is often associated with a specific cell type, tissue, stage of cell differentiation [16] and results in various isoforms of transcripts from one gene [17]. Current analysis also revealed that number of genes with alternative RNA splicing increases with complexity of the organism and ranges from 0.05 % in yeast up to 66.8 % in human. Thus, alternative RNA splicing is the driving force behind the complexity of proteome in higher organisms other than total number of the mapped genes.

Molecular Methods for Detection of RNA Splicing

A mRNA generated by RNA splicing is different from its pre-mRNA. First, mRNA is smaller in size than its pre-mRNA due to RNA splicing which removes the introns found in the pre-mRNA. In contrast, the pre-mRNA not only is larger than the

spliced mRNA but also has the same size as its DNA template. Second, mRNA contains exon–exon junctions with the sequence not present in DNA or its primary transcript allowing designing primers or probes to specifically identify a particular mRNA isoform due to alternative RNA splicing. Although an alternatively spliced mRNA may translate a truncated protein which could be detectable with a specific antibody, the molecular techniques based on detection of nucleic acids are more commonly used to detect RNA splicing.

Northern Blot

Northern blot is one of the oldest techniques to detect RNA splicing. First, RNA molecules isolated from samples are separated based on size by electrophoresis in agarose or polyacrylamide gel. After transfer to a nitrocellulose or nylon membrane, the individual RNA transcripts are detected by an antisense probe specific for the detecting RNA. The probes used for the Northern blot are usually labeled with ^{32}P isotope, enzyme (e.g. alkaline phosphatase), digoxigenin (DIG), or biotin and can be derived from a constitutive exon or an exon–exon junction (Fig. 38.3a). Constitutive exon-based probes would detect all spliced RNA isoforms and the remaining, unspliced pre-mRNA and are recommended when the size difference between spliced RNA isoforms and unspliced pre-mRNA is sufficient enough to be separated. If the size difference is too small for two RNA isoforms to be distinguished, an exon junction probe could be used to specifically detect a spliced product. In addition, a specific probe from an alternative exon or intron could be also designed for detection of individual splicing isoforms derived from exon/intron inclusion.

RNase Protection Assay

RNase protection assay (RPA) requires most often ^{32}P-labeled single-stranded antisense RNA probes complementary to the transcripts of interest. The prepared probe(s) is consequently hybridized with sample RNA to form an RNA–RNA hybrid. Unhybridized single-stranded RNA is then removed by RNases A and T1 which digest single-stranded RNA only. The protected RNA fragments are separated in the gel by electrophoresis and their sizes are determined by molecular markers. To distinguish a spliced RNA product the probe should contain at least a partial intron region which will be digested from the probe due to lack of the intron sequence in the spliced mRNA. As a result, the protected probe by the corresponding exon regions of the detecting mRNA is shorter and will run faster in the gel (Fig. 38.3b). In general, RPA is more sensitive than northern Northern blot in detection of RNA splicing.

Both Northern blot and RPA are commonly used in research laboratories. Their advantage is high specificity. However, both methods are very laborious and low-throughput requiring isolation of large amount (usually a few micrograms) of total RNA from samples and preparation of specific probes often labeled with radioisotopes and thus making their limited usage in clinical diagnostics.

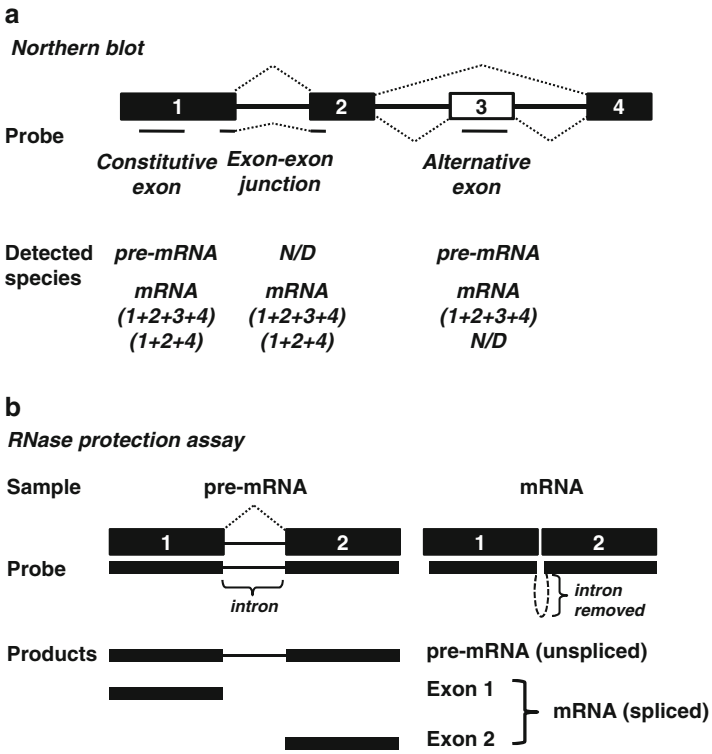


Fig. 38.3 Detection of RNA splicing products by Northern blot and RNase protection assays (RPA). (**a**) Diagram of a pre-mRNA with constitutive exons (*black boxes*), introns (*solid lines*) and an alternative exon (*empty box*). *Solid lines* below indicate positions of antisense probes commonly designed for northern blot to detect products by each probe. N/D not detectable. (**b**) Detection of spliced RNA products by RPA. Diagram shows an antisense riboprobe spanning over an intron region (a *solid thin line*) between two exons (*black boxes* or *solid thick lines*) and possible detection products. As an RNase used in the assay digests a single-stranded RNA region only, an RNA region base-paired with an antisense probe uniformly labeled with isotope ^{32}P will be protected from RNase digestion. In this diagram, the probe remains intact when binding to the pre-mRNA, while the probe binding to the spliced mRNA (lack of the intron) will cause the digestion of the probe intron region (single-stranded, dashed loop) resulting in production of smaller protected products corresponding to each exon

RT-PCR

RT-PCR (reverse transcription-polymerase chain reaction) is one of the most commonly used methods for detection and quantification of RNA molecules. During RT-PCR RNA transcripts are converted into complementary DNA (cDNA) by reverse transcription using random hexamers or oligo-dT or transcript-specific primers. The resulted cDNA is then used as a template in subsequent PCR with a pair of transcript-specific primers.

In principle the detection of spliced RNA transcripts by RT-PCR depends on amplicon selection and primer design. A most common approach is the amplification over the intron regions by a set of primers in flanking exons. The resulted RT-PCR products vary in sizes depending on how the detecting transcript is spliced. A larger product than predicted size may represent an unspliced pre-mRNA or the contaminating genomic DNA. The later can be determined by a minus RT amplification (PCR). A spliced mRNA always gives a smaller RT-PCR product than its pre-mRNA due to removal of intron sequences by RNA splicing (Fig. 38.4a). Another approach is to specifically amplify a spliced product by using an exon junction primer because the sequence at exon–exon junction occurs neither in pre-mRNA nor in genomic DNA. Similarly, a primer based on an alternative exon would amplify only transcript with the inclusion of that exon (Fig. 38.4b). After amplification the size and amount of RT-PCR products are analyzed by gel electrophoresis. Because of nonlinear nature of PCR amplification the classical PCR only provides semi-quantitative data on the abundance of various spliced RNA isoforms.

Introduction of real-time quantitative RT-PCR (qRT-PCR) with a broad (10^7) dynamic range has significantly improved the sensitivity of RT-PCR. Because of high sensitivity real-time RT-PCR enables to detect and amplify RNA directly from a single cell without RNA extraction. In addition it automates the quantification and does not require electrophoretic separation of RT-PCR products.

Currently there are four major chemistries widely used in real-time RT-PCR: SYBR green (Molecular Probes), *Taq*Man™ probes [18], Molecular Beacons [19], and Scorpions™ probes [20]. The principles of different chemistries are described in Fig. 38.5. As of today, SYBR green and *Taq*Man™ probes represent the most common chemistries. SYBR green is a fluorescent dye which has low fluorescence when in solution; however, it becomes highly fluorescent upon binding to double-stranded DNA. On the other hand, *Taq*Man™ probes, Molecular Beacons and Scorpions probes employ fluorescence resonance energy transfer or Förster resonance energy transfer (FRET, also known as resonance energy transfer [RET] or electronic energy transfer [EET]) to generate fluorescent signal. The FRET couples donor fluorescent dye (fluorophore) with nonfluorescent quenching moiety (quencher). When fluorescent dye is in close proximity of the quencher, the quencher molecule absorbs the energy and thus blocks fluorescence emission from the fluorophore when excited by light. TaqMan probes are 18–22 bp oligonucleotide probes which are labeled with a reporter fluorophore at the 5′ end and a quencher at the 3′ end and thus in close proximity. Each probe is complementary to a region in the middle of the detecting target between two primers during PCR reaction. When *Taq* polymerase extends the primer to synthesize the nascent strand, the 5′–3′ exonuclease activity of the *Taq* polymerase degrades the *Taq*Man™ probe annealed to the targeted region and releases the fluorophore from *Taq*Man™ probe and thereby breaks the close proximity to the quencher. As a result, the fluorophore when excited by cycler's light emits fluorescence which marks the presence of PCR product. The method determines the amount of product by generation of fluorescent signal, which is measured in "real time" during entire length of RT-PCR reaction enabling to calculation of the amount of PCR product after each amplification cycle. Similarly to *Taq*Man™ the Molecular Beacon and Scorpions™

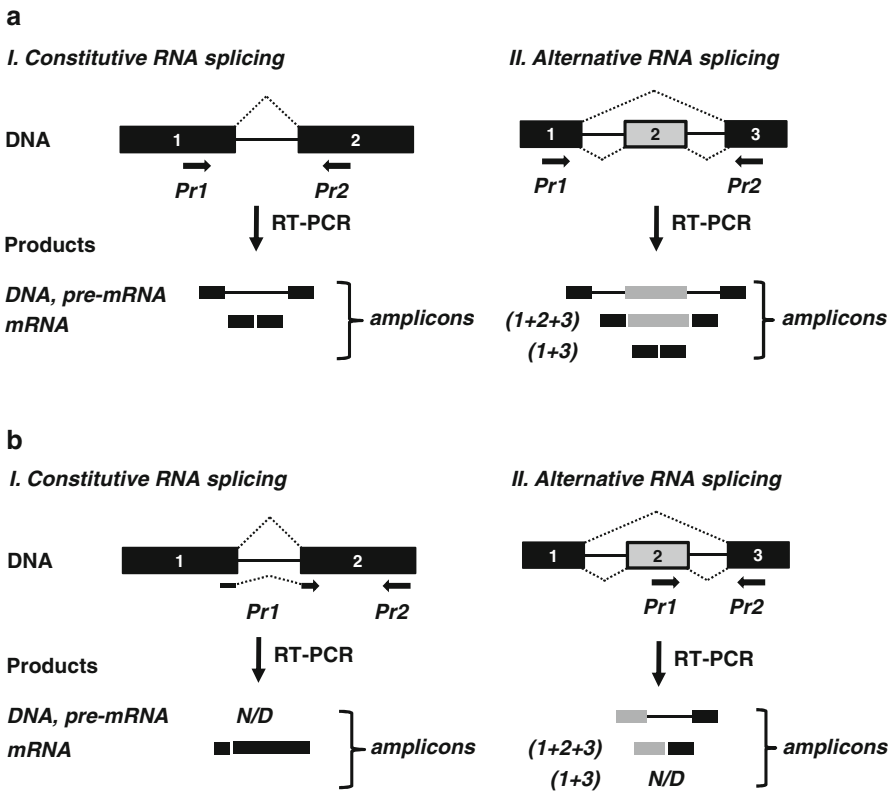


Fig. 38.4 Detection of spliced RNA products by RT-PCR. Intron removal from a pre-mRNA could be detected by RT-PCR based on the size of a product **(a)** or in a selective amplification **(a** and **b)**. **(a)** A pair of primers (Pr1 and Pr2) used for amplification are derived from two constitutive exons (*black boxes*) over an intron (*solid line*) and the amplified mRNA product (spliced) will be smaller in size than the products amplified from template DNA or unspliced pre-mRNA. In an alternative RNA splicing assay, a pair of primes is derived from exon 1 and exon 3 spanning over alternative exon 2 (*grey box*). Alternative RNA splicing will result in multiple spliced RNA products of various sizes amplified by RT-PCR. **(b)** Specific amplification of spliced RNA products during constitutive or alternative RNA splicing. The specific splicing products could be obtained by using a set of primers in which one represents a splicing junction (*Pr1*). Because of lack of this sequence in unspliced pre-mRNA or DNA, only the spliced product will be selectively amplified. A selective amplification could be also used to amplify by RT-PCR a specific RNA isoform derived by alternative RNA splicing by using one primer in an alternative exon (exon 2) in combination with a primer in exon 3. In this case, only the spliced product with exon 2 inclusion will be selectively amplified by RT-PCR

use probes to detect specific PCR product. However, instead of probe degradation the signal is generated by physical separation of fluorophore and quenching moieties after hybridization of specific probe to PCR product during amplification (Fig. 38.5).

Each chemistry has its own advantages and disadvantages which need to be considered during experimental design. SYBR green represents a simple, easy-to-use, and the most economical real-time RT-PCR method. Disadvantage of SYBR green

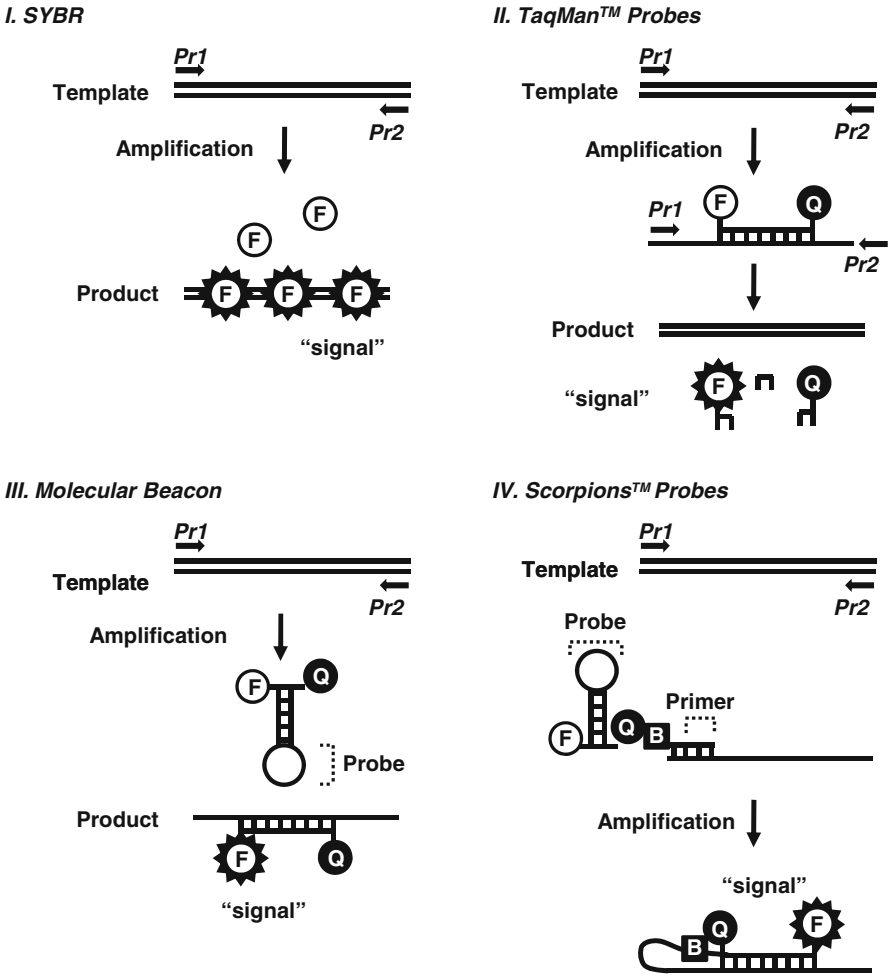


Fig. 38.5 Major chemistries of real-time PCR. PCR product detection by four major commercially available chemistries in real-time PCR: SYBR green (*I*), *Taq*Man™ (*II*), Molecular Beacon (*III*), and Scorpions™ probes (*IV*). The mechanism of each chemistry is described in details in other chapter. *Double lines* represent a double-stranded DNA template generated by RT-PCR while a *single line* is correspondent to single-strand DNA. *Horizontal arrows* mark primer (*Pr1* and *Pr2*) positions. F stands for quenched "fluorophore moiety" and could be present in two stages: quenched nonfluorescent stage (*empty circles*) or in activated stage (*empty circles with spikes*) with generation of detectable signal. Q in *black circles* represents "quencher." Scorpions probes contain amplification stop sequences named "blocker" (B in *black box*)

is its binding nonspecifically to any DNA including primer dimers and nonspecific PCR products and is not useful for multiplex amplification of several products in the same reaction. In contrast, *Taq*Man™, Molecular Beacons, and Scorpions™ probes specifically detect only a PCR product complementary to probe sequence enabling to distinguish specific from nonspecific products. Disadvantage of these chemistries

is that each PCR product requires synthesis of its own specific probe which increases the cost per reaction. On the other hand, labeling individual probes with fluorophores of different emission spectrum allows multiplexing and simultaneous detection of several products and reduces the cost and labor.

The usage of real-time RT-PCR for splicing detection requires special considerations. Since real-time RT-PRC techniques omit electrophoretic separation, the spliced product could not be distinguished based on size. Therefore it is important that only desired product is amplified. In this case, the usage of SYBR green chemistry is the most challenging because of the lack of specificity. Probe-based methods provide higher specificity due to probe hybridization to selected sequences which are not present in nonspecific products. To detect only desired spliced product the probe and primer should be complementary to a specific exon–exon junction or alternatively spliced region. Several other factors must be considered when using RT-PCR-based techniques in diagnostics including RNA sample quality and preparation, *Taq* polymerase inactivating contaminants in clinical samples, and amplification bias. Other considerations are false positivity and PCR cross-contamination.

Splicing Microarrays

DNA microarrays (also known as DNA chips) are composed of large number of probes, often in several thousands, spotted on very small area in 2D format on solid surface (glass or plastic). Probes represent DNA oligos of various length and chemistry. Each probe has specific DNA sequence allowing detection of corresponding DNA with complementary sequence. Currently, there are two major technologies of DNA arrays manufacturing of microarray: (a) direct synthesis of probes on the array or (b) printing arrays from library of presynthesized probes. Each DNA microarray allows rapid profiling of large number of DNA molecules at the same time. Today DNA microarrays are widely used to study gene expression profiling and RNA posttranscriptional modifications including RNA splicing [21].

The analysis of RNA transcripts by DNA microarrays requires a conversion of RNA samples to DNA by reverse transcription, following amplification and labeling with fluorescent dye. After labeling the samples are hybridized with the probes on the array. Unbound samples are washed away and the fluorescent signal is captured and analyzed by microarray readers (Fig. 38.6a). The intensity of fluorescent signal corresponding to the number of bound molecules allows determination of the level of RNA in the original sample by mathematical algorithm.

There are two different approaches in probe design to study of RNA splicing using DNA microarrays: tiling and exon arrays [22] (Fig. 38.6b). In tiling arrays the set of overlapping probes cover the full-length of nascent primary transcript including exons and introns. The analysis of florescence for each probe allows to identify exons and introns based on the difference in signal intensity (Fig. 38.6b*i*). The advantage of tiling arrays is their ability to identify known as well as new splice events. Therefore, the tilling arrays are often used as discovery tools. The disadvantages are

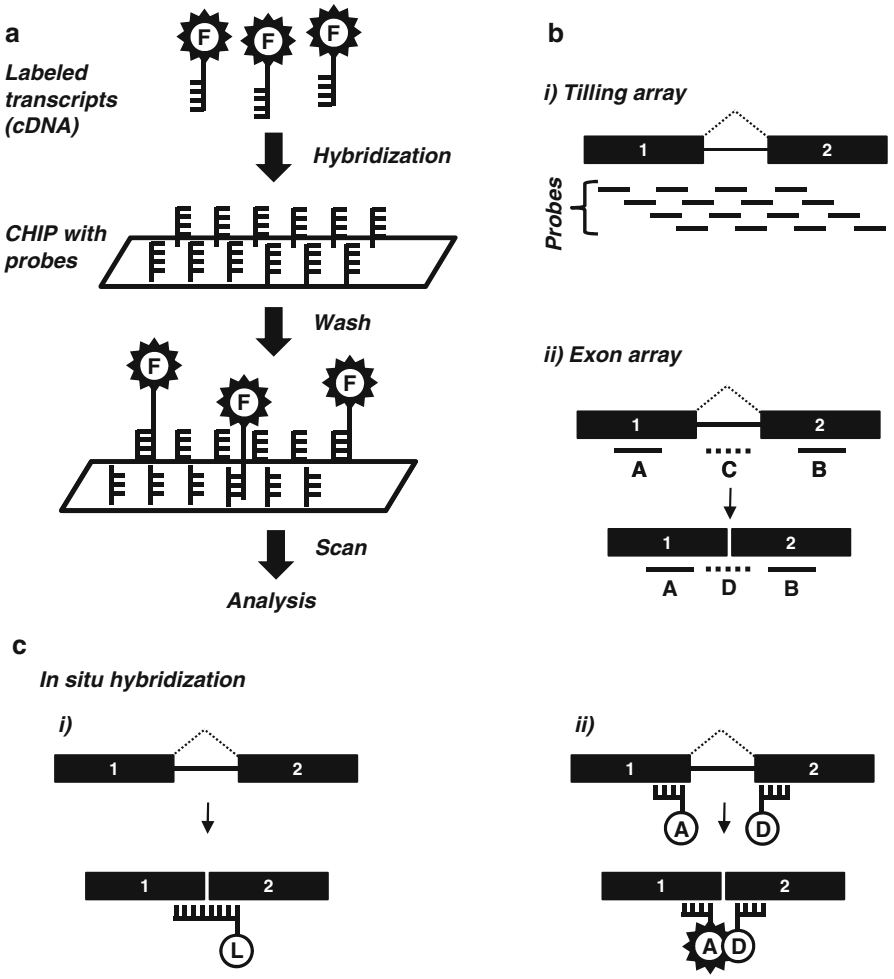


Fig. 38.6 Splicing microarrays and in situ hybridization. **(a)** A work flow for microarray assay. First, sample RNAs are converted into cDNAs simultaneously labeled with specific fluorophore (F). The labeled cDNAs are hybridized with oligonucleotide probes attached to a solid surface. The unbound cDNAs are washed away and the remaining fluorescence signals resulted from specific hybridization are collected by an array-scanning device and analyzed. **(b)** Microarrays in RNA splicing detection. (i) Tiling arrays represent a large set of overlapping probes (*short solid lines*) to cover the selected genes. Because an mRNA has no intron after RNA splicing, introns (*lines*) can be detected with drop on signal intensity when compared to neighboring exons (*solid boxes*). (ii) Exon arrays consist of combination of two types of probes: probes binding to exon regions (*short solid lines*) and probes binding to intron or exon–exon junction (*short dashed lies*). Splicing events are calculated by analysis of signal intensity between exon, intron, and junction probes. **(c)** Detection of spliced transcript by in situ hybridization. (i) Probe spanning over the exons (*solid boxes*) junction specifically binds to spliced product but not to unspliced pre-mRNA containing intron (*solid line*) or genomic DNA. The probe detection depends on type of labeling (L in *empty circle*) including isotope, biotin, digoxigenin, fluorophore, or others. (ii) Detection of RNA splicing by cohybridization of two probes labeled with acceptor (A in *cirle*) or donor (D in *circle*) fluorophores binding to exonic regions (*black boxes*) flanking the intervening intron (*solid line*). When bound to unspliced transcript the binding sites of two probes are separated by intron preventing energy transfer by FRET and thus, no signal is generated. After intron removal by RNA splicing, two probes are bought to close proximity for FRET to occur. The energy transfer from the donor to the acceptor leads to excitation of the acceptor fluorophore (A in *circle with spikes*) and generation of detectable signal. The diagram is modified from Blanco and Artero [31]

requirement of large amount of probes and are time-consuming data analysis. The exon arrays are more commonly used but require the knowledge of splicing events. Several types of probes hybridizing to flanking exons, intron, and exon–exon junctions are designed to detect each splicing event (Fig. 38.6b*ii*). The fluorescence intensity is detected for each probe and mathematical model is applied to determine the occurrence of splicing event. The advantages of exon arrays are: the requirement of smaller number of probes with simpler data analysis. However, the exon arrays detect only known or predicted splicing variants. Due to their large capacities the exon arrays could be designed to detect splicing in multiple viral pathogens.

In Situ Hybridization

Tissue sections historically represent an important tool in diagnostic of pathological changes during viral infection and detection of viral pathogens on cellular level. There are two major types of tissue sections: frozen and formalin-fixed, paraffin-embedded (FFPE). Both are routinely used for detection of viral antigens by various types of staining, but their use in detection of nucleic acids including spliced transcripts is still rather rare. Improved sensitivity of current nucleic acid isolation and amplification techniques allows the recovery of nucleic acid from tissue sections for further analysis by PCR and RT-PCR with selective isolation of only cells of interest by laser capture microdissection to bring additional level of specificity [23, 24]. However, detection of nucleic acids by in situ hybridization (ISH) directly on tissue sections can provide additional benefits about gene expression linked with spatial distribution of specific RNA transcripts at cellular and often even at subcellular level. In the past the nucleic acid molecules including RNA transcripts by ISH were detected by DNA probes labeled with radioisotope (^{35}S, ^{33}P, ^{3}H) [25], which were later replaced by nonradioactive DNA-probes labeled with biotin or digoxigenin and detected by chromogenic methods using enzymes-labeled antibodies (CISH) [26]. Labeling probes specific for different transcripts with different fluorophores (FISH) allow detection of multiple targets at the same tissue section. However, the sensitivity was always a limiting factor of ISH techniques. This was caused mainly by usage of DNA probes which suffer from low affinity to complementary RNA targets and sensitivity to degradation of RNA–DNA hybrids by RNase H. Development of tyramide signal amplification (TSA) has dramatically improved the sensitivity of DNA probes [27]. Further improvement came from by introduction of locked nucleic acids (LNA) and peptide nucleic acids (PNA) probes with high affinity to RNA molecules and resistance to RNase H degradation [28–30]. Detection of RNA splicing by ISH requires a probe to specifically bind only to spliced mRNA without binding to unspliced pre-mRNA or genomic DNA in the sample. Historically this was achieved by designing a probe over exon–exon junction containing sequences present only in spliced transcripts (Fig. 38.6c*i*). Another approach in detection of spliced transcripts by IHS represents the methods by cohybridization of two probes labeled with donor and acceptor fluorophore and generation of signals by FRET. In principle, each splicing event is monitored by a set of two probes complementary to exonic sequences flanking an intron region. One of

two probes carries a fluorophore acceptor while the second probe is labeled by fluorophore donor. When probes bind to genomic DNA or unspliced nascent transcript their binding sites are separated by intron regions resulting in distance between donor and acceptor too big for two fluorophores to engage in FRET. However, intron removal by splicing brings probe binding sites to proximity close enough for FRET to occur resulting in generation of measurable fluorescence [31] (Fig. 38.6c*ii*). This results in high specificity and low background. Using a set of probes with different fluorophores allows to detect multiple spliced transcripts or various spliced isoforms of the transcript. In summary, ISH hybridization methods provide us with a necessary tool to investigate the distribution not only of protein-encoding transcripts but also of rapidly growing number of virus-encoded noncoding RNAs, of which their role in viral pathogenesis remains often elusive [32]. In situ hybridization methods could be especially suitable in retrospective analysis of archived samples in collections.

RNA-seq

Next-generation sequencing (NGS) represents a new generation of analytical tools for genome and transcriptome analysis [33]. It bases on generation of a large amount of short sequences in parallel sequenced reactions. Advantages of NGS are the requirement of less amount of the initial sample, deep coverage, and nucleotide resolution. NGS also does not require any previous knowledge of detecting sequence. Currently there are several platforms including 454 Life Sciences (*Roche*), Genome Analyzer (*Illumina*), ABI Solid Sequencing (*ABI*), and many others. Each platform uses different technology to generate the data, but all provide the same information.

Sequencing of RNA samples converted to cDNA is called *RNA-seq*. *RNA-seq* provides a comprehensive picture of whole genome transcriptome and has been successfully used for analysis on gene expression and posttranscriptional processing including RNA splicing. However, NGS is costing and requires sophisticated data analysis, which makes it less suitable for clinical diagnostics. However, *RNA-seq* does not require any prior knowledge of detecting sequence composition and therefore allows to detect unknown or unpredicted RNA sequences. This may be especially beneficial in discovery of new pathogens including viruses [34]. In addition, *RNA-seq* instantly analyzes a transcriptome including spliced transcripts of any type of cells or tissues.

RNA Splicing in Clinical Virology

RNA splicing does not occur in prokaryotes and is a hallmark of the eukaryotic gene expression. In eukaryotes the number of genes which undergo splicing varies highly from organism to organism, with only about 5 % of all genes being spliced in yeasts to 95 % in human [35, 36]. Viruses as intracellular parasites replicate inside of host cells and hitchhike many cellular processes for their multiplication including RNA splicing. By using constitutive and/or alternative RNA splicing, most of DNA viruses and some of RNA viruses increase complexity of their proteome without requirement of additional genetic materials.

Detection of spliced viral mRNAs in clinical samples would provide several benefits. While detection of viral genomes in clinical samples would indicate virus infection, the result does not provide information about the stage and dynamic of virus infection. In many cases the progress of viral replication could be assumed from changes in viral load, but this approach requires multiple sampling in the course of infection and varies between individuals. One major advantage for detection of spliced viral transcripts is that viral RNA splicing reflects viral gene expression and thus active viral infection, providing important information about the status of infection without requiring multiple sampling. The production of viral transcripts and their RNA splicing are often the first sign of virus replication detectable before the increase of viral load or occurrence of viral-specific antigens or antibodies. Therefore the detection of active viral infection by RNA splicing may be especially important for the early viral diagnosis of the infection critical for successful treatment. Because of direct association of spliced viral transcripts with the level of active viral replication and by monitoring viral RNA one could provide essential information earlier enough for initiation of antiviral therapy. A rapid shut-off of viral transcription and RNA splicing could be also the first sign of the blockage of viral replication visible even before the change in viral load by genome copy numbers. In the case of ubiquitous viruses, such as members of herpesvirus or parvovirus family, which establish latent infection in the host, detection of RNA splicing of a viral early gene would assist to distinguish viral latent infection from active infection. Such a diagnosis is critical for recipients of the transplant organs where reactivation of latent viruses often leads to transplant rejection.

In addition, interpretation of RNA splicing detection results is straight forward without worrying carryover DNA contamination as a spliced RNA is smaller than its corresponding DNA template. As we describe above, there are many techniques available today for RNA splicing assay. These techniques are not only easy to set up with a low cost comparing to virus isolations and immunological methods, but could be quickly applied to detect new emerging viruses which cultivation of the virus is impossible and/or no immunological method is available. This is particularly true in combination of RNA-*seq* which can rapidly provide sequence information about a viral transcriptome and RNA splicing of viral messages.

RNA Splicing in RNA Viruses

Influenza Viruses

Influenza virus infection affects millions of people every year. Influenza viruses, including influenza virus A, B, and C, are the members of *Orthomyxoviridae* family. Influenza viruses are enveloped RNA viruses with a segmented, single-stranded RNA genome of negative polarity (ssRNA−). Number of segments may vary between virus species, with influenza viruses A and B genome having eight segments and influenza C seven segments. In contrast to the majority of RNA viruses the influenza viruses replicate in the nucleus of host cells because of their dependence

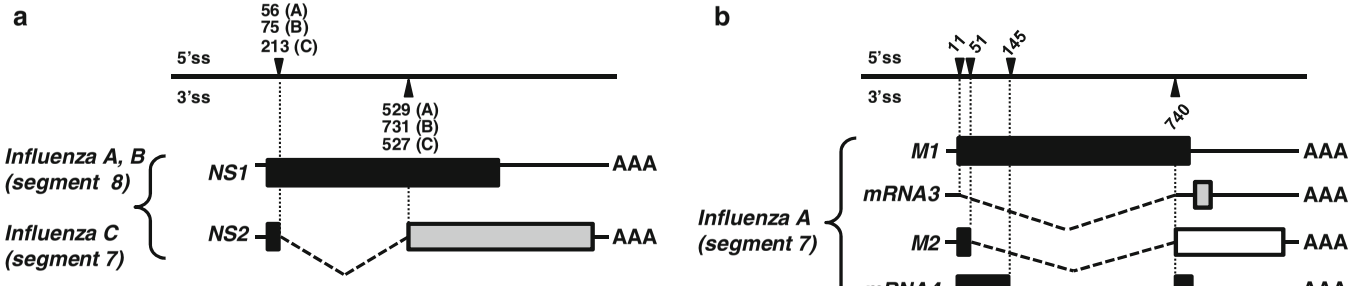
on cellular expression machinery [37]. During replication viral RNAs are produced by viral RNA-depended RNA polymerase. However, viral RNA genomes use short sequences with a cap structure generated by host RNA polymerase II for priming to initiate viral transcription. During infection viral polymerase produces two types of RNAs: one for protein synthesis and the other served as a template for viral genome replication (see review ref. [38]).

RNA splicing in influenza viruses was first detected in an RNA transcript from the smallest segment 8 in influenza A and B and their corresponding segment 7 in influenza C. This transcript encodes two nonstructural viral proteins: larger NS1 and smaller NS2 [39]. In influenza A, NS1 protein is encoded by an unspliced primary RNA transcript (~890 nts), whereas NS2 protein is expressed from a spliced RNA (~350 nts) generated by removal of a 473-nt intron from its primary RNA transcript. This leads influenza A NS1 and NS2 proteins sharing the same AUG start codon and first nine amino acid residues. Translation of NS2 protein continues in +1 frame after RNA splicing and this leads the C-terminal NS2 partially overlapping the NS1 by 70 amino acid residues [40] (Fig. 38.7a). Similar splicing event for production of NS1 and NS2 proteins has been detected from infections of influenza B [41] and influenza C [42] viruses.

Influenza A segment 7 which encodes M1 and M2 proteins produces three RNA species by alternative RNA splicing. The unspliced RNA which is collinear with the genome encodes M1 nucleoprotein with 252 amino acid residues and the two alternatively spliced RNAs M2 and mRNA3 [43] share the same 3′ splice site at nt 740 position, but use different 5′ splice sites for alternative RNA splicing (Fig. 38.7b). M2 RNA uses a 5′ splice site at nt 51 position, whereas mRNA3 employs another 5′ splice site at nt 11 position from the beginning of viral specific sequences. M2 protein bears ion channel activity and shares nine amino acid residues with the M1 N-terminus. The mRNA3 contains a short open reading frame in its exon 2 with potential to encode a short peptide of nine amino acid residues. However, the expression of this peptide was never experimentally confirmed. The role of this transcript during virus replication remains unknown.

While M2 and mRNA3 transcripts are detectable in cells infected with all influenza A viruses, some strains, like A/WSN/33, produce additional spliced transcript named mRNA4 [44]. mRNA4 is generated by usage of additional 5′ splice site at position nt 146 and share the same 3′ splice site with M2 and mRNA3 at position nt 740 (Fig. 38.7b). mRNA4 has potential to encode a peptide with 54 amino acid residues and its first 37 amino acid residues are identical with M1 protein. Sequence analysis of more than 6,000 influenza strains revealed that about 20 influenza A strains has conserved mRNA4 splice site [45]. Sequence information of all influenza viruses could be found at http://www.flu.lanl.gov/.

The primary RNA transcript of segment 7 in influenza virus B does not undergo alternative splicing to produce M2 protein as in case of influenza A. RNA splicing in M transcript takes place with segment 6 of influenza C [46]. Two transcripts generated from segment 6 in the infected cells are the full-length primary and single spliced transcript by removal of an intron located at the 3′ end of the primary transcript. The primary transcript contains a 374-aa ORF (P42), but the spliced message contains a shorter ORF encoding 242-aa residues (CM1) due to generation of STOP codon after RNA splicing from nt 751 5′ splice site to nt 982 3′ splice site



c

Detected transcripts	Primers
M1 mRNA, M vRNA (123 bp)	
(nt 171–188 M cRNA)	5'-GACCAATCCTGTCACCTC-3'
(nt 735–755 M vRNA)	5'-GATCTCCGTTCCCATTAAGAG-3'
M2 mRNA (276 bp)	
(nt 41–51/nt 740–745 M cRNA #)	5'-GAGGTCGAAAC/GCCTAT-3'
(nt 24–44 of M vRNA)	5'-CTCCAGCTCTATGTTGACAAA-3'
M3 mRNA (204 bp)	
(nt 1-11/nt 740–744 M cRNA #)	5'-CAT/AGCAAAAGCAG/GCCTA-3'
(nt 102–116 M vRNA)	5'-CAT/AGACTCTGGCACTCC-3'

Primers for M2, mRNA3 and mRNA4 are designed over exon junction

Fig. 38.7 Diagrams of RNA splicing of influenza virus NS and M transcripts. *Thin lines* represent noncoding sequences. *Dashed lines* indicate splicing directions. Transcripts are polyadenylated (AAA) at the 3′ end. *Black boxes* mark ORF in primary transcripts. *Small black boxes* and *white boxes* are ORF in other frames created by RNA splicing. RNA splicing of NS1 **(a)** and M1 **(b)** transcripts in influenza A, B, and C viruses are diagramed according to Lamb and Horvath [38]. (**c**) Oligo primers used for RT-PCR to detect spliced RNA products from segment 7 of influenza A/WSN/33 (H1N1) as described [207]. cRNA, complementary RNA; vRNA, virion RNA

(Fig. 38.7b). P42 protein is consequently processed by internal cleavage, resulting in production of a predominant CM2 protein containing the C-terminal 115-aa residues of P42 protein [47, 48].

In summary, there are two instances of viral RNA splicing in influenza infection: one conserved between all three species (NS1) and the second highly variable in each species (M). Thus, combination of NS1 and M RNA splicing assays would reveal not only active influenza virus infection, but also specify the infection with a specific influenza virus species.

Human Retroviruses

HIV-1 and -2

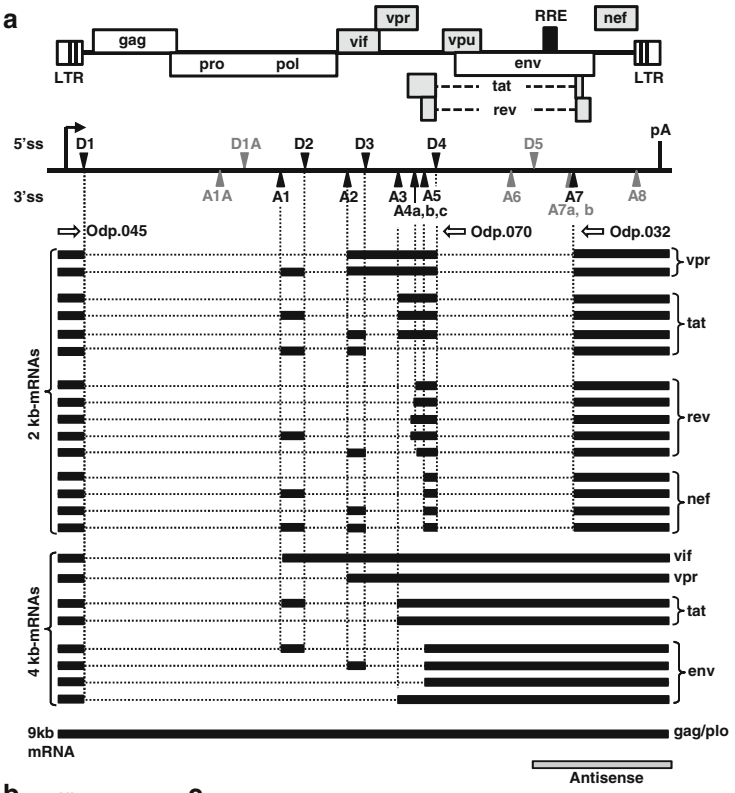
Human immunodeficiency virus (HIV) causing *acquired immunodeficiency syndrome* (AIDS) is a member of *Lentivirus* genus in retrovirus family. HIV infects cells of immune system consequently causing the failure of immunity associated with occurrence of opportunistic infections leading to death. HIV infection is considered pandemic with about 0.6 % of world population being infected. Two types of HIV viruses were characterized. Although closely related, HIV-1 differs from HIV-2 in infectivity and geographical distribution, with HIV-2 much less pathogenic in several West African countries.

HIV is an enveloped virus and carries a single-stranded RNA genome in size of 9-kb of positive polarity (ssRNA+). After initial infection the viral genomic RNA is converted by virus-encoded reverse transcriptase into DNA which then could integrate into host genome where the integrated viral genome resides as a provirus. Later, the integrated provirus serves as a template for transcription of viral transcripts.

In contrast to simple retroviruses, HIV genome has high coding capacity. Besides encoding viral structural and replication proteins (*gag*, *pol*, *env*) HIV expresses a large numbers of accessory proteins. This is achieved by production of over 40 RNA isoforms derived from single RNA transcript by extensive alternative RNA splicing [49] (Fig. 38.8a).

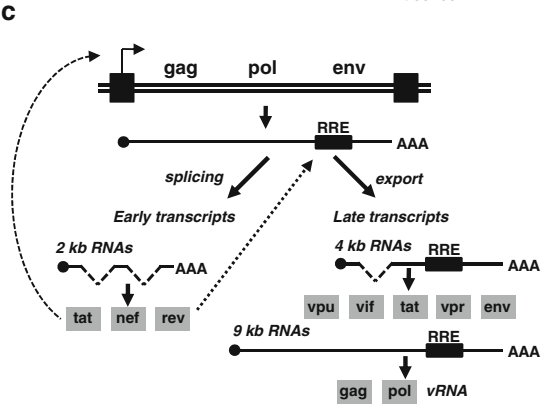
Three groups of HIV transcripts could be observed by size in Northern blot analysis. The first group represents an unspliced 9-kb transcript which serves a template for

Fig. 38.8 (continued) encodes gag and pol proteins. Alternatively spliced HIV-1 transcripts in size of ~2-kb and ~4-kb are grouped along with their protein coding potentials. *Solid boxes* represent exons and *dotted lines* are introns. A *grey box* below the 9-kb mRNA illustrates a recently discovered antisense transcript [52]. **(b)** Nucleotide positions of all mapped 5′ and 3′ splice sites in a prototype HIV genome, pNL 4-3 (GenBank Accs. No. AF324493), starting from the 5′ LTR [208]. **(c)** Alternative HIV RNA splicing is coupled with stages of HIV-1 infection. Multiple spliced transcripts (2-kb group) are expressed in the early stage of the infection resulting in the expression of accessory proteins: tat, rev, and nef. During the late stage of HIV-1 infection, rev protein translocation to the nucleus promotes nuclear export of single spliced (4-kb group) or unspliced HIV RNA via binding to an rev-responsive element (RRE) to express structural and replication proteins [51]. **(d)** Oligo primers used for RT-PCR, shown in (**a**), to detect spliced RNA products of HIV-1 pNL 4-3 [208]



b

pNL4-3	
D1	743
D2	4962
D3	5463
D4	6044
D5	6724
A1	4913
A2	5390
A3	5777
A4a	5936
A4b	5954
A4c	5960
A5	5976
A6	6611
A7a	8335
A7b	8341
A7	8369
A8	9167



d

Primers	Position (nt)	Sequence	Detected transcripts
Odp.045	477-499	5'-CTGAGCCTGGGAGCTCTCTGGC-3'	2.0 kb (early)
Odp.032	8477-8498	5'-CCGCAGATCGTCCCAGATAAG-3'	
Odp.045	477-499	5'-CTGAGCCTGGGAGCTCTCTGGC-3'	4.0 kb (late)
Odp.070	6094-6115	5'-ACTATTGCTATTATTATTGCTACTAC-3'	

Fig. 38.8 RNA splicing of HIV-1 transcripts. **(a)** Schematic diagram of HIV-1 genome with structural and replicatory protein ORFs marked with *empty boxes* and accessory protein ORFs with *grey boxes*. *LTR* long terminal repeats. Shown below the diagram are positions of 5′ splice sites (D1–D4) and 3′ splice sites (A1–A7) identified in a 9-kb full-length primary transcript which

expression of *gag* and *gag/pol* as well as a genomic RNA for newly formed virions. The second group represents single spliced RNA transcripts in size of ~4-kb which encode *env*, *vif*, *vpr*, and *vpu* proteins. The third group in size of ~2-kb consists of multiple spliced RNA transcripts to encode accessory proteins *tat*, *rev*, *nef*, and *vpr*. During virus infection, HIV generates such a wide variety of RNA transcripts by usage of at least five alternative 5′ splice sites and 8–9 alternative 3′ splice sites [50, 51] (Fig. 38.8b). In addition, several antisense transcripts initiating from several 3′ long terminal repeats (3′ LTR) were also detected in HIV-1-infected cells [52].

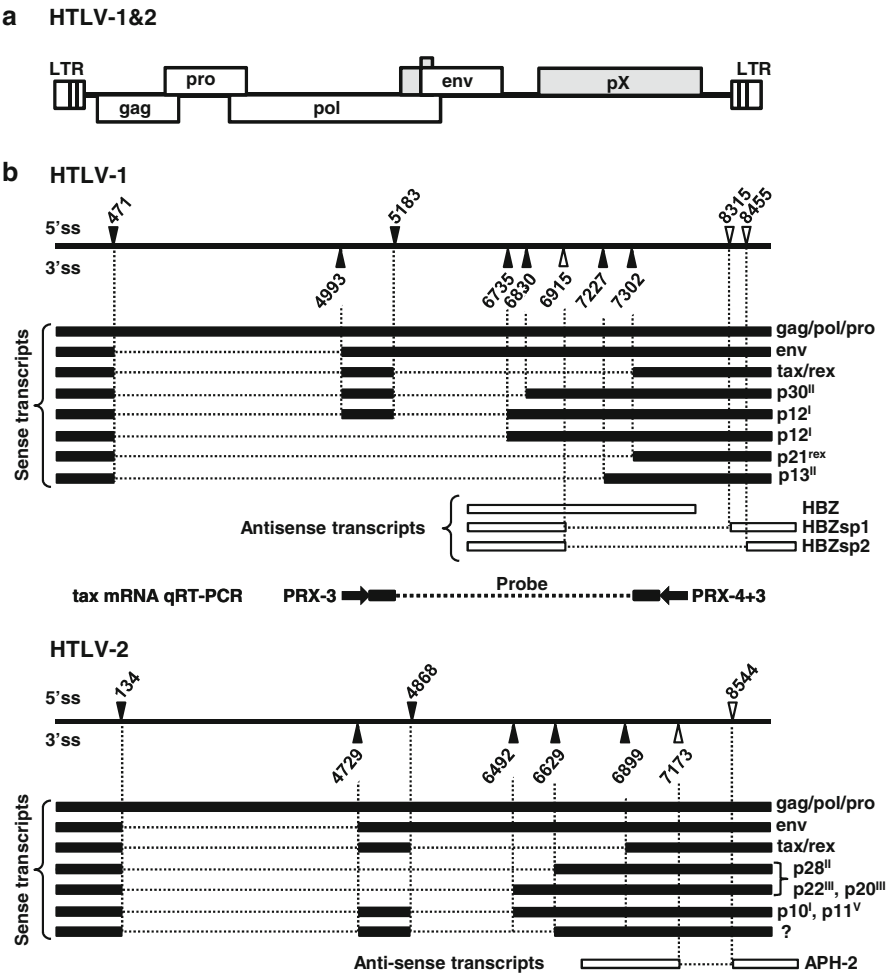
Recent studies showed that HIV RNA splicing is largely regulated by viral RNA *cis*-elements and cellular splicing factors and is orchestrated for completion of HIV life cycle in the course of virus infection. Multiple spliced transcripts of 2-kb family are expressed in the early stage of virus infection to express *tat*, *rev* and *nef*. This group of spliced RNAs is produced by using 3′ splice site A3–A5 located in the central part of the viral genome with A3 site for expression of *tat*, A4a–c for *rev*, and A5 for *nef* proteins. During late stage of HIV infection, nuclear import and accumulation of *tat* together with *rev* protein allows the *rev* protein bind to a *rev-responsive element* (RRE) in partially spliced 4-kb and unspliced 9-kb RNA transcripts located in *tat/rev* intron between D4 and A7 splice sites to mediate the later transcripts export into the cytoplasm for translation [53] (Fig. 38.8c). Sites A1A and D1A are involved in pre-mRNA stability [54]. Strains from IIIB family of HIV viruses use additional A6 and D5 to generate small exon in *env* region and the transcripts containing this exon express tripartite *tat-env-rev* fusion protein, *tev* [55, 56].

Regulation of HIV RNA splicing depends on the selection of 3′ splice sites which are, in general, weak in contrast to strong and highly active 5′ splice sites. In addition, numerous positive and negative splicing regulatory *cis*-elements identified in HIV RNA genome bind various cellular splicing factors and affect the selection of individual 3′ splice site (see review ref. [49]). Comparison of nucleotide sequences between various clades of HIV-1 has shown a high level of conservation of splice sites among different clades of HIV-1 strains (except D4a, b, c).

HTVL-1 and -2

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) were first two retroviruses discovered in human [57]. HTLV-1 is etiologically linked to adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of CD4+ T lymphocytes, as well as to a neurological disorder named HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [58–60]. HTLV-1 is endemic in Japan, Africa, Caribbean basin and South America. HTLV-2 is linked to HAM/TSP but not to ATLL. HTLV-2 infection occurs predominantly in parts of Africa and Americas [61].

HTLV-1 and -2 are two closely related complex retroviruses sharing about 70 % of nucleotide sequences. Their genome organization and replication is similar to HIV. Besides essential genes (*gag/pol/env*) expressed from the full-length or single spliced RNA, HTLVs also encode a number of accessory proteins from a pX region



c

Primer	Position (nt)	Sequence
RPX-3	5096-5115	5'-ATCCCGTGGAGACTCCTCAA-3'
RPX-4 + 3	7360-7338	5'-CCAAACACGTAGACTGGGTATCC-3'
Probe	5172-5183/7302-7312	5'-TCCAACACCATG/GCCCACTTCCC-3'

Fig. 38.9 RNA splicing of HTLV-1 & -2 transcripts. (**a**) Genomic organization of HTLV-1 and -2 with structural and replication genes (*white boxes*) on the 5′ end and regulatory gene pX (*grey boxes*) on the 3′ end of the genome flanked with long terminal repeats (LTR). (**b**) Viral transcripts generated by alternative RNA splicing in HTLV-1- and HTLV-2-infected cells. *Black boxes* indicate exons and *dotted line* are introns. Exons in antisense transcripts are represented by *empty boxes*. The 5′ and 3′ splice sites are indicated by their nucleotide positions in the virus genomes. The coding potentials of each transcript are shown on the right. (**c**) Positions and sequences of the primers and probes used for detection of HTLV-1 spliced tax mRNA as described [209]. Nucleotide positions are based on a full-length HTLV-1 cDNA (GenBank Accs. No. L03562.2)

located in the 3′ end of virus genome (Fig. 38.9a). Transcripts for encoding accessory proteins are generated by alternative RNA splicing of a full-length primary transcript using several 5′ splice sites and 3′ splice sites (Fig. 38.9b) (reviewed in ref. [62]). Recently transcripts antisense to pX region have been discovered in the infected cells either with HTLV-1 or HTLV-2 and these antisense transcripts encode HBZ and APH-2 proteins, respectively [63, 64]. Interestingly, these transcripts also undergo RNA splicing.

In contrast to HIV-1 the regulation of HTLVs RNA splicing and the roles of cellular splicing factors in HTVL RNA splicing are poorly understood.

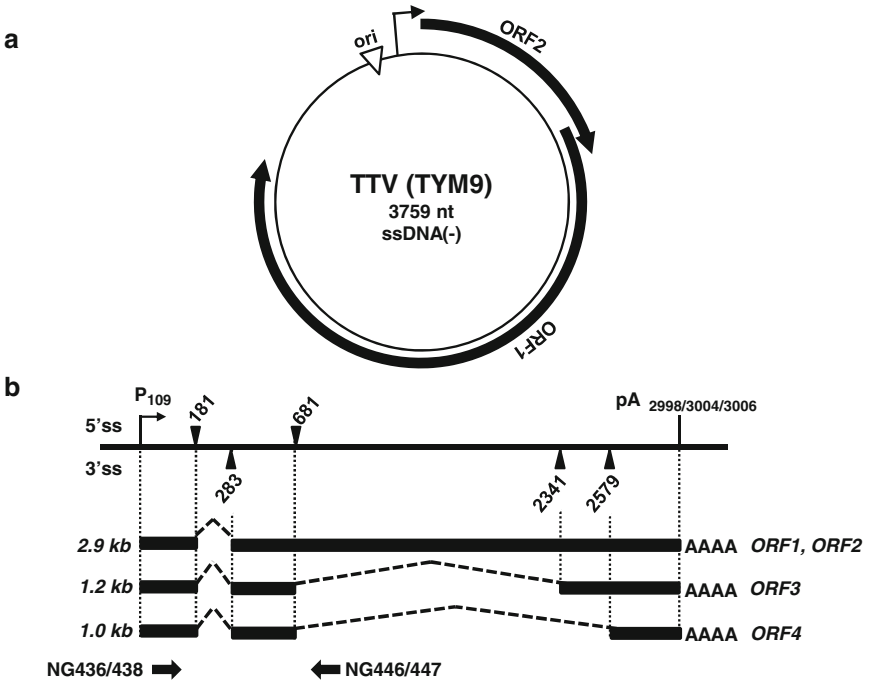
RNA Splicing in DNA Viruses

Circoviruses

Human circovirus TT virus (TTV) was originally discovered in the serum of a patient with posttransfusion non-A-G hepatitis [65]. Later studies showed that TTV is present in body fluids of healthy individuals and is not associated with any pathological disorder. However, the prevalence of TTV in general population appears highly variable from about 2 to 90 % of the incidence of TTV infection. These large variables among the reported studies are most likely attributable to primer selection and PCR performance [66]. TTV isolates have considerable diversity (about 30 %) which could be clustered in several genotypes groups without any particular geographical distribution, indicating that TTV represents a rather ubiquitous virus. Virus replication, route of TTV infection, and association with pathological manifestations remain unclear.

TTV is small nonenveloped virus of icosahedral architecture and contains a circular single-stranded DNA genome in size of 3.6–3.9 kb of negative polarity (ssDNA−) [67, 68]. TTV replication is not yet fully understood partially due to the lack of supportive tissue culture system. TTV genome consists of a GC-rich noncoding region and a protein coding region with two overlapping ORFs (Fig. 38.10a). Three species of RNAs in sizes of 2.8-, 1.2-, and 1.0-kb were detected in infected bone marrow cells as well as in an in vitro infected cell line [69, 70]. All transcripts originate from the same promoter and undergo alternative RNA splicing (Fig. 38.10b). Each RNA transcript could be translated into two different proteins by using two alternative start codons [71]. The presence of other ORFs (ORF3 and 4) in TTV genome was predicted, but has not confirmed yet.

The role of TTV RNA splicing and its regulation in virus infection and virus production remained largely unknown. Another human circovirus TTV-like mini virus (TLMV) has recently been identified in human sera [72]. TLMV shares same genetic organization with TTV and other circoviruses, but its genome is only about 2.9-kb in size.



c

Primer	Position (nt)	Sequence	Amplicons (bp)
NG436	111–133	5'-GTTTTCTACGCCCGTCCGCAGCG-3'	
NG438	132–155	5'-CGGCAGCACCACGGAGGGTGATCC-3'	DNA: (575)
NG446	718–740	5'-CCGCGTCTA AGGTATGTGCGTCG-3'	RNA: 2.9 kb (474)
NG447	686–706	5'-TTCCCCACCGCCTCCGTCTCC-3'	

Fig. 38.10 Human circovirus TTV. (**a**) Single-stranded genome of TTV virus with origin of replication (ori), promoter (*arrow*) and two identified overlapping ORFs. (**b**) Transcripts identified in cells infected with TTV virus with nucleotide positions of two 5′ splice sites (*above the line*) and three 3′ splice sites (*below the line*), together with positions of promoter (*arrow*) and polyadenylation site (pA). The *black boxes* represent exons and *dashes lines* are introns. The size of the spliced transcripts (in kb) is indicated on the left and the coding potentials are on the right. *Arrows* below the transcripts are a primer pair used to detect the 2.9-kb transcript [69] by RT-PCR as detailed in (**c**). Primers positions in (**c**) are based on Torque teno virus TYM9 strain (GenBank Accs. No. AB050448.1)

Hepadnaviruses

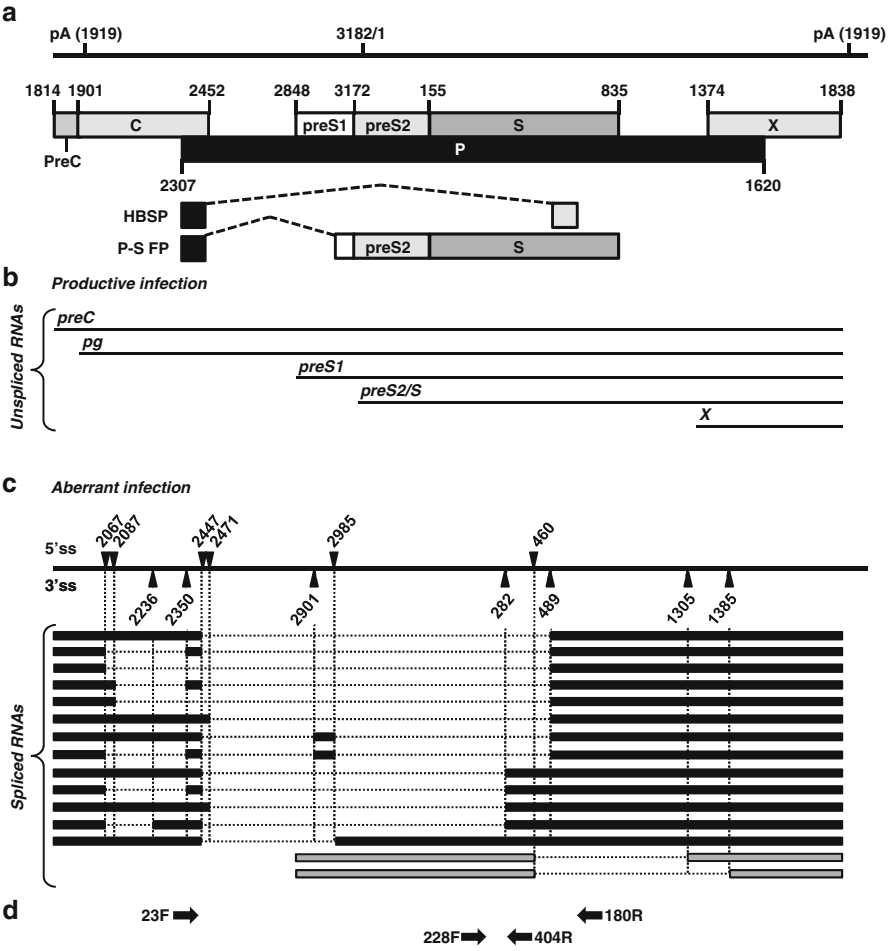
Hepatitis B virus (HBV) chronic infection leads to development of liver cirrhosis and hepatocellular carcinoma [73]. Despite an effective HBV vaccine is available in many countries, HBV infection remains epidemic in many parts of the world, particularly in Asia and sub-Saharan Africa. WHO estimates that HBV infects about two

billions of people worldwide, with chronic infection affecting 350 millions of people and causing death approximately one million people every year. While the vaccine can prevent HBV infection, there is no cure for already infected individuals.

HBV is a noncytopathic, hepatotropic virus. Its genome consists of a 3.2-kb long circular and partially double-stranded DNA. HBV encodes four viral proteins: core (C), reverse transcriptase-polymerase (P), surface (S) and X-protein (Fig. 38.11a). In infected hepatocytes viral DNA is converted to cccDNA (covalently closed circular DNA) and serves as a template for expression of viral pregenomic (pgRNA) and three subgenomic RNAs from several promoters. Despite the presence of several promoters, all transcripts used the same poly A signal for transcription termination (Fig. 38.11b). The core protein C and polymerase-transcriptase protein P are encoded by a bicistronic pgRNA. Subgenomic preS and S RNAs translate three surface antigens (large [preS1], middle [preS2], and small [S] surface antigens). A short RNA in size of 0.7 kb encodes X protein, a nonstructural viral protein presumably with oncogenic potentials [74]. A precore RNA initiated upstream of the pgRNA encodes HBeAg [75, 76]. Beside protein translation pgRNA is a template for reverse transcription into a genomic minus DNA strand during HBV genome replication.

All HBV transcripts from cccDNA are produced by cellular RNA polymerase II. A spliced 2.2-kb RNA transcript was first identified in transfected hepatoma cells [77] and contains a single 1,223-nt long intron starting from the end of core antigen ORF to the middle of S antigen ORF. Subsequently, other single and multiple spliced forms of pgRNA were discovered in sizes of 2.1–2.6-kb from cell cultures and liver tissues of HBV patients [78–80]. So far, 13 spliced variants of pgRNA and two spliced isoforms of pre-S2/S RNA have been identified during HBV gene expression infection and these spliced viral RNAs are produced by using six 5′ splice sites and seven 3′ splice sites (Fig. 38.11c). A viral *cis*-element PRE (posttranscriptional regulatory element) and cellular splicing factors such as PTB (polypyrimidine track-binding protein) and SR-proteins may play roles in regulation of HBV RNA splicing (see review ref. [81]).

Approximate 30–50 % of HBV RNA are spliced RNAs during HBV infection of human hepatoma cell lines Huh7 and HepG2, two popular cell lines for in vitro HBV replication studies [82]. Huh7 cells seem to produce more spliced RNA than Hep2G cells. The major spliced product is derived from 30 % of pgRNA using nt 2,447 5′ ss and nt 489 3′ ss in genotypes A, C, D, E. Serum of infected patients or hepatocarcinoma tumor samples frequently contain HBV DNA originated from spliced variants [83, 84]. Level of spliced HBV RNAs in patients varies widely from no splicing to extensive splicing and is related to viral genotype [85]. Role of HBV RNA splicing in HBV life cycle or pathogenesis remains to be elucidated. HBV spliced RNAs express two new proteins [86, 87]. A spliced mRNA derived from pgRNA with removal of a 454-nt intron from nt 2,447 to 2,901 encodes a structural polymerase-surface fusion protein (P-S FP) p43 with potential function in the entry [86]. Another single spliced pgRNA with removal of an intron from nt 2,447 to 489 translates a 93-aa fusion protein in size of 10.4-kDa, of which its first 46-aa residues are identical to the N-terminus of viral polymerase protein followed by the 47-aa residues generated by the frameshift from the second exon. This protein has been



Primers	Position (nt)	Sequence	Detected transcripts/genomes
23F	2411-2428	5'-CCGCGTCGCAGAAGATCT-3'	**spliced/defective**
180R	609-587	5'-ATGGGAATACAGGTGCAATTTCC-3'	
228F	247-271	5'-TCTAGACTCGTGGTGGACTTCTCTC-3'	**unspliced/full length**
404R	423-401	5'-CATAGCAGCAGGATGAAGAGGAA-3'	

Fig. 38.11 Expression and RNA splicing of HBV transcripts. (**a**) Diagram of linear HBV genome structure. *Numbers* indicate the beginning and the end of each ORF. (**b**) Full length viral transcripts generated from viral genome during productive HBV infection. (**c**) Alternatively spliced viral transcripts of preC (*black*) or preS1 (*grey*) primary transcripts, with exons in *black* or *grey boxes* and introns in *dotted lines*. Numbers above or below the linear genome are nucleotide positions of mapped 5′ and 3′ splice sites. It has been assumed that alternative RNA splicing of HBV preC and preS1leads to production of defective HBV viral particles. Diagrams are modified from Sommer and Heise [81]. *Arrows* below the transcripts are two sets of primer pairs used to detect spliced pgRNA products of HBV by RT-PCR [210] as detailed in **(d)** with primer positions and sequences derived from HBV TK113 genome (GenBank Accs. No. JF754635)

referred as hepatitis B splice-generated protein or HBSP [87] and could be associated with HBV chronic infection, viral cytopathogenic effect, and immune evasion (see review ref. [88]).

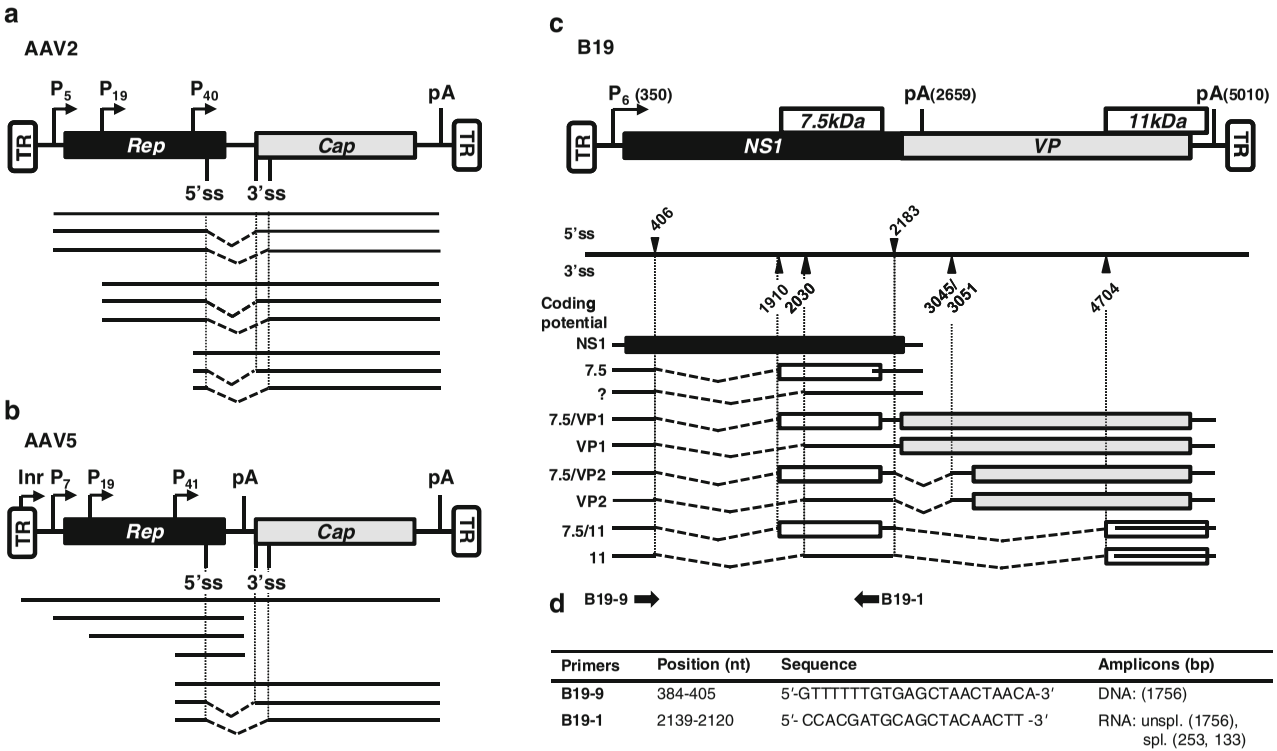
Parvoviruses

Parvoviruses are a group of small nonenveloped viruses containing a single-stranded DNA genome (ssDNA) in size of ~6-kb. The palindromic inverted terminal repeats at the ends of virus genome function as an origin of replication. Parvoviruses replicate via a double-stranded DNA intermediate which serves as a template for viral transcription [89]. Replication of some parvoviruses relies on "helper" virus such as adenovirus, herpesviruses, vaccinia virus, and human papillomaviruses [90–92].

Parvoviruses are ubiquitous viruses and infect a wide range of animals. As of today, there are at least four members of *Parvoviridae* family that are infectious to humans: adeno-associated viruses (AAV), parvovirus B19 (B19V), human bocaviruses (HuBoV), and human Parv4 [93]. Despite of structural and genetic similarity, different parvoviruses use different replication and transcription strategies during virus infection and have different host tropism to initiate a productive infection in the presence of a helper virus.

Adeno-associated viruses (AAV), currently classified as *Dependoviruses*, were first human parvoviruses identified in the group. AAVs infect wide range of species with AAVs-1, -2, -3, -8, and -9 being found in human [94]. Currently no disease or pathological condition is associated with AAV infection in human. The correlation between AAV infection and fetal loss and male infertility was proposed due to high prevalence of AAV DNA in placental tissues and in genital tissues of men with abnormal semen [95, 96]. Because AAV lacks pathogenicity, induces low immune response, infects both dividing and nondividing cells with capability of viral DNA integration into the host genome, AAV has gained attention as a vector for gene therapy (see review ref. [97]).

All AAV genomes consist of two open reading frames, *Rep* and *Cap*, with *Rep* for virus replication and *Cap* for structural capsid protein. AAVs use several different strategies to produce viral products. First group represents AAV1, AAV2, AAV3, AAV4, and AAV6 and their viral transcripts originate from one of three viral promoters on the left hand side of viral genome and are terminated on a single polyadenylation site on the right hand side of the genome. The middle part of the transcripts contains a ~300-nt long intron with a nonconsensus 5′ splice site and two 3′ splice sites (Fig. 38.12a).The efficient splicing requires the presence of both helper virus and large *Rep* protein [98]. While *Rep* protein seems to be essential for AAV2 splicing, several adenovirus proteins (E1A, E1B, E2a, E4 or f6, and VA RNA) as well as some products of herpes simplex virus (UL5, UL8, UL52, and UL29) have stimulatory effect on AAV2 splicing [99]. AAV5 and some animal AAVs are in the second group which utilizes three upstream promoters for their transcription, but their genome contains additional polyadenylation sites in the intron region. Transcripts



Primers	Position (nt)	Sequence	Amplicons (bp)
B19-9	384-405	5′-GTTTTTTGTGAGCTAACTAACA-3′	DNA: (1756)
B19-1	2139-2120	5′- CCACGATGCAGCTACAACTT -3′	RNA: unspl. (1756), spl. (253, 133)

Fig. 38.12 RNA splicing of human parvoviruses. Genome organizations of AAV2 **(a)**, AAV5 **(b)**, and human *Erythrovirus* B19 **(c)** with terminal repeats (TR) on the ends of each genome, along with viral promoters (P), polyadenylation sites (pA), splice sites (ss), and open reading frames (*boxes*). Shown below each genome are viral transcripts generated by alternative transcription initiation, RNA splicing, and polyadenylation. In each panel, *black boxes* represent coding regions, *solid lines* for noncoding regions and *dashed lines* for splice directions to remove the corresponding introns. (**d**) Oligo primers used for RT-PCR to detect spliced RNA products of B19 [211]. The primer positions and sequences are based on a partial genome sequence of B19-Au strain (GenBank Accs. No. M13178.1)

from two upstream promoters are polyadenylated on the internal polyA site, whereas spliced transcripts from P41 promoter use a poly A site at the right side of the genome (Fig. 38.12b). The only spliced transcript in AAV5 infections is the *Cap* transcript which contains a smaller (~240-nt) intron. Interestingly, the splicing of AAV5 *Cap* transcript is constitutive and highly efficient even in the absence of helper virus infection [100].

Human B19 virus, member of *Erythrovirus* genus, was first identified in the serum of blood donor [101]. Three of B19 viruses have been identified from different geographic regions [102]. After acute infection the virus persists in host for the rest of the life. The infection by B19 virus is in general asymptomatic, but several pathological conditions are associated with B19 infection and these include *erythema infectiosum* (the "fifth disease") [103], *polyarthropathy syndrome* [104], transient aplastic crisis (TAC) [105], and persistent anemia/pure red cell aplasia (PRCA). B19 infection during pregnancy may associate with spontaneous miscarriage and development of nonimmune *hydrops fetalis* [106].

Similar to other parvoviruses, B19 virus genome encodes two large open reading frames. NS1 ORF on the left-side genome translates a 77-kDa nonstructural protein and a VP ORF on the right-side genome produces two capsid proteins (84-kDa VP1 and 58-kDa VP2). At least nine virus-specific transcripts have been detected following B19 infection [107] which are all transcribed solely from a single promoter P6 located upstream of NS1 gene, but are alternatively spliced and terminated at two alternative polyadenylation sites (Fig. 38.12c) either in the middle or on the far right-side of the genome. By using the poly (A) site in the middle of virus genome, the P6 transcript has an intron in the NS1 ORF and splicing of this intron from NS1 transcript may create a novel ORF to encode a small accessory 7.5-kDa protein. However, if the poly (A) site on the right-side genome is used for RNA polyadenylation, the P6 transcript become a bicistronic (NS1 and VP) transcript with two introns. By splicing to remove the intron 1 from the bicistronic RNA, the single-spliced P6 transcript is capable to encode both 7.5-kDa and VP1 proteins. Double RNA splicing to remove both intron 1 and intron 2 from the P6 bicistronic transcript disrupts both ORFs for NS1 and VP1, but creates a VP2 or a novel ORF for another accessory 11-kDa protein, depending on which alternative 3′ splice site being selected (Fig. 38.12c). Thus, all detected B19 transcripts are derived from a P6 pre-mRNA containing one or two introns with two alternative 3′ splice site, depending on the selection of which one of two alternative poly A sites, and are alternatively spliced RNA transcripts, except the unspliced full-length NS1RNA. The *cis*-elements in the central exon and intron 2 are regulatory elements to control the alternative P6 RNA splicing, with the double spliced P6 RNAs being the predominant species in the infected cells [107].

Adenoviruses

The most common infection by adenoviruses in humans occurs in upper respiratory tract causing bronchitis and pneumonia. Adenovirus infection can also induce a wide range of other symptoms including conjunctivitis, ear infection, gastroenteritis,

myocarditis, hemorrhagic cystitis, meningitis, and encephalitis. There are 56 adenovirus types belonging to seven species (human adenovirus A-G). Types belonging to B and C are responsible for most respiratory infections, B and D for conjunctivitis, and F and G for gastroenteritis [108]. Adenoviruses were also found in other vertebrates.

Even though the human adenoviruses are not etiologically linked to any human cancer, some adenoviruses (types 2, 5, 12, 18, and 31) can, under special circumstances, transform rodent cells in vitro and induces tumors in small animals. Transformation activities are linked to two oncogenes E1A, which bind tumor suppressor pRB, and E1B, which binds tumor suppressor p53 [109].

Adenoviruses are nonenveloped viruses with icosahedral architecture and contain a linear, nonsegmented, double-stranded DNA genome in size of ~26–45-kb which is capable to encode 22–40 different gene products [109]. Adenoviruses replicates in the nucleus of infected cells (Fig. 38.13a). The early stage of virus infection is characteristic with the expression of nonstructural early protein, while viral structural proteins are expressed in the late stage of viral DNA replication marking switch between two infection phases.

Almost all adenoviral early and late transcripts undergo RNA splicing in order to produce their corresponding viral products [110]. Viral E1A and L1 transcripts are exemplified in this chapter for alternative RNA splicing seen in adenovirus infections.

Adenovirus E1A primary transcript contains three 5′ splice donor sites and two 3′ acceptor sites and is composed of three exons and two introns. The first intron between by D1 and A1 is a suboptimal, minor intron. The second intron is a major intron which uses two alternative donor sites D2 and D3 and one acceptor site A2 for RNA splicing. Alternative splicing of E1A RNA through usage of various combinations of splice donor and acceptor sites leads to formation of five species (13S, 12S, 11S, 10S, and 9S) of E1A mRNAs according to their sedimentation coefficient (Fig. 38.13b) and expression of individual unique protein [111].

Transcription of late genes starts predominantly from a major late promoter. The primary late transcript is then polyadenylated at one of five polyadenylation sites, forming five groups of late transcripts (L1–L5). Each late mRNA contains a 201-nt "leader" sequence derived from three noncoding exons which function as a translational enhancer [112]. There are two variants of leader sequence with or without *i*-leader exon. Beside the leader sequence region, L1 transcripts are also alternatively spliced by using a common 5′ splice site in combination with two alternative 3′ splice sites. Selection of a proximal 3′ splice site results in formation of 52, 55K RNA and selection of a distal 3′ splice site produces IIIa mRNA (Fig. 38.13c).

Characteristic feature of adenovirus splicing is depending on the stage of virus infection. For example, E1A 13S and 12S mRNA are two major spliced products during early virus infection. In contrary, 9S RNA is highly accumulated in the late stage of infection [113]. Similar phenomenon was observed in the expression of late mRNAs. Inclusion of the *i*-leader exon is generally a signature of early transcripts, but most of the late transcripts contain the classical tripartite leader. While 52,55K L1 RNA is produced during both early and late infection, the IIIa splice site is used

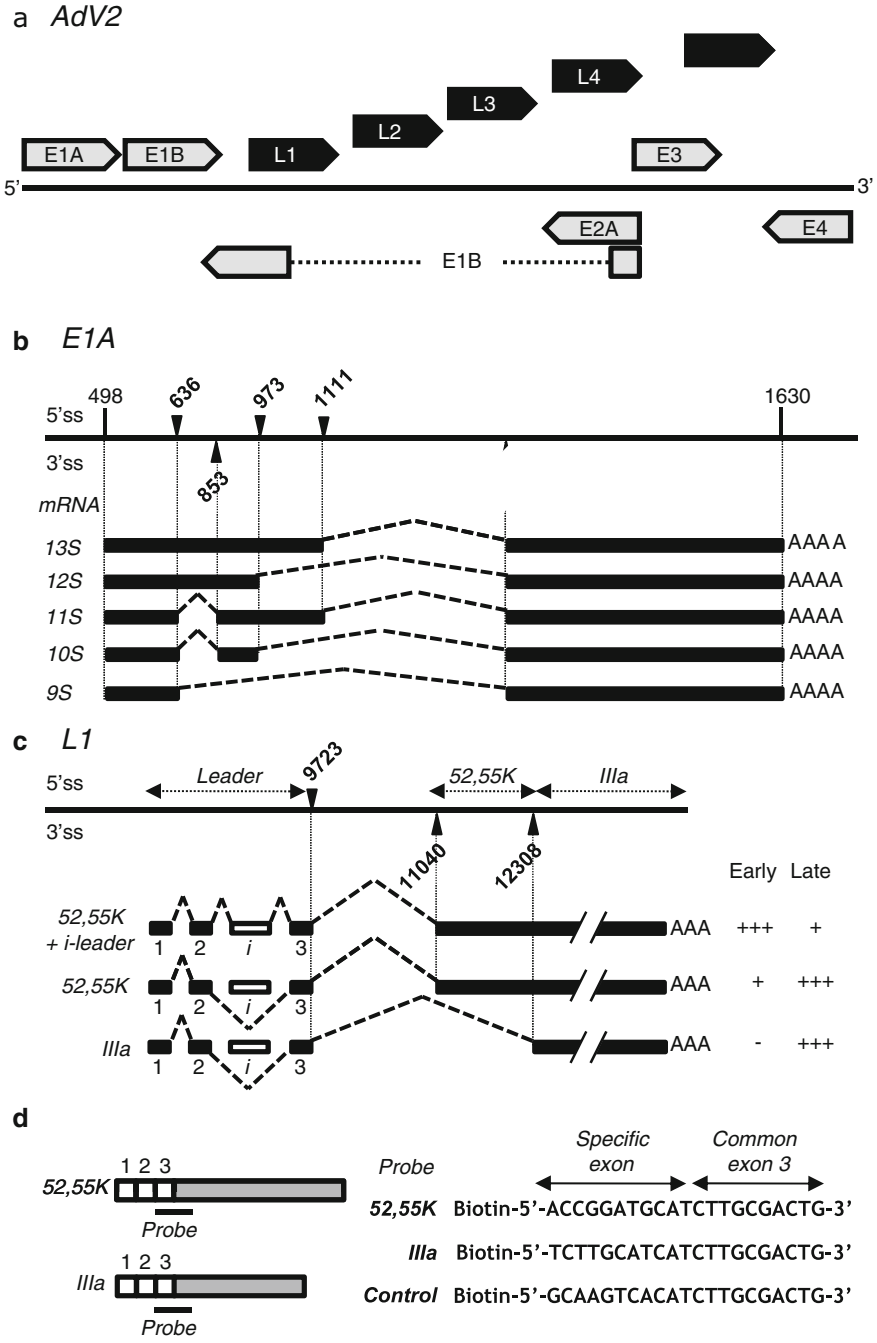


Fig. 38.13 Alternative RNA splicing of adenovirus early and late transcripts. (**a**) Simplified adenovirus genome with positions and orientations of viral early (*grey arrows*) and late (*black arrows*) genes. (**b** and **c**) Alternatively spliced RNA transcripts of adenovirus early E1A gene (**b**) and late L1 gene (**c**). *Black boxes*, exons; *white boxes*, alternative exons; *dashed lines*, introns or splice directions. Nucleotide positions of each splice site are based on a complete genome sequence of human adenovirus type 2 (GenBank Acss. No. AC_000007.1). (**d**) Schematic exon compositions of 52, 55K and IIIa transcripts and exon junction probes for specific detection of spliced L1 isoforms from adenovirus type 2 by in situ hybridization as described [212]

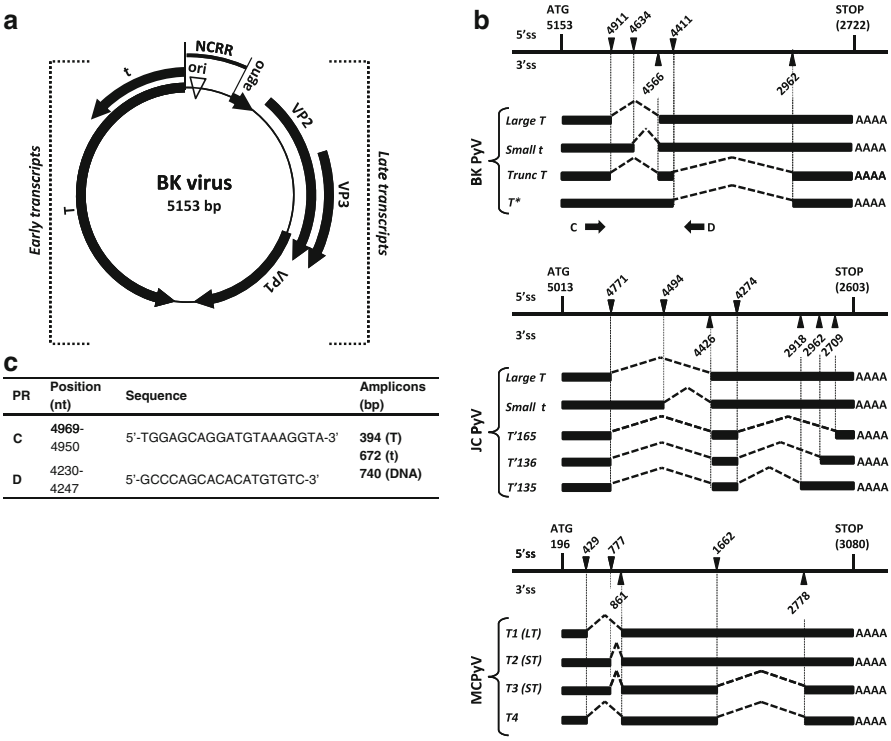
only in the late stage of viral infection [114]. Both cellular splicing machinery and viral products have been found to regulate alternative splicing of adenoviral transcripts in the course of viral infection (see reviews refs. [110, 115]).

Polyomaviruses

Polyomaviruses are small nonenveloped viruses which contains a circular double-stranded DNA (dsDNA) genome in size of ~5,000-bps. Polyomaviruses infect wide range of mammalian and avian species, but each virus exhibits a limited host range and narrow tissue tropism. The *Polyomaviridae* family contains only one genus *Polyomavirus* (*PyV*) which has nine members of human polyomaviruses: BKPyV [116], JCPyV [117], KI PyV [118], WU PyV [119], Merkel cell PyV (MCPyV) [120], HPyV6, HPyV7 [121], trichodysplasia spinulosa-associated PyV (TSV) [122], and most recently discovered HPyV9 [123]. Simian vacuolating virus 40 (SV40), a prototype virus of the family, was introduced into human population as a contaminant in early trials of poliovirus vaccine [124]. Serological data indicate that polyomavirus infection is widespread in general human population with initial infection occurring in childhood [125]. After infection polyomaviruses persist in host for the rest of the life. While initial infection is mostly asymptomatic, several human polyomaviruses are associated with various pathological conditions in immunocompromised patients including nephropathy and cystitis associated with BK PyV and progressive multifocal leukoencephalopathy associated with JC PyV [126], or trichodysplasia spinulosa presumably associated with TSV infection. Polyomaviruses express an oncoprotein T antigen and may lead to development of human cancer by abortive infection as has recently been confirmed in a rare but aggressive Merkel cell carcinoma [127–129].

Polyomavirus genome consists of three functional regions: two protein-coding regions (early and late) divided by a noncoding control region (NCCR) (Fig. 38.14a). Early and late transcripts are expressed in an opposite direction from promoters located in the NCCR which also contains origin of replication. Early transcripts encode nonstructural viral regulatory proteins (T [tumor] antigens) important for virus replication and modulation of cell cycle. Viral DNA replication initiates transcription of viral late genes to encode several viral capsid proteins.

