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Antibodies and Immunoassays for Detection of Bacterial Pathogens

Padmapriya P. Banada and Arun. K. Bhunia

Abstract

Antibody, also known as immunoglobulin, is normally made in the body in defense of foreign antigen or invading pathogen. Highly specific biorecognition property of antibody with antigen has made antibody as one of the most indispensable molecules for broad application, not only in the diagnosis or detection but also in prevention or curing of diseases. Animals are routinely used for production of both polyclonal and monoclonal antibodies; however, recombinant and phage display technologies are being adopted to improve antibody specificity and to cut cost for antibody production. Available genome sequence of pathogens is also allowing researchers to find and select suitable target antigens for production of antibody with improved specificity. In recent years, however, demand for antibody is even greater as novel biosensor or nanotechnology-based methods continue to utilize antibody for analyte capture and interrogation. Conventional immunoassay methods such as lateral flow and enzyme-linked immunoassays, though lack sensitivity, are available commercially and are widely used. While biosensor-based methods such as time-resolved fluorescence immunoassay, chemiluminescence assay, electrochemical immunoassay, surface plasmon resonance sensor, fiber optic sensor, and microfluidic biochip have, in some cases, demonstrated improved sensitivity, they require further optimization with real-world samples. Furthermore, environmental stress and the growth media are known to affect the physiological state of microorganism and antigen expression, often rendering unsatisfactory signal response from immunoassays. Thus, one must understand the microorganisms' response to these factors before designing an immunoassay to avoid false results. With the advent of microfluidics and nanotechnology, the adaptation of lab-on-chip concept in immunoassays will soon be a reality for near real-time detection of pathogens from food or clinical specimens.

1. Introduction

Our ability to produce specific antibodies against target analytes by conventional animal immunization methods or by cloning or recombinant DNA technology has revolutionized the field of immunoassay-based detection. Antibodies are among the most important molecules, with limitless applications in the field of biology, microbiology, medicine, and agriculture. For example, the most rapid diagnostic tests used in food or clinical laboratories are based on antigen-antibody reactions. Antibodies are used in the discovery of new bioactive molecules, the prevention of diseases, and the treatment of cancer. In recent years, however, antibodies have been found to be invaluable molecules in biosensor and nanotechnology applications for

Padmapriya P. Banada and Arun. K. Bhunia • Molecular Food Microbiology Laboratory, Department of Food Science, Purdue University, West Lafayette, Indiana

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the interrogation of analytes in macro- to nano-scale, including pathogens (bacteria, viruses) and toxins. In this chapter we discuss the significant role antibodies play in bacterial pathogen detection. Bacteria are prokaryotes, and their distribution is ubiquitous, including humans, animals, and the environment. They are the oldest living organisms in the history of this planet and play a profound role in maintaining the ecosystem. Humans, animals, and plants harbor bacteria in large numbers, and mostly they have a symbiotic relationship. Only a small fraction of bacteria are harmful and can cause diseases. It is estimated that on the average, about 1–2 billion people around the world are infected by bacteria each year, with 70 % of the cases being foodborne (Allos et al. 2004). Annually, foodborne diseases are estimated to affect about 1 in 4 persons in the United States and 1 in 5 persons in England (Kendall et al. 2006). In recent years there has been a significant decline in the number of outbreaks and illnesses due to foodborne bacterial pathogens. However, during late 2006 the US experienced multiple outbreaks caused by *Escherichia coli* O157:H7 that was associated with spinach and lettuce. A total of 199 people were infected in 26 states by consuming tainted spinach, with 3 fatalities (CDC 2006). A *Salmonella* outbreak involving tomatoes and peanut butter resulted in several illnesses too. Peanut butter contamination resulted in 288 cases in 39 states in early 2007. Bacteria also pose a concern because of an increase in antibiotic resistance during clinical infections (for example, methicillin- and vancomycin-resistant staphylococci). Additionally, they cause stomach ulcers (eg., *Helicobacter pylori*), tuberculosis (*Mycobacterium tuberculosis*), meningitis (species of *Streptococci, Neisseria*), cholera (*Vibrio cholerae*), sexually transmitted diseases, and nosocomial or hospital-acquired infection. In recent years, several bacterial pathogens have been considered as potential threats for bioterrorism: *Bacillus anthracis* (anthrax bacteria); botulinum toxin, staphylococcal enterotoxin B (SEB), *Yersinia pestis*, etc. (Bhunia 2006).

Consequently, rapid detection and diagnostic tools are being developed as a measure to combat these pathogenic bacteria. Culturing methods continue to be the "gold" standard; followed by nucleic acid-based assays, ranked "silver;" and the immunoassays, ranked "bronze" (Gracias and McKillip 2004; Bhunia 2006). However, immunoassay is by far the most rapid compared to the other two methods. Culture methods require 24–48 h or more in order to get the bacterial colonies on a petri dish. Some of the bacteria like *M. tuberculosis* take 7–14 days to grow on the selected media (Cheng et al. 2005). A nucleic acid-based assay requires good technical expertise and a nucleic acid extraction step. Currently attempts are being made to automate the system for on-site application. In contrast, most of the antibody-based detection methods, such as lateral flow immunoassays, dipstick assays, and slide agglutination tests, can be done outside the convenience of a laboratory, with little technical knowledge; and the results can be obtained relatively quickly, in 10–15 minutes.

In this chapter, we also discuss the methods or strategies for developing a "good" antibody against bacterial pathogens for use in the capture and concentration of bacterial cells using immunomagnetic separation (IMS) technology, and their detection by various immunoassay procedures, including immunosensors and biosensors.

2. Antibodies

Antibodies (immunoglobulins) are glycoproteins belonging to the immunoglobulin (Ig) supergene family. An antibody (Ab) molecule has been viewed as a "Y" shaped molecule consisting of two pairs of identical polypeptide chains, called light and heavy chains, joined by disulphide bonds. The two variable domains on light (V_L) and heavy (V_H) chains make up the antigen (Ag) recognition and binding site (Fig. 21.1). The amino acid sequence of this region

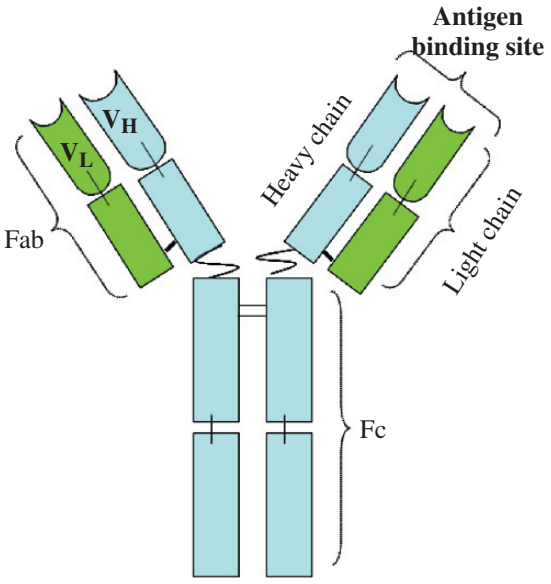

Figure 21.1. Structure of an immunoglobulin molecule.

is highly variable, and this contributes to the broad recognition power of the antibody to a wide range of target molecules. When digested with the enzyme papain, a mammalian antibody molecule yields two 50 kDa Fab (fragment antibody) fragments and one 50 kDa Fc (fragment crystalline) fragment. The Fab fragment binds to the antigen, while the Fc fragment binds to the Fc receptors located on many mammalian cells. Antibodies consist of different classes: IgA, IgG, IgM, IgE, and IgD (rare); and subclasses which slightly vary between humans and mice, primarily for IgG. Both human and mouse IgA consists of IgA1 and IgA2; while mouse IgG consists of IgG1, IgG2a, IgG2b, and IgG3, and human IgG consists of IgG1, IgG2, IgG3, and IgG4. The generation of a specific class or subclass of antibodies in a host depends largely on the nature of the antigens, the type of adjuvants, and the route of immunization, such as intramuscular vs. intradermal vs. subcutaneous. For all practical purposes, certain classes (or subclasses) are highly desirable for immunoassay applications because of their stability, binding affinity, and low cross reactions.

The antibody's ability to recognize and bind with high affinity to specific antigenic sites (epitopes), even in a complex mixture, is exploited for qualitative and quantitative measurement of the antigens. Thus antibody application is broad—it is not only used for detection and classification of the antigens, but also for understanding the microheterogeneity among proteins resulting from recombinant or somatic mutations.

The production and selection of a suitable antibody is imperative for the successful design of an immunoassay, which depends on the assay parameters: the choice of a polyclonal or monoclonal antibody; of purified or native sera; of fragmented, bispecific, or fusion proteins; and the relative cost (Liddell 2005).

Several reviews have described in depth the available polyclonal and monoclonal antibodies for various bacteria (Macario and Macario 1988; Bhunia 1997). Along with traditional polyclonal and monoclonal antibody production, in this chapter we have addressed different methodologies and selection approaches for the successful production of a polyclonal or monoclonal antibody for downstream applications in immunoassays.

2.1. Polyclonal Antibody

A polyclonal antibody (PAb) is a heterogeneous mixture of antibody molecules arising from a variety of constantly evolving B-lymphocytes, so that even successive bleeds from one animal are unique (Kane and Banks 2000). The assortment of antibodies present in a PAb preparation may consist of different classes and subclasses, and they may recognize multiple antigens or multiple epitopes located on the same antigen. In contrast, a monoclonal antibody recognizes only a specific epitope on an antigen. Polyclonal antibodies are produced for bacterial detection and were widely used by early immunologists and microbiologists for their ability to react with a variety of epitopes to characterize an antigen. Most of the commercial assays use polyclonal antibodies, and the assay format could be in the form of agglutination, precipitation, or an enzyme–linked immunosorbent assay (ELISA). Polyclonal antibodies are also found to be superior to monoclonal antibodies in capturing and concentrating target molecules and are used in immunomagnetic- or immunobead-based captures. Sheep, goats, and rabbits are the most common animals used for polyclonal antibody production, although chickens have been used occasionally (Kovacs-Nolan and Mine 2005).

Unlike mammalian antibodies, chicken antibodies are composed of three immunoglobulin subclasses: IgA, IgM, and IgY. The IgA and IgM are similar to mammalian IgA and IgM, while the IgY is equivalent to mammalian IgG. These antibodies are found in serum as well as in eggs. In eggs, IgA and IgM are primarily present in the albumen in trace amounts, while IgY is found in the yolk in large quantities ($\sim$25 mg/ml). Structurally, IgY (180 kDa) is larger than IgG (150 kDa) and can be readily harvested in large quantities from egg yolks (Kovacs-Nolan and Mine 2005).

Recently, several mammalian PAbs were used for the capture and detection of *Salmonella* (Kramer and Lim 2004; Hahm and Bhunia 2006), *E. coli* (Geng et al. 2006b), and *L. monocytogenes* (Geng et al. 2004; Gray and Bhunia 2005) on biosensor platforms, which will be discussed further in later sections.

