Chapter 7
Infectious Diseases, Vibrational Spectroscopic Approaches to Rapid Diagnostics

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Glossary

Chemometrics	A term to describe the use of multivariate statistics used to extract chemical information.
Fourier-transform infrared spectroscopy (FTIR)	A specific technique for acquiring IR absorption spectra in which all wavelengths are simultaneously measured.
Infrared spectroscopy	An absorption-based vibrational spectroscopic technique which primarily probes non-polar bonds.
Polymerase chain reaction (PCR)	An enzymatic method for amplifying a specific nucleic acid sequence.
Raman spectroscopy	A scattering vibrational spectroscopic technique which primarily probes polar bonds.
Surface-enhanced Raman spectroscopy (SERS)	A technique used to amplify Raman scattered signal via adsorption to a nanometer-scale metallic surface.
Vibrational (molecular) spectroscopy	A general term for the use of light to probe vibrations in a sample as a means of determining chemical composition and structure.

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Definition of the Subject and Its Importance

Importance of Rapid Diagnosis of Infectious Diseases

Infectious diseases are a major burden on human health with the World Health Organization (WHO) reporting that infectious diseases are responsible for one in ten deaths in the world's richest nations. The impact of infectious diseases is even greater in poorer regions of the world where six of every ten deaths are caused by a spectrum of infectious diseases that include bacteria, viruses, parasites and fungi. These infectious agents can further be described as classical pathogens, e.g., tuberculosis and malaria, seasonal epidemics, e.g., influenza and rhinoviruses, emerging infectious disease, e.g., highly pathogenic avian influenza and hemorrhagic fevers, or global pandemics such as the most recent outbreak of novel H1N1 influenza virus. Central to the management of each of these diseases are diagnostics. Early and rapid detection of an infectious agent is not only imperative to prevent the spread of disease, but it is also an essential first step to identify appropriate therapeutics that target the disease, as well as to overcome inappropriate administration of ineffective drugs that may drastically lead to drug-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA). This is just a succinct example which highlights the importance of diagnostic testing; however, the sections that follow discuss the current status of diagnostics and introduce an emerging approach to diagnostics based on vibrational spectroscopy which has tremendous potential to significantly advance the field.

Introduction

Classical Culture-Based Diagnostics

Despite the importance of diagnostic tests for infectious diseases, relatively few technological advances have supplanted classical microbiological approaches, e.g., in vitro culture, as a diagnostic standard. Clinical laboratories routinely rely on selective and chromogenic growth media to identify bacterial agents. For example, an $\alpha\beta-$chromogenic medium, which includes two substrates, has been developed to selectively isolate *Salmonella* spp. with 100% sensitivity and 90.5% specificity [1]. More recently chromogenic media have been developed to identify *Staphylococcus aureus* and distinguish methicillin-resistant strains (MRSA) [2, 3]. Culture-based diagnostics provide a method for definitive identification of many bacteria, and the tests are relatively inexpensive; however, the identification process has generally low throughput and substantial time is required for diagnostics. Typically, culture requires 24–72 h to allow the bacteria to grow while slow-growing organisms such as mycobacteria require substantially longer (6–12 week) incubation times.

Obviously, this is not ideal as the time frame can delay patient treatment. It should also be noted that not all pathogens can be cultured in a laboratory environment, thus the technique cannot be universally applied. There are several additional drawbacks of culture-based diagnostic methods including the requirement for species specific reagents, appropriate culture and storage environments, and labor intensive procedures.

Antibody-Based Diagnostics

Antigen detection and serology are common approaches used in clinical laboratories as alternative or complementary tools to culture-based detection. Common to both of these methods is the use of antibodies either to directly label and detect the antigen or to capture the host response, e.g., antibody responses to infection. Typically, an enzyme-linked immunosorbent assay (ELISA) is employed for antigen detection in diagnostic laboratories. As a first step, ELISA requires an unknown amount of antigen in a sample to be specifically, via a capture antibody in a sandwich assay format, or nonspecifically, via adsorption, immobilized to a solid phase such as a microtiter plate. After removing excess antigen, a known amount of detection antibody specific to the pathogen is then introduced to bind any immobilized antigen. The detection antibody is either directly labeled with an enzyme, or in an additional step, detected with an enzyme-labeled secondary antibody. After removal of excess reagent, a substrate is introduced to react with the enzyme producing a quantifiable color change. A slight modification of this approach replaces the enzyme with a fluorophor for fluorescence-based readout, eliminating the final substrate incubation step. A similar approach is taken for ELISA-based serological assays in which a known amount of purified antigen is immobilized onto the solid phase and incubated with serum to detect the presence of antibodies. While the procedure requires multitudinous steps, reagents, and substantial labor, ELISA is considered rapid relative to culture-based diagnostics as in many cases the assay can be completed within several hours. ELISA-based assays continue to be an integral part of laboratory diagnostics, but in their original form they are limited to the laboratory.

Lateral flow immunoassays, also called dipstick assays, immunochromatography, sol particle immunoassays, or rapid diagnostic tests, have been developed to overcome many limitations of ELISAs by eliminating the complex multi-step procedure, reducing labor, and allowing field or point-of-care testing. Lateral flow assays, like ELISAs, utilize pathogen-specific antibodies for the direct detection of antigen or detection of antibody response. However, for the case of lateral-flow assays the labeled detection antibody, capture antibody, and control reagents are dried on a prefabricated carrier strip. By design, these assays overcome diffusion-limited kinetics to exploit the rapid kinetics of antibody-antigen recognition [4, 5] to yield results in 10–20 min. Thus, because of the “reagentless” nature and rapid results, these assays are well suited for field use and resource-poor regions where

reagent storage and test sites are severely limited. It should be noted, however, that these benefits are at the loss of quantitative information and often a lower threshold of detection.