In polyomavirus-infected cells, multiple isoforms of T antigen are detectable as a result of alternative RNA splicing. The primary transcript of T antigen contains two introns, but its intron 1 has two alternative 5′ splice sites. During RNA splicing, the intron 2 retention is important for production of both large T and small t antigens. However, selection of a proximal 5′ splice site in the intron 1 for RNA splicing leads to production of large T antigen, whereas selection of a distal 5′ splice site in the intron 1 results in small t production. Because the sequence region between the proximal 5′ splice site and the distal 5′ splice site has a stop codon, retention of this region in small t RNA splicing makes the small t RNA larger than the large T RNA, but introduction of a premature stop codon in the small t RNA results in production



c

PR	Position (nt)	Sequence	Amplicons (bp)
C	4969-4950	5'-TGGAGCAGGATGTAAAGGTA-3'	394 (T) 672 (t)
D	4230-4247	5'-GCCCAGCACACATGTGTC-3'	740 (DNA)

Fig. 38.14 RNA splicing of polyomavirus T antigen transcripts. **(a)** Genome structure of BKPyV virus, a representative of human polyomaviruses. *Black arrows* represent open reading frames for early viral regulatory proteins (large and small T antigens) and late viral capsid proteins (VP1-3). *NCRR* noncoding repeat region, *ori* origin of replication. Agno, auxiliary agnoprotein. **(b)** Alternative RNA splicing of large T and small t antigens among BKPyV, JCPyV and MCPyV viruses. *Black boxes*, exons; *dashed lines*, introns or splice directions; *numbers*, nucleotide positions of splice donor and acceptor sites. The diagrams are modified from White et al. [213] and Shuda et al. [127]. Nucleotide positions for BKPyV are strain Dunlop (GenBank Accs. No. V01108), for JCPyV are strain Mad-1 (GenBank Accs. No. J02226) and for MCPyV are strain TSK (GenBank Accs. No. FJ173815). *Arrows* below the BKPyV transcripts are oligo primers used to detect spliced large T and small t antigen transcripts of strain Dunlop by RT-PCR as detailed in (**c**) [131]

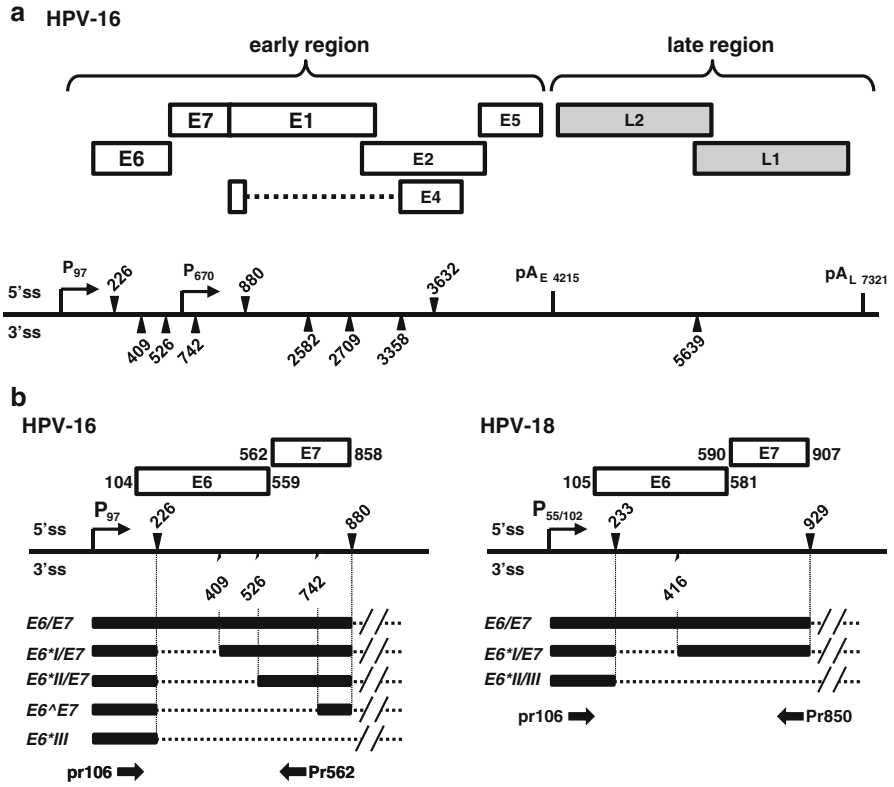
of a smaller protein (Fig. 38.14b). In addition, a rare tiny-*t* antigen in size of ~17-kDa has been attributed to double RNA splicing in SV40-infected cells [130]. In this case, the transcript encoding the 17-kDa antigen shows splicing of both introns, but splicing of the intron 1 by selection of the proximal 5′ ss. Similar to SV40, the multiple-spliced RNA species of early transcripts were detected also in other polyomaviruses such as truncated T-antigen (truncTAg) in BKPyV [131], T′135, T′136 and T′165 in JCPyV [132] and T3 and T4 early transcripts in MCPyV [127] (Fig. 38.14b). Alternative splicing of polyomavirus early transcripts allows expression of multiple T-antigens with distinguished function in viral life cycle. Beside the cells with actively replicating virus the early viral transcripts are expressed also in the cells

with nonproductive infection or in the polyomavirus-transformed cells. These cells often do not expressed late gene due to integration of viral DNA into host genome resulting in disregulated viral gene expression and cell transformation.

Papillomaviruses

Human papillomaviruses (HPVs) are a group of small DNA tumor viruses and has a genome in size of ~8-kb surrounded by a viral capsid. HPV genome consists of three regions: viral early, late, and noncoding regions and in general encodes eight viral genes (E1, E2, E4, E5, E6, E7, L1, and L2). Viral early gene products are regulatory proteins responsible for virus multiplication and pathogenesis during a productive infection, whereas L1 and L2 genes encode two viral capsid proteins for virus particle formation. Interestingly, almost all viral early genes are expressed from an early promoter upstream of viral E6 gene and are polyadenylated at an early poly (A) signal downstream of E5 gene. Thus, viral early gene transcripts are polycistronic, with several ORFs in a single RNA molecule, and undergo extensive alternative RNA splicing during viral RNA maturation. In contrast, viral L1 and L2 are commonly transcribed from E7 ORF and polyadenylated at a late poly (A) site downstream of L1 ORF (Fig. 38.15a). As a result, the 5′ sequences of viral L1 and L2 are part of the viral early transcript sequences. RNA splicing to removal the most of these early gene sequences from the RNA by RNA splicing is important for viral L1 and L2 expression [133].

HPVs are the etiological agent of cervical cancer and presumably of other anogenital cancers. HPV is present in >95 % of all cervical cancer and is required for initiation of cervical carcinogenesis and maintenance of the cervical cancer cells. Cervical cancer is a leading cause for women death in the developing world, with about 493,000 new cases and nearly 273,000 deaths each year (www.who.int/hpvcentre). More than 120 genotypes of HPVs have been identified to date and are grouped into two major groups according to their pathogenesis and association with cervical cancer [134]. The high-risk or oncogenic HPV types are present in cervical cancers, whereas low-risk or nononcogenic HPVs are not found in cervical or other anogenital cancers [135]. In general, women acquire HPV infection by sexual contact. Various epidemiology studies indicate that women with repeat exposure to oncogenic HPVs and with persistent cervical infection of oncogenic HPVs are in high risk to develop cervical cancer [136, 137]. Infection with oncogenic HPV-16 and HPV-18, two most common oncogenic HPV types, leads to development of almost 70 % of all cervical and other anogenital cancers. Viral E6 and E7 of oncogenic HPVs are two viral oncoproteins that inactivate, respectively, cellular p53 and pRB, two tumor suppressor proteins essential for cell cycle control [138, 139]. In cervical cancer tissues and cervical cancer-derived cell lines, E6 and E7 oncogenes are highly expressed and majority of the E6/E7 bicistronic RNA are alternatively spliced as diagramed for HPV-16 and HPV-18 (Fig. 38.15b). A major spliced RNA isoform of viral E6/E7 bicistronic RNA is E6*I derived from splicing of nt 226 5′ splice site to nt 409 3′ splice site for HPV-16 and of nt 233 5′ splice site to nt 416 3′ splice site



c

Primer	Position (nt)	Sequence	Amplicons (bp)
HPV16- Pr106	106-125	5'-GTTTCAGGACCCACAGGAGC-3'	DNA:(457), RNA:E6/E7 unspl. (457), E6*I/E7 (275), E6*II (158)
HPV16-Pr562	545-562	5'-TGATTACAGCTGGGTTTC-3'	
HPV18-Pr121	121-140	5'-ATCCAACACGGCGACCCTAC-3'	DNA: (730) RNA: E6/E7 unspl. (730), E6*I/E7 (548)
HPV18-Pr850	833-850	5'-CTGGAATGCTCGAAGGTC-3'	

Fig. 38.15 RNA splicing of viral oncogene E6 and E7 transcripts in high-risk human papillomavirus infections. **(a)** Genome structure of high-risk human papillomavirus type 16 divided by early (genes E1–E7, *open boxes*) and late (L1–L2, *grey boxes*) regions and positions of splice sites in HPV-16 genome. *P* promoter, pA_E early polyadenylation site, pA_L late polyadenylation site. (**b**) Alternative splicing of HPV-16 and HPV-18 E6–E7 regions. *Open boxes* represent E6 and E7 ORFs with their corresponding start and stop codon positions. Transcripts derived from promoter P97 have an intron (*dashes*) in the E6 and E7 ORF with three alternative 3′ splice sites as diagramed. *Filled black boxes* are exons. Coding potentials for each transcript are showed on the *left*. *Arrows* below the transcripts are the primers used for detection of spliced E6E7 transcripts detailed in (**c**). The diagrams are modified from Zheng and Baker [133] and Wang et al. [141]. **(c)** Sequences and nucleotide positions of two sets of primer pairs in (**b**) for RT-PCR amplification of alternatively spliced E6–E7 transcripts expressed in HPV-16 and -18 infections [140, 141]. Primer nucleotide positions and sequences are based on corresponding HPV reference strains available on http://pave.niaid.nih.gov

for HPV-18 (Fig. 38.15b). It has been demonstrated that this RNA splicing is necessary for viral E7 translation [140] and can be easily detected by RNase protection assay (RPA) or RT-PCR methods [140, 141].

The presence of high-grade premalignant lesions (CIN, cervical intraepithelial neoplasia) caused by oncogenic HPV infection is a sign of increased risk of developing cervical cancer. These lesions can be detected by routine cervical examination and treated by surgery to prevent progression to cervical cancer. Papanicolaou test (also called Pap smear) is a screening test used in gynecology to detect premalignant and malignant cells in cervical swabs. A woman who has Pap smear with abnormal cells may also be referred for HPV DNA testing by two FDA-approved assays: Hybrid Capture 2 DNA test to detect 13 high-risk HPVs (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) from Qiagen [142] or Cobas 4800 System to detect 14 high-risk HPVs (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) from Roche [143]. More recently, a few of HPV E6/E7 RNA tests have been introduced. APTIMA HPV Assay from Gen-Probe was designed to detect HPV E6/E7 mRNA from 14 high-risk types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68) [144] with a sensitivity and specificity similar to or better than Hybrid Capture 2 DNA test [145, 146]. PreTect HPV-Proofer from NorChip was designed to detect E6 and E7 RNA from HPV types 16, 18, 31, 33, and 45 [147, 148], with more specific than HC2 in identifying women with CIN 2+ but has a lower sensitivity [149]. By using the primers detailed in Fig. 38.15c for RT-PCR assays, the spliced E6/E7 RNAs of HPV-16 and HPV-18 can be detected easily based on the amplicon size smaller than E6/E7 DNA, without worry of carryover viral DNA contamination encountered by HPV DNA tests.

Herpesviruses

Herpesviruses are large DNA viruses with complex life cycle. Their relatively large linear double-stranded DNA (dsDNA) genome (~100–200-kb) is encapsulated in a capsid with icosahedral architecture. Capsid is covered with heterogeneous layer of viral proteins and RNAs called tegument. Outside of the tegument is a lipid bilayer membrane (envelope) containing several virus-encoded glycoproteins. A hallmark of herpesvirus infection is to establish life-long "latent" infection in their host following initial infection. Latent virus is often reactivated by various stimuli causing recurrent infections—a typical feature of all herpesviruses.

Currently there are more than 100 known herpesviruses infecting wide range of animal species. All human herpesviruses belong to *Herpesviridae* family which is further grouped into four subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, *Gammaherpesvirinae*, and unassigned viruses. As of today, eight herpesvirus species have been isolated from humans and they are assigned to three subfamilies of *Herpesviridae*. These include the following: herpes simplex virus type 1 [HSV-1, also referred as human herpesvirus 1 (HHV1)], herpes simplex virus type 2 (HSV-2 or HHV2), Varicella-zoster virus (VZV or HHV3), Epstein-Barr virus (EBV or

HHV4), human cytomegalovirus (CMV or HCMV or HHV5), human herpesvirus 6 (HHV6), human herpesvirus 7 (HHV7), and Kaposi sarcoma-associated herpesvirus (KSHV or HHV8).

Herpesviruses replicate in the nucleus. After virus entry the viral genome is translocated to the nucleus of infected cells where expression of viral genes occurs. All herpesviruses have two types of viral life cycle, latent and lytic, with distinctive transcriptional profile. Latent infection is characterized with the expression of a few viral genes (latent transcripts) to maintain viral genome in latently infected cells. Lytic infection is associated with viral genome replication and production of infectious virions and generally leads to destruction of infected cell. In contrast to latent infection almost all of viral lytic genes in viral lytic infection are expressed in timely regulated fashion and are divided, based on their dependence on viral protein expression and viral genome replication, into three kinetic classes: immediate early, early, and late. At some circumstances the virus in latently infected cells may be reactivated and proceeded to lytic infection. The mechanisms controlling the establishment of latency and reactivation of herpesviruses are not fully understood. Herpesviral genome encodes up to 100 different genes including a variable number of noncoding genes for noncoding RNAs or viral miRNAs [150–152].

Most human herpesviruses are highly prevalent in general population. Initial infection generally occurs in childhood or early adolescence through body contact and follows by establishment of latent infection. Some herpesviruses are sexually transmitted. Blood transfusion, tissue transplantation or congenital transmission represents other ways of acquiring the virus. Primary infection often occurs at epithelia, the point of entry, followed by establishment of latent infection generally in a specialized cell type (neurons or lymphocytes) which serves as a virus reservoir. Recurrence of infection is caused by virus reactivation from latency when virus escapes from host immunological surveillance. Overall symptoms of herpesvirus infections in healthy individuals are generally mild, but may be life threatening in immunocompromised patients. While infections of some herpesviruses such as EBV and KSHV are clearly etiologically linked to the development of several types of cancer, the role of other human herpesviruses in cell transformation remains unknown [153]. Several compounds are used to treat acute herpesvirus infection. No vaccine against herpesviruses is currently available.

The infections by herpesviruses are most commonly diagnosed by the presence of specific antibodies and antigens or by detection of viral DNA by PCR. However, without quantification in multiple time points these techniques cannot distinguish virus carriers from patients with active virus replication. Detection of viral transcripts associated with virus lytic phase by RT-PCR provides indication of active virus replication, but often leads to a false-positive result due to viral DNA contamination. DNA contamination problem could be avoided by selection of an amplicon over the intron in spliced viral transcripts, a specific product of the spliced RNA could be distinguished from its corresponding to DNA based on its size. The number of spliced viral transcripts varies from one herpesvirus to another, with only handful split genes in HSV-1 to about 30 % in KSHV [154]. Both latent and lytic genes could have an intron and sometimes are alternatively spliced.

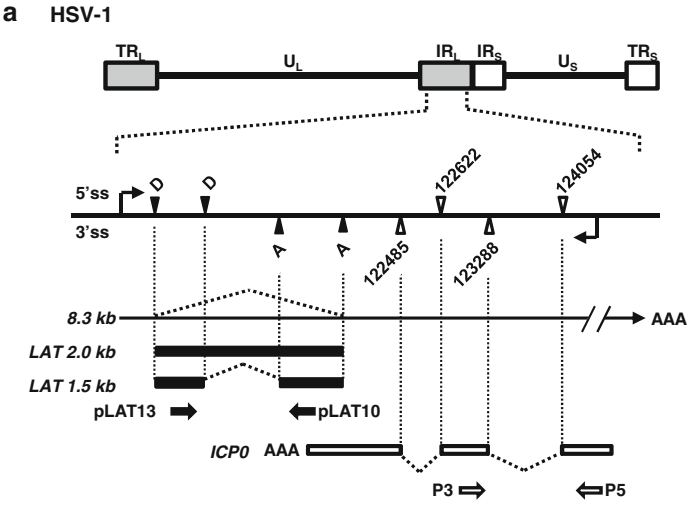
Herpes Simplex Viruses

HSV-1, a member of *Alphaherpesvirinae* subfamily, is an human neurotropic herpesvirus associated with "herpes labialis," Bell's palsy and vestibular neuritis [155]. After initial infection, HSV-1 establishes the latent infection in sensory neural ganglia from where it periodically reactivates. Viral genome consists of two unique regions (long and short) flanked with inverted repeat regions (internal or terminal) (Fig. 38.16a). HSV-1 encodes at least 84 genes. The majority of gene are named based on their position within a particular part of viral genome like UL1 (unique long region ORF1) or US3 (unique short region ORF3) while others have alternative historical manes, like ICP0 (infected cells protein 0). Only few HSV-1 transcripts are spliced (LAT, ICP0, UL15, US1, US12/ICP47) (see review ref. [156]) including both latent and lytic transcripts.

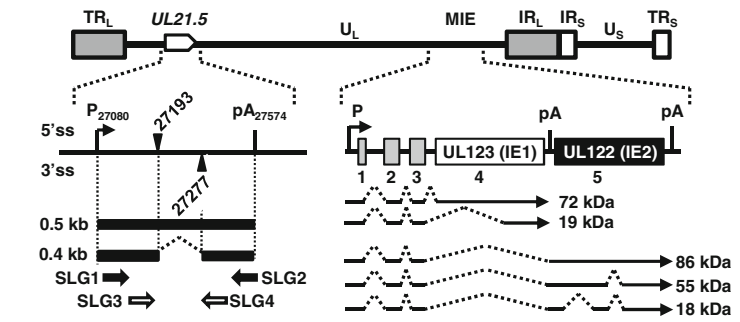
During latency HSV-1 expressed LAT (*l*atency-*a*ssociated *t*ranscript) RNA from a repeat region of viral genome called LAT-DNA [157, 158]. Two forms of LAT RNAs are detectable in latently infected neurons. A major 2.0-kb RNA is produced by splicing of a capped and polyadenylated 8.3-kb primary transcript and represents a unique stable intron while spliced exonic RNA is unstable and quickly degraded [159]. A minor 1.5-kb RNA is generated by further splicing of the 2.0-kb RNA by removing an intron of 559 or 556-bp depending on the virus strain [160] (Fig. 38.16a). Both LAT RNAs are uncapped without a poly A tail and accumulates in the nucleus of infected cells. HSV-1 LAT RNA is a noncoding regulatory RNA for establishment and maintenance of viral latency by inhibiting expression of viral lytic genes and interfering with cellular apoptosis pathway [161]. Recent studies showed that LAT transcript functions as a precursor for generation of virus-encoded miRNAs [162]. Expression from LAT-DNA was observed also during lytic infection. Lytic LAT transcripts differ from latent LAT RNA by the presence of a poly A tail [163].

ICP0 (IE110) is encoded by a gene located in a viral genome repeat region and partially overlaps with LAT transcripts. Antisense expression of LAT transcripts inhibits the expression of ICP0 during latency. ICP0 is an immediate early gene expressed in the early stage of lytic infection. ICP0 functions as a nonspecific transactivator and a cofactor of another viral transactivator ICP4 [164]. ICP0 initiates lytic replication in both newly infected cells as well as after reactivation in cells with latent infection. ICP0 is transcribed in reverse orientation from viral genome and its pre-mRNA contains three exons separated by two introns [165] (Fig. 38.16a). After splicing, the mature mRNA encodes ICP0 protein with 775 aa residues. An alternatively spliced ICP0 transcript retaining intron 2 is detectable in the infected cells [166] and encodes a truncated ICP0R in size of 262-aa residues due to the presence of a stop codon in the intron 2. Thus, both ICP0 and ICP0R have the same aa sequences in the N-terminal part. ICP0R functions as a repressor of viral expression [167].

HSV-2 represents another important human pathogen belonging to the alpha herpesvirus subfamily. Genital infection with HSV-2 causes genital herpes which is considered as a sexually transmitted disease. HSV-2 is also neurotropic and establishes latent infection in sacral ganglia. HSV-1 and HSV-2 are two closely related viruses with a similar genome and gene structures including their LAT and ICP0 regions [168].



Primer	Position (nt)	Sequence	Amplicons (bp)
pLAT10	120408-120389	5'-ATGGAGCCAGAACCACAGTG-3'	DNA: (947)
pLAT13	119464 -119484	5'-GTAGGTTAGACACCTGCTTCT-3'	RNA: LAT 2.0 kb (947), LAT 1.5 kb (297)
P3	123214-123233	5'-TTCGGTCTCCGCCTGAGAGT-3'	DNA: (922)
P5	124116-124135	5'-GACCCTCCAGCCGCATACGA-3'	RNA: ICP0 unspl. (922), ICP0 spl. 1.5 kb (157)



Primer	Position (nt)	Sequence	Amplicons (bp)
SLG1	27120-27139	5'-CTATGGATCTTGAGCTTACT-3'	
SLG2	27410-27429	5'-TCGCTGCCATCTCCGTCTGT-3'	DNA: (258)
SLG3	27144-27144	5'-GTGACCTTGACGGTGGCTTT-3'	RNA: unsliced (258) spliced (175)
SLG4	27382-27401	5'-CGTCATACTCCCCGGAGTAA-3'	

Fig. 38.16 RNA splicing of LAT and ICP0 transcripts in HSV-1 infections and UL21.5, UL122, and UL123 transcripts in HCMV infections. **(a)** Genome of herpes simplex virus type 1 (HSV-1) consists of two unique regions (long-U_L and short-U_S) flanked with terminal (TR) and internal (IR) repeats.

In general the infection of HSV-1 and -2 is controlled by host immune system. Thus initial or recurrent infections are associated with only mild symptoms. Infection in immunocompromised patients could cause several severe diseases including encephalitis [169]. Genital infections or reactivation of HSV-2 during pregnancy could lead to congenital infection [170]. Detection of viral DNA may not provide sufficient information about virus replication status due to the permanent presence of viral DNA in the infected cells. Detection viral lytic products, such as spliced ICP0 RNA, may be a better predictor of virus reactivation even before occurrence of clinical symptoms for early diagnosis enabling of early treatment. Disappearance of the detectable lytic products could be a sign of treatment efficiency since the viral transcripts disappear earlier than viral DNA.

Human Cytomegalovirus

Human cytomegalovirus (HCMV) together with HHV-6 and HHV-7 belongs to *Betaherpesvirnae*. High prevalence of CMV infection has been noticed in 50–80 % human population. In majority of healthy individuals, the primary CMV infection occurs asymptomatically, but in some cases could be associated with sore throat, prolong fever or syndrome similar to infectious mononucleosis. After initial infection the virus remains latent in T cells for the rest of the host life without apparent symptoms. In contrast, the CMV infection in immunocompromised individuals, such as newborns, transplant recipients, people with AIDS or cancer patients, could lead to severe disease and even death. The symptoms include hepatitis, retinitis, colitis, pneumonia, encephalitis and others.

CMV has a large genome of about 220 kb capable to encode around 200 genes (reviewed in ref. [171]). While the majority of CMV transcripts are intronless, the presence of several split genes has been identified in all kinetic classes of viral genes [172]. A major immediate early region (MIE) located within a unique long (UL) region of CMV genome contains several genes highly expressed at the early stage of viral lytic infection. These include UL123 (IE1), UL122 (IE2), and UL119-115. MIE transcripts contain multiple introns and undergo complex alternative RNA

Fig. 38.16 (continued) Shown below are two representatives of HSV-1 RNA splicing, latency associated transcript (LAT) and immediate-early ICP0 transcripts with positions of 5′ and 3′ splice sites, and primer pairs (*arrows below*) used for detection of spliced transcripts as detailed in the table further below. *Boxes* (full for LAT and empty for ICP0) represent exons divided by introns (*dashed lines*). The primer pairs in the table were described by Tanaka et al. [214], with nucleotide positions in HSV-1 genome (GenBank Accs. No. X14112.1). (**b**) Genome structure of human cytomegalovirus (see other details in (**a**)). Shown below the genome structure are spliced transcripts of late UL21.5 gene and two immediate early genes UL123 (IE1) and UL122 (IE2) expressed from a major immediate early region (MIE). *Boxes* or *solid lines* are exons and *dashes* are introns or splicing directions. *P* promoter, *pA* polyadenylation site. *Arrows* below UL21.5 transcripts are primer pairs used for detection of spliced UL21.5 transcripts by nested RT-PCR as described [174] and detailed in the table below, with the nucleotide positions in HCMV genome (strain AD169, GenBank Accs. No. X17403.1)

splicing. MIE transcripts IE1 and IE2 are expressed from the same promoter, but alternatively polyadenylated. These transcripts have five major exons and can be alternatively spliced to express additional isoforms of IE1 and IE2 proteins (Fig. 38.16b). Splicing was detected also in transcripts from other CMV genes such as TRL4, UL89, US3, R160461, and R27080 [172]. Gene UL21.5 (previously named as R27080) is one of the known CMV split late genes (SLG). The UL21.5 transcript which encodes viral glycoprotein is expressed from the UL region posited from nt 27,080 to nt 27,574 of CMV genome [173], and has a short intron with 83 nts. Removal of this intron leads to production of a mature mRNA in size of ~0.4-kb (Fig. 38.16b). Both spliced and unspliced UL21.5 RNAs are easily detectable by RT-PCR from infected cells [174].

Allogenic bone marrow transplant recipients are in high risk to develop CMV diseases. Historically, viremia was used as an indicator of CMV disease to guide preemptive treatment. Multiple approaches have been developed to detect CMV viremia in circulating lymphocytes by direct virus isolation with cultivation or by detection of viral antigens or viral DNA [175, 176]. However, the detection of viremia is not sufficient in disease prediction since many viremic patients never develop symptoms. An active CMV replication in peripheral blood lymphocytes can be verified by analyzing viral mRNAs [177]. Amplification of spliced viral transcripts has some advantages in comparison to intronless transcripts, with no worry by DNA contamination. Detection of spliced immediate early transcripts had been reported in good correlation with detection of viral DNA or viral antigen [178–180]. Detection of late gene UL21.5 has better prediction value and significantly correlates with disease progression [174, 181, 182].

Epstein-Barr Virus

Epstein-Barr virus (EBV), a well-characterized member of *Gammaherpesvirinae* subfamily, is an important human pathogen. EBV infection is highly prevalent, with more than 95 % of human population become seropositive in early life. While primary infection during childhood is unremarkable, the virus acquisition in adolescence and adulthood is often associated with the development of infectious mononucleosis. In healthy individuals the EBV infection is well controlled by immune system. However, EBV remains in long-living memory B cells where it establishes a latent infection. EBV is an oncogenic virus capable to transform the infected B cells [183]. EBV infections have been associated with the development of several human malignancies, including nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's lymphoma, gastric carcinoma, and others (see review ref. [184]). Active EBV replication due to immunosuppression may cause posttransplant lymphoproliferative disease [185]. During latent infection, EBV expresses six nuclear antigens (EBNAs-1, -2, -3A, -3B, -3C, and -LP), three latent membrane proteins (LMPs-1, -2A, and -2B) and several noncoding transcripts (EBERs-1 and -2 and BARTs). Many EBV latent products are defined as oncogenes responsible

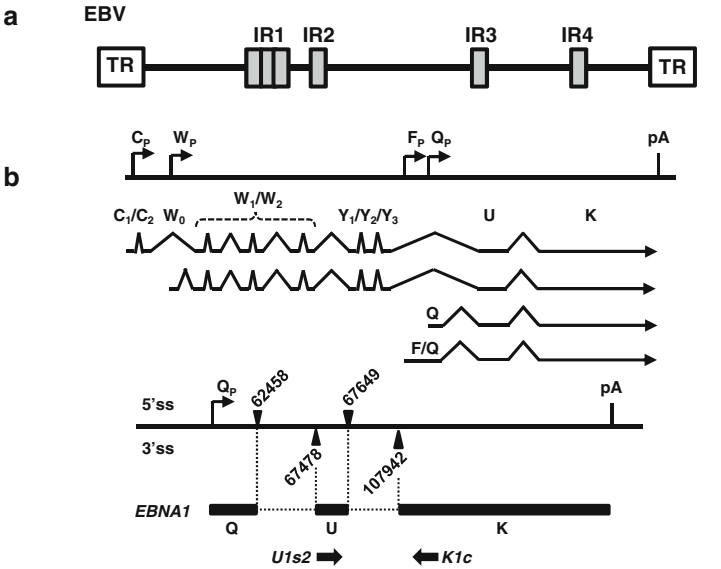
for EBV-mediated cell transformation [115]. Several types of EBV latency were defined by variable expression of latent genes in various malignancies [186].

EBV genome (~172-kb) is flanked by multiple terminal repeats (TR) and can be divided into a long and a short unique region (U_L and U_S) by internal repeat 1 (IR1) (Fig. 38.17a). EBV encodes at least 80 viral proteins [187] and several noncoding RNAs including viral miRNAs [188, 189]. The ORF names are derived from their positions in *Bam*HI fragment (from A to Z) by orientation (L-left or R-right) and a digital number representing the frame (F) order (for example BZLF1). Other genes retain their historical names based on the gene product function. Number of split genes in EBV is significantly higher than that found in alpha- and beta-herpesviruses. Extensive alternative RNA splicing is prominent especially for almost all EBV latent transcripts, but the transcripts of many lytic genes are also spliced.

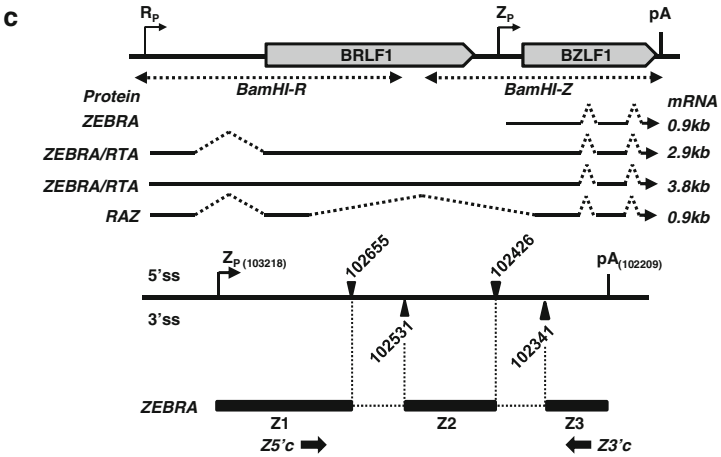
EBNA-1 is a multifunctional viral protein critical for establishing and maintaining EBV latency and for regulation of viral promoter activities [190]. In infected cells, EBNA-1 is expressed from a spliced mRNA derived from a primary transcript in size of ~100 kb. This transcript is originated from one of two alternative promoters, Cp or Wp, which are named by their localization in a particular *Bam*HI fragment of viral genome (Fig. 38.17b). At the early stage of latent infection, the Wp is initially used, but the expressed EBNA1 and EBNA2 from the Wp transactivate the Cp promoter and cause a switch of transcription from Wp to Cp [191]. Usage of Cp promoter is associated with EBV "latency type III." In Burkitt's lymphoma and Burkitt's lymphoma-derived cell lines, EBNA1 expression is initiated from a distal Qp promoter rather than from Cp and Wp and is associated with "latency type I" [192]. EBNA is expressed also in the lytic phase from additional Fp promoter closely localized upstream to the Qp promoter [193].

The establishment of active EBV replication after virus reactivation from latency is dependent on the expression of two immediate-early genes BZLF1 and BRLF1 [194] to encode viral transactivators ZEBRA (BZLF1) and RTA (BRLF1). Although BRLF1 and BZLF1 are transcribed separately from a different promoter with the Rp for BRLF1 and the Zp for BZLF1, both gene transcripts utilize the same polyadenylation site for RNA polyadenylation [195] (Fig. 38.17c). Thus, the Zp promoter transcript is a monocistronic ZEBRA RNA containing two constitutive introns and splicing of these two introns results in production of a 0.9-kb mRNA to encode ZEBRA protein. Transcription from the Rp promoter leads to produce a 3.8-kb bicistronic transcript, ZEBRA/RTA, which contains two additional introns over the ZEBRA RNA. Splicing of the intron 1 in the 5′ noncoding region of ZEBRA/RTA transcript leads to production of a 2.9-kb RNA as a major RNA isoform. However, both isoforms of ZEBRA/RTA RNA have a potential to encode ZEBRA and RTA proteins. A third, minor isoform of ZEBRA/RTA transcript is derived from splicing of additional internal intron spanning over BRLF1 ORF to BZLF1 ORF and this splicing produces a RAZ transcript in size of ~0.9-kb to encode a RTA-ZEBRA fusion protein, RAZ. RAZ may function as an inhibitor to ZEBRA during EBV infection [196].

Transcripts for EBNA-1 are believed to be expressed in all forms of EBV latent infection except of latently infected nondividing B-cells with "latency type 0."



Primer	Position (nt)	Sequence	Amplicons (bp)
U1s2	67595-67614	5'-ATAGACCGCCAGTAGACCTG-3'	DNA: (~40kb)
K1c	108064-108045	5'-GGTTATCACCCCCTCTTCTT-3'	RNA: EBNA1 unspliced (~40kb), EBNA1 spliced (178)



Primer	Position (nt)	Sequence	Amplicons (bp)
Z5'c	102676-102657	5'-GAAACCACAACAGCCAGAAT-3'	DNA: (457)
Z3'c	102220-102239	5'-AAGAGATCCTCGTGTAAAAC-3'	RNA: ZEBRA unspliced (457), ZEBRA spliced (250)

Fig. 38.17 RNA splicing of EBNA1 and ZEBRA transcripts in EBV infections. **(a)** Organization of EBV genome with terminal repeats (TR) and internal repeats (IR1-4). (**b**) Multiple transcripts of EBV latency-associated EBNA1 transcribed from several alternative promoters (C_p-Q_p).

This makes the detection of EBNA-1 expression as a good marker for the presence of EBV in tumors. The expression of ZEBRA during lytic infection could be used to monitor a productive EBV infection and EBV reactivation.

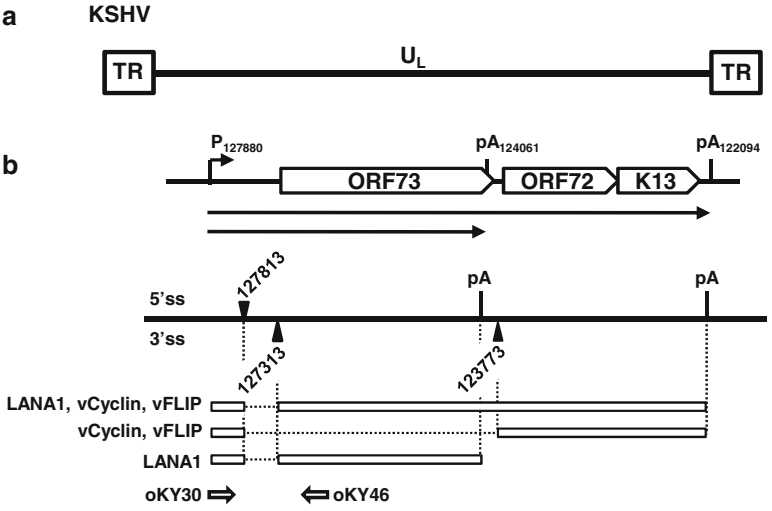
Kaposi Sarcoma-Associated Herpesvirus

Kaposi sarcoma-associated herpesvirus (KSHV) is the latest discovered human herpesvirus [197]. After primary infection, KSHV establishes latent infection in endothelial cells and B cells [198]. In healthy individuals both primary and latent KSHV infections are generally asymptomatic. Suppression of immune system in KSHV-positive individuals, such as AIDS patients or tissue transplant recipients, is associated with the development of several cancers including all forms of Kaposi sarcoma (a solid tumor of endothelial origin) or B-cell lymphomas [primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD)] (see review ref. [199]). Presence of viral genome and expression of viral-encoded products in all cancer cells support active roles of KSHV in cell transformation.

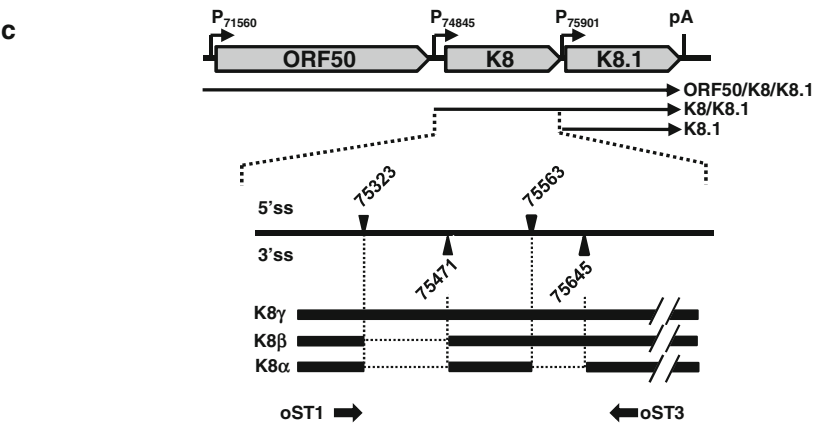
KSHV belongs to *Gammaherpesvirinae* and has similar genome organization as EBV with long unique region flanked with terminal repeats (Fig. 38.18a). KSHV genome (~165 kb) encodes up to 90 genes named by their position in viral genome from left to right (for example ORF47) [200]. Some genes are designated with digital K number, like K2, while some have alternative names based on their function (ORF57 or MTA-mRNA transcript accumulation). KSHV transcripts derived from ~30 % viral genes, including both latent and lytic genes, undergo RNA splicing [154].

During latency, KSHV genome expresses a latency-associated nuclear antigen-1 (LANA-1) [201] from ORF73. The gene ORF73 posits along with ORF72 and K13 in a larger latent locus of the virus genome. The latter two genes encode viral homologues of cellular proteins vCyclin (ORF72) and vFLICE (K13). ORF73/72/K13 are transcribed from a single promoter (P_{127880}) as a tricistronic RNA which has an intron containing two alternative 3′ splice site. Alternative RNA splicing and alternative

Fig. 38.17 (continued) EBNA-1 RNA contains multiple exons (C_1-K, *lines*) and introns (*half triangles*). A detailed EBNA-1 transcript derived from the Q_p promoter is shown below with splice sites (*black triangles*) and splice directions (*dashed lines* for introns). A primer pair used to detect the spliced EBNA1 transcript from exon U to exon K [185] are detailed in the table below. **(c)** Gene structures of BRLF1 (RTA) and BZLF1 (ZEBRA) (two EBV-immediate early genes) and their spliced RNA products. EBV RTA and ZEBRA are transcribed by two alternative promoters, R_P and Z_P, but polyadenylated by using the same polyadenylation signal downstream of ZEBRA ORF. Thus, the bicistronic RTA transcript derived from Rp promoter contains multiple introns and has potentials to encode RTA, ZEBRA, and RAZ proteins by alternative RNA splicing, whereas the monocistronic ZEBRA transcript derived from the Zp promoter encodes only ZEBRA protein and also contains multiple introns as detailed further below with nucleotide positions of splice sites, exons (Z1–Z3, *black boxes*) and introns (*dashed lines*). *Arrows* below exons Z1 and Z3 are a primer pair used for detection of spliced ZEBRA mRNA [185] and detailed in the table, with nucleotide positions in EBV genome (strain B95-8, GenBank Accs. No. V01555.2)



Primer	Position (nt)	Sequence	Amplicons (bp)
oKY30	127828-127846	5'-AGAGCAGCAGCTTGGTCCG-3'	DNA: (952)
oKY46	126877- 126895	5'-CTGGAGACTGCGTGGGTGG-3'	RNA: LANA1 unspliced (952), LANA1 spliced (453)



Primer	Position (nt)	Sequence	Amplicons (bp)
oST1	75182-75200	5'-ACCACCAAGAGGACCACACATTTC-3'	DNA:(657)
oST3	75813-75838	5'-CACACAAAGTCTGGCATGGTTCTCCC-3'	RNA: K8γ (657) K8β (510), K8α (428)

Fig. 38.18 RNA splicing of representative latent transcripts and early transcripts in KSHV infections. **(a)** Genome of KSHV contains a long unique region (U_L) flanked by terminal repeats (TR). **(b)** Gene structure of a latent gene locus containing ORF73/ORF72/K13 genes. Three genes are

RNA polyadenylation of the tricistronic pre-mRNA results in production of three mature mRNAs (5.4-, 3.3-, and 1.7-kb) [202] (Fig. 38.18b). The 5.4-kb transcript most likely for LANA-1 expression is produced by usage of the proximal 3′ splice site, whereas usage of the distal 3′ splice site leads to express the 1.7-kb transcript for vCyclin and vFLICE. Both transcripts are polyadenylated at the same distal polyadenylation site. The minor 3.3-kb transcript use the proximal splice site for RNA splicing, but is polyadenylated at a proximal noncanonical polyadenylation signal (Fig. 38.18b).

KSHV lytic replication is controlled by a major viral transactivator, ORF50 (also referred as Rta) [203, 204]. Similarly to LANA1, ORF50 posits along with K8 and K8.1 in a larger gene locus (ORF50/K8/K8.1 cluster) (Fig. 38.18c) and is expressed as an immediately early transcript during lytic virus replication. K8 encoding a viral k-bZIP protein is an early gene and K8.1 encoding a glycoprotein is a late gene. Although each of the three genes bears its own promoter, all of their RNA transcripts use a single polyadenylation site located downstream of K8.1 gene and undergo alternative RNA splicing (see review ref. [154]). Thus, the 3′ portion of ORF50 transcript is homologous to K8 and K8.1 and has the same intron and exon structures as seen in K8 and K8.1 transcripts. The ORF50 transcript is tricistronic, K8 is bicistronic, and K8.1 is monocistronic in nature. The bicistronic K8 full transcript composes of four exons separated by three introns (Fig. 38.18c). A functional K8α protein is expressed from a fully spliced mRNA, but retention of the intron 2 in K8β mRNA results in the expression of a minor form K8β protein [205]. An unspliced K8 RNA, K8γ, is also detectable, but rare in lytically infected cells.

In summary, LANA-1 expression is a hallmark of KSHV latent infection. Transcripts originated from the ORF73/72/K13 gene cluster are expressed in latently infected, KSHV-transformed cells and are detectable by RT-PCR. Active virus replication is associated with the expression of viral lytic genes. Amplification of spliced K8 region which detects the expression of both ORF50 tricistronic and K8 bicistronic transcripts could be used to monitor viral lytic replication [206].

Fig. 38.18 (continued) transcribed from a single promoter (P) as a polycistronic RNA. This polycistronic primary transcript is processed by alternative RNA splicing and alternative polyadenylation (pA, see *arrows* below). Nucleotide positions of splice sites (*triangles*) and polyadenylation site in the KSHV genome (GenBank Accs. No. U75698.1) are diagramed further below with coding potentials of each spliced product on the left. *Open boxes* are exons and *dashed lines* are introns or splicing directions. oKY30 and oKY46 are a primer pair used to detect spliced LANA transcripts [215] as detailed in the table below. **(c)** Gene structures of a KSHV lytic locus consisting of immediate early RTA (ORF50), early K8 and late K8.1 genes. Three genes are expressed from three separate promoters (P), but all of their transcripts (*full lines with arrows* immediately below to the right) are polyadenylated at the same polyadenylation site (pA), resulting in RTA as a tricistronic, K8 as a bicistronic, and K8.1 as a monocistronic transcript. The enlarged K8 coding region contains three exons (*filled boxes*) and two introns (*dashed lines*), with nucleotide positions of each splice site in the KSHV genome (GenBank Accs. No. U75698.1). Names of three common forms of K8 transcripts from alternative RNA splicing are shown on the left. oST1 and oST3 are two primers used to detect spliced K8 RNAs as described [215, 216] and are detailed in the table below

Conclusion

The major aim of this chapter is to provide our readers with knowledge of viral RNA splicing during viral infection and the detection of spliced viral RNA transcripts as a new approach in diagnostic virology. In the first part, we provided basic information about the mechanisms of RNA splicing and methodological approaches of specific detection of spliced RNA molecules. The core of these techniques represents an amplification and detection of nucleic acids. The advantage of nucleic acid-based techniques is the application of the same platform for detection various viral pathogens often in the same time by multiplexing. The rapid setup of these methods is especially important for quick response to emerging viruses as has recently been proven in the case of SARS and avian flu pandemic, when nucleic acid amplification was rapidly employed to detect and to confirm the infection. The less material requirement and simplicity make these detection methods suitable for applications in low resources setting such laboratories of the first contact and field laboratories. Because amplification of nucleic acid molecules as a routine in many diagnostic laboratories is used to detect the genomic sequences from many viruses, the detection of spliced viral transcripts could be performed simultaneously to already existing methods.

The second part of the chapter summarized our current knowledge about viral RNA splicing events in the majority of known human viruses. We also included some viral agents, such as human circoviruses and adeno-associated viruses, where a direct link between infection and pathological manifestation remains to be determined. In addition, we have provided examples of each virus where the detection of spliced viral RNA could bring additional benefit to current techniques to improve the disease prognosis or better monitoring of efficiency of therapeutic intervention. We believe that systematic study of RNA splicing events during viral infection could lead to better viral diagnosis, better therapy management, and eventually leading to our better understanding of pathogenesis of human viral pathogens.

References

1. Wiedbrauk DL (2009) Nucleic acid amplification and detection methods. In: Specter S, Hodinka RL, Young SA, Wiedbrauk DL (eds) Clinical virology manual, 4th edn. ASM Press, Washington, DC, pp 156–184
2. Berget SM, Moore C, Sharp PA (1977) Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. Proc Natl Acad Sci U S A 74(8):3171–3175
3. Chow LT, Gelinas RE, Broker TR, Roberts RJ (1977) An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. Cell 12(1):1–8
4. Fong N, Bentley DL (2001) Capping, splicing, and 3′ processing are independently stimulated by RNA polymerase II: different functions for different segments of the CTD. Genes Dev 15(14):1783–1795
5. Maniatis T, Reed R (2002) An extensive network of coupling among gene expression machines. Nature 416(6880):499–506
6. Tarn WY, Steitz JA (1996) A novel spliceosome containing U11, U12, and U5 snRNPs excises a minor class (AT-AC) intron in vitro. Cell 84(5):801–811

7. Wu Q, Krainer AR (1999) AT-AC pre-mRNA splicing mechanisms and conservation of minor introns in voltage-gated ion channel genes. Mol Cell Biol 19(5):3225–3236
8. Wachtel C, Manley JL (2009) Splicing of mRNA precursors: the role of RNAs and proteins in catalysis. Mol Biosyst 5(4):311–316
9. Toor N, Rajashankar K, Keating KS, Pyle AM (2008) Structural basis for exon recognition by a group II intron. Nat Struct Mol Biol 15(11):1221–1222
10. Chasin LA (2007) Searching for splicing motifs. Adv Exp Med Biol 623:85–106
11. Long JC, Caceres JF (2009) The SR protein family of splicing factors: master regulators of gene expression. Biochem J 417(1):15–27
12. Han SP, Tang YH, Smith R (2010) Functional diversity of the hnRNPs: past, present and perspectives. Biochem J 430(3):379–392
13. Kornblihtt AR (2007) Coupling transcription and alternative splicing. Adv Exp Med Biol 623:175–189
14. Luco RF, Allo M, Schor IE, Kornblihtt AR, Misteli T (2011) Epigenetics in alternative pre-mRNA splicing. Cell 144(1):16–26
15. Zheng ZM (2004) Regulation of alternative RNA splicing by exon definition and exon sequences in viral and mammalian gene expression. J Biomed Sci 11(3):278–294
16. Wang ET, Sandberg R, Luo S et al (2008) Alternative isoform regulation in human tissue transcriptomes. Nature 456(7221):470–476
17. Croft L, Schandorff S, Clark F, Burrage K, Arctander P, Mattick JS (2000) ISIS, the intron information system, reveals the high frequency of alternative splicing in the human genome. Nat Genet 24(4):340–341
18. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 4(6):357–362
19. Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14(3):303–308
20. Whitcombe D, Theaker J, Guy SP, Brown T, Little S (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol 17(8):804–807
21. Hughes TR, Hiley SL, Saltzman AL, Babak T, Blencowe BJ (2006) Microarray analysis of RNA processing and modification. Methods Enzymol 410:300–316
22. Cuperlovic-Culf M, Belacel N, Culf AS, Ouellette RJ (2006) Microarray analysis of alternative splicing. OMICS 10(3):344–357
23. Pinzani P, Orlando C, Pazzagli M (2006) Laser-assisted microdissection for real-time PCR sample preparation. Mol Aspects Med 27(2–3):140–159
24. Esposito G (2007) Complementary techniques: laser capture microdissection—increasing specificity of gene expression profiling of cancer specimens. Adv Exp Med Biol 593:54–65
25. Niedobitek G, Herbst H (2006) In situ detection of Epstein-Barr virus and phenotype determination of EBV-infected cells. Methods Mol Biol 326:115–137
26. Knoll JH, Lichter P, Bakdounes K, Eltoum IE (2007) In situ hybridization and detection using nonisotopic probes. Curr Protoc Mol Biol Chapter 14: Unit 14.7
27. Speel EJ, Hopman AH, Komminoth P (2006) Tyramide signal amplification for DNA and mRNA in situ hybridization. Methods Mol Biol 326:33–60
28. Yamamichi N, Shimomura R, Inada K et al (2009) Locked nucleic acid in situ hybridization analysis of miR-21 expression during colorectal cancer development. Clin Cancer Res 15(12):4009–4016
29. Bonvicini F, Mirasoli M, Gallinella G, Zerbini M, Musiani M, Roda A (2007) PNA-based probe for quantitative chemiluminescent in situ hybridisation imaging of cellular parvovirus B19 replication kinetics. Analyst 132(6):519–523
30. Kang JG, Majerciak V, Uldrick TS et al (2011) Kaposi's sarcoma-associated herpesviral IL-6 and human IL-6 open reading frames contain miRNA binding sites and are subject to cellular miRNA regulation. J Pathol 225(3):378–389
31. Blanco AM, Artero R (2010) A practical approach to FRET-based PNA fluorescence in situ hybridization. Methods 52(4):343–351

32. de Planell-Saguer M, Rodicio MC, Mourelatos Z (2010) Rapid in situ codetection of noncoding RNAs and proteins in cells and formalin-fixed paraffin-embedded tissue sections without protease treatment. Nat Protoc 5(6):1061–1073
33. Su Z, Ning B, Fang H et al (2011) Next-generation sequencing and its applications in molecular diagnostics. Exp Rev Mol Diagn 11(3):333–343
34. Nakamura S, Yang CS, Sakon N et al (2009) Direct metagenomic detection of viral pathogens in nasal and fecal specimens using an unbiased high-throughput sequencing approach. PLoS One 4(1):e4219
35. Davis CA, Grate L, Spingola M, Ares M Jr (2000) Test of intron predictions reveals novel splice sites, alternatively spliced mRNAs and new introns in meiotically regulated genes of yeast. Nucleic Acids Res 28(8):1700–1706
36. Lander ES, Linton LM, Birren B et al (2001) Initial sequencing and analysis of the human genome. Nature 409(6822):860–921
37. Bouvier NM, Palese P (2008) The biology of influenza viruses. Vaccine 26(Suppl 4): D49–D53
38. Lamb RA, Horvath CM (1991) Diversity of coding strategies in influenza viruses. Trends Genet 7(8):261–266
39. Lamb RA, Choppin PW (1979) Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proc Natl Acad Sci U S A 76(10):4908–4912
40. Lamb RA, Lai CJ (1980) Sequence of interrupted and uninterrupted mRNAs and cloned DNA coding for the two overlapping nonstructural proteins of influenza virus. Cell 21(2):475–485
41. Briedis DJ, Lamb RA (1982) Influenza B virus genome: sequences and structural organization of RNA segment 8 and the mRNAs coding for the NS1 and NS2 proteins. J Virol 42(1):186–193
42. Nakada S, Graves PN, Palese P (1986) The influenza C virus NS gene: evidence for a spliced mRNA and a second NS gene product (NS2 protein). Virus Res 4(3):263–273
43. Lamb RA, Lai CJ, Choppin PW (1981) Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proc Natl Acad Sci U S A 78(7):4170–4174
44. Shih SR, Suen PC, Chen YS, Chang SC (1998) A novel spliced transcript of influenza A/WSN/33 virus. Virus Genes 17(2):179–183
45. Chiang C, Chen GW, Shih SR (2008) Mutations at alternative 5′ splice sites of M1 mRNA negatively affect influenza A virus viability and growth rate. J Virol 82(21):10873–10886
46. Yamashita M, Krystal M, Palese P (1988) Evidence that the matrix protein of influenza C virus is coded for by a spliced mRNA. J Virol 62(9):3348–3355
47. Pekosz A, Lamb RA (1998) Influenza C virus CM2 integral membrane glycoprotein is produced from a polypeptide precursor by cleavage of an internal signal sequence. Proc Natl Acad Sci U S A 95(22):13233–13238
48. Hongo S, Sugawara K, Muraki Y et al (1999) Influenza C virus CM2 protein is produced from a 374-amino-acid protein (P42) by signal peptidase cleavage. J Virol 73(1):46–50
49. Stoltzfus CM (2009) Chapter 1. Regulation of HIV-1 alternative RNA splicing and its role in virus replication. Adv Virus Res 74:1–40
50. Schwartz S, Felber BK, Benko DM, Fenyo EM, Pavlakis GN (1990) Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J Virol 64(6):2519–2529
51. Tazi J, Bakkour N, Marchand V, Ayadi L, Aboufirassi A, Branlant C (2010) Alternative splicing: regulation of HIV-1 multiplication as a target for therapeutic action. FEBS J 277(4): 867–876
52. Landry S, Halin M, Lefort S et al (2007) Detection, characterization and regulation of antisense transcripts in HIV-1. Retrovirology 4:71
53. Fischer U, Pollard VW, Luhrmann R et al (1999) Rev-mediated nuclear export of RNA is dominant over nuclear retention and is coupled to the Ran-GTPase cycle. Nucleic Acids Res 27(21):4128–4134

54. Lutzelberger M, Reinert LS, Das AT, Berkhout B, Kjems J (2006) A novel splice donor site in the gag-pol gene is required for HIV-1 RNA stability. J Biol Chem 281(27):18644–18651
55. Benko DM, Schwartz S, Pavlakis GN, Felber BK (1990) A novel human immunodeficiency virus type 1 protein, tev, shares sequences with tat, env, and rev proteins. J Virol 64(6): 2505–2518
56. Salfeld J, Gottlinger HG, Sia RA, Park RE, Sodroski JG, Haseltine WA (1990) A tripartite HIV-1 tat-env-rev fusion protein. EMBO J 9(3):965–970
57. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC (1980) Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci U S A 77(12):7415–7419
58. Yoshida M, Miyoshi I, Hinuma Y (1982) Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci U S A 79(6):2031–2035
59. Gessain A, Barin F, Vernant JC et al (1985) Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet 2(8452):407–410
60. Osame M, Usuku K, Izumo S et al (1986) HTLV-I associated myelopathy, a new clinical entity. Lancet 1(8488):1031–1032
61. Gessain A, de TG (1996) Geographic and molecular epidemiology of primate T lymphotropic retroviruses: HTLV-I, HTLV-II, STLV-I, STLV-PP, and PTLV-L. Adv Virus Res 47:377–426
62. Bindhu M, Nair A, Lairmore MD (2004) Role of accessory proteins of HTLV-1 in viral replication, T cell activation, and cellular gene expression. Front Biosci 9:2556–2576
63. Cavanagh MH, Landry S, Audet B et al (2006) HTLV-I antisense transcripts initiating in the 3′LTR are alternatively spliced and polyadenylated. Retrovirology 3:15
64. Halin M, Douceron E, Clerc I et al (2009) Human T-cell leukemia virus type 2 produces a spliced antisense transcript encoding a protein that lacks a classic bZIP domain but still inhibits Tax2-mediated transcription. Blood 114(12):2427–2438
65. Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M (1997) A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun 241(1):92–97
66. Springfeld C, Bugert JJ, Schnitzler P, Tobiasch E, Kehm R, Darai G (2000) TT virus as a human pathogen: significance and problems. Virus Genes 20(1):35–45
67. Miyata H, Tsunoda H, Kazi A et al (1999) Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J Virol 73(5):3582–3586
68. Mushahwar IK, Erker JC, Muerhoff AS et al (1999) Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. Proc Natl Acad Sci U S A 96(6):3177–3182
69. Okamoto H, Nishizawa T, Tawara A et al (2000) TT virus mRNAs detected in the bone marrow cells from an infected individual. Biochem Biophys Res Commun 279(2):700–707
70. Kamahora T, Hino S, Miyata H (2000) Three spliced mRNAs of TT virus transcribed from a plasmid containing the entire genome in COS1 cells. J Virol 74(21):9980–9986
71. Qiu J, Kakkola L, Cheng F et al (2005) Human circovirus TT virus genotype 6 expresses six proteins following transfection of a full-length clone. J Virol 79(10):6505–6510
72. Takahashi K, Iwasa Y, Hijikata M, Mishiro S (2000) Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch Virol 145(5):979–993
73. Yang JD, Roberts LR (2010) Hepatocellular carcinoma: a global view. Nat Rev Gastroenterol Hepatol 7(8):448–458
74. Studach LL, Rakotomalala L, Wang WH et al (2009) Polo-like kinase 1 inhibition suppresses hepatitis B virus X protein-induced transformation in an in vitro model of liver cancer progression. Hepatology 50(2):414–423
75. Seeger C, Mason WS (2000) Hepatitis B virus biology. Microbiol Mol Biol Rev 64(1): 51–68

76. Fang ZL, Sabin CA, Dong BQ et al (2009) The association of HBV core promoter double mutations (A1762T and G1764A) with viral load differs between HBeAg positive and anti-HBe positive individuals: a longitudinal analysis. J Hepatol 50(2):273–280
77. Su TS, Lai CJ, Huang JL et al (1989) Hepatitis B virus transcript produced by RNA splicing. J Virol 63(9):4011–4018
78. Suzuki T, Masui N, Kajino K, Saito I, Miyamura T (1989) Detection and mapping of spliced RNA from a human hepatoma cell line transfected with the hepatitis B virus genome. Proc Natl Acad Sci U S A 86(21):8422–8426
79. Chen PJ, Chen CR, Sung JL, Chen DS (1989) Identification of a doubly spliced viral transcript joining the separated domains for putative protease and reverse transcriptase of hepatitis B virus. J Virol 63(10):4165–4171
80. Suzuki T, Kajino K, Masui N, Saito I, Miyamura T (1990) Alternative splicing of hepatitis B virus RNAs in HepG2 cells transfected with the viral DNA. Virology 179(2):881–885
81. Sommer G, Heise T (2008) Posttranscriptional control of HBV gene expression. Front Biosci 13:5533–5547
82. Abraham TM, Lewellyn EB, Haines KM, Loeb DD (2008) Characterization of the contribution of spliced RNAs of hepatitis B virus to DNA synthesis in transfected cultures of Huh7 and HepG2 cells. Virology 379(1):30–37
83. Rosmorduc O, Petit MA, Pol S et al (1995) In vivo and in vitro expression of defective hepatitis B virus particles generated by spliced hepatitis B virus RNA. Hepatology 22(1):10–19
84. Lin X, Ma ZM, Yao X, Zhang YP, Wen YM (2002) Replication efficiency and sequence analysis of full-length hepatitis B virus isolates from hepatocellular carcinoma tissues. Int J Cancer 102(5):487–491
85. Lee GH, Wasser S, Lim SG (2008) Hepatitis B pregenomic RNA splicing—the products, the regulatory mechanisms and its biological significance. Virus Res 136(1–2):1–7
86. Huang HL, Jeng KS, Hu CP, Tsai CH, Lo SJ, Chang C (2000) Identification and characterization of a structural protein of hepatitis B virus: a polymerase and surface fusion protein encoded by a spliced RNA. Virology 275(2):398–410
87. Soussan P, Garreau F, Zylberberg H, Ferray C, Brechot C, Kremsdorf D (2000) In vivo expression of a new hepatitis B virus protein encoded by a spliced RNA. J Clin Invest 105(1):55–60
88. Assrir N, Soussan P, Kremsdorf D, Rossignol JM (2010) Role of the hepatitis B virus proteins in pro- and anti-apoptotic processes. Front Biosci 15:12–24
89. Berns KI (1990) Parvovirus replication. Microbiol Rev 54(3):316–329
90. Myers MW, Laughlin CA, Jay FT, Carter BJ (1980) Adenovirus helper function for growth of adeno-associated virus: effect of temperature-sensitive mutations in adenovirus early gene region 2. J Virol 35(1):65–75
91. Handa H, Carter BJ (1979) Adeno-associated virus DNA replication complexes in herpes simplex virus or adenovirus-infected cells. J Biol Chem 254(14):6603–6610
92. Geoffroy MC, Salvetti A (2005) Helper functions required for wild type and recombinant adeno-associated virus growth. Curr Gene Ther 5(3):265–271
93. Brown KE (2010) The expanding range of parvoviruses which infect humans. Rev Med Virol 20(4):231–244
94. Gao G, Vandenberghe LH, Alvira MR et al (2004) Clades of adeno-associated viruses are widely disseminated in human tissues. J Virol 78(12):6381–6388
95. Erles K, Rohde V, Thaele M, Roth S, Edler L, Schlehofer JR (2001) DNA of adeno-associated virus (AAV) in testicular tissue and in abnormal semen samples. Hum Reprod 16(11):2333–2337
96. Tobiasch E, Rabreau M, Geletneky K et al (1994) Detection of adeno-associated virus DNA in human genital tissue and in material from spontaneous abortion. J Med Virol 44(2): 215–222
97. Blechacz B, Russell SJ (2004) Parvovirus vectors: use and optimisation in cancer gene therapy. Exp Rev Mol Med 6(16):1–24

98. Qiu J, Pintel DJ (2002) The adeno-associated virus type 2 Rep protein regulates RNA processing via interaction with the transcription template. Mol Cell Biol 22(11):3639–3652
99. Weindler FW, Heilbronn R (1991) A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. J Virol 65(5): 2476–2483
100. Qiu J, Nayak R, Tullis GE, Pintel DJ (2002) Characterization of the transcription profile of adeno-associated virus type 5 reveals a number of unique features compared to previously characterized adeno-associated viruses. J Virol 76(24):12435–12447
101. Cossart YE, Field AM, Cant B, Widdows D (1975) Parvovirus-like particles in human sera. Lancet 1(7898):72–73
102. Candotti D, Etiz N, Parsyan A, Allain JP (2004) Identification and characterization of persistent human erythrovirus infection in blood donor samples. J Virol 78(22):12169–12178
103. Anderson MJ, Lewis E, Kidd IM, Hall SM, Cohen BJ (1984) An outbreak of erythema infectiosum associated with human parvovirus infection. J Hyg (Lond) 93(1):85–93
104. Woolf AD, Campion GV, Chishick A et al (1989) Clinical manifestations of human parvovirus B19 in adults. Arch Intern Med 149(5):1153–1156
105. Potter CG, Potter AC, Hatton CS et al (1987) Variation of erythroid and myeloid precursors in the marrow and peripheral blood of volunteer subjects infected with human parvovirus (B19). J Clin Invest 79(5):1486–1492
106. Jordan JA (1996) Identification of human parvovirus B19 infection in idiopathic nonimmune hydrops fetalis. Am J Obstet Gynecol 174(1 Pt 1):37–42
107. Ozawa K, Ayub J, Hao YS, Kurtzman G, Shimada T, Young N (1987) Novel transcription map for the B19 (human) pathogenic parvovirus. J Virol 61(8):2395–2406
108. Lynch JP III, Fishbein M, Echavarria M (2011) Adenovirus. Semin Respir Crit Care Med 32(4):494–511
109. Berk AJ (2005) Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. Oncogene 24(52):7673–7685
110. Akusjarvi G (2008) Temporal regulation of adenovirus major late alternative RNA splicing. Front Biosci 13:5006–5015
111. Stephens C, Harlow E (1987) Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30 kd and 35 kd proteins. EMBO J 6(7):2027–2035
112. Cuesta R, Xi Q, Schneider RJ (2000) Adenovirus-specific translation by displacement of kinase Mnk1 from cap-initiation complex eIF4F. EMBO J 19(13):3465–3474
113. Akusjarvi G, Stevenin J (2003) Remodelling of the host cell RNA splicing machinery during an adenovirus infection. Curr Top Microbiol Immunol 272:253–286
114. Akusjarvi G, Persson H (1981) Controls of RNA splicing and termination in the major late adenovirus transcription unit. Nature 292(5822):420–426
115. Zheng ZM (2010) Viral oncogenes, noncoding RNAs, and RNA splicing in human tumor viruses. Int J Biol Sci 6(7):730–755
116. Gardner SD, Field AM, Coleman DV, Hulme B (1971) New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet 1(7712):1253–1257
117. Padgett BL, Walker DL, ZuRhein GM, Eckroade RJ, Dessel BH (1971) Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. Lancet 1(7712):1257–1260
118. Allander T, Andreasson K, Gupta S et al (2007) Identification of a third human polyomavirus. J Virol 81(8):4130–4136
119. Gaynor AM, Nissen MD, Whiley DM et al (2007) Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog 3(5):e64
120. Feng H, Shuda M, Chang Y, Moore PS (2008) Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 319(5866):1096–1100
121. Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB (2010) Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. Cell Host Microbe 7(6):509–515

122. van der ME, Janssens RW, Lauber C, Bouwes Bavinck JN, Gorbalenya AE, Feltkamp MC (2010) Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient. PLoS Pathog 6(7):e1001024
123. Scuda N, Hofmann J, Calvignac-Spencer S et al (2011) A novel human polyomavirus closely related to the African green monkey-derived lymphotropic polyomavirus. J Virol 85(9): 4586–4590
124. Sweet BH, Hilleman MR (1960) The vacuolating virus, S.V. 40. Proc Soc Exp Biol Med 105:420–427
125. Knowles WA, Pipkin P, Andrews N et al (2003) Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40. J Med Virol 71(1):115–123
126. Krumbholz A, Bininda-Emonds OR, Wutzler P, Zell R (2009) Phylogenetics, evolution, and medical importance of polyomaviruses. Infect Genet Evol 9(5):784–799
127. Shuda M, Feng H, Kwun HJ et al (2008) T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. Proc Natl Acad Sci U S A 105(42):16272–16277
128. Shuda M, Kwun HJ, Feng H, Chang Y, Moore PS (2011) Human Merkel cell polyomavirus small T antigen is an oncoprotein targeting the 4E-BP1 translation regulator. J Clin Invest 121(9):3623–3634
129. Houben R, Adam C, Baeurle A et al (2012) An intact retinoblastoma protein-binding site in Merkel cell polyomavirus large T antigen is required for promoting growth of Merkel cell carcinoma cells. Int J Cancer 130(4):847–856
130. Zerrahn J, Knippschild U, Winkler T, Deppert W (1993) Independent expression of the transforming amino-terminal domain of SV40 large I antigen from an alternatively spliced third SV40 early mRNA. EMBO J 12(12):4739–4746
131. Abend JR, Joseph AE, Das D, Campbell-Cecen DB, Imperiale MJ (2009) A truncated T antigen expressed from an alternatively spliced BK virus early mRNA. J Gen Virol 90(Pt 5): 1238–1245
132. Trowbridge PW, Frisque RJ (1995) Identification of three new JC virus proteins generated by alternative splicing of the early viral mRNA. J Neurovirol 1(2):195–206
133. Zheng ZM, Baker CC (2006) Papillomavirus genome structure, expression, and post-transcriptional regulation. Front Biosci 11:2286–2302
134. Bernard HU, Burk RD, Chen Z, van DK, Hausen H, de Villiers EM (2010) Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology 401(1):70–79
135. Munoz N, Bosch FX, de SS et al (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348(6):518–527
136. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S (2007) Human papillomavirus and cervical cancer. Lancet 370(9590):890–907
137. Chen HC, Schiffman M, Lin CY et al (2011) Persistence of type-specific human papillomavirus infection and increased long-term risk of cervical cancer. J Natl Cancer Inst 103(18):1387–1396
138. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63(6):1129–1136
139. Dyson N, Howley PM, Munger K, Harlow E (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243(4893):934–937
140. Tang S, Tao M, McCoy JP Jr, Zheng ZM (2006) The E7 oncoprotein is translated from spliced E6*I transcripts in high-risk human papillomavirus type 16- or type 18-positive cervical cancer cell lines via translation reinitiation. J Virol 80(9):4249–4263
141. Wang X, Meyers C, Wang HK, Chow LT, Zheng ZM (2011) Construction of a full transcription map of human papillomavirus type 18 during productive viral infection. J Virol 85(16):8080–8092
142. Khan MJ, Castle PE, Lorincz AT et al (2005) The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. J Natl Cancer Inst 97(14):1072–1079

143. Stoler MH, Wright TC Jr, Sharma A, Apple R, Gutekunst K, Wright TL (2011) High-risk human papillomavirus testing in women with ASC-US cytology: results from the ATHENA HPV study. Am J Clin Pathol 135(3):468–475
144. Getman D, Aiyer A, Dockter J, Giachetti C, Zhang F, Ginocchio CC (2009) Efficiency of the APTIMA HPV Assay for detection of HPV RNA and DNA targets. J Clin Virol 45(Suppl 1):S49–S54
145. Ratnam S, Coutlee F, Fontaine D et al (2011) Aptima HPV E6/E7 mRNA test is as sensitive as Hybrid Capture 2 Assay but more specific at detecting cervical precancer and cancer. J Clin Microbiol 49(2):557–564
146. Clad A, Reuschenbach M, Weinschenk J, Grote R, Rahmsdorf J, Freudenberg N (2011) Performance of the Aptima high-risk human papillomavirus mRNA assay in a referral population in comparison with Hybrid Capture 2 and cytology. J Clin Microbiol 49(3):1071–1076
147. Molden T, Kraus I, Skomedal H, Nordstrom T, Karlsen F (2007) PreTect HPV-Proofer: real-time detection and typing of E6/E7 mRNA from carcinogenic human papillomaviruses. J Virol Methods 142(1–2):204–212
148. Benevolo M, Vocaturo A, Caraceni D et al (2011) Sensitivity, specificity, and clinical value of human papillomavirus (HPV) E6/E7 mRNA assay as a triage test for cervical cytology and HPV DNA test. J Clin Microbiol 49(7):2643–2650
149. Ratnam S, Coutlee F, Fontaine D et al (2010) Clinical performance of the PreTect HPV-Proofer E6/E7 mRNA assay in comparison with that of the Hybrid Capture 2 test for identification of women at risk of cervical cancer. J Clin Microbiol 48(8):2779–2785
150. Steitz J, Borah S, Cazalla D et al (2011) Noncoding RNPs of viral origin. Cold Spring Harb Perspect Biol 3(3):a005165
151. Cullen BR (2011) Herpesvirus microRNAs: phenotypes and functions. Curr Opin Virol 1(3):211–215
152. Plaisance-Bonstaff K, Renne R (2011) Viral miRNAs. Methods Mol Biol 721:43–66
153. Taylor GS, Blackbourn DJ (2011) Infectious agents in human cancers: lessons in immunity and immunomodulation from gammaherpesviruses EBV and KSHV. Cancer Lett 305(2):263–278
154. Zheng ZM (2003) Split genes and their expression in Kaposi's sarcoma-associated herpesvirus. Rev Med Virol 13(3):173–184
155. Arduino PG, Porter SR (2008) Herpes Simplex Virus Type 1 infection: overview on relevant clinico-pathological features. J Oral Pathol Med 37(2):107–121
156. Rajcani J, Andrea V, Ingeborg R (2004) Peculiarities of herpes simplex virus (HSV) transcription: an overview. Virus Genes 28(3):293–310
157. Javier RT, Stevens JG, Dissette VB, Wagner EK (1988) A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology 166(1):254–257
158. Croen KD, Ostrove JM, Dragovic LJ, Smialek JE, Straus SE (1987) Latent herpes simplex virus in human trigeminal ganglia. Detection of an immediate early gene "anti-sense" transcript by in situ hybridization. N Engl J Med 317(23):1427–1432
159. Farrell MJ, Dobson AT, Feldman LT (1991) Herpes simplex virus latency-associated transcript is a stable intron. Proc Natl Acad Sci U S A 88(3):790–794
160. Spivack JG, Woods GM, Fraser NW (1991) Identification of a novel latency-specific splice donor signal within the herpes simplex virus type 1 2.0-kilobase latency-associated transcript (LAT): translation inhibition of LAT open reading frames by the intron within the 2.0-kilobase LAT. J Virol 65(12):6800–6810
161. Henderson G, Peng W, Jin L et al (2002) Regulation of caspase 8- and caspase 9-induced apoptosis by the herpes simplex virus type 1 latency-associated transcript. J Neurovirol 8(Suppl 2):103–111
162. Umbach JL, Kramer MF, Jurak I, Karnowski HW, Coen DM, Cullen BR (2008) MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature 454(7205):780–783
163. vi-Rao GB, Goodart SA, Hecht LM, Rochford R, Rice MK, Wagner EK (1991) Relationship between polyadenylated and nonpolyadenylated herpes simplex virus type 1 latency-associated transcripts. J Virol 65(5):2179–2190

164. Chen J, Silverstein S (1992) Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. J Virol 66(5):2916–2927
165. Perry LJ, Rixon FJ, Everett RD, Frame MC, McGeoch DJ (1986) Characterization of the IE110 gene of herpes simplex virus type 1. J Gen Virol 67(Pt 11):2365–2380
166. Everett RD, Cross A, Orr A (1993) A truncated form of herpes simplex virus type 1 immediate-early protein Vmw110 is expressed in a cell type dependent manner. Virology 197(2):751–756
167. Spatz SJ, Nordby EC, Weber PC (1996) Mutational analysis of ICP0R, a transrepressor protein created by alternative splicing of the ICP0 gene of herpes simplex virus type 1. J Virol 70(11):7360–7370
168. Tang S, Bertke AS, Patel A, Wang K, Cohen JI, Krause PR (2008) An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. Proc Natl Acad Sci U S A 105(31):10931–10936
169. Berger JR, Houff S (2008) Neurological complications of herpes simplex virus type 2 infection. Arch Neurol 65(5):596–600
170. Desselberger U (1998) Herpes simplex virus infection in pregnancy: diagnosis and significance. Intervirology 41(4–5):185–190
171. Bankier AT, Beck S, Bohni R et al (1991) The DNA sequence of the human cytomegalovirus genome. DNA Seq 2(1):1–12
172. Rawlinson WD, Barrell BG (1993) Spliced transcripts of human cytomegalovirus. J Virol 67(9):5502–5513
173. Bresnahan WA, Shenk T (2000) A subset of viral transcripts packaged within human cytomegalovirus particles. Science 288(5475):2373–2376
174. Nelson PN, Rawal BK, Boriskin YS et al (1996) A polymerase chain reaction to detect a spliced late transcript of human cytomegalovirus in the blood of bone marrow transplant recipients. J Virol Methods 56(2):139–148
175. Gerna G, Revello MG, Percivalle E, Morini F (1992) Comparison of different immunostaining techniques and monoclonal antibodies to the lower matrix phosphoprotein (pp 65) for optimal quantitation of human cytomegalovirus antigenemia. J Clin Microbiol 30(5): 1232–1237
176. Fernando S, Booth J, Boriskin Y et al (1994) Association of cytomegalovirus infection with post-transplantation cardiac rejection as studied using the polymerase chain reaction. J Med Virol 42(4):396–404
177. Greijer AE, Adriaanse HM, Kahl M et al (2001) Quantitative competitive NASBA for measuring mRNA expression levels of the immediate early 1, late pp 67, and immune evasion genes US3, US6 and US11 in cells infected with human cytomegalovirus. J Virol Methods 96(2):133–147
178. Randhawa PS, Manez R, Frye B, Ehrlich GD (1994) Circulating immediate-early mRNA in patients with cytomegalovirus infections after solid organ transplantation. J Infect Dis 170(5):1264–1267
179. Velzing J, Rothbarth PH, Kroes AC, Quint WG (1994) Detection of cytomegalovirus mRNA and DNA encoding the immediate early gene in peripheral blood leukocytes from immunocompromised patients. J Med Virol 42(2):164–169
180. Meyer T, Reischl U, Wolf H, Schuller C, Arndt R (1994) Identification of active cytomegalovirus infection by analysis of immediate-early, early and late transcripts in peripheral blood cells of immunodeficient patients. Mol Cell Probes 8(4):261–271
181. Boriskin YS, Fuller K, Powles RL et al (2002) Early detection of cytomegalovirus (CMV) infection in bone marrow transplant patients by reverse transcription-PCR for CMV spliced late gene UL21.5: a two site evaluation. J Clin Virol 24(1–2):13–23
182. Andre E, Imbert-Marcille BM, Cantarovich D, Besse B, Ferre-Aubineau V, Billaudel S (1999) Use of reverse transcription polymerase chain reaction with colorimetric plate hybridization to detect a cytomegalovirus late spliced mRNA in polymorphonuclear leukocytes from renal transplant patients. Diagn Microbiol Infect Dis 34(4):287–291

183. Yao QY, Ogan P, Rowe M, Wood M, Rickinson AB (1989) Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. Int J Cancer 43(1):67–71
184. Kutok JL, Wang F (2006) Spectrum of Epstein-Barr virus-associated diseases. Annu Rev Pathol 1:375–404
185. Qu L, Green M, Webber S, Reyes J, Ellis D, Rowe D (2000) Epstein-Barr virus gene expression in the peripheral blood of transplant recipients with persistent circulating virus loads. J Infect Dis 182(4):1013–1021
186. Young LS, Arrand JR, Murray PG (2007) EBV gene expression and regulation. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, Yamanishi K (eds) Human herpesviruses: biology, therapy, and immunoprophylaxis. Cambridge University Press, Cambridge. pp 461–489
187. Baer R, Bankier AT, Biggin MD et al (1984) DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 310(5974):207–211
188. Swaminathan S (2008) Noncoding RNAs produced by oncogenic human herpesviruses. J Cell Physiol 216(2):321–326
189. Barth S, Meister G, Grasser FA (2011) EBV-encoded miRNAs. Biochim Biophys Acta 1809(11–12):631–640
190. Kennedy G, Sugden B (2003) EBNA-1, a bifunctional transcriptional activator. Mol Cell Biol 23(19):6901–6908
191. Woisetschlaeger M, Yandava CN, Furmanski LA, Strominger JL, Speck SH (1990) Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes. Proc Natl Acad Sci U S A 87(5):1725–1729
192. Nonkwelo C, Ruf IK, Sample J (1997) The Epstein-Barr virus EBNA-1 promoter Qp requires an initiator-like element. J Virol 71(1):354–361
193. Nonkwelo C, Henson EB, Sample J (1995) Characterization of the Epstein-Barr virus Fp promoter. Virology 206(1):183–195
194. Miller G, El-Guindy A, Countryman J, Ye J, Gradoville L (2007) Lytic cycle switches of oncogenic human gammaherpesviruses. Adv Cancer Res 97:81–109
195. Manet E, Gruffat H, Trescol-Biemont MC et al (1989) Epstein-Barr virus bicistronic mRNAs generated by facultative splicing code for two transcriptional trans-activators. EMBO J 8(6):1819–1826
196. Furnari FB, Zacny V, Quinlivan EB, Kenney S, Pagano JS (1994) RAZ, an Epstein-Barr virus transdominant repressor that modulates the viral reactivation mechanism. J Virol 68(3): 1827–1836
197. Chang Y, Cesarman E, Pessin MS et al (1994) Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 266(5192):1865–1869
198. Ganem D (2006) KSHV infection and the pathogenesis of Kaposi's sarcoma. Annu Rev Pathol 1:273–296
199. Ablashi DV, Chatlynne LG, Whitman JE Jr, Cesarman E (2002) Spectrum of Kaposi's sarcoma-associated herpesvirus, or human herpesvirus 8, diseases. Clin Microbiol Rev 15(3): 439–464
200. Russo JJ, Bohenzky RA, Chien MC et al (1996) Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci U S A 93(25):14862–14867
201. Verma SC, Lan K, Robertson E (2007) Structure and function of latency-associated nuclear antigen. Curr Top Microbiol Immunol 312:101–136
202. Dittmer D, Lagunoff M, Renne R, Staskus K, Haase A, Ganem D (1998) A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. J Virol 72(10):8309–8315
203. Sun R, Lin SF, Gradoville L, Yuan Y, Zhu F, Miller G (1998) A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc Natl Acad Sci U S A 95(18):10866–10871
204. Lukac DM, Kirshner JR, Ganem D (1999) Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. J Virol 73(11):9348–9361

205. Yamanegi K, Tang S, Zheng ZM (2005) Kaposi's sarcoma-associated herpesvirus K8beta is derived from a spliced intermediate of K8 pre-mRNA and antagonizes K8alpha (K-bZIP) to induce p21 and p53 and blocks K8alpha-CDK2 interaction. J Virol 79(22):14207–14221
206. Majerciak V, Pripuzova N, McCoy JP, Gao SJ, Zheng ZM (2007) Targeted disruption of Kaposi's sarcoma-associated herpesvirus ORF57 in the viral genome is detrimental for the expression of ORF59, K8alpha, and K8.1 and the production of infectious virus. J Virol 81(3):1062–1071
207. Cheung TK, Guan Y, Ng SS et al (2005) Generation of recombinant influenza A virus without M2 ion-channel protein by introduction of a point mutation at the 5′ end of the viral intron. J Gen Virol 86(Pt 5):1447–1454
208. Purcell DF, Martin MA (1993) Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. J Virol 67(11):6365–6378
209. Yamano Y, Nagai M, Brennan M et al (2002) Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). Blood 99(1):88–94
210. Preiss S, Littlejohn M, Angus P et al (2008) Defective hepatitis B virus DNA is not associated with disease status but is reduced by polymerase mutations associated with drug resistance. Hepatology 48(3):741–749
211. Nguyen QT, Wong S, Heegaard ED, Brown KE (2002) Identification and characterization of a second novel human erythrovirus variant, A6. Virology 301(2):374–380
212. Gama-Carvalho M, Condado I, Carmo-Fonseca M (2003) Regulation of adenovirus alternative RNA splicing correlates with a reorganization of splicing factors in the nucleus. Exp Cell Res 289(1):77–85
213. White MK, Safak M, Khalili K (2009) Regulation of gene expression in primate polyomaviruses. J Virol 83(21):10846–10856
214. Tanaka S, Minagawa H, Toh Y, Liu Y, Mori R (1994) Analysis by RNA-PCR of latency and reactivation of herpes simplex virus in multiple neuronal tissues. J Gen Virol 75(Pt 10):2691–2698
215. Majerciak V, Yamanegi K, Allemand E, Kruhlak M, Krainer AR, Zheng ZM (2008) Kaposi's sarcoma-associated herpesvirus ORF57 functions as a viral splicing factor and promotes expression of intron-containing viral lytic genes in spliceosome-mediated RNA splicing. J Virol 82(6):2792–2801
216. Tang S, Zheng ZM (2002) Kaposi's sarcoma-associated herpesvirus K8 exon 3 contains three 5′-splice sites and harbors a K8.1 transcription start site. J Biol Chem 277(17): 14547–14556

Chapter 39
Application of Microarrays for Laboratory Detection and Identification of Medically Important Fungi

Tsung Chain Chang

Introduction

Infections caused by fungi have been increasing over the past several decades due to advanced medical treatments as well as the increase in immunocompromised patients. For example, invasive aspergillosis is now a leading cause of infection among patients undergoing treatment for hematological malignancies, solid organ transplantation, and hematopoietic stem cell transplantation (HSCT), and among patients with AIDS [1–4]. *Aspergillus fumigatus* remains the most important cause of invasive aspergillosis, followed by *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, and *A. versicolor* [5]. However, Husain et al. [6] prospectively studied 53 heart and liver transplant recipients and found that invasive infections due to non-*Aspergillus* fungi were significantly more likely to be associated with central nervous system and disseminated infections than were those due to *Aspergillus*. The associated mortality rates were 100, 80, 54, and 20 % for zygomycosis, non-*Aspergillus* hyalohyphomycosis, aspergillosis, and phaeohyphomycosis, respectively. Non-*Aspergillus* molds causing severe infections in organ transplant recipients include zygomycetes, *Fusarium* spp., *Scedosporium apiospermum*, *Scedosporium prolificans*, and dematiaceous molds [7].

Accurate and rapid identification of fungal pathogens clearly is important for appropriate treatment with antifungal agents, some of which are specific for certain type of fungi. The identification of filamentous fungi (molds) can be challenging and inaccurate [8]. Conventional methods for fungal identification in the clinical

T.C. Chang (✉)
Department of Medical Laboratory Science and Biotechnology,
School of Medicine, National Cheng Kung University,
1 University Road, Tainan 701, Taiwan, Republic of China
e-mail: tsungcha@mail.ncku.edu.tw

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microbiology laboratory are primarily based on morphological characteristics (especially the characteristics of reproductive structures) and a limited number of phenotypic biochemical tests. These methods often require several days or even weeks, and may be subjective and inaccurate [9]. Since invasive fungal infections are often associated with a high morbidity and mortality, early, rapid, and accurate identification of these fungal pathogens is important for timely and appropriate antifungal therapy.

Infections caused by yeasts also have increased in the past several decades. Of medically important yeasts, *Candida albicans* remains the most important pathogen. However, the use of antifungal agents for prophylaxis as well as the use of broad-spectrum antibiotics is producing a shift in the epidemiology of yeast infections [10]. Infections caused by non-*albicans Candida* and other less common yeasts, such as *Cryptococcus*, *Pichia*, *Rhodotorula*, *Saccharomyces*, and *Trichosporon*, have been widely reported [11–18]. Identification of less encountered yeasts by conventional phenotypes, especially carbon source assimilation, can be difficult and sometimes ambiguous [19, 20].

To date, numerous DNA-based methods have been developed to diagnose mycotic infections and to identify specific pathologic fungi [7]. PCR methods are particularly promising because of their sensitivity, specificity, and simplicity. Genes of the 18S rRNA [21–23] and 28S rRNA [24–26] have been commonly used as the targets for molecular identification. Hall et al. [8] evaluated the commercial MicroSeq D2 large-subunit rDNA fungal sequencing kit (Applied Biosystems, Foster City, CA, USA); they found that 33 % of clinical isolates could not be identified, and this might be due to limited sequence database in this commercial system. Some studies have described hybridization probes, restriction fragment length polymorphism, or other methods to identify unique rRNA gene sequences [27, 28]. In addition, the ribosomal internal transcribed spacer (ITS) regions have been extensively used as targets for fungal identification or detection [29–36].