2.2. Monoclonal Antibody

Development of monoclonal antibodies was first introduced by Kohler and Milstein in 1975 (Fig. 21.2), which revolutionized the understanding of the structure, nature, and distribution of antigens. Conventionally, during the production of polyclonal antibodies, a target-specific antibody is present in a pool of B-lymphocytes expressing unique antibodies. Separation of the target-specific lymphocytes would yield a highly specific antibody, which in practice is impossible because the lymphocytes cannot be regenerated in vitro. During MAb production, the B-lymphocytes are collected from the spleen of immunized mice, rats, or rabbits (in humans, from blood) and fused with myeloma cells (Sp2/0, NS1), producing the hybrid cells that in turn produce the antibodies and proliferate infinitely in a flask (Bhunia et al. 1991; Liddell 2005) (Fig. 21.2).

Target-specific antibodies are developed either for the whole-cell detection of bacteria such as *Salmonella, Listeria, E. coli, Campylobacter, Clostridium, Staphylococci, Pseudomonas,* etc., or their toxins. For whole-cell detection, the heat-killed cells (Bhunia 1997; Warschkau and Kiderlen 1999), or whole cellular antigens (Zhao and Liu 2005), or surface antigens like flagella (Kim et al. 2005), cell wall-associated lipopolysaccharides (Thirumalapura et al. 2005), cell surface antigens (Kunhe et al. 2004), and surface-expressed virulence proteins (Hearty et al. 2006) have been used as antigens for monoclonal antibody production. The epitope-masking method, using an antibody to block undesirable epitopes in an antigen, has been used to successfully develop a specific monoclonal antibody against *L. monocytogenes* (Bhunia and Johnson 1992).

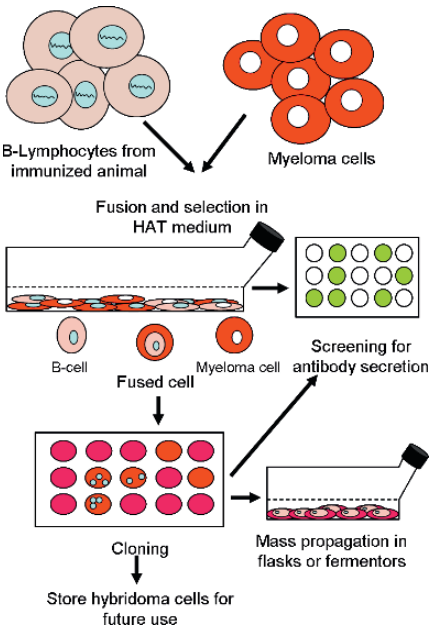

Figure 21.2. Flow diagram showing steps involved in monoclonal antibody production. HAT = hypoxanthine, aminopterin, and thymidine.

The type of antibodies (PAb vs. MAb) to be used depends on the specific application. Whenever possible, monoclonal antibodies are preferred. However, due to the high cost involved in the production of monoclonal antibodies, polyclonal antibodies still have a broader appeal. Irrespective of the type, the production of a target-specific, high performance antibody depends on the proper strategy in selecting and delivering the antigenic molecules such as small peptides or polypeptides as immunogens. Some proteins are not very easy to isolate in pure form in sufficient amounts from pathogens. In some situations, only a small fraction of the potential target proteins are isolated and characterized. Therefore, under these conditions the native proteins cannot be used to generate the desired antibodies. Thus several other strategies like the use of synthetic peptides, recombinant DNA technology, and phage display were introduced as alternative methods for antibody production.

2.3. Use of Synthetic Peptides for Antibody Production

The use of synthetic peptides as immunogens was first reported in 1982 by Young and Atassi and have since been widely used. Such use for the production of antibodies has been a popular choice for vaccine research and development (Tsurumi et al. 2003; Cauchard et al. 2006; Maruta et al. 2006). The major advantage of using this technique is to avoid the high degree of cross-reactivity which often is a major concern in many immunoassay experiments. Furthermore, peptides can be conveniently purified by using liquid chromatography. These peptides are then used for generating polyclonal or monoclonal antibodies. In order to design a good peptide, one should bear in mind that its optimal length should be 10–20 residues long, it should possess an antigenic region or domain, and it should be stable. Too long, too short, or unstable molecules result in steric or conformational changes in the molecule during conjugation or synthesis, resulting in cross-reactivity. Since peptides are too small to induce an immune response, a suitable carrier molecule such as bovine serum albumen (BSA) or keyhole limpet hemocyanine (KLH) is attached, or sometimes peptides are synthesized as multiple antigen

peptides (MAPs) (Angeletti 1999). MAPs are 13–17 kDa protein, composed of 4–8 identical peptides linked by a poly L-lysine core, and are suitable for antibody production without the aid of a carrier protein. Maruta et al. (2006) used alum along with inactivated *Bordetella pertusis* for eliciting antipeptide antibodies against an array of peptides. They showed that this combination increased antibody production in mice. A novel approach that utilized nanoparticles as adjuvants elicited a strong immunologic response in foals against virulence associated protein A (Vap A) peptides of *Rhodococcus equi*, and the resulting antibody showed a strong reaction with the organism (Cauchard et al. 2006).

Several researchers have developed antibodies against peptides to neutralize the bacterial superantigens or toxins (Visvanathan et al. 2001; Dale et al. 2002) and against various epitopes on the cell surface proteins of *S. aureus*, Streptococci, *Helicobacter pylori*, and others (Huesca et al. 2000; Shin, Roe and Kim 2004; Bialek et al. 2006). IgY antibodies were produced in hens using the synthetic peptides of urease epitope from *Helicobacter pylori* (Shin et al. 2004). Subsequent characterization of antibodies using ELISA revealed that among the five peptides, UreB peptide containing 15- aminoacid residues was highly specific for IgY-*Hp* antibodies.

In our laboratory, we identified from the database several unique 20-mer amino acid sequence long peptides (epitopes) from surface-associated proteins of *L. monocytogenes* that are likely to be present on the surface of cells in their native configuration. The goal was that when the antibodies were developed, they should bind to the surface-exposed epitope on the bacterial cell for reliable detection of whole cells (Lathrop 2005; Lathrop et al. 2008). Sequence analysis of the published genome sequences of *L. monocytogenes* (Glaser et al. 2001) revealed 22 unique surface proteins of *L. monocytogenes*. Of these 22 proteins, 5 belonged to the internalin (invasion protein)-multigene family; one ActA (actin polymerization) protein and the remaining 16 were unknown (Lathrop 2005). Nine 20-mer peptides, representing each of nine proteins, were synthesized with an extra cysteine residue that was used to form a disulfide cross-link with the KLH. KLH-peptides were purified by HPLC and the identity of each was confirmed by mass spectrometry. The peptides were injected subcutaneously into rabbits for antibody production. In spite of careful and thorough analysis, not all peptides produced desirable antibodies. Some antibodies showed highly specific reactions against *L. monocytogenes* cells, while some showed reactions against the multiple species within the genus *Listeria* or other bacterial species (Lathrop 2005; Lathrop et al. 2008). One of the major problems that arose from this study was the presence of nonspecific antibodies in the pool of rabbit serum immunized with *Listeria*-specific peptides that were showing reactions with several nontarget pathogens. Further testing of preimmune serum revealed the presence of nonspecific antibodies against an array of bacterial pathogens. This indicated that the rabbits had previous exposure to nontarget microorganisms that induced immune responses. Therefore, for the production of peptide-specific PAbs in rabbits, thoroughly testing the preimmune serum of each animal for the presence of antibodies to the target pathogens as well as to common microorganisms is advised before initiating the immunization regimen.

In a test for the presence of antisera against 27 microorganisms involving 19 rabbits raised under conventional or pathogen-free environments, Lathrop et al. (2006) reported that 17 rabbits were positive for at least one pathogen, and 14 of 27 cultures showed positive reactions with 50 % or more preimmune sera. If these rabbits were used for the production of pathogen-specific antibodies for the purpose of diagnostic immunoassay, they would produce cross-reactive low quality antibodies, which might not be suitable for use in diagnostic immunoassays. To overcome this problem, monoclonal antibodies could be developed against the synthetic peptides. As the genome sequences of several microbial pathogens are currently available, the synthetic peptide approach continues to be an attractive and highly desirable method for the development of specific antibodies.

2.4. Recombinant DNA Technology

Orlandi et al. (1989) introduced a breakthrough method of antibody production when they demonstrated an innovative approach to clone antibody genes through a recombinant DNA technology. Recombinant antibodies are similar to monoclonal antibodies, except that they consist of only antigen-binding domains, without the Fc domain (Fig. 21.2). The mRNA is isolated from the lymphocytes of immunized animals or nonimmune donors and a cDNA library is constructed. These genes are amplified by polymerase chain reactions (PCR), using the antibody-specific primers designed for heavy and light chain fragments of the antibody. The size of the fragment depends on whether a whole Fab fragment or a single chain variable fragment (ScFv) is to be made (Emanuel et al. 2000; Liddell 2005). The PCR-amplified products are inserted into phagemid vectors and a combinatorial library is created by cloning into *E. coli* and infecting it with a helper bacteriophage, which helps express the antibody fragments on the surface (Fig. 21.3).

Several different platforms are used to select the recombinant antibodies: (1) a phage display, (2) a protein-mRNA link using a ribosome or mRNA display, and (3) a microbial cell display on yeast, bacteria, or retrovirus. Other selection methods may include a microbead display by in vitro compartmentalization, a display based on protein-DNA linkage, and an in vivo-based growth selection based on the protein fragment complementation (Hoogenboom 2005). However, the phage display, which is discussed more in this chapter, seems to be the most often applied approach for bacterial antibody production.

The major improvement of recombinant antibodies over polyclonal or monoclonal antibodies is that they are produced by bacteria, which offer a stable genetic source. Furthermore, screening is less time–consuming, and clones that are not specific or not producing antibodies against the target pathogen are discarded. The advantages are that the bacteria can be genetically manipulated and the recombinant antibodies can be produced in abundance in a relatively short period of time without the need for a cell culture system or the requirement of a surrogate animal (mice are often used to grow hybridoma in their peritoneal cavity) for monoclonal antibody production in large quantities (Emanuel et al. 2000).

2.4.1. Phage Display

Phage display was first reported in 1985 by George P. Smith, who demonstrated that peptide fragments can be expressed on the surface of bacteriophages. It was introduced as a method for antibody selection in 1990 by McCafferty and his colleagues, and it has since become a widely used method for recombinant antibody production. The method is based on the expression of functional antibody fragments (Fab) on the surface of a filamentous phage. A large number of antibodies can be quickly selected from libraries on the basis of the antigen-binding behavior of individual clones (Posner et al. 1993; Hoogenboom 2005).

Random peptide phage display has been successfully employed to identify bacteria (Williams et al. 2003; Paoli et al. 2004; Nanduri et al. 2007a, 2007b), new receptor molecules, receptor ligands (Lu et al. 2003) in epitope mapping and mimicking of protein antigens, antibodies (Khuebachova et al. 2002; Muhle et al. 2004), and drug discovery (Pan et al. 2006).

The most commonly used phages are the filamentous phages M13, f1, and fd. A phage-display library consists of a group of filamentous phages carrying genes encoding foreign proteins (antigens), which are expressed as fused proteins in their outer coat (Smith and Petrenko 1997). Briefly, the specific peptide represented by the DNA is fused to the coat protein on the surface of the virion, and each phage displays a single peptide. When the infective carrier phages are cloned individually into a suitable host, e.g., *E. coli*, they propagate indefinitely (Fig. 21.3).