Numerous lateral flow immunoassays have been developed commercially for clinical diagnostics. Several competing manufacturers offer rapid diagnostic tests for influenza virus in which a conserved antigen is detected in a lateral flow assay format. Some detect influenza A and influenza B without distinction of the subtypes, e.g., QuickVue Influenza Test (Quidel), others detect and differentiate A and B strains, e.g., QuickVue Influenza A + B Test (Quidel) and 3 M™ Rapid Detection Flu A + B Test, and some only identify A or B strains, e.g., SAS Influenza A Test, (SA Scientific). Similarly, commercial rapid diagnostic tests are available for detection of a conserved protein for rotavirus A, e.g., IVD Rotavirus A Testing Kit. Not all lateral flow assays are designed for antigen detection; a rapid diagnostic test developed for leptospirosis diagnosis target anti-*Leptospira* IgM antibodies [6].

Despite continued advancements in antibody-based diagnostics these platforms will always be limited by the need for species-specific reagents, i.e., antibodies where the assays can only perform with the sensitivity and specificity inherent to the antibody. For example, commercial lateral flow assays for influenza only provide 50–70% sensitivity and 90–95% specificity with respect to culture-based diagnosis [7]. While the lateral flow assays may be performed rapidly, their low sensitivity may preclude early diagnosis due to low levels or unsustained levels of antigen through disease available for detection. Moreover, serological-based assays developed to detect agent-specific antibodies require that the infection elicit a detectable sustained immune response before the assay can be performed, a feature which substantially delays diagnosis.

Molecular Diagnostics

Nucleic acid and sequence-based methods for diagnostics offer significant advantages over conventional culture- and antibody-based diagnostics with regard to sensitivity, specificity, speed, cost, and portability. Central to molecular diagnostics is the use of a complementary nucleic acid probe that hybridizes to a unique species-specific region of the infectious agents RNA or DNA. While several molecular platforms have been developed for infectious diseases diagnostics, e.g., fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR) is the most commonly employed molecular method for diagnostics. PCR is a method of amplifying targeted segments of nucleic acid by several orders of magnitude to facilitate detection. In principal, complementary primer sequences are used to hybridize to a target nucleic acid sequence. A thermostable DNA polymerase, e.g., Taq polymerase, is then employed to extend the primer sequence. Thermal control facilitates extension, melting, and annealing, and via temperature-controlled cycling, the number of target sequences increases exponentially with each cycle. Amplification of the target sequence can be read out in an ethidium

bromide-stained agarose gel or in real-time via cleavage of a fluorescent tag from the primer during the extension step. Appropriate selection of the primers provides extremely specific detection, while the amplification of the target nucleic acid provides excellent sensitivity.

PCR has been demonstrated to be sensitive to single-copy numbers of DNA/RNA targets. In these most sensitive PCR assays, primers are chosen to fully complement a region of the target sequence. Perfect complement probes are also ideal for maximizing the assay specificity to a particular infectious agent. However, in practice, genetic mutations, particularly prevalent in RNA viruses such as influenza, can render a primer/probe set ineffective for diagnosis. Thus, degenerate probes are sometimes chosen to encompass some genetic diversity at the expense of sensitivity.

Multiplexed PCR utilizes multiple primer/probe sets that target different pathogens. Multiplexed assays are implemented when the sample size is limited, preventing multiple individual singleplex PCR analyses, and the clinician is unable to determine the most likely causative agent based on early clinical presentation. Multiplexed PCR assays are not quantitative due to target competition for reagents, are typically less sensitive than singleplex assays, and because of increased reagents, are more expensive to perform than singleplex assays. Moreover, multiple PCR products cannot be simultaneously read out by fluorescence, thus microarray analysis or electrophoresis to identify PCR products of different lengths is required to detect multiple PCR products. However, breakthroughs in multiplexed detection and quantitation are forthcoming [8–10].

Thus, for detection and diagnosis of many diseases such as viruses, PCR offers many advantages over classical methods of diagnostics, and its role will continue to expand in clinical diagnostic laboratories. However, there are challenges associated with PCR. For pathogens in which culture and microscopy can be used, molecular diagnostics are not the most cost effective. For example, the cost of a commercial PCR assay for tuberculosis ranges from $40 to $80, whereas microscopy and culture can be implemented for $1 and $5, respectively. Another consideration is how to interpret PCR results. Due to the sensitivity afforded by PCR, extremely low levels of infectious agent can be detected which may be below clinically relevant thresholds for disease presentation. Thus, quantitative PCR rather than qualitative PCR is typically more informative when correlating to clinical diagnosis. PCR assays developed in the laboratory are not always translational to analysis of clinical samples. In general, PCR cannot be performed directly on biological fluids such as blood because compounds such as hemoglobin, lactoferrin, heme, and IgG inhibit amplification [11–13]. Therefore, DNA and RNA are extracted as a first step, prior to PCR, but inefficiency of extraction kits often lead to a decrease in analytical performance compared to laboratory cultures [14, 15]. Moreover, isolation of nucleic acids is time consuming and technically challenging unless automated, which requires expensive equipment and reagents. A final consideration is the need for temperature-sensitive reagents, thermocyclers, skilled workers, and a clean laboratory environment to prevent contamination leading to false-negative results. While tremendous efforts are focused on PCR automation, incorporation of microfluidics [16, 17], and isothermal amplification [18–21], e.g., loop-mediated

isothermal amplification (LAMP), to overcome these challenges, the current status of PCR precludes widespread use of PCR diagnostics in point-of-care settings and developing nations.