Although the published methods described above are useful for fungal diagnosis, most of these methods can identify only one or a very limited number of fungal species at a time. DNA microarray (or DNA chip) technology has been found to be a useful tool to identify a wide spectrum of bacterial pathogens, especially for bacteria that are difficult to differentiate by phenotypic methods or where the identification procedures may take a long time [37–40]. For fungal identification, an early study was conducted by Wu et al. [23]; these investigators developed an oligonucleotide array to detect 31 species (15 genera) of airborne fungi, by using probes designed from the 18S rRNA genes. After this study, additional investigators have studied the possibility of applying the array technique for diagnosis of molds and yeasts that may cause human infections; feasible results have been obtained. The current chapter discusses the application of the array method to identify (or detect) clinically relevant fungi, including molds and yeasts. Aspects concerning the probe design, target genes, and the application of the array for direct detection of fungi in clinical specimens are discussed.

Solid Support for Fabrication of Arrays

Currently, there are two major types of solid support for immobilization of probes. The first one is glass slide and the other is synthetic membrane. Applications of the two solid supports for fungal diagnosis are addressed as follows.

Glass Slides

For glass arrays, the probes are immobilized on the slide surface by covalent bonding, while the amplified target DNA is usually labeled by a fluorescent dye, such as carbocyanine 3 (Cy3) fluorochrome. Spiess et al. [41] designed oligonucleotide probes targeting the ITS1 region of the rRNA operon to detect 14 species of fungi in clinical samples from neutropenic patients. The 14 species were *A. fumigatus*, *A. flavus*, *A. terreus*, *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. lusitaniae*, *C. tropicalis*, *Fusarium oxysporum*, *F. solani*, *Mucor racemosus*, *Rhizopus microsporus*, *S. prolificans*, and *Trichosporon asahii*. Clinical samples used for fungal detection included blood, bronchoalveolar lavage, and tissue specimens [41]. The capture oligonucleotide probes were bound to the glass surface by covalent bounding and the amplified PCR product was labeled with Cy3 fluorochrome [42]. Two multiplex PCR reactions were evaluated by Spiess et al. [41] in a study that amplified fungal DNA for array hybridization. The first multiplex primer mix, including nine sense primers derived from the conserved 18S and three antisense primers from the 5.8S rRNA gene regions, was for ITS1 amplification, while the second multiplex primer set was used to amplify the internal standard, a positive, and a negative control [41]. Different species were identified by their hybridization patterns after hybridization, rather than by species-specific probes. A total of 14 fungal pathogens were detected from 46 clinical samples from neutropenic patients with proven, probable, or possible invasive fungal infection (IFI) or without IFI. The detection limits of the array were 300 pg DNA for *C. tropicalis*, 500 pg for *F. oxysporum*, and 300 pg for *Rhizopus microsporus* [41]. The different detection limits for different species might be caused by various probes having different secondary structures and binding strength with their respective target DNAs. It was interesting to find that two or more fungal pathogens were detected in a large proportion of patients with IFI, highlighting the potential of the array method to concurrently detect multiple fungal pathogens in a single patient [41]. However, the medical indication of IFI caused by multiple fungal pathogens is still limited. In view of the changing spectrum of fungal pathogens causing IFI, the authors concluded that their array might meet urgent clinical needs, especially the high-risk group of patients having immunocompromised conditions or hematologic malignancies [41].

Leinberger et al. [43] also described a glass array method for identification of 12 common pathogenic *Candida* and *Aspergillus* species; this method used oligonucleotide

probes against the ITS1 or ITS2 regions of the rRNA gene cassette. The 12 species were *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis*, *A. flavus*, *A. fumigatus*, *A. niger*, and *A. terreus*. A total of 51 control and species- or genus-specific probes were used, with each probe having a 14-thymidine spacer at the 3′ end and an amino modification at the 5′ end. The probes were spotted onto epoxy-coated glass slides and covalent bonding was achieved by incubating at 60 °C for 30 min. The target DNA was amplified using fluorochrome-labeled fungal specific primers (ITS1 and ITS4) targeting the 3′ end of the 18S and 5′ end of the 28S rRNA genes, respectively. The hybridization procedure took about 4 h after DNA extraction from cultured isolates.

In another study, Huang et al. [44] immobilized 22 probes on a glass slide to identify 22 fungal species (eight genera), including yeasts, dermatophytes, and other molds. The specific oligonucleotide probes were designed from the ITS2 region and were modified with amino group at the 3′ ends to facilitating binding to the glass surface. The detection limit of the array was 15 pg/ml of genomic DNA.

Synthetic Membrane

Reading of the hybridization result of fluorochrome-labeled DNA on the array requires a fluorescence scanner that is expensive and is not routine equipment in the clinical laboratory. Furthermore, most clinical microbiologists are not familiar with the chemical reactions for immobilizing probes on glass slide that involved specific chemical reactions. The use of synthetic membrane as the solid support for probes is much simple and straightforward. After the probes are spotted (by an automatic arrayer), they can be fixed on the membrane by simply exposed to shortwave UV irradiation (e.g., Stratalinker 1800, Stratagen, La Jolla, CA, USA) for a very short time (30 s) [45–48]. The 5′ end of one or both primers can be labeled with a digoxigenin molecule (or a biotin molecule), thus producing a digoxigenin-labeled (or biotin-labeled) amplicon after PCR amplification. After hybridization, alkaline phosphatase-conjugated anti-digoxigenin antibodies (or alkaline phosphatase-conjugated streptavidin) are used to react with the digoxigenin-labeled DNA, followed by the addition of alkaline phosphatase substrate (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate) to reveal the hybridization signal. The hybridized spot can be read by the naked eye if the diameter of the spotted probe is larger than 0.2–0.3 mm. The detection limits of membrane arrays ranged from 1 to 10 pg fungal genomic DNA; [46, 48] the results were almost as sensitive as those obtained by glass arrays using fluorescent labeling [41, 43, 44]. If the DNA content of a yeast cell is 37 fg [49], the detection limits of the membrane arrays were equivalent to 27–270 cells per assay. The advantages of using membrane arrays are low production cost and easy to fabricate.

By targeting the ITS1 and ITS2 regions, Hsiao et al. [46], developed a nylon membrane array (1.1 by 0.9 cm) to identify a wide spectrum (64 species in 32 genera)

Table 39.1 The 64 species (32 genera) of molds that can be identified by the membrane array of Hsiao et al. [46].

Microorganism	Microorganism	Microorganism
Absidia corymbifera	*Acremonium falciforme*	*Acremonium kiliense*
Acremonium strictum	*Alternaria alternata*	*Arthroderma cajetanum*
Arthroderma grubyi	*Arthroderma obtusum*	*Aspergillus clavatus*
Aspergillus flavus	*Aspergillus fumigatus*	*Aspergillus nidulans*
Aspergillus oryzae	*Emericella nidulans* var. *echinulata*	*Aspergillus niger*
Aspergillus terreus	*Aspergillus versicolor*	*Aureobasidium pullulans*
Beauveria bassiana	*Bipolaris spicifera*	*Blastomyces dermatitidis*
Chaetomium cochlioides	*Chaetomium funicola*	*Chaetomium globosum*
Cladophialophora bantiana	*Cladophialophora carrionii*	*Cokeromyces recurvatus*
Cunninghamella bertholletiae	*Epidermophyton floccosum*	*Exophiala dermatitidis*
Exophiala jeanselmei	*Hortaea* (*Exophiala*) *werneckii*	*Fonsecaea compacta*
Fonsecaea pedrosoi	*Fusarium moniliforme*	*Fusarium oxysporum*
Fusarium pallidoroseum	*Fusarium solani*	*Geotrichum candidum*
Geotrichum capitatum	*Malbranchea filamentosa*	*Microsporum audouinii*
Microsporum canis	*Microsporum ferrugineum*	*Microsporum gypseum*
Paecilomyces javanicus	*Paracoccidioides brasiliensis*	*Penicillium marneffei*
Phialophora richardsiae	*Phialophora verrucosa*	*Piedraia hortai* var. *hortai*
Pseudallescheria boydii	*Rhizomucor pusillus*	*Rhizopus oryzae*
Scedosporium prolificans	*Scopulariopsis brevicaulis*	*Scytalidium dimidiatum*
Scytalidium hyalinum	*Trichophyton mentagrophytes*	*Trichophyton mentagrophytes* var. *interdigitale*
Trichophyton rubrum	*Trichophyton schoenleinii*	*Trichophyton soudanense*
Trichophyton tonsurans	*Trichophyton verrucosum*	*Trichophyton violaceum*
Ulocladium consortiale		

of filamentous (or dimorphic) fungi causing superficial, cutaneous, subcutaneous, and invasive infections (Table 39.1). The coverage of species was much wider than those of previously published paper [41, 43, 44, 50]. The layout of all probes and the hybridization patterns of *A. flavus* and *F. solani*, just named a few, on the array are shown in Fig. 39.1. A collection of 397 fungal strains (290 target and 107 non-target strains) were analyzed by the array, resulting in a sensitivity of 98.3 % (285/290) and a specificity of 98.1 % (105/107). Several misidentified strains were usually species belonging to the same genus of the target species. Since some closely related species have high ITS sequence homology, a few group-specific probes were designed to identify a group of related species [46], rather than to identify each individual species. For example, the probe (Chcgf1) was used to identify three species (*Chaeotomium cochlioides*, *C. globosum*, and *C. fumicola*) as a group. In contrast, multiple probes were constructed to identify a single species since high intraspecies sequence divergence in the ITS region was observed for the species. For example, two probes (Ackil2 and Ackil3) were synthesized to identify *Acremonium kiliense*.

a

	1	2	3	4	5	6	7	8	9	10	
A	Abcor3	Acfal1	Ackil2	Ackil3	Acstr2	M	Alalt3	Aupul2	Aupul3	Aupul4	A
B	Ascla4	Asfla4	Asfum2	Asnid2	Asnig2	M	Aster2	Asver4	Bebas5	Bihs1	B
C	Blder2	Chcgf1	Chfun2	Clban5	Clcar2	M	Cocor1	Corec2	Cun4	Cuber3	C
D	Epflo2	Exder1	Exjea	Focp	Fumon	M	Fumop	Fusol2	Gecan	Gecap3	D
E	M	M	M	M	M	NC	M	M	M	M	E
F	Howe2	Mafil4	Macf1	Migyp3	Micok4	M	Migal3	Minan1	Pabra2	Pihor2	F
G	Pajav	Palil3	Pamar1	Pavir3	Pemar2	M	Phric3	Phver3	Psboy3	PC	G
H	Ripu4	Riory4	Scbre3	Scpro4	Sydh	M	Trmmist1	Trver	Trrsv3	Ulcon2	H
	1	2	3	4	5	6	7	8	9	10	

b

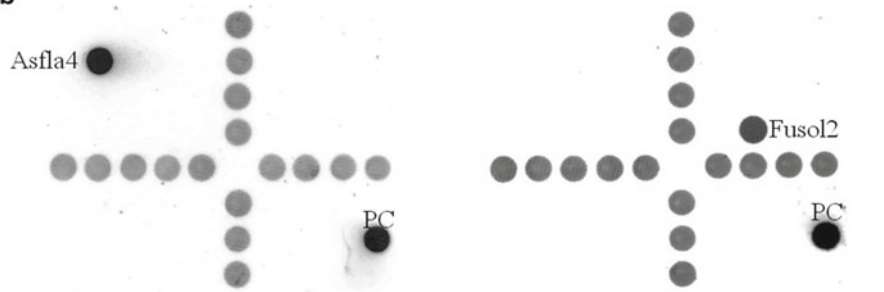

Fig. 39.1 (**a**) The layout of probes on the membrane array (1.1 by 0.9 cm) for identification of medically important molds. The probe codes for different molds and the corresponding probe sequences are published previously [46]. "M" is a position marker. "PC" a fungus-specific positive control probe. "NC" a negative control probe. (**b**) Hybridization patterns of *Aspergillus flavus* BCRC 30007 (probe Asfla4) and *Fusarium solani* CBS 109028 (probe Fusol2) on the array

Dermatophytes are keratinophilic fungi that typically cause dermatophytoses and are among the most adaptable microbial associates of humans. Species of dermatophytes are classified into three anamorphic (asexual) genera, *Epidermophyton*, *Microsporum*, and *Trichophyton*. Many of the dermatophyte species are phylogenetically and taxonomically closely related [51–53]. Although most typical isolates of dermatophytes can be identified from the isolation media, the phenotypic features can be influenced by culture medium and temperature variation [54] and occasionally the absence of reproductive structures of some species poses additional identification problem. However, fingerprinting (or single nucleotide polymorphism) sequences were found to present in the ITS regions of the *Microsporum canis* complex (*Microsporum audouinii*, *M. canis*, and *M. ferrugineum*) [55] and *Trichophyton rubrum* complex (*T. rubrum*, *T. soudanense*, *T. violaceum*, and other closely related species) [56]. Based on the fingerprinting sequences, Li et al. [48] was able to construct a membrane array to identify 17 species of dermatophytes; these fungi were *Epidermophyton floccosum*, *M. audouinii*, *M. canis*, *M. cookie*,

M. ferrugineum, *M. gallinae*, *M. gypseum*, *M. nanum*, *M. persicolor*, *Trichophyton mentagrophytes*, *T. rubrum*, *T. schoenleinii*, *T. soudanense*, *T. terrestre*, *T. tonsurans*, *T. verrucosum*, and *T. violaceum*. The dermatophyte array demonstrated the potential of the array technique to differentiate closely related molds that are difficult to be distinguished by phenotypic characteristics.

Principle of Probe Design

Oligonucleotide probes used for fungal identification normally have lengths ranging from 20 to 30 nucleotides [46–48, 50]. In general, probes shorter than 20 nucleotides tend to have low melting temperature (the temperature at which a DNA double helix dissociates into two single strands) and hence will produce a weak hybridization signal, while probes longer than 30 nucleotides may have a higher possibility of cross-reactions with other microorganisms. The designed probes should be checked for internal repeat, self-biding, melting temperature (T_m), secondary structure, and GC content by software, such as Vector NTI (Invitrogen Corporation, Carlsbad, CA, USA) or Oligo (Oligo 6.0; MedProbe AS, Oslo, Norway). The specificity of a designed probe must be compared with sequences in the GenBank database (or other public nucleotide sequence databases) using Basic Local Alignment Search Tool (BLAST) or other relevant algorithms.

Since all probes on an array hybridize with target DNA at a fixed hybridization temperature and washed with the same buffers (in other words, under the same stringency), all probes should have a narrow range of melting temperatures (T_m) and the T_m is higher than the hybridization temperature. However, exceptions were found in several cases. For example, even when the T_m values of probes were lower than the hybridization temperature (55 °C), clear hybridization signals were still obtained by several probes in the study of Hsiao et al. [46]. Another example involves an array developed to identify bacterial pathogens (*Listeria*); in this array, several probes having T_m (40–44 °C) lower than the hybridization temperature (45 °C) still displayed good hybridization signals [40]. In contrast, weak hybridization signals were observed for some probes having relatively high T_m [46]. The secondary structure of probes may play an important role on the hybridization efficiency [57].

Probe specificity is a major concern of developing an array for microbial identification. Cross-reactions are most frequently caused by other species in the same genus and may not be able to avoid under some conditions. To solve this problem, 1- or 2-base mismatches can be intentionally incorporated into a probe to eliminate cross-reactions [38, 47, 50], while the sensitivity of the probe is still maintained. The effect of incorporating a mismatched base into an oligonucleotide probe was carefully evaluated by Ikuta et al. [58]. They found that the G-T and G-A mismatches only slightly destabilize a duplex, while the A-A, T-T, C-T, and C-A mismatches have profound destabilization effect. Another interesting finding is that sometimes an antisense probe may have a much better hybridization efficiency

than the sense probe or vice versa, even the two complementary probes have the same length, GC content, and T_m [43, 47, 50]. This might be caused by the difference in secondary structures of the sense and antisense probes.

Normally, one [46] or both primers [47] can be labeled by a tag at the 5′ end when performing PCR. Dual labeling can increase the hybridization signal to some degree (unpublished data). It is thought that the two denatured single strands of an amplicon may form a partial duplex before hybridization to probes on an array. Following array hybridization, the partial duplex complex has two digoxigenin (or biotin) molecules, instead of one, available for binding with enzyme-linked antibodies. Random labeling with digoxigenin-dUTP (DIG-dUTP, Roche, Mannheim, Germany) during PCR can further increase the hybridization signal, since multiple digoxigenin molecules are available on an amplicon for antibody binding. Random labeling is especially useful for direct detection of fungal DNA in clinical specimens. Furthermore, the addition of multiple (5–20) thymine bases to one end of a probe has the benefit of reducing steric hindrance and enhancing the hybridization signal [59].

Finally, one, two, or multiple probes can be constructed to identify a single species of fungus, depending on the availability of divergent sequences in the target DNA region. The advantage of using multiple probes is the increased coverage of different strains within a species, but the inherent disadvantage is the potential decrease of specificity due to unexpected cross-hybridizations caused by nontarget species [50].

Target Genes

Most arrays, whether based on glass slide or synthetic membrane, utilize probes derived from the rRNA operon. Based on the 18S rRNA gene, Wu et al. [23] synthesized 33 probes to identify 31 species of airborne fungi that may cause health problems such as allergy or infection. The ITS1 and ITS2 regions are widely used as targets for fungal identification [29, 32, 36, 41, 50, 51]. The ITS regions have low intraspecies sequence variation and high interspecies sequence divergence and the regions are good targets for molecular identification of molds and yeasts. A variety of membrane arrays based on the ITS1 and ITS2 sequences have been developed in my laboratory to identify or detect a wide range of fungi, including medically important molds [46] and airborne fungi [50], dermatophytes [48], fungi that colonize (or true pathogens) the respiratory tract of patients with cystic fibrosis [45], and yeasts [47]. In addition, based on the ITS sequence, Huang et al. [44] developed a microarray that was able to identify 20 fungal species (6 genera), while Leinberger et al. [43] constructed an array to diagnose 12 common pathogenic *Aspergillus* and *Candida* species. To accelerate diagnosis of invasive fungal infection in immunocompromised patients, Spiess et al. [41] used a microarray targeting the ITS1 region to detect 14 fungal pathogens in clinical samples including blood, bronchoalveolar

lavage, and tissue from neutropenic patients. So far, the ITS region is the most commonly used target to develop molecular diagnostic methods for fungi.

The rRNA operon has a high copy number (40–80 copies per haploid genome); [60] therefore molecular methods targeting the ITS have higher sensitivity (or lower detection limit) than targeting genes that have only one or a few copies in a genome. The whole ITS region can be amplified using the fungus-specific universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTG ATATGC-3′) [60]. The amplicon includes the 3′ portion of the 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and the 5′ portion of the 28S rRNA gene. Alternatively, the ITS1 region can be separately amplified by universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCATCGATGAAGAACG CAGC-3′), while the ITS2 region can be amplified by the primer pair ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-GCATATCAATAAGCGG AGGA-3′) [60]. Occasionally, the ITS region cannot be amplified by the forward primer ITS1, therefore the primer ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) can replace primer ITS1 for amplification. Hung et al. [50] used a mixture of ITS1 and ITS5 as the forward primers and ITS4 as the reverse primer for amplification of the ITS regions of airborne fungi.

Hybridization Procedures

The hybridization stringency (hybridization temperature and buffer strength) could be optimized according to individual applications. A typical hybridization protocol of membrane array is presented here [46]. The hybridization procedures are carried out at room temperature with a shaking speed of 60 rpm, except otherwise indicated. Most reagents, except buffers, can be found in the DIG Nucleic Acid Detection kit (Roche, Mannheim, Germany). Each array, put in an individual well of a 24-well cell culture plate, is prehybridized at 50 °C for 2 h with 1 ml of hybridization solution [5× SSC, 1 % (w/v) blocking reagent, 0.1 % *N*-laurylsarcosine, and 0.02 % sodium dodecyl sulfate (SDS); 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]. The digoxigenin-labeled (or biotin-labeled) PCR product amplified from a target fungus is heated in a boiling water bath (or heating block) for 5 min and immediately cooled on an ice bath. Ten to 20 μl of the denatured PCR product are diluted with 0.3 ml of hybridization solution and added to each array in a well. Hybridization is conducted at 55 °C for 90 min. After removing the nonhybridized DNA, the array is washed two to four times (5 min each) in 1 ml of maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), followed by blocking for 1 h with 1 ml of blocking solution [1 % (w/v) blocking reagent dissolved in maleic acid buffer]. The blocking solution is removed and 0.5 ml of alkaline phosphatase-conjugated anti-digoxigenin antibodies (diluted 1:2,500 in blocking solution) is added to each well and incubated for 1 h. The array is washed three times (each 15 min) in 1 ml of washing solution [0.3 % (v/v) Tween 20 in maleic acid buffer],

followed by one wash for 5 min in 1 ml of detection buffer (0.1 M Tris–HCl, 0.15 M NaCl, pH 9.5). The hybridized probe is revealed by adding 0.15 ml of alkaline phosphatase substrate (stock solution of nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate diluted 1:50 in detection buffer) to the top of each array and incubated at 37 °C (or room temperature) in the dark without shaking. The hybridized spot will be visible within 15 and 30 min after the addition of enzyme substrates. The individual steps of array hybridization can be optimized or shortened without causing a decrease of either sensitivity or specificity. For example, the prehybridization and hybridization steps probably can be combined into a single step, and the blocking step and adding of the enzyme-conjugated antibody to the array can be integrated into a step (our unpublished data).

Identification of Multiple Fungal Species

A prominent advantage of using the array method for microbial identification is that multiple species can be simultaneously identified on a single array. The DNAs from different fungal species can be amplified and then concurrently hybridized with probes on the array. For example, the PCR products of *A. flavus* and *Penicillium marneffei* could be identified on an array at the same time. Alternatively, the DNAs of colonies from two or three species can be coextracted in a tube, amplified by PCR, and hybridized with an array [50]. Bouchara et al. [45] found that their array could detect as many as five to seven species of fungi in the sputum specimens from patients with cystic fibrosis, although some of these fungi might be only colonizers in the respiratory tracts (Fig. 39.2). Since universal primers are available for amplification of the fungal ITS and bacterial intergenic spacer regions [38], it is possible that mixed isolates (or cultures) of fungi and bacteria could be identified at the same time with a single array (our unpublished data).

Direct Detection of Fungi in Clinical Specimens

Most published arrays have been developed for fungal identification [44, 46–48, 50]; therefore, isolated colonies are a prerequisite for the purpose of identification. However, several studies have reported the successful application of array techniques for direct detection of fungi from clinical specimens; a few examples are discussed here. Cystic fibrosis is the major genetic disease among Caucasian populations, caused by mutations in the gene *CFTR* (cystic fibrosis transmembrane conductance regulator). The disease occurs in one in several thousand live births in people of European ancestry [61]. The mutations result in a thickening of the bronchial mucus, which facilitates the colonization and infection of bacteria and fungi [62, 63]. By targeting the ITS regions and using nested PCR for ITS amplification, Bouchara et al. [45] constructed an oligonucleotide array to detect fungi in the

a

	1	2	3	4	5	6	
A	Acfus2a&2b	Asfla4	Asfum2a	M	Asnid2	Asnig2	A
B	Aster2	CAB5	CDU1a	M	CGL1	CLUS1	B
C	CP6	CP8	CP10	M	CT3c	Exder1	C
D	M	M	M	NC	M	M	D
E	Fopin1	Palil4	Pavar2	M	Psboy3	Scbre3	E
F	Scpro4	Taeme4	Taeme6	M	NC	PC	F
	1	2	3	4	5	6	

b

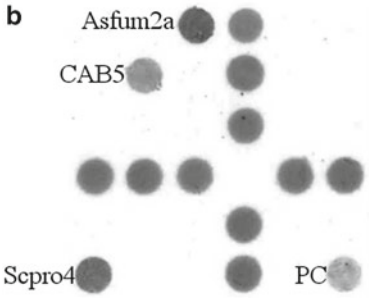

Fig. 39.2 (**a**) The layout of probes on the membrane array (0.5 by 0.5 cm) for detection of fungi in sputum samples from cystic fibrosis patient. The probe codes for different target microorganisms and the corresponding probe sequences are published previously [45]. "M" is a position marker. "PC" a fungus-specific positive control probe. "NC" a negative control probe. (**b**). Simultaneous detection of *Aspergillus fumigatus* (probe Asfum2a), *Candida albicans* (probe CAB5), and *Scedosporium prolificans* (probe Scpro4) by array hybridization in a sputum sample from a cystic fibrosis patient. The hybridization results were in concordant with that obtained by culture

sputum samples from cystic fibrosis patients. The array was used to analyze fungi in 57 specimens (39 patients), and the results were compared to those obtained by culture methods. For 16 specimens, the results of the array corresponded with those obtained by culture (Fig. 39.2). For 33 samples, the array detected more fungal species than did cultures, while the reverse was found in eight samples. In general, the array detected more fungi than those isolated by culture. The culture method employed five agar plates (CHROMAgar Candida, Sabouraud dextrose agar supplemented with chloramphenicol and gentamicin, Sabouraud dextrose agar supplemented with chloramphenicol and cycloheximide, Dichloran-Rose Bengal agar supplemented with benomyl, and erythritol-chloramphenicol agar) and two incubation temperatures (37 °C for the first two media and 25 °C for the others). However, neither the array nor the culture method is able to differentiate between colonization and infection [45].

Invasive fungal infections (IFI) can cause severe morbidity and high mortality rates in immunocompromised patients, especially patients with allogeneic stem cell transplantation or with acute leukemia after intensive chemotherapy. Spiess et al. [41] designed a DNA microarray targeting the ITS1 region to detect fungal pathogens in clinical specimens from neutropenic patients. By testing samples from 46 patients with proven, probable, or possible IFI or without IFI, the array detected *A. flavus*, *A. fumigatus*, *C. albicans*, *C. dubliniensis*, *C. glabrata*, *F. oxysporum*, *F. solani*, *R. microsporus*, *S. prolificans*, and *T. asahii* in blood, bronchoalveolar lavage, and tissue specimens. The authors concluded that the microarray could directly detect fungal DNAs in clinical samples and demonstrated a significant improvement for the diagnosis of IFIs [41].

Yeast Identification by an Oligonucleotide Array

Like mold infections, yeast infections also have increased in the past decades; *C. albicans* is the most common pathogen. However, infections caused by non-*C. albicans* and non-*Candida* yeasts, such as *Cryptococcus*, *Pichia*, *Rhodotorula*, *Saccharomyces*, and *Trichosporon*, have been widely reported [11, 12, 16, 17]. Identification of yeasts with this increasing diversity by biochemical methods may be challenging and inconclusive [19]. The introduction of reliable molecular methods that can identify taxonomically quite different opportunistic yeasts is imperative, since these emerging yeasts may have different susceptibilities to antifungals [64–66]. Commercially available identification kits, such as the Vitek Yeast Biochemical Card (bioMe´rieux Vitek, Taipei, Taiwan), API 20C (bioMe´rieux Vitek), and API ID32C (bio-Me´rieux Vitek), are able to identify common yeast pathogens to the species level with relatively high accuracy. However, a recent study showed that only 87 % of clinical yeast isolates were identified correctly to the species or genus level by the ID32C kit, and the remaining 13 % isolates were either unidentified or misidentified [19].

Leaw et al. [47] constructed an oligonucleotide array based on nylon membrane to identify 77 species (16 genera) of clinically relevant yeasts (Table 39.2). As the mold array [46], the method consisted of ITS amplification by universal primers followed by hybridization of the digoxigenin-labeled PCR product to a panel of oligonucleotide probes on the array for yeast identification. The layout of probes on the membrane array and identification of *C. albicans* and *C. glabrata* are shown in Fig. 39.3. After testing 452 yeast strains (419 target and 33 nontarget strains), the sensitivity and specificity of the array were 100 and 97 %, respectively. For *C. albicans*, the array had a detection limit of 10 pg genomic DNA per assay. Yeasts were directly detected from clinical specimens as demonstrated with sputum samples from patients with cystic fibrosis [45].

In order to rapidly identify fungi in fungus-positive blood culture bottles (Becton-Dickinson Microbiology Systems, Sparks, MD, USA), Hsiue et al. [67] used mold [46] and yeast [47] arrays to analyze 116 positive cultures (105 patients) and the results

Table 39.2 The 77 species (16 genera) of yeast that can be identified by the membrane array of Leaw et al. [47].

Microorganism (Teleomorph)	Microorganism (Teleomorph)	Microorganism (Teleomorph)
Candida albicans	*Candida boidinii*	*Candida cacaoi* (*Yamadazyma farinosa*)
Candida cantarelli	*Candida catenulata*	*Candida chodatii* (*Pichia burtonii*)
Candida colliculosa (*Torulaspora delbrueckii*)	*Candida dattila* (*Lachancea thermotolerans*)	*Candida dubliniensis*
Candida famata (*Debaryomyces hansenii*)	*Candida freyschussii*	*Candida glabrata*
Candida globosa (*Citeromyces matritensis*)	*Candida guilliermondii* (*Pichia guilliermondii*)	*Candida haemulonii*
Candida holmii (*Kazachstania exigua*)	*Candida inconspicua*	*Candida intermedia* (*Kluyveromyces cellobiovorus*)
Candida kefyr (*Kluyveromyces marxianus*)	*Candida krusei* (*Issatchenkia orientalis*)	*Candida lambica* (*Pichia fermentans*)
Candida lipolytica (*Yarrowia lipolytica*)	*Candida lusitaniae* (*Clavispora lusitaniae*)	*Candida maltosa*
Candida melibiosica	*Candida membranifaciens*	*Candida norvegensis* (*Pichia norvegensis*)
Candida norvegica	*Candida parapsilosis*	*Candida pelliculosa*
Candida pintolopesii	*Candida robusta* (*Saccharomyces cerevisiae*)	*Candida rugosa*
Candida sake	*Candida santamariae*	*Candida silvicola* (*Pichia holstii*)
Candida sphaerica (*Kluyveromyces lactis*)	*Candida steatolytica* (*Zygoascus hellenicus*)	*Candida tannotolerans* (*Vanderwaltozyma yarrowii*)
Candida tropicalis	*Candida utilis* (*Pichia jadinii*)	*Candida valida* (*Pichia membranifaciens*)
Candida viswanathii	*Candida zeylanoides* (*Pichia dubia*)	*Arthroascus schoenii*
Brettanomyces bruxellensis (*Dekkera bruxellensis*)	*Cryptococcus albidus*	*Cryptococcus curvatus*
Cryptococcus laurentii	*Cryptococcus neoformans* (*Filobasidiella neoformans*)	*Cryptococcus uniguttulatus* (*Filobasidium uniguttulatum*)
Debaryomyces carsonii	*Debaryomyces etchellsii*	*Debaryomyces maramus*
Kloeckera apiculata (*Hanseniaspora uvarum*)	*Kloeckera apis* (*Hanseniaspora guilliermondii*)	*Kloeckera japonica* (*Hanseniaspora valbyensis*)

(continued)

Table 39.2 (continued)

Microorganism (Teleomorph)	Microorganism (Teleomorph)	Microorganism (Teleomorph)
Kluyveromyces delphensis (*Nakaseomyces delphensis*)	*Kodamaea ohmeri*	*Lachancea cidri*
Lachancea fermentati	*Lodderomyces elongisporus*	*Pichia spartinae*
Rhodotorula glutinis (*Rhodosporidium diobovatum*)	*Rhodotorula minuta*	*Rhodotorula mucilaginosa*
Saccharomyces kluyveri	*Saccharomycopsis fibuligera*	*Sporobolomyces salmonicolor* (*Sporidiobolus salmonicolor*)
Trichosporon aquatile	*Trichosporon asahii*	*Trichosporon cutaneum*
Trichosporon inkin	*Trichosporon pullulans*	*Williopsis saturnus*
Zygosaccharomyces bisporus	*Zygotorulaspora florentinus*	

a

	1	2	3	4	5	6	7	8	9	10	11	12	
A	CAB5	CB1	CCA1	CCT1	CCH3	CCO5b	M	AS1	CAL5	CCUR2	CLR4	CN4b	A
B	CDAT2a	CDU1a	CFAM1a	CFRE2	CGL1	CGLO1a	M	CUNI2	DMAR1c	DB3	EF4	HS2	B
C	CGU2	CHAE1a	CHO2	CINC3	CIT2	CKEF2	M	KAP2	KAPIS2b	KJAP1	KDEL3	KYAR1	C
D	CK3	CLAM3a	CLI1a	CLUS1	CML4	CMEL1	M	LEL2	PCAR1b	PETC2	PF1	PC	D
E	M	M	M	M	M	M	NC	M	M	M	M	M	E
F	CMB1	CNO2b	CNOR1a	CP6	CP8	CP10	M	PO2	PSPA3	RGLU1	RM4	RRUB1	F
G	CPEL3	CPI2	CRUG2	CSAK3	CSAN1	CSIL2	M	SC2	SKLU1	SSAL3	ZB5	ZCI3	G
H	CSPH3a	CST3	CT3c	CUT5	CVAL2a	CVIS4a	M	ZF6a	ZFLO1a	TAQAS1	TAQU3	TASA1b	H
A	PC					CZEY2c	M	TCUT3b	TINK2	TP1a			A
	1	2	3	4	5	6	7	8	9	10	11	12	

b

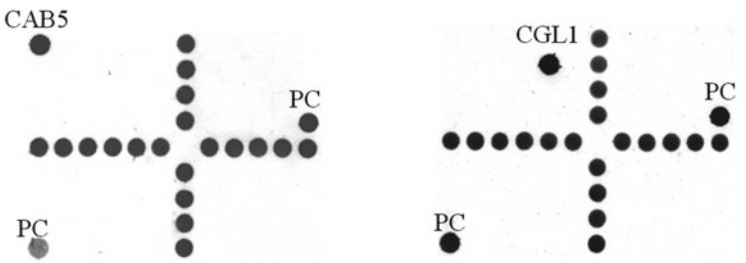

Fig. 39.3 (**a**) The layout of probes on the membrane array (0.75 by 0.9 cm) for identification of medically important yeasts. The probe codes for different target microorganisms and the corresponding probe sequences are published previously [47]. "M" is a position marker. "PC", the positive control probe. "NC": the negative control probe. (**b**) Identification of *C. albicans* (probe CAB5) and *Candida glabrata* (probe CGL1) by array hybridization

were compared with those obtained by the conventional methods. A total of 124 yeast isolates and two mold isolates were detected by the two arrays; the species (isolate number) included *C. albicans* (50), *C. tropicalis* (26), *C. glabrata* (18), *C. parapsilosis* (14), *Cryptococcus neoformans* (9), *T. asahii* (2), *Rhodotorula mucilaginosa* (2), *P. marneffei* (2), and three other species. Fungemia due to multiple species was detected in 10 blood bottles, while the conventional method only found six mixed cultures. An isolate identified as *Rhodotorula glutinis* by biochemical tests was identified as *R. mucilaginosa* by the array; the identification of the isolate as *R. mucilaginosa* by hybridization was found to be correct as revealed by sequencing of the ITS region of the isolate [68]. A test sensitivity of 100 % was obtained by the two arrays. The identification procedure could be completed within 16–24 h, while 2–3 days are normally required by culture once a blood culture bottle is found to be growth-positive. The results indicated that the array method has the advantages of rapidity, accuracy, and the extraordinary capability to detect multiple species fungemia.

Concluding Remarks

Identification of clinically important fungi by arrays is reliable and can be used as an accurate alternative to the conventional methods. The whole procedure of hybridization can be completed within 8–24 h after starting with isolated colonies. Fungal identification by array hybridization does not necessarily require knowledge of the fungal morphological characteristics, which are essential for conventional identification of filamentous fungi. The array technique permits a shorter time to achieve results as well as the correct identification of some species that are morphologically indistinguishable from each other. The array technique also has a potential for direct detection of fungi in clinical specimens as demonstrated in the studies of Bouchara et al. [45] and Spiess et al. [41], provided the fungal DNAs in clinical samples are effectively amplified by PCR or other amplification methods. The prominent feature of the array technique for fungal identification is the use of a common protocol encompassing DNA extraction, target gene amplification, and array hybridization. The technique is relatively simple and straightforward [41, 45–47] and the published arrays can be expanded by including more probes to cover more fungal species. However, it is not easy for clinical laboratory staffs to prepare their own arrays. Therefore, laboratories would greatly benefit by the availability of commercial kits based on the array technology for fungal identification in the near future.

References

1. Baddley JW, Stroud TP, Salzman D, Pappas PG (2001) Invasive mold infections in allogeneic bone marrow transplant recipients. Clin Infect Dis 32:1319–1324
2. Cornet M, Fleury L, Maslo C, Bernard JF, Brucker G, Invasive Aspergillosis Surveillance Network of the Assistance Publique-Hopitaux de Paris (2002) Epidemiology of invasive

aspergillosis in France: a six-year multicentric survey in the Greater Paris area. J Hosp Infect 51:288–296
3. Dasbach EJ, Davies GM, Teutsch SM (2000) Burden of aspergillosis-related hospitalizations in the United States. Clin Infect Dis 31:1524–1528
4. McNeil MM, Nash SL, Hajjeh RA et al (2001) Trends in mortality due to invasive mycotic diseases in the United States, 1980–1997. Clin Infect Dis 33:641–647
5. Denning DW (1998) Invasive aspergillosis. Clin Infect Dis 26:781–803
6. Husain S, Alexander BD, Munoz P et al (2003) Opportunistic mycelial fungal infections in organ transplant recipients: emerging importance of non-*Aspergillus* mycelial fungi. Clin Infect Dis 37:221–229
7. Chen SC, Halliday CL, Meyer W (2002) A review of nucleic acid-based diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays. Med Mycol 40:333–357
8. Hall L, Wohlfiel S, Roberts GD (2004) Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of filamentous fungi encountered in the clinical laboratory. J Clin Microbiol 42:622–626
9. Larone DH (2002) Medically important fungi: a guide to identification, 4th edn. ASM, Washington, DC
10. Pfaller MA, Diekema DJ (2004) Rare and emerging opportunistic fungal pathogens: concerns for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. J Clin Microbiol 42:4419–4431
11. Davies F, Logan S, Johnson E, Klein JL (2006) Sternal wound infection by *Trichosporon inkin* following cardiac surgery. J Clin Microbiol 44:2657–2659
12. Enache-Angoulvant A, Hennequin C (2005) Invasive *Saccharomyces* infection: a comprehensive review. Clin Infect Dis 41:1559–1568
13. Fanci R, Pecile P (2005) Central venous catheter-related infection due to *Candida membranaefaciens*, a new opportunistic azole-resistant yeast in a cancer patient: a case report and a review of literature. Mycoses 48:357–359
14. Holland SM, Shea YR, Kwon-Chung J (2004) Regarding *Trichosporon pullulans* infection in 2 patients with chronic granulomatous disease. J Allergy Clin Immunol 114:205–206
15. Mele G, Musci M, Musto C, D'Amato L, Traficante A, Di Renzo N (2005) Pneumonia caused by *Trichosporon pullulans* in an autologous peripheral blood stem cell transplant recipient: possible misidentification. Bone Marrow Transpl 35:1219–1220
16. Panagopoulou P, Evdoridou J, Bibashi E et al (2002) *Trichosporon asahii*: an unusual cause of invasive infection in neonates. Pediatr Infect Dis J 21:169–170
17. Pasqualotto GC, Copetti FA, Meneses CF, Machado AR, Brunetto AL (2005) Infection by *Rhodotorula* sp. in children receiving treatment for malignant diseases. J Pediatr Hematol Oncol 27:232–233
18. Taj-Aldeen SJ, Doiphode SH, Han XY (2006) *Kodamaea* (*Pichia*) *ohmeri* fungaemia in a premature neonate. J Med Microbiol 55:237–239
19. Ciardo DE, Schar G, Bottger EC, Altwegg M, Bosshard PP (2006) Internal transcribed spacer sequencing versus biochemical profiling for identification of medically important yeasts. J Clin Microbiol 44:77–84
20. Pfaller MA, Diekema DJ, Colombo AL et al (2006) *Candida rugosa*, an emerging fungal pathogen with resistance to azoles: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program. J Clin Microbiol 44:3578–3582
21. Kobayashi M, Sonobe H, Ikezoe T, Hakoda E, Ohtsuki Y, Taguchi H (1999) In situ detection of *Aspergillus* 18S ribosomal RNA in invasive pulmonary aspergillosis. Intern Med 38:563–569
22. Skladny H, Buchheidt D, Baust C et al (1999) Specific detection of *Aspergillus* species in blood and bronchoalveolar lavage samples of immunocompromised patients by two-step PCR. J Clin Microbiol 37:3865–3871
23. Wu Z, Tsumura Y, Blomquist G, Wang X-R (2003) 18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for the detection of fungal isolates. Appl Environ Microbiol 69:5389–5397

24. Evertsson U, Monstein HJ, Johansson AG (2000) Detection and identification of fungi in blood using broad-range 28S rDNA PCR amplification and species-specific hybridisation. Acta Pathol Microbiol Immunol Scand 108:385–392
25. Kurtzman CP, Robnett CJ (1997) Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol 35:1216–1223
26. Ninet B, Isabell J, Bontems O et al (2003) Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit. J Clin Microbiol 41:826–830
27. Kappe R, Okeke CN, Fauser C, Maiwald M, Sonntag H-G (1998) Molecular probes for the detection of pathogenic fungi in the presence of human tissue. J Med Microbiol 47: 811–820
28. Velegraki A, Kambouris ME, Skiniotis G, Savala M, Mitroussia-Ziouva A, Legakis NJ (1999) Identification of medically significant fungal genera by polymerase chain reaction followed by restriction enzyme analysis. FEMS Immunol Med Microbiol 23:303–312
29. Chang HC, Leaw SN, Huang AH, Wu TL, Chang TC (2001) Rapid identification of yeasts in positive blood cultures by a multiplex PCR method. J Clin Microbiol 39:3466–3471
30. Chen Y, Eisner JD, Kattar MM et al (2001) Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. J Clin Microbiol 39:4042–4051
31. De Aguirre L, Hurst SF, Choi JS, Shin JH, Hinrikson HP, Morrison CJ (2004) Rapid differentiation of *Aspergillus* species from other medically important opportunistic molds and yeasts by PCR-enzyme immunoassay. J Clin Microbiol 42:3495–3504
32. Henry T, Iwen PC, Hinrichs SH (2000) Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. J Clin Microbiol 38:1510–1515
33. Iwen PC, Freifeld AG, Bruening TA, Hinrichs SH (2004) Use of a panfungal PCR assay for detection of fungal pathogens in a commercial blood culture system. J Clin Microbiol 42:2292–2293
34. Iwen PC, Hinrichs SH, Rupp ME (2002) Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med Mycol 40: 87–109
35. Li YL, Leaw SN, Chen JH, Chang HC, Chang TC (2003) Rapid identification of yeasts commonly found in positive blood cultures by amplification of the internal transcribed spacer regions 1 and 2. Eur J Clin Microbiol Infect Dis 22:693–696
36. Turenne CY, Sanche SE, Hoban DJ, Karlowsky JA, Kabani AM (1999) Rapid identification of fungi by using the ITS 2 genetic region and an automated fluorescent capillary electrophoresis system. J Clin Microbiol 37:1846–1851
37. Adamczyk J, Hesselsoe M, Iversen N et al (2003) The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Appl Environ Microbiol 69:6875–6887
38. Lin YT, Vaneechoutte M, Huang AH et al (2010) Identification of clinically important anaerobic bacteria by an oligonucleotide array. J Clin Microbiol 48:1283–1290
39. Fukushima M, Kakinuma K, Hayashi H, Nagai H, Ito K, Kawaguchi R (2003) Detection and identification of *Mycobacterium* species isolates by DNA microarray. J Clin Microbiol 41:2605–2615
40. Volokhov D, Rasooly A, Chumakov K, Chizhikov V (2002) Identification of *Listeria* species by microarray-based assay. J Clin Microbiol 40:4720–4728
41. Spiess B, Seifarth W, Hummel M et al (2007) DNA microarray-based detection and identification of fungal pathogens in clinical samples from neutropenic patients. J Clin Microbiol 45:3743–3753
42. Seifarth W, Krause U, Hohenadl C, Baust C, Hehlmann R, Leib-Mösch C (2000) Rapid identification of all known retroviral reverse transcriptase sequences with a novel versatile detection assay. AIDS Res Hum Retroviruses 16:721–729
43. Leinberger DM, Schumacher U, Autenrieth IB, Bachmann TT (2005) Development of a DNA microarray for detection and identification of fungal pathogens involved in invasive mycoses. J Clin Microbiol 43:4943–4953

44. Huang A, Li JW, Shen ZQ, Wang XW, Jin M (2006) Highthroughput identification of clinical pathogenic fungi by hybridization to an oligonucleotide microarray. J Clin Microbiol 44:3299–3305
45. Bouchara JP, Shieh HY, Croquefer S et al (2009) Development of an oligonucleotide array for direct detection of fungi in sputum samples from patients with cystic fibrosis. J Clin Microbiol 47:142–152
46. Hsiao CR, Huang L, Bouchara J-P, Barton RH, Li C, Chang TC (2005) Identification of medically important molds by an oligonucleotide array. J Clin Microbiol 43:3760–3768
47. Leaw SN, Chang HC, Barton R, Bouchara JP, Chang TC (2007) Identification of medically important *Candida* and non-*Candida* yeast species by an oligonucleotide array. J Clin Microbiol 45:2220–2229
48. Li HC, Bouchara JP, Hsu MM-L, Barton R, Chang TC (2007) Identification of dermatophytes by an oligonucleotide array. J Clin Microbiol 45:3160–3166
49. Riggsby WS, Torres-Bauza LJ, Wills JW, Townes TM (1982) DNA content, kinetic complexity, and the ploidy question in *Candida albicans*. Mol Cell Biol 2:853–862
50. Hung W-T, Su S-L, Shiu L-Y, Chang TC (2011) Rapid identification of allergenic and pathogenic molds in environmental air by an oligonucleotide array. BMC Infect Dis 11:91
51. Gräser Y, El Fari M, Vilgalys R et al (1999) Phylogeny and taxonomy of the family *Arthrodermataceae* (dermatophytes) using sequence analysis of the ribosomal ITS region. Med Mycol 37:105–114
52. Jackson CJ, Barton RC, Evans EGV (1999) Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J Clin Microbiol 37:931–936
53. Makimura K, Mochizuki T, Hasegawa A, Uchida K, Saito H, Yamaguchi H (1998) Phylogenetic classification of *Trichophyton mentagrophytes* complex strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J Clin Microbiol 36: 2629–2633
54. Weitzman I, Summerbell RC (1995) The dermatophytes. Clin Microbiol Rev 8:240–259
55. Kaszubiak A, Klein S, de Hoog GS, Gräser Y (2004) Population structure and evolutionary origins of *Microsporum canis*, *M. ferrugineum* and *M. audouinii*. Infect Genet Evol 4:179–186
56. Li HC, Bouchara JP, Hsu MM-L, Barton R, Su S, Chang TC (2008) Identification of dermatophytes by sequence analysis of the rRNA gene internal transcribed spacer regions. J Med Microbiol 57:592–620
57. Diaz MR, Fell JW (2004) High-throughput detection of pathogenic yeasts of the genus *Trichosporon*. J Clin Microbiol 42:3696–3706
58. Ikuta S, Takagi K, Wallace RB, Itakura K (1987) Dissociation kinetics of 19 base paired oligonucleotide-DNA duplexes containing different single mismatched base pairs. Nucleic Acids Res 15:797–811
59. Brown TJ, Anthony RM (2000) The addition of low numbers of 3′ thymine bases can be used to improve the hybridization signal of oligonucleotides for use within arrays on nylon supports. J Microbiol Methods 42:203–207
60. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gefland DH, Sninsky JJ, White TJ (eds) PCR protocols, a guide to methods and applications. Academic, San Diego, CA, pp 315–322
61. Gilligan PH, Kiska DL, Appleman MD (2006) Cumitech 43, Cystic fibrosis microbiology. In: Appleman MD (ed) ASM, Washington, DC
62. Gilligan PH (1991) Microbiology of airway disease in patients with cystic fibrosis. Clin Microbiol Rev 4:35–51
63. Moss RB (1995) Cystic fibrosis: pathogenesis, pulmonary infection and treatment. Clin Infect Dis 21:839–851
64. Gomez-Lopez A, Mellado E, Rodriguez-Tudela JL, Cuenca-Estrella M (2005) Susceptibility profile of 29 clinical isolates of *Rhodotorula* spp. and literature review. J Antimicrob Chemother 55:312–316

65. Rodero L, Cuenca-Estrella M, Cordoba S et al (2002) Transient fungemia caused by an amphotericin B-resistant isolate of *Candida haemulonii*. J Clin Microbiol 40:2266–2269
66. Serena C, Pastor FJ, Ortoneda M, Capilla J, Nolard N, Guarro J (2004) In vitro antifungal susceptibilities of uncommon basidiomycetous yeasts. Antimicrob Agents Chemother 48:2724–2726
67. Hsiue HC, Huang YT, Kuo YL, Liao CH, Chang TC, Hsueh PR (2010) Rapid identification of fungal pathogens in positive blood cultures using oligonucleotide array hybridization. Clin Microbiol Infect 16:493–500
68. Leaw SN, Chang HC, Sun HF, Barton R, Bouchara JP, Chang TC (2006) Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions. J Clin Microbiol 44:693–699

Chapter 40
Laboratory Technical Advances in the Diagnosis of *Clostridium difficile*

Karen C. Carroll

Microbiology and Pathogenesis

Clostridium difficile (*C. difficile*) is a spore-forming, anaerobic, gram positive rod that colonizes the colon of 5 % of healthy adults and 30–70 % of healthy infants [1]. The organism can also be found in a variety of environmental sources including soil, river water, domestic animals, and home and healthcare environments [2]. *C. difficile* acquired its name from the observations by Hall and O'Toole [3] in the difficulty of isolating the organism because of its slow growth (doubling time 40–70 min) compared to other *Clostridium* spp. During logarithmic growth, when vegetative cells predominate, the organism is very aerointolerant. In 1978, prior knowledge of the organism and the observation that antibiotic associated diarrhea was associated with a cytotoxin in the hamster model, converged in the work by Bartlett et al. that demonstrated that *C. difficile* caused disease in humans through the elaboration of a cytotoxin [2, 4, 5]. Later it was established that the organism produces two toxins, toxin A, a 308 kDa enterotoxin and toxin B, a 270 kDa cytotoxin. Both toxins cause disease by glycosylating small GTPases such as Rho, Rac, and Cdc42 in the cell [2, 6]. Glycosylation of these small proteins disrupts signaling pathways causing irreversible changes in cellular morphology and consequent inhibition of cell division and membrane trafficking, leading to cell death [2, 6].

The genes that encode toxins A and B, *tcdA* and *tcdB,* are found along with three other genes (*tcdC*, *tcdE*, *tcdR*) on the pathogenicity locus (PaLoc), a conserved 19.6 kb region of the bacterial genome [6]. *tcdE* encodes a protein whose pore-forming activity allows the release of TcdA and TcdB from the cell [6, 7]. *tcdR*, found upstream of *tcdB*, is a positive regulator of *tcdA* and *tcdB* expression [6, 7].

K.C. Carroll (✉)
Division of Medical Microbiology, The Johns Hopkins University School of Medicine, The Johns Hopkins Medical Institutions, Meyer B1-193, 600 N. Wolfe St., Baltimore, MD 21287, USA
e-mail: Kcarrol7@jhmi.edu

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tcdC is found downstream of *tcdA* and this gene has been shown to be a negative regulator of toxin production that prevents transcription of the PaLoc [6–10]. Mutations in many of these various genes have a significant impact on expression of one or both toxins and have been shown to be responsible for the emergence of hypervirulent toxin variant strains (see Epidemiology section).

In addition to toxins A and B, about 6–12 % of strains produce a binary toxin, *C. difficile* transferase, encoded by *cdtA* and *cdtB* which are not located on the PaLoc [11, 12]. Binary toxin may contribute to virulence by enhancing cytotoxicity and also by increasing adherence of *C. difficile* in vivo [13].

The transmissible form of *C. difficile* is a spore which contributes to survival of the organism in the host and is responsible for recurrence of disease when therapy is withdrawn. Like other bacterial spores *C. difficile* spores are metabolically dormant, survive for long periods of time, and are resistant to harsh physical or chemical treatments such as 70 % ethanol and ultraviolet light [14]. Failure to remove spores from contaminated hospital environments contributes to nosocomial spread in healthcare facilities.

Epidemiology

Among hospitalized patients *C. difficile* is responsible for 25–30 % of cases of antibiotic associated diarrhea and greater than 95 % of cases of pseudomembranous colitis [1, 2, 4]. The incidence of *C. difficile* infections (CDI) has more than doubled since 1996, making it not only the most common cause of healthcare associated diarrhea, but possibly the most common bacterial cause of diarrhea in the USA [15].

The increased incidence of CDI is likely related to a variety of organism and host factors. In 2000, hospitals in both the USA and Canada began to see outbreaks of CDI that were associated with more severe disease and an increase in mortality [16, 17]. In the USA, eight facilities in six states reported that the *C. difficile* isolates recovered from the patients with more severe illness belonged to a particular clone designated North American pulsed field type 1 (NAP-1), ribotype 027, and restriction endonuclease analysis (REA) type B1 (hereafter referred to as NAP-1) [16]. Prior to 2001, this particular strain accounted for less than 1 % of all infections. More importantly, the "wild type" strain was quinolone susceptible, but the outbreak strain was found to be fluoroquinolone resistant [16]. Upon further characterization, NAP-1 was shown to be a toxin variant strain, toxinotype III, and to have an 18 bp deletion at nucleotides 330–347 in *tcd*C, the negative regulator of toxins A and B [16]. Subsequently the 18 bp deletion was shown not to be important with respect to organism virulence [9, 10]. Other investigators have provided additional phylogenetic characterization of the novel NAP-1 clone. Spigaglia et al. confirmed the 18 bp deletion, detected an additional 39 bp deletion as well as nonsense mutations in *tcdC* that reduced the TcdC protein from 232 to 61 amino acids [18]. There is a correlation between truncation of TcdC, due to the single base-pair deletion at position 117, which results in the formation of a stop codon, and increased toxin production

due to derepression of the Pathogenicity Locus [9, 18–20]. These mutations and others have correlated with observations of increased quantity of toxins A and B, increased duration of toxin production and increased sporulation that explain the enhanced virulence of NAP-1 [20–22].

Depending upon the geographic location, other strains have been implicated in outbreaks and severe disease. Ribotype 017, prevalent in Asia, is a toxin A negative, toxin B positive strain that is quinolone and macrolide resistant and has been associated with hospital outbreaks and increase rates of pseudomembranous colitis [23, 24]. Ribotype 078, more common in piglets and other food animals, has increased in incidence as a cause of human disease in the Netherlands and has been isolated in the USA as well [1, 2]. Similar to the NAP-1 strain, this ribotype also has deletions in *tcdC* responsible for more severe symptoms although mortality has been less than with NAP-1 [2]. The enhanced virulence of some of these toxin variant strains, combined with an increasingly susceptible population has created the perfect storm for the epidemic of CDI that has slowly spread across the globe. The emerging epidemiology and potential risk factors including age, quinolone antibiotics, and potentially, proton pump inhibitors, for acquisition of *C. difficile,* have received much attention as well. Additional details on the epidemiology of *C. difficile* are provided in the review by Freeman et al. [25].

Against this backdrop of evolving strains and emerging epidemiology has been the impetus to implement better and faster diagnostic methods for the detection of *C. difficile.* Although controversy still exists about the optimum method of diagnosis, the epidemic of CDI has clearly forced laboratories to scrutinize their practices. Practice guidelines from professional societies have been published to guide the clinical and laboratory approaches to diagnosis [26, 27] and a variety of molecular assays have been approved for diagnosis. This chapter focuses on the rationale for the diagnostic guidelines and the performance of new methods and algorithms for *C. difficile* detection.

Diagnostic Methods

The Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA) in the USA, as well as the European Society of Clinical Microbiology have published recommendations regarding the diagnosis and management of *C. difficile* infection [26, 27]. Both sets of practice guidelines emphasize that testing should only be performed on unformed stool specimens in patients at risk for CDI. Both groups recommend testing using a two-step algorithm. In the case of IDSA, the recommendation is to begin with EIA testing for glutamate dehydrogenase (GDH) followed by a test for toxin production if the GDH result is positive (see GDH section below) [26]. In the case of the ESCMID document, initial testing might be an EIA for GDH testing, an EIA that detects toxins A and B, or a molecular test for the detection *tcdB* [27]. If the result is negative then no further testing is needed. If the result is positive then the specimen should be retested with

Table 40.1 Performance characteristics of various test methods for *C. difficile* diagnosis

	Performance characteristics	
Methods/assays	Sensitivity (%, range)	Specificity (%, range)
Toxigenic anaerobic culture	N/A	N/A
Enzyme immunoassays[a]	34–99	84–100
Cell culture cytotoxicity assays[b]	67–86	97–100
Glutamate dehydrogenase[c]	71–100	76–98
Nucleic acid amplification tests[d]		
BD-GeneOhm	84–96	94–100
Prodesse ProGastro	77–92	95–99
GeneXpert	94–100	93–99
illumigene®	99	98

Modified with permission from [2]
[a]Derived from [27, 43, 46] and includes both solid phase and membrane assays combined. Data is not stratified by comparative method
[b]Compiled from [42–44, 65]
[c]Compiled from [27, 47, 50–52]
[d]Compiled from [42, 43, 45, 54, 56, 57, 63–70]

a method that detects either free toxins, a molecular test, or GDH and different from the assay used for the initial screening test. Both documents agree that toxigenic culture is the most sensitive method for detection of *C. difficile* in stool specimens [26, 27]. Table 40.1 lists the test methods currently available for the diagnosis of *C. difficile* infections and their performance characteristics.

Toxigenic Bacterial Culture

Many laboratories have resurrected bacterial culture for CDI to assist with outbreak investigations by determining whether NAP-1 is circulating in a particular environment. In addition, culture has become the new diagnostic "gold standard" for evaluation of the plethora of available and developing molecular assays for CDI detection. Finally, culture is useful for surveillance of drug resistance and is sometimes helpful in patient management [2]. However, it is too slow and impractical to be used in clinical microbiology laboratories as the primary diagnostic method for CDI.

Toxigenic anaerobic culture requires inoculation of the stool to anaerobic media, incubating the media anaerobically for 2–5 days, and once recovered, determining whether the *C. difficile* isolate is a toxin producer. There is no agreed upon standard method, but a well done culture has been shown to significantly increase the yield of *C. difficile* detection by 15–25 % when performed after a negative direct toxin test [28, 29]. Factors to consider when developing a culture method include (1) the need for and type of spore enrichment, (2) the type of media, and (3) the best method for confirming toxin production in the recovered isolate. Each of these factors is briefly addressed.

A variety of media, both selective and nonselective, is available for culturing *C. difficile*. Nonselective anaerobic media have the advantages of being less expensive and more readily available in clinical labs than selective agars or broths, but do not allow for easy presumptive identification of *C. difficil*e [30]. The original cycloserine, cefoxitin, fructose agar (CCFA) as described by George et al., contained an egg yolk fructose agar base with 500 μg/ml of cycloserine and 16 μg/ml of cefoxitin [30]. On this medium, the *C. difficile* organisms produced yellow, fluorescent filamentous colonies that were easy to distinguish from other organisms [30].

Over the years incorporation of substances to enhance germination of spores such as horse blood in place of the egg yolk, taurocholate, and lysozyme have been shown to improve recovery [2, 31]. In some studies, CCFA variants with reduced concentrations of the antimicrobial agents were less sensitive compared to the George formulation [32, 33]. However, in the study by Levett et al. using CCFA with cycloserine and cefoxitin at concentrations half that of the George formulation were less inhibitory to *C. difficile* when combined with a protocol that used alcohol shock for spore enrichment [34]. Whatever medium is chosen, it is important to use prereduced media as the failure to do so can impact the sensitivity of the culture method [33].

Other studies have examined the utility of broth enrichment compared to direct plating on solid media as well as spore enrichment techniques. In the former situation, fecal specimens are inoculated to an enrichment broth that contains taurocholate, antibiotics, increased carbohydrates and/or lysozyme to reduce normal fecal flora and enhance recovery of small concentrations of *C. difficile*. Several studies have shown enhanced recovery of enrichment broth compared to direct plating on solid media [35–37].

Spore enrichment involves treating the fecal specimen with either heat or ethanol to reduce competing normal flora. When subjected to heat or ethanol, *C. difficile* develops spores which then germinate when the specimen is planted on anaerobic media. Both heat and alcohol have been shown to reduce competing normal flora. With heat shock, an aliquot of the stool specimen is incubated in a 70 °C water bath or heat block for 20 min prior to plating [38]. In the case of alcohol shock, 0.5 ml of stool sample is added to an equal volume of ethanol and mixed by vortexing. After standing at room temperature for 1 h, the specimen is then plated on selective *C. difficile* media or other anaerobic media [39].

Once *C. difficile* is recovered from culture, the isolate should be tested for toxin production, as culture will recover both nontoxigenic as well as toxigenic isolates. At least one study demonstrated that an enzyme immunoassay should not be used to test the isolate for toxin production as it may be too insensitive [40]. A cell culture cytotoxicity assay (CCCA) or polymerase chain reaction (PCR) is preferable [40].

In summary, there is no agreed upon standard method for culturing *C. difficile.* A combination of some type of enrichment (whether it be spore enrichment or broth enrichment) and direct plating on solid media seems to provide the most sensitive approach and should be used in circumstances where low quantities of organisms may be present, as may be the case in an epidemiological study.

Cell Culture Cytotoxicity Assays

Until this decade, CCCAs were considered the "gold standard" and preferred method for *C. difficile* diagnosis. This method was the comparator against which many of the enzyme immunoassays were assessed. Performance of cytotoxin tests involves multiple steps and lack of adherence to these factors can significantly affect performance. The fecal sample is first suspended in phosphate buffered saline (PBS). Then the buffered sample is centrifuged to remove debris and the supernatant is filtered. The diluted filtrate is applied to a cell monolayer usually in a macrotiter or microtiter multiwelled plate. After incubation at 37 °C for 24 h the plates are assessed for cytopathic affect that is characterized by rounding of cells. The cytopathic effect (CPE) is neutralized by *C. difficile* or *C. sordelli* antitoxin. If the monolayer does not show CPE, it is reincubated for another 24 h before calling the sample negative.

As mentioned, there are many factors that affect optimum performance of CCCAs. While many different cell types can be used, such as MRC-5, CHO-K1, WI-38, Vero, HEp2, and HFF, HFF are preferred in the USA and were the most sensitive cell line in one study [41]. Cells should be fresh (5–14 days old) and of low passage [41]. The specimen should be diluted 1:40 to 1:200 to prevent nonspecific toxicity by substances in fecal samples. Criteria used to interpret CPE can also affect the results. In some instances, CPE can be seen as early as 4 h [41].

Recent comparisons of CCCAs to toxigenic culture have shown that the overall sensitivity of this method, whether a user developed assay or a commercial product, is surprisingly low, well below 90 %, which is suboptimal for a "gold standard" [42–45]. The need for more rapid answers in the era of hypervirulent strains and the disappearance of cell culture techniques for viral diagnosis in clinical labs has seen the decrease in availability of this method as a primary diagnostic test. It is also not endorsed by professional societies as a test of choice for direct specimen testing [26]. However, CCCAs are still very useful for confirmation of toxin production in clinical isolates recovered from anaerobic culture.

Toxin Enzyme Immunoassays

Enzyme immunoassays became available in the mid to late 1980s to replace the more labor-intensive cell culture cytotoxicity assays. Solid phase microtiter plate formats, that were coated with monoclonal or polyclonal antibodies against toxins A and/or B, allowed for batch testing and the ability to report same day results. Later, rapid immunoassays in chromatographic cassettes, immunocard, and lateral flow membrane formats became available. These assays have an overall sensitivity of 75–85 % compared to CCCAs, which is suboptimal performance in the era of NAP-1 epidemic disease [1, 2, 43, 46]. In some studies the performance of these assays is well below this range as low as 38 and 43 % [47, 48]. Two comprehensive reviews of these assays have recently confirmed that these tests are suboptimal as primary diagnostic methods. The results of these studies are summarized in

Table 40.2 Summary of two comprehensive reviews of EIA performance for detection of *C difficile*

	Planche et al.[a]		Eastwood et al.[b]			
	Comparison to any reference method		Comparison to CCCA		Comparison to toxigenic culture	
Assays Evaluated	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Premier toxin A+B[c]	94.8	97	91.7	97.1	80.8	97.5
TechLab ToxA/B II[d]	83.3	98.7	90.7	95.7	80.0	96
TechLab Tox A/B Quik Chek[d]	83.9	99.7	84.3	98.6	74.4	98.9
Remel Xpect[e]	82.0	96.2	77.8	98.8	68.8	99.4
bioMerieux VIDAS[f]	75.7	93.1	89.8	96.7	80	97.3
Meridian Immunocard[c]	89.6	99.3	77.8	92.8	68.8	93
GA C. diff antigen[g]	N/A	N/A	76.8	90.9	68.8	91.4
Rida Screen Tox A/B[h]	N/A	N/A	66.7	95.1	60.0	95.6
Remel ProsSpecT[e]	N/A	N/A	89.8	92.6	81.6	93.3

[a]Data are from [46] and are reprinted with permission. The numbers represent median values from multiple studies where the reference method was either CCCA or toxigenic culture
[b]Data are from [43] and are reprinted with permission. The authors tested each assay in their clinical laboratory on the same set of 600 samples. The sensitivity and specificity of the CCCA compared to toxigenic cultures are 86.4 and 99.2 %, respectively
[c]Meridian Bioscience, Inc. Cincinnati, OH
[d]TechLab, Blacksburg, VA
[e]Remel, Lenexa, KS
[f]bioMerieux, Durham, NC
[g]The Binding Site Ltd. Birmingham, UK
[h]R-Biopharm AG Darmstadt, Germany

Table 40.2. In the study by Planche et al., the authors reviewed the literature on the six most commonly used EIAs and only included those reports where the assays were compared to a reference method [46]. The authors defined acceptability criteria as a sensitivity of 90 % and a positive predictive value of ≤3 %. Using diagnostic odds ratios (Kruskal–Wallis test) and logistic regression, the authors determined that there was no difference in performance among the various assays [46]. However, none of the assays met the acceptability criteria [46].

In the study by Eastwood et al., the authors tested 600 diarrheal samples using nine commercial methods for detection of *C. difficile* and compared the results of those assays to toxigenic culture [43]. The sensitivities and specificities for the eight EIAs that were evaluated ranged from 60 to 82 % and 93–99 %, respectively [43]. None of the assays evaluated fulfilled the criteria for acceptability as outlined in the Planche study [43]. Given the extensive data on EIAs demonstrating inadequate performance, combined with literature that shows that strain variation may impact the performance of these tests [49] (see GDH Discussion below), professional societies no longer endorse toxin EIAs as a primary diagnostic methods for CDI [26, 27].

Glutamate Dehydrogenase Testing

Given the poor performance of toxin EIAs and the lack of timeliness and poor sensitivity of CCCAs, prior to the availability of molecular tests, laboratories developed two step algorithms predicated upon screening for *C. difficile* glutamate dehydrogenase. This enzyme, also called the common antigen, is present in high levels in all strains of *C. difficile*, both toxigenic and nontoxigenic isolates. The initial microtiter plate enzyme immunoassay test called the C DIFF CHEK (TechLab, Blacksburg, VA) showed good sensitivity and high negative predictive values in earlier studies [47, 50–52], making the assay a very useful first step in screening for the presence of the organism. Depending upon the institution's prevalence of *C. difficile*, 85–90 % of results can be finalized after the GDH test because a negative result reliably means the absence of *C. difficile*. Those specimens that are positive have toxin testing performed using CCCA or more recently, a molecular assay. A newer version of the test called the C DIFF QUIK CHEK COMPLETE™ (TechLab) combines the GDH test and an EIA for toxins A and B into a single immunochromatographic membrane device that can yield a result in 30 min. Several published studies have shown excellent sensitivity (100 %) for the GDH portion of the assay, but the toxin component of the assay has a sensitivity of 61–78 % [53, 54]. Therefore, some laboratories have decided to confirm GDH positive, toxin negative specimens with another more sensitive method such as PCR [54, 55].

While many laboratories have been happy with the performance of either version of the GDH assay, more recent studies have reported sensitivities below 90 % [56, 57]. At least one study suggests that the variability in GDH and toxin EIA performance may be related to strain type [49]. Certain non-NAP-1 ribotypes such as 002 and 106 were less likely to be detected by GDH and commercial EIA kits than NAP-1 strains [49]. Such results have not been duplicated in other clinical studies. A recent in vitro study failed to demonstrate an impact of strain type on GDH performance when clinical isolates grown on CCFA were suspended in sterile water at a 0.5 McFarland concentration and tested neat and diluted [58]. In addition, a recent meta-analysis of 13 manuscripts that met inclusion criteria showed a high accuracy of GDH for detection of *C. difficile* in stool where the sensitivity and specificity compared to culture were each above 90 % [59]. Given the mixed literature on the performance of GDH tests, laboratories that implement it should verify that the assay has acceptable test performance for their patient population.

Molecular Assays

Molecular tests for detection of *C. difficile* directly from fecal samples were tried in the early 1990s [60, 61]. Most of these assays used conventional polymerase chain reaction techniques and lengthy cumbersome nucleic acid extraction methods [60, 61]. Later in the decade, reports of improved fecal extraction methods and success with real-time platforms were published [62]. In 2008, the first qPCR assay, the

BD-GeneOhm Cdiff assay, received FDA approval. This assay is based upon TaqMan chemistry and targets conserved regions of *tcdB*. This assay in its current format has a manual extraction. A swab is inserted into an aliquot of liquid or soft stool. The swab containing specimen is then placed into a sample buffer tube provided by the manufacturer and is vortexed for 1 min. Uninoculated sample buffer is added to a lysis tube with glass beads, then 10 μl of stool in buffer is added to the lysis tube. Following centrifugation and inactivation at 95 °C in a dry heating block, lysed sample is added to a SmartCycler tube (Cepheid, Inc., Sunnyvale, CA) containing reconstituted master mix. An internal control is contained in the master mix. Uninoculated sample buffer can serve as a negative control and a positive DNA control provided by the manufacturer comes with the kit. The SmartCycler tubes are placed in the SmartCycler instrument and after amplification, the software provides a qualitative result of "negative," absence of *tcdB* or "positive," *tcdB* present. Other possible results include the following: "unresolved," indicating possible inhibition, or "invalid assay run," indicating that one or both controls failed, and "not determined," in the case of instrument malfunction. This assay has been extensively evaluated in the literature where it has been compared to toxigenic culture, CCCAs, EIAs, and a GDH two-step algorithm [42, 43, 45, 57, 64]. When compared directly to toxigenic culture the sensitivity ranges from 84 to 94 % and the specificity from 95 to 98 % [42, 43, 45].

The Prodesse ProGastro™ Cd Assay (Gen-Probe, Inc., San Diego, CA) was the second assay to obtain FDA clearance in the USA. In this assay 100 μl of stool is diluted 1:5, then clarified by adding the sample to a proprietary buffer called S.T.A.R. buffer. An internal control is then added to each clarified stool specimen. Isolation and purification of the DNA occurs on the bioMerieux NucliSENS easyMag (bioMerieux, Durham, NC) automated extractor. This assay targets conserved regions of *tcdB* using Taqman chemistry. Amplification is performed on a Cepheid SmartCycler or Rotorgene instrument. Three controls are required per run—a negative control, a positive matrix control and a negative matrix control. The assay requires about 3–4 h in total to complete [65]. To date there have been two published reports on the performance of this assay. Stamper et al. compared the ProGastro CD assay to both an EIA for toxins A and B and toxigenic culture [65]. The sensitivities and specificities were 88.3 and 77.3 % and 95.6 and 99.2 %, respectively [65]. More recently Karre et al. using 346 samples compared the Prodesse assay to a user-developed PCR method and the BD-GeneOhm assay [66]. Discrepant analysis was performed by culture. The sensitivity of the Prodesse assay was 91.9 % and the specificity was 99 % [66].

The Cepheid Gene Xpert® *C. difficile* assay is easier to perform than the above mentioned commercial products. A swab is dipped into the liquid or soft stool specimen, and then it is placed into a buffer vial. The vial is vortexed then the sample is pipetted into the sample port of the Xpert™ C difficile assay cartridge. The cartridge is then placed into the Xpert™ instrument. A positive result may be available in as short a time as 29 min. This assay targets the toxin B gene, and it is the only commercially available molecular assay to have FDA clearance for detection of the deletion in *tcdC* that is present in NAP-1 strains and some other less common

Table 40.3 Features of the four FDA cleared molecular assays available in the USA

Assay	Targets	Extraction	Internal control	TAT (min)	Cost/test (US $)[a]	References
BD GeneOhm®	*tcdB*	Manual	Yes	75–90	25–49	[43, 45, 57, 64]
Prodesse ProGastro™	*tcdB*	EasyMag	Yes	180	25	[65, 66, 71]
GeneXpert®	*tcdB* nt 117 del *tcdC*	Automated (infinity platform)	Yes	29–45	45	[49, 54, 56, 67, 68]
illumigene®	*tcdA*	Manual	Yes	70	25–49	[69, 70]

tcbB toxin B gene, *tcdC* toxin C gene, *nt* nucleotide, *del* deletion, *tcdA* toxin A gene
[a]Prices are variable and are negotiated based upon volume of testing

ribotypes. As is true for the other assays mentioned above, this assay has been extensively evaluated in the literature [54–56, 67, 68]. Those studies that have compared this assay to toxigenic culture report sensitivities ranging from 94.4 to 100 % and specificities of 96.3–99.2 % [54–56].

As mentioned above in the discussion of the GDH algorithms, the impact of strain typing on assay performance was an important observation to emerge from the multicenter clinical trial of the Xpert C difficile assay [49, 56]. When comparing the performance of the GDH assay to the Xpert test for NAP-1(ribotype 027) strains, there was no difference in performance. However, when the performance was assessed for non-NAP-1 strains, the overall sensitivity of the GDH assay was 72.2 % compared to 91.7 % for the Xpert assay ($p<0.001$) [49]. The results were more dramatic when comparing the Xpert Test to EIAs for toxin A and B detection. EIA sensitivity varied from 18.8 % for ribotype 106 isolates, which are more common in the UK, to 78.4 % for ribotype 027 (NAP-1) [49]. These observations are important because they can explain, in part, geographical variation in assay performance.

The most recent assay to obtain FDA clearance for the detection of *C. difficile* is the *illumigene*® (Meridian Bioscience, Cincinnati, OH). This molecular assay is the only one to have FDA approval for testing specimens from children. It is also unique in that it is based upon loop-mediated isothermal amplification (LAMP) of a conserved region of the toxin A gene. It will detect toxin A negative, toxin B positive strains. In this assay, a proprietary sample brush is dipped into the stool specimen then placed into a diluent after which it is vortexed for 10 s. Five drops of the specimen in diluent is squeezed into an extraction tube, heated to 95 °C for 10 min, then vortexed for 10 s. Fifty microliters of this extracted mixture is then added to a reaction buffer tube and vortexed for 10 s. The final step involves adding 50 μl of the extracted mixture to both a test vial and a control vial of the amplification device. The device is then placed into a small desktop instrument, the run is created and results are generated in 1 h. Total assay time is about 70 min. Compared to toxigenic culture the sensitivity has been reported to range from 91.8 to 98 % and the specificity ranges from 98 to 99 % [69, 70]. Table 40.3 summarizes the features and performance characteristics of the four FDA-cleared molecular assays that are currently available in the USA.

The trend in many clinical laboratories in adoption of molecular methods for detection of *C. difficile* has resulted in a response that ranges from consternation to ready acceptance among clinicians. There is no doubt that in all of the available publications on these tests, molecular assays are more sensitive than EIAs, algorithms that incorporate GDH, and CCCAs, but are not as sensitive as toxigenic culture. There are a few practical concerns that have been raised. One is that these assays do not detect the toxins, but the genes that encode for toxins, raising the issue of clinical specificity. For this reason it is extremely important that physicians not send specimens to the laboratory on patients who do not have diarrhea or otherwise meet a clinical case definition of *C. difficile*. In addition, laboratories should monitor positivity rates and assess their environments for contamination. The other concern for laboratories is the expense of these assays, which in general are two- to fourfold more expensive than EIAs for toxin or GDH detection.

To reduce the expense that may be incurred with widespread implementation of these assays, several investigators have adopted three step algorithms [54, 55, 71, 72]. In these studies, the authors look at screening with the C diff CHEK Complete assay (GDH plus toxin EIA, see GDH section). If the results are concordantly positive or negative for GDH and toxin, then no additional testing is required. However, if the GDH is positive and the toxin portion of the device is negative, then those specimens (approximately 12 % in one study) [55], are tested by a rapid molecular method. Such an algorithm can produce same day results and potentially save money, but this does require maintenance of multiple test methods, training, and the required proficiency, and raises other regulatory compliance issues such as whether reimbursement is allowed for multiple test methods [73].

Other questions that surround implementation of molecular testing for CDI include the theoretical concerns of genetic drift in the gene targets, the impact of emerging strain variation on assay performance, the frequency with which the gene is present but not expressed, among other questions. Other desirable information includes the impact of rapid molecular testing on infection control and patient management.

Summary

C. difficile remains an important cause of antibiotic associated diarrhea and data have shown that the incidence has increased over the last decade. The increase is multifactorial, but has largely been driven by the emergence of multidrug resistant, toxin variant strains and an increasingly susceptible population. The increased frequency of more severe disease and higher mortality rates has forced laboratories to critically evaluate diagnostic testing algorithms. There is a movement away from insensitive toxin A, B EIAs toward more sensitive multistep algorithms and rapid molecular assays. There are currently four FDA cleared molecular assays in the USA all of which have been shown to have superior performance to all methods

except toxigenic culture. The latter is now perceived as the new "gold standard" against which other methods are compared. More data is needed regarding the impact of molecular assays on infection control and patient management.