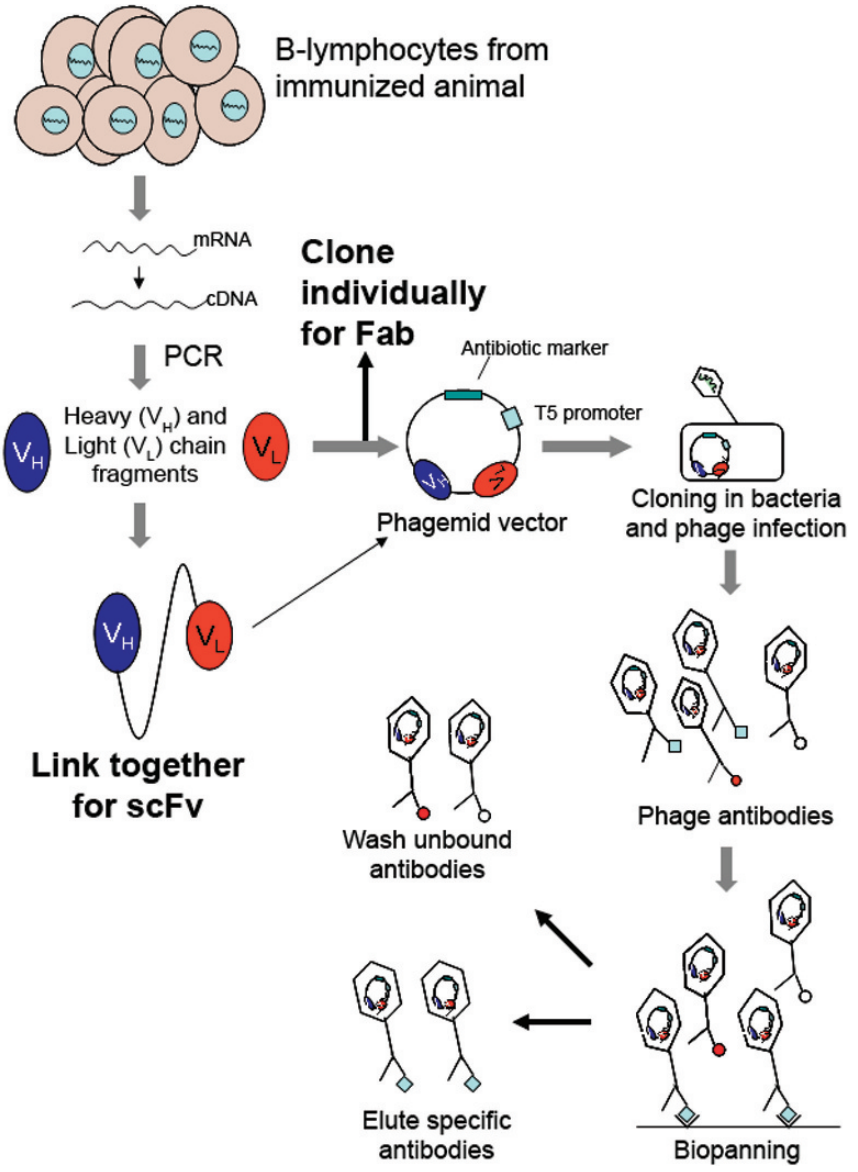

Figure 21.3. Recombinant antibody production through phage display (adapted from references: Liddell 2005; Smith and Petrenko 1997; Petrenko and Vodyanoy 2003; Emanuel et al. 2000).

The phage antibodies carry the sites for antigen recognition and binding domains. Through the affinity selection, a specific antibody is recognized and a particular phage is isolated and propagated by infecting a suitable host. Thus, a single-phage antibody library could be constructed and useful quantities of the antibody could be made (Petrenko and Vodyanoy 2003). The screening of the library for the selection of suitable phage-bearing peptides is called biopanning. Traditionally, 2–10 biopannings are done to select one phage antibody.

The development of several phage antibodies for numerous pathogens has been reported. Goldman et al. (2000) developed a phage antibody against staphylococcal enterotoxin B (SEB). They labeled the whole phage with the Cy5 fluorescent tag, and used it in a fiber-optic biosensor and a fluorescence microplate assay for the direct detection of SEB from samples. They were

able to detect 1.4 ng of SEB. A phage display antibody was also developed to detect botulinum neurotoxin (Emanuel et al. 1996). Williams et al. (2003) screened commercial phage-display libraries for the presence of peptide ligand for *Bacillus* spores. They identified phage peptides that were specific for the spores of *Bacillus anthracis* and the spores of other *Bacillus* spp. Using those two peptides in tandem, they were able to detect *B. anthracis* spores. Kanitpun et al. (2004) developed single chain variable fragment (ScFv) molecules from hybridoma clones that produced immunoglobulins specific for the LPS and flagellar antigen of *E. coli* O157:H7, using phage display technology. Single chain variable fragment (ScFv) antibodies have been shown to be successful for the detection of *L. monocytogenes* (Paoli et al. 2004; Nanduri et al. 2007a) and *E. coli* (Nanduri et al. 2007b). Phage display methods for detection of bacterial pathogens are described in chapter 28.

3. Capture and Concentration of Cells by Immunomagnetic Separation

Paramagnetic beads coated with antibodies are currently used for the detection as well as for the concentration of bacterial cells from complex environmental samples (Fig. 21.4). Paramagnetic beads show a magnetic property only when placed under a magnetic field, and the magnetism disappears when the magnetic field is removed. This is an important property for their application in immunomagnetic separation (IMS), since magnetic beads would be free to interact with the target antigens (cells) in liquid suspension without being attracted to each other by inter-magnetic force.

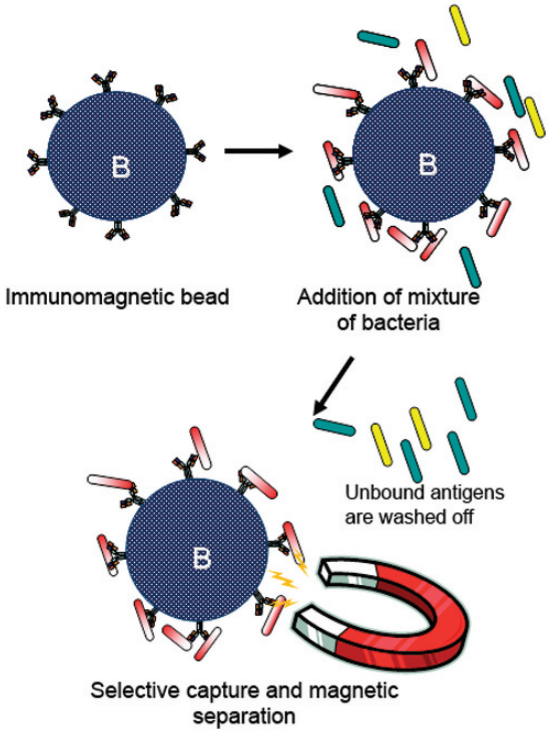

Figure 21.4. Schematic diagram showing immunomagnetic separation technology for capture and concentration of target antigens. The magnetic beads are conjugated with the specific antibodies when added to a mixture of bacteria; the antibody binds specifically to the target antigen. Under the magnetic field, the paramagnetic bead concentrates in the area of magnetism and unbound antigens are washed off.

The IMS step is considered a selective enrichment step since it allows bacterial capture and concentration from liquid food matrices. Since the capture rate varies (10–70 %) depending on the type of microorganisms and the antibody used, the food suspension must carry a significant number of cells. Most often, IMS is suitable for use with food suspensions that have been subjected to a pre-enrichment or a selective enrichment step to bolster bacterial counts. Captured microbes (antigens) are then plated on nutrient/selective agar plates or further tested using other assays such as PCR, immunoassay, flow cytometry, and chemiluminescent- or pathogenicity-based assays (Feng 2001). Over the past two decades, IMS has been widely used to separate bacterial cells from complex materials including food, water, and clinical samples. Conventional methods of bacterial cell isolation require growing on a selective agar medium, which takes about 2–5 days, depending on the bacteria. IMS offers a rapid recovery of the cells and improves the sensitivity of the downstream microbiological analysis.

The Dynal Biotech company (Oslo, Norway) initially designed the paramagnetic beads called Dynabeads® and commercialized them by coating them with specific antibodies. Their Dynabeads® Anti-*Salmonella* and anti-*E. coli* antibodies have been shown to be superior to the conventional ISO or culturing methodologies for isolation of these pathogens. Culturing methods coupled with IMS increased the sensitivity of detection of many bacterial pathogens like *Salmonella* (Hara-Kudo et al. 2001; Jordan et al. 2004), *Staphylococcus aureus* (Yazdankhah et al. 1999), and *Listeria* (Bauwens et al. 2003).

IMS coupled with immunoassays or PCR assays was shown to significantly improve the speed and sensitivity of *E. coli* O157 detection to as low as 1 CFU in 25 g of the food sample (Chapman and Ashton 2003). IMS-PCR combinations for the detection of *Salmonella* (Soumet et al. 1999; Jenikova et al. 2000; Mercanoglu and Griffiths 2005), *L. monocytotenes* (Hudson et al. 2001; Amagliani et al. 2006; Ueda et al. 2006), and *E. coli* (Fu et al. 2005) have become popular in recent years. IMS coupled with multiplex PCR was used to simultaneously detect *L. monocytogenes* and *Salmonella* from food samples, and the detection limit was established to be about 1000 cells (Li et al. 2000; Hsih and Tsen 2001). They suggested that additional pre-enrichment with universal pre-enrichment broth would enhance the detection sensitivity. Hudson et al. (2001) showed that IMS-PCR could detect as little as 1.1 CFU/g of *L. monocytogenes* in a ham sample.

IMS has also found a wide application in flow cytometry (Jung et al. 2003; Splettstoesser et al. 2003) and fiber-optic biosensors (DeMarco and Lim 2002; Liu et al. 2003) for the detection of bacterial pathogens.

Nonmagnetic immunobeads, such as the protein A-conjugated sepharose bead (Sigma, St. Louis, MO) coated with an anti-*Listeria* monoclonal antibody (MAb-C11E9), were developed in our laboratory for the detection of *L. monocytogenes* (Gray and Bhunia 2005). First, the immunobeads and magnetic Dynabeads were used to capture bacteria from food matrices; and second, the captured bacteria were tested for cytotoxicity on a mammalian cell line to differentiate pathogenic from nonpathogenic *Listeria* species. This immunobead method was highly specific for *L. monocytogenes*, and performed better than the Dynabeads because the antibody (MAb-C11E9) on the immunobead is specific for *L. monocytogenes* and some strains of *L. innocua* (Bhunia et al. 1991; Lathrop et al. 2003; Gray and Bhunia 2005), while the antibody in Dynabeads reacts with all species of *Listeria* (Vytrasova et al. 2005). When tested in a cytotoxicity assay, the immunocaptured cells *L. monocytogenes* (captured with an immunobead assay) gave a positive cytotoxicity; while *L. monocytogenes, L. ivanovii*, and *L. seeligeri* (captured by Dynabeads) also showed a positive cytotoxicity (Gray and Bhunia 2005).

Recently, an improved IMS technique was described by Varshney et al. (2005), in which magnetic nanoparticle conjugates (MNCs) were coated with polyclonal anti-*E. coli* antibodies using a biotin and streptavidin system and then used to capture *E. coli* O157:H7. Capture efficiency values increased from 69 to 94.5 %, while bacterial concentrations decreased from

3.4×10^7 to 8.0 CFU/ml in ground beef samples. This technology requires minimal sample preparation and results in higher capture efficiency compared to the traditional Dynal bead-based IMS.