Limitations of Classical and Conventional Diagnostics

As discussed above, diagnostics are essential to healthcare and disease management. While there is merit in further advancing current diagnostic methods, there are shortcomings for each approach. As noted above, culture is sensitive, but the length of time prevents rapid and early diagnosis. Antibody-based techniques are often limited in sensitivity, and like molecular diagnostics, are expensive, and require species-specific detection reagents. It is likely that any improvements in these methods to address these challenges will be incremental; however there are several next generation diagnostics that offer potential paradigm shifting approaches to detection and diagnoses that are currently being investigated. One important area is the use of molecular or vibrational spectroscopy for "whole-organism" fingerprinting. This innovative approach to diagnostics promises to be rapid, specific, and truly reagentless.

Spectroscopy-Based Diagnostics

Vibrational spectroscopy includes a number of nondestructive analytical techniques which provide molecular information about the chemical makeup, e.g., functional groups, of a sample. Subtle changes in the frequency of a particular functional group vibration, e.g., group frequency, provides additional details of chemical structure, local environment surrounding the bond, bond angle, length, geometry, and conformation. These attributes of vibrational spectroscopy have led to the development of vibrational spectroscopic approaches to generate whole-organisms fingerprints to serve as unique biochemical signatures for pathogen identification. Unique to this approach of infectious agent identification is rapid readout, and perhaps most importantly, there is no need for species-specific reagents or other reagents of any kind. Three of the most developed vibrational spectroscopic techniques include infrared absorption spectroscopy, Raman scattering spectroscopy, and surface-enhanced Raman spectroscopy (SERS). These three methods, as well as their development for diagnosing infectious diseases are described in detail below.

Infrared Spectroscopy

Infrared spectroscopy is an absorption technique in which the sample is irradiated most commonly with mid-infrared light with wavelengths in the range of

2.5–50 μm. Photons of appropriate energy to bring about a transition from one vibrational state to an excited vibrational state are absorbed by the analyte. Selection rules govern which vibrations are allowed to absorb IR photons, providing chemical and structural information. These rules require a net change in the dipole moment of the molecule as a result of the vibration. Thus, IR absorption spectra are dominated by asymmetrical vibrations. With consideration to these selection rules, proteins and nucleic acids (building blocks of bacteria, viruses, etc.) are excellent absorbers of IR radiation making IR spectroscopy an ideal tool for characterization of infectious agents.

The chemical complexity and sheer size of bacteria and viruses tend to produce complex spectra with broad and overlapping bands. Yet subtle changes in band shape, slight shifts in band peak positions, and variation in relative band intensities provide significant insight into chemical structure. Thus, careful evaluation of the full spectral fingerprint of whole-organisms, rather than analysis of single peaks common to small molecule studies, can lead to identification and classification of microorganisms.

IR spectroscopy as a technique for whole-organism fingerprinting dates back to 1952 [22]. In this early study, Stevenson and Boulduan showed that the IR spectra for *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and six other species of cultured bacteria are unique to each species. In addition to species identification, six strains of *Bacterium tularense* were spectroscopically differentiated. This initial work did not immediately translate to the diagnostic applications of IR spectroscopy. Two major breakthroughs that did not occur for several decades after the original findings were essential to further increase the utility of IR for whole-organism fingerprinting. First, prior to the 1980s when Fourier transform infrared spectroscopy (FTIR) was introduced, dispersive instruments were typically used. Dispersive instruments do not provide the speed or analytical performance required for IR-based diagnostics. FTIR instruments provide much better signal-to-noise spectra with improved spectral resolution, and are acquired in less time. These advantages provided by FTIR were essential to accurately analyze these complex biological spectra by distinguishing subtle changes in spectral band shape and to rapidly collect data. Additional developments in the methods of data analysis were also essential to move IR whole-organism fingerprinting forward. The large number of variables, i.e., wavelengths, each containing relevant information, inherent to IR spectra and rather subtle changes in intensity prevented traditional single-peak analysis for bacterial identification. Moreover, visual inspection of the spectra by the analyst is too labor intensive, prone to operator error, and unrealistic for large numbers of spectra and/or organisms from which to identify. The introduction of chemometrics, i.e., multivariate statistics applied to spectroscopy, led to the continued interest and advancement of IR-based diagnostics. These methods, including principal component analysis [23, 24], discriminant analysis, multiple regression analysis [25], and artificial neural networks [26, 27], function to simplify the high dimensional dataset by identifying the most significant variables with the ultimate goal of sample identification or quantification.

FTIR has received the greatest attention with respect to vibrational spectroscopic techniques over the last 2 decades and has been developed to the point that it can be considered an established method for the identification of both bacterial species and strains [28–33]. Researchers continue to investigate laboratory cultures of bacteria in an effort to standardize sampling protocols so that spectral databases can be shared among laboratories and to standardize the methods of analysis including spectral preprocessing, feature selection, and classification algorithms. As the method has matured, and while it continues to be tweaked and standardized, FTIR is now being applied to the analysis of clinical specimens. For example, FTIR has been successfully used to analyze clinical sputum samples collected from cystic fibrosis patients [26]. This FTIR capability is important as historically, lung infection caused by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Haemophilus influenzae* were the major causes of morbidity and mortality in cystic fibrosis patients; however, the number of emerging nonfermenting species is on the rise [34], and many of these species are closely related and not appropriately identified using typical clinical diagnostics and microbiological approaches [35]. Using a FTIR spectral library and an artificial neural network built for pathogen identification, the results from the FTIR method were compared to conventional microbiology detection methods. A two-tiered ANN classification scheme was built in which the top-level network identified *P. aeruginosa*, *S. maltophilia*, *Achromobacter xylosoxidans*, *Acinetobacter* spp., *R. pickettii*, and *Burkholderia cepacia* complex (BCC) bacteria. The second-level network differentiated among four species of BCC, *B. cepacia*, *B. multivorans*, *B. cenocepacia*, and *B. stabilis*. Ultimately, this method resulted in identification success rates of 98.1% and 93.8% for the two ANN levels, respectively. However, before this optimized method was established, the research highlighted three important considerations. First, not all bacterial isolates produce poly-β-hydroxybutyric acid (PHB) which contributes to the IR spectra and confounds classification. To overcome this, each isolate was cultured on TSA medium and harvested after 5 h of growth, prior to the expression of PHB. This step enriches the bacteria for analysis and eliminates interference from PHB. Second, flagella or pilus fibers were determined to contribute to spectral heterogeneity. Vigorous vortexing and subsequent centrifugation removes the fibers to significantly improve spectral reproducibility and classification results (Fig. 7.1). Third, the classification algorithm significantly affects the classification results. The authors show that hierarchical clustering algorithms (HCA) discriminate between reference and clinical strains rather than based on bacterial identity. Advanced methods, such as ANN, that determine spectral variables that vary only as a result of the bacteria was necessary to correctly classify according to strain. This example work demonstrates the power of IR-based diagnostics, but suggests that these methods may require problem-specific standardization of experimental protocols and data analysis.