References

1. Kachrimanidou M, Malisiovas N (2011) *Clostridium difficile* infection: a comprehensive review. Crit Rev Microbiol 37:1–10
2. Carroll KC, Bartlett JG (2011) Biology of *Clostridium difficile*: implications for epidemiology and diagnosis. Annu Rev Microbiol 65:501–521
3. Hall IC, O'Toole E (1935) Intestinal flora in newborn infants: with a description of a new pathogenic anaerobe, *Bacillus difficilis*. Am J Dis Child 49:390
4. Bartlett JG (1994) *Clostridium difficile*: history of its role as an enteric pathogen and the current state of knowledge about the organism. Clin Infect Dis 18(Suppl 4):S265–S272
5. Bartlett JG, Chang TW, Gurwith M, Gorbach SL, Onderdonk AB (1978) Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N Engl J Med 298:531–534
6. Voth DE, Ballard JD (2005) *Clostridium difficile* toxins: mechanism of action and role in disease. Clin Microbiol Rev 18:247–263
7. Deneve C, Janoir C, Poilane I, Fantinato C, Collignon A (2009) New trends in *Clostridium difficile* virulence and pathogenesis. Int J Antimicrob Agents 33:S24–S28
8. Cohen SH, Tang YJ, Silva J Jr (2000) Analysis of the pathogenicity locus in *Clostridium difficile* strains. J Infect Dis 181:659–663
9. Matamouros S, England P, Dupuy B (2007) *Clostridium difficile* toxin expression is inhibited by the novel regulator TcdC. Mol Microbiol 64:1274–1288
10. Dupuy B, Govind R, Antunes A, Matamouros S (2008) *Clostridium difficile* toxin synthesis is negatively regulated by TcdC. J Med Microbiol 57:685–689
11. Geric B, Carman RJ, Rupnik M, Genheimer CW, Sambol SP et al (2006) Binary toxin-producing large clostridial toxin-negative *Clostridium difficile* strains are enterotoxic but do not cause disease in hamsters. J Infect Dis 193:1143–1150
12. Sundriyal A, Roberts AK, Ling R, McGlashan J, Shone CC, Acharya KR et al (2010) Expression, purification and cell cytotoxicity of actin-modifying binary toxin from *Clostridium difficile*. Prot Expr Purif 74:42–48
13. Schwan C, Stecher B, Tzivelekidis T, van Hamm M, Rhode M et al (2009) *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. PLoS Pathog 5:1–14
14. Lawley TD, Croucher NJ, Yu L, Clare S, Sebaihia M et al (2009) Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores. J Bacteriol 191:5377–5386
15. DuPont HL (2011) The search for effective treatment of *Clostridium difficile* infection. N Engl J Med 364:473–475
16. McDonald LC, Kilgore GE, Thompson A et al (2005) An epidemic, toxin gene-variant strain of *Clostridium difficile*. N Engl J Med 353:2433–2441
17. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD et al (2005) A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. N Engl J Med 353:2442–2449
18. Spigaglia P, Mastrantonio P (2002) Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J Clin Microbiol 40:3470–3475
19. Curry SR, Marsh JW, Muto CA, O'Leary MM, Pasculle AW, Harrison LH (2007) *tcdC* genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile*. J Clin Microbiol 45:215–221

20. Stabler RA, Gerding DN, Songer JG, Drudy D, Brazier JS et al (2006) Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains. J Bacteriol 188:7297–7305
21. Warny M, Pepin J, Fang A, Killgore G, Thompson A et al (2005) Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet 366:1079–1084
22. Akerlund T, Persson I, Unemo M, Noren T, Svenungsson B et al (2008) Increased sporulation rate of epidemic *Clostridium difficile* type 027/NAP1. J Clin Microbiol 46:1530–1533
23. Kim H, Riley TV, Kim M, Kim CK, Yong D, Lee K, Chong Y, Park JW (2008) Increasing prevalence of toxin A-negative, toxin B-positive isolates of *Clostridium difficile* in Korea: impact on laboratory diagnosis. J Clin Microbiol 46:1116
24. Huang H, Fang H, Weintraub A, Nord CE (2009) Distinct ribotypes and rates of antimicrobial drug resistance in *Clostridium difficile* from Shanghai and Stockholm. Clin Microbiol Infect 15:1170–1173
25. Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B et al (2010) The changing epidemiology of *Clostridium difficile* infections. Clin Microbiol Rev 23:529–549
26. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG et al (2010) Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the infectious diseases society of America. Infect Control Hosp Epidemiol 31:431–455
27. Crobach MJT, Dekkers OM, Wilcox MH, Kuijper EJ (2009) European Society of Clinical Microbiology and Infectious Diseases (ESCMID): Data review and recommendations for diagnosing *Clostridium difficile*-infection (CDI). Clin Microbiol Infect 15:1053–1066
28. Bouza E, Pelaez T, Alonso R, Catalan P, Munoz P, Creixems MR (2001) "Second-look" cytotoxicity: an evaluation of culture plus cytotoxin assay of *Clostridium difficile* isolates in the laboratory diagnosis of CDAD. J Hosp Infect 48:233–237
29. Reller ME, Lema CA, Perl TM, Cai M, Ross TL et al (2007) Yield of stool culture with isolate toxin testing versus a two-step algorithm including stool toxin testing for detection of toxigenic *Clostridium difficile*. J Clin Microbiol 45:3601–3605
30. George WL, Sutter VL, Citron D, Finegold SM (1979) Selective and differential medium for isolation of *Clostridium difficile*. J Clin Microbiol 9:214–219
31. Bliss DZ, Johnson S, Clabots CR, Savik K, Gerding DN (1997) Comparison of cycloserine-cefoxitin-fructose agar (CCFA) and taurocholate-CCFA for recovery of *Clostridium difficile* during surveillance of hospitalized patients. Diagn Microbiol Infect Dis 29:1–4
32. Mundy LS, Shanholtzer CJ, Willard KE, Gerding DN, Peterson LR (1995) Laboratory detection of *Clostridium difficile*. A comparison of media and incubation systems. Am J Clin Pathol 103:52–56
33. Peterson LR, Kelly PJ, Nordbrock HA (1996) Role of culture and toxin detection in laboratory testing for diagnosis of *Clostridium difficile*-associated diarrhea. Eur J Clin Microbiol Infect Dis 15:330–336
34. Levett PN (1985) Effect of antibiotic concentration in a selective medium on the isolation of *Clostridium difficile* from faecal specimens. J Clin Pathol 38:233–234
35. Buchanan AG (1984) Selective enrichment broth culture for detection of *Clostridium difficile* and associated cytotoxin. J Clin Microbiol 20:74–76
36. O'Farrell S, Wilks M, Nash JQ, Tabaqchali S (1984) A selective enrichment broth for the isolation of *Clostridium difficile*. J Clin Pathol 37:98–99
37. Arroyo LG, Rousseau J, Willey BM, Low DE, Staempfli H et al (2005) Use of a selective enrichment broth to recover *Clostridium difficile* from stool swabs stored under different conditions. J Clin Microbiol 43:5341–5343
38. Hanff PA, Zaleznik DF, Kent KC, Rubin MS, Kelly E, Cote J, Rosol-Donoghue J (1993) Use of heat shock for culturing *Clostridium difficile* in rectal swabs. Clin Infect Dis 16(Suppl 4):S245–S247
39. Borriello SP, Honour P (1981) Simplified procedure for the routine isolation of *Clostridium difficile* from faeces. J Clin Pathol 34:1124–1127

40. She RC, Durrant RJ, Petti CA (2009) Evaluation of enzyme immunoassays to detect *Clostridium difficile* toxin from anaerobic stool culture. Am J Clin Pathol 131:81–84
41. Tichota-Lee J, Jaqua-Stewart MJ, Benfield D, Simmons JL, Jaqua RA (1987) Effect of age on the sensitivity of cell cultures to *Clostridium difficile* toxin. Diagn Microbiol Infect Dis 8:203–214
42. Barbut F, Braun M, Burghoffer B, Lalande V, Eckert C (2009) Rapid detection of toxigenic strains of *Clostridium difficile* in diarrheal stools by real-time PCR. J Clin Microbiol 47:1276–1277
43. Eastwood K, Else P, Charlett A, Wilcox M (2009) Comparison of nine commercially available *Clostridium difficile* toxin detection assays, a real-time PCR assay for *C. difficile tcdB*, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods. J Clin Microbiol 47:3211–3217
44. Peterson LR, Manson RU, Paule SM, Hacek DM, Robicsek A et al (2007) Detection of toxigenic *Clostridium difficile* in stool samples by real-time polymerase chain reaction for the diagnosis of *C. difficile*-associated diarrhea. Clin Infect Dis 45:1152–1160
45. Stamper PD, Alcabasa A, Aird D, Babiker W, Wehrlin J, Ikpeama I, Carroll KC (2009) Comparison of a commercial real-time PCR assay for *tcdB* detection to a cell culture cytotoxicity assay and toxigenic culture for direct detection of toxin-producing *Clostridium difficile* in clinical samples. J Clin Microbiol 47:373–378
46. Planche T, Aghaizu A, Holliman R, Riley P, Poloniecki J et al (2008) Diagnosis of *Clostridium difficile* infection by toxin detection kits: a systematic review. Lancet Infect Dis 8:777–784
47. Ticehurst JR, Aird DZ, Dam LM, Borek AP, Hargrove JT, Carroll KC (2006) Effective detection of toxigenic *Clostridium difficile* by a two-step algorithm including tests for antigen and cytotoxin. J Clin Microbiol 44:1145–1149
48. Gilligan PH (2008) Is a two-step glutamate dehydrogenase antigen-cytotoxicity neutralization assay algorithm superior to the Premier toxin and B enzyme immunoassay for laboratory detection of *Clostridium difficile*? J Clin Microbiol 46:1523–1525
49. Tenover FC, Novak-Weekley S, Woods CW, Peterson LR, Davis T et al (2010) Impact of strain type on detection of toxigenic *Clostridium difficile*: comparison of molecular diagnostic and enzyme immunoassay approaches. J Clin Microbiol 48:3719–3724
50. Fenner L, Widmer AF, Goy G, Rudin S, Frei R (2008) Rapid and reliable diagnostic algorithm for detection of *Clostridium difficile*. J Clin Microbiol 46:328–330
51. Snell H, Ramos M, Longo S, John M, Hussain Z (2004) Performance of the TechLab C DIFF CHEK-60 enzyme immunoassay (EIA) in combination with the *C. difficile* Tox A/B II EIA kit, the Triage C. difficile panel immunoassay, and a cytotoxin assay for diagnosis of *Clostridium difficile*-associated diarrhea. J Clin Microbiol 42:4863–4865
52. Zheng L, Keller SF, Lyerly DM, Carman RJ, Genheimer CW et al (2004) Multicenter evaluation of a new screening test that detects *Clostridium difficile* in fecal specimens. J Clin Microbiol 42:3837–3840
53. Quinn CD, Sefers SE, Babiker W, He Y, Alcabasa R et al (2010) C diff Quik Chek complete enzyme immunoassay provides a reliable first-line method for detection of *Clostridium difficile* in stool specimens. J Clin Microbiol 48:603–605
54. Swindells J, Brenwald N, Reading N, Oppenheim B (2010) Evaluation of diagnostic tests for *Clostridium difficile* infection. J Clin Microbiol 48:606–608
55. Sharp SE, Ruden LO, Pohl JC, Hatcher PA, Jayne LM, Ivie WM (2010) Evaluation of the Cdiff Quik Chek Complete Assay, a new glutamate dehydrogenase and A/B toxin combination lateral flow assay for use in rapid, simple diagnosis of *Clostridium difficile* disease. J Clin Microbiol 48:2082–2086
56. Novak-Weekley SM, Marlowe EM, Miller JM, Cumpio J, Nomura JH, Vance PH, Weissfeld A (2010) *Clostridium difficile* testing in the clinical laboratory by use of multiple testing algorithms. J Clin Microbiol 48:889–893
57. Kvach EJ, Ferguson D, Riska PF, Landry ML (2010) Comparison of BD GeneOhm C diff real-time PCR assay with a two-step algorithm and a toxin A/B enzyme-linked immunosorbent assay for diagnosis of toxigenic *Clostridium difficile* infection. J Clin Microbiol 48:109–114

58. Goldenberg SD, Gumban M, Hall A, Patel A, French GL (2011) Lack of effect of strain type on detection of toxigenic *Clostridium difficile* by glutamate dehydrogenase and polymerase chain reaction. Diagn Microbiol Infect Dis 70:417–419
59. Shetty N, Wren MW, Coen PG (2011) The role of glutamate dehydrogenase for the detection of *Clostridium difficile* in faecal samples: a meta-analysis. J Hosp Infect 77:1–6
60. Kato N, Ou CY, Kato H, Bartley SL, Luo CC, Killgore GE, Ueno K (1993) Detection of toxigenic *Clostridium difficile* in stool specimens by the polymerase chain reaction. J Infect Dis 167:455–458
61. Gumerlock PH, Tang YJ, Meyers FJ, Silva J (1991) Use of the polymerase chain reaction for the specific and direct detection of *Clostridium difficile* in human feces. Rev Infect Dis 13:1053–1060
62. Belanger SD, Boissinot M, Clairoux N, Picard FJ, Bergeron MG (2003) Rapid detection of *Clostridium difficile* in feces by real-time PCR. J Clin Microbiol 41:730–734
63. van den Berg RJ, van Coppenraet LSB, Gerritsen HJ, Endtz HP, van der Vorm ER, Kuijper EJ (2005) Prospective multicenter evaluation of a new immunoassay and real-time PCR for rapid diagnosis of *Clostridium difficile*-associated diarrhea in hospitalized patients. J Clin Microbiol 43:5338–5340
64. Terhes G, Urban E, Soki J, Nacsa E, Nagy E (2009) Comparison of a rapid molecular method, the BD GeneOhm Cdiff assay to the most frequently used laboratory tests for detection of toxin-producing *Clostridium difficile* in diarrheal feces. J Clin Microbiol 47:3478–3481
65. Stamper PD, Babiker W, Alcabasa R et al (2009) Evaluation of a new commercial TaqMan PCR assay for direct detection of the *Clostridium difficile* toxin B gene in clinical stool specimens. J Clin Microbiol 47:3846–3850
66. Karre T, Sloan L, Patel R, Mandrekar J, Rosenblatt J (2010) Comparison of two commercial molecular assays to a laboratory developed molecular assay for diagnosis of *Clostridium difficile* infection. J Clin Microbiol 49:725–727
67. Huang H, Weintraub A, Fang H, Nord CE (2009) Comparison of a commercial multiplex real-time PCR to the cell cytotoxicity neutralization assay for diagnosis of *Clostridium difficile* infections. J Clin Microbiol 47:3729–3731
68. Babady NE, Stiles J, Ruggiero P, Khosa P, Huang D et al (2010) Evaluation of the Cepheid Xpert *Clostridium difficile* EPI assay for diagnosis of *Clostridium difficile* infection and typing of the NAP1 strain at a cancer hospital. J Clin Microbiol 48:4519–4524
69. Noren T, Alriksson I, Andersson J, Akerlund T, Unemo M (2011) Rapid and sensitive loop-mediated isothermal amplification (LAMP) test of *Clostridium difficile* diagnosis challenges cytotoxin B cell test and culture as gold standard. J Clin Microbiol 49:710–711
70. Lalande V, Barrault L, Wadel S, Eckert C, Petit JC, Barbut F (2011) Evaluation of a loop-mediated isothermal amplification assay for diagnosis of *Clostridium difficile* infections. J Clin Microbiol 49:2714–2716
71. Doing KM, Hintz MS, Keefe C, Horne S, LeVasseur S, Kulikowski ML (2010) Reevaluation of the Premier *Clostridium difficile* toxin A and B immunoassay with comparison to glutamate dehydrogenase common antigen testing evaluating Bartels cytotoxin and Prodesse ProGastro Cd polymerase chain reaction as confirmatory procedures. Diagn Microbiol Infect Dis 66:129–134
72. Larson AM, Fung AM, Fang FC (2010) Evaluation of *tcdB* real-time PCR in a three-step diagnostic algorithm for detection of toxigenic *Clostridium difficile*. J Clin Microbiol 48:124–130
73. Carroll K, Loeffelholz M (2011) Conventional versus molecular methods for the detection of *Clostridium difficile*. J Clin Microbiol 49:S49–S52

Chapter 41
Molecular Diagnosis of HIV-1 Infections: Current State of the Art

Yi-Wei Tang

Introduction

In the management of HIV infections, laboratorians or clinical microbiologists determine whether a patient is infected with HIV, evaluate the status of the HIV infection, and monitor antiretroviral therapy. Theoretically, an HIV infection can be diagnosed and monitored by any of five possible ways: (1) direct microscopic examination such as visualization of an HIV virion by electronic microscopy, (2) cultivation and identification of HIV by suspension lymphocyte culture, (3) detection of HIV viral antigens, (4) measurement of HIV-specific immune responses, and (5) detection and quantification of HIV-specific nucleic acids [1, 2]. Practically speaking, the diagnosis and monitoring of HIV infection is done by serologic and molecular methods. Molecular methods were first used by Ou et al. from the US Centers for Disease Control and Prevention in 1988 to directly amplify HIV-1 specific nucleic acids from peripheral blood mononuclear cells of HIV-1 seropositive individuals [3]. In this sentinel article, the authors concluded that "the method may therefore be used to complement or replace virus isolation as a routine means of determining HIV-1 infection [3]." This has certainly proven to be correct.

Indeed, the diagnostic capabilities for HIV infections have improved rapidly and have expanded greatly thanks to the molecular technology revolution. Molecular assays along with serology have become the mainstay used in the HIV laboratory diagnostic field. HIV-1 plasma viral load assays are routinely used in combination with CD4 cell counts to determine when to initiate therapy and when a regimen is failing. In addition, unlike serologic assays, these assays, especially the qualitative

Y.-W. Tang (✉)
Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 37212, USA
e-mail: tangy@mskcc.org

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ones, have been used to diagnose perinatal and acute HIV-1 infections. HIV-1 antiretroviral susceptibility tests, which include a genotyping assay detecting mutations known to confer resistance in viral genomes and a phenotypic assay measuring recombinant viral replication in the presence of antiretroviral drugs, has become routine patient care in the management of antiretroviral treatment. Assessing host gene polymorphisms and immune responses have emerged in clinical laboratories for better diagnosis and monitoring of HIV-1 infections. Point-of-care testing (POCT) based on molecular techniques has been actively developed and will be used for diagnosis and monitoring in the near future (Table 41.1).

Qualitative Detection of HIV

The primary diagnosis of HIV infection is most often accomplished by serology via detection of HIV antibody/antigen using a fourth generation screening enzyme immunoassay (EIA) or a rapid assay, followed by a subsequent confirmatory Western blot (WB) test. In addition to serology assays, molecular methods are now routinely used to minimize the window period for the diagnosis of acute or early infection in special populations. In newborns, the HIV proviral DNA detection method is used to rule out maternal antibody and confirm HIV infection [4, 5]. Seronegative HIV-1 infected cases have been reported even for common HIV clades [6, 7]. In immunocompromised hosts, serology may be limited, likely due to the inability to mount an effective immune response.

Molecular technologies based on in vitro nucleic acid amplification can be utilized in the diagnosis of acute or primary infection due to the fact that viral RNA can be detected earlier than the antibody or p24 antigen. Qualitative RNA molecular assays have been developed and commercially available for blood-donor screening in the blood bank [8]. The APTIMA HIV-1 RNA Qualitative Assay and the Procleix HIV-1/HCV assay (Gen-Probe, San Diego, CA), which incorporates transcription-mediated amplification technology, has been approved by the FDA to aid in the diagnosis of HIV-1 infection, including acute or primary infection [9]. Recent data indicated that the Aptima HIV assay readily discriminated between HIV-1-infected and -uninfected individuals and effectively reduced the number of indeterminate results relative to Western blot analysis [10]. This assay has been evaluated recently as being a more sensitive screening tool for HIV-positive samples from a sexually transmitted disease clinic than typical antibody testing followed by pooled RNA testing. The data indicate that screening and confirmation of HIV infection by the qualitative molecular method alone may constitute an effective alternative HIV diagnostic algorithm in certain settings [11]. The qualitative format can detect a lower amount of viral RNA than quantitative tests, i.e., less than 100 copies of HIV-1 RNA per ml [12]. Such rapid screening by using molecular qualitative assays has been used in pooled plasma specimens [13–15] and alternative specimen types [12, 16]. Since HIV viral loads in patients with acute or primary infection are usually high, other HIV RNA quantification assays have been used as an alternative for diagnostic purposes [17–22].

Table 41.1 Current molecular methods for laboratory diagnosis and monitoring of HIV infections

Method	Applicability	Turnaround time	Main devices (manufacturer)	Applications	Comments	Exemplary references
Molecular assays, qualitative	B	1–2 days	APTIMA HIV-1 RNA Qualitative Assay or Procleix HIV-1/HCV Assay (Gen-Probe)	Blood donor screening and aid in acute or primary HIV infection diagnosis	Used for blood donor screening in pooled specimens; test of choice for HIV infections in newborns and infants	[4–7, 9, 10]
Molecular assays, viral load testing	A	1–2 days	COBAS AmpliPrep/TaqMan HIV-1 (Roche); VERSANT HIV-1 RNA (Siemens); NucliSens HIV-1 RNA QT (bioMerieux) RealTime m2000 HIV-1 (Abbott Molecular)	Antiretroviral therapy monitoring	Used to guide HAART initiation and monitor the treatment efficacy in conjunction with CD4 counting	[37, 39, 62–65, 69, 70, 78–80]
Genotyping	A	1–3 days	Trugene HIV-1 Genotyping (Siemens); ViroSeq HIV-1 Genotyping (Abbott Molecular)	Antiretroviral drug resistance determination	Indirect determination by detecting resistance-related HIV gene mutations; may miss low level mutations	[83, 85–87, 94]
Phenotyping	B	3–6 weeks	AntiVirogram assay (VIRCO Lab); PhenoSense HIV (MonoGram Biosciences)	Antiretroviral drug resistance determination	Direct determination by measuring HIV ability to grow in presence of drugs; time-consuming and expensive tests	[116–119]
Molecular near-the-patient testing	C	1–4 h	IsoAmp HIV-1 Assay (BioHelix); ExaVir Load Assay (Cavidi); Liat HIV Quant Assay (Iquum)	Diagnosis and monitoring of HIV infection in point of care	Still in research and development; include HIV RNA detection and quantification	[146, 175–178]

(continued)

Table 41.1 (continued)

Method	Applicability	Turnaround time	Main devices (manufacturer)	Applications	Comments	Exemplary references
Host polymorphism testing	D	1–3 days	None	Determine host susceptibility to HIV infection	Used to determine HIV infection susceptibility and monitoring treatment efficacy and side effects	[183–199]
Host response and transcriptome testing	D	1–3 days	Human Genome U133 Array Strip (Affymetrix)	Assess disease progression and outcome; monitor antiretroviral therapy	Used to determine HIV infection susceptibility and monitoring treatment efficacy and side effects	[210–216, 219, 224–226]

A, molecular assay is widely used for clinical diagnosis and/or monitoring of HIV-1 infections; B, molecular assay is useful under certain circumstances or for the diagnosis of particular populations; C, molecular assay is seldom useful for general diagnostic purposes but may be useful in specific researches and trials; D, molecular assay is not available or not used for laboratory diagnosis and monitoring

In addition to blood donor screening, the qualitative molecular assays, especially those for the detection of HIV proviral DNA, have become the test of choice for establishing the diagnosis of infection in infants born to HIV-1 infected mothers [4, 5]. The persistence of maternal antibodies against HIV in exposed infants up to 18 months of age prevents the use of antibody-based assays for the early diagnosis of HIV infection. It is, on the other hand, important to promptly establish the infection status of an HIV-exposed infant, since the effectiveness of highly active antiretroviral therapy at an early age has been demonstrated. Thus, approaches to the early diagnosis of infection in infants lean towards molecular techniques that amplify target HIV DNA [23], RNA [5, 21], or total nucleic acid [19, 20, 22]. Detection of HIV-1 DNA can be used to differentiate primary infection during the diagnostic window period or in newborns of infected mothers. In the diagnosis of HIV in infants, the DNA PCR assays possess sensitivities of ≥95 % and even higher specificities [4, 24, 25]. At this time, only one qualitative HIV-1 DNA PCR assay is commercially available from Roche and has yet to be approved by the FDA [5, 26].

HIV RNA Viral Load Assays

HIV-1 infection results in lifelong persistence of the virus, independent of antiretroviral treatment. In chronically infected patients, the HIV RNA viral load in plasma in conjunction with the CD4 T-lymphocyte cell numbers are the routine laboratory markers used to guide both initiation of the highly active antiretroviral therapy (HAART) and to monitor treatment effectiveness and the likelihood of clinical progression [27–30]. Viral load assays, which measure the quantity of HIV-1 RNA present in plasma, are used as prognostic markers, to monitor response to therapy and to guide HIV treatment decisions [31–33]. Characterization of HIV-1 RNA levels as being below the limit of detection indicates HAART adherence and effectiveness [27, 34, 35]. Periodic monitoring of HIV-1 viral loads can be performed by either HIV RNA amplification or branched chain DNA (bDNA) tests [36]. Technically, less than a threefold variation (0.5 $\log_{10}$ copies) is considered as intraassay or biological variabilities; however, an over tenfold (1 $\log_{10}$ copies) change is considered clinically significant [37–40]. In the clinical setting, 1 month after an effective regimen, viral load should fall by at least 1 log. By 4–6 months into therapy, viral load should have fallen below the detection limit of the test, usually less than 50–75 copies/ml [31–33].

Sensitive measurement of viral load with broad dynamic range of detection, and enhanced ability to quantify HIV-1 Group M subtypes A-G of the virus are two major requirements for the quantitative assay for HIV RNA. While HIV-1 subtype B continues to predominate in Western countries, studies now confirm that the incidence of HIV-1 non-B subtypes is increasing all over the world. The ability of a test to detect a broader range of these genetically diverse viruses is therefore crucial to HIV patient care on a global basis. In the USA, five commercial assays are FDA approved for the quantification of HIV-1 RNA in plasma (Table 41.2). All of these

Table 41.2 Commercial, FDA-cleared HIV-1 viral load assays

Device	Manufacturer	Amplification format	Genome target(s)	Quantification range (copies/ml)	Selected references
COBAS AmpliPrep/ TaqMan HIV-1	Roche Diagnostics, Indianapolis, IN	Real-time reverse transcriptase PCR with TaqMan hydrolysis probe	*gag* and LTR	48–10,000,000	[37, 39, 43, 44, 62–68, 80]
VERSANT HIV-1 RNA	Siemens Diagnostics, Tarrytown, NY	Branched DNA-based signal amplification	*pol*	75–500,000	[36, 37, 62, 69–73, 80]
NucliSens HIV-1 RNA QT	bioMerieux, Durham, NC	Nucleic acid sequence-based amplification	*gag*	176–3,470,000	[37, 62, 73–77]
RealTime m2000 HIV-1	Abbott Molecular, Des Plaines, IL	Real-time reverse transcriptase PCR with partially double-stranded linear DNA probe	Intergrease	40–10,000,000	[36, 43, 44, 65, 66, 78–81]

assays are licensed for monitoring HIV-1 infected patients; they are not proposed to be used for HIV screening tests nor for confirmatory HIV tests, but have often been used in this context [18–22, 36, 41]. Performance may significantly vary between HIV viral load assay platforms according to subtype. HIV viral diversity in the population being tested must be considered in selection of the viral load platform and the same format for the monitoring of HIV-1-infected patients should be kept for the routine laboratory services [42–44]. None of the assays detect HIV-2.

Plasma is the main specimen type for HIV-1 viral load testing. Plasma collected by plasma preparation tubes should be transferred to a secondary tube before freezing and transportation [45–48]. HIV viral RNA is relatively unstable and thus requires that blood samples (plasma) be processed within 4–6 h of collection and stored in a deep freezer. Nonplasma specimens such as peripheral blood mononuclear cells (PBMCs), saliva, cerebrospinal fluid, seminal plasma, dried plasma, and dried blood spots have been evaluated for HIV-1 viral load testing [16, 41, 49–53]. Dried blood spots have been used at rural and remote healthcare facilities to collect and transport specimens for HIV-1 RNA viral load monitoring [54–58]. When specimens are carefully processed, viral load results are stable and reproducible, and cross-contamination is rare and avoidable [59–61].

The Cobas Amplicor HIV-1 Monitor Assay (Roche Diagnostics, Indianapolis, IN) is a reverse transcription (RT)-PCR-based system targeting HIV-1 RNA [37, 39, 62]. Two basic assay platforms exist: (1) the Amplicor HIV-1 Monitor assay, which is a manual test performed in microwell plates and (2) the Cobas AmpliPrep/Cobas TaqMan HIV-1 assay, which provides full automation of the nucleic acid extraction followed by real-time PCR amplification. Its first FDA approved HIV viral load test in 1996 measures viral loads at levels as low as 400 HIV-1 RNA copies/ml. The Amplicor Ultra Sensitive test, approved in 1999, uses a slightly different sample-processing protocol and measures viral loads down to 50 HIV-1 RNA copies/ml. The fully automated version of the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 system (version 1.0) has been in use in the United States with a reported upper limit of quantification and lower limit of detection of 1,000,000 and 50 RNA copies/ml, respectively [63–65]. The system targets the HIV-1 *gag* gene and has been designed to quantify all group M and N viruses and many circulating recombinant forms [64, 66]. Recently, a version 2.0 of this assay has become available, which targets both *gag* and LTR regions to further improve test sensitivity and has better HIV-1 Group M subtype coverage including Group O [43, 44, 67, 68].

The bDNA-based test known as VERSANT HIV-1 RNA 3.0 Assay (Siemens Healthcare Diagnostics, Tarrytown, NY) provides good reproducibility since no amplification variation is expected due to its signal amplification technology [69, 70]. Without extraction steps to isolate HIV-1 RNA, the reproducibility of the bDNA assay has been reported to be a superior test, particularly at the low end of the dynamic range [37, 70]. The influence of inhibitory substances contained in a variety of clinical specimens is much lower in comparison with other methods and the risk of contamination is reduced as well. This test has good precision across a wide reporting range and can distinguish three-fold ($0.5 \log_{10}$) changes across the entire assay range [37, 62, 71]. The bDNA test can also be used to determine quantification

of the viral load down to 75 copies/ml [72, 73]. The disadvantages from the intrinsic bDNA technique include the requirement for a large volume of plasma, the absence of an internal quantification standard for each sample tested, and lower specificity compared to target amplification methods. The recently available Bayer System 440 provides more extensive automation [42].

The NucliSens HIV-1 RNA QT assay (bioMerieux, Inc., Durham, NC) incorporates three key technologies: silica-based nucleic acid extraction; nucleic acid sequence-based amplification (NASBA) for HIV RNA amplification; and electrochemiluminescence detection and quantification of the amplified RNA [62, 74]. The NASBA technology is a sensitive, isothermal amplification method that does not require a thermocycler, so there is no need for heat-stable enzymes. The NucliSens assay is more sensitive in detecting HIV-1 RNA at lower concentrations than the standard Roche AMPLICOR test and reaches a broad linear dynamic ranging from 51 to 5,390,000 copies/ml [75, 76]. The second generation assay currently in use cannot reliably quantify subtypes A and G [42, 75]. The assay can be used for measuring viral loads at other body sites because the RNA extraction procedure consistently generates RNA products that are free of interfering substances [37, 65, 73, 77]. The isothermal process runs at 41 °C which is lower than the annealing temperature of the primers used, resulting in a lower specificity of the amplification process.

The RealTime HIV-1 assay in the *m*2000 system (Abbott Molecular, Des Plaines, IL) consists of two components: the m2000sp for nucleic acid extraction and loading of sample and master mix into the 96-well optical reaction plate, and the m2000rt for amplification and detection [65, 78–80]. Incorporated with a partially double-stranded linear DNA probe [81], this assay offers several advantages over conventional viral load assays, including a broad linear coverage, improved sequence variation tolerance, and decreased risk of carryover contamination. The system is fully automated with a wide coverage of HIV-1 genotypes including groups M and N circulating recombinant forms, as well as Group O virus [43, 65, 66, 79, 80]. The assay possesses a wide linear dynamic range of between 40 and 10 million HIV-1 RNA copies/ml and the limit of detection of the assay is 40 copies/ml for a 1.0 ml sample volume, 75 copies/ml for a 0.5 ml sample volume, and 150 copies/ml for a 0.2 ml sample volume [36, 44].

Genotypic Antiretroviral Susceptibility Testing

The goal of HAART is to completely suppress viral replication below the detection limit of the most sensitive assay in order to avoid the emergence of drug-resistant virus mutants and to delay clinical progression [27, 34, 35, 82]. Antiretrovial drug resistance is defined as the ability of HIV-1 to multiply in the presence of antiretroviral drugs. In the clinical setting, if a viral load fails to fall adequately, or if it rebounds to greater than 1,000 copies/ml, tests for antiretroviral resistance are recommended. Two types of formats are available to determine antiretroviral susceptibilities.

Phenotypic resistance assays directly measure viral replication of the patient's virus in the presence of antiretroviral drugs while genotypic resistance assays detect viral genome mutations known to confer decreased sensitivity to antiretroviral drugs. Both genotypic and phenotypic-based tests are available, but the latter is much more expensive and is usually reserved for patients with prior viral resistance. The clinical utility of HIV susceptibility testing has been evaluated in a number of prospective randomized clinical trials [83–90]. The use of HIV-1 susceptibility testing to guide antiretroviral treatment has been reported to be cost-effective [82, 91, 92].

Genotypic drug-resistance testing has been implemented in clinical guidelines as an important tool to guide therapy changes, overall therapy, and more recently, initiation of therapy [83, 85–87, 93, 94]. There are currently two commercial assays available for HIV-1 genotyping: the TruGene HIV-1 Genotyping Kit and OpenGene DNA Sequencing System (Siemens Healthcare Diagnostics, Tarrytown, NY), and the ViroSeq HIV-1 Genotyping System (Abbott Molecular, Des Plaines, IL) (Table 41.3). Both systems are based on a PCR amplification of reverse transcriptase and protease genes followed by amplification product nucleic acid sequencing by the traditional Sanger method. One major difference between the two systems is the sequencing chemistry: ViroSeq uses a four-dye termination system while the TrueGene uses the dye primer system. In addition, six samples are needed to analyze one patient for ViroSeq, compared with 12 samples for TruGene. The ViroSeq system requires an additional purification step for removal of the dye terminators [95]. A parallel validation revealed that both assays generated an accurate sequence with similarity in overall complexity. While the OpenGene system is limited in throughput, it provides an interpretative report containing information relating mutations to drug resistance [96]. Both systems work well for the USA dominantly circulating HIV-1 groups with varied performance in minor groups. One recent study used the ViroSeq HIV-1 genotyping system to determine drug resistance on a panel of diverse HIV-1 group M isolates circulating in Cameroon. The data revealed that the performance of this assay can be altered by the sequence variation of non-B HIV-1 strains that predominate in African settings [97].

Both systems detect mutations in the reverse transcriptase and protease genes, but do not detect mutations associated with resistance to the fusion inhibitor, integrase inhibitors, and CCR5 inhibitors. Another limitation of the two genotypic assays is that they are only able to detect mutants that comprise major fractions of the patient's virus; resistant variants must constitute at least 25 % of the virus population [93, 98]. Resistant mutations present at low levels missed by standard population-based genotyping assays can lead to failure of subsequent treatments [99]. Two other genotyping formats, the HIV PRT GeneChip assay (Affymetrix, Santa Clara, CA) and the HIV-1 RT Line Probe Assay (Innogenetics, Ghent, Belgium) have been reported for the rapid detection of drug resistance-related mutation in HIV genomes, which have the potential to detect multiple mutations at lower levels [100–103]. Allele-specific PCR [104, 105], single-genome sequencing [99, 104, 106] and ultra-deep sequencing (UDS) [104, 107, 108] have been reported to increase the sensitivity of minority mutation detection. However, due to the innate error rate of reaction enzymes, it has been reported that UDS had limited sensitivity

Table 41.3 Commercial HIV-1 antiretroviral resistance assays

Device	Manufacturer	Platform	Scope	Additional comments	Selected references
Trugene HIV-1 genotyping kit	Siemens Diagnostics, Tarrytown, NY	Genotypic	Detection of protease and reverse transcriptase gene mutations	FDA cleared; accuracy varies due to sequence variations of non-B HIV-1 strains	[85, 86, 88, 94, 95]
ViroSeq HIV-1 genotyping system	Abbott Molecular, Des Plaines, IL	Genotypic	Detection of protease and reverse transcriptase gene mutations	FDA cleared; accuracy varies due to sequence variations of non-B HIV-1 strains	[88–90, 94, 95]
HIV PRT GeneChip assay	Affymetrix, Santa Clara, CA	Genotypic	Detection of protease and reverse transcriptase gene mutations	Potential to detect multiple mutations at lower levels	[102, 103]
HIV-1 RT Line Probe Assay	Innogenetics, Ghent, Belgium	Genotypic	Detection of protease and reverse transcriptase gene mutations	Potential to detect multiple mutations at lower levels	[100, 101, 103]
PhenoSense, universal, entry and integrase	Monogram Biosciences, South San Francisco, CA	Phenotypic	Susceptibility determination for reverse transcriptase, protease, entry and integrase inhibitors	Three separate devices for different drugs	[113–115, 117, 127, 129]
Trofile	Monogram Biosciences, South San Francisco, CA	Phenotypic	Susceptibility determination for CCR5 inhibitors	Used prior to initiating therapy	[132, 135, 136]
SensiTrop II HIV coreceptor tropism	Pathway Diagnostics, Malibu, CA	Phenotypic	Susceptibility determination for CCR5 inhibitors	Used prior to initiating therapy	
Virco TYPE HIV-1	VIRCO Lab, Inc., Titusville, NJ	Virtual phenotype	Susceptibility determination by using genetic data	Accuracy depends on constant database updates	[121, 122]

to about 0.5 % and may have less utility in treatment-experienced patients with persistent viremia on therapy [109–111]. The majority of low-frequency drug resistance-related mutations detected using UDS are likely errors inherent to UDS methodology or a consequence of error-prone HIV-1 replication [112].

Phenotypic Antiretroviral Susceptibility Testing

Phenotyping assays measure the ability of HIV-1 to grow in the presence of various concentrations of antiretroviral agents. Phenotyping assays are considered a molecular method as well, since the recombinant viruses are generated and used in the testing. Phenotyping uses clinical cutoffs associated with treatment outcome data and estimates the net effect of multiple mutations more directly [113–115]. The current procedure involves recombinant DNA technology. HIV-1 RNA from patient's plasma is extracted and protease and reverse transcriptase genes are amplified by RT-PCR. The amplified gene fragments from the patient's specimen are then inserted into HIV-1 vectors. The recombinant HIV-1 replication is measured by a reporter gene system [116–119]. Data from these assays are relatively simple to interpret, and a report format of 50 or 90 % inhibitory concentrations (IC_{50} or IC_{90}) is familiar to clinicians. However, phenotyping requires accurate fold-change clinical cut-off values for prediction of response. The test also requires longer turnaround time as it is labor intensive and technically complex. As with genotypic testing, the phenotypic assays can only detect mutant variants that comprise at least 25 % of the viral population. More data need to be collected for evidence of clinical utility for phenotyping rather than for genotyping [120]. Currently, two HIV-1 phenotyping assays are available at two commercial laboratories: the AntiVirogram assay at VIRCO Lab (Bridgewater, NJ) [116] and the PhenoSense HIV assay at MonoGram Biosciences (South San Francisco, CA) [117]. A virtual phenotyping has been described that provides an estimation of the phenotype by averaging viruses with similar genotypes [121, 122].

As integrase and entry/fusion inhibitors become available in antiretroviral therapy, HIV-1 genotyping assays have been extended to cover resistance-related mutations related to integrase and entry/fusion inhibitor resistances [123–126]. Since inhibition by entry/fusion inhibitor Enfuvirtide blocks HIV-1 before it enters the human immune cell, genotyping alone provides insufficient resistance information. A PhenoSense Entry assay (MonoGram) has been developed to assess resistance to entry inhibitors [127, 128]. Natural-resistance to Enfuvirtide among different HIV-1 subtypes have been studied [129], while HIV-2 is intrinsically resistant to the fusion inhibitor Enfuvirtide, and other culture-based and cell-free phenotyping assays should be used to determine resistance profiles [130]. Maraviroc, a CCR5 inhibitor drug, is only effective against viruses that use CCR5 as a coreceptor for entry [131, 132]. Prior to Maraviroc administration, a tropism assay must be performed to determine whether the virus is CCR5-tropic [133, 134]. Two commercial tropism assays are available at the Monogram Biosciences [132, 135, 136] and Pathway Diagnostics (Malibu, CA).

Point-of-Care Testing

POCT is performed in homes, workplaces, pharmacies, physicians' offices, outpatient clinics, emergency rooms, disaster sites, and patient bedsides. The need of POCT has been driven by the practitioner's need to make rapid, evidence-based, therapeutic action at or near the site of patient care [137]. This form of diagnosis has also been available for several years for problems related to infectious diseases. Numerous products are commercially available for the POC diagnosis of viral, bacterial, and parasitic infections [138]. Efforts have been spent in developing POCT devices including HIV antibody and nucleic acid detection as well as CD4 and HIV viral load quantification for the diagnosis and monitoring of HIV infections in resource-limited settings [139]. Because early diagnosis profoundly affects the health care and survival of infected/high-risk individuals, and because the time required for conventional testing remains a barrier in many settings, rapid HIV antibody testing has been developed for use at the point of care. An ideal molecular POCT in the diagnosis of HIV-1 infections should possess relatively good sensitivity with low cost and a very short completion time [140].

Molecular tests are sensitive and shorten the window of detection period to 8–10 days [141, 142]. At the time of preparation, there are no real molecular POC devices approved by the FDA yet; some nucleic acid detection platforms have potential applications as an "instrument-free" means of HIV nucleic acid amplification and detection. A helicase-dependent amplification (HDA) was developed in which helicases are used to separate DNA strands rather than heat; it simply relies on DNA polymerase to amplify DNA rather than on the combinations of polymerases with other enzymes [143, 144]. This greatly simplifies the enzymology involved in the amplification process, while keeping the advantage of all isothermal amplification technologies. An IsoAmp HIV-1 assay (BioHelix Corp, Beverly, MA) was developed targeting the HIV-1 *gag* gene using the isothermal RT-HDA and a disposable amplicon containment device with an embedded vertical-flow DNA detection strip to detect the presence of HIV-1 amplicons [145]. The vertical-flow DNA detection strip has a control line to validate the performance of the device as well as a test line to detect the analyte. The preliminary limit of detection of the IsoAmp HIV assay is 50 copies of the HIV-1 Armored RNA (Assuragen, Austin, TX) that were input into the IsoAmp HIV reaction [146].

In addition to the rapid diagnosis of HIV infections by instrument-free molecular methods, other simple and user-friendly systems have been developed for antiretroviral therapy monitoring near patients. Two smaller flow cytometers (Point-Care and Easy CD4 Analyzer) have been created targeting global health and point-of-care applications with limited functionality [147]. Another simple image cytometer for CD4 enumeration has recently been described and used on HIV-1-infected patients in Thailand [148]. Simple microfluidic approaches merged with rapid detection and counting can capture CD4 cells selectively by fluorescent labeling or label-free techniques [149]. Two groups have reported the development of counting

microfluidic chips with lensless imaging to target CD4 cell counts for HIV POCT in resource-limited settings [150, 151]. Semiconductor quantum dots are integrated into a nanobiochip for enumeration of CD4+ T cell counts at the point-of-care [152]. A recent study used three POCT devices, including Pima CD4 (Alere Inc., Waltham, MA, USA), for CD4 counting for accurate CD4 T-cell enumeration and antiretroviral drug toxicity monitoring in primary healthcare clinics in Mozambique. The results indicated that POC diagnostics to monitor antiretroviral therapy at primary healthcare level is technically feasible and should be utilized in efforts to decentralize HIV care and treatment [153].

Each molecular diagnostic device can be divided into nucleic acid extraction, amplification, and detection components [154]. A rapid, point-of-care extraction of HIV-1 proviral DNA from whole blood was reported and used for detection by real-time PCR [155]. Simple and inexpensive molecular assays based on dipstick and zipper technology have been described [156, 157]. The Cepheid GeneXpert System (Sunnyvale, CA), a single-use sample processing cartridge system with integrated multicolor real-time PCR capacity [158], has the potential to greatly simplify nucleic acid amplification tests. Microarrays have also been incorporated with nucleic acid probes and peptides to detect and quantify HIV-1 [159–163]. Miniaturized PCR "POCT" devices have been reported for microbial agent detection and identification. Integration of microfluidics and lensless imaging for POCT has been reported in HIV-1 point-of-care clinical diagnostics [164]. With incorporation of micro/nano- fabrications/crystals (e.g., quantum dots), microfluidics and array based systems will enable the development of more feasible immunological and molecular tests for HIV POCT in resource-limited settings [165–170].

There is an urgent need for low-cost, simple, and accurate HIV-1 viral load monitoring technologies in resource-limited settings, particularly when scaling-up first and second-line highly active antiretroviral therapies [171–173]. The Liat HIV Quant Assay (IQuum, Marlborough, MA) is comprised of two components, the Liat Analyzer and the Liat Tube (IQuum), that provide rapid and automated sample-to-result HIV load tests in the near-patient setting within 1.5 h. The assay demonstrated linearity of 6 logs and a limit of detection of 57 copies/ml and covered HIV-1 group M (clades A-H), group O, and HIV-2 [174]. Besides nucleic acid amplification techniques, the ExaVir Load assay (Cavidi, Sweden), which requires simple equipment to perform the modified enzyme-linked immunosorbent assay (ELISA) format to measure viral reverse transcriptase (RT) activity in a simple laboratory environment, correlates with plasma RNA levels [175–179]. Only standard ELISA equipment, together with the ExaVir Load start-up equipment, is required for analysis. The separation equipment used in the current Version 3 makes the procedure less time-consuming, more efficient, and easier to handle than ever. Using a similar principle, the system has been used for HIV phenotypic susceptibility testing (ExaVir Drug assay), which worked well for efavirenz but not for nevirapine [179].

Host Genome Polymorphism Testing

Enhanced by the human genome programs, diagnostic virologists envisioned the utilization of genetics beyond HIV-1 genomes to help manage HIV infections [180]. If infections, especially chronic and persistent HIV-1 infections, can be viewed as "horizontally acquired" genetic diseases, it makes sense to view pathogen and host as an integrated system. Increasing evidence indicates that the outcome of HIV infections is influenced by the genetic background of the host [181, 182]. Earlier pioneer studies revealed that resistance to HIV-1 infection, both in vitro and in vivo has been associated with an internal 32 base-pair deletion in the human chemokine receptor CCR-5 gene [183, 184]. Polymorphisms in AIDS restriction genes, e.g., CCR5, CCR2, and CXCR4, influence the risk of becoming infected with HIV, as well as the rate of AIDS progression once one is infected [185–189]. CCR5-Δ32 and CCR5 P1 haplotypes had the opposite effects on therapy efficacy and subsequent progression to AIDS while on HAART: the CCR5-Δ32 decreased time to viral suppression and was protective against AIDS, whereas the CCR5 P1 haplotype was associated with delayed viral suppression and accelerated time to AIDS [190]. Pine and colleagues revealed that polymorphisms in toll-like receptors 4 and 9 influence viral load in a seroincident cohort of HIV-1-infected individuals [191]. A recent study in high-risk South African black women indicated that the risk of acquiring HIV infection was threefold greater in those with the trait of Duffy-null-associated low neutrophil counts, compared with all other study participants. Because of the high prevalence of this trait among persons of African ancestry, it may contribute to the dynamics of the HIV epidemic in Africa [192].

Accordingly, detection of host polymorphisms in the HIV diagnostic field can help identify those at risk of rapid disease progression and help with the timing of the initiation of treatment. Allele frequencies and relative hazard values of CCR5-Δ32, CCR2 64I, CCR5 P1, IL-10 5'A, HLA-B*35, and HLA homozygosity were determined to generate a composite relative hazard of progression to AIDS [193]. Possession of a CCL3L1 copy number lower than the population average is associated with markedly enhanced HIV/AIDS susceptibility; which is enhanced in individuals who possess the CCR5-Δ32 genotype [194]. A model was defined that retained CCR5-Δ32, CCR2 64I, CCR5 59029AA, CCL3 495TT, SDF1 3′A, PML—225TT, PPIA 1650G, and TSG101—183 °C where the differences between carrying opposing genetic variants would translate into lengthening or shortening the time from 500 CD4 T cells/μl to <200 CD4 T cells/μl by up to 2.8 years [195]. Genetically, polymorphic profiles in cytochrome P450s and transporters facilitate the optimal chemotherapy for HIV infections. An association between *CYP2D6* genetic variants and plasma levels of EFV and NVP in treatment-naïve individuals with HIV infection was observed, in which patients carrying a loss-of-function *CYP2D6* allele had higher median plasma levels of both drugs [196]. Polymorphisms in CYP2B6 correlate with high Efavirenz concentrations in plasma and the central nervous system [197, 198]. A recent study identified more than 300 genome-wide significant SNPs within the MHC, and the data implicated that the nature of the

HLA–viral peptide interaction as the major factor modulating durable control of HIV infections [199].

Microarray has become a powerful technique to screen many genes for multiple polymorphisms on hundreds of samples [163]. With recent technological advances, it is now possible to genotype over one million polymorphisms for thousands of samples by using either the Illumina or Affymetrix system [200, 201]. While more and more HIV infection resistance and disease progression-related host gene polymorphisms have been demonstrated, simple, user-friendly techniques for the detection of such known mutations will soon be adapted into the clinical diagnostic field. Currently used techniques include allele-specific nucleotide amplification [202, 203], single nucleotide primer extension [204], and the oligonucleotide ligation assay [205, 206]. PCR-led amplification technology has been important for these methods since it is either used for the generation of DNA fragments, or is part of the detection method. Real-time PCR assays based on TaqMan hydrolysis probes have been used as confirmatory methods, which are very robust but less cost effective for larger scale studies [207, 208]. DNA sequencing remains the gold standard and is enhanced by high-throughput processing and deep production scaling [209] and is now considered the most powerful procedure for polymorphism detection.

Host Response and Transcriptome Analysis

In addition to CD4 cell counting, other host responses can be used for monitoring therapy efficacies and side-effects in HIV-infected patients receiving antiretroviral therapy. T-cell-receptor-chain rearrangement excision circles (TREC) are episomal circles which are generated during T cell maturation in the thymus. TREC are stable and persist in newly matured T cells, and, after entering the peripheral bloods, they are diluted out during mitosis of these cells. Quantification of TREC present in naïve T cells is considered to be an accurate measure of thymic function. Although thymic function declines mainly with age, substantial output is maintained into late adulthood. HIV infection leads to a decrease in thymic function that can be measured in the peripheral blood and lymphoid tissues. In adults treated with HAART, there is a rapid and sustained increase in thymic output in most subjects, indicating that the adult thymus can contribute to immune reconstitution following antiretroviral therapy [210–212]. In addition to CD4 cell counts and HIV-1 viral loads, TREC has been described as another biomarker to monitor the treatment effectiveness and the feasibility of clinical progression [210, 212–216].

Mitochondrial toxicity of antiretroviral drugs, particularly the nucleoside reverse transcriptase inhibitor (NRTI), has been postulated to be responsible for the pathogenesis of many secondary effects of HAART, including hyperlactatemia [217, 218]. During HIV antiretroviral therapy, clinically symptomatic mitochondrial dysfunction has been associated with mitochondrial DNA depletion, and a real-time PCR was developed to determine a mitochondrial DNA versus nuclear DNA ratio as a biomarker of NRTI toxicity [219]. The observed increases in mitochondrial

DNA and RNA content during the first year of treatment may represent a restorative trend resulting from suppression of HIV-1 infection, independent of the treatment used. Mitochondrial DNA and RNA content in individual cell subtypes, rather than in peripheral leukocytes, may be better markers of toxicity and deserve further investigation [220]. Other assays, which include mitochondrial RNA quantification by real-time PCR [221] and mitochondrial protein synthesis by Western blot immunoanalysis [222], have been described to measure mitochondrial toxicity-related functional changes. Recently, a flow cytometric assay has been developed to gauge mitochondrial function. Flow cytometric quantification of a mitochondrial DNA-encoded mitochondrial protein and a nuclear DNA-encoded mitochondrial protein was optimized and validated, which allows simultaneous detection of mitochondrial DNA and nuclear DNA encoded proteins at the single cell level, offering a method to monitor for mitochondrial function [223].

New microarray techniques, especially those from Affymetrix (Santa Clara, CA), have recently allowed the host transcriptome analyses in individuals with HIV-1 [163, 224]. A comprehensive review of the 34 studies involving HIV-1 and microarrays in the 2000–2006 period concluded that these studies yielded important data on HIV-1-mediated effects on gene expression, providing new insights into the intricate interactions occurring during infection [225]. Several recent studies have demonstrated progress in expanding the pool of target genes and understanding the functional correlates of gene modulation to HIV-1 pathogenesis in vivo [180, 226]. It is predicted that these host transcriptome profiles will be used for the assessment of disease progression and prognosis. The precision of transcriptome analyses will be greatly improved through the added resolution of the RNA-Seq approach, which uses deep-sequencing technologies for transcriptome profiling [227, 228].

Concluding Remarks

The development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis methodology and monitoring strategy of infectious diseases. Molecular techniques have quickly become the mainstay for laboratory diagnosis and assessment of HIV-1 infections. Qualitative molecular assays have been used as the test of choice to diagnose perinatal and acute HIV-1 infections. HIV-1 plasma viral load assays are routinely used in combination with CD4 cell counts to determine when to initiate therapy and when a regimen is failing. HIV-1 antiretroviral susceptibility tests, which include a genotyping assay that detects mutations known to confer resistance in viral genomes and a phenotypic assay measuring recombinant viral replication in the presence of antiretroviral drugs, have become routine patient care in the management of antiretroviral treatment. Point-of-care or near-the-patient molecular assays are being developed with the potential to make rapid, evidence-based, therapeutic action at or near the site of patient care. Assessing host gene polymorphisms and immune responses have emerged in research and clinical laboratories by initiating alternative tools for better diagnosis and monitoring of HIV-1 infections.

References

1. Tang YW, Persing DH (2009) Diagnostic microbiology. In: Schaechter M (ed) Encyclopedia of microbiology, 3rd edn. Elsevier, Oxford, pp 308–320
2. Branson BM (2007) State of the art for diagnosis of HIV infection. Clin Infect Dis 45(Suppl 4):S221–S225
3. Ou CY, Kwok S, Mitchell SW et al (1988) DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 239(4837):295–297
4. Owens DK, Holodniy M, McDonald TW, Scott J, Sonnad S (1996) A meta-analytic evaluation of the polymerase chain reaction for the diagnosis of HIV infection in infants. JAMA 275(17):1342–1348
5. Stevens WS, Noble L, Berrie L, Sarang S, Scott LE (2009) Ultra-high-throughput, automated nucleic acid detection of human immunodeficiency virus (HIV) for infant infection diagnosis using the Gen-Probe Aptima HIV-1 screening assay. J Clin Microbiol 47(8):2465–2469
6. Novitsky V, Gaolathe T, Woldegabriel E, Makhema J, Essex M (2007) A seronegative case of HIV-1 subtype C infection in Botswana. Clin Infect Dis 45(5):e68–e71
7. Sullivan PS, Schable C, Koch W et al (1999) Persistently negative HIV-1 antibody enzyme immunoassay screening results for patients with HIV-1 infection and AIDS: serologic, clinical, and virologic results. Seronegative AIDS Clinical Study Group. Aids 13(1):89–96
8. Fiebig EW, Wright DJ, Rawal BD et al (2003) Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS 17(13):1871–1879
9. Giachetti C, Linnen JM, Kolk DP et al (2002) Highly sensitive multiplex assay for detection of human immunodeficiency virus type 1 and hepatitis C virus RNA. J Clin Microbiol 40(7):2408–2419
10. Pierce VM, Neide B, Hodinka RL (2011) Evaluation of the Gen-Probe Aptima HIV-1 RNA qualitative assay as an alternative to Western blot analysis for confirmation of HIV infection. J Clin Microbiol 49(4):1642–1645
11. Ren A, Louie B, Rauch L et al (2008) Screening and confirmation of human immunodeficiency virus type 1 infection solely by detection of RNA. J Med Microbiol 57(Pt 10):1228–1233
12. Kerr RJ, Player G, Fiscus SA, Nelson JA (2009) Qualitative human immunodeficiency virus RNA analysis of dried blood spots for diagnosis of infections in infants. J Clin Microbiol 47(1):220–222
13. Pilcher CD, McPherson JT, Leone PA et al (2002) Real-time, universal screening for acute HIV infection in a routine HIV counseling and testing population. JAMA 288(2):216–221
14. Ethridge SF, Hart C, Hanson DL et al (2010) Performance of the Aptima HIV-1 RNA qualitative assay with 16- and 32-member specimen pools. J Clin Microbiol 48(9):3343–3345
15. Stramer SL, Glynn SA, Kleinman SH et al (2004) Detection of HIV-1 and HCV infections among antibody-negative blood donors by nucleic acid-amplification testing. N Engl J Med 351(8):760–768
16. Nugent CT, Dockter J, Bernardin F et al (2009) Detection of HIV-1 in alternative specimen types using the APTIMA HIV-1 RNA Qualitative Assay. J Virol Methods 159(1):10–14
17. Kebe K, Ndiaye O, Ndiaye HD et al (2011) RNA versus DNA (NucliSENS EasyQ HIV-1 v1.2 versus Amplicor HIV-1 DNA Test v1.5) for Early Diagnosis of HIV-1 Infection in Infants in Senegal. J Clin Microbiol 49(7):2590–2593
18. Busch MP, Glynn SA, Wright DJ et al (2005) Relative sensitivities of licensed nucleic acid amplification tests for detection of viremia in early human immunodeficiency virus and hepatitis C virus infection. Transfusion 45(12):1853–1863
19. Cunningham CK, Charbonneau TT, Song K et al (1999) Comparison of human immunodeficiency virus 1 DNA polymerase chain reaction and qualitative and quantitative RNA polymerase chain reaction in human immunodeficiency virus 1-exposed infants. Pediatr Infect Dis J 18(1):30–35

20. Lambert JS, Harris DR, Stiehm ER et al (2003) Performance characteristics of HIV-1 culture and HIV-1 DNA and RNA amplification assays for early diagnosis of perinatal HIV-1 infection. J Acquir Immune Defic Syndr 34(5):512–519
21. Rouet F, Montcho C, Rouzioux C et al (2001) Early diagnosis of paediatric HIV-1 infection among African breast-fed children using a quantitative plasma HIV RNA assay. AIDS 15(14):1849–1856
22. Stevens W, Erasmus L, Moloi M, Taleng T, Sarang S (2008) Performance of a novel human immunodeficiency virus (HIV) type 1 total nucleic acid-based real-time PCR assay using whole blood and dried blood spots for diagnosis of HIV in infants. J Clin Microbiol 46(12):3941–3945
23. Sherman GG, Cooper PA, Coovadia AH et al (2005) Polymerase chain reaction for diagnosis of human immunodeficiency virus infection in infancy in low resource settings. Pediatr Infect Dis J 24(11):993–997
24. Dunn DT, Brandt CD, Krivine A et al (1995) The sensitivity of HIV-1 DNA polymerase chain reaction in the neonatal period and the relative contributions of intra-uterine and intra-partum transmission. AIDS 9(9):F7–F11
25. Kovacs A, Xu J, Rasheed S et al (1995) Comparison of a rapid nonisotopic polymerase chain reaction assay with four commonly used methods for the early diagnosis of human immunodeficiency virus type 1 infection in neonates and children. Pediatr Infect Dis J 14(11):948–954
26. Germer JJ, Gerads TM, Mandrekar JN, Mitchell PS, Yao JD (2006) Detection of HIV-1 proviral DNA with the AMPLICOR HIV-1 DNA Test, version 1.5, following sample processing by the MagNA Pure LC instrument. J Clin Virol 37(3):195–198
27. Ford N, Nachega JB, Engel ME, Mills EJ (2009) Directly observed antiretroviral therapy: a systematic review and meta-analysis of randomised clinical trials. Lancet 374:2064–2071
28. Mellors JW, Munoz A, Giorgi JV et al (1997) Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. Ann Intern Med 126(12):946–954
29. O'Brien WA, Hartigan PM, Martin D et al (1996) Changes in plasma HIV-1 RNA and CD4+ lymphocyte counts and the risk of progression to AIDS. Veterans Affairs Cooperative Study Group on AIDS. N Engl J Med 334(7):426–431
30. Saag MS, Holodniy M, Kuritzkes DR et al (1996) HIV viral load markers in clinical practice. Nat Med 2(6):625–629
31. Hughes MD, Johnson VA, Hirsch MS et al (1997) Monitoring plasma HIV-1 RNA levels in addition to CD4+ lymphocyte count improves assessment of antiretroviral therapeutic response. ACTG 241 Protocol Virology Substudy Team. Ann Intern Med 126(12):929–938
32. Kitchen CM, Kitchen SG, Dubin JA, Gottlieb MS (2001) Initial virological and immunologic response to highly active antiretroviral therapy predicts long-term clinical outcome. Clin Infect Dis 33(4):466–472
33. Mellors JW, Rinaldo CR Jr, Gupta P, White RM, Todd JA, Kingsley LA (1996) Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science 272(5265):1167–1170
34. Gross R, Bilker WB, Friedman HM, Strom BL (2001) Effect of adherence to newly initiated antiretroviral therapy on plasma viral load. AIDS 15(16):2109–2117
35. Wood E, Yip B, Hogg RS et al (2000) Full suppression of viral load is needed to achieve an optimal CD4 cell count response among patients on triple drug antiretroviral therapy. AIDS 14(13):1955–1960
36. Church D, Gregson D, Lloyd T et al (2011) Comparison of the RealTime HIV-1, COBAS TaqMan 48 v1.0, Easy Q v1.2, and Versant v3.0 assays for determination of HIV-1 viral loads in a cohort of Canadian patients with diverse HIV subtype infections. J Clin Microbiol 49(1):118–124
37. Lin HJ, Pedneault L, Hollinger FB (1998) Intra-assay performance characteristics of five assays for quantification of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 36(3):835–839
38. Martin D (2000) Appropriate laboratory monitoring of HIV. S Afr Med J 90(1):33–36
39. Muyldermans G, Debaisieux L, Fransen K et al (2000) Blinded, multicenter quality control study for the quantification of human immunodeficiency virus type 1 RNA in plasma by the Belgian AIDS reference laboratories. Clin Microbiol Infect 6(4):213–217

40. Schuurman R, Descamps D, Weverling GJ et al (1996) Multicenter comparison of three commercial methods for quantification of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 34(12):3016–3022
41. Tang YW, Huong JT, Lloyd RM Jr, Spearman P, Haas DW (2000) Comparison of human immunodeficiency virus type 1 RNA sequence heterogeneity in cerebrospinal fluid and plasma. J Clin Microbiol 38(12):4637–4639
42. Church D, Gregson D, Lloyd T et al (2011) Comparison of the RealTime HIV-1, COBAS TaqMan 48 v1.0, Easy Q v1.2, and Versant v3.0 assays for determination of HIV-1 viral loads in a cohort of Canadian patients with diverse HIV subtype infections. J Clin Microbiol 49(1):118–24
43. Sire JM, Vray M, Merzouk M et al (2011) Comparative RNA quantification of HIV-1 group M and non-M with the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 v2.0 and Abbott Real-Time HIV-1 PCR assays. J Acquir Immune Defic Syndr 56(3):239–243
44. Bourlet T, Signori-Schmuck A, Roche L et al (2011) HIV-1 load comparison using four commercial real-time assays. J Clin Microbiol 49(1):292–297
45. Griffith BP, Mayo DR (2006) Increased levels of HIV RNA detected in samples with viral loads close to the detection limit collected in Plasma Preparation Tubes (PPT). J Clin Virol 35(2):197–200
46. Rebeiro PF, Kheshti A, Bebawy SS et al (2008) Increased detectability of plasma HIV-1 RNA after introduction of a new assay and altered specimen-processing procedures. Clin Infect Dis 47(10):1354–1357
47. Salimnia H, Moore EC, Crane LR, Macarthur RD, Fairfax MR (2005) Discordance between viral loads determined by Roche COBAS AMPLICOR human immunodeficiency virus type 1 monitor (version 1.5) Standard and ultrasensitive assays caused by freezing patient plasma in centrifuged becton-dickinson vacutainer brand plasma preparation tubes. J Clin Microbiol 43(9):4635–4639
48. Stosor V, Palella FJ Jr, Berzins B et al (2005) Transient viremia in HIV-infected patients and use of plasma preparation tubes. Clin Infect Dis 41(11):1671–1674
49. Ayele W, Schuurman R, Messele T et al (2007) Use of dried spots of whole blood, plasma, and mother's milk collected on filter paper for measurement of human immunodeficiency virus type 1 burden. J Clin Microbiol 45(3):891–896
50. Bourlet T, Cazorla C, Berthelot P et al (2001) Compartmentalization of HIV-1 according to antiretroviral therapy: viral loads are correlated in blood and semen but poorly in blood and saliva. AIDS 15(2):284–285
51. Lloyd RM Jr, Burns DA, Huong JT et al (2009) Dried-plasma transport using a novel matrix and collection system for human immunodeficiency virus and hepatitis C virus virologic testing. J Clin Microbiol 47(5):1491–1496
52. Shepard RN, Schock J, Robertson K et al (2000) Quantitation of human immunodeficiency virus type 1 RNA in different biological compartments. J Clin Microbiol 38(4):1414–1418
53. Gil C, Garcia MT, Garcia F et al (2011) Evaluation of the Roche COBAS(R) TaqMan(R) HIV-1 test for quantifying HIV-1 RNA in infected cells and lymphoid tissue. J Virol Methods 174(1–2):69–76
54. Lofgren SM, Morrissey AB, Chevallier CC et al (2009) Evaluation of a dried blood spot HIV-1 RNA program for early infant diagnosis and viral load monitoring at rural and remote healthcare facilities. AIDS 23(18):2459–2466
55. Mbida AD, Sosso S, Flori P et al (2009) Measure of viral load by using the Abbott Real-Time HIV-1 assay on dried blood and plasma spot specimens collected in 2 rural dispensaries in Cameroon. J Acquir Immune Defic Syndr 52(1):9–16
56. van Deursen P, Oosterlaken T, Andre P et al (2010) Measuring human immunodeficiency virus type 1 RNA loads in dried blood spot specimens using NucliSENS EasyQ HIV-1 v2.0. J Clin Virol 47:120–125
57. Zhang Q, Wang L, Jiang Y et al (2008) Early infant human immunodeficiency virus type 1 detection suitable for resource-limited settings with multiple circulating subtypes by use of nested three-monoplex DNA PCR and dried blood spots. J Clin Microbiol 46(2):721–726

58. Nkenfou CN, Elong EL, Ouwe-Missi-Oukem-Boyer O et al (2012) Implementation of HIV Early Infant Diagnosis and HIV-1 RNA viral load determination on Dried Blood Spots in Cameroon: challenges and propositions. AIDS Res Hum Retroviruses 28:176–181
59. Driver GA, Patton JC, Moloi J, Stevens WS, Sherman GG (2007) Low risk of contamination with automated and manual excision of dried blood spots for HIV DNA PCR testing in the routine laboratory. J Virol Methods 146(1–2):397–400
60. Leelawiwat W, Young NL, Chaowanachan T et al (2009) Dried blood spots for the diagnosis and quantitation of HIV-1: stability studies and evaluation of sensitivity and specificity for the diagnosis of infant HIV-1 infection in Thailand. J Virol Methods 155(2):109–117
61. Mitchell C, Kraft K, Peterson D, Frenkel L (2010) Cross-contamination during processing of dried blood spots used for rapid diagnosis of HIV-1 infection of infants is rare and avoidable. J Virol Methods 163:489–491
62. Revets H, Marissens D, de Wit S et al (1996) Comparative evaluation of NASBA HIV-1 RNA QT, AMPLICOR-HIV monitor, and QUANTIPLEX HIV RNA assay, three methods for quantification of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 34(5):1058–1064
63. Damond F, Roquebert B, Benard A et al (2007) Human immunodeficiency virus type 1 (HIV-1) plasma load discrepancies between the Roche COBAS AMPLICOR HIV-1 MONITOR Version 1.5 and the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 assays. J Clin Microbiol 45(10):3436–3438
64. Schumacher W, Frick E, Kauselmann M, Maier-Hoyle V, van der Vliet R, Babiel R (2007) Fully automated quantification of human immunodeficiency virus (HIV) type 1 RNA in human plasma by the COBAS AmpliPrep/COBAS TaqMan system. J Clin Virol 38(4):304–312
65. Scott LE, Noble LD, Moloi J, Erasmus L, Venter WD, Stevens W (2009) Evaluation of the Abbott m2000 RealTime human immunodeficiency virus type 1 (HIV-1) assay for HIV load monitoring in South Africa compared to the Roche Cobas AmpliPrep-Cobas Amplicor, Roche Cobas AmpliPrep-Cobas TaqMan HIV-1, and BioMerieux NucliSENS EasyQ HIV-1 assays. J Clin Microbiol 47(7):2209–2217
66. Gueudin M, Plantier JC, Lemee V et al (2007) Evaluation of the Roche Cobas TaqMan and Abbott RealTime extraction-quantification systems for HIV-1 subtypes. J Acquir Immune Defic Syndr 44(5):500–505
67. Pas S, Rossen JW, Schoener D et al (2010) Performance evaluation of the new Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test version 2.0 for quantification of human immunodeficiency virus type 1 RNA. J Clin Microbiol 48(4):1195–1200
68. Sizmann D, Glaubitz J, Simon CO et al (2011) Improved HIV-1 RNA quantitation by COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 using a novel dual-target approach. J Clin Virol 49(1):41–46
69. Dewar RL, Highbarger HC, Sarmiento MD et al (1994) Application of branched DNA signal amplification to monitor human immunodeficiency virus type 1 burden in human plasma. J Infect Dis 170(5):1172–1179
70. Nolte FS, Boysza J, Thurmond C, Clark WS, Lennox JL (1998) Clinical comparison of an enhanced-sensitivity branched-DNA assay and reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 36(3):716–720
71. Gleaves CA, Welle J, Campbell M et al (2002) Multicenter evaluation of the Bayer VERSANT HIV-1 RNA 3.0 assay: analytical and clinical performance. J Clin Virol 25(2):205–216
72. Berger A, Scherzed L, Sturmer M, Preiser W, Doerr HW, Rabenau HF (2005) Comparative evaluation of the Cobas Amplicor HIV-1 Monitor Ultrasensitive Test, the new Cobas AmpliPrep/Cobas Amplicor HIV-1 Monitor Ultrasensitive Test and the Versant HIV RNA 3.0 assays for quantitation of HIV-1 RNA in plasma samples. J Clin Virol 33(1):43–51
73. Holguin A, Lopez M, Molinero M, Soriano V (2008) Performance of three commercial viral load assays, Versant human immunodeficiency virus type 1 (HIV-1) RNA bDNA v3.0, Cobas AmpliPrep/Cobas TaqMan HIV-1, and NucliSens HIV-1 EasyQ v1.2, testing HIV-1 non-B subtypes and recombinant variants. J Clin Microbiol 46(9):2918–2923
74. van Gemen B, Kievits T, Schukkink R et al (1993) Quantification of HIV-1 RNA in plasma using NASBA during HIV-1 primary infection. J Virol Methods 43(2):177–187

75. Ginocchio CC, Kemper M, Stellrecht KA, Witt DJ (2003) Multicenter evaluation of the performance characteristics of the NucliSens HIV-1 QT assay used for quantitation of human immunodeficiency virus type 1 RNA. J Clin Microbiol 41(1):164–173
76. Weusten JJ, Wouters PA, van Zuijlen MC, van de Wiel PA (2002) Stochastic processes defining sensitivity and variability of internally calibrated quantitative NASBA-based viral load assays. Nucleic Acids Res 30(24):e137
77. Fiscus SA, Brambilla D, Coombs RW et al (2000) Multicenter evaluation of methods to quantitate human immunodeficiency virus type 1 RNA in seminal plasma. J Clin Microbiol 38(6):2348–2353
78. Pyne MT, Konnick EQ, Phansalkar A, Hillyard DR (2009) Evaluation of the Abbott investigational use only realtime HIV-1 assay and comparison to the Roche Amplicor HIV-1 monitor test, version 1.5. J Mol Diagn 11(4):347–354
79. Schutten M, Fries E, Burghoorn-Maas C, Niesters HG (2007) Evaluation of the analytical performance of the new Abbott RealTime RT-PCRs for the quantitative detection of HCV and HIV-1 RNA. J Clin Virol 40(2):99–104
80. Swanson P, Huang S, Abravaya K et al (2007) Evaluation of performance across the dynamic range of the Abbott RealTime HIV-1 assay as compared to VERSANT HIV-1 RNA 3.0 and AMPLICOR HIV-1 MONITOR v1.5 using serial dilutions of 39 group M and O viruses. J Virol Methods 141(1):49–57
81. Huang S, Salituro J, Tang N et al (2007) Thermodynamically modulated partially double-stranded linear DNA probe design for homogeneous real-time PCR. Nucleic Acids Res 35(16):e101
82. Raboud JM, Seminari E, Rae SL et al (1998) Comparison of costs of strategies for measuring levels of human immunodeficiency virus type 1 RNA in plasma by using Amplicor and Ultra Direct assays. J Clin Microbiol 36(11):3369–3371
83. Baxter JD, Mayers DL, Wentworth DN et al (2000) A randomized study of antiretroviral management based on plasma genotypic antiretroviral resistance testing in patients failing therapy. CPCRA 046 Study Team for the Terry Beirn Community Programs for Clinical Research on AIDS. AIDS 14(9):F83–F93
84. Cohen CJ, Hunt S, Sension M et al (2002) A randomized trial assessing the impact of phenotypic resistance testing on antiretroviral therapy. AIDS 16(4):579–588
85. Durant J, Clevenbergh P, Halfon P et al (1999) Drug-resistance genotyping in HIV-1 therapy: the VIRADAPT randomised controlled trial. Lancet 353(9171):2195–2199
86. Meynard JL, Vray M, Morand-Joubert L et al (2002) Phenotypic or genotypic resistance testing for choosing antiretroviral therapy after treatment failure: a randomized trial. AIDS 16(5):727–736
87. Tural C, Ruiz L, Holtzer C et al (2002) Clinical utility of HIV-1 genotyping and expert advice: the Havana trial. AIDS 16(2):209–218
88. Beddows S, Galpin S, Kazmi SH et al (2003) Performance of two commercially available sequence-based HIV-1 genotyping systems for the detection of drug resistance against HIV type 1 group M subtypes. J Med Virol 70(3):337–342
89. Sturmer M, Berger A, Doerr HW (2003) Modifications and substitutions of the RNA extraction module in the ViroSeq HIV-1 genotyping system version 2: effects on sensitivity and complexity of the assay. J Med Virol 71(4):475–479
90. Cunningham S, Ank B, Lewis D et al (2001) Performance of the applied biosystems ViroSeq human immunodeficiency virus type 1 (HIV-1) genotyping system for sequence-based analysis of HIV-1 in pediatric plasma samples. J Clin Microbiol 39(4):1254–1257
91. Sendi P, Gunthard HF, Simcock M, Ledergerber B, Schupbach J, Battegay M (2007) Cost-effectiveness of genotypic antiretroviral resistance testing in HIV-infected patients with treatment failure. PLoS One 2(1):e173
92. Weinstein MC, Goldie SJ, Losina E et al (2001) Use of genotypic resistance testing to guide hiv therapy: clinical impact and cost-effectiveness. Ann Intern Med 134(6):440–450
93. Dunn DT, Coughlin K, Cane PA (2011) Genotypic resistance testing in routine clinical care. Curr Opin HIV AIDS 6(4):251–257

94. Hammer SM (2002) HIV drug resistance: implications for management. Top HIV Med 10(5):10–15
95. Sturmer M, Berger A, Preiser W (2004) HIV-1 genotyping: comparison of two commercially available assays. Expert Rev Mol Diagn 4(3):281–291
96. Erali M, Page S, Reimer LG, Hillyard DR (2001) Human immunodeficiency virus type 1 drug resistance testing: a comparison of three sequence-based methods. J Clin Microbiol 39(6):2157–2165
97. Aghokeng AF, Mpoudi-Ngole E, Chia JE, Edoul EM, Delaporte E, Peeters M (2011) High failure rate of the ViroSeq HIV-1 genotyping system for drug resistance testing in Cameroon, a country with broad HIV-1 genetic diversity. J Clin Microbiol 49(4):1635–1641
98. Gunthard HF, Wong JK, Ignacio CC, Havlir DV, Richman DD (1998) Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. AIDS Res Hum Retroviruses 14(10):869–876
99. Palmer S, Kearney M, Maldarelli F et al (2005) Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. J Clin Microbiol 43(1):406–413
100. Servais J, Lambert C, Fontaine E et al (2001) Comparison of DNA sequencing and a line probe assay for detection of human immunodeficiency virus type 1 drug resistance mutations in patients failing highly active antiretroviral therapy. J Clin Microbiol 39(2):454–459
101. Stuyver L, Wyseur A, Rombout A et al (1997) Line probe assay for rapid detection of drug-selected mutations in the human immunodeficiency virus type 1 reverse transcriptase gene. Antimicrob Agents Chemother 41(2):284–291
102. Vahey M, Nau ME, Barrick S et al (1999) Performance of the Affymetrix GeneChip HIV PRT 440 platform for antiretroviral drug resistance genotyping of human immunodeficiency virus type 1 clades and viral isolates with length polymorphisms. J Clin Microbiol 37(8):2533–2537
103. Wilson JW, Bean P, Robins T, Graziano F, Persing DH (2000) Comparative evaluation of three human immunodeficiency virus genotyping systems: the HIV-GenotypR method, the HIV PRT GeneChip assay, and the HIV-1 RT line probe assay. J Clin Microbiol 38(8):3022–3028
104. Halvas EK, Aldrovandi GM, Balfe P et al (2006) Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. J Clin Microbiol 44(7):2612–2614
105. Toni TA, Brenner BG, Asahchop EL, Ntemgwa M, Moisi D, Wainberg MA (2010) Development of an allele-specific PCR for detection of the K65R resistance mutation in patients infected with subtype C human immunodeficiency virus type 1. Antimicrob Agents Chemother 54(2):907–911
106. Van Laethem K, De Munter P, Schrooten Y et al (2007) No response to first-line tenofovir + lamivudine + efavirenz despite optimization according to baseline resistance testing: impact of resistant minority variants on efficacy of low genetic barrier drugs. J Clin Virol 39(1):43–47
107. Hedskog C, Mild M, Jernberg J et al (2010) Dynamics of HIV-1 quasispecies during antiviral treatment dissected using ultra-deep pyrosequencing. PLoS One 5(7):e11345
108. Simen BB, Simons JF, Hullsiek KH et al (2009) Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. J Infect Dis 199(5):693–701
109. D'Aquila RT, Geretti AM, Horton JH et al (2011) Tenofovir (TDF)-selected or abacavir (ABC)-selected low-frequency HIV type 1 subpopulations during failure with persistent viremia as detected by ultradeep pyrosequencing. AIDS Res Hum Retroviruses 27(2):201–209
110. Delobel P, Saliou A, Nicot F et al (2011) Minor HIV-1 variants with the K103N resistance mutation during intermittent Efavirenz-containing antiretroviral therapy and virological failure. PLoS One 6(6):e21655
111. Varghese V, Wang E, Babrzadeh F et al (2010) Nucleic acid template and the risk of a PCR-Induced HIV-1 drug resistance mutation. PLoS One 5(6):e10992

112. Gianella S, Delport W, Pacold ME et al (2011) Detection of minority resistance during early HIV-1 infection: natural variation and spurious detection rather than transmission and evolution of multiple viral variants. J Virol 85:8359–8367
113. Call SA, Saag MS, Westfall AO et al (2001) Phenotypic drug susceptibility testing predicts long-term virologic suppression better than treatment history in patients with human immunodeficiency virus infection. J Infect Dis 183(3):401–408
114. Dunne AL, Mitchell FM, Coberly SK et al (2001) Comparison of genotyping and phenotyping methods for determining susceptibility of HIV-1 to antiretroviral drugs. AIDS 15(12):1471–1475
115. Zhang M, Versalovic J (2002) HIV update. Diagnostic tests and markers of disease progression and response to therapy. Am J Clin Pathol 118(Suppl):S26–S32
116. Hertogs K, de Bethune MP, Miller V et al (1998) A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. Antimicrob Agents Chemother 42(2):269–276
117. Petropoulos CJ, Parkin NT, Limoli KL et al (2000) A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. Antimicrob Agents Chemother 44(4):920–928
118. Qari SH, Respess R, Weinstock H et al (2002) Comparative analysis of two commercial phenotypic assays for drug susceptibility testing of human immunodeficiency virus type 1. J Clin Microbiol 40(1):31–35
119. Zhang J, Rhee SY, Taylor J, Shafer RW (2005) Comparison of the precision and sensitivity of the Antivirogram and PhenoSense HIV drug susceptibility assays. J Acquir Immune Defic Syndr 38(4):439–444
120. Munoz M, Carmona R, Perez-Alvarez L et al (2005) Analysis of discrepancies in the interpretation of antiretroviral drug resistance results in HIV-1 infected patients of Basque Country, Spain. J Clin Virol 33(3):224–229
121. Gallego O, Martin-Carbonero L, Aguero J, de Mendoza C, Corral A, Soriano V (2004) Correlation between rules-based interpretation and virtual phenotype interpretation of HIV-1 genotypes for predicting drug resistance in HIV-infected individuals. J Virol Methods 121(1):115–118
122. Perez-Elias MJ, Garcia-Arota I, Munoz V et al (2003) Phenotype or virtual phenotype for choosing antiretroviral therapy after failure: a prospective, randomized study. Antivir Ther 8(6):577–584
123. Cooper DA, Steigbigel RT, Gatell JM et al (2008) Subgroup and resistance analyses of raltegravir for resistant HIV-1 infection. N Engl J Med 359(4):355–365
124. Eshleman SH, Hudelson SE, Smith P et al (2009) Analysis of pol integrase sequences in diverse HIV type 1 strains using a prototype genotyping assay. AIDS Res Hum Retroviruses 25(3):343–345
125. Hearps AC, Greengrass V, Hoy J, Crowe SM (2009) An HIV-1 integrase genotype assay for the detection of drug resistance mutations. Sex Health 6(4):305–309
126. Hudelson SE, Marlowe N, Huang W et al (2009) Analysis of HIV type 1 gp41 and enfuvirtide susceptibility among men in the United States who were HIV infected prior to availability of HIV entry inhibitors. AIDS Res Hum Retroviruses 25(7):701–705
127. Coakley E, Petropoulos CJ, Whitcomb JM (2005) Assessing chemokine co-receptor usage in HIV. Curr Opin Infect Dis 18(1):9–15
128. Veazey RS, Ketas TA, Klasse PJ et al (2008) Tropism-independent protection of macaques against vaginal transmission of three SHIVs by the HIV-1 fusion inhibitor T-1249. Proc Natl Acad Sci U S A 105(30):10531–10536
129. Carmona R, Perez-Alvarez L, Munoz M et al (2005) Natural resistance-associated mutations to Enfuvirtide (T20) and polymorphisms in the gp41 region of different HIV-1 genetic forms from T20 naive patients. J Clin Virol 32(3):248–253
130. Smith RA, Anderson DJ, Pyrak CL, Preston BD, Gottlieb GS (2009) Antiretroviral drug resistance in HIV-2: three amino acid changes are sufficient for classwide nucleoside analogue resistance. J Infect Dis 199(9):1323–1326

131. Westby M, Lewis M, Whitcomb J et al (2006) Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. J Virol 80(10):4909–4920
132. Recordon-Pinson P, Soulie C, Flandre P et al (2010) Evaluation of the genotypic prediction of HIV-1 coreceptor use versus a phenotypic assay and correlation with the virological response to maraviroc: the ANRS GenoTropism study. Antimicrob Agents Chemother 54(8):3335–3340
133. Low AJ, Swenson LC, Harrigan PR (2008) HIV coreceptor phenotyping in the clinical setting. AIDS Rev 10(3):143–151
134. MacArthur RD, Novak RM (2008) Reviews of anti-infective agents: maraviroc: the first of a new class of antiretroviral agents. Clin Infect Dis 47(2):236–241
135. Whitcomb JM, Huang W, Fransen S et al (2007) Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. Antimicrob Agents Chemother 51(2):566–575
136. Wilkin TJ, Goetz MB, Leduc R et al (2011) Reanalysis of coreceptor tropism in HIV-1-infected adults using a phenotypic assay with enhanced sensitivity. Clin Infect Dis 52(7):925–928
137. Lee-Lewandrowski E, Lewandrowski K (2009) Perspectives on cost and outcomes for point-of-care testing. Clin Lab Med 29(3):479–489
138. Blyth CC, Booy R, Dwyer DE (2011) Point of care testing: diagnosis outside the virology laboratory. Methods Mol Biol 665:415–433
139. Campbell S, Fedoriw Y (2009) HIV testing near the patient: changing the face of HIV testing. Clin Lab Med 29(3):491–501
140. Hsieh YH, Gaydos CA, Hogan MT et al (2011) What qualities are most important to making a point of care test desirable for clinicians and others offering sexually transmitted infection testing? PLoS One 6(4):e19263
141. Grant PR, Busch MP (2002) Nucleic acid amplification technology methods used in blood donor screening. Transfus Med 12(4):229–242
142. Scuracchio PS, Poli MC, Lemos MM et al (2007) Detection of HIV-1 infection in blood donors during the immunological window period using the nucleic acid-amplification technology. Transfus Med 17(3):200–204
143. An L, Tang W, Ranalli TA, Kim HJ, Wytiaz J, Kong H (2005) Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification. J Biol Chem 280(32):28952–28958
144. Vincent M, Xu Y, Kong H (2004) Helicase-dependent isothermal DNA amplification. EMBO Rep 5(8):795–800
145. Goldmeyer J, Li H, McCormac M et al (2008) Identification of Staphylococcus aureus and determination of methicillin resistance directly from positive blood cultures by isothermal amplification and a disposable detection device. J Clin Microbiol 46(4):1534–1536
146. Tang W, Chow WH, Li Y, Kong H, Tang YW, Lemieux B (2010) Nucleic acid assay system for tier II labs and moderately complex clinics to detect HIV in low-resource settings. J Infect Dis 201:S46–S51
147. Spacek LA, Shihab HM, Lutwama F et al (2006) Evaluation of a low-cost method, the Guava EasyCD4 assay, to enumerate CD4-positive lymphocyte counts in HIV-infected patients in the United States and Uganda. J Acquir Immune Defic Syndr 41(5):607–610
148. Li X, Breukers C, Ymeti A et al (2010) Clinical evaluation of a simple image cytometer for CD4 enumeration on HIV-infected patients. Cytometry B Clin Cytom 78(1):31–36
149. Rodriguez WR, Christodoulides N, Floriano PN et al (2005) A microchip CD4 counting method for HIV monitoring in resource-poor settings. PLoS Med 2(7):e182
150. Cheng X, Gupta A, Chen C, Tompkins RG, Rodriguez W, Toner M (2009) Enhancing the performance of a point-of-care CD4+ T-cell counting microchip through monocyte depletion for HIV/AIDS diagnostics. Lab Chip 9(10):1357–1364
151. Moon S, Keles HO, Ozcan A et al (2009) Integrating microfluidics and lensless imaging for point-of-care testing. Biosens Bioelectron 24(11):3208–3214

152. Jokerst JV, Floriano PN, Christodoulides N, Simmons GW, McDevitt JT (2008) Integration of semiconductor quantum dots into nano-bio-chip systems for enumeration of CD4+ T cell counts at the point-of-need. Lab Chip 8(12):2079–2090
153. Jani IV, Sitoe NE, Chongo PL et al (2011) Accurate CD4 T-cell enumeration and antiretroviral drug toxicity monitoring in primary healthcare clinics using point-of-care testing. AIDS 25(6):807–812
154. Tang YW, Procop GW, Persing DH (1997) Molecular diagnostics of infectious diseases. Clin Chem 43(11):2021–2038
155. Jangam SR, Yamada DH, McFall SM, Kelso DM (2009) Rapid, point-of-care extraction of human immunodeficiency virus type 1 proviral DNA from whole blood for detection by real-time PCR. J Clin Microbiol 47(8):2363–2368
156. Guttikonda S, Wang W, Suresh M (2004) Molecular zipper assays: a simple homosandwich with the sensitivity of PCR. J Pharm Pharm Sci 7(4):7–16
157. Panhotra BR, Hassan ZU, Joshi CS, Bahrani A (2005) Visual detection of multiple viral amplicons by dipstick assay: its application in screening of blood donors a welcome tool for the limited resource settings. J Clin Microbiol 43(12):6218, author reply -9
158. Raja S, Ching J, Xi L et al (2005) Technology for automated, rapid, and quantitative PCR or reverse transcription-PCR clinical testing. Clin Chem 51(5):882–890
159. Perrin A, Duracher D, Perret M, Cleuziat P, Mandrand B (2003) A combined oligonucleotide and protein microarray for the codetection of nucleic acids and antibodies associated with human immunodeficiency virus, hepatitis B virus, and hepatitis C virus infections. Anal Biochem 322(2):148–155
160. Hsia CC, Chizhikov VE, Yang AX et al (2007) Microarray multiplex assay for the simultaneous detection and discrimination of hepatitis B, hepatitis C, and human immunodeficiency type-1 viruses in human blood samples. Biochem Biophys Res Commun 356(4):1017–1023
161. Khodakov DA, Zakharova NV, Gryadunov DA, Filatov FP, Zasedatelev AS, Mikhailovich VM (2008) An oligonucleotide microarray for multiplex real-time PCR identification of HIV-1, HBV, and HCV. Biotechniques 44(2):241–246, 248
162. Burgess ST, Kenyon F, O'Looney N et al (2008) A multiplexed protein microarray for the simultaneous serodiagnosis of human immunodeficiency virus/hepatitis C virus infection and typing of whole blood. Anal Biochem 382(1):9–15
163. Miller MB, Tang YW (2009) Basic concepts of microarrays and potential applications in clinical microbiology. Clin Microbiol Rev 22(4):611–633
164. Lee SH, Kim SW, Kang JY, Ahn CH (2008) A polymer lab-on-a-chip for reverse transcription (RT)-PCR based point-of-care clinical diagnostics. Lab Chip 8(12):2121–2127
165. Edgar R, McKinstry M, Hwang J et al (2006) High-sensitivity bacterial detection using biotin-tagged phage and quantum-dot nanocomplexes. Proc Natl Acad Sci U S A 103(13):4841–4845
166. Kopp MU, Mello AJ, Manz A (1998) Chemical amplification: continuous-flow PCR on a chip. Science 280(5366):1046–1048
167. Liu RH, Lodes MJ, Nguyen T et al (2006) Validation of a fully integrated microfluidic array device for influenza A subtype identification and sequencing. Anal Chem 78(12):4184–4193
168. Michalet X, Pinaud FF, Bentolila LA et al (2005) Quantum dots for live cells, in vivo imaging, and diagnostics. Science 307(5709):538–544
169. Mokkapati VK, Sam Niedbala R, Kardos K et al (2007) Evaluation of UPlink-RSV: prototype rapid antigen test for detection of respiratory syncytial virus infection. Ann N Y Acad Sci 1098:476–485
170. Sista R, Hua Z, Thwar P et al (2008) Development of a digital microfluidic platform for point of care testing. Lab Chip 8(12):2091–2104
171. Rouet F, Rouzioux C (2007) The measurement of HIV-1 viral load in resource-limited settings: how and where? Clin Lab 53(3–4):135–148
172. Calmy A, Ford N, Hirschel B et al (2007) HIV viral load monitoring in resource-limited regions: optional or necessary? Clin Infect Dis 44(1):128–134
173. Fiscus SA, Cheng B, Crowe SM et al (2006) HIV-1 viral load assays for resource-limited settings. PLoS Med 3(10):e417

174. Tanriverdi S, Chen L, Chen S (2010) A rapid and automated sample-to-result HIV load test for near-patient application. J Infect Dis 201(Suppl 1):S52–S58
175. Iqbal HS, Balakrishnan P, Cecelia AJ et al (2007) Use of an HIV-1 reverse-transcriptase enzyme-activity assay to measure HIV-1 viral load as a potential alternative to nucleic acid-based assay for monitoring antiretroviral therapy in resource-limited settings. J Med Microbiol 56(Pt 12):1611–1614
176. Labbett W, Garcia-Diaz A, Fox Z et al (2009) Comparative evaluation of the ExaVir Load version 3 reverse transcriptase assay for measurement of human immunodeficiency virus type 1 plasma load. J Clin Microbiol 47(10):3266–3270
177. Malmsten A, Shao XW, Aperia K et al (2003) HIV-1 viral load determination based on reverse transcriptase activity recovered from human plasma. J Med Virol 71(3):347–359
178. Greengrass V, Lohman B, Morris L et al (2009) Assessment of the low-cost Cavidi ExaVir Load assay for monitoring HIV viral load in pediatric and adult patients. J Acquir Immune Defic Syndr 52(3):387–390
179. Napravnik S, Cachafeiro A, Stewart P, Eron JJ Jr, Fiscus SA (2010) HIV-1 viral load and phenotypic antiretroviral drug resistance assays based on reverse transcriptase activity in comparison to amplification based HIV-1 RNA and genotypic assays. J Clin Virol 47(1):18–22
180. Fellay J, Shianna KV, Telenti A, Goldstein DB (2010) Host genetics and HIV-1: the final phase? PLoS Pathog 6(10):e1001033
181. Vannberg FO, Chapman SJ, Hill AV (2011) Human genetic susceptibility to intracellular pathogens. Immunol Rev 240(1):105–116
182. Telenti A, Goldstein DB (2006) Genomics meets HIV-1. Nat Rev Microbiol 4(11):865–873
183. Dean M, Carrington M, Winkler C et al (1996) Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study [see comments]. Science 273(5283):1856–1862
184. Samson M, Libert F, Doranz BJ et al (1996) Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene [see comments]. Nature 382(6593):722–725
185. Kostrikis LG (2000) Impact of natural chemokine receptor polymorphisms on perinatal transmission of human immunodeficiency virus type 1. Teratology 61(5):387–390
186. Marmor M, Hertzmark K, Thomas SM, Halkitis PN, Vogler M (2006) Resistance to HIV infection. J Urban Health 83(1):5–17
187. O'Brien SJ, Moore JP (2000) The effect of genetic variation in chemokines and their receptors on HIV transmission and progression to AIDS. Immunol Rev 177:99–111
188. Philpott S, Weiser B, Tarwater P et al (2003) CC chemokine receptor 5 genotype and susceptibility to transmission of human immunodeficiency virus type 1 in women. J Infect Dis 187(4):569–575
189. Reiche EM, Bonametti AM, Voltarelli JC, Morimoto HK, Watanabe MA (2007) Genetic polymorphisms in the chemokine and chemokine receptors: impact on clinical course and therapy of the human immunodeficiency virus type 1 infection (HIV-1). Curr Med Chem 14(12):1325–1334
190. Hendrickson SL, Jacobson LP, Nelson GW et al (2008) Host genetic influences on highly active antiretroviral therapy efficacy and AIDS-free survival. J Acquir Immune Defic Syndr 48(3):263–271
191. Pine SO, McElrath MJ, Bochud PY (2009) Polymorphisms in toll-like receptor 4 and toll-like receptor 9 influence viral load in a seroincident cohort of HIV-1-infected individuals. AIDS 23(18):2387–2395
192. Ramsuran V, Kulkarni H, He W et al (2011) Duffy-null-associated low neutrophil counts influence HIV-1 susceptibility in high-risk South African black women. Clin Infect Dis 52(10):1248–1256
193. Carrington M, O'Brien SJ (2003) The influence of HLA genotype on AIDS. Annu Rev Med 54:535–551

194. Gonzalez E, Kulkarni H, Bolivar H et al (2005) The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science 307(5714):1434–1440
195. Bleiber G, May M, Martinez R et al (2005) Use of a combined ex vivo/in vivo population approach for screening of human genes involved in the human immunodeficiency virus type 1 life cycle for variants influencing disease progression. J Virol 79(20):12674–12680
196. Fellay J, Marzolini C, Meaden ER et al (2002) Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. Lancet 359(9300):30–36
197. Haas DW, Smeaton LM, Shafer RW et al (2005) Pharmacogenetics of long-term responses to antiretroviral regimens containing Efavirenz and/or Nelfinavir: an Adult Aids Clinical Trials Group Study. J Infect Dis 192(11):1931–1942
198. Tsuchiya K, Gatanaga H, Tachikawa N et al (2004) Homozygous CYP2B6 *6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens. Biochem Biophys Res Commun 319(4):1322–1326
199. Pereyra F, Jia X, McLaren PJ et al (2010) The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. Science 330(6010):1551–1557
200. Kerr JR, Kaushik N, Fear D, Baldwin DA, Nuwaysir EF, Adcock IM (2005) Single-nucleotide polymorphisms associated with symptomatic infection and differential human gene expression in healthy seropositive persons each implicate the cytoskeleton, integrin signaling, and oncosuppression in the pathogenesis of human parvovirus B19 infection. J Infect Dis 192(2):276–286
201. Pavy N, Pelgas B, Beauseigle S et al (2008) Enhancing genetic mapping of complex genomes through the design of highly-multiplexed SNP arrays: application to the large and unsequenced genomes of white spruce and black spruce. BMC Genomics 9:21
202. Chehab FF, Kan YW (1989) Detection of specific DNA sequences by fluorescence amplification: a color complementation assay. Proc Natl Acad Sci U S A 86(23):9178–9182
203. Saiki RK, Walsh PS, Levenson CH, Erlich HA (1989) Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc Natl Acad Sci U S A 86(16):6230–6234
204. Kornher JS, Livak KJ (1989) Mutation detection using nucleotide analogs that alter electrophoretic mobility. Nucleic Acids Res 17(19):7779–7784
205. Alves AM, Carr FJ (1988) Dot blot detection of point mutations with adjacently hybridising synthetic oligonucleotide probes. Nucleic Acids Res 16(17):8723
206. Landegren U, Kaiser R, Sanders J, Hood L (1988) A ligase-mediated gene detection technique. Science 241(4869):1077–1080
207. Kwok PY (2000) Approaches to allele frequency determination. Pharmacogenomics 1(2):231–235
208. Shi MM (2002) Technologies for individual genotyping: detection of genetic polymorphisms in drug targets and disease genes. Am J Pharmacogenomics 2(3):197–205
209. Margulies M, Egholm M, Altman WE et al (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437(7057):376–380
210. Douek DC, McFarland RD, Keiser PH et al (1998) Changes in thymic function with age and during the treatment of HIV infection. Nature 396(6712):690–695
211. Ribeiro RM, de Boer RJ (2008) The contribution of the thymus to the recovery of peripheral naive T-cell numbers during antiretroviral treatment for HIV infection. J Acquir Immune Defic Syndr 49(1):1–8
212. Zhang L, Lewin SR, Markowitz M et al (1999) Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. J Exp Med 190(5):725–732
213. Dion ML, Bordi R, Zeidan J et al (2007) Slow disease progression and robust therapy-mediated CD4+ T-cell recovery are associated with efficient thymopoiesis during HIV-1 infection. Blood 109(7):2912–2920
214. Hatzakis A, Touloumi G, Karanicolas R et al (2000) Effect of recent thymic emigrants on progression of HIV-1 disease. Lancet 355(9204):599–604

215. Marchetti G, Gori A, Casabianca A et al (2006) Comparative analysis of T-cell turnover and homeostatic parameters in HIV-infected patients with discordant immune-virological responses to HAART. AIDS 20(13):1727–1736
216. Saitoh A, Singh KK, Sandall S et al (2006) Association of CD4+ T-lymphocyte counts and new thymic emigrants in HIV-infected children during successful highly active antiretroviral therapy. J Allergy Clin Immunol 117(4):909–915
217. Haugaard SB, Andersen O, Pedersen SB et al (2005) Depleted skeletal muscle mitochondrial DNA, hyperlactatemia, and decreased oxidative capacity in HIV-infected patients on highly active antiretroviral therapy. J Med Virol 77(1):29–38
218. Swartz MN (1995) Mitochondrial toxicity—new adverse drug effects. N Engl J Med 333(17):1146–1148
219. Cote HC, Brumme ZL, Craib KJ et al (2002) Changes in mitochondrial DNA as a marker of nucleoside toxicity in HIV-infected patients. N Engl J Med 346(11):811–820
220. Casula M, Weverling GJ, Wit FW et al (2005) Mitochondrial DNA and RNA increase in peripheral blood mononuclear cells from HIV-1-infected patients randomized to receive stavudine-containing or stavudine-sparing combination therapy. J Infect Dis 192(10):1794–1800
221. Garrabou G, Moren C, Gallego-Escuredo JM et al (2009) Genetic and functional mitochondrial assessment of HIV-infected patients developing HAART-related hyperlactatemia. J Acquir Immune Defic Syndr 52(4):443–451
222. Miro O, Lopez S, Rodriguez de la Concepcion M et al (2004) Upregulatory mechanisms compensate for mitochondrial DNA depletion in asymptomatic individuals receiving stavudine plus didanosine. J Acquir Immune Defic Syndr 37(5):1550–1555
223. Lin CH, Sloan DD, Dang CH et al (2009) Assessment of mitochondrial toxicity by analysis of mitochondrial protein expression in mononuclear cells. Cytometry B Clin Cytom 76(3):181–190
224. Wang Z, Trillo-Pazos G, Kim SY et al (2004) Effects of human immunodeficiency virus type 1 on astrocyte gene expression and function: potential role in neuropathogenesis. J Neurovirol 10(Suppl 1):25–32
225. Giri MS, Nebozhyn M, Showe L, Montaner LJ (2006) Microarray data on gene modulation by HIV-1 in immune cells: 2000–2006. J Leukoc Biol 80(5):1031–1043
226. Rotger M, Dalmau J, Rauch A et al (2011) Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque. J Clin Invest 121(6):2391–2400
227. Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10(1):57–63
228. Cirulli ET, Singh A, Shianna KV et al (2011) Screening the human exome: a comparison of whole genome and whole transcriptome sequencing. Genome Biol 11(5):R57

Chapter 42
Bead-Based Suspension Arrays for the Detection and Identification of Respiratory Viruses

Sherry A. Dunbar

Introduction

The clinical signs and symptoms associated with many infectious diseases are often too nonspecific to discriminate between causative agents, and thus, definitive diagnosis requires specific laboratory tests for all of the suspected pathogens. In particular, respiratory tract infections can be caused by numerous different viral, bacterial, and fungal pathogens that are indistinguishable by clinical diagnosis. Respiratory tract infections are also among the most common infections in humans, with approximately 6–9 episodes per year in children and 2–4 episodes per year in adults [1]. These infections cause considerable morbidity and mortality as well as high healthcare costs associated with doctor visits, hospitalizations, treatment, and absences from work and school. Early diagnosis of the etiological agent in a respiratory infection permits effective antimicrobial therapy and appropriate management of the disease.