Immunomagnetic beads were also used together with quantum dots for their ability to detect bacterial pathogens quantitatively (Su and Li 2004; Yang and Li 2005; Tully et al. 2006). Quantum dots naturally emit fluorescence and are used as the source of tracer fluorophor. Magnetic bead-captured bacterial cells can be quantitatively detected using antibody conjugated to quantum dots. Yang and Li (2005) used streptavidin-coated fluorescent semiconductor quantum dots to detect *Salmonella* cells at 10^3–10^7 CFU/ml bound to the magnetic beads conjugated with biotin-labeled anti-*Salmonella* antibody. The measure of fluorescence intensity was correlated to the number of cells present in the test sample.

3.1. Automated IMS Systems

Dynal Biotech Ltd. (Wirral, UK) automated the IMS procedure, and the commercial system is called BeadRetriever™. This system works on the inverse magnetic particle processing principle, in which the dynabeads are moved among tubes containing specific reagents with the aid of a magnetic bar, rather than by moving the liquids. The tube strips with their compartments contain reagents such as bacteria, secondary antibody, washing fluids, and the substrate. The system can process 15 pre-enriched samples in 20 minutes. BeadRetriever™ has been applied widely for the detection of *E. coli* (Chapman and Cudjoe 2001; Reinders et al. 2002; Fegan et al. 2004), *Salmonella* (Duncanson et al. 2003), and *L. monocytogenes* (Amagliani et al. 2006). Table 21.1 lists some of the commercialized and laboratory-developed IMS-integrated models.

4. Immunoassays for Pathogen Detection

Immunoassays are developed to measure the presence of an analyte (usually proteins) through antigen-antibody interaction. The detection signal can be radioactive, colorimetric, or fluorescent. The sensitivity and specificity of the immunoassays is highly dependent on the choice of antibodies.

4.1. Radioimmunoassay

The radioimmunoassay (RIA) was developed in 1960 by Yalow and Berson, who received the Nobel Prize in medicine in 1977 and demonstrated the application of RIA for the detection of insulin. In this method, a gamma-radioactive isotope (of iodine or tyrosine) was labeled to the antigen and then mixed with the antibody. A nonlabeled antigen was then added to this mixture at higher concentrations, to compete with the labeled antigen. Antibody preferentially binds to nonlabeled antigen, and the radioactive signal from the displaced labeled antigen is measured and the binding curve is plotted. Although this method is very sensitive and highly specific for bacterial cells or toxins, the cost and high risks associated with radioisotopes prohibited its widespread use. In recent years, this method has been replaced by enzyme immunoassays (EIA), where the detection is colorimetric or fluorescence-based.

4.2. Enzyme Immunoassays

Among the enzyme immunoassays, enzyme-linked immunosorbent assay (ELISA) has emerged as the most reliable quantitative detection method. ELISA is a rapid immunochemical method in which an antigen-antibody reaction is catalyzed by an enzyme producing a

Table 21.1. Partial list of automated or semi-automated IMS integrated systems (AIMS)

Name of the Assay OR Commercial Name	Antibody Used	Integrated Method to AIMS	Detection	Detection Limit	Commercialized (yes or no) and Company Name	Reference
Pathatrix system*	Anti-*E. coli*, anti-*Salmonella* Anti-Listeria	Plating	Characteristic colonies on the plate, PCR	1CFU/25 g	Yes/ Matrix microsciene, CO	cfsan.fda.gov
Immunomagnetic particle- Enzyme Linked ImmunoSorbent Assay (IMP-ELISA) using BeadRetriever™	Dynal® Anti-*Salmonella, E. coli* and *Listeria*	ELISA	Colorimetric	10^5 CFU/mL	Yes/ Invitrogen Corporation, Carlsbad, CA	invitrogen.com
Enhanced Automated Immunomagnetic Separation (eAIMS)	Dynabeads anti-E.coli O157	Charge Switch® Technology (CST)	qPCR	10 CFU/25 g	Yes/ Invitrogen, CA	invitrogen.com
SI-RSC (sequential injection renewable separation column) IMS	Anti-*E. coli* O157	TaqMan PCR and DNA Microarray	Real time amplification Fluorescence	10–10^3 CFU/mL without enrichment	No	Chandler et al. 2001
EIAFOSS™*	Anti-*E. coli* O157	ELISA	Colorimetric	10^5 CFU/mL	Yes/ Foss Electric A/S, Hillerød, Denmark	Reinders et al. 2002
Automated Immunomagnetic Separation-Enzyme Immuno Assay (AIMS-EIA)	Dynal® Anti-*Salmonella*	ELISA	Chemiluminiscence	ND	No	Duncanson, Wareing and Jones 2003
IMS/PCR Biodetection Enabling Analyte Delivery System (IMS/PCR BEADS)	Dynabeads anti-*E.coli* O157	PCR	PCR	10 cells/mL	No	Straub et al. 2005

*AOAC certified; ND- Not determined; Most or all of these have been applied in detection of *E. coli, Listeria* and *Salmonella* unless specified.

chromogenic or fluorescent signal. ELISA is performed in different formats depending on the location of antigen or the antibody on the solid surface (Fig. 21.5).

In competitive ELISA, the primary antibody is mixed in a separate tube with various dilutions of bacteria (or antigen) and added to the wells containing immobilized antigen. Only the free unbound antibody will bind the immobilized antigen. A secondary antibody-enzyme conjugate and substrate system is added for color development. The highest dilution of cells showing the minimum reaction or equivalent to a background control is considered positive (Fig. 21.5a).

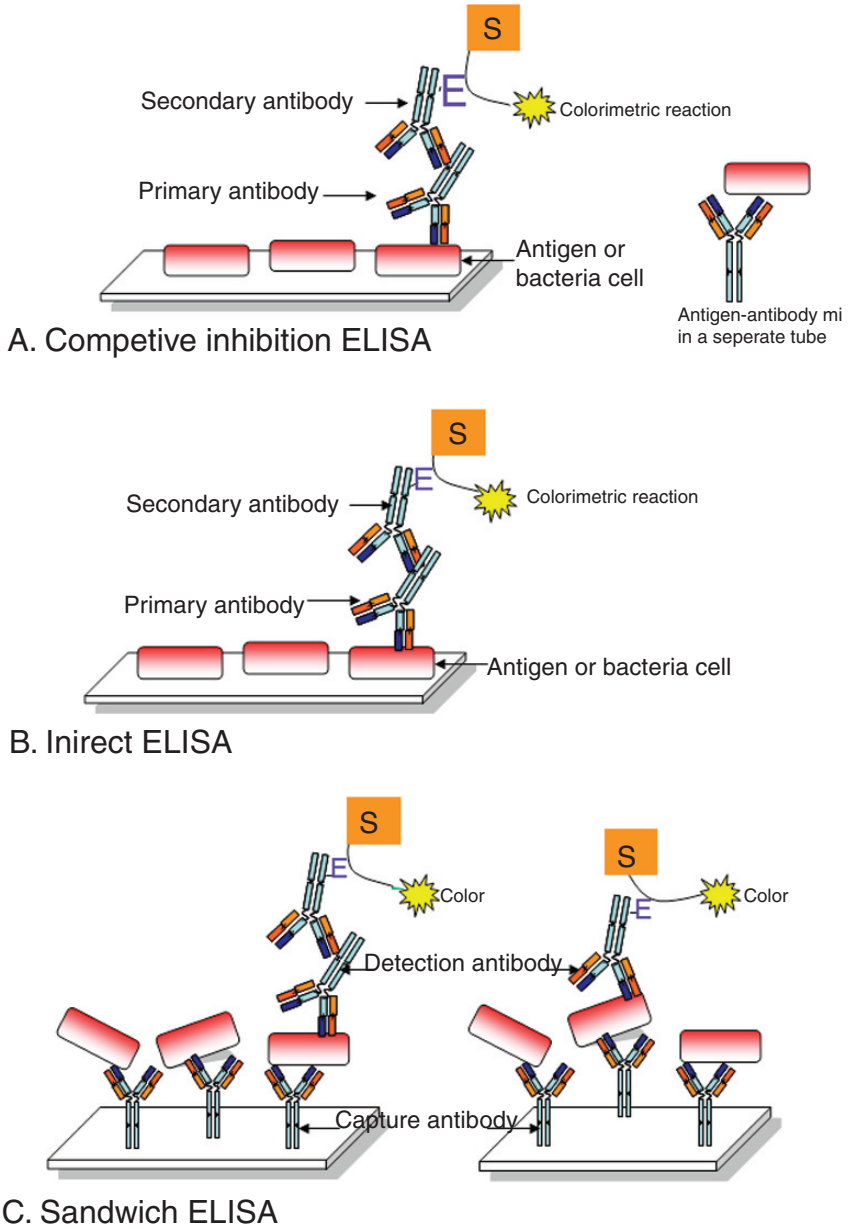

Figure 21.5. Diagrammatic representation of different ELISA formats for colorimetric detection of bacteria (E = enzyme; S = substrate).

In indirect ELISA (Fig. 21.5b), the antigen is first immobilized in the wells of a microtiter plate and is then added to the antibody solution. A secondary antibody conjugated with substrate modifying enzyme, e.g., horseradish peroxidase (HRP) or alkaline phosphatase (AP), is added to bind to the primary antibody, and the reaction is developed with a suitable substrate to give a color reaction. If a fluorescent molecule (e.g., FITC, Cy-5, Alexa-Flour) is used, the reaction is quantified by the amount of fluorescence emitted.

Sandwich ELISA works similarly to indirect ELISA, except that the microtiter plates are first coated with a capture antibody and then the antigen is added. The reaction is developed using the labeled detection or tracer antibody in one step, or using an additional secondary antibody conjugate in a two-step reaction (Fig. 21.5c).

ELISA has been a popular choice among microbiologists for the sensitive and quantitative detection of bacteria or their toxins in food, water, and environmental and clinical samples (Yeh et al. 2002; Gracias and McKillip 2004; Haggerty et al. 2005). Some of the commercially available immunoassay kits are listed in Table 21.2. ELISA assays available for some foodborne pathogens are discussed below.

4.2.1. *Escherichia coli*

Bacterial surface antigens, like proteins, lipopolysachharides (LPS), and flagella, have been used as targets for detection of whole cells present in water and food samples. Low molecular weight outer membrane proteins (<10,000 kDa) and LPS from *E. coli* O157:H7 have been targeted in ELISA using polyclonal antibodies. Blais et al. (2006) used polymyxin B, which binds LPS more efficiently, to immobilize the *E. coli* cells. They used commercially available anti-*E. coli* O111 or anti-*E. coli* O26 antisera to detect the respective strains in ground beef.