These groundbreaking efforts to develop IR for bacterial analysis have led to the realization that spectroscopic methods have advantages for exploring detection of other pathogens. For example, FTIR has been employed for the distinction of yeast and fungi with success [36, 37]. More recently IR has been investigated as a method

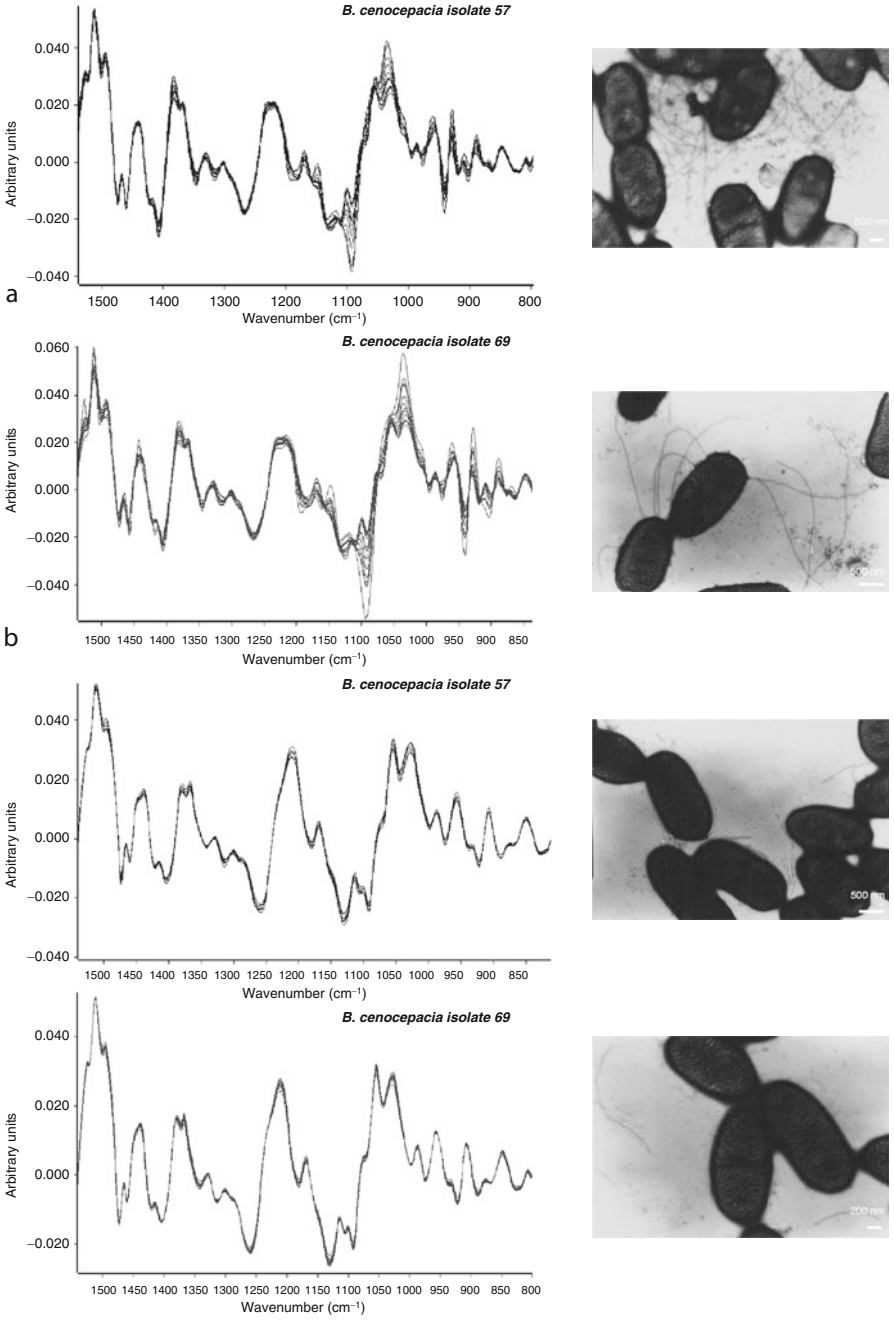

Fig. 7.1 Vector-normalized first-derivative spectra of two *B. cenocepacia* clinical isolates (isolates 57 and 69) in the 1,500–800 cm^{-1} range. (**a**) The heterogeneity of 15 replicate measurements for each strain in the spectral ranges of 1,200–900 cm^{-1} and 1,500–1,300 cm^{-1} and the corresponding micrographs obtained by TEM are shown. (**b**) Vector-normalized first-derivative spectra measured after vortexing of similar cells at the maximum intensity for 15 min and subsequent centrifugation at 8,000 × g for 5 min to separate the cells from free pilus appendages in the supernatants. Micrographs of the cells obtained by TEM after they were vortexed without centrifugation show the small fragments of pili or fibers suspended in the supernatants (From [26])

to detect viral infections, although current experiments are limited to viral infection of cells in culture [38–41]. While mock-infected and herpes simplex virus type 1-infected Vero cells are readily distinguished via IR, infection-induced spectral changes are inconsistent [39, 40]. Thus, substantially more research effort is necessary to standardize protocols and correlate the spectral response to the biochemical response upon infection.