Molecular assays designed to directly detect microbial nucleic acid sequences from patient specimens have allowed for more rapid diagnosis and treatment of infectious diseases with high accuracy and reduced turnaround time as compared to traditional immunological and culture-based methods. Further, molecular testing methodologies that permit multiplexing have the advantage that they allow for simultaneous detection of multiple nucleic acid sequences from the same sample in a single reaction vessel. Multiplexed tests reduce the time, labor, and cost of laboratory testing as compared to single reaction detection methods, and in addition to improved efficiency, also have a higher diagnostic yield by the ability to detect multiple infections. Thus, multiplexed molecular assays are an efficient method for the definitive diagnosis of respiratory infections and can also provide information on coinfections and secondary infections. Among the various multiplexing technologies

S.A. Dunbar (✉)
Luminex Corporation, 12212 Technology Blvd., Austin, TX 78727, USA
e-mail: sdunbar@luminexcorp.com

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available, microsphere- or bead-based suspension arrays have emerged as a standard molecular multiplexing technology in the clinical microbiology laboratory. As compared to planar arrays, some of the benefits of bead-based arrays include ease of use, low cost, statistical superiority, excellent sensitivity and specificity, faster hybridization kinetics, flexibility in array preparation, and rapid data acquisition [2, 3].

The Luminex® xMAP® Technology platform was the first commercial bead-based array platform to use differentially dyed microspheres of the same size to achieve multianalyte profiling for proteins and nucleic acids. Key drivers for adoption of the xMAP platform include cost savings, labor and workflow efficiencies, sample conservation, high sensitivity, and broad dynamic range. The system has the added benefit of being an open platform in that assays can be rapidly developed, optimized, and implemented by the end user. The versatility of this open architecture is evidenced by approximately 10,000 peer-reviewed publications describing a variety of applications. As a result, the platform was rapidly adopted for nucleic acid and protein analysis in various laboratory settings, and further, the Luminex® 200™ system has achieved a 510(k) clearance status for in vitro diagnostic (IVD) applications. The breadth of bioanalytical applications on the platform includes hundreds of commercially available analytes and a vast number of custom assays developed for a global install base of more than 7,000 instruments. This chapter describes the principle and uses of the xMAP Technology platform for infectious disease nucleic acid detection with an emphasis on applications for the detection and identification of respiratory viruses.

Technology Overview

The Luminex xMAP system incorporates polystyrene microspheres (beads) that are internally dyed with two or three spectrally distinct fluorochromes. Using precise amounts of each of these fluorochromes, an array is created consisting of different bead sets with specific spectral addresses. The unique spectral characteristics within individual bead sets allow each bead region to be specifically differentiated from all others in a multiplex. Each bead set can possess a different reactant on its surface and because bead sets can be distinguished by their spectral addresses and each address is associated with a specific analyte or target, they can be combined in a single reaction to measure up to 500 different analytes simultaneously. An additional fluorochrome coupled to a reporter molecule quantifies the biomolecular interaction that has occurred at the bead surface. The configuration of an xMAP nucleic assay consists of a suspension array where specific capture oligonucleotides are covalently coupled to the surfaces of the internally dyed bead sets. After completion of assay incubations with a nucleic acid sample and a detectable reporter reagent, the reactions are analyzed within a Luminex reader, classifying the beads based on the spectral address or bead identity (region) and quantifying the bound fluorophore in the reporter detection channel. The potential for simultaneous detection of 1–500 protein or nucleic acid targets provides for rapid, sensitive, and specific multiplexed molecular analyses.

xMAP Microspheres

The first generation of xMAP microspheres utilized two internal fluorescent dyes to produce a 100-membered array of spectrally distinct bead sets. Inclusion of a third internal dye has allowed the expansion from the original 100–500 bead regions (Fig. 42.1). Several varieties of fluorescent bead reagents are available from Luminex. The fundamental MicroPlex® Microspheres are 5.6 μm polystyrene beads functionalized with surface carboxyl groups for covalent attachment of capture ligands. Most of the currently available xMAP assays were developed on MicroPlex Microspheres. MagPlex® Microspheres are 6.5 μm superparamagnetic beads functionalized with surface carboxyl groups for covalent attachment of ligands. MagPlex Microspheres exhibit both high performance and low nonspecific binding, and they can be magnetically separated from solution, allowing easy automation of assay processes and simplifying assay wash steps. Microsphere reagents precoupled with unique capture oligonucleotides (oligos) are also available for nucleic acid assay development. These reagents incorporate the use of xTAG® Technology, a proprietary universal tag sorting system that allows easy optimization, development, and expansion of molecular diagnostic assays and eliminates the need to couple content-specific capture probes to the beads. xTAG oligos are optimized to be an isothermal set and have minimum cross-reactivity. Earlier assays used MicroPlex-TAG™ Microspheres which are nonmagnetic MicroPlex beads, containing two internal fluorescent dyes and precoupled with xTAG oligo capture sequences (anti-TAGs). These beads have been replaced with MagPlex-TAG™ Microspheres which are superparamagnetic MagPlex beads, containing two or three internal fluorescent dyes and precoupled with anti-TAG capture oligos.

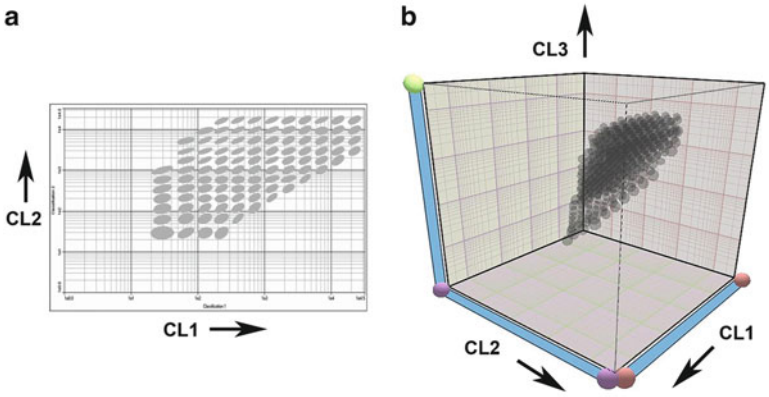

Fig. 42.1 Luminex bead maps. (**a**) Two-dimensional representation of the 100-plex bead map with Classification 1 dye (CL1) on the *x*-axis and Classification 2 dye (CL2) on the *y*-axis. *Gray ovals* indicate the positions of the 100 bead regions. (**b**) Three-dimensional representation of the 500-plex bead map with CL2 on the x- axis, Classification 3 dye (CL3) on the *y*-axis, and CL1 on the *z*-axis. *Gray ovals* show the positions of the 500 bead regions. The software allows the user to zoom in to specific areas ("slices") of the bead map for a more detailed view

Luminex Analyzers

There are currently three xMAP analyzers available through Luminex or its partners: the Luminex 200, the FLEXMAP 3D®, and the MAGPIX® (Fig. 42.2). The Luminex 200 and the FLEXMAP 3D are flow analyzers capable of multiplexing up to 100- or 500-plex, respectively, with fluidics and laser-based optics similar to traditional flow cytometry instruments. The MAGPIX utilizes a flow cell and CCD-based optics with magnetic beads for multiplexing up to 50-plex. In the flow analyzers, the beads are introduced into a rapidly flowing fluid stream and through hydrodynamic focusing, are interrogated individually as they pass by two separate lasers. A 635-nm, 10-mW red diode laser excites the fluorochromes contained within the microspheres and a 532-nm, 13-mW yttrium aluminum garnet (YAG) laser excites the reporter fluorochrome (R-phycoerythrin, Alexa 532, or Cy-3) bound to the bead surface. High-speed digital signal processing classifies the microsphere based on its spectral address and quantifies the reaction on the microsphere surface. Multiple readings are made per microsphere set, providing valid and robust statistics. Thousands of microspheres are interrogated per second resulting in a system capable of analyzing and reporting up to 500 different reactions in a single reaction vessel in just a few seconds per sample.

The Luminex 200 is designed to meet the multiplex testing needs of clinical and research laboratories. Built on proven, existing technologies including flow cytometry, microspheres, lasers, digital signal processing, and traditional chemistry, the Luminex 200 analyzer allows multiplex analysis of up to 100 analytes per reaction. The system is versatile and can be used for a variety of applications such as, immunoassays, genotyping, gene expression, and enzymatic assays. A few microliters of sample in a 20–200 μL reaction can provide up to 100 results, thus the platform is well suited for applications where sample size is limited. Robust optics and fluidics afford quantitative results over a 3–4 log dynamic range with strong concordance to enzyme-linked immunosorbent assay (ELISA), real-time polymerase chain reaction (PCR) and mass spectrometry. An intuitive template-based software, xPONENT®, operates the system and is designed for use with commercial kits or custom protocols and has simple assay set up, plate reading, and data analysis capabilities. The 21 CFR Part 11 compatible upgrade offers multilevel user management, full audit trail, electronic records, and electronic signatures. The system is approved for IVD use, with more than 50 xMAP-based 510(k) cleared kits available and numerous existing laboratory-developed tests (LDTs).

The FLEXMAP 3D is an enhanced flow-based multiplexing system utilizing xMAP Technology. The FLEXMAP 3D system assures rapid high-throughput analyses without compromising flexibility or performance and optimizes workflow through automation of routine tasks and integration with front-end sample preparation platforms. Enhanced optics permit multiplexing of up to 500 analytes per well and provide enhanced sensitivity with dynamic range extended to 4.5 logs. The system is compatible with both 96-well and 384-well plates and has a piercing probe which allows sealed plates to be analyzed. The dual syringe configuration processes plates 2–3 times faster than the Luminex 200. The system software, xPONENT, has

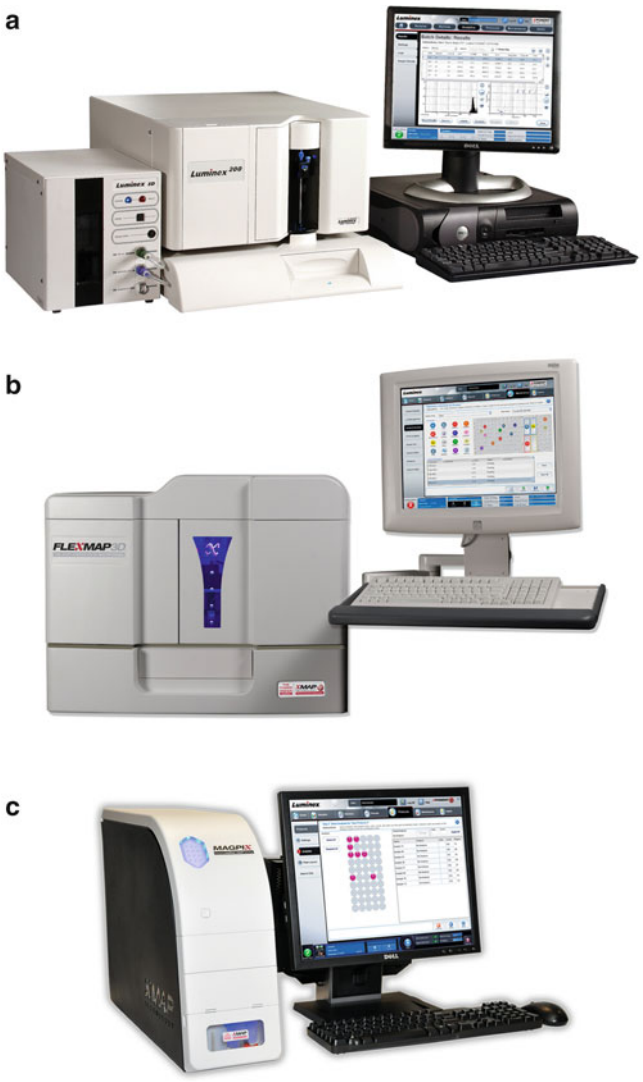

Fig. 42.2 Luminex analyzers. (**a**) The Luminex® 200™ total system includes the Luminex 200 flow analyzer, the Luminex XYP™ plate handling platform, the Luminex SD™ sheath fluid delivery system, xPONENT® software, and computer. (**b**) The FLEXMAP 3D™ system includes analyzer, plate handling, and fluid delivery systems integrated within a single unit. Also included are xPONENT® software and computer with an articulating arm to house the monitor and keyboard. (**c**) The MAGPIX® system is a compact system based on CCD imaging technology. xPONENT® software provides stream-lined start-up and shutdown protocols and minimal maintenance requirements

automation and LIS interface components and includes walk-away maintenance and calibration functions. This combination of advanced features and capabilities can help to accelerate the discovery and development process and make the FLEXMAP 3D an ideal platform for multiplexing analytes that may have broadly dissimilar levels and eliminating the need for sample reanalysis due to out-of-range results.

The MAGPIX, which utilizes a flow cell and robust LED/CCD-based optics, was released in July 2010. The MAGPIX system supports multiplexing of up to 50 tests in a single reaction volume using MagPlex or MagPlex-TAG beads. In MAGPIX, the reacted magnetic beads are sent through a flow cell into an imaging flow cell where a magnetic actuator pulls the beads out of suspension and holds them in place for optical analysis. Red LEDs (630-nm) excite the fluorescent dyes contained within the microspheres and green LEDs (515–521-nm) excite the reporter fluorochrome bound to the bead surface. A CCD imager identifies the bead region and quantifies the bound reporter. xPONENT for MAGPIX operates the system and can be used with commercial kits or user-developed assays. With a lower cost and a compact size (requiring only 64.8 cm bench space), MAGPIX provides an affordable multiplexing solution ideal for the low- to medium-throughput laboratory and remote laboratory testing sites.

Nucleic Acid Chemistries and Assay Development

xMAP Technology provides a flexible, open platform where users can easily develop their own custom assays. Various assay chemistries and assay development techniques have been used for nucleic acid detection on the xMAP Technology platform. Detailed sample protocols and recommendations to guide the user through the assay development process are available as downloads in the Support section of the Luminex website [4]. Assay development training is available from Luminex and custom assay development services are also available from Luminex and its partners. A general workflow for nucleic acid assay development can be described as follows: (1) Acquire materials and reagents (e.g., oligos for amplification and capture/detection, enzymes, buffers, reporter fluorophore, nucleic acid samples, and standards/controls); (2) Purify the reagent to be coupled of extraneous primary amines (e.g., Tris), if needed; (3) Conjugate the microspheres with the capture oligo; (4) Evaluate coupling efficiency using an appropriate target and detection reagent (e.g., biotinylated reverse complementary oligo); (5) Assess assay performance (background, sensitivity, specificity) using positive and negative control samples; and (6) Optimize amplification conditions and/or hybridization time, temperature, and sample input.

Microsphere Coupling

Capture oligos are modified with a spacer and terminal amine (e.g., 5′-Amino-Modifier C12) and covalently coupled to carboxylated beads using a carbodiimide

coupling procedure [5, 6]. Capture oligos should be resuspended and diluted in dH_2O as Tris, azide, or other amine-containing buffers can interfere in the coupling process. If oligos were previously solubilized in an amine-containing buffer, they should be desalted by column or precipitation and resuspended into dH_2O. Stock uncoupled carboxylated beads are washed and resuspended in 100 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 4.5. The capture oligo is added, followed by addition of *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and incubation in the dark for 30 min. The EDC addition and incubation are repeated and then the coupled beads are washed once with 0.02 % Tween-20 and once with 0.1 % SDS. Coupled microspheres are stored in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0 (TE) at 2–8 °C in the dark. When properly stored, coupled microspheres are usually stable for more than 1 year [7]. Coupling reactions can be easily scaled up or down according to user needs. Initial coupling and optimization of coupling amount (i.e., nmol oligo per million microspheres) is usually done in small coupling reactions of 2.5–5 million microspheres. Coupling reactions can then be scaled up for manufacturing and preparation of bulk reagents. For optimal coupling efficiency, it is important to perform oligo coupling reactions in as small a reaction volume as possible, typically 25–100 μL for 2.5–100 million beads [8].

Verification of coupling can be performed using a biotinylated reverse complementary positive control oligo [9]. Coupled microspheres are resuspended by vortex and sonication and combined to a final concentration of 100–150 beads of each set/μL in hybridization buffer. Target biotinylated oligos are added (5–200 fmol) and hybridized to the coupled beads for 15 min, followed by labeling with reporter (streptavidin-R-phycoerythrin) for 5 min. The reactions are then ready for analysis. Effective coupling is demonstrated by a dose response increase in median fluorescent intensity (MFI) with increasing concentration of oligo target [10].

Direct Hybridization

Several assay chemistries have been used for nucleic acid detection on the xMAP system. One approach is to use direct hybridization of a labeled PCR-amplified target DNA to bead sets bearing oligonucleotide capture probes specific for each sequence (Fig. 42.3). Direct hybridization is the simplest assay chemistry that can provide single nucleotide discrimination and takes advantage of the fact that the melting temperature for hybridization of a perfectly matched template compared to one with a single base mismatch can differ by several degrees for capture oligos approximately 15–20 nucleotides in length [11, 12]. The reaction kinetics can be adversely affected by immobilization of the reactant on a solid surface but these effects are less severe for bead-based suspension arrays; however, the diffusion rate of the immobilized capture probe can be slower and the effective concentration is reduced as compared to free DNA in solution [13]. Design of sequence-specific capture probes and PCR primers for a direct hybridization assay on the xMAP suspension array can be facilitated through the use of a tetramethylammonium chloride

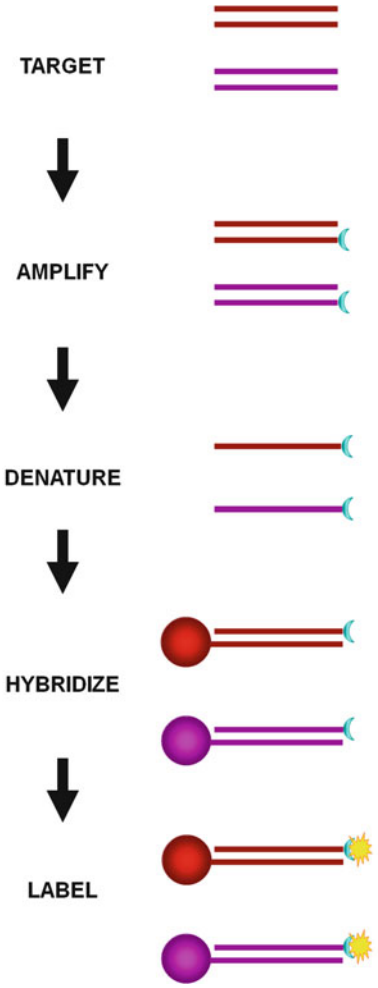

Fig. 42.3 Direct hybridization. Target DNA is PCR-amplified with one biotinylated and one unlabeled primer. The PCR products are denatured, hybridized to probe-coupled bead sets, and labeled for detection with streptavidin-R-phycoerythrin

(TMAC)-containing hybridization buffer. TMAC stabilizes AT base pairs, minimizing the effect of base composition on hybridization [14, 15]. For oligos less than 200 base pairs in length, hybridization efficiency in TMAC is a function of the length of the perfect match and less dependent on base composition. Thus, hybridization buffers incorporating 3 or 4 M TMAC equalize the melting points of different probes, allowing probes with different characteristics to be used under identical conditions with high duplex yields [16, 17]. Typically for single nucleotide discrimination, capture probes are designed to be matched in length at approximately 20 nucleotides. The probes are complementary in sequence to the labeled strand of the PCR product and the polymorphic nucleotide is located at the center of the probe, as mismatches in the center have a more profound effect on the equilibrium state than mismatches near the 5′ or 3′end [18, 19].

Optimal assay conditions are determined by evaluating the effect of hybridization temperature, probe length, and input target concentration on assay sensitivity and specificity. The probe melting temperature is influenced by length, sequence, and type and position of the mismatched base. The effect of a mismatch on hybridization is greater with increasing temperature and decreasing probe length [12, 20]. Thus, discrimination can be improved by increasing the hybridization temperature and/or decreasing the probe length. After testing known DNA samples under a set of standard hybridization conditions, nucleotides are added to the 5′ and/or 3′ ends of the probe to improve sensitivity or removed from the 5′ and/or 3′ ends of the probe to increase specificity. The position of the polymorphism within the probe sequence can be adjusted when necessary to avoid formation of secondary structures and adequate specificity can usually be achieved when the polymorphic nucleotide is between positions 8 and 14 of a 20-nucleotide probe [19].

PCR primers are typically designed to amplify 100–300 base pair regions of target sequence with one primer of each pair biotinylated at the 5′ end for labeling the target strand of the amplicon. Using a small target DNA minimizes the potential for steric hindrance to affect hybridization efficiency. In some cases, larger targets (400–1,200 base pairs) have been used successfully, suggesting that hybridization efficiency is also dependent on the sequence and overall secondary structure of target [21]. Hybridization kinetics and thermodynamic affinities of matched and mismatched sequences can be driven in a concentration-dependent manner [22]. At concentrations beyond the saturation level, the hybridization efficiency can decrease presumably due to competition of the complementary strand and renaturation of the PCR product [20, 23]. Therefore, it is also important to determine the range of target concentrations that yield efficient hybridization without sacrificing discrimination.

Solution-Based Chemistries with Bead Capture

Another approach is to use a sequence-specific enzymatic reaction in solution to determine the target sequence followed by capture onto the bead surface for detection (Fig. 42.4). This format involves the incorporation of a specific capture sequence during the enzymatic step that allows hybridization to a complementary “address” sequence on the bead surface. Commonly used enzymatic methods for sequence determination rely on the discriminating ability of DNA polymerases and DNA ligases, and include allele-specific or target-specific primer extension (ASPE or TSPE), oligonucleotide ligation assay (OLA), single base chain extension (SBCE), and target-specific PCR (TS-PCR) [24–26]. This approach takes advantage of solution-phase kinetics and permits the addressed bead sets to be used in many different assays where new sequences can be targeted by adding the appropriate capture sequence to the target-specific oligo used in the enzymatic step. Hybridization buffer and reaction conditions for detection are dependent upon the capture sequences attached to the microspheres. Commercially available Luminex MagPlex-TAG Microspheres, precoupled with xTAG capture oligos (anti-TAGs) are compatible with

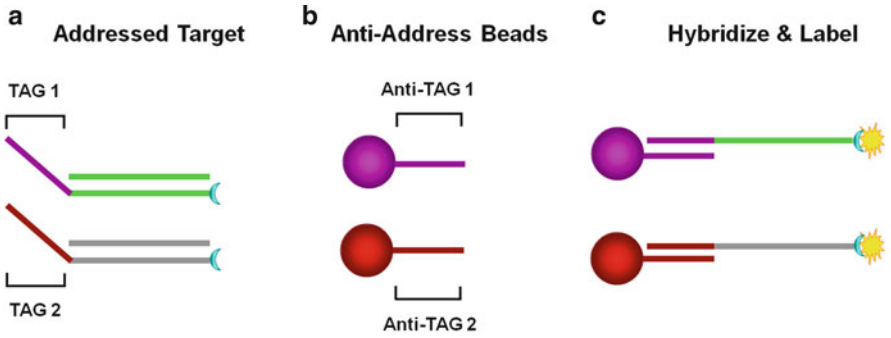

Fig. 42.4 Capture of "addressed" targets onto precoupled beads. (**a**) Solution-based enzymatic assay amplifies targets, incorporating a unique address sequence into each product. (**b**) Bead sets coupled with complementary capture sequences ("anti-address"). (**c**) Products are captured onto beads through hybridization of the anti-address capture and address sequences, and labeled with streptavidin-R-phycoerythrin

solution-based enzymatic chemistries. The assay developer appends an appropriate complementary TAG sequence to each target-specific oligo to allow capture onto the corresponding anti-TAG coupled to the bead.

Assay development parameters for enzymatic solution-based nucleic acid assays on the xMAP platform have been described and various procedures are illustrated in Fig. 42.5 [27–29]. Briefly, target-specific oligos are designed to be matched in melting temperature at 51–56 °C. For TSPE, TS-PCR, and OLA, the discriminating nucleotide is positioned at the 3′ end of the oligo, whereas for SBCE the 3′ end of the primer is positioned one nucleotide upstream. The unique capture sequence for each target is incorporated at the 5′ end of the oligo. Optimization, when needed to improve sensitivity or specificity, can be achieved by adding or removing nucleotides from the 5′ end of the target-specific sequence (immediately downstream from the capture sequence) or by targeting the opposite DNA strand.

Templates containing the target sequences for the TSPE, OLA, and SBCE reactions are generated by PCR using unlabeled primers. In TSPE, a thermostable polymerase is used to extend the primer by incorporation of dNTPs, including one which is biotin-labeled. Extension only occurs if the 3′ nucleotide of the primer is complementary and can anneal to the template DNA. OLA employs the same oligo design but a thermostable ligase is used to ligate a biotin-labeled oligo (reporter probe)

Fig. 42.5 (continued) Sample DNA is amplified and annealed to addressed target-specific primers in a reaction containing a DNA polymerase and a biotinylated ddNTP (in separate reactions for each nucleotide). Targets are extended and addressed extension products are captured onto complementary beads sets, and labeled with streptavidin-R-phycoerythrin. (**d**) TS-PCR. Sample DNA is amplified using addressed target-specific upstream primers paired with downstream biotinylated primers. Addressed PCR products are simultaneously hybridized to complementary bead sets and labeled with streptavidin-R-phycoerythrin. This example depicts a spacer between address and target-specific primer sequences to prevent amplification of the capture sequence

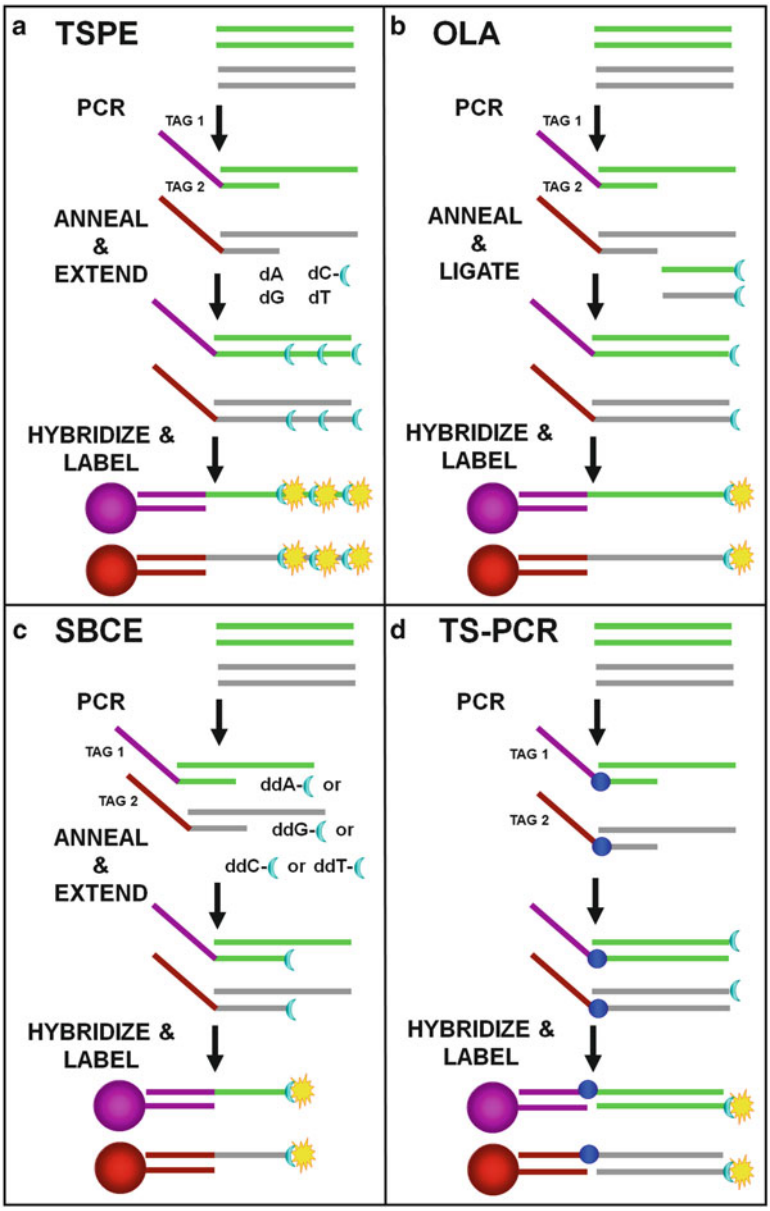

Fig. 42.5 Chemistries used for addressed microsphere capture assays. (**a**) TSPE. Sample DNA is amplified and annealed to addressed target-specific primers in a reaction containing a DNA polymerase and dNTPs (one with biotin label). Targets are extended and addressed extension products are captured onto complementary beads sets, and labeled with streptavidin-R-phycoerythrin. (**b**) OLA. Sample DNA is amplified and annealed to addressed target-specific primers in a reaction containing a DNA ligase and biotinylated reporter probe. Addressed ligation products are captured onto complementary beads sets, and labeled with streptavidin-R-phycoerythrin. (**c**) SBCE.

that is complementary to the sequence downstream from the target nucleotide. The reporter probe is designed to be matched in melting temperature at 51–56 °C, is phosphorylated at the 5′ end to provide a substrate for ligase, and is biotin-labeled at the 3′ end for fluorescent detection with streptavidin-R-phycoerythrin. For SBCE, individual reactions must be set up for each of the four possible nucleotides and a thermostable polymerase is used to incorporate a single biotin-labeled ddNTP. Extension occurs only if the nucleotide complementary to the sequence immediately downstream of the primer is present in the reaction. Targets for SBCE can be combined for each of the nucleotide reactions and if different capture sequences are used for each, the products can be multiplexed for capture onto the addressed microsphere sets. For TS-PCR, target sequences are directly amplified and labeled by PCR using one primer containing both capture and target-specific sequences paired with a biotinylated target-specific primer. This method requires a strategy which minimizes or eliminates production of the capture sequence in the nontarget strand, which would compete for hybridization of the target to the addressed bead. Some methods include asymmetric PCR, post-PCR treatment with phosphatase and exonuclease, or incorporating a spacer between the capture-specific and target-specific sequences of the primer [29]. TS-PCR chemistry eliminates the need for separate amplification and target-specific enzymatic steps, thus simplifying the assay procedure and reducing reagent costs and turnaround time.

Bead-Based Applications for Respiratory Virus Detection and Identification

Molecular analysis of infectious organisms is widely applied in healthcare and numerous applications using xMAP technology have been described for the detection of bacterial, viral, fungal and parasitic pathogens. Several commercial products are available for multiplexed detection and identification of respiratory viruses using xMAP bead-based suspension arrays, including the ResPlex™ II Panel from Qiagen, Inc. (Valencia, CA), and the xTAG RVP and xTAG RVP FAST assays from Luminex Molecular Diagnostics, Inc. (Toronto, ON). Since their development, these assays are being increasingly used for clinical diagnostics and epidemiological research, and numerous studies utilizing bead-based suspension array assays for respiratory virus detection have been reported.

The ResPlex II Panel is a for research use only (RUO) product that employs a novel multiplex reverse transcription-polymerase chain reaction (RT-PCR) strategy, called Tem-PCR (Target Enriched Multiplexing-PCR), combined with direct hybridization for capture onto target-specific probe-coupled bead sets. Tem-PCR uses nested gene-specific primers at low concentrations to enrich the specific targets during initial PCR cycles, followed by universal forward and reverse primers at high but unequal concentrations to achieve exponential asymmetric amplification [30]. The primer in excess is biotinylated to yield a surplus of labeled target strands that can be hybridized and detected on the complementary bead sets. The ResPlex II Panel

detects 18 respiratory viral targets: respiratory syncytial viruses (RSV) A and B, influenza A virus (Flu-A), influenza B virus (Flu-B), parainfluenza viruses (PIV) 1–4, human metapneumoviruses (hMPV) A and B, coxsackieviruses/echovirus (CVEV), rhinovirus (RhV), adenoviruses (AdV) A and E, coronaviruses (CoV) NL63, HKU1, 229E and OC43, and bocavirus (BocV) [31]. The panel also includes an internal control to monitor viral RNA isolation and PCR inhibition, and a positive control for human genomic DNA to monitor sample integrity and success of the run.

Li et al. evaluated the ResPlex II panel in a retrospective study using 360 frozen respiratory specimens and the results were compared to those obtained by a reference standard of culture and individual real-time TaqMan® RT-PCR assays [32]. Sensitivity and specificity for Flu-A, Flu-B, PIV-1, PIV-3, and RSV were 72.2–90.0 % and 99.7–100 %, respectively, and the assay could process 96 samples in 6 h. Brunstein et al. used the ResPlex II panel in combination with the ResPlex I assay, which tests for bacterial pathogens and AdV A and E, in parallel with a DFA and culture testing protocol to investigate the incidence of coinfections and the potential for complex interactions between multiple pathogens in respiratory infections [33, 34]. Coinfections were found in 27 % of the specimens tested. Using multiple statistical methods, they determined patterns of association between pathogens found in individual specimens. Potential positive correlations were found for specimens coinfected with the following: *Streptococcus pneumoniae* and *Haemophilus influenzae*, *Neisseria meningitidis* and Flu-B, and CVEV and RhV. In addition, while overall prevalence for *S. pneumoniae* was 20 %, prevalence was 26 % in RSV B positive specimens. Although the results were preliminary, the authors concluded that the data support the hypothesis that coinfection with multiple respiratory pathogens is medically relevant and that effective treatment for severe respiratory infections may require diagnosis of all pathogens involved, rather than single-pathogen reporting protocols where diagnostic testing is discontinued after identification of the first relevant pathogen.

The xTAG respiratory viral panel (RVP) assay utilizes a TSPE chemistry with xTAG Technology to identify all major respiratory viruses commonly encountered, including Flu-A subtypes H1 and H3, in a single test. The assay is approved for in vitro diagnostic use (IVD) in the United States, Canada, and Europe for 12–19 virus types and subtypes, depending on geographical location. The regulatory clearance status for each target in each region is shown in Table 42.1. The assay also includes an internal (extraction control), a positive (run) control, and xTAG Data Analysis Software (TDAS) to interpret the MFI results and establish the presence or absence of each target. RVP is compatible with the Luminex 100/200 instrument and can provide results for 96 samples in a single work day (approximately 8 h). The RVP assay was first described by Mahony et al. in a prospective evaluation using 294 nasopharyngeal swab (NP) specimens and compared to direct fluorescent antibody assay (DFA) and culture. RVP detected 112 positives as compared to 119 positives detected by DFA/culture for a sensitivity of 97 %, and further, detected an additional 61 positive specimens that were either negative by DFA/culture or positive for viruses not tested by DFA/culture [35]. Following resolution of discordants by PCR, RVP detected 180 of 183 true positives (98.5 % sensitivity) as compared to 126 of

Table 42.1 xTAG RVP targets and regulatory clearances

	RVP		
Viral family and subtype	US-IVD	Health Canada IVD	CE-IVD Europe
Respiratory syncytial virus (RSV)			
RSV A	•	•	•
RSV B	•	•	•
Influenza A (Flu-A)			
Nonspecific Flu-A	•	•	•
H1 subtype	•	•	•
H3 subtype	•	•	•
H5 subtype			•
Influenza B (Flu-B)	•	•	•
Parainfluenza 1 (PIV-1)	•	•	•
Parainfluenza 2 (PIV-2)	•	•	•
Parainfluenza 3 (PIV-3)	•	•	•
Parainfluenza 4 (PIV-4)		•	•
Metapneumovirus (hMPV)	•	•	•
Adenovirus	•	•	•
Enterovirus-Rhinovirus (EV-RhV)	•	•	•
Coronavirus (CoV) NL63		•	•
CoV HKU1		•	•
CoV 229E		•	•
CoV OC43		•	•
CoV SARS			•
MS2 bacteriophage internal control			
Lambda phage positive control			

183 true positives by DFA/culture (68.8 % sensitivity). RVP detected the presence of two viruses in 5.2 % of the specimens tested. The performance of RVP was also compared to real-time PCR in a parallel study using the same sample extracts [36]. The results were comparable for samples with Ct values less than or equal to 35 and RVP demonstrated signal to noise ratios of greater than or equal to 50.

For IVD clearance, the performance characteristics of the RVP assay were established in a multicenter clinical trial involving six sites in North America and Europe using a total of 1,464 specimens collected and tested during the 2005/2006 influenza season [37–39]. For 544 prospectively collected NP swabs, sensitivity was greater than 91 % (91.5–100 %) for all analytes except PIV-3 (84.2 %), PIV-4 (50.0 %), and AdV (78.3 %). However, only two positive specimens were available for PIV-4, and sequence analysis revealed that low overall sensitivity for AdV was mainly due to poor detection of serotypes in the AdV C species. In addition, few positive samples were available for CoV, thus sensitivity for CoV types ranged from 0.0 to 100 % or could not determined. Specificity was greater than 91 % (91.3–100 %) for all analytes, the limit of detection (LoD) was from 6×10^{-2} to 5×10^{4} median tissue culture infective dose ($TCID_{50}$)/mL, and overall site-to-site reproducibility was 98.2 %. No interference was detected in 16 combinations of analyte plus potential bacterial or

viral interferent, and no cross-reactivity was observed for 26 other bacterial and viral pathogens tested. One BocV sample yielded a false positive call for RhV but was subsequently determined to be contaminated with RhV. In addition, there were no significant differences in the sensitivity of the target analytes in medicated versus unmedicated patients.

In a comparison study, ResPlex II and RVP suspension array assays detected more viruses than culture for 202 adult respiratory specimens, including NP and other swabs, bronchoalveolar lavages (BAL), lung biopsies, pleural fluids and sputa [40]. RVP showed 100 % sensitivity and negative predictive value (NPV), 93 % specificity, and near perfect agreement with culture. ResPlex II had slightly higher specificity (94 %), 80 % positive predictive value (PPV), and 89 % sensitivity. Coinfections were detected in 1–4.5 % of samples, most of which included a PIV, furthermore PIV-1 and PIV-2 were only detected in multiple infections. The ResPlex II was easiest to use with one reagent preparation/amplification step and one hybridization/detection step, generating results in 5.5 h, whereas RVP was more labor intensive with five reagent preparation steps and two thermal cycler programs, resulting in a 7–8 h turnaround time. The ResPlex II (RUO) offered a broader coverage of virus types than the RVP (IVD) assay, but RVP had the ability to differentiate human from nonhuman Flu-A subtypes. The authors concluded that as compared to culture, multiplexed molecular assays could provide enhanced sensitivity, faster turnaround time, and broader coverage, and should prove to be a useful tool for respiratory virus identification as an aid in patient management and outbreak investigations.

Multiplexed molecular assays can be useful as a screening tool for the detection of novel or emerging viral strains and for outbreak investigations. Wong et al. used the RVP assay in a retrospective study of 1,108 specimens from 244 suspected respiratory virus outbreaks to assess utility for enhanced respiratory outbreak investigation [41]. When used in combination with DFA and various in-house nucleic acid amplification tests (NATs), RVP testing improved detection of a viral etiological agent from 72.5 to 90.8 % of outbreaks. The RVP assay also proved to be an effective aid for the detection of 2009 Flu-A/H1N1 strains [42–44]. While the assay can detect the matrix gene of 2009 Flu-A/H1N1, it cannot identify the hemagglutinin (HA) gene of 2009 Flu-A/H1N1 in clinical specimens. In New York during the 2009 Flu-A/H1N1 outbreak, the RVP assay was used to test 375 samples that initially tested positive for Flu-A by rapid antigen tests, DFA, and culture, or were from patients with suspected infection. RVP identified 201 samples as Flu-A, with 60 identified as seasonal (H1 and H3) strains but the remaining 141 were unsubtypeable (negative for both H1 and H3). The CDC real-time RT-PCR assay specific for 2009 Flu-A/H1N1 was used to further test 101 of these specimens and 99 were identified as positive for 2009 Flu-A/H1N1. These data indicated that the predictability of unsubtypeable Flu-A identified by the RVP assay to be pandemic Flu-A/H1N1 was high. Implementation of RVP screening in the testing algorithm enabled the laboratory to rapidly subtype Flu-A, rule out seasonal H1 and H3 subtypes, detect additional respiratory viruses, and identify probable pandemic Flu-A/H1N1 cases.

The xTAG RVP FAST assay is a newer version of the xTAG RVP assay, modified to have a simpler protocol with a faster turnaround time. The assay has been

Table 42.2 xTAG RVP FAST targets and regulatory clearances

	RVP		
Viral family and subtype	US-IVD	Health Canada IVD	CE-IVD Europe
Respiratory syncytial virus (RSV)		•	•
Influenza A (Flu-A)			
Nonspecific Flu-A	•	•	•
H1 subtype	•	•	•
H3 subtype	•	•	•
Influenza B (Flu-B)	•	•	•
Parainfluenza 1 (PIV-1)		•	•
Parainfluenza 2 (PIV-2)		•	•
Parainfluenza 3 (PIV-3)		•	•
Parainfluenza 4 (PIV-4)		•	•
Metapneumovirus (hMPV)	•	•	•
Adenovirus	•	•	•
Enterovirus-Rhinovirus (EV-RhV)	•	•	•
Coronavirus (CoV) NL63		•	•
CoV HKU1		•	•
CoV 229E		•	•
CoV OC43		•	•
Bocavirus (BocV)		•	•
MS2 bacteriophage internal control			
Lambda phage positive control			

approved for IVD use in the United States, Canada, and Europe for 8–17 virus types and subtypes (Table 42.2). The targets detected have been modified slightly from the RVP assay and, depending on geographical location, can additionally detect BocV. RVP FAST uses TS-PCR chemistry where a multiplexed RT-PCR reaction is performed using target-specific upstream primers containing a TAG capture sequence paired with biotinylated downstream primers to amplify the target sequences in a single reaction. The amplified product is added to a combined hybridization/detection reaction containing the corresponding anti-TAG bead sets and streptavidin-R-phycoerythrin reporter, and then the completed reactions are read on the Luminex analyzer to determine the MFI signal. The raw fluorescence values are then analyzed using TDAS for RVP FAST software and each target interpreted as present or absent. The RVP FAST assay combines amplification with target-specific extension, and uses a combined hybridization and detection step, resulting in a turnaround time of less than 5 h (including nucleic acid extraction). Further, the risk of contamination from post-PCR manipulation is minimized by the reduction of operator handling steps to a single post-PCR transfer.

The performance characteristics of RVP FAST for IVD use were determined from two datasets resulting from multicenter clinical studies of 1,518 total specimens (EU/Canada study) and 1,191 NP swab specimens (US study) submitted for viral testing at three independent laboratories [45, 46]. For the combined dataset ($n = 1518$), sensitivity was greater than 91 % (91.2–100 %) for all analytes with the exception of PIV where sensitivity was 76 % for PIV-2, 76.29 % for PIV-3, and 80 % for PIV-1, respectively. Specificity was 89.2–99.33 % and LoD ranged from

0.1 to 1,000 $TCID_{50}$/mL, depending on analyte. An additional 285 specimens were collected and tested for a subset of targets and showed sensitivity of 96.49–100 % and specificity of 89.47–98.57 % when compared to real-time RT-PCR [45]. For the NP swab dataset ($n=1,191$), sensitivity and specificity was 90.9–97.2 and 92.5–99.3 %, respectively [46]. A coinfection was identified in 3 % of these specimens and was most commonly due to RSV/RhV at 1.1 % prevalence and Flu-A/RhV at 0.4 % prevalence. The LoDs for the viral analyte targets were determined by serial dilution of high titer stocks in Universal Transport Medium (UTM) and ranged from 1.4×10^{-2} to 3.9×10 [2] $TCID_{50}$/mL. No cross-reactivity was observed for 30 (20 bacterial and 10 viral) pathogens tested and no interference was detected in 14 combinations of analyte plus potential bacterial or viral interferent. In addition, there were no significant differences in the sensitivity of the target analytes in medicated versus unmedicated patients.

RVP FAST was compared to culture, DFA, and a combination of single and multiplex real-time RT-PCR assays in a retrospective study of 286 respiratory specimens [47]. At least one respiratory virus was detected in 13.6 % of specimens by culture and DFA combined, in 49.7 % by real-time RT-PCR, and in 46.2 % by RVP FAST. Using real-time RT-PCR results as the gold standard, RVP FAST had 78.8 % sensitivity and 99.6 % specificity. Specimens not detected by RVP FAST generally had low viral loads or contained AdV. For some viral targets, few positive samples were available in the set tested, which suggests that further study using larger numbers of positive samples is needed to fully assess sensitivity of RVP FAST for all targets. Overall, the investigators found RVP FAST to be rapid and easy to perform in comparison to the multiple real-time RT-PCR assays that would be required for equal target coverage. Pabbaraju et al. compared RVP FAST with the original RVP assay on 334 respiratory specimens that had been previously characterized for a variety of respiratory virus targets [48]. Samples were tested in parallel by both assays and the RVP assay was found to be more sensitive than RVP FAST for the combined targets, with sensitivities of 88.6 and 77.5 %, respectively. However, RVP FAST was tenfold more sensitive for the detection of RSV A. Targets not detected by RVP FAST were primarily Flu-B, PIV-2, and CoV 229E. In addition, a small number of samples positive for Flu-A, RSV B, hMNV, and PIV-1 were not detected by the RVP assay and generally had low viral loads. Reproducibility was similar between the two assays but RVP FAST exhibited better reproducibility for detection of AdV at the LoD. The authors concluded that RVP FAST met the diagnostic needs for sensitivity and specificity in their laboratory with the exception of Flu-B, which had decreased sensitivity for Flu-B in specimens collected in 2010. Reduced sensitivity for PIV-2 and CoV 229E was of lesser concern because PIV-2 has low prevalence and detection of CoV 229E does not affect established patient management protocols. Their preliminary studies also indicated that use of RVP FAST will lead to cost savings and improved turnaround time as a result of the shorter protocol and a reduction in hands-on time to 1.5 h.

The openness of the xMAP platform lends itself to the rapid development of new assays as analytes of interest are identified and described. As an example, the emergence of antiviral resistance in influenza has raised concerns about use of antiviral drugs and treatment in response to future pandemic outbreaks. Amantadine resistance

has increased dramatically for Flu-A/H3N2 and Flu-A/H1N1, and increasing oseltamivir resistance for seasonal Flu-A/H1N1 has been reported worldwide since 2007 [49–51]. Currently, resistance testing is done by sequencing or phenotypic assays which can be costly and time consuming; however, a multiplexed bead-based array could be applied for this purpose in combination with or in addition to a comprehensive respiratory viral panel. This is illustrated in a report of a multiplexed bead-based LDT that was developed for simultaneous identification of Flu-A subtypes and the oseltamivir resistance genotype [52]. The assay employed the use of degenerate primers for amplification of the HA and neuraminidase (NA) genes and eight target-specific primers with TAG modifications for TSPE and subsequent detection on MicroPlex-xTAG beads. The assay was evaluated using 54 NP specimens that were Flu-A positive by DFA or real-time RT-PCR for the matrix gene, and was capable of correctly identifying the Flu-A subtype and the oseltamivir sensitive (H275) and resistance (H275Y) alleles in all samples. Of the 17 seasonal Flu-A/H1N1 isolates, H275Y was identified in 15, and of the 24 pandemic 2009 Flu-A/H1N1 isolates, 12 were H275 (sensitive), 3 were H275Y (resistant), and 9 revealed a combination of both alleles. The assay was also inexpensive to run with a combined reagent cost of $6.95 Canadian dollars (CAD) per test.

Cost analysis studies indicate that use of a multiplexed bead-based array for diagnosis of respiratory viral infections can be less expensive than routine strategies using DFA and/or culture. A 2009 cost analysis revealed that the average diagnostic cost per pediatric inpatient was lowest for a strategy using xTAG RVP testing alone as compared to DFA alone, DFA plus culture, and DFA plus RVP assay [53]. When all four diagnostic strategies were compared, RVP alone was the least costly strategy when the prevalence of infection was 11 % or higher but DFA alone was less costly when the prevalence was less than 11 %. They estimated a savings of $291 CAD per case if the xTAG RVP test alone was used to replace DFA plus culture, and a savings of $529,620 CAD per year in direct costs for the four hospitals included in the study. An operational workflow analysis using lean methodology principles was employed in another study to evaluate potential advantages of a multiplexed bead-based array for laboratory workflow and associated cost [54]. Implementation of the RVP assay resulted in a standardized workflow that decreased laboratory costs and improved efficiency as compared to DFA and culture for diagnosis of respiratory viruses. Workflow was evaluated in terms of total hands-on time and number of operator steps, and all hospital and laboratory costs associated with testing were calculated for each test for both positive and negative result scenarios. Combining the scores for all samples included in the analysis revealed that the hands-on time to completion for 1,015 samples was 80 h for RVP as compared to 503 h for DFA and culture. Total cost per sample was $99.75 United States dollar (USD) for DFA-positive samples (no culture or additional testing performed) and ranged from $329.68 to $429.07 USD for DFA-negative samples which are reflexed to culture with variable time to completion. The total cost for RVP was $135.03 USD, regardless of result. Further, although RVP required more time, steps, and was slightly more expensive per test than DFA screening alone, only 23 % of the sample population studied were DFA-positive. The additional time and costs associated

with follow-up culture for the large percentage of DFA-negative specimens were far greater. Thus, the bead-based array assays not only provide increased diagnostic capability for respiratory viral infections but can also maximize efficiency and reduce the costs associated with diagnosis.

Conclusions

Recent advances in molecular diagnostics have provided the clinical laboratory with new methods that allow rapid diagnosis and treatment of infectious diseases. Among these, multiplexed bead-based suspension arrays have emerged as a rapid, high-throughput, and cost-effective means to provide physicians with reliable and actionable results for timely and improved patient care. In particular, bead-based suspension array assays have been demonstrated as a method of choice for the comprehensive detection and identification of respiratory viruses and have improved diagnosis, allowed identification of coinfections, provided valuable epidemiological data, and aided in the response to outbreaks of respiratory infections. With the availability of assays cleared for IVD use, this technology should become easy to implement, even in diagnostic laboratories with limited experience in molecular techniques. It can be anticipated that as new assays are developed and existing assays are continually enhanced and improved with simpler protocols, streamlined workflows, and automation, these assays will continue to prove valuable for diagnosis of respiratory viruses and other infectious diseases.

References

1. Templeton KE (2007) Why diagnose respiratory viral infection? J Clin Virol 40(Suppl 1):S2–S4
2. Nolan JP, Sklar LA (2002) Suspension array technology: evolution of the flat-array paradigm. Trends Biotechnol 20(1):9–12
3. Kellar KL (2003) Applications of multiplexed fluorescent microsphere-based assays to studies of infectious disease. J Clin Ligand Assay 26(2):76–86
4. Luminex Corporation. Support resources. http://www.luminexcorp.com/Support/SupportResources/. Accessed 26 June 2011
5. Luminex Corporation. Sample protocol for coupling oligonucleotides to MicroPlex Microspheres. http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/oligonucleotide-coupling-proto.pdf. Accessed 26 June 2011
6. Luminex Corporation. Sample protocol for coupling oligonucleotides to MagPlex Microspheres. http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/magnetic-oligo-coupling-protoc.pdf. Accessed 26 June 2011
7. Luminex Corporation. Oligo-coupled microsphere stability. (Accessed June 26, 2011, at http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/oligo-coupled-microsphere.pdf.)
8. Luminex Corporation. Recommendations for scaling up or scaling down oligonucleotide coupling reactions. http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/oligo-coupling-scaling-recom.pdf. Accessed 26 June 2011
9. Luminex Corporation. Sample protocol for oligonucleotide hybridization. http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/oligonucleotide-hybridization.pdf. Accessed 26 June 2011

10. Luminex Corporation. Oligonucleotide hybridization results. http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/oligo-hybridization-results.pdf. Accessed 26 June 2011
11. Ikuta S, Takagi K, Wallace RB, Itakura K (1987) Dissociation kinetics of 19 base paired oligonucleotide-DNA duplexes containing different single mismatched base pairs. Nucleic Acids Res 15(2):797–811
12. Livshits MA, Mirzabekov AD (1996) Theoretical analysis of the kinetics of DNA hybridization with gel-immobilized oligonucleotides. Biophys J 71(5):2795–2801
13. Peterson AW, Wolf LK, Georgiadis RM (2002) Hybridization of mismatched or partially matched DNA at surfaces. J Am Chem Soc 124(49):14601–14607
14. Wood WI, Gitschier J, Lasky LA, Lawn RM (1985) Base-composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc Natl Acad Sci U S A 82(6):1585–1588
15. Jacobs KA, Rudersdorf R, Neill SD et al (1988) The thermal stability of oligonucleotide duplexes is sequence independent in tetraalkylammonium salt solutions: application to identifying recombinant DNA clones. Nucleic Acids Res 16(10):4637–4650
16. Maskos U, Southern EM (1992) Parallel analysis of oligodeoxyribonucleotide (oligonucleotide) interactions. I. Analysis of factors influencing duplex formation. Nucleic Acids Res 20(7):1675–1678
17. Maskos U, Southern EM (1993) A study of oligonucleotide reassociation using large arrays of oligonucleotides synthesized on a large support. Nucleic Acids Res 21(20):4663–4669
18. Gotoh M, Hasegawa Y, Shinohara Y, Schimizu M, Tosu M (1995) A new approach to determine the effect of mismatches on kinetic parameters in DNA hybridization using an optical biosensor. DNA Res 2(6):285–293
19. Luminex Corporation. Effect of mismatch position and type on SNP genotyping. http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/snp-genotyping-effect-of-misma.pdf. Accessed 26 June 2011
20. Armstrong B, Stewart M, Mazumder A (2000) Suspension arrays for high throughput, multiplexed single nucleotide polymorphism genotyping. Cytometry 40(2):102–108
21. Diaz MR, Fell JW (2004) High-throughput detection of pathogenic yeasts of the genus *Trichosporon*. J Clin Microbiol 42(8):3696–3706
22. Wetmur JG (1991) DNA probes: applications of the principles of nucleic acid hybridization. Crit Rev Biochem Mol Biol 26(3–4):227–259
23. Nolan JP, Mandy FF (2001) Suspension array technology: new tools for gene and protein analysis. Cell Mol Biol 47(7):1241–1256
24. Syvanen AC, Aalto-Setala K, Harju L, Kontula K, Soderlund H (1990) A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. Genomics 8(4):684–692
25. Ugozzoli L, Wahlqvist JM, Ehsani A, Kaplan BE, Wallace RB (1992) Detection of specific alleles by using allele-specific primer extension followed by capture on solid support. Genet Anal Tech Appl 9(4):107–112
26. Landegren U, Kaiser R, Sanders J, Hood L (1988) A ligase-mediated gene detection technique. Science 241(4869):1077–1080
27. Taylor JD, Briley D, Nguyen Q et al (2001) Flow cytometric platform for high-throughput single nucleotide polymorphism analysis. Biotechniques 30(3):661–669
28. Ye F, Li M-S, Taylor JD et al (2001) Fluorescent microsphere-based readout technology for multiplexed human single nucleotide polymorphism analysis and bacterial identification. Hum Mutat 17(4):305–316
29. Luminex Corporation. Custom nucleic acid detection. http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/custom-nucleic-acid-detection.pdf. Accessed 26 June 2011
30. Han J, Swan DC, Smith SJ et al (2006) Simultaneous amplification and identification of 25 human papillomavirus types with Templex technology. J Clin Microbiol 44(11):4157–4162
31. Qiagen, Inc. (2008) ResPlex™ II Panel v2.0 handbook
32. Li H, McCormac MA, Wray Estes R et al (2007) Simultaneous detection and high-throughput identification of a panel of RNA viruses causing respiratory tract infections. J Clin Microbiol 45(7):2105–2109

33. Brunstein JD, Cline CL, McKinney S, Thomas E (2008) Evidence from multiplex molecular assays for complex multipathogen interactions in acute respiratory infections. J Clin Microbiol 46(1):97–102
34. Qiagen, Inc. (2010) ResPlex™ I handbook
35. Mahony J, Chong S, Merante F et al (2007) Development of a respiratory virus panel test for detection of twenty human respiratory viruses by use of multiplex PCR and a fluid microbead-based assay. J Clin Microbiol 45(9):2965–2970
36. Merante F, Yaghoubian S, Janeczko R (2007) Principles of the xTAG™ respiratory viral panel assay (RVP assay). J Clin Virol 40(Suppl 1):S31–S35
37. Krunic N, Yager TD, Himsworth D et al (2007) xTAG™ RVP assay: analytical and clinical performance. J Clin Virol 40(Suppl 1):S39–S46
38. Luminex Molecular Diagnostics, Inc. xTAG™ RVP (Respiratory Viral Panel) CE Package Insert. 2009
39. Luminex Molecular Diagnostics, Inc. (2011) xTAG® RVP (Respiratory Viral Panel) IVD Package Insert
40. Balada-Llasat JM, LaRue H, Kelly C, Rigali L, Pancholi P (2011) Evaluation of commercial ResPlex II v2.0, MultiCode®-PLx, and xTAG® respiratory viral panels for the diagnosis of respiratory viral infections in adults. J Clin Virol 50(1):42–45
41. Wong S, Pabbaraju K, Lee BE, Fox JD (2009) Enhanced viral etiological diagnosis of respiratory system infection outbreaks by use of a multitarget nucleic acid amplification assay. J Clin Microbiol 47(12):3839–3845
42. Ginocchio CC, St. George K (2009) Likelihood that an unsubtypeable Influenza A virus result obtained with the Luminex xTAG respiratory virus panel is indicative of infection with novel A/H1N1 (swine-like) influenza virus. J Clin Microbiol 47(7):2347–2348
43. Ginocchio CC, Zhang F, Manji R et al (2009) Evaluation of multiple test methods for the detection of novel 2009 influenza A (H1N1) during the New York City outbreak. J Clin Virol 45(3):191–195
44. Crawford JM, Stallone R, Zhang F et al (2010) Laboratory surge response to pandemic (H1N1) 2009 outreak, New York City metropolitan area, USA. Emerg Infect Dis 16(1):8–13
45. Luminex Molecular Diagnostics, Inc. (2009) xTAG® Respiratory Viral Panel Fast CE Package Insert
46. Luminex Molecular Diagnostics, Inc. (2011) xTAG® Respiratory Viral Panel Fast IVD Package Insert
47. Gadsby NJ, Hardie A, Claas ECJ, Templeton KE (2010) Comparison of the Luminex respiratory virus panel fast assay with in-house real-time PCR for respiratory viral infection diagnosis. J Clin Microbiol 48(6):2213–2216
48. Pabbaraju K, Wong S, Tokaryk KL, Fonseca K, Drews SJ (2011) Comparison of the Luminex xTAG respiratory viral panel with xTAG respiratory viral panel fast for diagnosis of respiratory virus infections. J Clin Microbiol 49(5):1738–1744
49. Besselaar TG, Naidoo D, Buys A et al (2008) Widespread oseltamivir resistance in influenza A viruses [H1N1], South Africa. Emerg Infect Dis 14(11):1809–1810
50. Meijer A, Lackenby A, Hungnes O et al (2009) Oseltamivir-resistant influenza virus A [H1N1], Europe, 2007–08 season. Emerg Infect Dis 15(4):552–560
51. Centers for Disease Control and Prevention (2011) Antiviral agents for the treatment and chemoprophylaxis of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb Mortal Wkly Rep 60(No. RR-1):1–24
52. Mahony JB, Chong S, Luinstra K, Petrich A, Smieja M (2010) Development of a novel bead-based multiplex PCR assay for combined subtyping and oseltamivir resistance genotyping (H275Y) of seasonal and pandemic H1N1 influenza A viruses. J Clin Virol 49(4):277–282
53. Mahony JB, Blackhouse G, Babwah J et al (2009) Cost analysis of multiplex PCR testing for diagnosing respiratory virus infections. J Clin Microbiol 47(9):2812–2817
54. Dundas NE, Ziadie MS, Revell PA et al (2011) A lean laboratory: operational simplicity and cost effectiveness of the Luminex xTAG™ respiratory viral panel. J Mol Diagn 13(2):175–179

Chapter 43
Molecular Diagnosis and Monitoring of Human Papillomavirus Infections

Bruce K. Patterson

Introduction

Though the incidence of cervical cancer is low in the USA, cervical cancer is still the second leading cause of cancer and cancer-related deaths worldwide [1]. The Papanicolaou (PAP) smear has been the hallmark of cervical cancer screening since 1949. Following the discovery that HPV was the causative agent in >99 % of cervical cancers [2], the detection of high-risk HPV types (HRHPV) that cause cervical cancer in cervical samples became an important adjunct in the cervical cancer screening algorithm [3, 4]. Currently, HPV DNA testing is recommended in cervical cancer screening as a co-test to the Pap test and as a reflex test for cytology samples determined to be atypical squamous cells of undetermined significance (ASCUS) [5]. Both of these recommendations are only in women over 30 years old (>30). Though HRHPV types were universally found in cervical cancer, infection by HRHPV types results in cervical cancer in a very small percentage of infections [6]. More recently, new- or second-generation cervical cancer diagnostics targeting different aspects of the mechanism of cervical cancer pathogenesis have been brought into clinical use and added much needed specificity to the cervical cancer screening algorithm [7]. The purpose of this chapter is to understand the utility of HPV/cervical cancer diagnostics. In particular, discussion focuses on the relationship between the diagnostic target and the pathogenesis of cervical cancer as the field attempts to direct diagnostics toward detection of lesions requiring treatment and minimize the number of women sent to unnecessary, invasive procedures. In other words, cervical cancer diagnostics to date have focused on the mere presence of HRHPV and the associated risk of developing cervical cancer rather than the

B.K. Patterson (✉)
IncellDx, Inc., 1700 EI Camino Real, Menlo Park, CA 94027, USA
e-mail: brucep@incelldx.com

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HPV-driven mechanism by which HPV causes cervical cancer. In this chapter, consideration is also given to other uses of HPV diagnostics including anal and head and neck cancers.

Specimen Collection for HPV Diagnostics

Collection of cervical cytology specimens changed dramatically in the mid-1990s as new technologies were introduced that allowed the cervical cytology sample collected by spatula or brush to be placed in a liquid preservative [8, 9]. Liquid cytology preservatives are typically alcohol based (ethanol or methanol) and, in general, contain compounds that dissolve mucus and disaggregate cells. All technologies create a slide that contains a monolayer of representative cells from the ecto- and endocervix stained by the method of Pap. SurePath liquid (Becton Dickinson Burlington, NC) is used for collection of cervical cytology specimens and involves pressing cell suspensions through the small orifice of a syringe which disaggregates cell clusters. Isolation of epithelial cells is accomplished by removing interfering debris and inflammatory cells by centrifugation through a density gradient. After centrifugation, the tubes including cell pellets are placed on the PrepStain System for staining using the Pap stains. Thin Prep (Hologic, Marlborough, MA) uses a filter pressed through a cell suspension until a monolayer is created on the filter membrane. The filter is then pressed onto a treated slide and stained by the method of Pap. These technologies improved the quality and uniformity of cells on slides and reduced but did not eliminate unsatisfactory slides [8, 9]. A third liquid-based cervical cytology solution, LiquiPrep (LP), has also been used extensively around the world. Liqui Prep was the first LBC technology not requiring a dedicated processor. Through the use of its proprietary separation chemistry, this LBC system can be run on any swing bucket centrifuge allowing for broad adoption. The LP Preservative solution contains no hazmat chemicals which allows for lower shipping costs and simple disposal, the first truly “green” LBC system. The LP Preservative also does not cause morphology changes as seen with the first-generation system utilizing high concentration of methanol. Through the use of LP Cell Base, the cells are affixed to the slide maintaining classic morphology which allows labs to quickly adopt reading of the technology. To maximize cost-effectiveness the LP system offers the selective use of LP Cleaner to remove mucus in those samples where inflammatory cells are an issue. To provide further savings, no special coated slides are required; so any lab slide can be used.

As is discussed in subsequent sections, liquid-based cervical cytology has significantly aided the use of automated screening devices and the automation of advanced molecular and proteomic assays. Some studies suggest that liquid cytology preservatives can preserve RNA for up to 12 months [10]. This has led to the use of liquid-based cervical cytology samples as well as swabs for HPV diagnostics that target either DNA or RNA.

HPV and Cervical Carcinogenesis

In this section, the role of HPV in cervical carcinogenesis is discussed with an overlay of the commercially available diagnostics that detect different phases of HPV infection through transformation leading to cancer (Fig. 43.1). In HRHPV-associated lesions, HPV DNA exists as an episome in the cytoplasm of epithelial cells (Diagnostic Correlate: *Hybrid Capture 2 (Qiagen), Cervista (Hologic) , cobas 4800 HPV test (Roche), INFINITI (Autogenomics), CLART (Genomica), RealTime High Risk HPV test (Abbott)*). Under certain circumstances HPV DNA linearizes and integrates into the host cell chromosome [11]. During integration, the E2 gene, which is a negative regulator of E6 and E7, (Diagnostic Correlate: *Aptima (GenProbe),NucliSense Easy Q (BioMerieux), PreTect Proofer (NorChip)*) is deleted leading to overexpression of E6 and E7 mRNA (Diagnostic *Correlate*: *HPV OncoTect* (*IncellDx*)) [12–15]. This derepression of E6 and E7 mRNA is different from the small amounts of E6 and E7 that are required for replication and expressed during a "normal life cycle" [16]. E6 and E7 and certain host factors are clearly involved in the transformation of cells leading to cell cycle abnormalities and proliferation (Diagnostic Correlate: *HPV OncoTect 3Dx (IncellDx), CINTec plus (mtm Laboratories), ProEx C (Becton Dickinson*), and eventually cancer) [17] (Fig. 43.1). Mechanistically, the E6 protein causes degradation of p53, BAK, and activation of

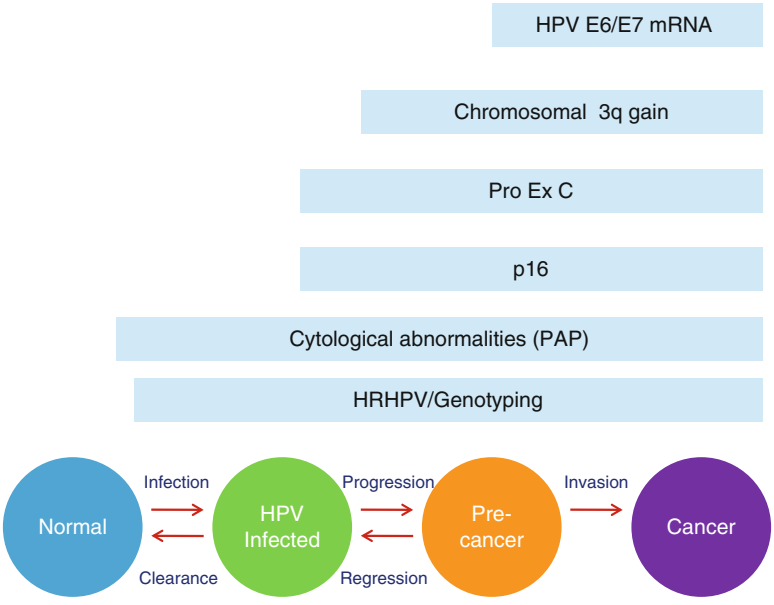

Fig. 43.1 Overlay of cervical cancer pathogenesis and diagnostic tests. This figure illustrates the detection of various stages of disease and where in the disease process various diagnostic tests detect changes

SRC-family kinases in addition to the activation of telomerase (Diagnostic Correlate:*3q addition (Ikonysis)*). Similarly and synergistically, the E7 protein degrades the RB releasing the transcription factor E2F from inhibition and upregulating the cellular protein $p16^{INK4A}$ (Diagnostic Correlate: *CINTec p16* (*mtm Laboratories*)) [17–28]. Several reports have shown not only the ubiquitous presence of E6 and E7 mRNA regardless of the high-risk type in cervical cancer but also a quantitative difference in the overexpression of E6/E7 depending on the severity of the cervical lesion, CIN 0, 18 %; CIN 1, 58 %; CIN 2, 77 %; and CIN 3, 84 %, and 100 % of cervical cancers [29, 30].

HPV and Cervical Cancer Diagnostics

As discussed above, the indication for HPV testing in the cervical cancer algorithm is for co-testing with cervical cytology and for ASCUS reflex testing. The metric by which cervical cancer diagnostics are measured is histology. In particular, high-grade dysplasia at the level of cervical intraepithelial neoplasia 2 (CIN 2) or worse, CIN 3 and squamous cell carcinoma, is considered the "Gold Standard." For several reasons this standard is not perfect because it is subject to sampling bias and the reproducibility of calling a lesion CIN 2 is poor relative to pathologist's ability to call a lesion CIN 1 or CIN 3 [31]. The CIN 2 or higher (CIN 2+) standard was also chosen since it was an actionable metric. In other words, clinicians generally treat a lesion of CIN2+ severity though recently the diagnosis and immediate treatment of CIN 2 are debated. A summary of the performance of commercially available tests follows and the attributes of each test are summarized in Table 43.1.

HPV DNA Tests (Consensus)

The first test on the market as an adjunct to cervical cytology was the Digene (now Qiagen) Hybrid Capture (HC2) technology which is a liquid hybridization assay that detects 13 high-risk strains of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). The performance of HC2 for the detection of high-grade disease (CIN 2+) is well documented but in general the sensitivity is >95 % and the specificity is <30 % [32]. Similarly, the negative predictive value (NPV) is 99 % and the positive predictive value is 15–25 %. Though not approved by the US FDA, newer versions of the Qiagen offering will reflex to HPV 16, 18, 45 genotyping. The Cervista test, like HC2, is approved by the FDA in the USA and detects the same strains of HPV as HC2 in addition to HPV type 66, now considered to be a high-risk type. Cervista also includes an HPV type 16, 18 reflex component which is discussed under HPV genotyping. In general, Cervista has an identical performance profile as HC2 though some publications have indicated that the positivity rate of Cervista on normal cytology specimens may be as high as 18 % [33].

Table 43.1 Summary of HPV/cervical cancer test attributes by diagnostic category

	PAP	P16	HPV DNA		L1 genotyping			E6 E7 genotyping		E6 E7 mRNA quantification
	Hologic; BD	CINtec	Qiagen Digene HC2	Hologic Cervista	Roche cobas	Genomica	Autogenomics	GenProbe	BioMerieux	HPV OncoTect
Performance attributes										
Technology	Cytostain	ICC/IHC	hybrid capture	Invader	PCR	Array	Array	TMA	NASBA	SUSHI
Specific (by Biopsy)	90 %	60 %	30 %	30 %	40 %	40 %	40 %	30–40 %	30–60 %	90 %
Sensitivity (by Biopsy)	50 %	80 %	95 %	95 %	90 %	90 %	90 %	80–95 %	80–85 %	93 %
Sample controls	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Cell type differentiation	Yes	Yes	No	No	No	No	No	No	No	Yes
Women <30 effective	Yes	Yes	No	No	No	No	No	No	No	Yes
Laboratory workflow attributes										
Subjectivity	Yes	Yes	No	No	No	No	No	No	No	No
Use of slides	Yes	Yes	No	No	No	No	No	No	No	No
Nucleic Acid Extraction	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Assay completion time	1-2 days	6–24 h	6 h	6 h	6 h	8 h	8 h	6 h	6 h	3.5 h
Automated	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Reagent stability	Ambient	4–8 °C	4–8 °C	−20 °C	4–8 °C	−20 °C	Ambient	4–8 °C	Ambient	Ambient

HPV DNA (Genotyping)

Many HPV genotyping assays exist in the market worldwide including *INFINITI* (Autogenomics), CLART (Genomica), PapilloCheck (Greiner Bio-One), *RealTime High Risk HPV test* (Abbott), and Cobas 4800 HPV test (Roche) which was recently FDA approved for cervical cancer screening in the USA. All of these tests will report at least 15 high-risk types (16, 18, 31, 33, 35, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68). These assays are important from an epidemiologic standpoint and more recent evidence has suggested that detection of HPV 16, 18 in particular increases the risk of a high-grade lesion (CIN 3+) from approximately 5 to 11 % [34, 35]. In a study with >40,000 women enrolled with an ASCUS diagnosis, the cobas 4800 HPV test demonstrated 93.5 % sensitivity, 69.3 % specificity, 8.4 % PPV, and 99.7 % NPV. By comparison, HC2 showed 91.3 % sensitivity, 70 % specificity, 8.5 % PPV, and 99.6 % NPV [34, 35].

HPV E6, E7 mRNA (Qualitative/Genotyping)

Aptima (GenProbe), NucliSENS EasyQ HPV (BioMerieux), and PreTect HPV Proofer (NorChip) are E6, E7 mRNA assays that provide a genotype based on qualitative detection of genotypic sequences within the E6, E7 genes. The Aptima test detects genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 and the other two tests detect 16, 18, 31, 33, and 45. Since expression of E6, E7 mRNA is an event that would indicate cellular transformation, the intent of this approach was to improve on the low specificity and low PPV of HPV DNA assays by increasing the specificity for CIN2+ lesions. Interestingly, these two tests have very different performance profiles. The GenProbe Aptima assay has a performance profile similar to HPV DNA assays with sensitivity for CIN2+ >95 % and specificity of 40 % and a PPV of 40 % [32]. The other two assays that detect only five high-risk types have sensitivities of 70–75 % and specificities of 70–75 % with a PPV of 55 % [32].

HPV E6, E7 mRNA (Quantitative)

HPV OncoTect is a quantitative assay that uses flow or image cytometry to quantify both the overexpression of E6, E7 mRNA on a cell-by-cell basis, which is the hallmark of cellular transformation, and the quantity of cells overexpressing E6, E7 mRNA in a liquid-based cervical cytology specimen [36]. Because flow cytometry has high analytic sensitivity and can analyze thousands of cells with high throughput, HPV OncoTect has a unique performance profile with both high sensitivity of 90–93 % and high specificity of 80–90 % [37]. The PPV of HPV OncoTect is between 60 and 80 % depending on the age of the woman [38].

Other HPV-Related Diagnostics

As described above, the cervical cancer screening filed is moving toward more specific markers that indicate that HPV is actually causing disease. The CINTec p16 marker is a protein that is upregulated following E7 overexpression [39]. Reactivity for the p16 marker was 68 % in CIN 2 and 82 % of CIN 3 in a meta-analysis of clinical studies. Because p16 staining is a slide-based test, the authors concluded that subjectivity affected reproducibility [39]. The ProEx C test detects the markers mtm2 and Top2a that are produced as a result of cell cycle dysregulation [18] and the 3q addition cytogenetics test reflects the activation of telomerase as a result of HPV transformation [40].

Comments, Future Approaches, and Summary

Because HPV has been implicated in a number of other epithelial cancers including anal cancer, head and neck cancers, and even breast cancer, HPV diagnostics are gaining importance in sites other than the cervix. Both HPV DNA assays such as HC2 and PCR (Roche Linear Array) and HPV RNA (HPV OncoTect) have been used to screen for anal intraepithelial neoplasia (AIN) in a similar manner to cervical cancer screening. The results vary with sensitivities for AIN 2, 3 between 50 % (Roche Linear Array) and 75 % (HC2 and HPV OncoTect) [41, 42]. The specificity of HPV DNA for AIN 2,3 is similar to the specificity for cervical disease around 30–40 % and as expected the specificity of HPV RNA by HPV OncoTect was higher at 60–70%. Other studies have suggested that HPV RNA may be a better marker for anal screening because of the potential reservoir of HPV in the anus that does not cause disease [43].

For head and neck squamous cell carcinomas, molecular markers that differentiate HPV-positive tumors from HPV-negative tumors correlate with tumor prognosis with increased survival in transcriptionally active HPV infections [44, 45]. For example, oropharyngeal squamous cell carcinomas that have p16 expression exhibit a 79 % 5-year survival, compared with 20 % 5-year survival in individuals with HPV-negative tumors, and 18 % 5-year survival in persons with HPV-16-positive tumors with no evidence of transcriptional activity [46].

In summary, the trend toward more specific HPV/cervical cancer tests either singly or in combination suggests a movement of the field to replace the Pap smear—so-called “Primary Screening.” Studies addressing whether HPV DNA can be used as a primary screen for cervical cancer have arisen extensively in Europe [47]. In fact a new technology, HPV OncoTect 3Dx technology, allows for simultaneous E6, E7 mRNA quantification, cell cycle (ploidy analysis), and imaging of the cells while still in suspension for high-throughput analysis without ever producing a slide (Fig. 43.2) [48]. Using this technology, studies showed that ASCUS cytology specimens clearly fell into normal, LSIL or rarely, HSIL diagnostic categories, thus demonstrating the potential of eliminating this ambiguous category [48].