Most of the detection kits for *E. coli* O157 use sandwich ELISA with polyclonal antibodies that are raised against the whole cells. LMD-ELISA (LMD Laboratories, Carlsbad, CA) for *E. coli* O157 has been shown to detect *E. coli* cells within 1 h (Park et al. 1996). In the LMD-ELISA kit for *E. coli*, the microwell test strips were coated with polyclonal antibodies to *E. coli* O157. Test samples were prepared by mixing 0.2 ml of the sample with 0.1 ml of 10 % buffered formalin, and 0.1 ml of this suspension was then added to the appropriate wells. The plates were incubated at room temperature for 20 min, then washed, and two drops of enzyme conjugate (peroxidase-labeled anti-*E. coli* O157 antibodies) were added to each well. Plates were then incubated at room temperature for 10 min, washed again with the buffer, and rinsed with distilled water. One drop each of substrate A (tetramethylbenzidine) and substrate B (peroxide) were added, and the plates were incubated at room temperature for 5 min. The reaction was stopped using the stop solution (phosphoric acid), and the plates were read spectrophotometrically at 450 nm.

At least 12 other ELISA-based kits are available for the detection of *E. coli* cells (Feng 2001). Several ELISA-based assay kits have been developed for the detection of enterotoxins or Shiga-like toxins (Stx). *E. coli* O157:H7 produces either Stx1 or Stx2, or both. Of these, Stx2 is reported to be the most significant pathogenic factor. The Premier EHEC test (Meridian Diagnostics, Inc.) is a sandwich ELISA which uses monoclonal antibodies directed against Stx1 and Stx2 as capture antibodies and a polyclonal anti-Stx antibody conjugated with horseradish peroxidase for detection. It is easy to perform and is suitable for the routine analysis of food and stool samples (Nataro and Kaper 1998).

Competitive ELISA has been developed for the detection of enterotoxins, and as few as 3–20 pg of heat stable (ST) and heat labile (LT) enterotoxins from *E. coli* were detected using this method (Germani et al. 1994). Oxoid (Hampshire, UK) commercialized an ELISA-based kit for the detection of ST toxins. ELISA-based VEROTEST (MicroCarb) and Premier EHEC (Meridian) kits have also been developed for the detection of Shiga toxins in *E. coli* (Feng 2001).

Table 21.2. Partial list of commercially available immunoassay kits for bacterial pathogens (expanded from Feng 2001)

Target Bacteria/Toxin	The Commercial Name and the Company	Assay Principle
Helibacter pylori	***GAP-IgG,*** BioRad	ELISA
	HM-CAP, Enteric products	
	Pylori test EIA, Orion	
	Premier Platinum HpSA ELISA, The Meridian (Cincinnati, OH)	
	Pylori test, Oxoid	LA
Salmonella	***Salmonella-Tek***, Organon Teknika Durham, NC	
	Equate, Binax	
	BacTrace, KPL	
	LOCATE, Rhone-Poulenc	ELISA
	Assurance, Biocontrol Inc, Belleview, Wash.	
	TRANSIA plate Salmonella Gold, Diffchamb	
	Bioline, Bioline	
	TECRA* and *OPUS, Tecra	
	VIDAS	
	VIDAS automatic ELFA, BioMerieux	ELFA
	Seobact, Remel	
	Welcolex, Laboratoire Wellcome	LA
	Bactigen, Wampole labs	
	Spectate, Rhone-Poulenc	
	Microscreen, Mercia	
	***Salmonella* Latex test**, Oxoid	
	Singlepath *Salmonella*, EMD, Germany	
	Reveal, Neogen	
	Clearview, Unipath	Ab-ppt
	PATH-STIK, LUMAC	
Salmonella serotype typhi	Sanofi qualitative agglutination test kits (Bio-Rad, CA)	Tube agglutination
	TyphiDot™	Dot-ELISA
	Multi-Test Dip-S-Ticks, PANBIO INDX, Inc., Baltimore, Md.	Lateral flow
	TUBEX IDL Biotech, Sollentuna, Sweden	Agglutination
E. coli	**EHEC-TEK**, Organon Teknika	ELISA
	HEC O157ELISA, 3M Company (St. Paul, MN)	
	TRANSIA Card *E. coli* O157, DiffChamb	
	TECRA, TECRA	
	Assurance, Biocontrol	
	E. coli O157, LMD lab	
	Premier O157, The Meridian	
	E. coli O157:H7, Binax	
	E. coli Rapitest, Microgen	
	VIDAS (ELFA), BioMerieux	
	RIM, Remel	LA
	E. coli O157 latex test and Dryspot E. coli O157 Oxoid	
	Prolex, Prolab	
	Ecolex, Orion diagnostica	
	Welcolex, Murex	

E. coli	**VIP,** Biocontrol	Ab-ppt
	Reveal, Neogen	
	QuixRapid O157, Universal Health watch	
	ImmunoCardSTAT, The Meridian	
	Agilent 2100 Bioanalyzer (Agilent Technologies)	Microfluidic-flowcytometry
E. coli Shiga toxin (Verotoxin)	***Verotest,*** Microcarb	ELISA
	Premier EHEC The Meridian	
	Verotox-F, Denka Seiken	RPLA
	VTEC-RPLA, Oxoid	
Enterotoxigenic *E. coli*		
E. coli heat stable toxin (ST)	**E. coli *ST* EIA,** Oxoid	ELISA
E. coli heat labile toxin (LT)	***Vet-RPLA***, Oxoid,	RPLA
Listeria	**Listeria-*TEK***, Organon Teknika	
	***TRANSIA Plate* Listeria**, DiffChamb	
	TECRA, TECRA	ELISA
	Assurance, Biocontrol	
	Pathalert, Merck	
	EIAFOSS, VIDAS Listeria express (ELFA), BioMerieux	
	Microscreen, Listeria latex, Microgen	RPLA
	VIP, BioControl	Ab-ppt
	Clearview and ***Rapidtest***, Unipath	
Staphylococcus aureus	*S. aureus VIA*, TECRA	ELISA
	Enterotoxins:	
	SET-EIA, Toxin technology	
	TRANSIA Plate SE, DiffChamb	
	TECRA and *OPUS,* TECRA	
	VIDAS (ELFA), BioMerieux	
	RIDASCREEN, R-Biopharm	
	Staphyloslide, BD	LA
	Aureustest, Trisum	
	StaphLatex, DIFCO	
	Dry Spot Staphytect Plus, Oxoid	
	PBP2′ test (MRSA), Oxoid	
	SET-RPLA, Oxoid	RPLA
	TSST-1:	
	TST-RPLA, Oxoid	
Campylobacter	VIDAS, BioMerieux	
	EIAFOSS, Foss Electric A/S, Hillerød, Denmark	ELISA
	TECRA, TECRA	
	Campyslide, BD, Cockeysville, Md.	
	Meritechcampy, Meridian	LA
	Microscreen, Mercia	
Bacillus cereus diarrhoeal *toxin*	TECRA, TECRA	ELISA
	BceT, Oxoid	RPLA
Clostridium botulinum toxin	ELCA, ELCA Tech	ELISA
Vibrio cholerae	CholeraSMART, New Horizon	Ab-ppt
	BengalSMART	
	CholeraScreen	
	BengalScreen	Agglutination
	Enterotoxin	
	VET-RPLA	RPLA

Note: Analyte 2000 is a fiberoptic system. SPREETA-2000 (Texas Instruments) is a fiber optic SPR system. BIAcore SPR systems have been used for detection of various bacteria with some modifications.

Premier EHEC is an EIA which utilizes monoclonal anti-Stx antibodies adsorbed to microtitre wells to capture Stx, and a polyclonal anti-Stx antibody to detect bound toxin. Bennett-Wood et al. (2004) reported 100 % sensitivity and specificity for this kit after an overnight enrichment of stool samples to detect EHEC.

4.2.2. *Listeria monocytogenes*

Listeria monocytogenes is an intracellular bacterial pathogen which primarily affects immunocompromised patients, pregnant women, and children. A zero tolerance limit imposed by U.S. regulatory agencies on this bacterium has resulted in the development of numerous sensitive detection methods (Gasanov et al. 2005). The commercial kits for *Listeria* (*Listeria* Unique, TECRA International, Frenchs Forest, Australia; and VIDAS *Listeria* Express, bioMerieux, Marcy Etoile, France) claim equal sensitivity to traditional culture methods and enable a result within 30 h of sample receipt (Gasanov et al. 2005).

Bhunia (1997) has extensively reviewed different antibodies developed against *L. monocytogenes* surface antigens and their potential application. Kim et al. (2005) reported the development of five different monoclonal antibodies and a polyclonal IgY antibody against flagella from the 4b strain of *L. monocytogenes*. They showed that a combination of HRP-conjugated MAb7A3 and MAb 2B1 in a sandwich ELISA format was able to detect 10^5 cells/mL, compared to HRP-labeled IgY and MAb 2B1, which detected 10^6–10^7 cells/mL. Yu et al. (2004) designed a sandwich ELISA using the monoclonal antibodies against p60, a highly immunogenic murein hydrolase (essential for cell division), encoded by the *iap* (invasion associated protein) gene. Their two monoclonal antibodies, p6007 and p6017, were highly specific to *L. monocytogenes* and *Listeria* spp., respectively, in ELISA. Similar studies showed that monoclonal antibodies against Internalin A (MAb2B3; Hearty et al. 2006) and unknown proteins (Lin et al. 2006) reacted specifically with *L. monocytogenes* in ELISA. Polyclonal antibodies have been developed against Phospholipase C (Chaudhari et al. 2004), Listeriolysin O (Barbuddhe et al. 2002; Boerlin et al. 2003), Internalin B (Leonard et al. 2005) and Internalin A (Boerlin et al. 2003).

4.2.3. *Salmonella*

Salmonella Newport was detected with a commercially available ELISA kit (TECRA *Salmonella* Visual Immunoassays, International Bioproducts Inc., Vaughn, Ontario, Canada) (Bohaychuk et al. 2005). The VIDAS *Salmonella* test, a commercial ELISA kit, has been shown to be a good screening kit for the detection of *Salmonella* in fecal, tissue, feed, or meat samples (Uyttendaele et al. 2003; Sommerhauser and Failing 2006). However, the TRANSIA card showed a poor specificity for *Salmonella* serovars in food samples (Fratamico 2003). Veling et al. (2001) evaluated two ELISA methods in milk, using the antibodies raised against LPS and the flagellar antigen of *Salmonella*. The specificity for both the assays were 95–100 %; however, the sensitivity was very poor.

4.2.4. Staphylococcal Enterotoxins

Immunoassays have been the major choice for staphylococcal enterotoxin (SE) detection in food and clinical samples (Bhunia 2006). ELISA-based kits for enterotoxin detection are commercially available from various manufacturers like TECRA (*S. aureus* VIA, Tecra, OPUS), bioMerieux (VIDAS), Diffchamb AB (Transia Plate SE- Official Methods recommended by Ministere de l'Agriculture, France, Transia Tube SE), R-Biopharm (RIDASCREEN-A, B, C, D, E) and Toxin Technology (SET-EIA) (Feng 2001).

Since as little as 100–200 ng of the toxin can cause symptoms of staphylococcal intoxication, the development of highly sensitive detection systems is obligatory. Studies have indicated that VIDAS SET2 has a greater sensitivity (< 0.5 ng/g of toxins A and B; and < 1 ng/g of toxins C, D, and E) and specificity (100 %) than VIDAS SET and TRNASIA PLATE SE (Vernozy-Rozand et al. 2004) and RIDASCREEN could detect SEs A through E as low as 0.35 ng/mL of food extract or 0.5–0.75 ng/g of foods within 3 h (Park et al. 1994). Later, Schotte et al. (2002) demonstrated that a rapid immunochromatographic-based handheld assay can detect as little as 50 pg/g of SEB within 15 min. Staphylococcal superantigens (SEA, SEB, and SEC), toxic shock syndrome toxin-1 (TSST-1), and streptococcal pyrogenic toxin A (SPEA) were detected by ELISA and were quantifiable at picogram levels within 2.5 h (Miwa et al. 2000).