Reports continue to support the utility of FTIR-based diagnostics in the clinical laboratory, but there are certain limitations to consider. First, water is a particularly strong absorber of IR light. Thus, care must be taken to completely dehydrate the sample prior to data acquisition. This obviously does not prevent IR-based diagnostics, it is merely an inconvenience. Second, IR absorption spectroscopy is not an inherently sensitive method and trace levels of a pathogen are not readily apparent. Hence, clinical samples will likely require a culture step to generate sufficient biomass for IR analysis. As noted above, this sample enrichment can be as short as 5 h, and with IR data acquisition on the order of minutes, the total analysis time is still more rapid, less labor intensive, and more informative in many cases than conventional diagnostic methods and does not require species-specific reagents.

Raman Spectroscopy

Raman spectroscopy is a scattering technique, in which the sample is irradiated with a monochromatic light source, almost always a laser. The majority of the scattered photons are elastically scattered and maintain the same frequency as the excitation source; however, a small fraction of the photons are shifted in frequency relative to the excitation source. The difference in the energy between the excitation and inelastically, i.e., Raman, scattered photons correspond to the energy necessary to bring about a transition from one vibrational state to an excited vibrational state. Thus, much like IR spectroscopy, Raman spectra provide insight into the chemical structure, local environment, geometry, and conformation of the sample and can serve as a whole-organism fingerprinting method. Selection rules also govern which vibrations are Raman active. These rules require a change in the polarizability during the vibration to be Raman active. Thus, Raman spectra are dominated by symmetrical vibrations and the technique is often seen as a complementary rather than competing technique with IR spectroscopy. However, for application to the analysis of biological materials and whole-organism fingerprinting methods, Raman offers many inherent advantages over IR spectroscopy.

Because of the selection rules, the main chain and aromatic side chains of peptides rather than aliphatic side chains are probed via Raman scattering in contrast to IR. Raman bands of nucleic acids are limited to heterocyclic bases or phosphodiester groups making up the backbone. Raman bands are narrower and less likely to overlap, thus the spectra are much less complicated compared to IR spectra because of the many more nonsymmetric vibrations that are possible.

Another major advantage of Raman is that water does not interfere since its vibrations do not fit the selection rules criteria. This is an extremely important consideration when analyzing biological samples which are endemic to aqueous environments. Other advantages of Raman include the flexibility to analyze samples in any state, e.g., gas, liquid, or solid, and the ability to analyze small sample volumes and masses because of the tight focus of incident laser light (square microns) compared to the incident IR beams (square centimeters).

Viruses were the first infectious agent analyzed by Raman spectroscopy, although not in a diagnostic capacity [42]. In this first work, Raman spectroscopy was used to probe the RNA and protein structure upon viral packaging. In the 1970s, Raman spectroscopy suffered from poor sensitivity due to instrument limitations. The first evaluation of Raman spectroscopy for pathogen detection was not until 1987 when spectra were collected for five species of bacteria including *E. coli*, *P. fluorescens*, *S. epidermidis*, *B. subtilis*, and *E. cloacae* [43]. To overcome the limited sensitivity of the instruments at the time, an ultraviolet laser was used for excitation to enhance spectral features of RNA, DNA, tyrosine, and tryptophan via resonance Raman. Unique spectra were observed for each bacterium, although analysis relied on visual interpretation since chemometrics had not been implemented for spectral analysis yet. UV Raman instruments, while producing the requisite sensitivity for pathogen analysis, is quite expensive and nonresonant vibrations are not observed which results in a significant loss in information that is valuable for differentiation.

Despite the recognized benefits of Raman-based diagnostics, particularly when compared to conventional and IR-based diagnostics, instrumentation has limited the maturation of Raman-based diagnostics. After development of UV Raman for pathogen detection [43–46], Fourier transform Raman (FT-Raman) instruments were introduced for microbiological studies which increased instrument sensitivity [47, 48]. Raman instruments have now evolved to include NIR lasers to reduce fluorescence from biological and NIR-sensitive CCD detectors. These modern instruments have only been developed in this decade to fully explore the potential of Raman as a diagnostic technique [49–55]. Thus Raman-based whole-organism fingerprinting is less developed than IR-based methods and examples are generally limited to the analysis of laboratory cultures.

In an early study, Maquelin et al. [54] utilized Raman spectroscopy to directly analyze five bacterial strains, including three strains of *Staphylococcus* spp., *E. coli*, and *E. faecium*, on solid culture medium. The flexibility in sample type afforded by Raman spectroscopy allowed direct measurement on the culture plate that would not be possible using IR spectroscopy. The background Raman spectrum resulting from the culture medium was subtracted from those spectra collected from the bacterial microcolonies. Hierarchical cluster analysis yielded two major groupings, one consisting of the three *Staphylococcus* strains and one consisting of the *E. coli* and *E. faecium*. The *E. coli* and *E. faecium* spectra clearly grouped according to species within the latter subcluster while spectra in the *Staphylococcus* subcluster grouped according to strain. While chemometric analysis of these spectra collected from same-day cultures yielded a successful classification rate of 100% for external

validation samples, combined data collected from 3 days dropped the accuracy to 83% for classification of two *S. aureus* strains (ATCC 29213 and UHR 28624). However, these two strains are extremely similar and in general the results demonstrate the utility of Raman-based diagnostics.