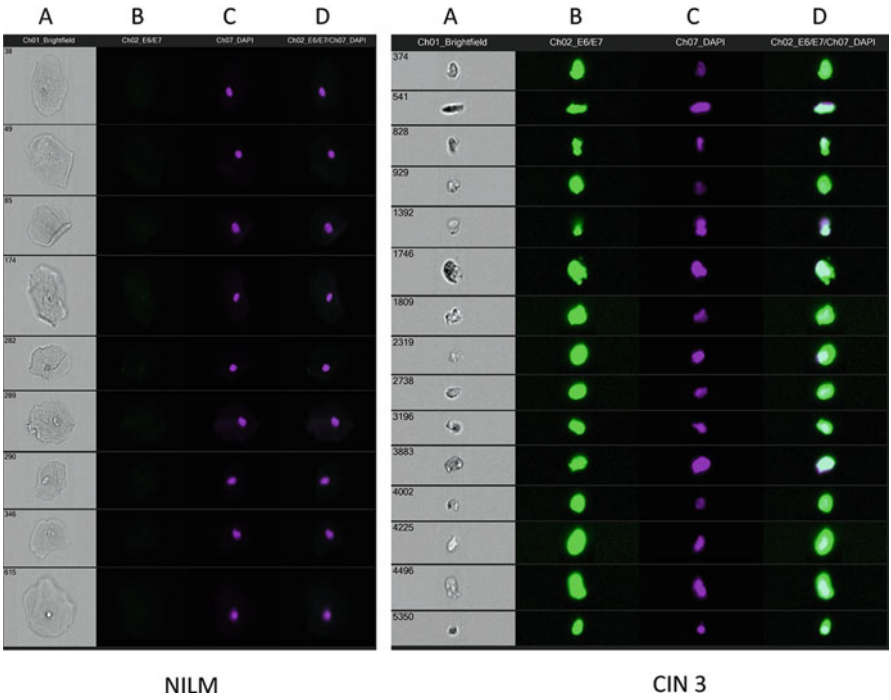

Fig. 43.2 Simultaneous morphology using nuclear to (**a**) cytoplasmic ratio (N/C), (**b**) E6, E7 mRNA expression (*green*), (**c**) cell cycle/nuclear pleiomorphism (*purple*), and (**d**) overlay on cervical cytology cells in suspension (HPV OncoTect 3Dx). Use of these three parameters allows the determination of normal, low-grade, and high-grade samples without the use of the Pap smear

References

1. ACS (2009) Cancer facts and figures
2. Walboomers JM, Jacobs MV, Manos MM et al (1999) Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189:12–19
3. Cox JT (2009) History of the use of HPV testing in cervical screening and in the management of abnormal cervical screening results. J Clin Virol 45(Suppl 1):S3–S12
4. Schiffman M, Solomon D (2009) Screening and prevention methods for cervical cancer. JAMA 302:1809–1810
5. Cox JT (2006) Human papillomavirus testing in primary cervical screening and abnormal Papanicolaou management. Obstet Gynecol Surv 61:S15–25
6. Sherman ME, Lorincz AT, Scott DR et al (2003) Baseline cytology, human papillomavirus testing, and risk for cervical neoplasia: a 10-year cohort analysis. J Natl Cancer Inst 95:46–52
7. Reynolds J (2011) HPV: a look into new methods for high-risk testing (2011). Med Lab Obs 43:10–12
8. Wilbur DC, Facik MS, Rutkowski MA, Mulford DK, Atkison KM (1997) Clinical trials of the CytoRich specimen-preparation device for cervical cytology. Preliminary results. Acta Cytol 41:24–29

9. Corkill M, Knapp D, Martin J, Hutchinson ML (1997) Specimen adequacy of ThinPrep sample preparations in a direct-to-vial study. Acta Cytol 41:39–44
10. Tarkowski TA, Rajeevan MS, Lee DR, Unger ER (2001) Improved detection of viral RNA isolated from liquid-based cytology samples. Mol Diagn 6:125–130
11. Zur Hausen HH (2002) Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2:342–350
12. Romanczuk H, Howley PM (1992) Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. Proc Natl Acad Sci U S A 89:3159–3163
13. McBride AA, Romanczuk H, Howley PM (1991) The papillomavirus E2 regulatory proteins. J Biol Chem 266:18411–18414
14. Munger KM, Scheffner M, Huibregtse JM, Howley PM (1992) Interactions of HPV E6 and E7 oncoproteins with tumour suppressor gene products. Cancer Surv 12:197–217
15. Munger K, Howley PM (2002) Human papillomavirus immortalization and transformation functions. Virus Res 89:213–228
16. Cuscherieri K, Wentzensen N (2008) HPV mRNA and p16 detection as biomarkers for the improved diagnosis of cervical neoplasia. Cancer Epidemiol Biomarkers Prev 17:2536–2545
17. Doorbar J (2006) Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci 110:525–541
18. Kelly D, Kincaid E, Fansler Z, Rosenthal DL, Clark DP (2006) Detection of cervical high grade squamous intraepithelial lesions from cytologic samples using a novel immunocytochemical assay (ProEx C). Cancer 108:494–500
19. Bedell MA, Jones KH, Laimins LA (1987) The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and rat-1 cells. J Virol 61:3635–3640
20. Longworth MS, Wilson R, Laimins LA (2005) HPV31 E7 facilitates replication by activating E2F2 transcription through its interaction with HDACs. EMBO J 24:1821–1830
21. Howley PM, Scheffner M, Huibregtse J, Munger K (1991) Oncoproteins encoded by the cancer-associated human papillomaviruses target the products of the retinoblastoma and p53 tumor suppressor genes. Cold Spring Harb Symp Quant Biol 56:149–155
22. Chellappan S, Kraus VB, Kroger B et al (1992) Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc Natl Acad Sci U S A 89:4549–4553
23. McIntyre MC, Ruesch MN, Laimins LA (1996) Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. Virology 215:73–82
24. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63:1129–1136
25. Scheffner M, Munger K, Byrne JC, Howley PM (1991) The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc Natl Acad Sci U S A 88:5523–5527
26. Scheffner M, Takahashi T, Huibregtse JM, Minna JD, Howley PM (1992) Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. J Virol 66:5100–5105
27. Song S, Liem A, Miller JA, Lambert PM (2000) Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis. Virology 267:141–150
28. Werness BA, Levine AJ, Howley PM (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248:76–79
29. Sotlar K, Stubner A, Diemer D et al (2004) Detection of high-risk human papillomavirus E6 and E7 oncogene transcripts in cervical scrapes by nested RT-polymerase chain reaction. J Med Virol 74:107–116
30. Nakagawa S, Yoshikawa H, Yasugi T et al (2000) Ubiquitous presence of E6 and E7 transcripts in human papillomavirus-positive cervical carcinomas regardless of its type. J Med Virol 62:251–258

31. Stoler M, Schiffman M (2001) Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study. JAMA 285:1500–1505
32. Szarewski A, Ambroisine L, Cadman L et al (2008) Comparison of predictors for high-grade cervical intraepithelial neoplasia in women with abnormal smears. Cancer Epidemiol Biomarkers Prev 17:3033–3042
33. Kinney W, Stolar MH, Castle PE (2010) Patient safety and the next generation of HPV DNA tests. Am J Clin Pathol 134:193–199
34. Wright TC Jr, Stolar MH, Sharma A, Zhang G, Behrens CM, Wright TL (2011) Evaluation of HPV-16`and HPV-18 genotyping for the triage of HPV-positive cytology-negative women. Am J Clin Pathol 136:578–586
35. Stolar MH, Wright TC Jr, Sharma A, Apple R, Gutekunst K, Wright TL (2011) High-risk human papillomavirus testing in women with ASC-US cytology: results from the ATHENA HPV study. Am J Clin Pathol 135:468–475
36. Narimatsu R, Patterson B (2005) High throughput cervical cancer screening using intracellular HPV E6, E7 mRNA quantification by flow cytometry. Am J Clin Pathol 123:716–723
37. Coquillard G, Palao B, Patterson B (2011) Quantification of intracellular HPV E6/E7 mRNA expression increases the specificity and positive predictive value of cervical cancer screening compared to HPV DNA. Gynecol Oncol 120:89–93
38. Pierry D, Lack B, Chen V, Fusco J, Weiss G. Intracellular HPV E6, E7 mRNA quantification (HPV Oncotect) predicts CIN 2+ in cervical biopsies better than pap screening for women regardless of age. Arch Pathol Lab Med (in press).
39. Tsoumpou I, Arbyn M, Kyrgiou M, Wentzensen N, Koliopoulos G, Martin-Hirsch P, Paraskevaidis E (2009) p16INK4a immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. Cancer Treat Rev 35:210–220
40. Seppo A, RezaJalali G et al (2009) Gain of 3q26: a genetic marker in low-grade squamous intraepithelial lesions (LSIL) of the uterine cervix. Gynecol Oncol 114:80–83
41. Phanuphak P (2011) Screening for anal intraepithelial neoplasia (AIN) using HPV Oncotect increases sensitivity and specificity compared to HPV DNA. Eurogin 2011, Lisbon, Portugal
42. Vardas E, Giuliano AR, Goldstone S, et al. External genital human papillomavirus prevalence and associated factors among heterosexual men on 5 continents
43. Valari O, Koliopoulos G, Karakitsos P et al (2011) Human papillomavirus DNA and mRNA positivity of the anal canal in women with lower genital tract HPV lesions: predictors and clinical implications. Gynecol Oncol 122:505–508
44. Licitra L, Perrone F, Bossi P et al (2006) High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. J Clin Oncol 24:5630–5636
45. Reimers N, Kasper HU, Weissenborn SJ et al (2007) Combined analysis of HPV-DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. Int J Cancer 120:1731–1738
46. Klussmann JP, Gultekin E, Weissenborn SJ et al (2003) Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. Am J Pathol 162:747–753
47. Ronco G, Giorgi-Rossi F, Carozzi F et al (2008) Results at recruitment from a randomized controlled trial comparing human papillomavirus testing alone with conventional cytology as the primary cervical cancer screening test. J Natl Cancer Inst 100:492–501
48. Patterson BK, Lack B, Shults K (2011) Combined nuclear-to-cytoplasmic ratio (N/C), E6, E7 mRNA Quantification, and DNA ploidy in suspension cervical cells. Eurogin 2011, Lisbon, Portugal

Chapter 44
Molecular Niches for the Laboratory Diagnosis of Sepsis

Donna M. Wolk

Introduction

Bloodstream infections are among the top causes of death in the US, and substantial mortality is attributed to testing delays in determination of the microbial cause(s) and selection of the appropriate antibiotic. In this review, the human and financial impact of sepsis, are summarized as well as the predisposing factors, symptoms, and common modes of bacterial pathogenesis. Important clinical and laboratory criteria for the diagnosis of sepsis and key aspects of the Surviving Sepsis Campaign Guidelines are detailed as they relate to diagnosis, therapy, and resuscitation from the septic event. Clinical laboratories must expand their understanding of the complexities related to diagnosing and treating sepsis in order to expand their role as productive members of interdisciplinary health care teams focused on improving survival from sepsis and limiting the financial impact that sepsis imparts on our health care systems.

Bloodstream infections and sepsis are among the top causes of mortality in the US, killing nearly 600 people per day. Many septic patients are treated in Emergency Medicine Departments (EMDs), or Critical Care Units (CCU), settings in which rapid administration of targeted antibiotic therapy drastically reduces mortality. Unfortunately, current microbiology laboratory methods are too slow to support rapid interventions, typically requiring >24 h to detect the presence of bloodstream pathogens (hematopathogens) and at least 3–5 days to confirm selection of appropriate antimicrobial therapy. Moreover, cultures from septic patients are often falsely negative due to preemptive therapy, the presence of fastidious organisms, or microbes that are present in low density. As a result, empiric, broad-spectrum treatment is common, and is a costly approach that may fail to effectively target the

D.M. Wolk (✉)
Department of Pathology, University of Arizona College of Medicine,
1501 N. Campbell Ave., P.O. Box 245059, Tucson, AZ 85724-5059, USA
e-mail: dwolk@email.arizona.edu

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correct microbe, may inadvertently harm patients via antimicrobial toxicity, and may contribute to the evolution of drug-resistant microbes. Clearly new rapid laboratory methods, which enhance the laboratory capabilities to diagnose bloodstream infections, will be a useful and welcome addition to clinical microbiology laboratories.

The Impact of Sepsis

Morbidity and Mortality

Bloodstream infections can lead to sepsis, a multi-symptom manifestation of bloodstream infection that causes rapid fatalities across all demographic populations. Sepsis is among the top ten causes of death for patients in the US with over 750,000 episodes each year, not; at times, exceeding 50 % mortality. Over 500 US patients die per day, nearly 183,000 per year [1–7]. The estimated sepsis incidence rate is approximately 3 in every 1,000 patients; of these patients, 51 % require ICU care, with additional 17 % requiring mechanical ventilation in an intermediate care or coronary care unit setting [4]. Cases of sepsis, primarily caused by bloodstream infections, are increasing dramatically, recently doubling in incidence [1–3], which is expected to increase by as much as 1.5–10 % per year [1, 2, 4, 8]. Sepsis is a growing problem to health care systems worldwide, affecting 18 million people and equaling the number of fatalities from acute myocardial infarction [9].

Economic Burden of Sepsis

Sepsis occurs in approximately 2 % of all hospitalizations and 75 % of intensive care patients [1], with enormous concomitant social and economic burden. In the US, the current per capita incidence is at least 240 patients per 100,000 people and estimated US health care costs exceed $17 billion dollars per year [2, 4, 8]. Emergency Medicine Departments (EMDs) are a common interface with septic patients, who account for approximately 571,000 EMD visits per year [4, 8, 10]. Sepsis is commonly associated with prolonged length of stay in the hospital and the ICU, up to 8 days longer, according to *MEDPAR Hospital Discharge Databases 2004 through 2005* and critical care reviews [4, 11]. With reports of 2.26 cases per 100 hospital discharges, costs to care for septic patients impart a large economic burden, averaging $22,100–40,890 per case [2, 4, 8]. In a study of medication costs in the ICU, half of the cost of medications and nutrition was attributed to antibiotics [12]. A common precursor to sepsis, bacteremia, acquired in the ICU, is associated with increased mortality, a longer ICU stay, and a 25 % increase in costs of hospital care ($85,137 vs. $67,879) [13].

The Attributes of Sepsis

Predisposing Factors and Underlying Diseases Increase Risks for Sepsis

Contributing factors are varied and include the following.

Critical care and surgery. Sepsis can be a fatal outcome for post-surgical patients in a variety of settings including, transplantation, wound surgery, splenectomy, intra-abdominal surgery, and cancer surgery [14–19].

Cancer. An estimated 5 % of cancer patients acquire severe sepsis. Hospitalized patients with cancer are more than five times more likely to die (37.8 %) than cancer patients without sepsis. Patients with hematologic cancers are 15 times more likely than the average person to suffer from severe sepsis [20]. The use of cytotoxic agents is largely responsible for immune suppression in these patients, which predisposes them to sepsis. In addition, malignant neoplasms can provide entry for bacteria into the bloodstream.

Age. People over 65 years old account for only 1/8 of the US population, but account for 2/3 of all sepsis cases. Age over 40 is a risk factor for sepsis [21–23]. The poorest outcomes are observed in patients over age 85, with a mortality rate over 38.4 % [4]. At the other end of the age spectrum, children share elevated mortality rates [11].

Other. Rheumatic or congenital heart disease; septic abortion; pelvic infection; intravenous drug abuse; other infections such as severe CAP (community-acquired pneumonia), abdominal infection, and urinary tract infection (with risk in descending order); alcoholism; meningitis; cellulitis; and chronic diseases (including diabetes, heart failure, chronic renal failure, and COPD); surgery; or cirrhosis are all risk factors for sepsis. Immunocompromised status due to HIV/AIDS increases risks, as do other immunosuppressive states. Indeed, due to hospital-associated drug-resistant infections, the simple act of hospitalization increases risk for bloodstream infections and sepsis.

The Systemic Inflammatory Response Syndromes (SIRS) and Sepsis

Bloodstream infections can lead to sepsis, in the following manner. At onset, a bacterial cell triggers the host immune response. For gram negative hematopathogens, the bacterial cell wall endotoxin, a lipopolysaccharide from the cell wall, initiates the human inflammatory response. For gram positive hematopathogens, it is the lipotechoic acid, peptidoglycan, and extracellular products (toxins) that trigger the response. An inflammatory response follows, functioning to mount protective host responses, such as vascular, cellular, and chemical. These responses are designed to

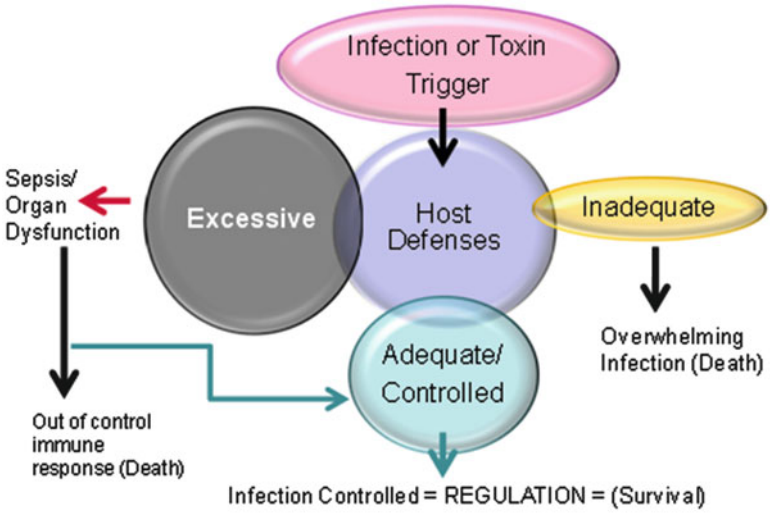

Fig. 44.1 Inflammation responses that occur in response to bacterial bloodstream infection. Reprinted with permission from Elsevier.

stop injury with edema, which dilutes toxins, and phagocytosis, which removes bacteria and cell debris.

The pathogenesis of sepsis involves pro-inflammatory mediators, anti-inflammatory mediators, and vaso-inflammatory mediators. There are several inflammatory responses of importance, which are linked to human cells that respond to bacterial invasion: (1) phagocytes (monocytes/macrophages, neutrophils, eosinophils, and to a lesser extent, mast cells), (2) mast cells, which are induced by lipopolysaccharide (LPS) and complement (C3a and C5a) to release immune mediators, and (3) natural killer cells, which cause lysis of target cells and production of cytokines like IFN-γ and TNF-α in a process called cell-mediated cytotoxicity. Other physiological changes include reduced protein C activity, micro-plugging of vessels, cellular necrosis (ischemic injury), fibrinolysis inhibition, apoptosis, leukocyte-mediated tissue injury, endothelial dysfunction, and cytopathic hypoxia.

While some patients may die of infection due to their lack of ability to mount an effective immune response, sepsis also results from a human immune response to bacteria (or other pathogens), which goes awry and out of control (Fig. 44.1). Patient survival is dependent on whether or not physicians can balance the patient immune response to microbial pathogens and bring patients' systems back to homeostasis.

Pathophysiology of Sepsis, a Disease Gradient

Due to the widespread inflammatory response [23], disease symptoms are widely variable and include fever, chills, hypotension, neutrophilic leukocytosis or neutropenia, hypothermia (especially in the elderly), diaphoresis, apprehension, change in

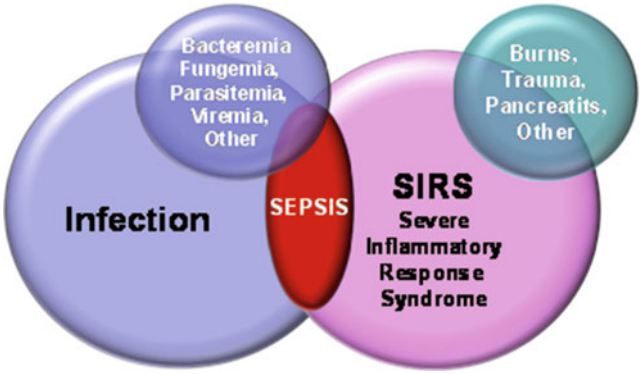

Fig. 44.2 There is substantial overlap between infection, inflammatory response, and sepsis. There are also noninfectious causes of inflammation, which must be eliminated before treatment for microbial sepsis (not drawn to scale). Reprinted with permission from Elsevier

mental status, tachypnea, tachycardia, hyperventilation and respiratory alkalosis, reduced vascular tone, and ultimately organ dysfunction. Hematologic findings are also extremely important as the septic patient can present with thrombocytopenia, toxic granulations of neutrophils, or disseminated intravascular coagulation (DIC). Renal and gastrointestinal signs include acute tubular necrosis, oliguria, anuria, upper GI bleeding, cholestatic jaundice, increased transaminase levels, or hypoglycemia.

There are consensus definitions that define the serial stages of sepsis [21], a progression of disease detailed below.

1. Bacteremia: The presence of viable bacteria in blood.
2. Systemic Inflammatory Response Syndrome (SIRS), a systemic inflammatory response to insults (e.g., infection, burns, trauma) that requires two or more of the following:
   (a) Temperature of >38° C or <36° C.
   (b) Heart rate of >90 bpm.
   (c) Respiratory rate of >20 or $PaCO_2$ <32 mmHg.
   (d) WBC count $>12 \times 10^9$/l or $<4 \times 10^9$/l or 10 % immature forms (bands).
3. Sepsis: The systemic inflammatory response to infection (SIRS) secondary to infection, suspected or proven by culture.
4. Severe sepsis, which is sepsis plus signs of hypoperfusion, hypotension, or organ dysfunction.
5. Septic shock, which is refractory arterial hypotension or hypoperfusion despite adequate intravascular fluid resuscitation. Hypoperfusion may be manifested as lactic acidosis, oliguria, or mental status changes.

Figure 44.2 illustrates the substantial overlap of the disease gradient that arises with bacteremia or another microbial infection and ends with septic shock. Nonspecific causes of inflammation can cause SIRS and must be considered in assessment of patients who present with inflammatory symptoms.

Rapid Antibiotic Therapy Saves More Lives than Any Other Intervention

In cases of sepsis, rapid intervention with appropriate antimicrobial therapy can be critical to patient survival [24, 25]. For aerobes, anaerobes, and fungi, appropriate antibiotic therapy increases survival by approximately 25–45 %. Eliminating delays in appropriate antibiotic administration increases survival by ~7–10 %/h [26]. Optimized antibiotic care requires intravenous (IV) broad-spectrum antibiotics with daily re-evaluation to optimize efficacy, prevent resistance, avoid toxicity, and minimize costs with the ultimate goal to discontinue broad-spectrum within 3–5 days and continue antibiotics targeted to the causative pathogen [27].

Diagnostic Approach to the Septic Patient

Unfortunately, despite the enormous human and financial impact of sepsis, this diagnosis remains largely a clinical one [28], due to the lack of rapid, sensitive, and specific laboratory tests to detect the causative pathogens. In order to provide a more accurate diagnosis, there is a significant need to improve the speed and diagnostic breadth of laboratory detection methods for hematopathogens, bloodstream infections, and sepsis.

Requirements for an Interdisciplinary Sepsis Team

As with all complex diseases, the diagnostic approach to sepsis is multifaceted. Laboratory collaboration with Emergency Medicine Departments and Critical Care Services is essential. Laboratories can participate by partnering with the entire health care team, setting goals to provide rapid laboratory testing to maximize effectiveness of early goal-directed therapy, improve targeted antibiotic therapy, shorten antibiotic treatment duration, avoid development of antibiotic resistance and side effects, decrease mortality and morbidity, decrease length of stay, and decrease overall hospital costs. The clinical microbiology laboratory must help drive antibiotic intervention in partnership with pharmacists and physicians.

Upon presentation of a patient with symptoms of infection, physicians will seek the primary site of infection and attempt to direct therapy to that primary site. A full history is important to define the potential source and risks. For instance, important factors include the source of infection, community- or hospital-acquired; prior or current medications; recent manipulations or surgery; underlying or chronic diseases; and travel history.

In addition, there are several standardized classification systems for ICU patients, created to assess severity of illness [29]; one common scoring system is

the APACHE II SCORE (acute physiology and chronic health evaluation). After admission of a patient to an ICU, an integer score from 0 to 71 is computed based on several measurements, including assessment of temperature, arterial blood gas pH, mean arterial pressure (MAP), serum sodium, serum creatinine, heart rate, hematocrit, respiratory rate, white blood cell counts, oxygenation parameters and the Glasgow Coma Score. Higher scores imply a more severe disease and a higher risk of death.

Combining a variety of clinical assessments with various laboratory tests from clinical microbiology, hematology, chemistry, point-of-care testing, and blood gas laboratories is an important aspect of the optimum care and treatment of septic patients.

Other Evidence-Based Sepsis Guidelines

The Surviving Sepsis Campaign is a worldwide consortium of health care providers committed to improving the outcomes for patients with sepsis [21, 30]. All components of the "Surviving Sepsis Campaign Guidelines" are focused on reducing mortality by using standardized criteria for patient assessment and treatment. The guidelines, aspects of which are also referred to as "Early Goal-Directed Therapy" (EGDT), often rely on laboratory data for optimal use. EGDT is a combination of prompt recognition of the symptoms of severe sepsis and septic shock, early antibiotic administration, and aggressive, protocol-driven resuscitation interventions, and subsequent continuous monitoring of patients.

River's landmark paper on EGDT demonstrated a 16 % absolute reduction in mortality in patients treated using EGDT compared to standard therapy [21]. A more recent review of the literature regarding EGDT continues to show significant improvement in mortality when EGDT is implemented [30]. Used appropriately, EGDT has been shown to effect a reduction in mortality from 46.5 to 30.5 % [25]. However, despite its success, implementing EGDT is both costly and resource-intensive. Physicians must place invasive lines and monitor resuscitation of patients closely. Nurses must perform frequent blood draws, manage multiple medications (including pressors), and tailor prescribed therapy based upon a number of parameters—some of which may be measured continuously, and some of which require periodic blood draws. In light of the resource and cost intensity of EGDT, there is concern that overly sensitive and insufficiently specific criteria for identifying patients with true bacterial sepsis may cause overuse of this intervention. Overly sensitive criteria may also lead to over-administration of antibiotics, increasing bacterial resistance and putting patients at risk of experiencing side effects from allergic reaction to organ toxicity.

To focus EGDT at the most appropriate patients, EMD and ICU services may use a "Sepsis Team," experts whose focus is implementation and follow up with practices called "sepsis bundles," as detailed below.

Sepsis Resuscitation Bundle (Often Triggered in EMD or ICU in What Is Sometimes Referred to as a "Code Sepsis" Response)

1. Serum lactate measured.
2. Blood cultures obtained prior to antibiotic administration.
3. From the time of presentation, broad-spectrum antibiotics administered within 3 h for ED admissions and 1 h for non-ED ICU admissions.
4. In the event of hypotension and/or lactate >4 mmol/l (36 mg/dl):
   (a) Deliver an initial minimum of 20 ml/kg of crystalloid (or colloid equivalent).
   (b) Apply vasopressors for hypotension not responding to initial fluid resuscitation to maintain mean arterial pressure (MAP) >65 mmHg.
5. In the event of persistent hypotension despite fluid resuscitation (septic shock) and/or lactate >4 mmol/l (36 mg/dl):
   (a) Achieve central venous pressure (CVP) of >8 mmHg.
   (b) Achieve central venous oxygen saturation ($ScvO_2$) of >70 %.

Sepsis Management Bundle (Follow-Up to the Resuscitation Bundle)

1. *Steroid replacement*: Low-dose steroids administered for septic shock, which is poorly responsive to adequate fluid resuscitation and vasopressors in accordance with a standardized ICU policy.
2. *Glycemic control*: Glucose maintenance approximating 150 mg/dl (8.3 mmol/l) (nutritional support and insulin control are often included in this focus) (*Note*: The aspects of this intervention are still controversial).
3. Inspiratory plateau pressures maintained <30 cm H_2O for mechanically ventilated patients.
4. Drotrecogin alfa (recombinant activated Protein C, aka Xigris) administration was previously listed here, but it has since been withdrawn from use after a major study showed no Xigris efficacy for the treatment of sepsis [31, 32].

Historic Laboratory Methods for Blood Cultures

Current laboratory methods for identification and characterization of bloodstream pathogens are slow to produce useful results and are ineffective for detection of some pathogens [33]. Rapid detection of bloodstream infections in the critically ill followed by appropriate antimicrobial therapy, can have a life-saving impact. According to a review of more than 2,600 cases from 15 intensive care units in five US and Canadian cities, the risk of death from sepsis increases by 6–10 % with every hour that passes, from the onset of septic shock until the start of effective antimicrobial therapy [34]. Thus, the development of a rapid, sensitive, and accurate

molecular diagnostic laboratory method to identify bacterial and fungal pathogens and characterize associated antimicrobial resistance has great potential to benefit diagnosis and therapy for septic patients, and save many lives.

While a variety of historical methods were grandfathered into laboratory protocols prior to CLIA regulations and new methods are implemented by validating them as laboratory-defined tests (LDTs), the clinical utility and accuracy of these off-label assays must be re-established in each laboratory where the method is used. In a recent ad hoc survey conducted with members of the American Society for Microbiology's LISTSERV communities, the clinmicronet and divC in May 2009, the survey participants reported use of the following methods, extrapolated from the published literature: the direct tube coagulase [35, 36], cefoxtin disk with reduced zones to predict MRSA [35, 37, 38], rapid PBP2a latex agglutination test [35, 39] and other latex agglutination for streptococci [40], direct germ tube [41], MRSA*Select* Agar [42], and pneumoslide for *Streptococcus pneumonia* [43], were performed directly from blood culture bottles. In addition, direct susceptibility testing for bacteria [44–49] and yeast [50] have been reported.

Despite attempts to rapidly identify pathogens and their associated susceptibility, these techniques are still not a common practice and patients and their physicians continue to wait for confirmatory results from blood cultures, the gold standard for diagnosis of bacterial sepsis, which can take several days to grow out a pathogen, limiting their usefulness in the management of the acutely ill patient. Moreover, blood cultures can frequently remain negative even in severe cases of sepsis. For example, routine methods are relatively ineffective for detection of certain pathogens, such as *Coccidioides* sp. and *Brucella* sp., causes of culture-negative fungemia and bacterial endocarditis, respectively. Despite its large societal impact, historic detection methods for bacteremia and sepsis remain largely unchanged for the past 20 years. Unfortunately, despite the enormous human and financial impact of sepsis, its diagnosis remains largely a clinical one [28], due to the lack of rapid, sensitive, and specific laboratory tests to detect the causative pathogens.

Emerging Molecular Methods for the Diagnosis of Sepsis and Bacteremia

In order to provide a more accurate diagnosis, there is a significant need to improve the speed and diagnostic breadth of laboratory detection methods for bloodstream infections and sepsis. Routine diagnostic methods, including routine cultures for bacteria, fungi, and rarely, viruses, almost all require subsequent subculture for organism identification, and the entire process can take days to weeks to produce a final result. Because of associated multiple-organ dysfunction in septic patients, death can occur rapidly, so improvements in diagnosis and treatment are focused on EGDT, which require rapid identification and characterization of complex symptoms [25, 30, 51]. Microbiology detection methods to characterize microbial pathogens and host response patterns are historically too slow, too insensitive, or too nonspecific to support differential diagnosis for EGDT strategies and do not fully assess the

complexities of the host immune response during sepsis, which appears critical to the understanding of associated multiple-organ dysfunction and death.

Because of the complex nature of sepsis, there are no single laboratory tests that can combine with clinical information to assess health outcomes or describe a time course of certain key biomarkers. Such a multicomponent test will be the key to unraveling the parallel and complex processes in sepsis and to providing clinicians with a tool for detection, prognosis, and therapeutic monitoring of sepsis. Clearly, historical immunological and molecular methods are impractical for detecting the complex patterns of immune responses seen in sepsis and the wide variety of hematopathogens that can cause disease; therefore clinical microbiology laboratories will need to consider the approach of "sepsis diagnostic panels," to combine detection of hematopathogens and key aspects of host's innate and systemic immune response. In addition, sensitive genotypic and phenotypic predictors of antibiotic resistance and phamacogenomic markers of potential drug toxicity will play a role in the future. Clearly, it will be critical to offset costs of new rapid methods with overall reduction of hospital costs. In order for the laboratory to effect these changes, a team approach will rely on interaction with pharmacists, physicians, and other health care staff to determine the most judicious use of these methods. One approach may include selective testing on only high-risk patients, which may benefit most from rapid testing. Discussion with health care finance and reimbursement teams as well as antimicrobial utilization teams are critical for the proper test utilization decisions to be made. In light of the complexity and urgency of the diagnostic challenges we face, this review summarizes the most recent published developments in the diagnosis of sepsis and bacteremia, which impact clinical microbiology laboratories.

FDA-Approved Molecular Methods for Blood Culture Bottles

Peptide Nucleic Acid Fluorescent In Situ Hybridization (PNA-FISH)

PNA Molecules Are DNA Mimics

Peptide nucleic acid fluorescence in situ hybridization (PNA FISH) is a new technology to test blood smears made directly from positive blood culture bottles and provides results in as little as 20 minutes after a positive blood culture is identified. The probes are commercially available from AdvanDx (Woburn, MA) for the direct identification of *Staphylococcus aureus*, *Candida albicans*, and *Enterococcus faecalis*/other enterococcus from positive blood cultures. Probe kit utilization is driven by the gram stain result, thus gram positive cocci in clusters would utilize the *S. aureus* probe, yeast would utilize the *C. albicans* probe, and gram positive cocci in pairs and chains would utilize the dual *E. faecalis*/other enterococci probe. Testing for Gram-negative microbes is also available.

PNA probes contain the same nucleotide bases as DNA, and follow standard base-pairing rules for hybridization for the following basepairs: adenine (A), thymine (T), cytosine (C), guanine (G). The difference between DNA hybridization

probes and PNA hybridization probes are that the negatively charged sugar–phosphate backbone of DNA is replaced with a non-charged polyamide or "peptide" backbone for the PNA probes. This replacement confers several advantages over conventional nucleic acid probes: (1) the strength of the FISH binding is greater than the DNA probe; PNA Chemistry creates very small dye-labeled probes, approximately 12–20 mers in size; therefore they are easy to hybridize to the target of interest, (2) because of the neutral charge, there is no natural repulsion with charged backbones, therefore they bind tightly, and (3) there is exquisite base-discrimination, allowing for more specific binding to target DNA or RNA. In addition, the PNA probes confer very low background, which allows the fluorescent signal to be visualized and confers greater sensitivity.

PNA FISH probes target rRNA in microbial chromosomes, offering advantages of a small subunit rRNA (ssRNA) target—(1) sequences are known and unique between species, (2) ssRNA targets are highly abundant target and multicopy, and (3) PNA probes, due to their small size, can bind in highly conserved regions that are not accessible to larger DNA probes.

PNA-FISH Assay Accuracy

According to product inserts and clinical publications, PNA FISH performance data has demonstrated accuracy. For *S. aureus* sensitivity and specificity are 98.8 and 99.6 % respectively, *C. albicans* 99.3 and 100 %, *E. faecalis* 96.3 and 98.3 %, and other *enterococcus* 93.1 and 99.3 % [52–60]. The compatibility of these probes has been proven with demonstrated accuracy for various blood culture media, including FAN and resin bottles [53–55, 60–62].

The accuracy of the staphylococcal PNA FISH probe was assessed by Chapin and Musgnug [63]. In this study, 157 blood cultures with *S. aureus* or coagulase-negative staphylococci (CoNS) were tested by the API RAPIDEC staph (API, bioMerieux, Durham, NC), tube coagulase test (TCT) read at 4 h, and the PNA FISH (AdvanDx, Woburn, MA) and compared to the results of AccuProbe *S. aureus* Culture Identification Test (Gen-Probe, San Diego, CA), revealing that API, TCT, and PNA FISH exhibited sensitivities of 96, 84, and 99 %, respectively, and specificities of 99, 100, and 100 %.

In a blinded comparison of *S. aureus* PNA FISH with standard identification methods, Oliveria et al. tested a total of 564 routine blood culture bottles positive for GPCC recovered from both aerobic and anaerobic media from three different manufacturers (ESP, BACTEC, and BacT/Alert) [64]. The sensitivity and specificity of *S. aureus* PNA FISH were 100 % (57 of 57) and 99.2 % (116 of 117), respectively, with 174 GPCC-positive ESP blood culture bottles, 98.5 % (67 of 68) and 98.5 % (129 of 131), respectively, with 200 GPCC-positive BACTEC blood culture bottles, and 100 % (74 of 74) and 99.1 % (115 of 116), respectively, with 190 GPCC-positive BacT/Alert blood culture bottles. It is concluded that *S. aureus* PNA FISH performs well with commonly used continuously monitoring blood culture systems.

There are three types of Candida probes. In a multicenter trial of the *C. albicans/Candida glabrata* probe published by Shepard et al., $n = 197$ the *C. albicans*, sensitivity

98.7 % (78/79), *C. glabrata* was 100 % (37/37); specificity for both was 100 % (82/82) [65]. Seventy fungal reference strains were spiked and tested with the BacT/ALERT system. In an evaluation of 5-color PNA-FISH assay with five Candida species, *C. albicans, glabrata, krusei, parapsilosis, and tropicalis*, -specific probes to identify Candida colonies, compared well to standard phenotypic identification methods. Sensitivity was 99 % (109/110), and specificity was 99 % (129/130) [66].

PNA-FISH Algorithms Used for Intervention

The following review details studies that have applied PNA FISH as an adjunct to Gram's stain from positive blood culture bottles. It is clear that when used appropriately with a team involving laboratory, pharmacy, and physicians, PNA FISH cannot only direct therapy but can also drive down antibiotic and hospital costs and save lives. Optimal outcomes for rapid intervention with PNA-FISH have been best documented when there is a strong collaborative effort between the laboratory and pharmacy. Holtzman et al. found no impact when their pharmacy was not involved. [67]. Others found success with physician intervention as detailed here. Gamage et al. found substantial decreases in mortality and hospital expenditures using a multidisciplinary team of laboratorians, pharmacists, and physicians [68].

Support of Antibiotic Selection for Bacteremia Due to *E. faecalis* or Other Enterococcus

The *E. faecalis* OE PNA FISH assay (AdvanDx, Woburn, MA) can be utilized to reduce the time required to administer targeted antibiotics therapy [69]. Recently the probe has been modified to shorten the hybridization time to 90 min [70, 71]. At present, *enterococci* such as *E. faecium* make up the majority of vancomycin-resistant *enterococcus* (VRE) while *E. faecalis* are rarely vancomycin resistant. Identification of *E. faecium* isolates can significantly reduce the time required to prescribe correct therapy with linezolid. Since delay in appropriate antimicrobial therapy is a risk factor for death in VRE bacteremia, rapid identification and appropriate intervention with targeted antibiotics is aimed to reduce mortality with *E. faecium* isolates. Alternately, rapid identification of *E. faecalis* isolates from blood cultures provides the opportunity to direct therapy toward an earlier switch to ampicillin, providing a cost benefit. Use of these algorithms requires targeted interventions based on local antibiograms and should not be undertaken without critical review of local antibiograms and pharmacy formulary issues.

Using a targeted algorithm directed by the *E. faecailis* PNA FISH probes, Toombs et al. demonstrated that appropriate treatment for VRE and non-VRE can be directed for bloodstream infections [69]. Use of the PNA FISH probes significantly reduced the number of days required to reach appropriate therapy, from 2.5 to 1.4 days ($p<0.05$). In turn, there was a trend toward less mortality (36 % vs. 14 %) ($p<0.05$).

Reduction in mortality was also observed in a quasi-experimental study performed by Forrest et al. in which PNA FISH was added to a treatment algorithm for

hospital-acquired enterococcal bacteremia [72]. In this study, the primary outcome assessed was defined as the "time from blood culture draw to implementation of effective antimicrobial therapy." Comparing 129 patients in the pre-intervention arm vs. 95 in the post intervention arm, using PNA FISH, the investigators identified *E. faecalis* in 1.1 day vs. 4.1 day without PNA FISH; $p < 0.001$. For *E. faecium*, results were obtained in 1.1 day vs. 3.4 day; $p < 0.001$. Comparisons between the pre and post intervention period revealed a decreased 30-day mortality (26 % vs. 45 %; $p = 0.04$) and an overall hospital savings of $20,000/year.

The Ability to Curtail Unnecessary Antibiotics When Blood Cultures are Contaminated

The *S. aureus*/CNS PNA FISH Culture Identification Kit (AdvanDx, Woburn, MA) may be beneficial in terms of reducing time to correct species identification and therapy for staphylococcal bacteremia. Forrest et al. demonstrated the use of PNA FISH for the rapid differentiation of *S. aureus* from CoNS (a common skin contaminate) in blood cultures [58]. The goal was to identify CoNS contaminants and to limit the use of vancomycin in these patients. In this study, an antimicrobial team evaluated the once daily PNA FISH results, and then made suggestions to physicians, which led to reduction in vancomycin use in patients with positive cultures that were most likely contamination. In this study, there were 223 episodes of bacteremia, 39 were tested with PNA FISH, 84 were matched controls and had traditional identification without the benefit of the rapid PNA FISH assay. With this algorithm, the use of PNA FISH significantly reduced the median hospital stay by 2 days ($p < 0.05$) and resulted in less vancomycin usage with an estimated cost saving per patient of roughly $4,000. Reduction in median length of stay was achieved by 2 days:6 days in the control group, 4 days in the PNA FISH group.

Recently, Ly et al. studied 202 patients with gram positive cocci in clusters in the blood culture gram stain [73]. In the intervention group, results and general organism information from the PNA FISH results were relayed to the treating clinician. In the control group, no call was made. For patients whose physicians received a PNA FISH result, an 80 % reduction in intensive care unit (ICU) related mortality was observed for bloodstream infections due to *S. aureus*. The median hospital cost savings of $19,441 per patient was observed as was a 61 % reduction in antibiotics for coagulase-negative staphylococci, when deemed a blood culture contaminant.

Testing a high percentage of skin contaminants with PNA FISH can be costly and labor-intensive; therefore, it is prudent to limit the percentage of skin contaminants recovered from blood culture bottles. Skin and line antisepsis is critical to prevent blood culture contamination and <3 % contamination rate is considered a benchmark of good blood culture collection practice.

Hensley et al. report a comparison of the *S. aureus*/CNS PNA FISH Culture Identification Kit (AdvanDx, Woburn, MA) to standard methods for presumptive identification of *S. aureus* and coagulase-negative staphylococcus (CoNS) in positive blood cultures. Blood cultures ($n = 301$) that signaled positive on the

BacT/Alert 3D (bioMerieux, Durham, NC) automated blood culture system and had GPCCC on Gram stain were tested with the PNA FISH assay. Overall agreement was 96.7 %. Sensitivity for *S. aureus* was 96.5 % (83/86); specificity was 100 % (215/215). Sensitivity for CoNS was 96.6 % (201/208) and specificity was 96.8 % (90/93). This study had more discordant samples than other published comparisons, and misidentifications were observed for *S. aureus*, CoNS, and *S. viridans* [74].

Support of Antifungal Selection for Candidemia

Candida species are the fourth most common cause of nosocomial bloodstream infections, commonly in the immunocompromised host population. Of all the *Candida* sp., *C. albicans* is the most common isolate, accounting for 55 % of all candidemias [61]. Guidelines defined by the Infectious Disease Society of America (IDSA), promote the use of fluconazole as the initial therapy for *C. albicans* without prior azole use. Alternatively, guidelines suggest that broad-spectrum agents should be considered for non-*C. albicans* because of the risk of possible fluconazole resistance [75]. To support rapid adherence to those guidelines, PNA FISH testing supports the ability to provide a direct identification from positive blood cultures in 2.5 h, as opposed to 1–5 days by conventional culture methods.

The ability of PNA FISH to support appropriate antifungal selection has been evaluated [61, 76, 77]. In one key study the cost savings realized with the *C. albicans* PNA FISH was $1,729–1,837 per patient. The majority of the cost savings were realized in antifungal expenditures [61, 76, 77]. Reporting of *C. albicans* by PNA FISH led to early switch to generic fluconazole without compromising patient safety.

Detection of Gram Negative Microbes

Using spiked blood cultures, both ATCC and clinical strains, Peleg et al. showed described the accuracy of PNA FISH for the detection of *Acinetobacter* spp. and *Pseudomonas aeruginosa*. PNA FISH had a sensitivity and specificity of 100 and 100 %, respectively, for 20 clinical strains of *Acinetobacter* spp. and 100 and 95 %, respectively, for 20 strains of *P. aeruginosa* and was able to detect spiked mixtures [78]. The study was limited by the small number of species tested for specificity and depicted a false-positive with an *Escherichia coli* strain and a *Stentotrophomonas* strain [78]; further advancement via FDA clinical trial shows improved results.

PCR to Confirm Identification of *S. aureus* Bacteremia

The rapid detection of *S. aureus* bacteremia and a swift determination of methicillin susceptibility has serious clinical implications affecting patient mortality.

GeneXpert®, Xpert™ MRSA/SA BC Assay

In a multicenter preclinical evaluation, Wolk et al. evaluated the performance of two Cepheid Xpert™ MRSA/SA assay for detection of methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) [79]. Using an integrated DNA extraction process coupled to real-time PCR, MSSA, and MRSA are identified directly from positive BC bottles in <1 h. A total of 114 wound specimens and 406 blood culture bottles were tested from study sites in the United States and Europe in order to characterize assay performance of these assays in a clinical setting. Newly positive blood culture broths with Gram's stains consistent with gram-positive cocci in clusters were tested. Broths from each of the following continuously monitoring blood culture instruments were included: bioMérieux BacT/ALERT SA standard aerobic, SN standard anaerobic; BACTEC™ PEDS PLUS™/F Medium, Plus Aerobic/F Medium, Plus Anaerobic/F Medium; Standard Anaerobic/F Medium, Standard/10 Aerobic/F Medium, LYTIC/10 Anaerobic/F Culture Vials; and VersaTREK REDOX 1® (aerobic), REDOX 2® (anaerobic). BacT/ALERT FAN aerobic and anaerobic broths were not included. The primers and probes in the Xpert MRSA/SA assays detect sequences within the staphylococcal protein A (spa) gene, the gene for methicillin resistance (mecA), and the staphylococcal cassette chromosome (SCCmec) inserted into the SA chromosomal attB insertion site [80, 81]. Inclusion of both the attB insertion site and the mecA gene targets enables the assays to identify the presence of SCCmec cassette variants with mecA gene deletions, thus reducing false-positive results that occur in molecular tests that only target the SCCmec cassette [82, 83]. For the Xpert™ MRSA/SA assay performed on Cepheid GeneXpert® system, sensitivity was 97.1 and 98.3 % for MRSA in wound and blood culture specimens, respectively. Sensitivity was 100 % for *S. aureus* from both specimen types [79]. This assay has since been removed from the market and a replacement is pending.

SmartCycler, BD GeneOhm StaphSR Assay

BD GeneOhm™ StaphSR Assay on the SmartCycler® is used for detection of *S. aureus* and MRSA directly from positive blood culture bottles. In a study by Stamper et al., a real-time PCR assay, the StaphSR assay (BD GeneOhm, San Diego, CA) was used for the identification of methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA), from positive blood culture bottles, $n = 300$ [84]. The sensitivity, specificity, and positive and negative predictive values of the BD GeneOhm StaphSR assay for MSSA detection were 98.9, 96.7, 93.6, and 99.5 %, respectively when compared to phenotypic identification with a variety of reference standards including oxacillin and cefoxitin Etests (AB Biodisk, Solna, Sweden), penicillin-binding protein 2′ (Denka Seiken Co., Tokyo, Japan), and *mecA* PCR. For the detection of MRSA, the BD GeneOhm StaphSR assay was 100 % sensitive and 98.4 % specific. Inhibition was seen with only one sample, and the issue was resolved upon retesting.

Recently Grobner et al. evaluated the BD GeneOhm StaphSR assay for its use in the detection of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) ($n=134$). Of 134 samples five discrepant results arose with this assay due to the presence of methicillin-susceptible, revertant MRSA strains (3/45) and MRSA strains that were not detected by the BD GeneOhm StaphSR assay (2/45) [85].

New Methods with Potential for Future Impact

The future of clinical diagnostics is anticipated to include a variety of rapid and multiplex methods. Review of all technologies is beyond the scope of this review; however, several will be discussed, including multiplex-PCR, DNA sequencing, liquid microarrays, and PCR-electrospray ionization mass spectrometry. It is known that blood culture bottles, positive for bacteria, do not always support cultivation of the pathogen to agar [33, 86]; therefore new molecular methods may allow microbiology laboratories to identify fastidious pathogens or those damaged by antibiotics. More detailed information about the following techniques may be found in other reviews [87, 88].

PCR Mass Spectrometry, Genetic Profiling Directly from Blood Culture Bottles

PCR/electrospray ionization time-of-flight mass spectrometry (PCR/ESI-MS) from Abbott/Ibis, has recently been adapted and developed for rapid identification, strain typing, and antibiotic-resistance assessment [89–92], including *S. aureus* genetic targets [93, 94]. In a recent publication, Baldwin et al. provided evidence for the technology's ability to identify a broad range of pathogens, such as those that may be encountered in blood cultures. With accuracy listed as 91 % for the genus level, there is room for improvement; however, the approach shows promise as a rapid method for identification of hematopathogens. A new RUO assay, the Bacterial Candida Assay is available commercially in the US.

In this approach, broad-range primers are used to amplify PCR products from groupings of microbes, rather than primers targeted to single target or species. Electrospray ionization time-of-flight mass spectrometry is used to determine the nucleic base composition (the number of adenosines (A), cytidines (C), guanosines (G) and thymidines (T)) of the PCR amplicon and this base composition signature is used to identify the pathogens present. Unlike nucleic acid probe assays or microarrays, mass spectrometry does not require prior knowledge of products analyzed, but simply measures the masses of the nucleic acids present in the sample. The platform is significantly different from previous technologies in its ability to detect virtually all microbes from a family and even microbes that have mutated

significantly. This technology has the potential for identification of all known human microbial pathogens in 4–6 h from blood or sterile body fluids. The system is more fully described elsewhere in this book, and while accurate for identification of a wide range of hematopathogens, and comparable to MALDI-ToF mass spectrometry, it has yet to be adapted for use in the clinical laboratory [95, 96].

Multiplex Real-Time PCR

The Roche Septifast™ assay is a real-time PCR assay, performed using the LightCycler 480 system, which is designed to identify the 19 most common bacteria and 6 most common fungi isolated from blood culture bottles and whole blood. The system has been evaluated by several investigators [97–99] but is not yet commercially available in the US.

While the SeptiFast assay may lead to a more rapid and targeted antibiotic therapy early after the onset of fever, the results for sensitivity are low and the cost benefit has yet to be determined. Bravo et al. compared the performance of the LightCycler SeptiFast Test MGRADE and conventional blood culture for microbes isolated from febrile episodes occurring in neutropenic and critically ill patients (in the intensive care unit; ICU). A total of 86 febrile episodes occurred in 33 neutropenic patients and 53 ICU patients. Blood samples for traditional and molecular methods were obtained at the onset of fever, before the implementation of empirical antimicrobial therapy. The overall agreement between the SeptiFast test and blood culture was 69 % (κ=0.37) in neutropenic patients and 75 % (κ=0.56) in ICU patients. The sensitivity of the SeptiFast assay for clinically relevant episodes of bacteremia and fungemia was 62 % in neutropenic patients and 70 % in ICU patients [100]. For community-onset bacteremias, Josephson et al. prospectively evaluated results from adult patients from an infectious diseases department. For the evaluation, one BC/PCR set (two BC bottles and one PCR tube) per patient was used. For n=1,093 patients, the BC was positive in 138 and PCR was positive in 107. Ten PCR results were supported by the clinical presentation. The following sensitivities and positive predictive values (PPVs) were noted: *S. aureus*, 67 and 43 %; *S. pneumoniae*, 12 and 67 %; other *Streptococcus* species, 43 and 77 %; *Escherichia coli*, 53 and 56 %; and *Klebsiella species,* 43 and 23 %. If support from other cultures and the clinical presentation were included in the reference standard, the PPVs for the detection of these bacteria were 57, 100, 92, 75, and 69 %, respectively. Low sensitivity discourages routine use of the test in its present form for the detection of community-onset bloodstream infections [101].

For endocarditis, the accuracy of the method is also suboptimal; however, it may have a use in cases of IE in patients treated with antibiotics before admission. Casalta et al., evaluated results from 63 patients with infectious endocarditis (IE). The SeptiFast test was less sensitive than blood cultures; however, some gains were observed for patients treated with antibiotics on admission; three isolates were identified that were not able to be cultivated [98].

For surgical patients at the ICU, a positive Septifast test showed a high coincidence with SIRS (75.8 %); however, the sample size was small and many discordant results confound interpretation. Nevertheless, surgical ICU may be a patient population in which systematic investigation may be warranted [102]. Likewise, in a specialized patient population, liver transplant, two cases of invasive Aspergillus infection were successfully identified by SeptiFast, which identified *Aspergillus fumigatus* DNA in blood and BAL fluid. Follow-up of the SeptiFast findings and GM index correlated with the clinical course, with blood clearing of DNA in parallel with galactomannan results. Further evaluation will be are necessary to evaluate the accuracy of SeptiFast in diagnosing IA [103].

Relative to the use of SeptiFast for identification of pathogens in blood culture bottles, Dierks et al. tested 101 cultures from 77 patients. In this limited sample set, 63 (62 %) yielded concordant negative results, 14 (13 %) concordant positive, and 9 (9 %) were BC positive only. In 14 (13 %) samples pathogens were detected by SF only, resulting in adjustment of antibiotic therapy in five patients (7.7 % of patients). In three samples a treatment adjustment would have been made earlier resulting in a total of eight adjustments in all 101 samples (8 %) [97].

Molzyme SepsiTest

Another PCR method is based on the detection of conserved sequences in whole blood for rapid diagnosis of bacteremia and fungemia. The SepsiTest is not commercially available in the US. The test uses a universal PCR from the 16S and 18S rRNA genes with subsequent identification of bacteria and fungi, respectively, from positive samples by sequence analysis of amplicons. The SepsiTest was evaluated in a prospective, multicenter study of 342 blood samples from 187 patients with systemic inflammatory response syndrome, sepsis, or neutropenic fever. Compared to blood culture the diagnostic sensitivity and specificity of the PCR were 87.0 and 85.8 %, respectively, achieving improved results in accuracy over the SeptiFast, and yielding results in a few hours. The concordance of PCR and BC for both positive and negative samples was 86.0 % [104, 105].

In another study employing 20 infective endocarditis (IE) patients and ten non-IE patients, heart valves and preoperative blood samples were analyzed by blood culture and SepsiTest. The diagnostic sensitivity of the molecular test was 85 % and that of blood culture only 45 %, remaining negative in many cases as a result of antibiotic treatment. Also, etiological agents were identified by SepsiTest in 5/10 non-IE patients, whereas cultures were negative throughout [106].

DNA Sequencing

Pyrosequencing® (Qiagen, Germantown, MD) is a rapid method for sequencing based on the detection of pyrophosphate released during DNA synthesis. These methods' advantages include speed and ease of use in comparison to traditional

sequencing technology; disadvantages include the short lengths of sequences that can be currently analyzed. Pyrosequencing® provides short sequence information roughly 30–50 bases; it is useful for short-read DNA and mutation/SNP analysis. It is ideally suited for applied genomics research including molecular applications for disease diagnosis, clinical prognosis and pharmacogenomics testing. After PCR amplification and amplicon clean-up, run times approach 1 h for 96 samples, with approximately 30–45 min for sequence analysis applications.

Jordan et al. evaluated pyrosequencing directly from blood culture bottles to assess the potential of pyrosequencing to differentiate between bacteria commonly associated with neonatal sepsis. An informative 15 bases within the 380-bp amplicon was targeted for pyrosequencing following enrichment culture and PCR amplification. A total of 643 bacterial isolates commonly associated with neonatal sepsis, and 15 PCR-positive, culture-positive neonatal whole blood samples were analyzed by pyrosequencing. Results of DNA sequencing and culture identification were compared and were successful at using PCR and pyrosequencing together to accurately differentiate between several bacterial groups, both gram positive and gram negative. The system had some difficulty with identification of viridians group strep and *S. pyogenes* [107]. The same group evaluated specimens from isolates from neonatal sepsis events, to support species identification that could lead to rapid de-escalation or targeting of antibiotic therapy. A total of 643 bacterial isolates and 15 PCR-positive, culture-positive neonatal whole blood samples were analyzed by pyrosequencing. Pyrosequencing was able to provide useful information on the identity of species based on the amplicon generated by PCR [107].

Quiles-Melero et al. tested a unique paper-based DNA preservation method prior to pyrosequencing of *Candida* species and found 100 % concordance to species identification for 48 positive blood cultures, containing 47 yeast and one filamentous fungus. Primers for *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. krusei*, and *A. niger* were included [104].

StaphPlex System

Tang et al. report the accuracy of the Qiagen StaphPlex system, which amplifies and detects 18 gene targets simultaneously for species-level identification of staphylococci, detection of genes encoding Panton-Valentine leukocidin (PVL), and antimicrobial resistance determinants of staphylococci [108]. The StaphPlex system was compared to phenotypic methods for organism identification and antimicrobial resistance detection for positive blood culture specimens in which GPCC were observed. Of 360 specimens containing staphylococci, 273 (75.8 %), 37 (10.3 %), 37 (10.3 %), 1 (0.3 %), 3 (0.8 %), and 9 (2.5 %) were identified by StaphPlex as coagulase-negative Staphylococcus (CoNS), MRSA, MSSA, or mixed infections of CoNS and MRSA, CoNS and MSSA, or nonstaphylococci, respectively, with an overall accuracy of 91.7 %.

The 277 CoNS-containing specimens were further identified as 203 (73.3 %) *Staphylococcus epidermidis* isolates, 10 (3.6 %) *Staphylococcus haemolyticus*

isolates, 27 (9.7 %) *Staphylococcus hominis* isolates, 1 (0.4 %) *Staphylococcus lugdunensis* isolate, and 36 (13.0 %) other CoNS isolates, with an overall accuracy of 80.1 % compared to an API STAPH test and CDC reference identification. Numerous "very major" errors were observed for several of the drug-resistance genes, but demonstrated 100 % sensitivity and specificity, ranging from 95.5 to 100.0 % when used for staphylococcal cassette chromosome mec typing and PVL detection. StaphPlex provides simultaneous staphylococcal identification and detection of PVL and antimicrobial resistance determinants within 5 h.

Molecular Testing for Drug Resistance

Rapid and accurate determination of microbial drug susceptibility is actually the most critical challenge; it is essential to facilitate successful antimicrobial therapy of any person, and particularly useful for those with immune function impairment. Rapid testing for genetic resistance markers is an emerging clinical practice that cannot only identify the potential for drug resistance, but also help distinguish ambiguous breakpoints associated with susceptibility testing. The application of molecular diagnostic methods to detect drug resistance is evolving. Future methods should facilitate educated choices for therapy, which can be initiated early in diagnosis to impact patient outcomes. Evidence-based studies will be key to the adoption of these new testing antimicrobial testing paradigms.

Targets for genetic testing require relative genetic stability of the target sequence, a requirement that demands national and international efforts for monitoring genetic mutations in these target regions. Together, MRSA and VRE are the two most important resistant bacterial pathogens in US hospitals and their rapid detection remains a critical necessity as antimicrobial resistance continues to increase in the US and worldwide [109]. Among bacteria, other useful antimicrobial resistance targets include resistance genes for β-lactams, aminoglycosides, chloramphenicol, fluoroquinolones, glycopeptides, isoniazids, macrolides, mupicurin, rifampin, sulfonamids, tetracyclines, and trimethaprim [110–112]. For a list of PCR primers used to target such resistance markers see Tenover and Rasheed [112]. For fungi, rapid detection of antifungal resistance is useful, primarily due to the increase in fungal infections among immunocompromised patients. Current antifungal assays rely on fungal susceptibility testing which is dependent on growth. The practical application of antifungal molecular testing is yet to be seen, as there is still much to learn about the genetic markers, which mediate resistance. The genetic information needed to examine fungal resistance at the molecular level is complex; a review of molecular mechanisms of antifungal resistance has been published [113].

The full potential of molecular diagnostics for drug resistance testing in microbiology has not reached its full potential—its application is still in its infancy. As the molecular mechanisms of antimicrobial resistance are described, newer tech nologies may enhance the utility of such an approach. Furthermore, microarray technology has the promise to impact the rapid and accurate detection of multiple

mutations associated with resistant bacteria, mycobacterium, viruses, and fungi. Until the full potential of drug resistant markers is understood, rapid molecular antimicrobial testing must still be combined with traditional microbial cultivation [109].

Summary

Bacteremia and sepsis are critically important syndromes with high mortality and associated costs. Bloodstream infections and sepsis are among the top causes of mortality in the US, killing over 500 people per day. Many septic patients are treated in Emergency Medicine Departments or Critical Care Units, settings in which rapid administration of targeted antibiotic therapy drastically reduces mortality. Unfortunately, routine blood cultures are too slow to support rapid therapeutic interventions. As a result, empiric, broad-spectrum treatment is common—a costly approach that may fail to effectively target the correct microbe, may inadvertently harm patients via antimicrobial toxicity, and may contribute to the evolution of drug-resistant microbes. To meet these diagnostic challenges, laboratories must understand the complexity of diagnosing and treating septic patients, in order to focus on creating algorithms that help direct targeted antibiotic therapy and synergize with existing EMD and CCU clinical practices put forth in Surviving Sepsis Guidelines.

Several methods for rapid molecular identification of pathogens from blood culture bottles are available. Laboratories can integrate with overall care to support local Surviving Sepsis Campaigns by providing rapid testing to facilitate targeted therapeutic interventions for infections with common hematopathogens. As a result, empiric, broad-spectrum, antibiotic therapy can be shortened to improve survival, reduce health care costs, and decrease antibiotic resistance. More evidence and clinical utility studies are needed to justify the added expense of molecular methods, to determine an appropriate niche in patient populations for which the cost benefit would be favorable. The PNA FISH methods show much promise for clinical utility and cost-effectiveness; however, the accuracy and cost benefit of multiplex PCR remains to be fully evaluated. Pyrosequencing and PCR/ESI-MS have proven accuracy and await practical adaption to routine microbiology laboratories. MALDI-TOF-MS shows promise and is currently being adapted to rapid identification of pathogen isolates and potentially to blood cultures.

The development of molecular diagnostic assays for detection of single pathogens from blood culture bottles has already shown to have impact on reducing mortality and costs. In the future, tests for multiple pathogens that could characterize gram positive, gram negative, and fungal infections would enable more rapid and targeted antimicrobial interventions for those with severe disease. Targeted drug-resistance genes testing of blood culture bottles will enable risk assessment and guide treatment options for sepsis. Ultimately, early intervention by molecular detection of bacteria and fungi directly from whole blood would provide the most patient benefit and contribute to a tailored antibiotic coverage of the patient early on in the course of the disease, allowing for more effective treatment and better outcomes of patients with sepsis and septic shock.

References

1. Esper A, Martin GS (2007) Is severe sepsis increasing in incidence AND severity? Crit Care Med 35(5):1414–1415
2. Danai P, Martin GS (2005) Epidemiology of sepsis: recent advances. Curr Infect Dis Rep 7(5):329–334
3. Cribbs SK, Martin GS (2007) Expanding the global epidemiology of sepsis. Crit Care Med 35(11):2646–2648
4. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR (2001) Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med 29(7):1303–1310
5. Balk RA (2000) Pathogenesis and management of multiple organ dysfunction or failure in severe sepsis and septic shock. Crit Care Clin 16(2):337–352, vii
6. Balk RA (2000) Severe sepsis and septic shock. Definitions, epidemiology, and clinical manifestations. Crit Care Clin 16(2):179–192
7. Sands KE, Bates DW, Lanken PN et al (1997) Epidemiology of sepsis syndrome in 8 academic medical centers. JAMA 278(3):234–240
8. Burchardi H, Schneider H (2004) Economic aspects of severe sepsis: a review of intensive care unit costs, cost of illness and cost effectiveness of therapy. Pharmacoeconomics 22(12): 793–813
9. Wenzel RP, Edmond MB (2001) Severe sepsis-national estimates. Crit Care Med 29(7): 1472–1474
10. Wang HE, Shapiro NI, Angus DC, Yealy DM (2007) National estimates of severe sepsis in United States emergency departments. Crit Care Med 35(8):1928–1936
11. Watson RS, Carcillo JA, Linde-Zwirble WT, Clermont G, Lidicker J, Angus DC (2003) The epidemiology of severe sepsis in children in the United States. Am J Respir Crit Care Med 167(5):695–701
12. Biswal S, Mishra P, Malhotra S, Puri GD, Pandhi P (2006) Drug utilization pattern in the intensive care unit of a tertiary care hospital. J Clin Pharmacol 46(8):945–951
13. Laupland KB, Lee H, Gregson DB, Manns BJ (2006) Cost of intensive care unit-acquired bloodstream infections. J Hosp Infect 63(2):124–132
14. Mokart D, Capo C, Blache JL et al (2002) Early postoperative compensatory anti-inflammatory response syndrome is associated with septic complications after major surgical trauma in patients with cancer. Br J Surg 89(11):1450–1456
15. Mokart D, Leone M, Sannini A et al (2005) Predictive perioperative factors for developing severe sepsis after major surgery. Br J Anaesth 95(6):776–781
16. Okabayashi T, Hanazaki K (2008) Overwhelming postsplenectomy infection syndrome in adults—a clinically preventable disease. World J Gastroenterol 14(2):176–179
17. Spelman D, Buttery J, Daley A et al (2008) Guidelines for the prevention of sepsis in asplenic and hyposplenic patients. Intern Med J 38(5):349–356
18. Olsen I (2008) Update on bacteraemia related to dental procedures. Transfus Apher Sci 39(2):173–178
19. Gurusamy KS, Kumar Y, Davidson BR (2008) Methods of preventing bacterial sepsis and wound complications for liver transplantation. Cochrane Database Syst Rev Oct 8;(4):CD006660
20. Williams MD, Braun LA, Cooper LM et al (2004) Hospitalized cancer patients with severe sepsis: analysis of incidence, mortality, and associated costs of care. Crit Care 8(5):R291–R298
21. Dellinger RP, Levy MM, Carlet JM et al (2008) Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2008. Intensive Care Med 34(1):17–60
22. Dellinger RP, Levy MM, Carlet JM et al (2008) Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2008. Crit Care Med 36(1):296–327

23. Rangel-Frausto MS, Pittet D, Costigan M, Hwang T, Davis CS, Wenzel RP (1995) The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study. JAMA 273(2):117–123
24. Nguyen HB, Rivers EP, Knoblich BP et al (2004) Early lactate clearance is associated with improved outcome in severe sepsis and septic shock. Crit Care Med 32(8):1637–1642
25. Rivers E, Nguyen B, Havstad S et al (2001) Early goal-directed therapy in the treatment of severe sepsis and septic shock. N Engl J Med 345(19):1368–1377
26. Kumar A, Haery C, Paladugu B et al (2006) The duration of hypotension before the initiation of antibiotic treatment is a critical determinant of survival in a murine model of *Escherichia coli* septic shock: association with serum lactate and inflammatory cytokine levels. J Infect Dis 193(2):251–258
27. Sharma S, Kumar A (2008) Antimicrobial management of sepsis and septic shock. Clin Chest Med 29(4):677–687, ix
28. Hunfeld KP, Bingold T, Brade V, Wissing H (2008) Molecular biological detection of pathogens in patients with sepsis. Potentials, limitations and perspectives. Anaesthesist 57(4):326–337
29. Knaus WA (1995) Mortality risk prediction in sepsis. Crit Care Med 23(10):1793–1794
30. Osborn TM, Nguyen HB, Rivers EP (2005) Emergency medicine and the surviving sepsis campaign: an international approach to managing severe sepsis and septic shock. Ann Emerg Med 46(3):228–231
31. Silva E, de Figueiredo LF, Colombari F (2010) Prowess-shock trial: a protocol overview and perspectives. Shock 34(Suppl 1):48–53
32. Thompson BT, Ranieri VM, Finfer S et al (2010) Statistical analysis plan of PROWESS SHOCK study. Intensive Care Med 36(11):1972–1973
33. Fenollar F, Raoult D (2007) Molecular diagnosis of bloodstream infections caused by non-cultivable bacteria. Int J Antimicrob Agents 30(Suppl 1):S7–S15
34. Kumar A, Roberts D, Wood KE et al (2006) Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med 34(6):1589–1596
35. Diab M, El-Damarawy M, Shemis M (2008) Rapid identification of methicillin-resistant staphylococci bacteremia among intensive care unit patients. Medscape J Med 10(5):126
36. Sturm PD, Kwa D, Vos FJ, Bartels CJ, Schulin T (2008) Performance of two tube coagulase methods for rapid identification of *Staphylococcus aureus* from blood cultures and their impact on antimicrobial management. Clin Microbiol Infect 14(5):510–513
37. Bennett K, Sharp SE (2008) Rapid differentiation of methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *Staphylococcus aureus* from blood cultures by use of a direct cefoxitin disk diffusion test. J Clin Microbiol 46(11):3836–3838
38. Qian Q, Eichelberger K, Kirby JE (2007) Rapid identification of *Staphylococcus aureus* in blood cultures by use of the direct tube coagulase test. J Clin Microbiol 45(7):2267–2269
39. Marlowe EM, Linscott AJ, Kanatani M, Bruckner DA (2002) Practical therapeutic application of the oxoid PBP2′ latex agglutination test for the rapid identification of methicillin-resistant *Staphylococcus aureus* in blood cultures. Am J Clin Pathol 118(2):287–291
40. Kristensen B, Hojbjerg T, Schonheyder HC (2001) Rapid immunodiagnosis of streptococci and enterococci in blood cultures. APMIS 109(4):284–288
41. Terlecka JA, du Cros PA, Orla MC, Spelman D (2007) Rapid differentiation of *Candida albicans* from non-albicans species by germ tube test directly from BacTAlert blood culture bottles. Mycoses 50(1):48–51
42. Harriau P, Ruffel F, Lardy JB (2006) Use of BioRad plating agar MRSASelect for the daily detection of methicillin resistant staphylococci isolated from samples taken from blood culture bottles. Pathol Biol (Paris) 54(8–9):506–509
43. Chan WW, Lovgren M, Tyrrell GJ (2009) Utility of the pneumoslide test in identification of *Streptococcus pneumoniae* in blood cultures and its relation to capsular serotype. J Clin Microbiol 47(5):1559–1561
44. Chung JW, Jeon HS, Sung H, Kim MN (2009) Evaluation of MicroScan and Phoenix system for rapid identification and susceptibility testing using direct inoculation from positive BACTEC blood culture bottles. Korean J Lab Med 29(1):25–34

45. Chen JR, Lee SY, Yang BH, Lu JJ (2008) Rapid identification and susceptibility testing using the VITEK 2 system using culture fluids from positive BacT/ALERT blood cultures. J Microbiol Immunol Infect 41(3):259–264
46. Wellinghausen N, Pietzcker T, Poppert S et al (2007) Evaluation of the Merlin MICRONAUT system for rapid direct susceptibility testing of gram-positive cocci and gram-negative bacilli from positive blood cultures. J Clin Microbiol 45(3):789–795
47. Funke G, Funke-Kissling P (2004) Use of the BD PHOENIX Automated Microbiology System for direct identification and susceptibility testing of gram-negative rods from positive blood cultures in a three-phase trial. J Clin Microbiol 42(4):1466–1470
48. Bruins MJ, Bloembergen P, Ruijs GJ, Wolfhagen MJ (2004) Identification and susceptibility testing of Enterobacteriaceae and *Pseudomonas aeruginosa* by direct inoculation from positive BACTEC blood culture bottles into Vitek 2. J Clin Microbiol 42(1):7–11
49. Ling TK, Liu ZK, Cheng AF (2003) Evaluation of the VITEK 2 system for rapid direct identification and susceptibility testing of gram-negative bacilli from positive blood cultures. J Clin Microbiol 41(10):4705–4707
50. Avolio M, Grosso S, Bruschetta G, De RR, Camporese A (2009) Direct antifungal susceptibility testing of positive Candida blood cultures by sensititre YeastOne. New Microbiol 32(2): 179–184
51. Rivers EP, McIntyre L, Morro DC, Rivers KK (2005) Early and innovative interventions for severe sepsis and septic shock: taking advantage of a window of opportunity. CMAJ 173(9): 1054–1065
52. Adelson ME, Feola M, Trama J, Tilton RC, Mordechai E (2005) Simultaneous detection of herpes simplex virus types 1 and 2 by real-time PCR and pyrosequencing. J Clin Virol 33(1): 25–34
53. Oliveira K, Haase G, Kurtzman C, Hyldig-Nielsen JJ, Stender H (2001) Differentiation of *Candida albicans* and *Candida dubliniensis* by fluorescent in situ hybridization with peptide nucleic acid probes. J Clin Microbiol 39(11):4138–4141
54. Oliveira K, Procop GW, Wilson D, Coull J, Stender H (2002) Rapid identification of *Staphylococcus aureus* directly from blood cultures by fluorescence in situ hybridization with peptide nucleic acid probes. J Clin Microbiol 40(1):247–251
55. Oliveira K, Brecher SM, Durbin A et al (2003) Direct identification of Staphylococcus aureus from positive blood culture bottles. J Clin Microbiol 41(2):889–891
56. Rigby S, Procop GW, Haase G et al (2002) Fluorescence in situ hybridization with peptide nucleic acid probes for rapid identification of *Candida albicans* directly from blood culture bottles. J Clin Microbiol 40(6):2182–2186
57. Sogaard M, Stender H, Schonheyder HC (2005) Direct identification of major blood culture pathogens, including *Pseudomonas aeruginosa* and *Escherichia coli*, by a panel of fluorescence in situ hybridization assays using peptide nucleic acid probes. J Clin Microbiol 43(4): 1947–1949
58. Stender H (2003) PNA FISH: an intelligent stain for rapid diagnosis of infectious diseases. Exp Rev Mol Diagn 3(5):649–655
59. Gonzalez V, Padilla E, Gimenez M et al (2004) Rapid diagnosis of *Staphylococcus aureus* bacteremia using *S. aureus* PNA FISH. Eur J Clin Microbiol Infect Dis 23(5):396–398
60. Wilson DA, Joyce MJ, Hall LS et al (2005) Multicenter evaluation of a Candida albicans peptide nucleic acid fluorescent in situ hybridization probe for characterization of yeast isolates from blood cultures. J Clin Microbiol 43(6):2909–2912
61. Alexander BD, Ashley ED, Reller LB, Reed SD (2006) Cost savings with implementation of PNA FISH testing for identification of *Candida albicans* in blood cultures. Diagn Microbiol Infect Dis 54(4):277–282
62. Hartmann H, Stender H, Schafer A, Autenrieth IB, Kempf VA (2005) Rapid identification of *Staphylococcus aureus* in blood cultures by a combination of fluorescence in situ hybridization using peptide nucleic acid probes and flow cytometry. J Clin Microbiol 43(9):4855–4857
63. Chapin KC, Musgnug MC (2003) Direct susceptibility testing of positive blood cultures by using Sensititre broth microdilution plates. J Clin Microbiol 41(10):4751–4754

64. Oliveria KM, Brecher SM, Shapiro DS, Stender H (2007) Rapid identification of *S. aureus* and differentiation between MRSA and MSSA from blood culture bottles using *S. aurues* PNA FISH and MRSA EVIGENE. In: IDSA 2005 Annual Meeting
65. Shepard JR, Addison RM, Alexander BD et al (2008) Multicenter evaluation of the *Candida albicans*/*Candida glabrata* peptide nucleic acid fluorescent in situ hybridization method for simultaneous dual-color identification of *C. albicans* and *C. glabrata* directly from blood culture bottles. J Clin Microbiol 46(1):50–55
66. Reller ME, Mallonee AB, Kwiatkowski NP, Merz WG (2007) Use of peptide nucleic acid-fluorescence in situ hybridization for definitive, rapid identification of five common Candida species. J Clin Microbiol 45(11):3802–3803
67. Holtzman C, Whitney D, Barlam T, Miller NS (2011) Assessment of impact of peptide nucleic acid fluorescence in situ hybridization for rapid identification of coagulase-negative staphylococci in the absence of antimicrobial stewardship intervention. J Clin Microbiol 49(4):1581–1582
68. Gamage DC, Olson DP, Stickell LH et al (2011) Significant decreases in mortality and hospital costs after laboratory testing with PNA FISH (Abstract 3339; #D1302b). In: Proceedings of the 51st International Conference on Antimicrobial Agents and Chemotherapy(ICAAC), Chicago, IL, 19 Sept 2011
69. Toombs L, Weeks E, Forrest GN, Licalis D, Johnson JK, Venezia RA (2007) Impact of Peptide Nucleic Acid (PNA) Fluorescence in situ Hybridization (FISH) for Enterococcocal blood stream infections. In: IDSA 2006 Annual Meeting
70. Morgan M, Marlowe E, Della-Latta P et al (2010) Multicenter evaluation of a new shortened peptide nucleic acid fluorescence in situ hybridization procedure for species identification of select Gram-negative bacilli from blood cultures. J Clin Microbiol 48(6):2268–2270
71. Morgan MA, Marlowe E, Novak-Weekly S et al (2011) A 1.5 hour procedure for identification of Enterococcus Species directly from blood cultures. J Vis Exp 48:e2616
72. Forrest GN, Roghmann MC, Toombs LS et al (2008) Peptide nucleic acid fluorescent in situ hybridization for hospital-acquired enterococcal bacteremia: delivering earlier effective antimicrobial therapy. Antimicrob Agents Chemother 52(10):3558–3563
73. Ly T, Gulia J, Pyrgos V, Waga M, Shoham S (2008) Impact upon clinical outcomes of translation of PNA FISH-generated laboratory data from the clinical microbiology bench to bedside in real time. Ther Clin Risk Manage 4(3):637–640
74. Hensley DM, Tapia R, Encina Y (2009) An evaluation of the advandx *Staphylococcus aureus*/CNS PNA FISH assay. Clin Lab Sci 22(1):30–33
75. Pappas PG, Rex JH, Sobel JD et al (2004) Guidelines for treatment of candidiasis. Clin Infect Dis 38(2):161–189
76. Forrest GN, Mehta S, Weekes E, Lincalis DP, Johnson JK, Venezia RA (2006) Impact of rapid in situ hybridization testing on coagulase-negative staphylococci positive blood cultures. J Antimicrob Chemother 58(1):154–158
77. Forrest GN, Mankes K, Jabra-Rizk MA et al (2006) Peptide nucleic acid fluorescence in situ hybridization-based identification of *Candida albicans* and its impact on mortality and antifungal therapy costs. J Clin Microbiol 44(9):3381–3383
78. Peleg AY, Tilahun Y, Fiandaca MJ et al (2009) Utility of peptide nucleic acid fluorescence in situ hybridization for rapid detection of Acinetobacter spp. and *Pseudomonas aeruginosa*. J Clin Microbiol 47(3):830–832
79. Wolk DM, Struelens MJ, Pancholi P et al (2009) Rapid detection of Staphylococcus aureus and methicillin-resistant *S. aureus* (MRSA) in wound specimens and blood cultures: multicenter preclinical evaluation of the Cepheid Xpert MRSA/SA skin and soft tissue and blood culture assays. J Clin Microbiol 47(3):823–826
80. Katayama Y, Zhang HZ, Chambers HF (2004) PBP 2a mutations producing very-high-level resistance to beta-lactams. Antimicrob Agents Chemother 48(2):453–459
81. Ito T, Kuwahara K, Hisata K, Okuma K, Cui L, Hiramatsu K (2004) Community-associated methicillin-resistant *Staphylococcus aureus*: current status and molecular epidemiological perspective. Kansenshogaku Zasshi 78(6):459–469

82. Donnio PY, Oliveira DC, Faria NA, Wilhelm N, Le CA, de Lencastre H (2005) Partial excision of the chromosomal cassette containing the methicillin resistance determinant results in methicillin-susceptible *Staphylococcus aureus*. J Clin Microbiol 43(8):4191–4193
83. Francois P, Bento M, Renzi G, Harbarth S, Pittet D, Schrenzel J (2007) Evaluation of three molecular assays for rapid identification of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 45(6):2011–2013
84. Stamper PD, Cai M, Howard T, Speser S, Carroll KC (2007) Clinical validation of the molecular BD GeneOhm StaphSR assay for direct detection of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* in positive blood cultures. J Clin Microbiol 45(7):2191–2196
85. Grobner S, Dion M, Plante M, Kempf VA (2009) Evaluation of the BD GeneOhm StaphSR assay for detection of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from spiked positive blood culture bottles. J Clin Microbiol 47(6):1689–1694
86. Karahan ZC, Mumcuoglu I, Guriz H et al (2006) PCR evaluation of false-positive signals from two automated blood-culture systems. J Med Microbiol 55(Pt 1):53–57
87. Marlowe EM, Wolk DM (2006) Pathogen detection in the genomic era. In: Tang Y (ed) Advanced techniques in diagnostic microbiology. Springer, New York, NY
88. Wolk DM, Marlowe EM (2008) Future trends in diagnosis of infections in the immunocompromised population. In: Hayden RT, Tang YW, Carroll KC, Wolk DM (eds) Diagnostic microbiology of the immunocompromised host, 1st edn. ASM Press, Washington, DC
89. Ecker DJ, Massire C, Blyn LB et al (2009) Molecular genotyping of microbes by multilocus PCR and mass spectrometry: a new tool for hospital infection control and public health surveillance. Methods Mol Biol 551:71–87
90. Hujer KM, Hujer AM, Endimiani A et al (2009) Rapid determination of quinolone resistance in Acinetobacter spp. J Clin Microbiol 47(5):1436–1442
91. Ecker DJ, Sampath R, Massire C et al (2008) Ibis T5000: a universal biosensor approach for microbiology. Nat Rev Microbiol 6(7):553–558
92. Baldwin CD, Howe GB, Sampath R et al (2009) Usefulness of multilocus polymerase chain reaction followed by electrospray ionization mass spectrometry to identify a diverse panel of bacterial isolates. Diagn Microbiol Infect Dis 63(4):403–408
93. Hall TA, Sampath R, Blyn LB et al (2009) Rapid molecular genotyping and clonal complex assignment of *Staphylococcus aureus* isolates by PCR coupled to electrospray ionization-mass spectrometry. J Clin Microbiol 47(6):1733–1741
94. Wolk DM, Blyn L, Hall TA et al (2009) Pathogen Profiling: Rapid Molecular Characterization of *Staphylococcus aureus* by PCR/Electrospray Ionization-Mass Spectrometry and Correlation with Phenotype. J Clin Microbiol 47(10):3129–3137
95. Kaleta EJ, Clark AE, Johnson DR et al (2011) Use of PCR coupled with electrospray ionization mass spectrometry for rapid identification of bacterial and yeast bloodstream pathogens from blood culture bottles. J Clin Microbiol 49(1):345–353
96. Kaleta EJ, Clark AE, Cherkaoui A et al (2011) Comparative analysis of PCR-electrospray ionization/mass spectrometry (MS) and MALDI-TOF/MS for the identification of bacteria and yeast from positive blood culture bottles. Clin Chem 57(7):1057–1067
97. Dierkes C, Ehrenstein B, Siebig S, Linde HJ, Reischl U, Salzberger B (2009) Clinical impact of a commercially available multiplex PCR system for rapid detection of pathogens in patients with presumed sepsis. BMC Infect Dis 9(1):126
98. Casalta JP, Gouriet F, Roux V, Thuny F, Habib G, Raoult D (2009) Evaluation of the LightCycler SeptiFast test in the rapid etiologic diagnostic of infectious endocarditis. Eur J Clin Microbiol Infect Dis 28(6):569–573
99. Mancini N, Clerici D, Diotti R et al (2008) Molecular diagnosis of sepsis in neutropenic patients with haematological malignancies. J Med Microbiol 57(Pt 5):601–604
100. Bravo D, Blanquer J, Tormo M et al (2011) Diagnostic accuracy and potential clinical value of the LightCycler SeptiFast assay in the management of bloodstream infections occurring in neutropenic and critically ill patients. Int J Infect Dis 15(5):e326–e331
101. Josefson P, Stralin K, Ohlin A et al (2011) Evaluation of a commercial multiplex PCR test (SeptiFast) in the etiological diagnosis of community-onset bloodstream infections. Eur J Clin Microbiol Infect Dis 30(9):1127–1134

102. Lodes U, Meyer F, Konig B, Lippert H (2009) Microbiological sepsis screening in surgical ICU patients with the "lightCycler" Septifast test—a pilot study. Zentralbl Chir 134(3):249–253
103. Steinmann J, Buer J, Rath PM, Paul A, Saner F (2009) Invasive aspergillosis in two liver transplant recipients: diagnosis by SeptiFast. Transpl Infect Dis 11(2):175–178
104. Quiles-Melero I, Garcia-Rodriguez J, Romero-Gomez MP, Gomez-Sanchez P, Mingorance J (2011) Rapid identification of yeasts from positive blood culture bottles by pyrosequencing. Eur J Clin Microbiol Infect Dis 30(1):21–24
105. Wellinghausen N, Kochem AJ, Disque C et al (2009) Diagnosis of bacteremia in whole-blood samples by use of a commercial universal 16S rRNA gene-based PCR and sequence analysis. J Clin Microbiol 47(9):2759–2765
106. Kuhn C, Disque C, Muhl H, Orszag P, Stiesch M, Haverich A (2011) Evaluation of commercial universal rRNA gene PCR plus sequencing tests for identification of bacteria and fungi associated with infectious endocarditis. J Clin Microbiol 49(8):2919–2923
107. Jordan JA, Butchko AR, Durso MB (2005) Use of pyrosequencing of 16S rRNA fragments to differentiate between bacteria responsible for neonatal sepsis. J Mol Diagn 7(1):105–110
108. Tang YW, Kilic A, Yang Q et al (2007) StaphPlex system for rapid and simultaneous identification of antibiotic resistance determinants and Panton-Valentine leukocidin detection of staphylococci from positive blood cultures. J Clin Microbiol 45(6):1867–1873
109. Diekema DJ, Dodgson KJ, Sigurdardottir B, Pfaller MA (2004) Rapid detection of antimicrobial-resistant organism carriage: an unmet clinical need. J Clin Microbiol 42(7):2879–2883
110. Fluit AC, Visser MR, Schmitz FJ (2001) Molecular detection of antimicrobial resistance. Clin Microbiol Rev 14(4):836–871 (Table)
111. Rasheed JK, Tenover FC (2003) Detection and characterization of antimicrobial resistance genes in bacteria. In: Murray PR, Murray EJ, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (eds) Manual of clinical microbiology, 8th edn. ASM Press, Washington, DC, pp 1196–1213
112. Tenover FC, Rasheed JK (2004) Detection of antimicrobial resistance genes and mutations associated with antimicrobial resistance in microorganisms. In: Persing DH, Tenover FC, Versalovic J, Tang Y, Unger ER, Relman DA et al (eds) Molecular microbiology: diagnostic principles and practice. ASM Press, Washington, DC, pp 391–406
113. Edlind TD (2004) Molecular detection of antifungal resistance. In: Persing DH, Tenover FC, Versalovic J, Tang Y, Unger ER, Relman DA et al (eds) Molecular microbiology: diagnostic principles and practice. ASM Press, Washington, DC, pp 569–575

Chapter 45
Advanced Pathology Techniques for Detecting Emerging Infectious Disease Pathogens

Wun-Ju Shieh and Sherif R. Zaki

Introduction

Detection and surveillance for emerging and reemerging pathogens need a multidisciplinary approach. The intertwining complexity of these pathogens with their diverse tissue tropisms, direct effects on host cells, multiphasic immunological responses, and additional influence of superimposed secondary agents is beyond the expertise of a single discipline in modern medicine. A combined evaluation of patient's history, clinical manifestations, and physical examination may suggest a list of differential diagnosis, but it is often insufficient to determine the specific infectious etiology. Laboratory methods are essential to identify an etiologic agent from testing clinical samples, such as blood, serum, nasopharyngeal swab, etc. These methods, including traditional microbiological techniques, conventional immunological assays, and modern molecular methods, remain the mainstay in today's practice of clinical microbiology and infectious disease medicine. Nevertheless, there are technical and logistic issues associated with these methods, and the test results often lack a clinicopathologic correlation that can confound the interpretation of their clinical significance. For example, microbiological culture may fail to grow a causative organism, while the organism isolated by the laboratory in vitro may arise from contamination and does not represent the actual infective agent in vivo.