4.2.5. *Clostridium botulinum* Toxins

ELISA formats have been the most common method for the sensitive detection of botulinum neurotoxins (Ferreira et al. 2003; Lindstrom and Korkeala 2006). Using a sandwich ELISA format with a polyclonal antibody (PAb) as the capture antibody and a monoclonal antibody (BA93) as the detection antibody, Type A neurotoxin was detected at a concentration of 4–8 pg/mL or 1–2 mouse lethal dose (MLD)/mL (Ekong et al. 1995). An antibotulinum polyclonal antibody in ELISA was able to detect very low concentrations of botulinum neurotoxins A, B, E, and F within 8 h (Lindstrom and Korkeala 2006). Ferreira et al. (2004) developed ELISA-based detection systems for botulinum and have successfully detected botulinum toxin from foods that were responsible for botulism outbreaks. Both polyclonal and monoclonal antibodies for *C. botulinum* toxin and toxoids A through F are commercially available for assay development from KPL Inc. (Gaithersburg, MD).

4.3. Lateral Flow Immunoassay

The lateral flow immunoassay (LFI), or immunochromatographic strip (ICS), or dipstick test is one of the most attractive and widely used popular immunoassay methods in food and clinical diagnostic work today. In this assay the capture antibody is immobilized on the nitrocellulose membrane in a predefined position. The detection antibody, coupled with colloidal gold or latex particles, is placed in an area near the sample application port. The absorbing blot membrane located at the opposite end of the sample application serves as the wick and facilitates fluid movement on the membrane. When a sample of bacteria, toxin, or antigen suspended in liquid is applied to the sample port of the device, it binds to the detection antibody conjugated to a gold or latex particle. The antigen-antibody complex migrates laterally on the membrane by capillary action to the opposite end of the strip and through a porous membrane that contains two capture zones, one specific for the bacterial pathogen and another specific for unbound antibodies coupled to the gold or latex (control line). The presence of only one (control) line on the membrane indicates a negative sample, and the presence of two lines indicates a positive result, which is visualized within 5–10 min (Chapman and Ashton 2003; Ray and Bhunia 2008). One drawback to this method is that it is less sensitive than the ELISA-based assays and requires a bacterial cell concentration of about 10^7–10^9 cells for a positive reaction. In recent years, however, attempts have been made to improve the sensitivity of the assay by introducing an automatic reader, which avoids the ambiguity in reading the positive reactive bands with human eyes; or by introducing chemiluminescent-based detection of the antigen-antibody complex on the membrane.

A large number of LFI kits have been introduced in recent years, especially for the detection of *E. coli* O157:H7, *Salmonella*, and *Listeria* from enriched food samples. Commercially available LFI kits include, VIP (Biocontrol); Immunocard STAT (Meridian); Singlepath®

and Duopath® (Merck); DuPont LFI strips (Dupont); Tecra UNIQUE (De Paula et al. 2002; Briggs et al. 2004); RapidChek® (Strategic Diagnostics Inc. Newark, DE); and REVEAL (Neogen Corporation, Lansing, MI) (Table 21.2).

The performance of LFI kits (REVEAL, Neogen Corp.) was compared with that of ELISA and of PCR for the detection of *Salmonella* Newport, *L. monocytogenes*, and *E. coli*, and the study demonstrated that the LFI produced no false negatives and thus would be a better choice when screening for these pathogens in meat or poultry (Bohaychuk et al. 2005). However, LFI strips take about 2–4 days for the recommended pre-enrichment of the bacterial test samples before analyses.

4.4. Other Immunoassays

4.4.1. Latex Agglutination (LA) and Reverse Passive Latex Agglutination (RPLA) Tests

LA- and RPLA-based commercial detection kits are the most rapid methods used for bacterial or toxin detection. Generally these methods require large amounts of antigen to show a positive reaction. In these methods, antigen-specific antibodies are immobilized on latex particles and mixed with a sample in wells of microtiter plates. If the specific antigen (toxin) is present in the sample in LA, a coagulated precipitate is observed. In RPLA, a diffuse pattern will appear in the bottom; in its absence, a ring or button will appear and the latex does not play a role here—thus it is called a "passive latex agglutination test." These methods have been successfully applied to detect somatic or flagellar antigens or toxins of several foodborne pathogens, including *Staphylococcus aureus, Clostridium perfringens, Bacillus cereus, Vibrio cholerae, E. coli,* and *Campylobacter* (On 1996; Feng 2001; Gasanov et al. 2005).

4.4.2. Enzyme-Linked Fluorescent Assay

Fluorescence-based detection by ELISA, called enzyme linked fluorescent assay (ELFA), became popular because of its improved sensitivity and quick results (Vernozy-Rozand et al. 2004; Ray and Bhunia 2008). In this assay, the enzyme (i.e., alkaline phosphatase) conjugated to the detection antibody breaks down the substrate (4-methyl umbellliferyl phosphate, MUP) to produce a fluorescent end product (methyl umbelliferyl), which can be sensitively detected by a spectrofluorometer. In a different format, a fluorophore molecule, instead of an enzyme, is attached to the detection antibody for direct interrogation of antigens or pathogens. The commonly used fluorescent molecules are rhodamine B, fluorecein isocyanate, and fluorescein isothiocyanate (FITC). Decory et al. (2005) used liposome nanovesicles encapsulating fluorescent dyes to increase the fluorescence signal, thus reducing the detection time and limit. They were able to detect < 1 CFU/mL of *E. coli* within 8 h by combining IMS and fluorescence detection.

4.4.3. Time-Resolved Fluorescence Immunoassay

Time-resolved fluorescence immunoassay (TRFIA) is commercially marketed as a dissociation enhanced lanthamide fluorescent immunoassay (DELFIA) by Perkin-Elmer Life Sciences (Akron, OH). A lanthamide chelate (europium, samarium, terbium, or dysprosium) is used as a label in the detection antibody. The method works similarly to ELISA in a microtiter plate, in which an antibody first captures an antigen which is then detected by using the lanthamide-labeled antibody. A low pH enhancement solution is added to dissociate the label from the antibody after the reaction, and these free molecules rapidly form a stable new

fluorescent chelate which can be read by a fluorescent reader. Unlike other fluorescent labels, lanthamide has a long fluorescence decay time and an exceptionally large Stokes' shift, thus can be read after the background noise has reduced. Europium (Eu^{3+}) is the label that has been commonly used in TRFIA (Peruski and Peruski 2003; Tu et al. 2004; Lim et al. 2005; Bhunia 2006). TRFIA in combination with IMS (Tu et al. 2002; Yu et al. 2002) and PCR (Watanabe et al. 2002) has been shown to increase the detection sensitivity for *E. coli* and *Salmonella enterica*. It has also been used to detect toxins of *S. aureus* and *C. botulinum* at 4–20 pg ranges (Peruski et al. 2002).

4.4.4. Chemiluminescent Immunoassay

Chemiluminescent immunoassay (CLIA) has been developed to detect pathogens from food samples. Detection antibodies conjugated to the chemiluminescent dyes such as 3(2′ spiroadamantane) 4 methoxy 4(3″ phosphoryloxy) phenyl 1,2 dioxetane (AMPPD), APS-5 (Gehring et al. 2004), and luminol (3-aminophthalhydrazide) enhanced with 4-iodophenol (Zamora and Hartung 2002) are shown to elicit a sensitive signal upon binding to target bacteria. CLIA has proved to be an efficient method for detecting botulinum and staphylococcal toxins (Kijek et al. 2000; Cadieux et al. 2005). However, for *Salmonella* detection, CLIA showed no improvement over the conventional ELISA in specificity and sensitivity (Zamora and Hartung 2002).

4.4.5. Capillary Microbead (Spheres) Immunoassay

Capillary microbead (spheres) immunoassay for staphylococcal enterotoxins (SEs) was developed individually by two different groups during 1997–1998 (Strachan et al. 1997; Giletto and Fyffe 1998). Strachan et al. (1997) used sandwich ELISA, in which microbeads coated with antibody were used to capture antigens in a capillary tube and the SEB was detected with fluorescently labeled anti-SEB antibody. They automated the immunosensor and showed that it was capable of detecting as little as 5 ng/g of toxin within 10 min. Giletto and Fyffe (1998) used a similar approach, except that the detection was chromogenic, yielding a localized coloration upon addition of the substrate mimicking the lateral flow strip reaction. They could detect 0.5 ng/g of SEB in certain foods, compared to 2–10 ng/g by TECRA and SET-RPLA.

4.4.6. Electrochemical-Immunoassay

Electrochemical-immunoassay was adopted for bacterial detection as an alternative to a label-free assay system, in which the antigen-antibody reaction is measured amperometrically using the redox electrodes. In practice, electrochemical immunosensors are an extension of conventional antibody-based enzyme immunoassays (ELISA), in which catalysis of substrates by an enzyme conjugated to an antibody causes pH change, produces ions, or allows oxygen consumption that generates electrical signals on a transducer (Warsinke et al. 2000). Amperometric, potentiometric, and capacitive transducers have been used for such applications. In amperometric detection, for example, alkaline phosphatase conjugated to an antibody hydrolyzes *p*-nitrophenyl phosphate to phenol, which is detected by voltammetry. In light-addressable potentiometric sensors (LAPS), urease-conjugated antibody hydrolyzes urea, resulting in the production of carbon dioxide and ammonia that changes the pH of the solution. A silicon chip coated with a pH-sensitive insulator and an electrochemical circuit measures the alternating photocurrent as a light emitting photodiode shines on the silicon chip. These sensors are very sensitive and have been used for detection of *Salmonella* and *E. coli* O157:H7 rapidly in 0.5 to 1.5 h (Ghering et al. 1998). Croci et al. (2004) developed an electrochemical immunoassay coupled with fluorescence to detect 10^3 CFU of *Salmonella* in 25g of the meat sample.

4.5. Optical Biosensors

4.5.1. Surface Plasmon Resonance

Surface plasmon resonance (SPR) measures the changes in the refractive index resulting from binding of the antigen molecule to an immobilized antibody on the surface of metal films (Au or Ag) (Hsieh et al. 1998; Geng and Bhunia 2007). The binding kinetics could be measured in as soon as a few seconds to a maximum of 15 min (Dmitriev et al. 2002; Lathrop et al. 2003). Label-free, quantitative detection is the major advantage of SPR. Several commercial SPR instruments are currently available: Biacore (Biacore International SA, Switzerland), SPR-670M (Nippon Laser and Electronic Lab, Nagoya, Japan), Spreeta™ (Texas Instruments, Dallas, TX) (Homola et al. 1999; Rich and Myszka 2006). The sensitivity of most of the SPR-based methods is equivalent to an ELISA (10^5–10^8 CFU/mL); however, the major advantage is that it is a label-free detection assay. SPR has been demonstrated to be an efficient optical detection system for *E. coli* O157:H7 (Fratamico et al. 1998), *L. monocytogenes* (Lathrop et al. 2003; Bergwerff and Van Knapen 2006; Hearty et al. 2006), and staphylococcal enterotoxins (Rasooly and Herold 2006). Hsieh et al. (1998) used monoclonal antibodies against *Clostridium botulinum* neurotoxin in a BIAcore instrument to detect the toxin within 20 min. A polyclonal antibody against Internalin B was used in an SPR to detect about 10^5 CFU/mL of *L. monocytogenes*, with coefficients of variation between 2.5 % and 7.7 % (Leonard et al. 2005). Thomas et al. (2006) developed an SPR assay to detect the lipopolysaccharide antigen of *S. enterica* serovar Enteritidis using IgY, and the sensitivity and specificity was determined to be 84 % and 100 %, respectively.