The most rigorous evaluation of Raman spectroscopy for reagentless detection and identification of pathogens was performed in collaboration with a US government laboratory. In this work, a comprehensive library of Raman spectra has been established for over 1,000 species, including 281 CDC category A and B biothreats, 146 chemical threats, 310 environmental interferents, and numerous others [52]. Spectral signatures were collected using Raman chemical imaging spectroscopy (RCIS) [56]. RCIS technology combines digital imaging and Raman spectroscopy. Digital imaging automatically discriminates against background particulates and identifies regions of interest on a sample platform that are then targeted for Raman analysis. Sample analysis is faster and completely automated using this approach. Two commercially available instruments were tested, one in the laboratory (ChemImage Corp., Falcon) and the other in the field (ChemImage Corp., Eagle). To test the robustness of the Raman spectral library and classification scheme, blinded samples containing one of four *Bacillus* strains were analyzed and identified. The predictive performance ranged from 89.4% to 93.1% for these closely related bacteria. It was concluded that key to the success of this diagnostic approach is the extensiveness of the spectral library. There are many more bacterial phenotypes than genotypes, and it has been found that Raman fingerprints correlate with cell phenotype, thus an all-inclusive library must contain spectra for each bacterial strain grown under different conditions and at different stages of development. In a subsequent study untrained personnel at the Armed Forces Institute of Pathology evaluated 14 bacteria to generate a spectral library and sent 20 blinded samples to ChemImage for external validation in which all 20 samples were correctly identified. This comprehensive study is the first to establish the true utility of automated Raman-based diagnostics carried out off-site by untrained personnel. However, these samples were prepared in water, cell culture media, or spiked nasal swabs, none of which are truly clinical samples.

An early study to evaluate clinical samples for *Acinetobacter* by Raman spectroscopy and compare the results with an established diagnostic method were among the first showing the power and speed of Raman-based detection [55]. In this study, 25 *Acinetobacter* isolates from five hospitals in three countries were analyzed using selective amplification of restriction fragments (AFLP), an established molecular technique for typing bacteria strains. Dendograms resulting from the hierarchical cluster analysis of Raman and AFLP fingerprints for the isolates were generated and compared (Fig. 7.2). Both dendograms resulted in five clusters that separate the strains according to the five outbreaks, with the exception of one Basildon isolate RUH 3242 which clustered with isolates from Venlo in the Raman-based dendogram. Overall results from Raman fingerprinting of these clinical isolates were very similar to those obtained for established methods, but with the advantage of faster analysis and less complicated procedures.

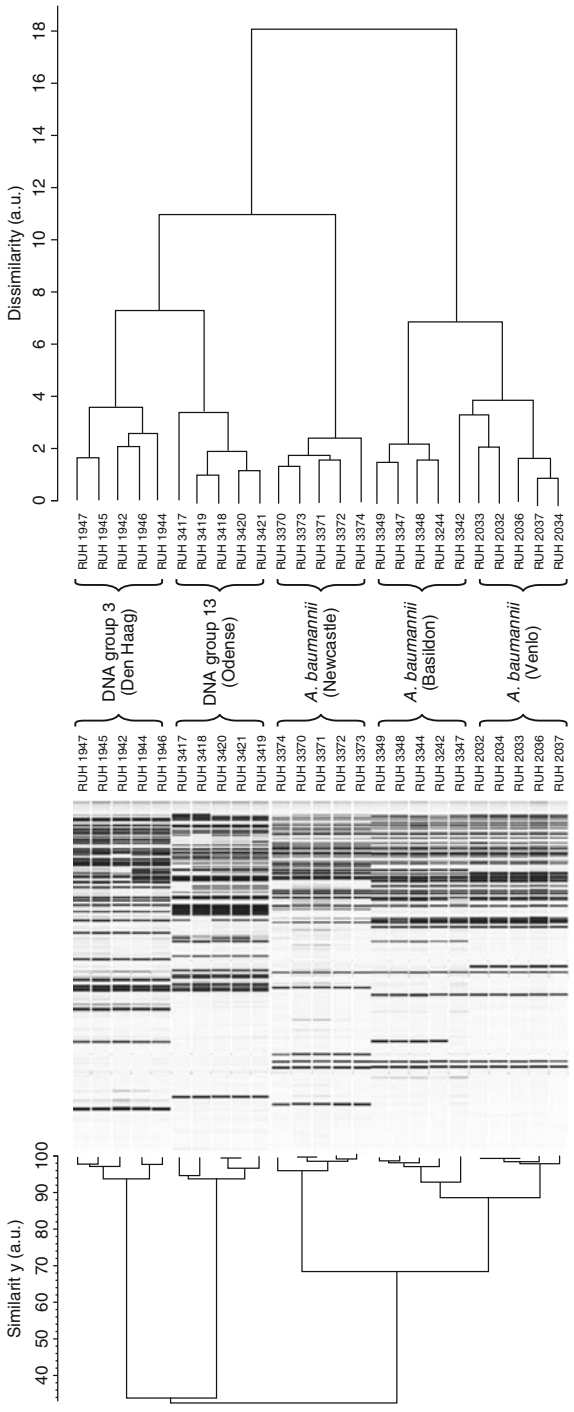

Fig. 7.2 Dendrograms resulting from the hierarchical cluster analysis of (*left*) AFLP analysis and (*right*) Raman analysis of the isolates. The asterisk marks the strain RUH 3242 misclassified via Raman fingerprinting (From [55])

Despite the advancement of Raman spectroscopy instrumentation and methods for pathogen fingerprinting, Raman is still often limited by poor sensitivity. Only ~1 in 10^6–10^8 photons are inelastically scattered as the vast majority are elastically scattered. This means that high quality spectra with the requisite signal-to-noise can take minutes to acquire. While this may not be a limitation in laboratory experiments, or developmental stages in research, it prohibits its usefulness in clinical diagnostic laboratories which analyze hundreds to thousands of samples per day. Thus, there is great interest in enhancing the Raman signal. One such method is to excite the sample with a frequency that resonates with an electronic transition, so called resonance Raman spectroscopy. For biological samples, this requires UV lasers for excitation, and as noted above, is cost prohibitive to widespread adoption of this method. Moreover, chemical information is lost when performing resonance Raman which would likely reduce classification accuracy of closely related pathogens. An alternative method to amplify Raman scattering is surface-enhanced Raman spectroscopy (SERS). SERS has received a great deal of attention, particularly with respect to whole-organism fingerprinting and is the subject of the next section.