Pathology plays a key role as a bridging subspecialty in such multidisciplinary approach. Pathologic examination, if available, can establish a more specific diagnosis correlated with clinical manifestations. Although general practice of pathology

W.-J. Shieh (✉) • S.R. Zaki
Infectious Diseases Pathology Branch, Division of High-Consequence Pathogens & Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, N.E., Mail Stop G-32, Atlanta, GA 30333, USA
e-mail: wshieh@cdc.gov

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Table 45.1 Examples of outbreaks caused by emerging pathogens initially identified or confirmed by pathologic studies

Year(s)	Disease outbreak	Country or geopolitical region
1993	Hantavirus pulmonary syndrome	USA
1995	Ebola hemorrhagic fever	Zaire
1995	Leptospirosis associated with pulmonary hemorrhage	Nicaragua
1996	Lassa hemorrhagic fever	Sierra Leone
1997	Enterovirus 71 hand-foot-and-mouth disease with encephalitis	Malaysia
1997	H5N1 influenza	Hong Kong
1998	Enterovirus 71 hand-foot-and mouth disease with encephalitis	Taiwan
1999	Nipah virus encephalitis	Malaysia
1999	West Nile encephalitis	USA
2000	Rift Valley fever	Saudi Arabia/Yemen
2000	Ebola hemorrhagic fever	Uganda
2001	Inhalational and cutaneous anthrax	USA
2002	Transplant-associated West Nile encephalitis	USA
2003	Sever acute respiratory syndrome	Global
2003	Monkeypox	USA
2003, 2005, 2007, 2010	Transplant-associated lymphocytic choriomeningitis virus	USA
2004	Transplant-associated rabies	USA
2006/2007	Rift Valley fever	Kenya/Somalia
2008	Lujo virus hemorrhagic fever	Zambia/South Africa
2009	H1N1 pandemic influenza	Global
2009	Transplant-associated *Balamuthia mandrillaris*	USA
2010	Dengue hemorrhagic fever	Puerto Rico
2011	Leptospirosis	Puerto Rico

is largely oriented toward diagnosis of neoplastic diseases, pathologists have been increasingly called upon to make diagnoses from tissue samples collected by cytology, biopsy, and autopsy procedures in response to the challenge of emerging infections [1–4]. Using these tissue samples as the source for laboratory workup, pathologists have made various contributions to our understanding of emerging infectious diseases in diagnostics, pathogenesis, epidemiology, and clinical aspects of these diseases (Table 45.1). In addition, results from pathologic studies can help design better strategies for control and prevention of these emerging infectious diseases, especially when they occur as an outbreak [5, 6]. Furthermore, pathologic studies also play an essential role in identifying the effects of secondary pathogens that commonly complicate the primary disease syndrome [7, 8].

Recent advances in molecular biology have revolutionized the practice of medicine, especially in the arena of diagnostic pathology and laboratory medicine [9–11]. The practice of pathology has evolved from using morphologic pattern

recognition as the main tool to a sophisticated medical subspecialty by applying a wide array of advanced immunologic and molecular techniques on top of the traditional methods. The so-called "traditional methods" include routine hematoxylin and eosin (H&E) stain, histochemical (special) stain, and electron microscopy (EM). The more commonly used advanced techniques include immunohistochemistry (IHC), in situ hybridization (ISH), polymerase chain reaction assay (PCR), and tissue microarrays. Other advanced techniques that are less standardized as diagnostic utilities include confocal microscopy, proteomics, laser capture microdissection (LCM), and in situ PCR. The results from these techniques provide different information regarding the infectious agents in the organ systems they involve (Table 45.2). Each technique has its respective advantages and limitations, and there is no single technique that can stand alone as the only method for etiologic diagnosis. The advanced techniques complement the traditional methods to confirm the diagnosis; therefore, it is always necessary to apply these techniques as an integrated laboratory utility to take full advantage of the pathology approach. A good example to illustrate such approach is the identification of a novel coronavirus during the global epidemic of severe acute respiratory syndrome (SARS) in 2003 [12–17]. By using traditional culture (Fig. 45.1a) and EM examinations (Fig. 45.1b) on clinical samples and tissue specimens, the morphologic evidence of coronavirus leads to subsequent anatomic localization of this novel virus in lung tissues by using a combination of IHC (Fig. 45.1c), ISH (Fig. 45.1d), and PCR. Ultimately, correlations of these data with serological and clinical findings confirmed the SARS-associated coronavirus (SARS-CoV) as the etiologic pathogen of the outbreak. This is a prime example of the contributions made by infectious disease pathology as part of a multidisciplinary approach to investigate emerging infections and disease outbreaks.

Table 45.2 Pathology techniques and their utilities for infectious disease diagnosis

Technique	Main utility	Remarks
Hematoxylin & Eosin Stain (H&E)	Shows histopathologic features of infectious process	* Illustrates the evidence of a microbial infection and provides guidance to subsequent laboratory testing * Does not highlight the pathogen per se * Can only suggest certain infections and not a specific etiologic organism
Histochemical stain (special stain)	Highlights organisms	* More useful for bacterial, mycobacterial, and fungal organisms * Only categorizes organisms within a broad classification but not a specific species * Can be difficult to interpret
Electron microscopy (EM)	Illustrates microbial ultrastructure	* The most direct evidence to show an infectious agent * Time consuming and limited to small areas of interest

(continued)

Table 45.2 (continued)

Technique	Main utility	Remarks
Immunohistochemistry (IHC)	Localizes microbial antigens	* Demonstrates antigens regardless the organism is intact or not * Provides histomorphologic correlation of infectious process * Many commercially available antibodies for common pathogens * Antibodies of novel pathogens may not be readily available * Formalin fixation may decrease sensitivity
In situ hybridization (ISH)	Localizes microbial nucleic acids	* Probes can be synthesized in-house with known sequence * Provides histomorphologic correlation of infectious process * Usually more specific but less sensitive than IHC * Formalin fixation may decrease sensitivity
Polymerase chain reaction assay (PCR)	Amplifies small amount of microbial nucleic acids	* Usually more sensitive than IHC and ISH * Contamination issues frequently encountered * Does not provide histomorphologic correlation of infectious process * Formalin fixation may decrease sensitivity
Tissue microarray	Detects multiple microbial nucleic acids	* Facilitate sequence analysis and pathogen identification * Can detect microbes and assess related host responses simultaneously * Biosafety concerns using frozen tissues * Less sensitive than conventional PCR
Confocal microscopy	Increases morphologic dimension	* Provides wider spectrum for histopathologic or cytologic interpretation * Limited diagnostic utility for emerging pathogens
Laser capture microdissection (LCM)	Dissect specific target cells for PCR or proteomic studies	* Useful in studies of pathogenesis * Limited diagnostic utility for emerging pathogens
In situ polymerase chain reaction assay	Localizes microbial nucleic acids with amplification process	* Combines amplification and in situ localization methods * Inherent technical issues with nonstandardized protocols * Formalin fixation may decrease sensitivity * Limited diagnostic utility for emerging pathogens
Proteomics	Detects microbial and host peptides	* Useful in studies of pathogenesis * Formalin fixation may decrease sensitivity * Limited diagnostic utility for emerging pathogens

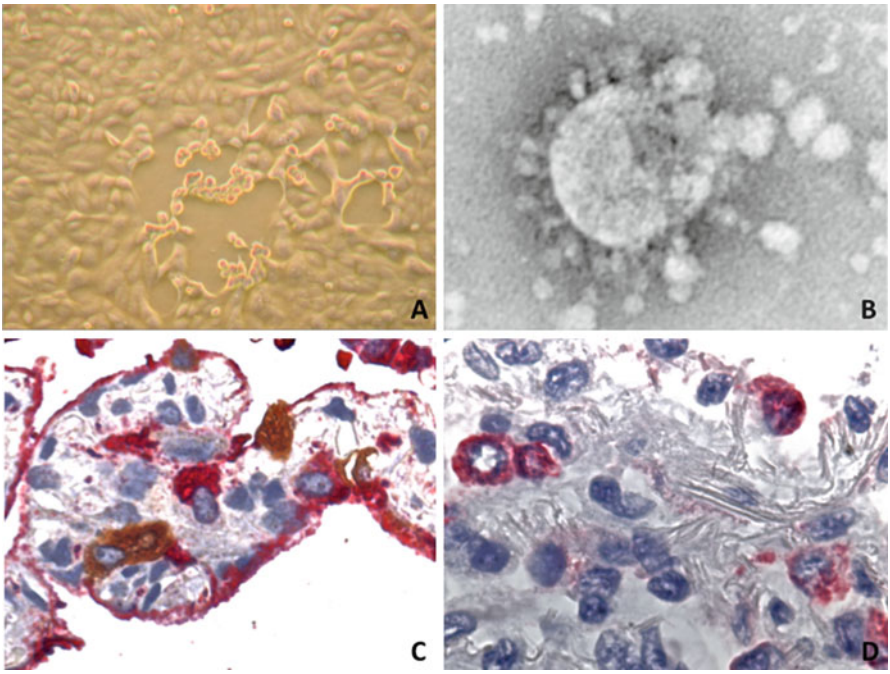

Fig. 45.1 (**a**) Vero E6 cells show early cytopathic effect with coronavirus isolates from patients with SARS. (Courtesy of Dr. Thomas G. Ksiazek). (**b**) Negative stain (methylamine tungstate stain) electron microscopy shows coronavirus particle with an internal helical nucleocapsid-like structure and club-shaped surface projections. (Courtesy of Dr. Charles D. Humphrey). (**c**) Double-stain IHC (immunoalkaline phosphatase polymer and peroxidase polymer) shows SARS-CoV (red) and surfactant antigens (brown) in type II pneumocytes. (**d**) ISH shows SARS-CoV nucleic acids in pneumocytes

Highlights of Techniques

Hematoxylin & Eosin Stain

Any pathology laboratory dealing with clinical diagnosis routinely performs H&E stain. It demonstrates the histologic and cytologic features in a tissue section and allows the pathologists to examine the microscopic changes related to infectious processes. This is the most unequivocal method to illustrate the evidence of a microbial infection and its consequence in the tissue. For example, the presence of abundant neutrophils in pulmonary alveoli is indicative of pneumonia (Fig. 45.2a), while neutrophils in meninges support the diagnosis of meningitis. However, these histopathologic findings shown by H&E stain are not specific because they can be caused by a variety of organisms; their importance is to pave the first step leading to further laboratory assays for detecting the causative agent.

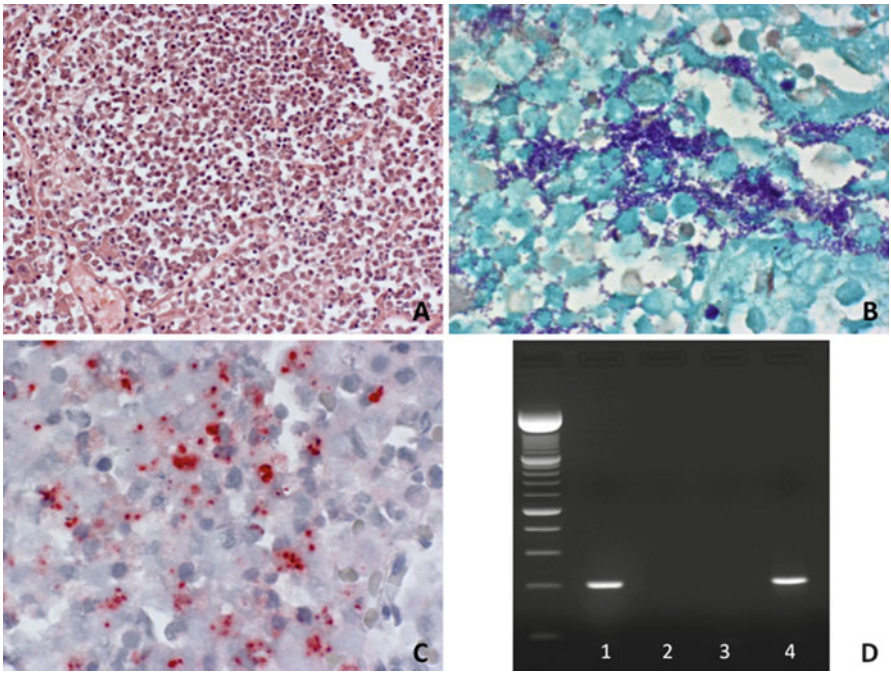

Fig. 45.2 (**a**) H&E stain shows abundant polymorphonuclear inflammatory cells in alveoli indicative of an acute pneumonia. (**b**) Gram stain highlights numerous gram-positive cocci mixed with inflammatory cells. (**c**) IHC with anti-S. *pneumoniae* antibody shows abundant extracellular and intracellular bacterial antigens. (**d**) PCR targeting pneumolysin gene of S. *pneumonia* shows positive amplicon. (lane 1: positive control; lane 2: negative control; lane 3: water control; lane 4: lung sample tested)

Histochemical Stains (Special Stains)

Many histochemical stains have been developed to highlight a variety of microbial organisms. Some of the common ones are tissue Gram stain (for bacteria), Grocott's methenamine silver stain (for fungi), acid-fast stain (for mycobacteria), periodic acid-Schiff stain (for organisms with high content of carbohydrate macromolecules), Warthin–Starry silver stain or Steiner's silver stain (for spirochetes and other bacteria). Interpretation of these special stains performed on tissue sections is usually more difficult than those performed on cultures because the coexistence of host tissue responses and accompanied histopathologic changes in the sections can confound the interpretation. It needs more expertise and effort to examine these special stains and usually requires a trained pathologist to carry out such examination. For example, *Streptococcus pneumoniae* can appear as gram-negative cocci in tissue sections because the host inflammatory responses, antibiotic treatment, or autolysin produced by the bacteria per se can damage the bacterial cell wall and render the

Gram stain appear negative. Even when these special stains properly highlight organisms of interest, they can only categorize them within a broad classification but not a specific species. For example, gram-positive cocci demonstrated by tissue Gram stain in a lung section (Fig. 45.2b) could represent different species of Streptococci or Staphylococci, and further testing with more specific assays is needed to reveal the true identity of these cocci.

Electron Microscopy

Four decades ago, EM was the only ancillary technique available to the pathologists when routine H&E and special stains failed to reveal diagnostic features in histopathology [18]. EM examination provides a direct visualization of microbial organisms at a high magnification. Ultrastructural finding is the most direct evidence to show the presence of an infectious agent in clinical specimens. Thin section and negative stain are two common EM methods used to study pathogen morphology and morphogenesis of the microorganisms with recognition of their cytoplasmic organelles and matrix constituents. Therefore, correlation of light and electron microscopic findings not only improves pathologist's diagnostic acumen but also allows for a more coherent explanation of the pathogenesis. Since the advent of immunohistochemical and molecular techniques, EM has been less often used for identifying infectious agents. However, EM still played an essential role in determining the specific family of the pathogen involved in several outbreaks caused by novel viruses, such as Sin Nombre virus [19, 20], Nipah virus [21, 22], SARS-CoV [12, 23], and monkeypox virus [24]. In these outbreak investigations, negative stain of virus isolated from tissue culture and thin-section preparation of tissue specimen facilitated the ultrastructural examination. The determination of etiologic agents guided subsequent laboratory, clinical, and epidemiologic investigations. Advanced EM methods, such as immuno-EM or EM in situ hybridization using colloidal gold labels, have been developed for a more specific ultrastructural diagnosis.

Immunohistochemistry

IHC has been widely used in all aspects of pathology diagnosis in the past three decades [25–27]. A large number of IHC is available that can be helpful in the identification of microorganisms. By using a variety of antibodies, IHC can detect the presence of microbial antigens in tissue specimens, whether they represent the intact or degraded pathogens, and whether they are intracellular or extracellular (Fig. 45.2c). Therefore, IHC has become a powerful technique used by pathologists for tissue diagnosis of infectious diseases. There are many ways to visualize an antibody–antigen interaction. The most common method is to apply an antibody conjugated to an enzyme, such as peroxidase [28–30] or alkaline

phosphatase [31, 32], which can further catalyze a reaction for colorimetric detection. The antibodies used for specific detection can be polyclonal or monoclonal. Polyclonal antibodies are a heterogeneous mixture of antibodies that recognize several epitopes of a specific organism or more commonly, a group of related organisms. Monoclonal antibodies are generated against a single epitope and hence more specific to the target antigen than polyclonal antibodies. Many of these antibodies are commercially available and are widely used in diagnostic pathology laboratories. Others, especially those antibodies for detecting novel emerging pathogens, are available only at highly specialized centers such as the Centers for Disease Control and Prevention. Development of new IHC is a worthwhile but usually labor-intensive task. Similar to all other laboratory assays, the sensitivity and specificity of any IHC always need a careful evaluation before establishing its status as a diagnostic assay.

Detection of two or more target antigens on one slide can be achieved with multiple staining IHC assays [33–35]. These assays can expand the information obtained from each slide and reduce turnaround-time compared to single staining or sequential staining methods. It is possible to assess the topographic relationship of the targets by using multiple staining IHC assays for determining the cellular tropism of viral infection with antibodies raised against virus and specific cellular markers respectively (Fig. 45.1c). These multiple staining methods not only help confirm the immunolocalization of pathogens but also enhance further understanding of pathogenesis in many emerging infections [7, 16, 19].

There are many advantages of using formalin-fixed tissues and IHC to detect etiologic pathogens. It is particular useful in detecting those fastidious or slow-growing organisms, such as mycobacteria [36, 37] or *Tropheryma whipplei* [38], and can improve the speed, sensitivity, and specificity of microbial diagnosis. It is also valuable for characterizing emerging infections, whose causes are initially unknown, such as those caused by Nipah virus [21] or SARS-CoV [12]. Immunolocalization of antigens by IHC provides histomorphologic correlation between the infectious pathogen and host tissue responses, which is not only crucial for diagnosis but also important to study the pathogenesis of those emerging infections [19, 21, 39, 40]. Additionally, IHC performed on fixed tissues can minimize laboratory worker's potential risk of exposure to infectious agents because of the deactivation of pathogens by formalin fixation. Another advantage of using IHC is its capability of detecting well-preserved microbial antigens in archived formalin-fixed, paraffin-embedded (FFPE) tissues, which allows retrospective studies of many emerging pathogens even after decades of archive [41, 42].

In Situ Hybridization

ISH is a technique that uses fluorescent or radiolabeled nucleic acid probes comprising complementary DNA or RNA strand to localize specific sequences in tissue sections [43, 44]. It has been applied in many medical diagnostics, such as gene

expression profiling, chromosomal integrity, and karyotyping, etc. There are also many ways to perform ISH in diagnosis of infectious pathogens with a variety of probes [45–50], including double-stranded DNA (dsDNA) probes, single-stranded DNA (ssDNA) probes, RNA probes (riboprobes), and synthetic oligonucleotides (oligoprobes). ISH can localize nucleic acids of microorganisms in tissues and provides histomorphologic correlation between the infectious pathogen and host tissue responses, similar to IHC. The advantages of using formalin-fixed tissues and ISH to detect etiologic pathogens are also similar to IHC, except it is usually less sensitive than IHC because of the potential fragmentation of target nucleic acids by formalin fixation [51, 52]. On the other hand, ISH can utilize in-house probes synthesized in a well-equipped laboratory with known sequences of the target nucleic acids, minimizing the need to depend on commercial resources.

Polymerase Chain Reaction Assay

PCR amplification undoubtedly is the most sensitive method available to detect microbial organisms in tissue specimens and has become a common practice in many pathology laboratories. PCR can be performed on FFPE samples [53–56]; therefore diagnoses can be made even if cultures were not obtained initially from biopsy or autopsy at the time of processing. In addition, molecular identification can accelerate definitive diagnosis of fastidious organisms that either grow slowly or does not grow at all with culture methods. When combined with other techniques mentioned above, PCR has markedly improved the capabilities of providing rapid and accurate detection of many emerging and reemerging pathogens as well as pathogens commonly encountered in medical practice.

PCR requires the isolation of nucleic acids from microorganisms in clinical samples and needs to apply adjunct techniques with restriction endonuclease enzymes, gel electrophoresis (Fig. 45.2d), and other nucleic acid hybridization methods. Degenerate primers can be employed in PCR assays at reduced stringency to facilitate detection of related but unknown organisms [12, 57, 58]. A vast number of PCR-based techniques have been developed in the past two decades and have been increasingly applied to clinical samples. For instance, multiplex PCR has been shown to increase the diagnostic yield in acute respiratory tract infections and contribute to overall improved outcome in patient care [59, 60]. New platforms such as real-time polymerase chain reaction (rt-PCR) combine nucleic acid amplification and fluorescent detection of the amplified product in the same closed system, resulting in an excellent technique that can diagnose a wide spectrum of infectious pathogens with tremendous flexibility, rapidity, and accuracy [55, 59, 61–63]. Nucleic acid sequence analysis has become highly automated and is now practical for use in many diagnostic and reference laboratories for the identification of a large number of microorganisms, whether they are cultivatable or not.

One particularly prevalent utility of PCR is the usage of wide-range paneubacteria 16S ribosomal RNA (16S rRNA) PCR for detecting unknown bacterial organisms in tissue specimens. 16S rRNA is 1,542 nt in length and is a component of the 30S subunit of prokaryotic ribosomes. The16S rRNA gene in bacteria contains well-conserved sequences that can be used as binding sites for universal PCR primers adjacent to variable sequences [64–66]. Subsequent analyses and comparisons of the sequences from amplicons to databases of known sequences can provide valuable information for etiologic diagnosis and further speciation. A set of broad-range PCR primers directed against conserved regions in the 16S rRNA gene was designed to specifically amplify either gram-positive or gram-negative bacteria [67]. These differential 16S rRNA gene PCR assays provide more specific information regarding the bacteria identity and are very useful for detecting bacterial pathogens in tissue samples in conjunction with histopathologic evaluation, special stains, and IHC.

Despite their high sensitivity, PCR techniques often face challenges from potential contamination issues. Processing of tissue samples, especially autopsy tissues, is often performed under a rather lax sterile condition and may enhance the chance of contamination. Many infectious pathogens can be present in the environment as commensals and their clinical relevance from PCR testing results can be confounded by such nature. Therefore, the PCR results should always be evaluated within the context of other diagnostic criteria. Moreover, any PCR testing of formalin-fixed tissues may be compromised by damage to DNA caused by the fixative. It is also important to know that identification to the species level may not be rigorous because the target gene may contain limited amount of sequence data available for comparison.

Microarrays

Microarrays can be performed on frozen tissue samples and may be helpful when multiplex PCR or other nucleic acid methods fail [68–70]. However, the sensitivity is generally lower than those multiplex PCR methods. Viral microarrays can be roughly divided into those targeting 10–100 agents and those designed for detection of thousands of agents, including unknown pathogens. Arrays designed to address a limited number of agents may employ multiplex consensus PCR to amplify specific genetic targets. Oligonucleotide microarrays with probes of up to 70 nt can offer a considerable advantage for detection of rapidly evolving targets, such as RNA viruses because these arrays are less likely to be confounded by minor sequence variation. Viral microarrays can facilitate sequence analysis and pathogen identification [68, 71–73]. Additionally, both microbial and host gene targets can incorporated in these high-density arrays, thus allows an opportunity to detect microbes and assess related host responses simultaneously for pathogenic features consistent with various classes of infectious agents.

Other Advanced Techniques

Other advanced pathology techniques such as confocal microscopy [74], proteomics [75–77], laser capture microdissection (LCM) [78, 79], and in situ PCR [80] have been used sparingly for detecting novel pathogens in a few specialized laboratories. Although they can become potentially powerful tools for diagnosis of emerging infections, most of them remain as pilot utilities and need further optimization to gain wide acceptance as mainstream techniques in practice of infectious diseases pathology.

General Guidelines of Using Pathology Techniques

Appropriate clinical specimen collection, transport, and processing are crucial to establish an accurate laboratory diagnosis of infectious diseases. Similarly, adequate tissue sampling is the first and the most important step to obtain an organism-specific diagnosis of infectious diseases by using pathology techniques. The pathology laboratory must have practical guidelines for optimal specimen collection and handling, and should communicate this information to the clinical staff and patient care sites. It is prudent to obtain biopsy or surgical samples from the precise site of infection and preferably before initiation of therapy to minimize the impact of treatment on subsequent diagnostic tests. This is particularly true for bacterial or fungal infections. Tissue specimens obtained surgically are acquired at great expense and pose considerable risk to the patient; therefore they should be procured with an amount of material adequate for both histopathologic and microbiological examination. Swabs are rarely adequate for this purpose. Representative samples from all major organs should be collected in autopsy cases, especially those unexplained fatal cases due to infectious causes.

Etiologic pathogens may be focally or sparsely present in involved organs and only a complete postmortem examination can attentively localize the causative organisms, as well as the full spectrum of their pathologic effects. In addition, the predilection site for infection may vary among different organisms. For example, herpes simplex virus tends to involve temporal lobe in the brain more frequently, while West Nile virus usually causes more severe infection in brain stem and spinal cord. Moreover, since multiple organs can be involved in the context of systemic diseases, collecting multiple representative portions of target organs with syndrome-based approach (Table 45.3) and tissue samples from any other organ system with findings suggestive of infection ensures the best chance of detecting the causative agent. Influenza-associated myocarditis is a good example to show the difficulty of identifying influenza virus in the heart tissue even with prominent histopathologic changes of myocarditis, while the evidence of infection is usually present in the respiratory tissues [81].

Table 45.3 Tissue sample collection with syndrome-based approach

Target system (syndrome)	Representative tissue sample collection
Central nervous system (meningitis, encephalitis, myelitis)	Cerebral cortex (frontal, parietal, temporal, and occipital), brain stem (midbrain, pons, medulla), spinal cord, cerebellum, basal ganglia, thalamus, hypothalamus, hippocampus, and meninges
Respiratory system (laryngitis, tracheiits, bronchitis, pneumonia, pulmonary hemorrhage)	Larynx, trachea, left and right main bronchi, hilar lung with segmental bronchi, and peripheral pulmonary parenchyma from both lungs
Cardiovascular system (myocarditis, endocarditis)	Ventricles, atrium, including endocardium, epicardium, and pericardium
Hepatobilliary system (hepatitis, cholecystitis, hepatic failure)	Different areas of liver, gall bladder
Gastrointestinal system (gastritis, enteritis, intestinal perforation, intussusception)	Esophagus, stomach, small intestine, large intestine, appendix, and mesenteric lymph nodes
Urinary system (nephritis, cystitis, renal failure)	Renal cortex and medulla, urinary bladder, and adrenal gland
Reproductive system (cervicitis, endometritis, pelvic inflammatory diseases, funisitis, chorioamnionitis)	Cervix, uterus (endometrium and myometrium), ovary, fallopian tube, umbilical cord, placenta
Cutaneous system (skin rashes, including macule, papule, vesicle, pustule, ulceration, and eschar)	Minimally, a 3 mm punch, deep shave, or excisional biopsy specimen from the representative rash lesion. Multiple biopsies should be obtained if multiple stages or forms of cutaneous lesions are identified

FFPE tissue samples are usually adequate for routine H&E stain, special stains, IHC, and ISH assays. However, prolonged formalin fixation can cause cross-linking of proteins and nucleic acids in tissues and hence decrease the sensitivity of IHC, ISH, or PCR assays. In general, antigens and nucleic acids in tissue samples can be well preserved in paraffin-embedded blocks if formalin fixation does not exceed 2 weeks. It is highly recommended to embed tissue samples in paraffin no longer than 72 h after adequate formalin fixation. Although FFPE blocks can also be used for ultrastructural examination, it is preferably to dissect tissue samples into small thin pieces (1 mm^3), placed in glutaraldehyde fixative, and stored in a refrigerator for optimal EM studies.

Sterile techniques are mandatory to obtain target tissue samples for microbiologic culture and PCR assays. While biopsy procedure is usually performed under a stringent sterile condition, autopsy is not. In addition, delay of postmortem examination will facilitate colonization by normal flora or contamination by environmental organisms and interfere subsequent diagnostic assays. Therefore, autopsy should be performed as soon as possible (preferably within 12 h after death) to minimize these postmortem confounding factors. Representative tissue samples for potential PCR assay should be obtained with sterile technique and frozen at −70 °C. It is noteworthy that FFPE can also be used for PCR testing if frozen samples are not readily available, but the sensitivity is usually lower because of the chemical property of formalin fixative mentioned earlier.

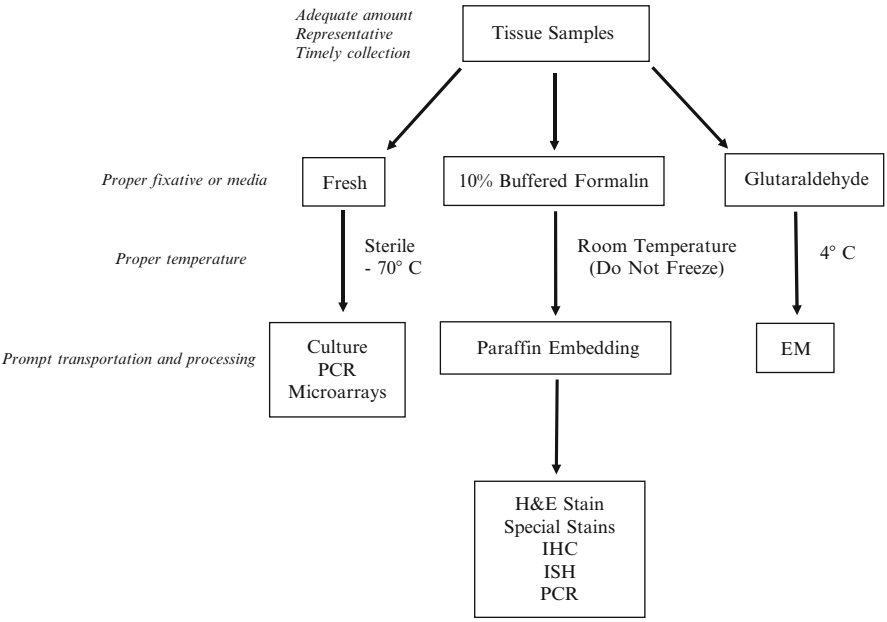

Fig. 45.3 Optimal tissue collections for pathologic studies

A diagram of optimal tissue collection for pathologic studies is shown in Fig. 45.3.

Summary

Diagnosis with pathologic techniques provides histomorphologic correlation for a specific infectious agent with the disease it causes and is essential for identifying the cause of death. It helps identify or confirm the etiology of an outbreak caused by a novel pathogen, especially from severe or fatal cases. It is crucial for management of clinical patient with unknown etiology of infection, control and prevention for emerging disease outbreak, epidemiologic surveillance, and study of pathogenesis. Tissue samples, especially postmortem specimens, should be collected adequately and promptly. They should be preserved in proper media and processed in a timely fashion. The histopathologic features identified in the tissue specimens in conjunction with relevant clinical and epidemiologic information should determine performance of specific IHC, ultrastructural, molecular, or other assays.

There are limitations of using pathologic techniques despite the advantages. Because immune mechanisms can greatly amplify the host response, the actual numbers of pathogens present in tissues can be relatively small. This means that many sections may need to be examined before a pathogen is identified. Topographic

issues related to tissue sampling can also affect the outcome of tests. If the tissue specimens are not obtained from relevant lesions or areas with histopathologic changes, the subsequent tests performed on such specimens can all result in false-negative outcomes. Timing of tissue sampling, as mentioned earlier, is another crucial element that can affect test results. Delayed autopsy procedure increases the chance of tissue autolysis and postmortem contamination, which can significantly interfere with histopathologic evaluation and all related pathologic tests. Technical issues, such as sensitivity and specificity, are universally present for each IHC, ISH, or PCR testing. A negative result cannot exclude the possibility of an infection caused by certain organisms because duration of illness, modalities of treatment, tissue sampling and fixation may affect the outcome of these assays. Therefore, a correlation of the test results with clinical history, epidemiological information, and other laboratory assays is highly recommended for a more accurate interpretation involving in patient care and public health management.

References

1. Schwartz DA, Bryan RT, Hughes JM (1995) Pathology and emerging infections—quo vadimus? Am J Pathol 147:1525–1533
2. Shieh W-J, Guarner J, Paddock C et al (2003) The critical role of pathology in the investigation of bioterrorism-related cutaneous anthrax. Am J Pathol 163:1901–1910
3. Walker DH, Dumler JS (1995) Will pathologists play as important a role in the future as they have in the past against the challenge of infectious diseases. Infect Agents Dis 4:167–170
4. Procop GW, Wilson M (2001) Infectious disease pathology. Clin Infect Dis 32:1589–1601
5. Zaki SR, Shieh WJ (1996) Leptospirosis associated with outbreak of acute febrile illness and pulmonary haemorrhage, Nicaragua, 1995. The Epidemic Working Group at Ministry of Health in Nicaragua. Lancet 347:535–536
6. Shieh WJ, Guarner J, Layton M et al (2000) The role of pathology in an investigation of an outbreak of West Nile encephalitis in New York, 1999. Emerg Infect Dis 6:370–372
7. Shieh W-J, Blau DM, Denison AM et al (2010) 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. Am J Pathol 177:166–175
8. Guarner J, Paddock CD, Shieh W-J et al (2006) Histopathologic and immunohistochemical features of fatal influenza virus infection in children during the 2003–2004 season. Clin Infect Dis 43:132–140
9. Naber SP (1994) Molecular pathology—diagnosis of infectious disease. N Engl J Med 331:1212–1215
10. Madea B, Saukko P, Oliva A, Musshoff F (2010) Molecular pathology in forensic medicine—introduction. Forensic Sci Int 203:3–14
11. Finn WG (2007) Diagnostic pathology and laboratory medicine in the age of “omics”: a paper from the 2006 William Beaumont Hospital Symposium on Molecular Pathology. J Mol Diagn 9:431–436
12. Ksiazek TG, Erdman D, Goldsmith CS et al (2003) A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 348:1953–1966
13. Kuiken T, Fouchier RAM, Schutten M et al (2003) Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. Lancet 362:263–270
14. Berger A, Drosten C, Doerr HW, Stürmer M, Preiser W (2004) Severe acute respiratory syndrome (SARS)—paradigm of an emerging viral infection. J Clin Virol 29:13–22

15. Franks TJ, Chong PY, Chui P et al (2003) Lung pathology of severe acute respiratory syndrome (SARS): a study of 8 autopsy cases from Singapore. Hum Pathol 34:743–748
16. Shieh W-J, Hsiao C-H, Paddock CD et al (2005) Immunohistochemical, in situ hybridization, and ultrastructural localization of SARS-associated coronavirus in lung of a fatal case of severe acute respiratory syndrome in Taiwan. Hum Pathol 36:303–309
17. Drosten C, Günther S, Preiser W et al (2003) Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 348:1967–1976
18. Sobrinho-Simoes M, Nesland JM, Johannessen JV (1981) Diagnostic ultrastructural pathology—sub-speciality or special stain? Diagn Histopathol 4:223–236
19. Zaki SR, Greer PW, Coffield LM et al (1995) Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. Am J Pathol 146:552–579
20. Goldsmith CS, Elliott LH, Peters CJ, Zaki SR (1995) Ultrastructural characteristics of Sin Nombre virus, causative agent of hantavirus pulmonary syndrome. Arch Virol 140:2107–2122
21. Wong KT, Shieh W-J, Kumar S et al (2002) Nipah virus infection: pathology and pathogenesis of an emerging paramyxoviral zoonosis. Am J Pathol 161:2153–2167
22. Goldsmith CS, Whistler T, Rollin PE et al (2003) Elucidation of Nipah virus morphogenesis and replication using ultrastructural and molecular approaches. Virus Res 92:89–98
23. Goldsmith CS, Tatti KM, Ksiazek TG et al (2004) Ultrastructural characterization of SARS coronavirus. Emerg Infect Dis 10:320–326
24. Bayer-Garner IB (2005) Monkeypox virus: histologic, immunohistochemical and electron-microscopic findings. J Cutan Pathol 32:28–34
25. Brandtzaeg P (1998) The increasing power of immunohistochemistry and immunocytochemistry. J Immunol Methods 216:49–67
26. Leong AS, Wright J (1987) The contribution of immunohistochemical staining in tumour diagnosis. Histopathology 11:1295–1305
27. Jaffer S, Bleiweiss IJ (2004) Beyond hematoxylin and eosin—the role of immunohistochemistry in surgical pathology. Cancer Invest 22:445–465
28. Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG (1970) The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen–antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315–333
29. Bosman FT, Cramer-Knijnenburg G, van Bergen Henegouw J (1980) A simplified method for the rapid preparation of peroxidase-anti peroxidase (PAP) complexes. Histochemistry 67:243–248
30. Mason DY, Sammons R (1978) Rapid preparation of peroxidase: anti-peroxidase complexes for immunocytochemical use. J Immunol Methods 20:317–324
31. Cordell JL, Falini B, Erber WN et al (1984) Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J Histochem Cytochem 32:219–229
32. Jackson R, Holme ER, Phimister GM, Kennedy A, McLay AL (1990) Immunoalkaline phosphatase technique applied to paraffin wax embedded tissues in diagnostic renal pathology. J Clin Pathol 43:665–670
33. Mason DY, Sammons R (1978) Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. J Clin Pathol 31:454–460
34. Tao Q (1994) Double-immunostaining method using biotin-conjugated primary antibodies from the same species. J Histochem Cytochem 42:439
35. Krenacs T, Krenacs L, Raffeld M (2010) Multiple antigen immunostaining procedures. Methods Mol Biol 588:281–300
36. Wiley EL, Mulhollan TJ, Beck B, Tyndall JA, Freeman RG (1990) Polyclonal antibodies raised against Bacillus Calmette-Guerin, *Mycobacterium duvalii*, and *Mycobacterium paratuberculosis* used to detect mycobacteria in tissue with the use of immunohistochemical techniques. Am J Clin Pathol 94:307–312

37. Carabias E, Palenque E, Serrano R, Aguado JM, Ballestin C (1998) Evaluation of an immunohistochemical test with polyclonal antibodies raised against mycobacteria used in formalin-fixed tissue compared with mycobacterial specific culture. APMIS 106:385–388
38. Baisden BL, Lepidi H, Raoult D, Argani P, Yardley JH, Dumler JS (2002) Diagnosis of Wihipple disease by immunohistochemical analysis: a sensitive and specific method for the detection of *Tropheryma whipplei* (the Whipple bacillus) in paraffin-embedded tissue. Am J Clin Pathol 118:742–748
39. Genrich GL, Guarner J, Paddock CD et al (2007) Fatal malaria infection in travelers: novel immunohistochemical assays for the detection of *Plasmodium falciparum* in tissues and implications for pathogenesis. Am J Trop Med Hyg 76:251–259
40. Burt FJ, Swanepoel R, Shieh WJ et al (1997) Immunohistochemical and in situ localization of Crimean-Congo hemorrhagic fever (CCHF) virus in human tissues and implications for CCHF pathogenesis. Arch Pathol Lab Med 121:839–846
41. Zaki SR, Khan AS, Goodman RA et al (1996) Retrospective diagnosis of hantavirus pulmonary syndrome, 1978–1993: implications for emerging infectious diseases. Arch Pathol Lab Med 120:134–139
42. Webb PR, Powell L, Denyer M et al (2009) A retrospective immunohistochemical study reveals atypical scrapie has existed in the United Kingdom since at least 1987. J Vet Diagn Invest 21:826–829
43. Jin L, Lloyd RV (1997) In situ hybridization: methods and applications. J Clin Lab Anal 11:2–9
44. Werner M, Wilkens L, Aubele M, Nolte M, Zitzelsberger H, Komminoth P (1997) Interphase cytogenetics in pathology: principles, methods, and applications of fluorescence in situ hybridization (FISH). Histochem Cell Biol 108:381–390
45. Ha Y, Chae C (2009) Optimal probe size and fixation time for the detection of porcine circovirus-2 DNA by in situ hybridization in formalin-fixed, paraffin-embedded tissue. J Vet Diagn Invest 21:649–654
46. Han KH, Hollinger FB, Noonan CA, Yoffe B (1992) Simultaneous detection of HBV-specific antigens and DNA in paraffin-embedded liver tissue by immunohistochemistry and in situ hybridization using a digoxigenin-labeled probe. J Virol Methods 37:89–97
47. Aksamit AJ, Mourrain P, Sever JL, Major EO (1985) Progressive multifocal leukoencephalopathy: investigation of three cases using in situ hybridization with JC virus biotinylated DNA probe. Ann Neurol 18:490–496
48. Choi YJ (1990) In situ hybridization using a biotinylated DNA probe on formalin-fixed liver biopsies with hepatitis B virus infections: in situ hybridization superior to immunochemistry. Mod Pathol 3:343–347
49. Saglie R, Cheng L, Sadighi R (1988) Detection of *Mycoplasma pneumoniae*-DNA within diseased gingiva by in situ hybridization using a biotin-labeled probe. J Periodontol 59:121–123
50. Shin JH, Molitor TW (2002) Localization of porcine reproductive and respiratory syndrome virus infection in boars by in situ riboprobe hybridization. J Vet Sci 3:87–96
51. Mulder WA, van Poelwijk F, Moormann RJ et al (1997) Detection of early infection of swine vesicular disease virus in porcine cells and skin sections. A comparison of immunohistochemistry and in-situ hybridization. J Virol Methods 68:169–175
52. Tatti KM, Gentsch J, Shieh W-J et al (2002) Molecular and immunological methods to detect rotavirus in formalin-fixed tissue. J Virol Methods 105:305–319
53. Hofman V, Selva E, Landraud L et al (2003) Value of PCR amplification from formalin-fixed paraffin-embedded tissues in the diagnosis of *Mycobacterium tuberculosis* infection. Annales de pathologie 23:206–215
54. Bhatnagar J, Guarner J, Paddock CD et al (2007) Detection of West Nile virus in formalin-fixed, paraffin-embedded human tissues by RT-PCR: a useful adjunct to conventional tissue-based diagnostic methods. J Clin Virol 38:106–111
55. Denison AM, Blau DM, Jost HA et al (2011) Diagnosis of influenza from respiratory autopsy tissues detection of virus by real-time reverse transcription-PCR in 222 cases. J Mol Diagn 13:123–128

56. Tang YW (2009) Duplex PCR assay simultaneously detecting and differentiating *Bartonella quintana*, *B. henselae*, and *Coxiella burnetii* in surgical heart valve specimens. J Clin Microbiol 47:2647–2650
57. Wintermantel WM, Hladky LL (2010) Methods for detection and differentiation of existing and new crinivirus species through multiplex and degenerate primer RT-PCR. J Virol Methods 170:106–114
58. Chua KB, Bellini WJ, Rota PA et al (2000) Nipah virus: a recently emergent deadly paramyxovirus. Science (New York, NY) 288:1432–1435
59. Abdeldaim GM, Stralin K, Korsgaard J, Blomberg J, Welinder-Olsson C, Herrmann B (2010) Multiplex quantitative PCR for detection of lower respiratory tract infection and meningitis caused by *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*. BMC Microbiol 10:310
60. Wang W, Ren P, Sheng J et al (2009) Simultaneous detection of respiratory viruses in children with acute respiratory infection using two different multiplex reverse transcription-PCR assays. J Virol Methods 162:40–45
61. Gadsby NJ, Hardie A, Claas EC, Templeton KE (2010) Comparison of the Luminex Respiratory Virus Panel fast assay with in-house real-time PCR for respiratory viral infection diagnosis. J Clin Microbiol 48:2213–2216
62. Agrawal AS, Sarkar M, Chakrabarti S et al (2009) Comparative evaluation of real-time PCR and conventional RT-PCR during a 2 year surveillance for influenza and respiratory syncytial virus among children with acute respiratory infections in Kolkata, India, reveals a distinct seasonality of infection. J Med Microbiol 58:1616–1622
63. Mentel R, Wegner U, Bruns R, Gurtler L (2003) Real-time PCR to improve the diagnosis of respiratory syncytial virus infection. J Med Microbiol 52:893–896
64. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703
65. Clarridge JE 3rd (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 17:840–862 (table of contents)
66. Greisen K, Loeffelholz M, Purohit A, Leong D (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol 32:335–351
67. Klausegger A, Hell M, Berger A et al (1999) Gram type-specific broad-range PCR amplification for rapid detection of 62 pathogenic bacteria. J Clin Microbiol 37:464–466
68. Jabado OJ, Conlan S, Quan P-L et al (2010) Nonparametric methods for the analysis of single-color pathogen microarrays. BMC Bioinform 11:354
69. Kellam P (2001) Post-genomic virology: the impact of bioinformatics, microarrays and proteomics on investigating host and pathogen interactions. Rev Med Virol 11:313–329
70. Fukui S, Feizi T, Galustian C, Lawson AM, Chai W (2002) Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. Nat Biotechnol 20:1011–1017
71. Lipkin WI (2008) Pathogen discovery. PLoS Pathog 4:e1000002
72. Lipkin WI, Palacios G, Briese T (2009) Diagnostics and discovery in viral hemorrhagic fevers. Ann N Y Acad Sci 1171(Suppl 1):E6–E11
73. Palacios G, Druce J, Du L et al (2008) A new arenavirus in a cluster of fatal transplant-associated diseases. N Engl J Med 358:991–998
74. Pawley JB (2006) Handbook of biological confocal microscopy, 3rd edn. Springer, New York, NY
75. List EO, Berryman DE, Bower B et al (2008) The use of proteomics to study infectious diseases. Infect Disord Drug Targets 8:31–45
76. Mazzulli T, Low DE, Poutanen SM (2005) Proteomics and severe acute respiratory syndrome (SARS): emerging technology meets emerging pathogen. Clin Chem 51:6–7

77. Ye Y, Mar E-C, Tong S et al (2010) Application of proteomics methods for pathogen discovery. J Virol Methods 163:87–95
78. Baisse B, Bian YS, Benhattar J (2000) Microdissection by exclusion and DNA extraction for multiple PCR analyses from archival tissue sections. Biotechniques 28:856–858, 860, 862
79. Xu BJ (2010) Combining laser capture microdissection and proteomics: methodologies and clinical applications. Proteomics Clin Appl 4:116–123
80. Nuovo GJ (1995) In situ PCR: protocols and applications. PCR Methods Appl 4:S151–S167
81. Nolte KB, Alakija P, Oty G et al (2000) Influenza A virus infection complicated by fatal myocarditis. Am J Forensic Med Pathol 21:375–379

Chapter 46
Diagnosis and Assessment of Microbial Infections with Host and Microbial microRNA Profiles

Lunbiao Cui, Charles W. Stratton, and Yi-Wei Tang

Introduction

Biomarkers are continuously being sought in the field of diagnostic microbiology for the laboratory diagnosis and assessment of microbial infections. A set of clinical and laboratory criteria necessary for an ideal diagnostic marker of infection have previously been proposed by Ng and his colleagues [1]. According these criteria, an ideal biomarker should possess at a minimum the following characteristics: (a) biochemically, a biomarker should be stable and remain significantly deregulated in the body fluid compartment for at least 12–24 h even after commencement of appropriate treatment that may allow an adequate time window for specimen collection or storage without significant decomposition of the active compound until laboratory processing; (b) its concentration should be determined quantitatively and the method of measurement should be automatic, rapid, easy, and inexpensive; (c) the collection of a specimen should be minimally invasive and require a small volume (e.g., <0.5 mL blood). Numerous biomarkers have been found and tested in clinical practice. Currently, microRNA (miRNA) molecules are without a doubt the biomarkers with the greatest potential capacities in the diagnostic microbiology field.

The first miRNA, lin-4, was inadvertently discovered in *Caenorhabditis elegans* in 1993 by Lee and colleagues [2]. However, recognition of the miRNA let-7 and its ability to regulate lin-14 by Ruvkun and colleagues [3] in 2000 resulted in the

L. Cui (✉)
Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China
e-mail: cui_lb@163.com

C.W. Stratton
Departments of Pathology and Medicine, Vanderbilt University Medical Center, Nashville, TN 37212, USA

Y.-W. Tang
Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 37212, USA

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establishment of this new class of regulatory nucleic acids and their potential value in diagnostic microbiology.

The majority of the characterized miRNA is intergenic and is located in introns. Primary miRNA transcripts, i.e., pri-miRNAs, are transcribed by RNA polymeraseII or III. In the nucleus, ribonuclease Drosha cleaves pri-miRNAs and releases 60–80-nt stem-loop intermediate structures named pre-miRNAs. The pre-miRNAs are transported to cytoplasm by exportin-5 protein, and then are cleaved by Dicer RNase III to form a mature double-stranded miRNA. One strand of the miRNA duplex is subsequently unwound and then incorporated into an effector protein complex termed RNA-induced silencing complex (RISC), which is responsible for the gene silencing in a posttranscriptional manner [4–6]. Mature miRNAs are single-stranded RNA molecules of about 19–25 nucleotides in length. Through partial homology to the 3′-untranscribed region (UTR) in target mRNAs, miRNAs control of gene expression via repression of translation as well as reducing mRNA levels directly.

A large number of miRNAs have been found in various animal and plant tissues. According to miRBase 18.0 (http://microrna.sanger.org/), which is a collective registry of currently known miRNA sequences and targets hosted by the Sanger Institute, there are currently 1,527 recognized miRNA sequences in the *Homo sapiens* genome; this number is constantly growing as new miRNA sequences are discovered. Recently, virus-encoded miRNAs have been discovered; these miRNAs may function as controls for viral replication and thus limit antiviral responses, inhibit apoptosis, and stimulate cellular growth [7]. Moreover, unique host cell miRNAs expression profiles have been revealed in response to various microbial infections [8, 9]. Host miRNA appears to play a role in viral replication and may be used by host cells to control viral infection.

Beginning in 2008, miRNAs have been found circulating in serum and plasma as well as other body fluids such as saliva, tears, and urine. Some of these miRNAs appear to be enriched in specific fluids [10, 11]. These circulating miRNAs subsequently have become the focus of ongoing research. The properties, origin, function, and relationship with disease of circulating miRNAs have been intensively explored. A number of important observations have been noted. Circulating miRNAs are present in a stable form that is protected from endogenous RNase activity [12, 13]. Some groups also reported a higher stability of miRNAs compared to mRNA in samples obtained from formalin-fixed paraffin-embedded tissues [14–16]. The expression level of miRNAs has been noted as consistent among individuals of the same species [12]. Expression alteration of circulating miRNAs has been reported to be associated with pathophysiological states including various cancers, heart disease, pregnancy, and diabetes [17, 18]. Needless to say, serum, plasma, and other body fluid specimens are generally available for clinical testing. Profiling hundreds of miRNA requires only 200 μL sera [13]. Thus, these unique and stable characteristics of circulating miRNAs potentially make them extremely useful biomarkers for disease diagnosis and prognosis.

Virus-Encoded miRNAs

The first virus-encoded miRNA was described by Pfeffer and his colleagues in 2004 when they identified five Epstein–Barr virus (EBV)-encoded pre-miRNAs [19]. Since then, hundreds of virus-encoded miRNAs have been described in humans, animals, and plants. Examples of human virus-encoded miRNA are shown in Table 46.1. Bewilderingly, more than 95 % of the virus-encoded miRNAs known today are of herpesvirus origin. Moreover, almost all virus-encoded miRNAs are encoded by DNA viruses except those encoded by retroviruses (HIV-1), which reverse-transcribe and integrate their genetic material into host DNA. Aberrantly expressed circulating miRNAs have been explored for the diagnosis and prognosis of several infectious diseases, including sepsis [20, 21], HBV [22], and HCV [23].

Viral miRNAs target perfectly complementary viral mRNAs as well as imperfectly complementary viral and/or cellular mRNAs. Viral miRNAs modulate expression of host gene involved in cell proliferation and survival, stress responses, and antiviral defense pathways, which are pivotal for viral replication. Another primary function of virus miRNAs is to regulate the latent-lytic switch. During latency, the host cell

Table 46.1 Virus-encoded miRNAs related to human infection (Adapted with permission from miRBase 18.0 (http://microrna.sanger.org/))

Virus family	Virus	No. of pre-miRNA	Names
α-Herpesviruses	HSV-1	16	hsv1-mir-H1 to hsv1-mir-H8, hsv1-mir-H11 to hsv1-mir-H18
	HSV-2	18	hsv2-mir-H2 to hsv2-mir-H7, hsv2-mir-H9 to hsv2-mir-H13, hsv2-mir-H19 to hsv2-mir-H25
β-Herpesviruses	hCMV	11	hcmv-mir-UL22A, hcmv-mir-UL36, hcmv-mir-UL70, hcmv-mir-UL112, hcmv-mir-UL148D, hcmv-mir-US4, hcmv-mir-US5-1, hcmv-mir-US5-2, hcmv-mir-US25-1, hcmv-mir-US25-2, hcmv-mir-US33
γ-Herpesviruses	EBV	25	ebv-mir-BART1 to ebv-mir-BART22, ebv-mir-BHRF1-1 to ebv-mir-BHRF1-3
	KSHV	13	kshv-mir-K12-1 to kshv-mir-K12-9, kshv-mir-K12-10a, kshv-mir-K12-10b, kshv-mir-K12-11, kshv-mir-K12-12
Polyomaviruses	Simian virus 40	1	sv40-mir-S1
	JC polyomavirus	1	jcv-mir-J1
	BK polyomavirus	1	bkv-mir-B1
	Merkel cell polyomavirus	1	mcv-mir-M1
Retroviridae	HIV-1	3	hiv1-mir-H1, hiv1-mir-N367, hiv1-mir-TAR

maintains the viral genome and only a limited portion of virus genome is expressed. Viral gene expression is restricted, but virus miRNAs and their precursors are regularly detected [24]. There is an accumulating amount of evidence that has demonstrated that virus-encoded miRNAs mediate evolutionarily conserved functions (e.g., immune evasion, cell cycle control, and promotion of latency, etc.). The miRNAs themselves show poorly primary sequence conservation [25]. These phenomena raise an important question: can viral miRNAs be used as the detection maker for virus infection during the latent infection phase despite the fact that little or no viral protein is being produced? Further studies are needed to evaluate this hypothesis.

Host miRNA Response in Relation to Microbial Infection

Microbial infections are known to downmodulate at least some cellular mRNAs and thereby exert physiological effects. Microbial infections induce changes in the host miRNA expression profile, which may also have a profound effect on the outcome of infection. Host miRNA may directly or indirectly affect virus replication and pathogenesis. For example, liver-specific miR-122 is required for HCV replication [26, 27]. Moreover, miR-28, miR-125b, miR-150, miR-223, and miR-382 are overexpressed in resting CD4+ T lymphocytes compared to their activated counterparts. These miRNAs are able to target sequences near the 3′ portion of HIV-1 mRNA. This finding suggests that miRNAs may contribute to viral latency [28]. However, it is unclear whether these miRNAs are actively inhibited by viral factors or whether their deregulation is due to host responses. Host miRNA expression profiles have been noted to represent specific pathophysiological states [17, 18]. Theoretically, a characteristic profile should be potential biomarkers for disease diagnosis and prognosis. A number of studies have been conducted to demonstrate this theory and promising results have been seen in a number of altered physiological states including various cancers, heart disease, pregnancy, diabetes, injury, and infection. The use of these miRNA profiles in infection diseases is discussed in next section.

HIV-1 and Other Human Retroviruses

Houzet and colleagues have profiled miRNAs in peripheral blood mononuclear cells (PBMCs) from HIV-1 infected patients. They found the T cell abundant miRNAs (miR-223, miR-150, miR-146, miR-16, and miR-191) were downregulated three- to ninefold compared to cells from uninfected controls, depending on the disease stage of the patient [29]. Triloubet et al. reported increased expression of 11 miRNAs including miR-122, miR-370, miR-373*, and miR-297 in HIV-1 infected Jurkat cells, whereas expression of the polycistronic miRNA cluster miR17/92 (comprises miR-17-(5p/3p), miR-18, miR-19a, miR-20a, miR-19b-1, and miR-92-1) was strongly decreased [30]. Like HIV-1, human T cell leukemia virus type 1

(HTLV-1) also infects CD4+ T cells. Two miRNA-profiling studies have been performed in infected cell lines and ATL (adult T-cell leukemia) cells [31, 32]. The studies find two common miRNAs that are consistently downregulated in the context of HTLV-1 infection.

Respiratory Viruses

The miRNA expression profile in bronchoalveolar stem cells (BASCs) infected with SARS coronavirus (CoV) has been determined using miRNA microarray [33]. A total of 116 miRNAs were found differentially expressed. Upregulated BASC miRNAs-17*, -574-5p, and -214 are co-opted by SARS-CoV to suppress its own replication and evade immune elimination until successful transmission takes place. In contrast, viral nucleocapsid and spike protein targets seem to co-opt downregulated miR-223 and miR-98 respectively. Differentially expressed miRNAs in chicken lung and trachea infected with a low pathogenic strain of H5N3 avian influenza virus were analyzed by a deep sequencing approach [34]. A total of 73 and 36 miRNAs are differentially expressed in lungs and trachea upon virus infection, respectively. Lung cellular "microRNAome" of mice infected by reconstructed 1918 influenza virus was compared with that of mice infected by a nonlethal seasonal influenza virus, A/Texas/36/91. A group of microRNAs, including miR-200a and miR-223, was differentially expressed in response to influenza virus infection and infection by these two influenza viruses induced distinct microRNA expression profiles [35].

Adenovirus

We have previously analyzed the miRNA expression profiles from adenovirus type 3 (AD3) infected human laryngeal epithelial (Hep2) cells using a SOLiD deep sequencing. A total of 44 miRNAs demonstrated high expression and 36 miRNAs showed lower expression in the AD3 infected cells than in control cells [36].

Human Herpesviruses

Wang et al. monitored the time course of cellular miRNA expression in human cytomegalovirus (CMV) infected cells using miRNA microarrays. Forty-nine miRNAs significantly changed on at least one time point [37]. There were no global unidirectional changes, with changes for these miRNAs sometimes being transient. The miR-199a/214 cluster (miR-199a-5p, miR-199a-3p, and miR-214) has recently been found to be downregulated in CMV-infected cells [38].

Herpes Simplex Viruses

Infection of human primary neural cells with a high phenotypic reactivator herpes simplex viruses-1 (HSV-1) (17syn+) can induce upregulation of a brain-enriched microRNA (miRNA)-146a [39]. Both miR-101 and miR-132 are also found to be highly upregulated after HSV-1 [40, 41]. Kaposi's sarcoma (KS) associated herpesvirus (KSHV) is the etiological agent of KS. The M type K15 protein of KSHV induces the expression of microRNAs miR-21 and miR-31 via this conserved motif [42], while K13 strongly stimulated upregulation of miR-146a [43].

Epstein–Barr Virus

EBV is an oncogenic herpes virus that is endemic in humans and also can be found in about 15 % of patients with diffuse large B-cell lymphoma (DLBCL). EBV de novo infection of primary cultured human B-cells results in a dramatic downregulation of cellular miRNA expression, with 99.5 % of the miRNAs detected being downregulated, with an average downregulation of 19.92-fold [44]. Imig et al. found that expression of hsa-miR-424, -223, -199a-3p, -199a-5p, -27b, -378, -26b, -23a, -23b were upregulated and those of hsa-miR-155, -20b, -221, -151-3p, -222, -29b/c, -106a were downregulated more than twofold due to EBV-infection of DLBCL [45]. Cameron et al. demonstrated differential expression of cellular miRNAs in type III versus type I EBV latency including elevated expression of miR-21, miR-23a, miR-24, miR-27a, miR-34a, miR-146a and b, and miR-155. In contrast, miR-28 expression was found to be lower in type III latency [46].

Bacterial Infections

In vitro infection assays have revealed that *Helicobacter pylori* infection can affect miRNA expression profiles: specifically, miRNAs such as miRNA-155, miRNA-16, and miRNA-146a are significantly upregulated in human gastric epithelial cells during infection [47]. More recently, expression patterns of miRNA in gastric mucosa infected with *H. pylori* using endoscopic biopsy specimens have been determined by microarray. There were 31 differentially expressed miRNAs between the *H. pylori*-infected and -uninfected mucosa (more than twofold) and miRNA expression profiling could distinguish *H. pylori* status, with the eight miRNAs yielding acceptable sensitivity and specificity [48]. Muscle-specific miRNAs miR-1 and miR-133 were significantly downregulated in the stomachs after long-term infection with *H. pylori* in mouse model [49].

Schulte et al. identified differentially regulated miRNAs by comparative deep sequencing of a total of 14 cDNA libraries prepared from the small RNA population

of host cells before or after *Salmonella* infection, or in mock-treated cells. In murine RAW 264.7 cells, upregulation of miR-21, miR-146a/b, and miR-155 was observed after infection, they also observed significant downregulation of several let-7 family members, namely, let-7a/c/d/f/g/i, and miR-98. In HeLa cells, a significant upregulation of miRNAs by *Salmonella* was limited to miR-1308. In contrast, miR-21, miR-146a/b, or miR-155 remained unaffected. Intriguingly, downregulation of let-7 miRNAs also occurred in HeLa cells [50].

Other Microbial Agents

Sharbati et al. performed miRNA as well as mRNA expression analysis of human monocyte-derived macrophages infected with several *Mycobacterium avium hominissuis* strains using microarrays as well as qRT-PCR. They found that the expression of let-7e, miR-29a, and miR-886-5p were increased in response to mycobacterial infection at 48 h [51]. Expression of miR-23b, miR-34a, and miR-218 are significantly reduced by human papillomavirus (HPV) E6 infection, while HPV E7 infection downregulates expression of miR-15a/miR-16-1 and miR-203 [52].

Methods of miRNA Detection

Accurate determination of miRNAs expression levels in a specific cell, tissue, or fluids is prerequisite to assess their biological, pathological, and clinical roles in health and disease. Theoretically, all mRNA detection methods should be useful for miRNA analysis. However, the following characteristics of miRNA sequences make quantification of miRNAs expression a technical challenge. First, mature miRNA are short (only 19–25 nucleotides; nts) and miRNAs within the same family may differ by a single nucleotide, which makes it difficult to design specific primers and probes and to reliably amplify or label each miRNA without introducing signal bias. Second, miRNAs are heterogeneous in their GC content, which results in melting temperatures (Tm) of these nucleic acid duplexes that vary widely. Finally, the target sequence is present in the primary miRNA transcript (pri-miRNA), the precursor miRNA (pre-miRNA), and the mature miRNA. It is therefore important to ensure that the nonactive pri-miRNA and pre-miRNA precursor species do not contribute to the detection signal [53]. Several standard methods for quantification of mRNA levels have been successfully adapted to miRNA including northern blotting, cloning, in situ hybridization, RT-PCR, and microarrays. In addition, emerging techniques based on colorimetric, fluorescence, bioluminescence, enzyme, and electrochemical, hold immense promise for the future of miRNA detection. However, technical issues must be addressed before they are accepted among the current standard methods [54].

Northern Blotting

Northern blotting was the first technique used to detect miRNAs and is considered the "gold standard" for characterizing miRNA expression. The basic procedures of miRNA northern blotting are similar to traditional blotting and are done as follows: (a) the small RNA molecules are separated by using high percentage denaturing urea–acrylamide gels rather than the usual agarose electrophoresis gels; (b) the small RNA molecules are transferred from the gel onto a membrane; (c) the miRNA molecules are fixed on the membrane through various cross-linking procedures; (d) the membrane is hybridized with radiolabeled oligonucleotide probes. Northern blotting methods are able to determine the absolute amount of miRNA in a sample by blotting a dilution series of synthetic oligo miRNA molecules of known concentrations in parallel with the sample. The concentration of miRNA in the sample can be calculated by a standard curve obtained from the dilution series. However, short length as well as low prevalence of mature miRNA molecules can lead to poor sensitivity of such routine northern analysis. It requires a large amount of total RNA for each sample (generally, more than 5 μg). Other disadvantages of this method include low throughput and potential environment hazards of radiolabeling. Several technical modifications have been used to improve detection sensitivity. LNA (locked nucleic acid)-modified oligonucleotide probes increases the affinity between LNA probes and target miRNA which results at least tenfold increase of sensitivity [55]. Using soluble carbodiimide cross-linking method increases the efficiency of miRNA that are fixed on the membrane, which can increase by 25–50-fold miRNA detection sensitivity compared to the traditional UV cross-linking method [56].

qRT-PCR

The most widely used method for detection and qualification of miRNA appears to be real-time quantitative RT-PCR (qRT-PCR). The small size of the mature miRNA sequences as well as sequence homology between the mature and precursor miRNA forms limits the direct application of conventional RT-PCR protocols to miRNA detection. To solve these problems, innovative solutions have been applied for each step of qRT-PCR used for the quantitative analysis of miRNAs. The first step in qRT-PCR of miRNAs is the accurate and complete conversion of miRNA into cDNA. Two different approaches for reverse transcription of miRNAs have been reported. In the first approach, miRNAs are reverse transcribed individually by using miRNAs-specific reverse transcription primers. Both stem-loop (Applied Biosystems Co, Fig. 46.1a) and a linear primer (Exiqon, Fig. 46.1b) containing partial complementary sequence of 3′-end of miRNA can be annealed to miRNA in order to prime the reverse transcription. The double stranded structure of the stem-loop primer prevents its nonspecific binding to pre- and pri-miRNAs, thereby increasing the specificity of the assay. Unlike stem-loop primer, the design of the linear primer is simpler. But the linear primer cannot discriminate mature miRNA from their precursors.

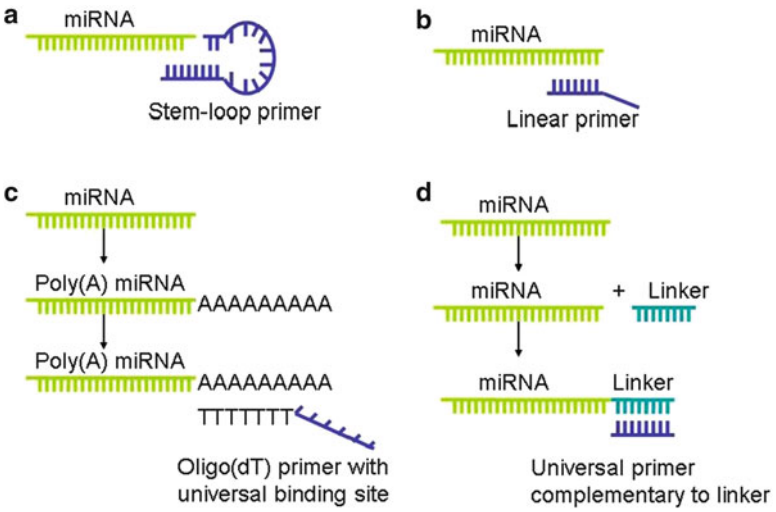

Fig. 46.1 Schematic representation of reverse transcription methods used to generate cDNA. Reverse transcription of individual mature miRNAs is done using stem-loop (**a**) or linear (**b**) primer. miRNAs are first tailed with adenosine nucleotides at 3′-end of miRNA with Poly(A) Polymerase (**c**) or linker adaptor with T4 RNA Ligase 1 (**d**). Then, a primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5′-end (**c**) or a universal primer complementary to the 3′-end of the linker (**d**) was used to prime reverse transcription

Another approach is done as follows: miRNAs are first tailed with adenosine nucleotides at 3′-end of miRNA with Poly(A) Polymerase (Fig. 46.1c) or linker adaptor with T4 RNA Ligase 1 (Fig. 46.1d). Then, a primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5′-end or a universal primer complementary to the 3′-end of the linker is used to prime reverse transcription [53]. The universal reverse primer sequence is introduced into the cDNA during reverse transcription. The design of the miRNA specific forward primer is critical for the specificity and sensitivity of the qRT-PCR assay. LNA modification is a widely used method for increasing the Tm and the specificity of primer. Each incorporated LNA monomer increases the Tm up to 2–8 °C, depending upon the position of the LNA moiety in the oligonucleotide primer [57].

There are two approaches available for detection of qRT-PCR products. One approach uses SYBR Green dye whose fluorescence increases approximately 100-times upon intercalating into dsDNA chain; this property is used to monitor real time amplification products as they accumulate during the PCR reaction. One limitation of the SYBR Green-based method is that target PCR products and nonspecific products cannot be discriminated. Therefore, a melting point analysis usually required following the PCR amplification, which can be reached by using a dual-labeled hydrolysis TaqMan probe. The basis for this type of TaqMan probe detection has been reviewed by Benes and Castoldi [53]. The characteristics of TaqMan miRNA assays make them ideally suited for detection of mature miRNAs. Moreover, these TaqMan miRNA assays can discriminate related miRNAs that differ in only

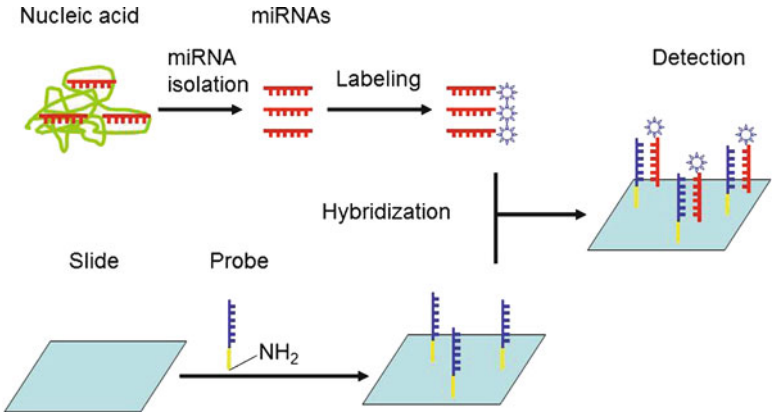

Fig. 46.2 Schematic flowchart of the microRNA profiling microarray. A miRNA microArray prepared by amine-modified microRNA probes that consist of "linker" sequences (*yellow*) and capture sequences (*blue*) spotting to amine-reactive glass slides. After miRNAs are isolated from samples and labeled with fluorescence dye, they are hybridized with microArray and then the expression signal can be detected by fluorescence detector

one nucleotide. However, the cost of the TaqMan probes is higher than the SYBR Green method, which limits its routine use in most laboratories.

The qRT-PCR method has demonstrated a high sensitivity and specificity with ability to accurately detect miRNAs in a single stem cell [58, 59]. Only low amounts of starting material (in the range of nanograms of total RNA) are needed and quantitative results can be acquired within 3 h. This method also has a considerably larger dynamic range compared to microarray analysis. Recently, the qRT-PCR technique has been adapted for increased throughput by developing a miRNA PCR array that can detect hundreds of miRNAs at one reaction tube. Examples include the TaqMan Low density microRNA Array (TLDA, Applied Biosystems) and the RT^2 miRNA PCR Arrays (SABioscience, a Qiagen Company).

Microarrays

Another widely used high-throughput technique for analysis of the expression level of miRNA molecules is microarray technology. The microarray technology was firstly applied to miRNA studies in 2003 [60]. Since then, numerous approaches based on different microarray platforms have been developed for miRNA quantification. The basic procedures of miRNA quantification using a microArray platform including (a) miRNA probe design and array preparation, (b) isolation of miRNA and labeling, (c) hybridization and signal detection. A schematic flow chart of the miRNA profiling microarray is shown in Fig. 46.2. The design of the microRNA probes, isolation of microRNA from samples and the labeling of miRNAs are the most critical procedures in the miRNA microarray assay.

The short length of miRNAs makes design of the probes more difficult because the design of the probe is almost exclusively determined by sequence of the miRNA itself. Sequence of the miRNA determines the wider Tm distribution of the probes. Therefore, Tm normalization of the full set of probes is absolutely required since the hybridization is usually carried out at one temperature. To solve this problem, two techniques, LNA utilization or adjusting the lengths of the probes, have been successfully used to normalize Tm value [61, 62]. Quantity of miRNA is also very important for miRNA microarray assay, since the abundance of miRNAs in total RNA is very low and a relatively large amount of miRNA is needed in comparison to a RT-PCR assay. Conventional denaturing urea–acrylamide gel electrophoresis combined with a commercialized kit (e.g., the mirVana™ microRNA isolation kit of Ambion, Inc. and the PureLink™ microRNA isolation kit of Invitrogen Co) have demonstrated excellent efficiency in isolating miRNA.

Labeling of miRNA is an absolutely key step for the overall sensitivity of the microarray. Many methods have been developed to label miRNA, and these can be classified into two main categories: direct labeling and indirect labeling. Direct labeling with fluorescent dye can be accomplished enzymatically. Currently used methods including labeling through poly(A) polymerase, labeling though T4 RNA ligase, guanine labeling, and labeling microRNA through a RNA-primed array-based Klenow enzyme assay (RAKE). Fluorescent dye also can conjugate with adjacent 3′-OH of mature miRNAs using a chemical reagent [63, 64]. Indirect labeling methods including labeling through miRNA reverse transcript, the RT-PCR product of miRNA or the in vitro transcript of miRNA. The advantages of indirect labeling are obvious, since the reverse transcription product of miRNAs are more stable and easy to preserve. In addition, miRNA can be amplified and labeled synchronously through PCR or in vitro transcription, which is very useful for low-abundance miRNAs. Although direct labeling is simple, this method has inherent problems including the fact that guanine labeling is not suitable for miRNAs lacking G residues, that T4 ligase labeling can introduce base bias, and that the procedure of chemical labeling is somewhat complicated. Though indirect labeling is more sensitive, this method may introduce artificial errors during the ligation and PCR amplification procedures.

Another microarray technique, which uses biotin labeled miRNAs to hybridize with LAN probes, has been coupled to xMAP suspension microspheres (Luminex Co) to offer more rapid and reproducible results than does solid planar array due to its favorable reaction kinetics in liquid phase. Using this technique, a single nt difference can be discriminated [65]. Microarray technology has proven to be standard technique for profiling miRNA expression.

Next-Generation Sequencing

Next-generation sequencing (NGS) is also called massively parallel or deep sequencing and is becoming the most effective method for miRNA analysis. Sequence

throughput of NGS is unapproachable by other miRNA analysis techniques, since they lay DNA fragments on a single chip and simultaneous sequencing up to millions of these fragments in parallel. The principle of NGS has been described in other chapter of this book and elsewhere [66, 67]. Currently, three main platforms are in widespread use for miRNA profiling and discovery: the Roche (454) GS FLX sequencer, the Illumina Genome Analyzer and the Applied Biosystems SOLiD sequencer. The methodologies of the NGS for miRNA analysis are similar and include sRNA isolation, library preparation, sequencing, and data analysis. In addition to increased throughput, NGS technique significantly reduce cloning biases observed with traditional capillary sequencing since sequence reads are generated from fragment libraries that do not need to be cloned and amplified.

Another key advantage of NGS over microArrays in miRNA study is that it can profile unknown genes since no sequence specific probes are needed for detection. Currently, most novel miRNAs have been discovered and characterized through NGS. NGS can also gauge miRNA expression level by counting clone frequencies, which has demonstrated more sensitivity than microArrays [66]. One disadvantage of NGS to comprehensively profile mRNA expression is that this technique is rather expensive compared to microArray though its cost has significantly decreased with the development of commercial platforms. Another limitation of NGS is that the read length is relatively short (35–500 bp) compared to traditional capillary sequencing (1,000–1,200 bp). This limits their use for de novo assembly of complete genomes but makes them become the ideal instruments for miRNA profiling since the length of miRNAs (21–35 nucleotides) is shorter than the read length of NGS.

As described above, each technique for miRNA detection has its advantages and disadvantages. The method used should best fit the research goal and experimental conditions. For example, for new miRNA discovery and identification, in addition to sequencing technique, cloning of miRNA may be the simplest method. In situ hybridization is more suitable for location of miRNA in tissue. Northern blotting is very sensitive, but it is very time-consuming and not practical in large clinical studies for routine detection of the expression of hundreds of miRNAs. qRT-PCR is able to detect low copy numbers with high sensitivity and specificity. When studying the expression levels of multi-miRNAs simultaneously, microarray and sequencing may be the best choice. Another important problem is that although each platform is relatively stable in terms of its own microRNA profiling intrareproducibility, the interplatform reproducibility among different platforms is low [68]. An “industry standard” for analysis of miRNA expression awaits further advances in both technology and computation [69].

Application of Circulating miRNAs for Diagnosis and Prognosis of Microbial Infection

Although altered miRNA expression profiles have been detected in various tissues or cells following microbial infection, these profiles have not yet been used as biomarkers in clinical practice because obtaining infected tissues and cells is difficult without

invasive procedures. As described above, circulating miRNAs in serum/plasma seem more suitable for biomarkers that can be easily used for the diagnosis or prognosis of these infections. Some potential uses are discussed in the next section.

Sepsis

Diagnosis and monitoring of sepsis can be difficult because many of its signs and symptoms can be caused by other noninfectious disorders. The current gold standard for diagnosing septicemia is the blood culture, which generally takes several days or longer. Other early biomarkers of sepsis are being investigated; these include acute phase proteins (C-reactive protein), cytokines (IL-1, -6, -10 and TNF-a), chemokines (IL-8, MCP-1, and G-CSF), procalcitonin, and metabonomic. To date, these biomarkers have not demonstrated sufficient sensitivity and/or specificity to guide clinical management. Host miRNA expression profiles have been intensively studied using both in vitro or in vivo models of inflammation that are triggered by *Escherichia coli* lipopolysaccharide (LPS) stimulation. For example, expression of miR-146, miR-155, and miR-132 increased in human acute monocytic leukemia cell line THP-1 in response to LPS stimulation [70]. Upregulation of miR-155, miR-223, miR-146a and downregulation of miR-125b, miR-144, and miR-142-5p have been observed in human monocyte-derived dendritic cells by Ceppi et al. [71]. Schmidt et al. screened for differentially expressed miRNAs in circulating leukocytes using an in vivo model of acute inflammation also triggered by LPS [72]. They found four miRNAs were downregulated (miR-146b, miR-150, miR-342, and let-7g) and one was upregulated (miR-143).

Vasilescu et al. profiled genome-wide miRNAs by microarray in peripheral blood leukocytes of sepsis patients and found that miR-150, miR-182, miR-342-5p, and miR-486 expression profiles differentiated sepsis patients from healthy controls [20]. Moreover, miR-150 levels were significantly reduced in plasma samples of sepsis patients and correlated with the level of disease severity. Finally, these investigators noted that the plasma levels ratio for miR-150/interleukin-18 can be used for assessing the severity of the sepsis. More recently, Wang et al. have analyzed seven miRNAs expression levels in patients diagnosed with sepsis, systemic inflammatory response syndrome (SIRS), and healthy controls using a qRT-PCR assay [21]. They determined serum miR-146a and miR-223 were significantly reduced in septic patients compared with SIRS patients and healthy controls. The areas under the receiver operating characteristic curve of miR-146a, miR-223, and IL-6 were 0.858, 0.804, and 0.785, respectively.

Viral Hepatitis B

HBV infection is also known to modulate the expression of host cellular miRNAs, which then participate in development of HBV-related liver diseases. The miRNA

profiles in chronic hepatitis B patient tissues or in HBV-expressing cells are reviewed by Liu et al. [73]. Li et al. profiled serum miRNAs of healthy controls, HBV-, HCV-, and HBV-positive hepatocellular carcinoma (HCC)-affected individuals by Solexa sequencing followed by validation with quantitative RT-PCR assay [21]. These investigators successfully identified 13 miRNAs that are differentially expressed in HBV serum. This 13-miRNA-based biomarker accurately discriminated not only HBV cases from controls and HCV cases, but also HBV-positive HCC cases from control and HBV cases. For example, when using four markers (miR-375, miR-10a, miR-223, and miR-423) to separate the control and HBV groups, the AUC was 99.9 ± 0.1 % (sensitivity: 99.3 %; specificity: 98.8 %). Similarly, two markers (miR-92a and miR-423) could separate the control and HCV groups with a high specificity and sensitivity (AUC: 99.6 ± 0.4 %; sensitivity: 97.9 %; specificity: 99.4 %). The control and HBV-positive HCC group could be clearly separated by 5 markers (miR-23b, miR-423, miR-375, miR-23a, and miR-342-3p; AUC: 99.9 ± 0.1 %; sensitivity: 96.9 %; specificity: 99.4 %). Similarly, the HBV and the HBV-positive HCC group could be separated by two markers (miR-10a and miR-125b; AUC: 99.2 ± 0.6 %; sensitivity: 98.5 %; specificity: 98.5 %).

Another study profiled miRNA expression on pooled sera obtained from identified groups of chronic asymptomatic carriers (ASC), patients with chronic hepatitis B (CHB) and HBV-associated acute-on-chronic liver failure (ACLF), as well as healthy controls (HC) using Applied Biosystems TaqmanArray assay [74]. A total of 37 miRNAs were amplified from HC, whereas 77, 101, and 135 were amplified from ASC, CHB, and ACLF, respectively. The expression levels of most miRNAs were also upregulated in HBV-infected patients when compared to HC. Furthermore, the level of miRNAs in the CHB serum was upregulated most in hepatitis B e antigen-positive patients. The expression of MiR-122, the most abundant miRNA in liver tissue, was significantly higher in HBV infected groups than in HC. The expression of miR-223 was similar between HC and ASC, but increased significantly in CHB and ACLF. The expression levels of miR-122 and miR-194 correlated negatively with the age of patients with CHB or ACLF.

Viral Hepatitis C

In an in vitro acute HCV infection model, 108 human miRNAs were identified whose expression levels changed for more than 2.0-fold in response to HCV infection [75]. Marquez et al. measured miR-122 and miR-21 levels in HCV-infected human liver biopsies relative to uninfected human livers and correlated these with clinical patient data [76]. They found that miR-21 expression correlated with viral load, fibrosis and serum liver transaminase levels. miR-122 expression inversely correlated with fibrosis, liver transaminase levels and patient age. While Morita et al. described hepatic miR-122 expression was weakly and positively correlated with the serum HCV load but was not correlated with HCV load in the human liver [77].

Bihrer et al. found sera from patients with chronic HCV infection contained higher levels of miR-122 than sera from healthy controls [23]. Serum miR-122 levels

correlated well with markers of liver inflammatory activity, that is, the serum levels of alanine leucine transaminase (ALT) and aspartate transaminase, and the histologic activity index (HAI) score. In patients with persistently normal ALT levels, serum miR-122 levels did not differ from healthy controls. There was no correlation of serum miR-122 levels with serum albumin, international normalized ratio, liver fibrosis, or serum HCV RNA. Thus, serum miR-122 appears to act as a biomarker of necroinflammation in patients with chronic Hepatitis C infection.

Enteroviral Infections

We previously performed a comprehensive miRNA profiling in EV71-infected Hep2 cells using deep sequencing. A total of 64 miRNAs were found whose expression levels changed for more than twofold in response to EV71 infection [78]. Ho et al. found upregulation of miR-141 upon enterovirus infection can facilitate viral propagation by expediting the translational switch [79]. We recently compared host serum miRNA levels in patients with hand-foot-and-mouth disease caused by enterovirus 71 (EV71) and coxsackievirus 16 (CVA16) as well as in other microbial infections and in healthy individuals. Among 664 different miRNAs analyzed using a miRNA array, 102 were upregulated and 26 were downregulated in sera of patients with enteroviral infections. Expression levels of ten candidate miRNAs were further evaluated by quantitative real-time PCR assays. A receiver operating characteristic (ROC) curve analysis revealed that six miRNAs (miR-148a, miR-143, miR-324-3p, miR-628-3p, miR-140-5p, and miR-362-3p) were able to discriminate patients with enterovirus infections from healthy controls with area under curve (AUC) values ranged from 0.828 to 0.934. The combined six miRNA using multiple logistic regression analysis provided not only a sensitivity of 97.1 % and a specificity of 92.7 % but also a unique profile that differentiated enteroviral infections from other microbial infections. Expression levels of five miRNAs (miR-148a, miR-143, miR-324-3p, miR-545, and miR-140-5p) were significantly increased in patients with CVA16 versus those with EV71 ($p<0.05$). Combination of miR-545, miR-324-3p, and miR-143 possessed a moderate ability to discrimination between CVA16 and EV71 with an AUC value of 0.761. These data indicate that sera from patients with different subtypes of enteroviral infection express unique miRNA profiles. Serum miRNA expression profiles may provide supplemental biomarkers for diagnosing and subtyping enteroviral hand-foot-and-mouth disease infections [80].

Concluding Remarks

Circulating miRNAs have been investigated as the diagnosis or prognosis marker for microbial infections. Studies on host miRNA profiles upon microbial infections are underway. There have a considerable way to go before being used in clinical

practice. Several issues should be refined in this field. (a) Source material should be fixed. Plasma, serum, whole blood, and isolated exosomes or microvesicles have been used as the source for miRNA profiling. Advantages and disadvantages of each approach should be considered ahead. (b) Though differences in circulating miRNAs between males and females have not been found with the exception of differences associated with pregnancy [81, 82], miRNA levels in plasma and serum from a large number of normal individuals of both genders and various ages, even same individual over time should be extensively studied. (c) No acknowledged reference genes have been found in serum/plasma. Frequently used endogenous controls, such as miR-16, dysregulated in some diseases and RNU6B degraded in serum. Spiking into RNA isolation process with synthetic exogenous miRNA only act as normalizers for differences in recovery between samples. (d) The methods of miRNA quantification including RNA isolation should be standardized, since interplatform reproducibility among different platforms is low. (e) There seems to be a lack of specificity when using single miRNA as biomarker, since the miRNA is commonly regulated in various diseases. A panel of miRNAs should be a best choice.