Surface plasmon resonance was used to detect staphylococcal enterotoxins (Homola et al. 2002) and surface proteins of *L. monocytogenes* at very low concentrations (Lathrop et al. 2003). Another SPR-based instrument, Spreeta™, has been evaluated for specific and sensitive detection of *E. coli* O157:H7 in near real time, with the total assay taking about 35 min (Meeusen et al. 2005). However, the detection limit was about 10^6–10^7 CFU/mL. Balasubramanian et al. (2007) applied Spreeta™ for label-free detection of *Staphylococcus aureus*, using lytic bacteriophages, with a detection limit of 10^4 CFU/mL. See chapter 5 for more information about the use of SPR for bacterial detection.

4.5.2. Fiber-Optic Biosensors

Fiber-optic biosensors utilize the total internal reflection (TIR) property of light when it travels through the waveguide and generates a boundary of evanescent waves on the surface of the waveguide. Antibody- or immunoassay-based fiber-optic biosensors provide increased sensitivity, selectivity, and speed compared to the conventional immunoassay techniques (Geng and Bhunia 2007; Bhunia et al. 2007).

Antibody-coupled fluorescence wave guide biosensors are commonly employed in bacterial detection. In principle, a specific antibody is first covalently linked to the optic fiber that captures the bacteria of interest and a fluorescently labeled (e.g., Cy-5 or Alexa-Fluor 647) detection antibody binds specifically to the bacteria. When a 635-nm laser light is launched at the proximal end of the waveguide, fluorescence molecules are excited and generate an evanescent wave. Part of the emitted light energy is transmitted through the fiber and detected by a photodetector at wavelengths of 670–710 nm (Fig. 21.6). Portable sensors, e.g., Analyte 2000 and RAPTOR, manufactured by Research International (Monroe, WA), are widely used for such applications (Anderson et al. 2000; Geng et al. 2004; Nanduri et al. 2006). These systems allow a qualitative detection of the target entity, and the signal is proportional to the amount of antigen or hapten present in the sample (Taitt et al. 2005).

In recent years there has been an increase in the use of fiber-optic sensors for bacterial pathogens and their toxins (Lim 2003). This sensor has been successfully used for the detection

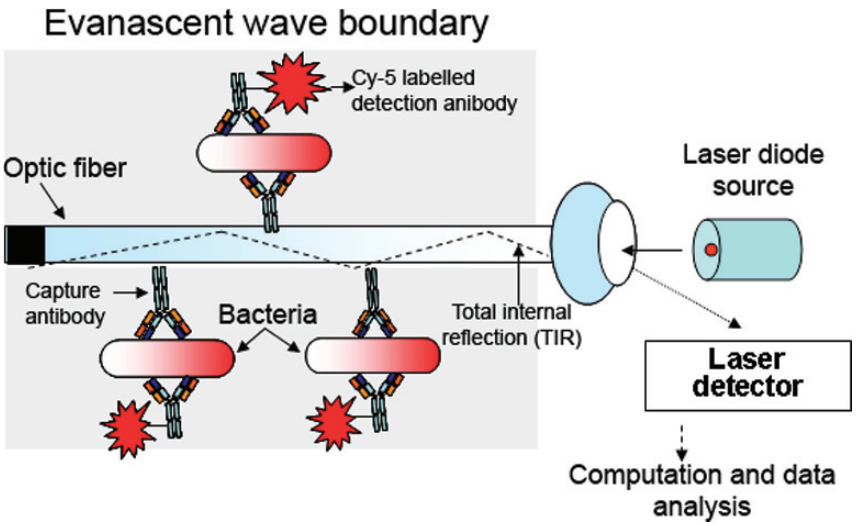

Figure 21.6. Schematic diagram showing bacteria detection using a fiber optic biosensor.

of *E. coli* (DeMarco and Lim 2002; Geng et al. 2006), *Streptococci* (Kishen et al. 2003), *Salmonella* (Kramer and Lim 2004), and *L. monocytogenes* (Geng et al. 2004; Nanduri et al. 2006). Geng et al. (2006) were able to detect *E. coli* O157:H7 with initial inoculation of 1 CFU/g of ground beef after only 4 h of enrichment. Using an automated fiber-optic–based detector, RAPTOR™, *Salmonella typhimurium* was detected in sprout rinse water at a concentration of 5×10^5 CFU/mL (Kramer and Lim 2004). Geng et al. (2004) reported a fiber-optic–based detection assay for *L. monocytogenes* with a detection limit of 10^3–10^4 CFU/mL in hot dogs and bologna, and it was later confirmed using the automated RAPTOR™ (Nanduri et al. 2006).

The combination of a fiber-optic biosensor with PCR has been shown to increase the sensitivity of detection of bacteria and dramatically improve the speed of detection from 10 h, needed for conventional fiber-optic sensors, to 2 h by conjugating with PCR (Simpson and Lim 2005). Chapter 6 covers the optical fibre biosensors for bacterial detection.

4.5.3. Antibody-Based Microfluidic Sensors

Research on microfluidics has burgeoned in the past decade from a fascinating concept to applications in clinical, molecular, biochemical, and medical diagnostics. The common materials used in the manufacture of microfluidic systems are silicon, glass, and polymers. The polymer, poly(dimethylsiloxane) (PDMS) has been widely used for designing immunoassays on chips (Bange et al. 2005).

A microfluidic immunosensor promises to improve analytical performance by reducing the assay time and reagent consumption, increasing sensitivity and reliability through automation, and integrating multiple processes in a single device (Bange et al. 2005; Lim and Zhang 2006). A number of researchers have demonstrated the ability of these micro- and nano-biochips to detect the presence of bacteria in food or clinical samples using optical or electrical methods (Stokes et al. 2001; Lin et al. 2005; Li and Su 2006).

Stokes et al. (2001) demonstrated the use of a microfluidic biochip with an integrated 2-dimensional photosensor array for the detection of *E. coli.* The chip contained an array of integrated, independently operating photodiodes, along with amplifiers, discriminators, and logic circuitry on a single platform.They used cellulosic membrane as the platform to capture *E. coli* cells with an antibody on an integrated circuit (IC) biochip and then detected the

bacteria using a Cy5-labeled polyclonal antibody in a sandwich immunoassay format. They demonstrated that the biochip had a linear dynamic range of three orders of magnitude greater than that observed for conventional assays, and can detect as few as 20 *E. coli* cells.

Gold nanoparticles, which are about 40–120 nm in diameter, have been exploited in the detection of protein analytes because of their basic ability to scatter a white light to yield a monochromatic light (Thanh and Rosenzweig 2002; Lin et al. 2005). Lin et al. (2005) coated the microchannels created on the PDMS with bacterial cell lysates, which reacted with biotin-labeled polyclonal antibodies raised against *Helicobacter pylori* and *E. coli* as the primary antibodies. The nanoparticles immobilized with antibiotin antibodies were used as a secondary antibody, and the positive reaction was recorded, as the amount of light emitted, under a dark-field stereo microscope. They were able to achieve a detection limit of 10–1000 ng in a 1.5 μL chamber, which is similar to the sensitivity achieved through a conventional ELISA. However, this technology permits small volumes and less time for detection.

Huang et al. (2003) showed that the specificity of the protein biochips could be enhanced using blocking agents such as biotinylated bovine serum albumin (BSA) on a C_{18}-derivatized SiO_2 surface. They used the monoclonal antibody C11E9 (Bhunia et al. 1999) to demonstrate the specific detection of *L. monocytogenes*, and minimized the nonspecific binding by other bacteria.

Alternatively, several studies have used antibodies to enhance the capture efficiency and quantification of bacteria on microfluidic biochips (Sakamoto et al. 2005; Yang et al. 2006). Yang et al. (2006) demonstrated a coupling of immunocapture with dielectrophoresis (DEP) on a microfluidic system to concentrate and detect *L. monocytogenes* cells. The microfluidic biochip consisted of an array of interdigitated microelectrodes on a flat oxidized silicon substrate, and an array of microelectrodes was made on a PDMS cover. A biotinylated anti-*L. monocytogenes* monoclonal antibody, C11E9, was immobilized on the surface of the SiO_2, and positive DEP (at 20Vpp and 1 MHz) was applied to concentrate the bacterial cells from the flowing sample in the chamber. DEP could capture 90 % of the cells during a continuous flow of the sample at the flow rate of 0.2 μL/min. DEP-concentrated cells were captured by the immobilized antibodies on the channel surface with an efficiency of 18–27 % when cells were present at 10^1–10^3/mL.

Accelr8 is a commercial microfluidic lab-on-chip device that allows immunochemical microscopic identification of bacterial cells (http://www.accelr8.com/index.php). The product BACcelr8r is in developmental stages, in which the bacterial cells from biological samples are concentrated on the surface of a chip by applying an electronic potential and are detected by an indirect ELISA format, using specific antibodies. The cells are detected fluorescently using microscopy and image analysis.

4.5.4. Serodiagnosis

Serodiagnosis refers to the serological identification of antibodies against a particular antigen in the serum or blood of the patients. Several kits have been developed based on the principle of ELISA, latex agglutination (LA), and reverse passive latex agglutination (RPLA) for the detection of antibodies against *M. tuberculosis* and *Salmonella typhi* (Perkins et al. 2003; Imaz et al. 2004; Jesudason and Sivakumar 2006). Typically, the ELISA plates or the latex particles are coated with the antigen and when reacted with the serum, the specific antibodies bind to the antigen and give an agglutination reaction.

Imaz et al. (2004) evaluated four different commercial kits (Omega Diagnostics Ltd., Alloa, Scotland) for the serological diagnosis of pulmonary tuberculosis (TB). The Panthozyme-TB Complex Plus detects IgG antibodies against recombinant forms of two antigens from the *M. tuberculosis* complex: r38 kDa (PstS-1, PhoS, antigen 5, antigen 78) and r16 kDa, a member of the alpha-crystalline family of low-molecular-weight heat shock proteins. The other tests, Pathozyme-Myco G (Myco G), Myco M, and Myco A, which utilize the r38 kDa antigen

and the lipoarabinomannan (LAM—a common lipoglycan component of the mycobacterial cell wall), detect human IgG, IgM, or IgA, respectively, in the serum of individuals infected with the tuberculosis bacilli (Imaz et al. 2004).

Typhidot® (Malaysian Biodiagnostics Research Sdn. Bhd, Selangor Darul Ehsan, Malaysia) uses an ELISA format, which detects IgG and IgM antibodies against *S. typhi* in the blood (Jesudason and Sivakumar 2006). Olsen et al. (2004) evaluated three different kits for *S. typhi*, Multi-Test Dip-S-Ticks, TyphiDot, and TUBEX, to detect immunoglobulin G (IgG), IgG and IgM, and IgM, respectively, and compared the results with those of the Widal test using the commercial kit from Bio-Rad, CA (Sanofi qualitative agglutination test kit). The Widal test is done as a tube agglutination test to detect the H and O antigens present on the surface of the typhoid bacteria. However, Olsen et al. (2004) observed that the Widal test was insensitive and displayed interoperator variability; and that the two rapid kits, TyphiDot and TUBEX, demonstrated promising results.