SERS

Surface-enhanced Raman spectroscopy is a technique in which the Raman signal of a sample is significantly amplified via adsorption onto a metallic nanostructured surface. A laser excitation frequency is selected such that it is in resonance with the collective oscillation of the conduction electrons in the nanostructures, i.e., surface plasmon resonance. When resonance conditions are met, the local electromagnetic field experienced by molecules in close proximity to the surface is significantly increased to yield rather large enhancements in the Raman scattering. While the signal enhancement is substrate and sample dependent, typical enhancements are on the order of 10^4–10^{14} with respect to normal Raman intensities, with several studies reporting the detection of single molecules using this technique [57, 58]. SERS offers the benefits of normal Raman compared to IR spectroscopy while providing a markedly improved sensitivity. Recent advances in nanofabrication methods and SERS theory has led to significant improvements in SERS substrates in the last several years and has driven increased efforts to develop SERS for whole-organism fingerprinting [59–78].

The major focus of whole-organism fingerprinting via SERS has been on bacteria identification [51, 64–74, 77, 78]. Most of these studies report differentiation among bacteria species, with many demonstrating discrimination of different strains of the same species. However, there are several inconsistencies that have been noted by researchers, particularly in the earlier studies. For example, Grow et al. found SERS spectra for strains that belong to the same species were sometimes less similar than spectra collected from different species [65], and Jarvis and Goodacre observed similar spectra for the same bacteria using different

preparations of silver nanoparticles, but noted subtle changes in signal intensities among nanoparticle batches [68]. These discrepancies evident in these early studies highlight the primary challenge of SERS-based diagnostics, i.e., the enhancing substrate. The SERS signal is highly dependent on the enhancing substrate, thus a reliable means of fabricating nanostructured materials is vital to the success of SERS-based diagnostics.

Several research laboratories have analyzed and published SERS spectra for both *Bacillus subtilis* and *E. coli*; however, each reported incongruent spectral fingerprints [67, 68, 71, 72]. The experimental protocols, however, varied among each study. For example, in two different reports Jarvis et al. used two different chemical synthesis preparations to generate colloidal silver, citrate reduction [67] and borohydride reduction [51], to serve as the SERS substrate. The SERS spectra were drastically different in each study. It is well known that spectra are dependent on the enhancing nanostructure, e.g., material, size, shape, interparticle spacing, etc., but given the same final nanostructure similar spectra were expected. The authors attributed the differences to the effect of diverse chemistries used to prepare each silver colloid [79]. However, it should be noted that different excitation sources, 7 nm and 785 nm, were employed in the two studies. For normal Raman, the Raman shifts should be independent of the excitation source, thus spectral fingerprinting should not be affected by the choice of the laser. SERS spectra, however, can be influenced by the excitation source because of the requisite pairing of the excitation frequency and plasmon resonance of the substrate. Therefore, it is perhaps more probable that spectral differences observed by Jarvis et al. are due to greater signal enhancement for the 7 nm excitation source rather than due to differences in chemical preparation of the colloidal silver. This interpretation is supported by a study in which a third variation in experimental parameters was implemented utilizing citrate-reduced silver colloid but acquired spectra with a 647 nm laser [71]. Results from this study closely resembled the results for *B. subtilis* obtained by Jarvis et al. employing borohydride reduced silver nanoparticles and 7 nm excitation. Collectively, these studies also demonstrate the need for procedural consistency.

In a pivotal study, scientists at a US Army research laboratory evaluated the SERS signatures for many bacteria using a standardized sampling protocol and instrumentation. To date, three SERS substrates were directly compared using the standardized protocol: silver nanoparticles, silver film over nanospheres (FONS), and commercially available Klarite. Interaction between substrate and bacteria vary significantly as visualized with electron microscopy which likely results in different spectral fingerprints. Moreover the signal intensities varied significantly among the substrates reflecting differences in enhancing quality. Details of these experiments are approved for public release as a technical report (ARL-TR-4957).

In another key study, SERS and Raman fingerprints were directly compared to assess the advantages of SERS analysis [72]. Raman and SERS spectra were collected for several bacteria, including four strains of *Bacillus*, *S. typhimurium*, and *E. coli*. As noted above, the substrate is a critical factor in SERS analysis, and in this study aggregated gold nanoparticle films were grown in-house and established

as a reliable means of substrate preparation for acquisition of repeatable spectra. As anticipated, SERS yielded much greater signal-to-noise spectra compared to normal Raman. The study also identified two unexpected benefits of SERS. Normal Raman signal for *Bacillus* species was overwhelmed by native fluorescence of the sample; however, in the SERS analysis, the metal substrate functioned to quench the fluorescence component in addition to enhancing the Raman signal. It was also observed that normal Raman spectra are more complex than SERS spectra. This is explained by the fact that bulk Raman interrogates all components throughout the entire bacterium equally, while the distance-dependence of SERS enhancement preferentially probes the region of the bacterium closest to the metal substrate and bands for the internal components are not detected. Fortunately, most chemical variation among bacterial strains and species are expressed on the cell surface, thus greater spectral differences are observed among SERS spectra of different samples than compared to bulk Raman spectra. This added advantage is exemplified by greater discrimination of bacteria when utilizing SERS spectra as compared to Raman spectra [72].