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References

1. Ng PC, Lam HS (2010) Biomarkers for late-onset neonatal sepsis: cytokines and beyond. Clin Perinatol 37:599–610
2. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854
3. Reinhart BJ, Slack FJ, Basson M et al (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403:901–906
4. Nelson KM, Weiss GJ (2008) MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther 7:3655–3660
5. Lee YS, Dutta A (2009) MicroRNAs in cancer. Annu Rev Pathol 4:199–227
6. Asli NS, Pitulescu ME, Kessel M (2008) MicroRNAs in organogenesis and disease. Curr Mol Med 8:698–710
7. Grassmann R, Jeang KT (2008) The roles of microRNAs in mammalian virus infection. Biochim Biophys Acta 1779:706–711
8. Gottwein E, Cullen BR (2008) Viral and cellular microRNAs as determinants of viral pathogenesis and immunity. Cell Host Microbe 3:375–387
9. Skalsky RL, Cullen BR (2010) Viruses, microRNAs, and host interactions. Annu Rev Microbiol 64:123–141
10. Weber JA, Baxter DH, Zhang S et al (2010) The microRNA spectrum in 12 body fluids. Clin Chem 56:1733–1741
11. Lawrie CH, Gal S, Dunlop HM et al (2008) Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 141:672–675
12. Chen X, Ba Y, Ma L et al (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18:997–1006

13. Mitchell PS, Parkin RK, Kroh EM et al (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 105:10513–10518
14. Nelson PT, Baldwin DA, Scearce LM, Oberholtzer JC, Tobias JW, Mourelatos Z (2004) Microarray-based, high-throughput gene expression profiling of microRNAs. Nat Methods 1:155–161
15. Tricoli JV, Jacobson JW (2007) MicroRNA: potential for cancer detection, diagnosis, and prognosis. Cancer Res 67:4553–4555
16. Wang H, Ach RA, Curry B (2007) Direct and sensitive miRNA profiling from low-input total RNA. RNA 13:151–159
17. Cortez MA, Calin GA (2009) MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. Expert Opin Biol Ther 9:703–711
18. Reid G, Kirschner MB, van Zandwijk N (2011) Circulating microRNAs: association with disease and potential use as biomarkers. Crit Rev Oncol Hematol 80(2):193–208
19. Pfeffer S, Zavolan M, Grasser FA et al (2004) Identification of virus-encoded microRNAs. Science 304:734–736
20. Vasilescu C, Rossi S, Shimizu M et al (2009) MicroRNA fingerprints identify miR-150 as a plasma prognostic marker in patients with sepsis. PLoS One 4:e7405
21. Wang JF, Yu ML, Yu G et al (2010) Serum miR-146a and miR-223 as potential new biomarkers for sepsis. Biochem Biophys Res Commun 394:184–188
22. Li LM, Hu ZB, Zhou ZX et al (2010) Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. Cancer Res 70:9798–9807
23. Bihrer V, Friedrich-Rust M, Kronenberger B et al (2011) Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. Am J Gastroenterol 106(9):1663–1669
24. Kurzynska-Kokorniak A, Jackowiak P, Figlerowicz M (2009) Human- and virus-encoded microRNAs as potential targets of antiviral therapy. Mini Rev Med Chem 9:927–937
25. Grundhoff A, Sullivan CS (2011) Virus-encoded microRNAs. Virology 411:325–343
26. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309:1577–1581
27. Randall G, Panis M, Cooper JD et al (2007) Cellular cofactors affecting hepatitis C virus infection and replication. Proc Natl Acad Sci U S A 104:12884–12889
28. Huang J, Wang F, Argyris E et al (2007) Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. Nat Med 13:1241–1247
29. Houzet L, Yeung ML, de Lame V, Desai D, Smith SM, Jeang KT (2008) MicroRNA profile changes in human immunodeficiency virus type 1 (HIV-1) seropositive individuals. Retrovirology 5:118
30. Triboulet R, Mari B, Lin YL et al (2007) Suppression of microRNA-silencing pathway by HIV-1 during virus replication. Science 315:1579–1582
31. Bellon M, Lepelletier Y, Hermine O, Nicot C (2009) Deregulation of microRNA involved in hematopoiesis and the immune response in HTLV-I adult T-cell leukemia. Blood 113:4914–4917
32. Yeung ML, Yasunaga J, Bennasser Y et al (2008) Roles for microRNAs, miR-93 and miR-130b, and tumor protein 53-induced nuclear protein 1 tumor suppressor in cell growth dysregulation by human T-cell lymphotrophic virus 1. Cancer Res 68:8976–8985
33. Mallick B, Ghosh Z, Chakrabarti J (2009) MicroRNome analysis unravels the molecular basis of SARS infection in bronchoalveolar stem cells. PLoS One 4:e7837
34. Wang Y, Brahmakshatriya V, Zhu H et al (2009) Identification of differentially expressed miRNAs in chicken lung and trachea with avian influenza virus infection by a deep sequencing approach. BMC Genomics 10:512
35. Li Y, Chan EY, Li J et al (2010) MicroRNA expression and virulence in pandemic influenza virus-infected mice. J Virol 84:3023–3032
36. Qi Y, Tu J, Cui L et al (2010) High-throughput sequencing of microRNAs in adenovirus type 3 infected human laryngeal epithelial cells. J Biomed Biotechnol 2010:915980
37. Wang FZ, Weber F, Croce C, Liu CG, Liao X, Pellett PE (2008) Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication. J Virol 82:9065–9074

38. Santhakumar D, Forster T, Laqtom NN et al (2010) Combined agonist–antagonist genome-wide functional screening identifies broadly active antiviral microRNAs. Proc Natl Acad Sci U S A 107:13830–13835
39. Hill JM, Zhao Y, Clement C, Neumann DM, Lukiw WJ (2009) HSV-1 infection of human brain cells induces miRNA-146a and Alzheimer-type inflammatory signaling. Neuroreport 20: 1500–1505
40. Zheng SQ, Li YX, Zhang Y, Li X, Tang H (2011) MiR-101 regulates HSV-1 replication by targeting ATP5B. Antiviral Res 89:219–226
41. Lagos D, Pollara G, Henderson S et al (2010) miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. Nat Cell Biol 12:513–519
42. Tsai YH, Wu MF, Wu YH et al (2009) The M type K15 protein of Kaposi's sarcoma-associated herpesvirus regulates microRNA expression via its SH2-binding motif to induce cell migration and invasion. J Virol 83:622–632
43. Punj V, Matta H, Schamus S, Tamewitz A, Anyang B, Chaudhary PM (2010) Kaposi's sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 suppresses CXCR4 expression by upregulating miR-146a. Oncogene 29:1835–1844
44. Godshalk SE, Bhaduri-McIntosh S, Slack FJ (2008) Epstein-Barr virus-mediated dysregulation of human microRNA expression. Cell Cycle 7:3595–3600
45. Imig J, Motsch N, Zhu JY et al (2011) microRNA profiling in Epstein-Barr virus-associated B-cell lymphoma. Nucleic Acids Res 39:1880–1893
46. Cameron JE, Fewell C, Yin Q et al (2008) Epstein-Barr virus growth/latency III program alters cellular microRNA expression. Virology 382:257–266
47. Xiao B, Liu Z, Li BS et al (2009) Induction of microRNA-155 during Helicobacter pylori infection and its negative regulatory role in the inflammatory response. J Infect Dis 200:916–925
48. Matsushima K, Isomoto H, Inoue N et al (2011) MicroRNA signatures in Helicobacter pylori-infected gastric mucosa. Int J Cancer 128:361–370
49. Saito Y, Suzuki H, Tsugawa H et al (2011) Dysfunctional gastric emptying with down-regulation of muscle-specific microRNAs in Helicobacter pylori-infected mice. Gastroenterology 140: 189–198
50. Schulte LN, Eulalio A, Mollenkopf HJ, Reinhardt R, Vogel J (2011) Analysis of the host microRNA response to Salmonella uncovers the control of major cytokines by the let-7 family. EMBO J 30:1977–1989
51. Sharbati J, Lewin A, Kutz-Lohroff B, Kamal E, Einspanier R, Sharbati S (2011) Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection. PLoS One 6:e20258
52. Zheng ZM, Wang X (2011) Regulation of cellular miRNA expression by human papillomaviruses. Biochim Biophys Acta 1809(11–12):668–677
53. Benes V, Castoldi M (2010) Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. Methods 50:244–249
54. Hunt EA, Goulding AM, Deo SK (2009) Direct detection and quantification of microRNAs. Anal Biochem 387:1–12
55. Valoczi A, Hornyik C, Varga N, Burgyan J, Kauppinen S, Havelda Z (2004) Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. Nucleic Acids Res 32:e175
56. Pall GS, Codony-Servat C, Byrne J, Ritchie L, Hamilton A (2007) Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. Nucleic Acids Res 35:e60
57. Ahmed FE (2007) Role of miRNA in carcinogenesis and biomarker selection: a methodological view. Expert Rev Mol Diagn 7:569–603
58. Chen C, Ridzon DA, Broomer AJ et al (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33:e179
59. Tang F, Hajkova P, Barton SC, Lao K, Surani MA (2006) MicroRNA expression profiling of single whole embryonic stem cells. Nucleic Acids Res 34:e9

60. Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS (2003) A microRNA array reveals extensive regulation of microRNAs during brain development. RNA 9:1274–1281
61. Baskerville S, Bartel DP (2005) Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA 11:241–247
62. Castoldi M, Schmidt S, Benes V et al (2006) A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). RNA 12:913–920
63. Beuvink I, Kolb FA, Budach W et al (2007) A novel microarray approach reveals new tissue-specific signatures of known and predicted mammalian microRNAs. Nucleic Acids Res 35:e52
64. Liang RQ, Li W, Li Y et al (2005) An oligonucleotide microarray for microRNA expression analysis based on labeling RNA with quantum dot and nanogold probe. Nucleic Acids Res 33:e17
65. Saber R (2007) Assay: perfect match versus single-base mismatch. MicroRNA detection combining xMAP® and locked nucleic acid (LNA™) technology. Genet Eng News 27:28–29
66. Mardis ER (2008) Next-generation DNA sequencing methods. Annu Rev Genomics Hum Genet 9:387–402
67. Zhang J, Chiodini R, Badr A, Zhang G (2011) The impact of next-generation sequencing on genomics. J Genet Genomics 38:95–109
68. Wang B, Howel P, Bruheim S et al (2011) Systematic evaluation of three microRNA profiling platforms: microarray, beads array, and quantitative real-time PCR array. PLoS One 6:e17167
69. Git A, Dvinge H, Salmon-Divon M et al (2010) Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA 16:991–1006
70. Taganov KD, Boldin MP, Chang KJ, Baltimore D (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A 103:12481–12486
71. Ceppi M, Pereira PM, Dunand-Sauthier I et al (2009) MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proc Natl Acad Sci U S A 106:2735–2740
72. Schmidt WM, Spiel AO, Jilma B, Wolzt M, Muller M (2009) In vivo profile of the human leukocyte microRNA response to endotoxemia. Biochem Biophys Res Commun 380:437–441
73. Liu WH, Yeh SH, Chen PJ (2011) Role of microRNAs in hepatitis B virus replication and pathogenesis. Biochim Biophys Acta 1809(11–12):678–685
74. Ji F, Yang B, Peng X, Ding H, You H, Tien P (2011) Circulating microRNAs in hepatitis B virus-infected patients. J Viral Hepat 18:e242–e251
75. Liu X, Wang T, Wakita T, Yang W (2010) Systematic identification of microRNA and messenger RNA profiles in hepatitis C virus-infected human hepatoma cells. Virology 398:57–67
76. Marquez RT, Bandyopadhyay S, Wendlandt EB et al (2010) Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans. Lab Invest 90:1727–1736
77. Morita K, Taketomi A, Shirabe K et al (2011) Clinical significance and potential of hepatic microRNA-122 expression in hepatitis C. Liver Int 31:474–484
78. Cui L, Guo X, Qi Y et al (2010) Identification of microRNAs involved in the host response to enterovirus 71 infection by a deep sequencing approach. J Biomed Biotechnol 2010:425939
79. Ho BC, Yu SL, Chen JJ et al (2011) Enterovirus-induced miR-141 contributes to shutoff of host protein translation by targeting the translation initiation factor eIF4E. Cell Host Microbe 9:58–69
80. Cui L, Qi Y, Li H et al (2011) Serum microRNA expression profile distinguishes enterovirus 71 and coxsackievirus 16 infections in patients with hand-foot-and-mouth disease. PLoS One 6:e27071
81. Chim SS, Shing TK, Hung EC et al (2008) Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 54:482–490
82. Gilad S, Meiri E, Yogev Y et al (2008) Serum microRNAs are promising novel biomarkers. PLoS One 3:e3148

Chapter 47
Interpretation and Relevance of Advanced Technique Results

Charles W. Stratton and Yi-Wei Tang

Introduction

Advanced techniques in the field of diagnostic microbiology have made amazing progress over the past two decades due largely to a technological revolution in the molecular aspects of microbiology [1, 2]. In particular, rapid molecular methods for nucleic acid amplification and characterization combined with automation and user-friendly software have significantly broadened the diagnostic capabilities of modern clinical microbiology laboratories. Molecular methods such as nucleic acid amplification tests (NAATs) rapidly are being developed and introduced in the clinical laboratory setting. Indeed, every section of the clinical microbiology laboratory, including bacteriology, mycology, mycobacteriology, parasitology, and virology, have benefited from these advanced techniques. Because of the rapid development and adaptation of these molecular techniques, the interpretation and relevance of the results produced by such molecular methods has lagged somewhat behind. The purpose of this chapter is to review and discuss the interpretation and relevance of results produced by these advanced molecular techniques. Moreover, this chapter will address the "myths" of NAATs, as these myths can markedly influence the interpretation and relevance of these results.

C.W. Stratton (✉)
Departments of Pathology and Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA
e-mail: charles.stratton@Vanderbilt.Edu

Y.-W. Tang
Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 37212, USA
e-mail: tangy@MSKCC.org

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Myths of Nucleic Acid Amplification Tests

Myth 1: NAATs Are Extremely Sensitive

There are two aspects on sensitivity: analytical, which is determined by the limit of detection in a given specimen, and diagnostic/clinical, which is determined by the percentage of target in a patient (true positive) population. Most of NAATs possess excellent analytical sensitivity reaching ten copies of target genomes per reaction. This has made the NAATs essential for the detection of microbial pathogens from specimens such as cerebrospinal fluid (CSF) where there may be extremely low microbial loads [3–6]. However, in certain situations, the sensitivities of NAATs are inferior to conventional culture techniques in which a large volume of a specimen easily can be evaluated. Examples include blood cultures and sputum cultures for *Mycobacterium tuberculosis* [7–10]. Current NAATs are usually performed using very small volumes; until the processing of such specimens is greatly improved, the sensitivity of these NAATs will remain lower than conventional culture techniques.

Currently, most NAATs are designed in a monoplex format, i.e., one primer set for one specific microbial pathogen. Although the use of multiplex PCR amplification techniques is increasing in the clinical diagnostic field, these multiplex methods account for the minority of molecular testing. In conventional microbial culture techniques, a chocolate agar plate or a mixed cell line would allow the recovery of many different pathogens and/or multiple pathogens if they were present in a tested specimen. From this perspective, the diagnostic sensitivity of a monoplex NAAT may not be sufficiently high since it only detects the one pathogen that is being tested for rather than many different pathogens and/or multiple pathogens.

Myth 2: Real-Time PCR Is Extremely Sensitive as well as Objective

The term "real-time PCR" indicates a PCR procedure in which nucleic acid amplification and amplification product detection happen simultaneously. Real-time PCR methods often incorporate a FRET procedure, which allows the amplicon detection and identification to occur in real time in relation to the nucleic acid amplification. This avoids carryover amplicon contamination since the reaction is occurring in closed system. Moreover, real-time PCR allows accurate quantification of the nucleic acid. However, NAATs based on real-time PCR methods are not necessarily more sensitive than other NAATs [11]. In contrast, due to the nonspecific spontaneous FRET procedure, real-time PCR assays may be less sensitive due to the raised cutoff values. The arithmetic, spontaneous increase in fluorescent background emissions interferes with the exponential, specific energy emissions during the simultaneous detection procedure, producing potentially false-positive results. To overcome this nonspecific issue, the system has to either increase the cycle threshold (Ct) cutoff value or decrease the cycle numbers during the amplification, which results in decreased sensitivity.

Real-time PCR does provide a nice qualification procedure with a wide range covered; however, it is not ideal for qualitative assay measurements as there are no objective criteria for determining the cutoff point. This can be problematic when the microbial load in the tested specimen is extremely low. For example, HSV and *Chlamydia pneumoniae* detection in CSF specimens by real-time PCR is not as objective and sensitive in comparison to end detection PCR procedures [4, 12]. In the later procedures, a separate detection and identification is used after amplification in which additional signal amplification (e.g., antigen–antibody linking) can be incorporated to further enhance the test sensitivity [12, 13].

Myth 3: NAATs Are Useful Tests for Assessing Therapeutic Efficacy

NAATs are often considered to be useful tests for assessing therapeutic efficacy. However, this is not true. NAATs detect microbial organism-specific nucleic acids; therefore, a positive NAAT result can occur with both alive and dead microorganisms, which is particularly true for those pathogens that have protective cell wall. The best example of this is the detection of *M. tuberculosis* DNA in sputum where the dead microbial pathogen DNA can remain un-degraded due to the fatty acid-rich cell walls [14, 15]. Unlike the results of a function-based testing method, such as mycobacterial cultures, in the clinical setting, a positive PCR result after anti-tuberculosis therapy does not necessarily mean treatment failure. Therefore, DNA-targeted NAATs are usually not considered to be tests of cure. This is also true for sexually transmitted pathogens such as *Chlamydia trachomatis* and *Neisseria gonorrhea* [16].

Although DNA-targeted NAATs are not useful for therapeutic monitoring, mRNA-targeted NAATs may be used for assessment of antimicrobial treatment therapy. For example, the application of mRNA-targeted NAATs has been demonstrated for monitoring of tuberculosis therapy. Anti-TB therapy regimen selection is largely empiric. Treatment may not be modified until weeks or months later as results of antimicrobial susceptibility tests become available. Because the half-life of bacterial mRNA is extremely short compared to rRNA or genomic DNA, molecular assays that target mycobacterial mRNA better reflect mycobacterial viability. The ability of mRNA-based assays to distinguish viable from nonviable organisms have demonstrated that such assays are useful in monitoring the efficacy of anti-TB therapy [14, 15].

Myth 4: NAAT Results Allow Direct and Objective Interpretation

Conventional microbiology methods are generally direct and objective in terms of interpretation of the results. A blood culture that is positive for *Staphylococcus aureus* strongly suggests that the patient has a staphylococcal bacteremia. A positive hepatitis A IgM antibody detected in serum implies a recent infection caused by this virus. However, positive NAAT results can be somewhat indirect and subjective

since the exact meaning of a positive NAAT result is only that the microbe-specific nucleic acid exists in the tested specimen. This can be caused by contamination, colonization and/or infection. Even when an infection is established, it can be either an acute or chronic infection.

Higher sensitivity may not always be clinically relevant. One good example is seen with the varicella-zoster virus (VZV). Previously, a positive VZV result, by either DFA or culture, would be interpreted as VZV being the causative pathogen and would be considered clinically relevant. PCR-based NAATs have increased VZV detection several hundred-fold such that a positive NAAT result for VZV from a skin or mucosal lesion specimen may be unclear as to the exact causative relationship of this virus to infection [17].

Clinical Relevance and Interpretation of Molecular Tests

The Use of Molecular Assays for Diagnosing Bloodstream Infections

Bloodstream infections have long been recognized as among the most severe manifestations of bacterial disease and were first described in 1940 by Keefer in his sentinel paper "The Clinical Significance of Bacteremia" [18]. The importance of the rapid diagnosis of bloodstream infections is not argued and serves to illustrate many of the issues involved in the interpretation and relevance of advanced techniques in diagnostic microbiology.

By 1940 when Keefer pointed out the clinical relevance of bacteremia, blood cultures were well established for the evaluation of febrile patients [19]. Since then, the techniques and pitfalls for blood cultures as well as the clinical implications of positive blood cultures have been well documented [20–23]. Not surprisingly, molecular and other non-culture-based methods for the rapid diagnosis of bloodstream infections have been widely evaluated [8, 10]. These studies along with earlier studies of blood cultures have illustrated some important points regarding the limitations of molecular assays for diagnosing bloodstream infections, which are described below:

Limitations of Molecular Assays for Diagnosing Bloodstream Infections

Interpretation of DNAemia

The detection of circulating microbial DNA (i.e., DNAemia) is, per se, a new diagnostic parameter that may or may not represent the presence of viable microorganisms in blood [10, 24]. For example, interpretation of DNAemia with coagulase-negative

staphylococci is problematic due to a false-positive rate that ranges from 60 to 80 % [22, 25]. In contrast, interpretation of DNAemia with *Ehrlichia* species is not a problem due to a true-positive rate of 100 % [26]. Interpretation of DNAemia has also been a problem in some studies where DNAemia is detected by PCR but not by blood cultures [24]. A number of these "false-positive" PCR results have been considered clinically significant, based on either retrospective chart review or subsequent isolation of the pathogen from other relevant clinical specimens [10, 27–32]. Clearly, the continued clinical investigation of microbial DNAemia during sepsis and other critical illnesses is needed and will provide a better understanding of the biology of the microbial circulating DNA that underpins such molecular diagnostic techniques [10, 24, 33].

Molecular Detection of Resistance Determinants

Another important issue for molecular diagnostic techniques is the need for molecular detection of resistance determinants [10, 24]. Antimicrobial susceptibility testing is recognized as important for confirming susceptibility to chosen empirical antimicrobial agents as well as for detecting resistance in individual microbial isolates [34]. Current methods for antimicrobial susceptibility testing continue to be based for the most part on the detection of microbial growth or lack of growth in the presence of the antimicrobial agent being tested [34, 35]. The direct detection of resistance genes by molecular methods such as PCR to date has limitations due to the fact that relatively few resistance genes are firmly associated with phenotypic resistance [34–36]. For example, resistance genes associated with phenotypic resistance that can be found in Gram-positive cocci include *mecA*, *vanA*, and *vanB*. In contrast, the lack of consensus sequences among acetyltransferases and adenyltransferase genes from Gram-negative bacilli makes the molecular detection of aminoglycoside resistance difficult. Although molecular methods for antimicrobial susceptibility testing are ascending, phenotypic methods for determining the level of susceptibility of bacterial isolates to antimicrobial agents are likely to remain clinically relevant for many years.

Volume of Blood Tested

The volume of blood cultured is known to be an important variable in blood cultures because the number of microorganisms in blood may small [37–41]. Typically in adults, there are fewer than 10 CFU/ml, and there may be less than 1 CFU/ml. In septic neonates, there is a sizeable subset with less than 4 CFU/ml [40]. Clearly the volume of blood tested by molecular methods will also be important. Moreover, the Poisson distribution of these microorganisms is such that they are not evenly distributed [37, 42]. This increases the likelihood that sampling a small volume of blood will miss a microorganism that is causing sepsis. Volume-related issues may explain the lower sensitivity seen with a molecular method (66.7 %) than seen with conventional

blood cultures in a study of neonatal sepsis [31]. The Poisson distribution may explain the moderate concordance between blood cultures and a molecular method reported in a study of post-surgical sepsis in adults [27].

Contamination of Blood Samples

The sample of blood collected to assess bacteremia and/or fungemia, whether this analysis is done by blood culture or by a molecular method, can be contaminated with microorganisms from the skin during venipuncture and/or from indwelling vascular devices if the blood is obtained from such a device [23]. False-positive blood cultures have been recognized as a troublesome issue for decades, and such contamination will be no less important for molecular methods.

The Use of Molecular Assays for Diagnosing Tuberculosis

Tuberculosis remains one of the most important public health issues in the world. Tuberculosis results in approximately 1.7 million deaths each year, and the number of new cases worldwide is estimated at more than nine million; this is higher than at any other time in history [43]. Yet control of this treatable infection has been handicapped until recently by the lack of new diagnostic tests for the detection of *M. tuberculosis* and drug resistance [44]. The development of molecular assays for the detection of *M. tuberculosis* as well as simultaneous detection of resistance to isoniazid and/or rifampin promises to greatly assist TB control efforts although there are important limitations of these molecular methods that must be understood when interpreting the results and considering the relevance of such molecular techniques [44–46]. Indeed, none of these molecular methods eliminates the need for mycobacterial cultures, and all require a laboratory infrastructure that can accommodate molecular testing. Specific limitations of these molecular methods in both interpretation and relevance will be described below.

The Limitations of Molecular Assays for Diagnosing Tuberculosis

Limited Sensitivities

There currently are a number of different molecular assays for detecting the presence of *M. tuberculosis* in sputum. These include PCR, transcription-mediated amplification, loop-mediated isothermal amplification [47], and Xpert MTB/RIF [47]. In comparison to mycobacterial culture, these molecular assays possess sensitivities approaching 90 %. In general, these molecular methods work better with

smear-positive than with smear-negative sputum specimens; none are more sensitive than mycobacterial cultures. The sensitivity for patients with smear-negative sputum can be increased by the use of bronchial aspirates [48] or bronchial lavage fluid [49], but is still not as sensitive as mycobacterial cultures.

Molecular Detection of Resistance Determinants

There currently are a number of different molecular assays for detecting gene mutations associated with resistance to a particular antituberculosis drug [50–54]. There are always gaps between basic research and clinical application as some of the drug-resistance mechanisms remain unknown while new resistance-related mutations are emerging. In addition, all molecular assays basically include a DNA amplification step and are categorized by the manner in which the amplified DNA is detected except for sequencing, which has some distinct advantages over the other methods. None of these methods, including sequencing, are able to detect all resistant strains although sequencing comes the closest to doing so. The major limitation of these molecular methods, except sequencing, is that they detect only known mutations in a defined site or region, as their design is dependent upon known mutations. The advantage of sequencing for molecular detection of mutations of drug resistance can be seen by a recent report from the Centers for Disease Control and Prevention [45]. This study used DNA sequencing to detect resistance to the first-line antituberculosis drugs isoniazid, rifampin, pyrazinamide, and ethambutol and to the second-line drugs amikacin, capreomycin, kanamycin, ciprofloxacin, and ofloxacin. The molecular data were compared to phenotypic data. Sensitivity and specificity values for the first-line and second-line drug loci were, in general, excellent and supported the use of DNA sequencing to detect drug resistance in the *M. tuberculosis* complex.

Misidentification

Although uncommon, misidentification has been reported with molecular assays for tuberculosis [55, 56]. In one of these reported cases [55], a patient presented with inguinal lymphadenopathy as well as erythema nodosum-like lesions on his legs and forearms. A biopsy of an enlarged inguinal lymph node demonstrated caseating granulomata and numerous acid-fast bacilli on Ziehl-Neelsen staining; a portion of this node was sent for mycobacterial culture and molecular analysis. In addition, a skin biopsy of a forearm nodule was done; this revealed acid-fast bacilli that were morphologically typical of *Mycobacterium leprae*. A diagnosis of leprosy was made based on the clinical presentation and the skin biopsy results. However, the lymph node sent for mycobacterial culture and molecular analysis was positive by the Gen-Probe Amplified *M. tuberculosis* Direct (MTD) test (BBL). Although leprosy was still considered to be a correct diagnosis due to the clinical presentation and the skin biopsy findings, the possibility of this patient also having tuberculosis

could not be ruled out until the culture results were known. Therefore, the patient was treated for both leprosy and tuberculosis until cultures at 7 weeks as well as additional PCR testing of lymph node material for *M. tuberculosis* were reported to be negative. A root cause analysis was done in order to investigate this misidentification. *M. leprae* culture material was obtained from the National Hansen's Disease Programs at Louisiana State University; these mycobacterial organisms were tested with the Gen-Probe MTD test and were positive at a concentration of 5×10 [5] organisms per ml, but were indeterminate at a concentration of 5×10 [4] organisms per ml. The investigators concluded that a high concentration of *M. leprae* in a clinical specimen could lead to a false-positive result with the Gen-Probe MTD test [55].

The Use of Molecular Assays for Diagnosing Respiratory Tract Infections

There is no doubt that respiratory tract infection other than those caused by *M. tuberculosis* also are of considerable clinical importance. Lower respiratory tract infections continue to be a leading cause of death due to infectious diseases in the United States as well as worldwide [57]. Hospital-acquired pneumonia is considered to be one of the most difficult treatment challenges in infectious diseases in part because results of culture and antimicrobial susceptibility testing can take 48 h or longer [58]. Viral respiratory tract infections caused by pathogens such as the severe acute respiratory syndrome coronavirus (SARS-CoV) [59] and novel A/H1N1 influenza virus [60] can cause epidemic viral pneumonia in which some patients have respiratory failure with a significant risk of mortality [61]. Respiratory tract infections are also important in the ambulatory setting because of the documented overuse of antimicrobial agents in this patient population [62].

Despite the obvious clinical importance of respiratory tract infections, the diagnosis of lower respiratory tract infections has always been problematic due, in large part, to issues related to the optimal collection and evaluation of sputum. The diagnostic value of detecting *M. tuberculosis* in the sputum was recognized as early as 1884 [63], and evaluation of sputum became the cornerstone for the diagnosis of tuberculosis [43, 44]. Post-mortem studies in the late 1890s and early 1900s then established the role of other microorganisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *S. aureus*, and *Klebsiella pneumoniae* in non-tuberculous infections of the respiratory tract [64–66]. In 1902, the use of the Gram's stain was described [67]. The microscopic examination of sputum was followed by the introduction of sputum cultures for the diagnosis of bacterial pneumonia [68–70]. Of note in these early reports describing sputum cultures was the recognition that collection of the sputum was important. For example, Hastings and Niles in a 1911 publication [69] point out that, "Exudates formed in portions of the respiratory tract that are normally sterile may be collected and treated in a way that will prevent contamination." These investigators further define a "*clean sputum*, i.e., one containing

only two or three types of bacteria and free from buccal squamous cells, and a *dirty sputum*, i.e., one containing a varied bacterial and fungoid flora and buccal squamous cells, are readily recognized on microscopic examination." They also state that, "A dirty sputum is not suitable for bacterial examination and should be discarded for a second or third clean specimen from the same patient." Luetscher opines in his paper [70] that, "The patient should be instructed to expectorate into the bottle or dish only what he is certain comes from his 'boots,' and also be made to understand that very little is wanted, but that that little must be choice." These astute observations remain relevant a century later.

Clearly, the pitfalls of collecting expectorated sputum specimens suitable for microscopic examination and cultures were recognized early in the twentieth century. In the 1960s, these pitfalls were again being articulated and addressed [71–76]. In particular, contamination by microorganisms present in the upper respiratory tract (i.e., nasal-oral-pharyngeal regions) was considered to be a major issue with expectorated sputum [77, 78]. Because of these pitfalls, a number of alternative methods have been used to obtain better sputum specimens. Bronchoscopy, although introduced early in the twentieth century and used on occasion for aspirating pus from larger airways [79], was not widely used for obtaining sputum for microscopy and culture until the 1970s when fiberoptic bronchoscopy became available [80]. Fiberoptic bronchoscopy also resulted in the use of bronchoalveolar lavage for diagnosing acute bacterial pneumonias [81]. Other methods adopted for obtaining uncontaminated sputum included transtracheal aspiration [72], percutaneous needle biopsy [76], and open-lung biopsy [71].

Despite these continued attempts to obtain appropriate sputum specimens that are more clinically relevant, the usefulness of sputum cultures has continued to be questioned in numerous reports [82–88]. Indeed, the Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults recommend that pretreatment Gram stain and culture should be performed only if a good quality sputum sample can be obtained and quality performance measures for collection, transport, and processing of this sputum sample can be assured [89]. It must be remembered that sputum collection is the "weakest link" in the "chain" of evidence that provides the etiologic diagnosis of pneumonia.

Assuming that sputum collection is done correctly, the next issue is making sure that any microbial pathogen present in the sputum can be identified. It is not surprising that molecular assays for the detection and characterization of microorganisms rapidly emerged in the clinical microbiology laboratory as an important adjunct to traditional culture methods [90, 91]. It was quickly realized that molecular assays such as NAATs offered significant advantages over conventional methods for the detection of *Mycoplasma pneumoniae* [92, 93], *Legionella* species [94], and *Chlamydia* species [95]; moreover, these three respiratory pathogens did not require concomitant susceptibility testing results from clinical isolates. Similarly, the advantage of NAATs for the laboratory diagnosis of pertussis was recognized very early [96, 97]; PCR testing is now considered by the CDC to be an important tool for diagnosis of pertussis especially in the setting of the current resurgence of pertussis

disease as it can provide timely results with improved sensitivity over culture [98]. The inherent problems associated with the detection and identification of respiratory viruses by culture and/or serologic methods also resulted in the early application of molecular assays for rapid detection and characterization of respiratory viruses [99]. Both user-developed and commercial molecular methods have quickly evolved and now allow rapid identification of multiple common viral pathogens causing respiratory tract infections [100–102]. In addition to identification of viral respiratory pathogens, it was appreciated that rapid molecular assays would also offer significant advantages for diagnosing recognized bacterial pulmonary pathogens causing community-acquired pneumonia [57, 93, 103, 104]. Indeed, initial studies in which rapid molecular assays were combined with conventional diagnostic methods have demonstrated that this approach increased the etiological diagnosis of lower respiratory tract infections considerably [105, 106]. This was especially true for patients with adequate collection of sputum [105]. Of interest was the observation that NAATs increased both the diagnostic and treatment costs [106]. Finally, the diagnosis of hospital-acquired pneumonia is another potential area where the use of rapid molecular assays for respiratory pathogens may prove useful [58]. Currently clinical trials are needed to provide evidence for which molecular assays are best as well as how this molecular information should be applied in the clinical setting.

The Limitations of Molecular Assays for Diagnosing Respiratory Tract Infections

Sputum/Specimen Collection

Clearly the same limitations of conventional sputum culture methods for diagnosing respiratory tract infections are also limitations for molecular methods. In particular, the collection of sputum continues to be the most important aspect for the diagnosis of lower respiratory tract infections even when molecular assays are used [58]. These new molecular methods will not guarantee that the microbiology laboratory will receive the optimal sputum sample to analyze.

Complexity of Pulmonary Microbiome

Another important aspect of molecular assays for the diagnosis of respiratory infections is that these methods are clearly going to reveal the complexities of the pulmonary microbiome. Indeed, recent applications of molecular assays have revealed a more diverse microbiota than previously recognized in the airways of patients with chronic pulmonary disease [107, 108]. For example, comprehensive profiling of the airway bacterial communities was accomplished using a culture-independent microarray, the 16S rRNA PhyloChip, of a cohort of COPD patients

requiring ventilatory support and antimicrobial therapy for exacerbation-related respiratory failure [109]. PhyloChip analysis demonstrated the presence of over 1,200 bacterial taxa representing 140 distinct families, including many that were not previously detected in airway diseases. A core community of 75 bacterial taxa was noted in all patients; many of these microorganisms were known pathogens in airway diseases.

Colonization Versus Infection

Given the fact that the pulmonary microbiome is more complex that previously appreciated, the obvious question then becomes which microorganisms are colonizing and which are causing infection. One might also ask if there is any real difference between colonization versus infection in the airways. Molecular identification of bacteria in the lower airways of preterm infants has revealed that early bacterial colonization of the airways with diverse species occurs within the first 3 days of life of intubated preterm infants [110]. Such neonatal airway colonization with Gram-negative bacilli is associated with a cytokine response as well as with severe bronchopulmonary dysplasia [111, 112]. The etiologic role of neonatal colonization in children with non-cystic fibrosis bronchiectasis is unclear at this time [113–115], but molecular methods may provide further insight into the pathogenesis of this disorder. Similarly, the etiologic role of bacterial colonization in the pathogenesis of chronic obstructive pulmonary disease [107–109, 116, 117] is currently being elucidated with the assistance of molecular methods.

Simultaneous Detection of Multiple Pathogens

The extreme sensitivity of molecular methods such as NAATs may result in simultaneous detection of multiple pathogens from sputum specimens. Detection of multiple pathogens in sputum by molecular methods has already been reported in community-acquired pneumonia [105] where mixed infections were frequently seen: these most commonly were *S. pneumoniae* together with a respiratory virus. These findings are not unexpected; a number of studies have reported an association between viral respiratory tract infections and invasive pneumococcal disease [118–120]. Molecular diagnostic methods employed in other studies of respiratory tract infections have confirmed the etiologic role viral respiratory tract infections and bacterial pneumonia [121–123].

Accuracy of Assay Development

An important issue for NAATs is whether the amplification products truly represent the target microorganism [104]. Molecular methods that employ DNA sequencing are often considered completely accurate with 100 % sensitivity and specificity.

This, unfortunately, is not the case. There are a variety of technical factors such as the influence of contaminating DNA from other sources on the sequencing template, the selection of the primers used for the amplification, the quality of the base-calling software, and the method used for compiling the "consensus sequence" from multiple forward and reverse reactions [104, 124–127]. Inappropriately chosen gene targets and regions will result in false-positives and negatives. The insertion sequence element IS*481*, found in several hundred copies in the *Bordetella pertussis* genome, is frequently used as a target for *B. pertussis* detection and has a much greater analytical sensitivity than assays with single-copy target sequences, such as that of the pertussis toxin promoter [128]. However, false-positive results have been reported due to the smaller copy numbers of IS481 existing in non-pertussis *Bordetella* species [129, 130]. The accuracy of assay development is often not appreciated by the non-molecular microbiologist or the clinician.

The Use of Molecular Assays for Diagnosing Enteric Infections

Most acute diarrheal illnesses are self-limited or viral [131]. For afebrile patients who present with watery non-bloody diarrhea of less than 24 h duration, microbiologic investigation is usually unnecessary [131, 132]. In contrast, patients with a diarrheal illness lasting for more than one day, especially when the illness is accompanied by fever, bloody stools, recent antimicrobial use, hospitalization, or systemic illness, should have a microbiologic evaluation of their diarrheal stool [131–134]. The microbiologic stool evaluation for such enteric infections has for many decades relied upon the analysis of bacterial cultures and/or microscopy to detect ova and parasites [134]. For nosocomial diarrhea or patients with a history of recent use of antimicrobial agents prior to the onset of diarrhea, the microbiologic stool evaluation should focus on the diagnosis of toxigenic *Clostridium difficile* [135]. For persistent diarrhea in patients with a history of international travel, the microbiologic stool evaluation may require special selective and differential agar such as thiosulfate citrate bile salts sucrose (TSCB) agar for *Vibrio* species [136]. Finally, the noroviruses are the most common cause of non-bacterial enteritis worldwide: the laboratory diagnosis of noroviruses depends on the detection of virus particles by EM, detection of viral antigens by EIA, or detection of viral RNA by real-time PCR [137].

Given the complexity of conventional methods for the microbiologic evaluation of a stool specimen from a patient with a diarrheal illness, it is not surprising that determining the microbiologic etiology of an enteric infection remains an elusive goal [138]. It is no wonder that molecular methods have been applied to the diagnosis of acute infectious diarrhea [138, 139]. Enteric infections due to the broad range of potential pathogens such as viruses, bacteria, protozoa, and helminths are well suited for multiplex molecular assays. Indeed, monoplex and multiplex molecular assays for many of these enteric pathogens have already been described [140–146]. Therefore, it will be important for both clinicians and microbiologists to appreciate the limitations of these molecular assays.

The Limitations of Molecular Assays for Diagnosing Enteric Infections

Lack of a Gold Standard for the Microbiologic Cause of Enteric Infections

The absence of a gold standard for the microbiologic cause of enteric infections means that the clinical significance of a detected pathogen may not always be clear [138]. Although conventional wisdom suggests that there should be one main pathogen causing an enteric infection in a patient, the detection of multiple pathogens in some patients will challenge this thinking [138]. This is apt to be particularly true for parasitic enteric infections. Moreover, the detection of RNA or DNA in a stool specimen does not necessarily mean a viable or an infectious pathogen.

Complexity of the Human Gut Microbiome

Molecular assays including high-throughput sequencing techniques have begun to identify the vast communities of bacteria that inhabit the skin and gut in humans [147]. Despite these methods, the human gut remains relatively unexplored [147, 148]. This complexity is likely to be a factor in the use of NAATs for diagnosing enteric infections if for no other reason than the influence of contaminating DNA from these gut microbes on the sequencing template.

Issues with Nucleic Acid Extraction

The molecular diagnosis of an enteric infection will usually begin with extraction of nucleic acid from the specimen. Because this specimen is generally a diarrheal stool sample, the extraction step becomes a critical step in this molecular diagnostic process. This is because stool is a complex mixture with multiple and diverse nucleic acids and amplification inhibitors. Investigators have noted that detection of a given target will be reduced several logs when the target is placed in a stool mixture [138]. This may result in enteric pathogens present in low numbers being missed. This is the reason that some investigators have used molecular methods following isolation of potential enteric bacterial pathogens from stool [149]. In addition, extraction of DNA from ova and parasites may be more difficult than extracting DNA from bacteria [140]. Concentration of ova and parasites that may be present in low numbers may be required, as it is for microscopic evaluation.

Requirement for Multiplex PCR

Over 50 pathogens currently are recognized a potential causes of enteric infections [138]. This means that a multiplex PCR such as the Luminex bead method must be used. Even a multiplex approach will likely require the use of a diagnostic algorithm

or the use of several multiplex assays. The use of multiplex assays will create several technical problems that include difficulty with discrimination of multiple targets in a single reaction and reduced sensitivity. Multiplex assays also will cause some problems with interpretation due to detection of multiple pathogens. For example, one study that reexamined stool samples using PCR found that the detection rate increased for both viral and bacterial pathogens, but the detection rate for multiple pathogens also increased [150].

Requirement for Quantitative PCR

Molecular assays due to their high sensitivity may detect low levels of enteric pathogens with unclear clinical significance. For example, *Giardia* species are known to occur in stool at high rates in persons without diarrhea [151]. Therefore, the use of quantitative PCR methods may be needed in order to provide information that will be useful for interpreting the clinical significance; the assumption being that a higher burden is more likely to be associated with disease [138]. Ultimately, this relationship of higher burden and symptoms of disease will need to be verified for many enteric pathogens for which this relationship has not yet been determined.

Molecular Detection of Resistant Determinants

Antimicrobial resistance is increasing for many bacterial pathogens and is likely to happen with enteric pathogens such as *Shigella*, *Salmonella*, and *Campylobacter*. Detection of resistance determinants may be necessary in the future and is likely to be difficult from stool samples due to the diversity of microorganisms present in stool [138].

The Use of Molecular Assays for Diagnosing Tissue Infections

The use of molecular assays for diagnosing tissue infections is another area that is rapidly evolving. For example, molecular assays have proven quite successful in the diagnosis of infectious endocarditis [151–160]. Indeed, a number of fastidious microorganisms causing endocarditis have been identified using molecular assays; these include *Tropheryma whippelii* [151], *Bartonella quintana* [153, 158, 160], *Bartonella henselae* [158], and *Coxiella burnetii* [158]. This success has resulted in molecular assays being included in the best practices and guidelines for identification of difficult-to-culture pathogens in infective endocarditis [161]. Molecular assays of tissue have been useful for diagnosing necrotizing fasciitis caused by group A streptococci when cultures were negative or not available [162, 163].

Finally, molecular assays for fungal pathogens also have been widely studied and have the potential to be useful in the diagnosis of fungal tissue infections [164–174]. Fungal pathogens identified from tissue by molecular assays include *Paracoccidioides*

brasiliensis [165], *Histoplasma capsulatum* [164], *Coccidioides immitis* [174], *Blastomyces dermatitidis* [174], *Aspergillus fumigatus* [166], *Absidia corymbifera* [166], *Rhisopus arrhizus* [166], and *Candida* species [171]. NNATs have been used to detect a variety of DNA and RNA viral pathogens in formalin-fixed, paraffin-embedded (FFPE) tissue specimens [175–180]. The use of molecular assays for diagnosing tissue infections will only increase over time; therefore, the limitations of these molecular assays should be appreciated.

Limitations in the Use of Molecular Assays for Diagnosing Tissue Infections

Fresh/Frozen Tissue Versus Formalin-Fixed, Paraffin-Embedded Tissue

Fresh/frozen tissue is best for molecular testing and should be available if molecular testing is considered at the time of biopsy [181, 182]. In contrast, FFPE tissue often is the only tissue available when molecular testing is considered as an afterthought. Accordingly, one of the most important limitations in the use of molecular assays for diagnosing tissue infections is considering these assays at the time of biopsy so that fresh tissue can be used or frozen for use later. The difference in sensitivity for PCR testing can be seen by a study in which fresh nonembedded tissues were found to have sensitivities for PCR detection of fungi of 97 % versus only 68 % for FFPE tissue [181]. The reason for this decreased sensitivity is that nucleic acids obtained from FFPE tissue are frequently damaged (i.e., cross-linked) and may contain PCR inhibitors [183, 184]. If FFPE tissue must be used, a housekeeping human gene must be amplified as a control [179, 184].

Wide Diversity of Potential Microbial Pathogens

The wide diversity of potential microbial pathogens that could potentially be detected in tissue is readily apparent. These pathogens could be viral, bacterial, fungal, or parasitic. This diversity will greatly influence the DNA targets and the PCR primers used as well as whether monoplex or multiplex PCR methods will be used. For example, species-specific identification of a wide range of clinically relevant fungal pathogens using Luminex technology required up to three different probes for each fungal pathogen using the internal transcribed spacer (ITS2) region, which is highly variable among genomes of individual fungal species [185].

Choice of DNA Target, PCR Primers, and Amplification Method

The choice of the DNA target is important [167]. In general, molecular assays that target multicopy genes provide the greatest sensitivity. Amplification methods

should provide objective endpoint assessments for the PDR test used. PCR primers are important. For example, there is insufficient variation in the internal transcribed spacer (ITS1) region to differentiate certain species of fungal pathogens [186]; therefore, analysis of other regions such as ITS2 should be considered. False-positive results have been described with certain primer for *H. capsulatum* [164]. False-negative results have been found for *C. immitis* from FFPE tissue (73 % sensitivity) versus fresh tissue (93 % sensitivity) suggesting a primer problem, degradation, or inhibitors [164]. Finally, it is estimated that approximately 10–20 % of the sequences in GenBank are misidentified [187]. Currently there are relatively few commercial kits available for molecular testing using tissue specimens. If laboratory-developed PCR assays for tissues are used, they must be evaluated, verified, and validated by the laboratory before the results can be used for clinical diagnosis and patient care [186, 188].

Issues with Nucleic Acid Extraction

DNA extraction from FFPE tissues is difficult and requires special protocols [187]. The amount of DNA extracted is usually quite small; reported methods show an amplification success rate between 60 % and 80 %. Commercial DNA extraction kits have been evaluated [187]; one method (TaKaRa) was noted to extract DNA for 69 of the 74 FFPE tissue samples from which a housekeeping gene could be amplified. Moreover, this method was cost-effective and had a non-laborious protocol. Successful extraction of RNA from FFPE specimens depends on the prompt original tissue processing and a well-developed extraction protocol [175, 179, 180, 184].

Low Number of Pathogens and/or Random Distribution in Tissue

When the number of pathogens is scant in tissues, the amount of DNA obtained may be insufficient to perform a PCR assay. Moreover, these pathogens are often randomly distributed in the tissue [37, 42]. When FFPE tissue is used, a punch biopsy can be used to take a sample from an area noted to have inflammation and/or microorganisms by a stained slide from the same tissue block. The stained slide can be marked and then used to direct the location for the punch biopsy sample from the tissue block. Use of fresh or frozen tissue is more problematic as the selection of tissue will be random and may not contain microorganisms.

Simultaneous Detection of Multiple Pathogens

As would be expected, molecular assays already have been noted to detect mixed infections. This may present difficulty in interpretation of the results. In particular, microbial diversity in endocarditis has been noted with cultivation-independent molecular techniques [188]. Multiple pathogens detected by molecular assays have also been reported in fungal infections [166].

Concluding Remarks

Outcomes from infectious diseases are directly related to the length of time required for identification of the microbial pathogen. Until recently, clinical microbiology laboratories have been handicapped by conventional, slow multistep culture-based techniques that require prolonged incubation times for many pathogens and are not able to isolate others. Clinicians unable by clinical judgment or diagnostic results to quickly and accurately identify a pathogen causing infection must adopt a conservative approach involving empiric therapy with broad-spectrum antimicrobial agents. Fortunately, this cumbersome approach is rapidly changing because of the introduction of molecular diagnostic techniques. Indeed, molecular assays such as NAATs have initiated a revolution in the field of diagnostic microbiology due to their high sensitivity, specificity, rapid test turnaround time as well as potential high throughput and automation. Molecular assays have been heralded as the "diagnostic tool for the millennium" [189, 190]. However, molecular assays also bring some uncertainty such as that caused by false-positive results due to background contamination from exogenous sources of DNA [190, 191]. For example, one study using a universal 16S rRNA PCR assays detected eubacterial DNA in blood samples from healthy subjects [192]. NAATs also may give false-negative results due to two principle reasons: (1) the relatively small sample required for PCR reactions and (2) technical problems associated with PCR processing [193]. Moreover, the results of molecular assays may be difficult to interpret and apply in the clinical setting. As NAATs are increasingly used in routine clinical microbiology laboratories, interpretation is expected to be more difficult as new tests are developed and more complicated multiplex assays emerge. For example, clinical relevance of positive NAATs in paraffin block specimens and multiple microbial organisms found in any specimen will need careful interpretation. As the usefulness of these molecular assays is determined by usage over time, communication between the clinician and the microbiology laboratory is always suggested whenever an interpretation is needed. Finally, both the clinical microbiologist and the clinician must acquire a working knowledge of the principles, diagnostic value, and limitations of these molecular assays [194, 195].

References

1. Tang YW, Stratton CW (2006) Advanced techniques in diagnostic microbiology. Springer Science, New York, NY
2. Tang YW, Persing DH (2009) Diagnostic microbiology. In: Schaechter M (ed) Encyclopedia of microbiology, 3rd edn. Elsevier, Oxford, pp 308–320
3. Tang YW (2007) Laboratory diagnosis of central nervous system infections by molecular amplification techniques. Exp Opin Med Diagn 1:489–509
4. Smalling TW, Sefers SE, Li HJ, Tang YW (2002) Molecular approaches to detecting herpes simplex virus and enteroviruses in the central nervous system. J Clin Microbiol 40:2317–2322
5. Tang YW, Mitchell PS, Espy MJ, Smith TF, Persing DH (1999) Molecular diagnosis of herpes simplex virus infections in the central nervous system. J Clin Microbiol 37:2127–2136

6. Yamamoto Y (2002) PCR in diagnosis of infection: detection of bacteria in cerebrospinal fluids. Clin Diagn Lab Immunol 9:508–514
7. Balasingham SV, Davidsen T, Szpinda I, Frye SA, Tonjum T (2009) Molecular diagnostics in tuberculosis: basis and implications for therapy. Mol Diagn Ther 13:137–151. doi:10.2165/01250444-200913030-00001
8. Ecker DJ, Sampath R, Li H et al (2010) New technology for rapid molecular diagnosis of bloodstream infections. Expert Rev Mol Diagn 10:399–415
9. Crump JA, Tuohy MJ, Morrissey AB et al (2011) Performance of nucleic acid amplification following extraction of 5 mL of whole blood for the diagnosis of Mycobacterium tuberculosis bacteremia. J Clin Microbiol 26:26
10. Mancini N, Carletti S, Ghidoli N, Cichero P, Burioni R, Clementi M (2010) The era of molecular and other non-culture-based methods in diagnosis of sepsis. Clin Microbiol Rev 23: 235–251
11. Bastien P, Procop GW, Reischl U (2008) Quantitative real-time PCR is not more sensitive than "conventional" PCR. J Clin Microbiol 46:1897–1900, Epub 9 Apr 2008
12. Tang YW, Sriram S, Li H et al (2009) Qualitative and quantitative detection of Chlamydophila pneumoniae DNA in cerebrospinal fluid from multiple sclerosis patients and controls. PLoS One 4:e5200, Epub 9 Apr 2009
13. Tang YW, Rys PN, Rutledge BJ, Mitchell PS, Smith TF, Persing DH (1998) Comparative evaluation of colorimetric microtiter plate systems for detection of herpes simplex virus in cerebrospinal fluid. J Clin Microbiol 36:2714–2717
14. Li L, Mahan CS, Palaci M et al (2010) Sputum Mycobacterium tuberculosis mRNA as a marker of bacteriologic clearance in response to antituberculosis therapy. Monitoring therapeutic efficacy by real-time detection of Mycobacterium tuberculosis mRNA in sputum. J Clin Microbiol 48:46–51, Epub 18 Nov 2009
15. Mdivani N, Li H, Akhalaia M et al (2009) Monitoring therapeutic efficacy by real-time detection of Mycobacterium tuberculosis mRNA in sputum. Clin Chem 55:1694–1700, Epub 2 Jul 2009
16. Gaydos CA, Crotchfelt KA, Howell MR, Kralian S, Hauptman P, Quinn TC (1998) Molecular amplification assays to detect chlamydial infections in urine specimens from high school female students and to monitor the persistence of chlamydial DNA after therapy. J Infect Dis 177:417–424
17. Schmid DS, Jumaan AO (2010) Impact of varicella vaccine on varicella-zoster virus dynamics. Clin Microbiol Rev 23:202–217
18. Keefer CS (1941) The clinical significance of bacteremia. NY State Med J 41:976–981
19. Fox H, Forrester JS (1940) Clinical blood culture. An analysis of over 5,000 cases. Am J Clin Pathol 10:493–504
20. Bryan CS (1989) Clinical implications of positive blood cultures. Clin Microbiol Rev 2: 329–353
21. Weinstein MP, Reller LB, Murphy JR, Lichtenstein KA (1983) The clinical significance of positive blood cultures: a comprehensive analysis of 500 episodes of bacteremia and fungemia in adults. I. Laboratory and epidemiologic observations. Rev Infect Dis 5:35–53
22. Weinstein MP, Towns ML, Quartey SM et al (1997) The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. Clin Infect Dis 24:584–602
23. Hall KK, Lyman JA (2006) Updated review of blood culture contamination. Clin Microbiol Rev 19:788–802
24. Struelens MJ (2010) Detection of microbial DNAemia: does it matter for sepsis management? Intensive Care Med 36:193–195
25. Schifman RB, Bachner P, Howanitz PJ (1996) Blood culture quality improvement: a College of American Pathologists Q-Probes study involving 909 institutions and 289 572 blood culture sets. Arch Pathol Lab Med 120:999–1002
26. Eshoo MW, Crowder CD, Li H et al (2010) Detection and identification of Ehrlichia species in blood by use of PCR and electrospray ionization mass spectrometry. J Clin Microbiol 48: 472–478

27. Bloos F, Hinder F, Becker K et al (2010) A multicenter trial to compare blood culture with polymerase chain reaction in severe human sepsis. Intensive Care Med 36:241–247
28. Dierkes C, Ehrenstein B, Siebig S, Linde HJ, Reischl U, Salzberger B (2009) Clinical impact of a commercially available multiplex PCR system for rapid detection of pathogens in patients with presumed sepsis. BMC Infect Dis 9:126
29. Louie RF, Tang Z, Albertson TE, Cohen S, Tran NK, Kost GJ (2008) Multiplex polymerase chain reaction detection enhancement of bacteremia and fungemia. Crit Care Med 36: 1487–1492
30. Mancini N, Clerici D, Diotti R et al (2008) Molecular diagnosis of sepsis in neutropenic patients with haematological malignancies. J Med Microbiol 57:601–604
31. Reier-Nilsen T, Farstad T, Nakstad B, Lauvrak V, Steinbakk M (2009) Comparison of broad range 16 S rDNA PCR and conventional blood culture for diagnosis of sepsis in the newborn: a case control study. BMC Pediatr 9:5
32. Westh H, Lisby G, Breysse F et al (2009) Multiplex real-time PCR and blood culture for identification of bloodstream pathogens in patients with suspected sepsis. Clin Microbiol Infect 15:544–551
33. Dark PM, Dean P, Warhurst G (2009) Bench-to-bedside review: the promise of rapid infection diagnosis during sepsis using polymerase chain reaction-based pathogen detection. Crit Care 13:217
34. Jorgensen JH, Ferraro MJ (2009) Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin Infect Dis 49:1749–1755
35. Holland TL, Woods CW, Joyce M (2009) Antibacterial susceptibility testing in the clinical laboratory. Infect Dis Clin North Am 23:757–790, vii
36. Tenover FC (1989) DNA probes for antimicrobial susceptibility testing. Clin Lab Med 9: 341–347
37. Forster LI (2003) Measurement uncertainty in microbiology. J AOAC Int 86:1089–1094
38. Hall MM, Ilstrup DM, Washington JA 2nd (1976) Effect of volume of blood cultured on detection of bacteremia. J Clin Microbiol 3:643–645
39. Mermel LA, Maki DG (1993) Detection of bacteremia in adults: consequences of culturing an inadequate volume of blood. Ann Intern Med 119:270–272
40. Schelonka RL, Chai MK, Yoder BA, Hensley D, Brockett RM, Ascher DP (1996) Volume of blood required to detect common neonatal pathogens. J Pediatr 129:275–278
41. Wilson ML, Weinstein MP (1994) General principles in the laboratory detection of bacteremia and fungemia. Clin Lab Med 14:69–82
42. Sun X, Kurosu S, Shintani H (2006) The expanded application of most probable number to the quantitative evaluation of extremely low microbial count. PDA J Pharm Sci Technol 60: 124–134
43. Lawn SD, Zumla AI (2011) Tuberculosis. Lancet 378:57–72
44. Wilson ML (2011) Recent advances in the laboratory detection of Mycobacterium tuberculosis complex and drug resistance. Clin Infect Dis 52:1350–1355
45. Campbell PJ, Morlock GP, Sikes RD et al (2011) Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrob Agents Chemother 55:2032–2041
46. Woods GL (1999) Molecular methods in the detection and identification of mycobacterial infections. Arch Pathol Lab Med 123:1002–1006
47. Boehme CC, Nabeta P, Henostroza G et al (2007) Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. J Clin Microbiol 45:1936–1940
48. Min JW, Yoon HI, Park KU, Song JH, Lee CT, Lee JH (2010) Real-time polymerase chain reaction in bronchial aspirate for rapid detection of sputum smear-negative tuberculosis. Int J Tuberc Lung Dis 14:852–858
49. Kibiki GS, Mulder B, van der Ven AJ et al (2007) Laboratory diagnosis of pulmonary tuberculosis in TB and HIV endemic settings and the contribution of real time PCR for M. tuberculosis in bronchoalveolar lavage fluid. Trop Med Int Health 12:1210–1217

50. Massire C, Ivy CA, Lovari R et al (2011) Simultaneous identification of mycobacterial isolates to the species level and determination of tuberculosis drug resistance by PCR followed by electrospray ionization mass spectrometry. J Clin Microbiol 49:908–917, Epub 29 Dec 2010
51. Gegia M, Mdivani N, Mendes RE et al (2008) Prevalence of and molecular basis for tuberculosis drug resistance in the Republic of Georgia: validation of a QIAplex system for detection of drug resistance-related mutations. Antimicrob Agents Chemother 52:725–729, Epub 10 Dec 2007
52. Cooksey RC, Morlock GP, Glickman S, Crawford JT (1997) Evaluation of a line probe assay kit for characterization of rpoB mutations in rifampin-resistant Mycobacterium tuberculosis isolates from New York City. J Clin Microbiol 35:1281–1283
53. Abebe G, Paasch F, Apers L, Rigouts L, Colebunders R (2011) Tuberculosis drug resistance testing by molecular methods: opportunities and challenges in resource limited settings. J Microbiol Methods 84:155–160
54. Boehme CC, Nabeta P, Hillemann D et al (2010) Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med 363:1005–1015
55. Chedore P, Broukhanski G, Shainhouse Z, Jamieson F (2006) False-positive amplified Mycobacterium tuberculosis direct test results for samples containing Mycobacterium leprae. J Clin Microbiol 44:612–613
56. Lefmann M, Moter A, Schweickert B, Gobel UB (2005) Misidentification of Mycobacterium leprae as Mycobacterium intracellulare by the COBAS AMPLICOR M. intracellulare test. J Clin Microbiol 43:1928–1929
57. Bartlett JG (2011) Diagnostic tests for agents of community-acquired pneumonia. Clin Infect Dis 52(Suppl 4):S296–S304
58. Endimiani A, Hujer KM, Hujer AM et al (2011) Are we ready for novel detection methods to treat respiratory pathogens in hospital-acquired pneumonia? Clin Infect Dis 52(Suppl 4): S373–S383
59. Cleri DJ, Ricketti AJ, Vernaleo JR (2010) Severe acute respiratory syndrome (SARS). Infect Dis Clin North Am 24:175–202
60. Ramsey C, Kumar A (2011) H1N1: viral pneumonia as a cause of acute respiratory distress syndrome. Curr Opin Crit Care 17:64–71
61. Lapinsky SE (2010) Epidemic viral pneumonia. Curr Opin Infect Dis 23:139–144
62. Grijalva CG, Nuorti JP, Griffin MR (2009) Antibiotic prescription rates for acute respiratory tract infections in US ambulatory settings. JAMA 302:758–766
63. Gabbett HS (1884) The diagnostic value of the discovery of Koch's Bacilli in sputum. Br Med J 1:805–808
64. Smith WH (1898) A Case of Pneumonia due to the Bacillus Mucosus Capsulatus. (B. of Friedlander.). J Boston Soc Med Sci 2:174–179
65. Smith WH (1899) The influenza bacillus and pneumonia. J Boston Soc Med Sci 3:274–289
66. Wollstein M (1905) The bacteriology oe broncho- and lobular pneumonia in infancy. J Exp Med 6:391–400
67. Smith WH (1902) A method of staining sputum for bacteriological examination. Bost Med Surg J 147:659–664
68. Hastings TW, Boehm E (1913) A study of cultures from sputum and blood in lobar pneumonia. J Exp Med 17:239–251
69. Hastings TW, Niles WL (1911) The bacteriology of sputum in common non-tuberculous infections of the upper and lower respiratory tracts, with special reference to lobar and broncho-pneumonia. J Exp Med 13:638–651
70. Leutscher JA (1915) A bacteriological and clinical study of the non-tuberculous infections of the respiratory tract, with special reference to sputum cultures as a means of diagnosis. Arch Int Med 16:657–680
71. Gaensler EA, Moister VB, Hamm J (1964) Open-lung biopsy in duffuse pulmonary disease. N Engl J Med 270:1319–1331
72. Kalinske RW, Parker RH, Brandt D, Hoeprich PD (1965) Transtracheal aspiration in diagnosis of lower respiratory tract diseases. Antimicrob Agents Chemother (Bethesda) 5:30–36

73. Morrow GW Jr, Andersen HA, Geraci JE (1964) The diagnosis and management of acute infectious pneumonia. Med Clin North Am 48:829–838
74. Mulder J (1964) Clinical significance of bacteriologic examination of sputum in cases of acute and chronic bacterial disease of respiratory tract. Adv Intern Med 12:233–255
75. Shulman JA, Phillips LA, Petersdorf RG (1965) Errors and hazards in the diagnosis and treatment of bacterial pneumonias. Ann Intern Med 62:41–58
76. Manfredi F, Rosenbaum D, Behnke RH (1963) Percutaneous needle biopsy of the lung in diffuse pulmonary diseases. Ann Intern Med 58:773–778
77. Hewitt WL (1970) Bacteria in sputum–contaminants or culprits? Calif Med 112:60–62
78. Hoeprich PD (1970) Etiologic diagnosis of lower respiratory tract infections. Calif Med 112:1–8
79. Jackson C (1925) Bronchoscopy for disease. Br Med J 2:699
80. Wimberley N, Faling LJ, Bartlett JG (1979) A fiberoptic bronchoscopy technique to obtain uncontaminated lower airway secretions for bacterial culture. Am Rev Respir Dis 119:337–343
81. Thorpe JE, Baughman RP, Frame PT, Wesseler TA, Staneck JL (1987) Bronchoalveolar lavage for diagnosing acute bacterial pneumonia. J Infect Dis 155:855–861
82. Coster JF, Barrett-Connor E (1972) The nonvalue of sputum culture in the diagnosis of pneumonia. Am Rev Respir Dis 105:139–140
83. Ewig S, Schlochtermeier M, Goke N, Niederman MS (2002) Applying sputum as a diagnostic tool in pneumonia: limited yield, minimal impact on treatment decisions. Chest 121:1486–1492
84. Heineman HS, Chawla JK, Lopton WM (1977) Misinformation from sputum cultures without microscopic examination. J Clin Microbiol 6:518–527
85. Lentino JR, Lucks DA (1987) Nonvalue of sputum culture in the management of lower respiratory tract infections. J Clin Microbiol 25:758–762
86. Lidman C, Burman LG, Lagergren A, Ortqvist A (2002) Limited value of routine microbiological diagnostics in patients hospitalized for community-acquired pneumonia. Scand J Infect Dis 34:873–879
87. Miyashita N, Shimizu H, Ouchi K et al (2008) Assessment of the usefulness of sputum Gram stain and culture for diagnosis of community-acquired pneumonia requiring hospitalization. Med Sci Monit 14:CR171–CR176
88. Theerthakarai R, El-Halees W, Ismail M, Solis RA, Khan MA (2001) Nonvalue of the initial microbiological studies in the management of nonsevere community-acquired pneumonia. Chest 119:181–184
89. Mandell LA, Wunderink RG, Anzueto A et al (2007) Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis 44(Suppl 2):S27–S72
90. Murdoch DR (2003) Nucleic acid amplification tests for the diagnosis of pneumonia. Clin Infect Dis 36:1162–1170, Epub 22 Apr 2003
91. Murdoch DR (2004) Molecular genetic methods in the diagnosis of lower respiratory tract infections. APMIS 112:713–727
92. Blackmore TK, Reznikov M, Gordon DL (1995) Clinical utility of the polymerase chain reaction to diagnose Mycoplasma pneumoniae infection. Pathology 27:177–181
93. Deng J, Zheng Y, Zhao R, Wright PF, Stratton CW, Tang YW (2009) Culture versus polymerase chain reaction for the etiologic diagnosis of community-acquired pneumonia in antibiotic-pretreated pediatric patients. Pediatr Infect Dis J 28:53–55
94. Jaulhac B, Nowicki M, Bornstein N et al (1992) Detection of Legionella spp. in bronchoalveolar lavage fluids by DNA amplification. J Clin Microbiol 30:920–924
95. Tong CY, Sillis M (1993) Detection of Chlamydia pneumoniae and Chlamydia psittaci in sputum samples by PCR. J Clin Pathol 46:313–317
96. Muller FM, Hoppe JE, Wirsing von Konig CH (1997) Laboratory diagnosis of pertussis: state of the art in 1997. J Clin Microbiol 35:2435–2443
97. van der Zee A, Agterberg C, Peeters M, Mooi F, Schellekens J (1996) A clinical validation of Bordetella pertussis and Bordetella parapertussis polymerase chain reaction: comparison with culture and serology using samples from patients with suspected whooping cough from a highly immunized population. J Infect Dis 174:89–96

98. Hauk L (2011) CDC releases best practices for the use of PCR testing for diagnosing pertussis. Am Fam Physician 84:1176
99. Yan Y, Zhang S, Tang YW (2011) Molecular assays for the detection and characterization of respiratory viruses. Semin Respir Crit Care Med 32:512–526, Epub 19 Aug 2011
100. Caliendo AM (2011) Multiplex PCR and emerging technologies for the detection of respiratory pathogens. Clin Infect Dis 52:S326–S330
101. Mahony JB (2008) Detection of respiratory viruses by molecular methods. Clin Microbiol Rev 21:716–747
102. Wu W, Tang YW (2009) Emerging molecular assays for detection and characterization of respiratory viruses. Clin Lab Med 29:673–693
103. IDSA (2011) An unmet medical need: rapid molecular diagnostics tests for respiratory tract infections. Clin Infect Dis 52:S384–S395
104. Tenover FC (2011) Developing molecular amplification methods for rapid diagnosis of respiratory tract infections caused by bacterial pathogens. Clin Infect Dis 52:S338–S345
105. Johansson N, Kalin M, Tiveljung-Lindell A, Giske CG, Hedlund J (2010) Etiology of community-acquired pneumonia: increased microbiological yield with new diagnostic methods. Clin Infect Dis 50:202–209
106. Oosterheert JJ, van Loon AM, Schuurman R et al (2005) Impact of rapid detection of viral and atypical bacterial pathogens by real-time polymerase chain reaction for patients with lower respiratory tract infection. Clin Infect Dis 41:1438–1444, Epub 13 Oct 2005
107. Cabello H, Torres A, Celis R et al (1997) Bacterial colonization of distal airways in healthy subjects and chronic lung disease: a bronchoscopic study. Eur Respir J 10:1137–1144
108. Huang YJ, Lynch SV (2011) The emerging relationship between the airway microbiota and chronic respiratory disease: clinical implications. Expert Rev Respir Med 5:809–821
109. Huang YJ, Kim E, Cox MJ et al (2010) A persistent and diverse airway microbiota present during chronic obstructive pulmonary disease exacerbations. Omics 14:9–59
110. Mourani PM, Harris JK, Sontag MK, Robertson CE, Abman SH (2011) Molecular identification of bacteria in tracheal aspirate fluid from mechanically ventilated preterm infants. PLoS One 6:e25959, Epub 10 Oct 2011
111. Cordero L, Ayers LW, Davis K (1997) Neonatal airway colonization with gram-negative bacilli: association with severity of bronchopulmonary dysplasia. Pediatr Infect Dis J 16:18–23
112. De Dooy J, Ieven M, Stevens W, Schuerwegh A, Mahieu L (2004) Endotracheal colonization at birth is associated with a pathogen-dependent pro- and antiinflammatory cytokine response in ventilated preterm infants: a prospective cohort study. Pediatr Res 56:547–552, Epub 4 Aug 2004
113. King P (2011) Pathogenesis of bronchiectasis. Paediatr Respir Rev 12:104–110, Epub 24 Nov 2010
114. Redding GJ (2009) Bronchiectasis in children. Pediatr Clin North Am 56:157–171, xi
115. Stafler P, Carr SB (2010) Non-cystic fibrosis bronchiectasis: its diagnosis and management. Arch Dis Child Educ Pract Ed 95:73–82
116. Sethi S, Maloney J, Grove L, Wrona C, Berenson CS (2006) Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 173:991–998, Epub 10 Feb 2006
117. van Alphen L, Jansen HM, Dankert J (1995) Virulence factors in the colonization and persistence of bacteria in the airways. Am J Respir Crit Care Med 151:2094–2099, discussion 9–100
118. Murdoch DR, Jennings LC (2009) Association of respiratory virus activity and environmental factors with the incidence of invasive pneumococcal disease. J Infect 58:37–46, Epub 29 Nov 2008
119. Stensballe LG, Hjuler T, Andersen A et al (2008) Hospitalization for respiratory syncytial virus infection and invasive pneumococcal disease in Danish children aged <2 years: a population-based cohort study. Clin Infect Dis 46:1165–1171
120. Watson M, Gilmour R, Menzies R, Ferson M, McIntyre P (2006) The association of respiratory viruses, temperature, and other climatic parameters with the incidence of invasive pneumococcal disease in Sydney, Australia. Clin Infect Dis 42:211–215, Epub 12 Dec 2005

121. Creer DD, Dilworth JP, Gillespie SH et al (2006) Aetiological role of viral and bacterial infections in acute adult lower respiratory tract infection (LRTI) in primary care. Thorax 61:75–79, Epub 14 Oct 2005
122. Johnstone J, Majumdar SR, Fox JD, Marrie TJ (2008) Viral infection in adults hospitalized with community-acquired pneumonia: prevalence, pathogens, and presentation. Chest 134:1141–1148, Epub 8 Aug 2008
123. Vu HT, Yoshida LM, Suzuki M et al (2011) Association between nasopharyngeal load of Streptococcus pneumoniae, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. Pediatr Infect Dis J 30:11–18
124. Bonfield JK, Staden R (1995) The application of numerical estimates of base calling accuracy to DNA sequencing projects. Nucleic Acids Res 23:1406–1410
125. Churchill GA, Waterman MS (1992) The accuracy of DNA sequences: estimating sequence quality. Genomics 14:89–98
126. Ewing B, Green P (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res 8:186–194
127. Richterich P (1998) Estimation of errors in "raw" DNA sequences: a validation study. Genome Res 8:251–259
128. Andre P, Caro V, Njamkepo E, Wendelboe AM, Van Rie A, Guiso N (2008) Comparison of serological and real-time PCR assays to diagnose Bordetella pertussis infection in 2007. J Clin Microbiol 46:1672–1677, Epub 26 Mar 2008
129. Loeffelholz MJ, Thompson CJ, Long KS, Gilchrist MJ (2000) Detection of Bordetella holmesii using Bordetella pertussis IS481 PCR assay. J Clin Microbiol 38:467
130. Register KB, Sanden GN (2006) Prevalence and sequence variants of IS481 in Bordetella bronchiseptica: implications for IS481-based detection of Bordetella pertussis. J Clin Microbiol 44:4577–4583, Epub 25 Oct 2006
131. Hoshiko M (1994) Laboratory diagnosis of infectious diarrhea. Pediatr Ann 23:570–574
132. Thielman NM, Guerrant RL (2004) Clinical practice. Acute infectious diarrhea. N Engl J Med 350:38–47
133. Guerrant RL, Van Gilder T, Steiner TS et al (2001) Practice guidelines for the management of infectious diarrhea. Clin Infect Dis 32:331–351, Epub 30 Jan 2001
134. Pawlowski SW, Warren CA, Guerrant R (2009) Diagnosis and treatment of acute or persistent diarrhea. Gastroenterology 136:1874–1886, Epub 7 May 2009
135. Kufelnicka AM, Kirn TJ (2011) Effective utilization of evolving methods for the laboratory diagnosis of Clostridium difficile infection. Clin Infect Dis 52:1451–1457
136. Ryan ET, Madoff LC, Ferraro MJ (2011) Case records of the Massachusetts General Hospital. Case 20–2011. A 30-year-old man with diarrhea after a trip to the Dominican Republic. N Engl J Med 364:2536–2541
137. Patel MM, Hall AJ, Vinje J, Parashar UD (2009) Noroviruses: a comprehensive review. J Clin Virol 44:1–8, Epub 11 Dec 2008
138. Platts-Mills JA, Operario DJ, Houpt ER (2011) Molecular diagnosis of diarrhea: current status and future potential. Curr Infect Dis Rep 26:26
139. Echeverria P, Sethabutr O, Serichantalergs O (1993) Modern diagnosis (with molecular tests) of acute infectious diarrhea. Gastroenterol Clin North Am 22:661–682
140. Calderaro A, Gorrini C, Montecchini S et al (2010) Evaluation of a real-time polymerase chain reaction assay for the laboratory diagnosis of giardiasis. Diagn Microbiol Infect Dis 66:261–267, Epub 10 Nov 2009
141. Liu J, Gratz J, Maro A et al (2011) Simultaneous detection of six diarrhea-causing bacterial pathogens with an in house PCR-Luminex Assay. J Clin Microbiol 9:9
142. Liu J, Kibiki G, Maro V et al (2011) Multiplex reverse transcription PCR Luminex assay for detection and quantitation of viral agents of gastroenteritis. J Clin Virol 50:308–313, Epub 21 Jan 2011
143. O'Leary J, Corcoran D, Lucey B (2009) Comparison of the EntericBio multiplex PCR system with routine culture for detection of bacterial enteric pathogens. J Clin Microbiol 47:3449–3453, Epub 2 Sept 2009

144. Taniuchi M, Verweij JJ, Noor Z et al (2011) High throughput multiplex PCR and probe-based detection with Luminex beads for seven intestinal parasites. Am J Trop Med Hyg 84: 332–337
145. Taniuchi M, Verweij JJ, Sethabutr O et al (2011) Multiplex polymerase chain reaction method to detect Cyclospora, Cystoisospora, and Microsporidia in stool samples. Diagn Microbiol Infect Dis 71:386–390, Epub 6 Oct 2011
146. Wolffs PF, Bruggeman CA, van Well GT, van Loo IH (2011) Replacing traditional diagnostics of fecal viral pathogens by a comprehensive panel of real-time PCRs. J Clin Microbiol 49:1926–1931, Epub 23 Mar 2011
147. Parfrey LW, Walters WA, Knight R (2011) Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions. Front Microbiol 2:153, Epub 11 Jul 2011
148. Walter J, Ley R (2011) The human gut microbiome: ecology and recent evolutionary changes. Annu Rev Microbiol 65:411–429
149. He Y, Li H, Lu X, Stratton CW, Tang YW (2010) Mass spectrometry biotyper system identifies enteric bacterial pathogens directly from colonies grown on selective stool culture media. J Clin Microbiol 48:3888–3892, Epub 15 Sept 2010
150. Amar CF, East CL, Gray J, Iturriza-Gomara M, Maclure EA, McLauchlin J (2007) Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case–control Infectious Intestinal Disease Study (1993–1996). Eur J Clin Microbiol Infect Dis 26:311–323
151. Goldenberger D, Kunzli A, Vogt P, Zbinden R, Altwegg M (1997) Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. J Clin Microbiol 35:2733–2739
152. Khulordava I, Miller G, Haas D et al (2003) Identification of the bacterial etiology of culture-negative endocarditis by amplification and sequencing of a small ribosomal RNA gene. Diagn Microbiol Infect Dis 46:9–11
153. Kotilainen P, Heiro M, Jalava J et al (2006) Aetiological diagnosis of infective endocarditis by direct amplification of rRNA genes from surgically removed valve tissue. An 11-year experience in a Finnish teaching hospital. Ann Med 38:263–273
154. Lang S, Watkin RW, Lambert PA, Bonser RS, Littler WA, Elliott TS (2004) Evaluation of PCR in the molecular diagnosis of endocarditis. J Infect 48:269–275
155. Lisby G, Gutschik E, Durack DT (2002) Molecular methods for diagnosis of infective endocarditis. Infect Dis Clin North Am 16:393–412, x
156. Madershahian N, Strauch JT, Breuer M, Bruhin R, Straube E, Wahlers T (2005) Polymerase chain reaction amplification as a diagnostic tool in culture-negative multiple-valve endocarditis. Ann Thorac Surg 79:e21–e22
157. Mencacci A, Leli C, Cardaccia A et al (2011) Comparison of conventional culture with SeptiFast real-time PCR for microbial pathogen detection in clinical specimens other than blood. J Med Microbiol 60:1774–1778, Epub 11 Aug 2011
158. Tang YW (2009) Duplex PCR assay simultaneously detecting and differentiating Bartonella quintana, B. henselae, and Coxiella burnetii in surgical heart valve specimens. J Clin Microbiol 47:2647–2650, Epub 24 Jun 2009
159. Wilck MB, Wu Y, Howe JG, Crouch JY, Edberg SC (2001) Endocarditis caused by culture-negative organisms visible by Brown and Brenn staining: utility of PCR and DNA sequencing for diagnosis. J Clin Microbiol 39:2025–2027
160. Rahimian J, Raoult D, Tang YW, Hanna BA (2006) Bartonella quintana endocarditis with positive serology for Coxiella burnetii. J Infect 53:e151–e153, Epub 27 Dec 2005
161. Houpikian P, Raoult D (2003) Diagnostic methods. Current best practices and guidelines for identification of difficult-to-culture pathogens in infective endocarditis. Cardiol Clin 21: 207–217
162. Louie L, Simor AE, Louie M, McGeer A, Low DE (1998) Diagnosis of group A streptococcal necrotizing fasciitis by using PCR to amplify the streptococcal pyrogenic exotoxin B gene. J Clin Microbiol 36:1769–1771

163. Muldrew KL, Simpson JF, Stratton CW, Tang YW (2005) Molecular diagnosis of necrotizing fasciitis by 16 S rRNA gene sequencing and superantigen gene detection. J Mol Diagn 7: 641–645
164. Bialek R, Feucht A, Aepinus C et al (2002) Evaluation of two nested PCR assays for detection of Histoplasma capsulatum DNA in human tissue. J Clin Microbiol 40:1644–1647
165. Bialek R, Ibricevic A, Aepinus C et al (2000) Detection of Paracoccidioides brasiliensis in tissue samples by a nested PCR assay. J Clin Microbiol 38:2940–2942
166. Bialek R, Konrad F, Kern J et al (2005) PCR based identification and discrimination of agents of mucormycosis and aspergillosis in paraffin wax embedded tissue. J Clin Pathol 58: 1180–1184
167. Chen SC, Halliday CL, Meyer W (2002) A review of nucleic acid-based diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays. Med Mycol 40:333–357
168. Hammond SP, Bialek R, Milner DA, Petschnigg EM, Baden LR, Marty FM (2011) Molecular methods to improve diagnosis and identification of mucormycosis. J Clin Microbiol 49: 2151–2153, Epub 20 Apr 2011
169. Lass-Florl C, Follett SA, Moody A, Denning DW (2011) Detection of Aspergillus in lung and other tissue samples using the MycAssay Aspergillus real-time PCR kit. Can J Microbiol 57:765–768, Epub 23 Aug 2011
170. Rickerts V, Just-Nubling G, Konrad F et al (2006) Diagnosis of invasive aspergillosis and mucormycosis in immunocompromised patients by seminested PCR assay of tissue samples. Eur J Clin Microbiol Infect Dis 25:8–13
171. Rickerts V, Khot PD, Myerson D, Ko DL, Lambrecht E, Fredricks DN (2011) Comparison of quantitative real time PCR with Sequencing and ribosomal RNA-FISH for the identification of fungi in formalin fixed, paraffin-embedded tissue specimens. BMC Infect Dis 11:202
172. Rickerts V, Loeffler J, Bohme A, Einsele H, Just-Nubling G (2001) Diagnosis of disseminated zygomycosis using a polymerase chain reaction assay. Eur J Clin Microbiol Infect Dis 20:744–745
173. Rickerts V, Mousset S, Lambrecht E et al (2007) Comparison of histopathological analysis, culture, and polymerase chain reaction assays to detect invasive mold infections from biopsy specimens. Clin Infect Dis 44:1078–1083, Epub 5 Mar 2007
174. Bialek R, Gonzalez GM, Begerow D, Zelck UE (2005) Coccidioidomycosis and blastomycosis: advances in molecular diagnosis. FEMS Immunol Med Microbiol 45:355–360
175. Bhatnagar J, Guarner J, Paddock CD et al (2007) Detection of West Nile virus in formalin-fixed, paraffin-embedded human tissues by RT-PCR: a useful adjunct to conventional tissue-based diagnostic methods. J Clin Virol 38:106–111, Epub 8 Dec 2006
176. Boyd AS, Stasko TS, Tang YW (2011) Basaloid squamous cell carcinoma of the skin. J Am Acad Dermatol 64:144–151
177. Sato M, Li H, Ikizler MR et al (2009) Detection of viruses in human adenoid tissues by use of multiplex PCR. J Clin Microbiol 47:771–773, Epub 30 Dec 2008
178. Smeets SJ, Hesselink AT, Speel EJ et al (2007) A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer 121:2465–2472
179. Ullrich T, Tang YW, Correa H, Hill M, Matta P, Weitkamp JH (2012) Absence of gastrointestinal pathogens in ileum tissue resected for necrotizing enterocolitits. Pediatr Infect Dis J 31(4):413–414
180. Vogt S, Schneider-Stock R, Klauck S, Roessner A, Rocken C (2003) Detection of hepatitis C virus RNA in formalin-fixed, paraffin-embedded thin-needle liver biopsy specimens. Am J Clin Pathol 120:536–543
181. Lau A, Chen S, Sorrell T et al (2007) Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. J Clin Microbiol 45: 380–385, Epub 22 Nov 2006
182. Mrazek C, Lass-Florl C (2011) Biopsy procedures for molecular tissue diagnosis of invasive fungal infections. Curr Infect Dis Rep 13:504–509

183. Cabaret O, Toussain G, Abermil N et al (2011) Degradation of fungal DNA in formalin-fixed paraffin-embedded sinus fungal balls hampers reliable sequence-based identification of fungi. Med Mycol 49:329–332, Epub 18 Oct 2010
184. Foss RD, Guha-Thakurta N, Conran RM, Gutman P (1994) Effects of fixative and fixation time on the extraction and polymerase chain reaction amplification of RNA from paraffin-embedded tissue. Comparison of two housekeeping gene mRNA controls. Diagn Mol Pathol 3:148–155
185. Landlinger C, Preuner S, Willinger B et al (2009) Species-specific identification of a wide range of clinically relevant fungal pathogens by use of Luminex xMAP technology. J Clin Microbiol 47:1063–1073, Epub 25 Feb 2009
186. CLSI (2008) Interpretive criteria for identification of bacteria and fungi by DNA target sequencing: approved guideline. Clinical and Laboratory Standards Institute, Wayne, PA
187. Munoz-Cadavid C, Rudd S, Zaki SR et al (2010) Improving molecular detection of fungal DNA in formalin-fixed paraffin-embedded tissues: comparison of five tissue DNA extraction methods using panfungal PCR. J Clin Microbiol 48:2147–2153, Epub 14 Apr 2010
188. Wolff TY, Moser C, Bundgaard H, Hølby N, Nielsen PH, Thomsen TR (2011) Detection of microbial diversity in endocarditis using cultivation-independent molecular techniques. Scand J Infect Dis 43:857–869, Epub 26 Aug 2011
189. Burd EM (2010) Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev 23:550–576
190. Pitt TL, Saunders NA (2000) Molecular bacteriology: a diagnostic tool for the millennium. J Clin Pathol 53:71–75
191. Borst A, Box AT, Fluit AC (2004) False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. Eur J Clin Microbiol Infect Dis 23:289–299, Epub 10 Mar 2004
192. Aslanzadeh J (2004) Preventing PCR amplification carryover contamination in a clinical laboratory. Ann Clin Lab Sci 34:389–396
193. Nikkari S, McLaughlin IJ, Bi W, Dodge DE, Relman DA (2001) Does blood of healthy subjects contain bacterial ribosomal DNA? J Clin Microbiol 39:1956–1959
194. Wolk D, Mitchell S, Patel R (2001) Principles of molecular microbiology testing methods. Infect Dis Clin North Am 15:1157–1204
195. Yang S, Rothman RE (2004) PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis 4:337–348

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