A number of novel nanofabrication methods have recently emerged for producing SERS substrates with the potential for addressing the issues noted above due to substrate heterogeneity. These include electron beam lithography [80, 81], nanosphere lithography [82–84], a template method [85–88], oblique angle vapor deposition (OAD) [89–91], and a proprietary wet-etching technology used to produce commercially available Klarite (D3 technologies). It should be noted, however, that with the exception of OAD and Klarite, these fabrication methods are not adaptable to large-scale production due to the complexity of the fabrication procedure. Not only is it likely that these substrates will lead to significant advances in SERS-diagnostics of bacteria, the use of OAD and Klarite substrates has already lead to successful application to virus identification [59, 60, 62, 63, 75, 76].

In the most recent investigation of SERS-based viral fingerprinting, eight strains of rotavirus were analyzed [63]. These isolates were recovered from clinical fecal samples and propagated in MA104 cells and represent the 5 G and 3 P genotypes responsible for the most severe infections. Unique SERS fingerprints were acquired for each strain when adsorbed onto OAD-fabricated silver nanorods. Representative spectra for each strain and negative control, as well as the difference spectra which subtract out the background cell lysate signal are displayed in Fig. 7.3. Classification algorithms based on partial least squares discriminant analysis were constructed to identify the samples according to (1) rotavirus positive or negative, (2) P4, P6, or P8 genotype, (3) G1, G2, G3, G4, or G9 genotype, or (4) strain. Respectively, these four classification models resulted in 100%, 98–100%, 96–100%, and 100% sensitivity and 100%, 100%, 99–100%, and 99–100% specificity.

Compilation and critical analysis of reports to date demonstrate the potential of Raman-based diagnostics and its advantages over IR, normal Raman spectroscopy, and convention diagnostic methods, but also highlight the need for standardization. The challenge in the future is standardization of substrates and sampling protocols since background can “quench” signal from the analyte. For example, blood

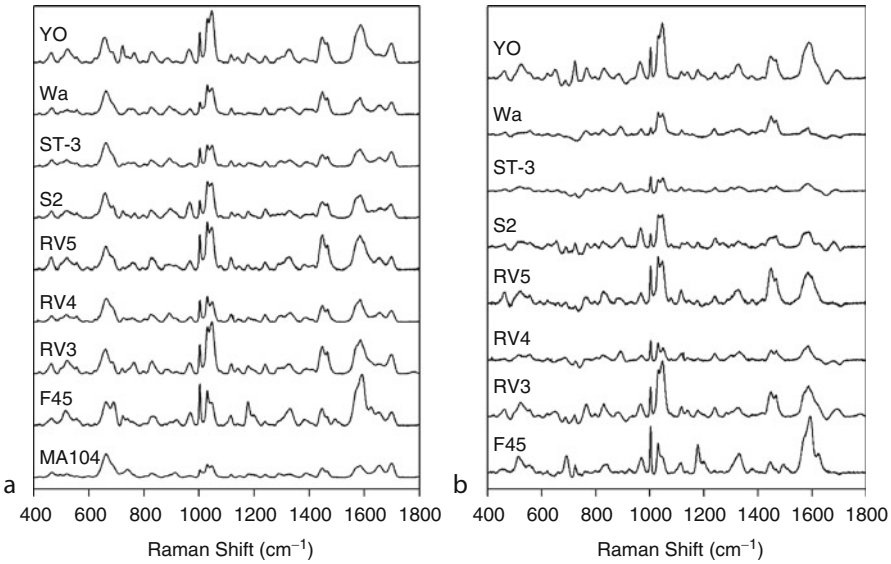

Fig. 7.3 (**a**) Average SERS spectra for eight strains of rotavirus and the negative control (MA104 cell lysate). Spectra were baseline corrected, normalized to the band at 633 cm^{-1}, and offset for visualization. (**b**) Difference SERS spectra for eight strains after subtraction of MA104 spectrum (From [63])

analysis requires sample processing to remove some competing elements [92], yet SERS spectra highly dependent on the sample pretreatment procedure as remaining chemical species will also contribute signal and degrade the performance of matching in spectral library databases. The outlook of SERS is not a question of spectral quality and reproducibility in a controlled environment, the question is how to control the environment across laboratories.

Future Directions

The future of spectroscopic-based diagnostics is bright as demonstrated by the many studies cited and discussed above. In addition to the success found in these studies, areas of improvement have also been identified. An important area of potential development is the methods used for statistical analysis. Well-established algorithms such as PCA, HCA, and discriminant analysis continue to provide high predictive accuracy, but recent examples have shown that more creative and novel approaches such as artificial neural networks, "bar-coding" [70], or innovative uses of PLS [59] can further improve the predictive value. A revolution in instrumentation is also occurring. Vibrational spectroscopy has recently filled niches in quality control of pharmaceuticals and raw materials as well as identification of chemical

threats. The nature of these applications and explosion in interest have driven the instrumentation industry to invest in the development and production of high-quality yet affordable handheld instruments for mobile, on-site analysis. This market-driven commercialization, in effect, is paving the way for point-of-care, mobile, and cost-effective spectroscopy-based diagnostics. The most important factor for widespread realization of spectroscopic diagnosis will be the emergence of a universal protocol for sampling, and for the case of SERS, a standard substrate. The accepted protocol must then be used to build a spectral database covering a variety of phenotypes and developmental stages as illustrated above. Implementing a standard practice is crucial for the success of the technique, but once developed this technology has the potential to become the first and immediate response to clinical cases in which infection is suspected.

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