5. Recent Developments in Immunoassays

Although the technologies described above yield sensitive and rapid detection of bacteria and their toxins, the need for portability in the instrumentation and even faster results, along with increased specificity and sensitivity, has led to the design of more sophisticated technologies. Here we review some of the novel immunobased assays which show promise in bacterial detection.

5.1. Protein/Antibody Microarrays

Microarrays were originally developed as a tool for genotyping and gene expression analyses. The success of DNA and mRNA microarrays led to more promising ventures into targeting proteins, either toxins or cells using an antibody array format. Unlike the nucleic acid arrays, in which the lysis of cells to release the DNA or RNA is required, protein microarrays have the advantage of detecting cells or toxins in one step. Although the protein microarray is a commercial success in medicine, it still remains in its infancy in respect to bacterial detection.

In recent years, patterned protein (Cai et al. 2005; Steller et al. 2005) or antibody microarrays (Morhard et al. 2000; Howell et al. 2003; Gehring et al. 2006) have been developed for the identification and detection of different bacteria, viz., *Salmonella* (Cai et al. 2005), *E. coli* O157:H7 (Gehring et al. 2006), *Renibacterium salmoninarum* (Howell et al. 2003), and *Neisseria meningitidis* (Steller et al. 2005).

In a protein microarray, the antigens or the recombinant proteins are spotted onto a coated glass slide using an automated spotter and then reacted with fluorescent-labeled antibodies, and the spots are read in the microarray reader. In an antibody microarray, antibodies are patterned, using microcontact printing or soft lithography, onto the substrates (such as glass or silicon), without the loss of biological activity (Inerowicz et al. 2002; Howell et al. 2003; Pavlickova et al. 2004). Binding of antigen to the captured antibodies is detected using fluorescent-labeled antibodies. Phage display technology has been used for antibody production against large arrays of antigens for the purpose of using them on a microarray platform (Pavlickova et al. 2004). Most commonly, different substrates like glass (Howell et al. 2003), gold (Morhard et al. 2000), and silicon (Nijdam et al. 2007) have been used to create patterns for bacteria.

Cai et al. (2005) developed antibody microarrays for serotyping of *Salmonella enterica* strains. With their model, they were able to identify 86 target strains, partially identify 30 more, and differentiate 73 nontarget strains from the targets. Gehring et al. (2006) developed antibody microarrays for detection of *E. coli* O157:H7 in a sandwich format. A linear signal was obtained when cell concentrations were between 3×10^6 and 9×10^7 cells/mL.

Microarrays were successfully used for the simultaneous detection of a multitude of different biowarfare agents: *Staphylococcus* enterotoxin B, ricin, Venezuelan equine encephalitis virus, St. Louis encephalitis virus, West Nile virus, yellow fever virus, orthopox virus, *Francisella tularensis, Yersinia pestis, Brucella melitensis*, *Burkholderia mallei*, and *Escherichia coli* EHEC O157:H7. A chip was developed for the ArrayTube platform, with classical sandwich ELISA and streptavidin HRP-based detection, which could be accomplished within 1–1.5 h (Huelseweh et al. 2006).

The most common problems associated with fluorescence or direct labeling are lower sensitivity and impaired protein solubility. Thus label-free detection systems like scattering and mass spectrometry are considered alternative methods of choice for the interrogation of antigens. Chapter 26 describes the protein microarray technologies for detection and identification of bacterial analytes.

5.2. Mass Spectrometric Immunodetection

Matrix-assisted laser desorption/ionization (MALDI) is the most common technique used for mass spectrometric analysis of proteins using laser pulses. MALDI coupled with time-of-flight (TOF) measures the mass of intact peptides. In recent years, MALDI-TOF has been applied to protein biochips, to study the interaction of recombinant antibody-antigen, and for direct detection of bacterial cells (Pavlickova et al. 2004). MALDI-TOF has been also shown to be an effective and rapid method for identification of whole bacterial cells (Madonna et al. 2001, 2003) and toxins (Nedelkov and Nelson 2003). *Salmonella* was detected at a concentration of $\sim 10^5$ cells/mL within 1 h using MALDI-TOF (Madonna et al. 2001). In order to increase the sensitivity of detection, IMS and phage-typing were integrated into MALDI-TOF, to rapidly and specifically identify very low numbers of bacterial cells (Madonna et al. 2003). Paramagnetic beads coated with anti-*E. coli* polyclonal antibodies were used to isolate and concentrate *E. coli* from a complex mixture. Captured cells were then infected with a specific lytic bacteriophage (MS2 phage), which produced a large number of phage progeny. Using MALDI-TOF MS, the capsid protein of the MS2 phage was detected, which correlated with the presence of the target bacteria. This integrated approach improved the detection limit twofold, to 10^4 cells/mL.

SPR in combination with MALDI-TOF was found to be highly sensitive for the detection of bacterial lysates and toxins, and was able to detect <1 ng/mL of staphylococcal enterotoxin B, corresponding to the mass analysis of $\sim$500 amol of SEB (Nedelkov and Nelson 2003). Chapter 36 describes the use of mass spectrometric techniques for bacterial detection.

5.3. μSERS Biochip Technology

μSERS is a novel label-free detection technology which uses surface-enhanced Raman scattering (SERS) microscopy. The chip comprises pixels of capture antibodies on a SERS active metal surface, which selectively binds the target bacteria in a sample. Using the Raman microscope, the SERS fingerprints are collected from the pixels on the chip. At each pixel, the bacteria are identified in the spectral domain by matching the unique SERS fingerprint against the library of known fingerprints (Grow et al. 2003). An array of microorganisms consisting of *Listeria*, *Legionella*, *Bacillus* spores, and *Cryptosporidium* oocysts were identified and differentiated in a mixed sample (Grow et al. 2003). See chapter 20 for more information about μSERS.

6. Limitations and Challenges

6.1. Specificity and Sensitivity

Universal questions about the immunoassays have been about how specific and sensitive the reaction is. The specificity of any immunoassay-based detection depends on the specificity

of the antibodies used. Monoclonal antibodies provide a high degree of specificity because they are specific for an epitope and the antibody-producing clone is selected to provide a limitless supply of homogeneous antibody. However, if that epitope is shared by pathogenic as well as nonpathogenic microorganisms, the assay may not be specific; thus detailed knowledge about an antibody is a prerequisite before it can be incorporated into an assay. On the other hand, polyclonal antibodies could be the source of nonspecific reactions in an immunoassay, since they are comprised of antibodies that react with various epitopes on the same antigen or multiple antigens, and the chances of epitope sharing among different microorganisms are very high. In addition, the animals that are used for antibody development may be the source of undesirable/ unintended antibodies, because they may harbor background antibodies against common microorganisms. In a survey, Lathrop et al. (2006) observed that more than 50 % of the pre-immune sera from 19 different rabbits cross-reacted with 14 different bacterial antigens, suggesting that the presence of cross-reactive antibodies in pre-immune serum is a common problem in the production of specific antibodies. Raising a highly specific antibody is still a challenge, and thus adopting improved methods in the selection and screening of antibodies using recombinant technologies would enhance the specificity of the immunoassays. Conversely, highly specific antibodies can also result in a number of false-negatives. Thus a thorough analysis of the antibodies and evaluation of the assay should be done, if the target is to detect all the pathogenic serotypes and strains in a particular bacterial species.

The sensitivity of the immunoassay still remains a limitation. Although it has shown to be highly sensitive in detecting pico- and femtogram quantities of bacterial toxins, the detection of whole cells below 1,000 cells/mL is still a challenge. With the USDA/FSIS-imposed regulations on "zero tolerance" for many bacterial pathogens (*Clostridium perfringens, Listeria monocytogenes, Salmonella*, and *E. coli*) in various foods, the demand for sensitivity in detection technologies is elevated. Thus, a short enrichment of the samples before use in immunoassays has become a common practice for sensitive detection of bacterial pathogens. Additionally, automated instruments, such as those developed for microtitre plates or biochips, may be a potential source of errors. Bak et al. (2006) noticed significant errors in his assay instrument, even when several data points were collected from over a short period of time.

6.2. Effect of Physical and Chemical Stresses on the Expression Profile of Antigens in Bacteria

6.2.1. Effect of Media Composition on the Expression of Proteins in Bacteria

Antibody-based immunoassays are rapid, but most commercially available assays to detect bacterial species are performed after the test samples are enriched for 24–48 h (Hitchins 1998; Donnelly 2002). Conventionally, USDA/FSIS procedure recommends enrichment of the food sample for isolation and detection using specific selective enrichment broths/media. However, these media may or may not support the expression of the proteins that are used as targets for immunoassays, since each component in the media can interfere with the regulation of gene expression in bacterial pathogens.

Selective enrichment broths have previously been shown to severely affect the expression of anti-*Listeria* monoclonal and polyclonal antibodies (Geng et al. 2003, 2006b; Nannapaneni et al. 1998a, 1998b). Studies in our lab have demonstrated that *L. monocytogenes* expression is differentially regulated under different media and stress conditions (Jaradat and Bhunia 2002; Geng et al. 2003; Lathrop 2005; Geng et al. 2006b; Hahm and Bhunia 2006; Lathrop et al. 2008). Virulence factors like Internalin A, Internalin B, Listeriolysin O, actin polymerization protein, and phospholipases are obvious target antigens for antibody development (Bhunia 1997) because they are associated with the pathogenicity of *L. monocytogenes*.

Glucose, through its active metabolism by *L. monocytogenes*, may be indirectly responsible for the repression of many pathogenic factors by lowering the pH of the media. Glucose-induced, low pH-mediated suppression of the virulence protein, LLO (Milenbachs et al. 1997) and expression of LAP (Jaradat and Bhunia 2002) have been reported earlier. Similar observations have been made by earlier researchers in *E. coli* strains, in which the carbohydrates and pH have resulted in differential expression of various proteins in *E. coli* (Stancik et al. 2002; Vanmaele and Armstrong 1997).

Differential protein expression among strains of *L. monocytogenes* under different growth and environmental conditions has been extensively studied (Sokolovic et al. 1996; Geng et al. 2003; Milohanic et al. 2003; Chatterjee et al. 2006). Geng et al. (2006b) showed that commonly used bacteriological media for *Listeria* isolation and recovery, such as brain-heart infusion broth (BHI), buffered *Listeria* enrichment broth (BLEB), *Listeria* repair broth (LRB), University of Vermont medium (UVM), and Fraser broth (FB) have affected the stress recovery and immunodetection of *L. monocytogenes*. Indirect ELISA using a monoclonal antibody, C11E9, and a polyclonal anti-*Listeria* PAb revealed that BLEB and LRB favorably supported increased expression of antigens and proved to be superior to UVM and FB for the immunodetection of stressed *L. monocytogenes* cells. An earlier study by Sokolovic et al. (1996) also demonstrated that the ActA expression was significantly higher in *L. monocytogenes* serogroup 4 cells grown in a mammalian cell culture medium, MEM (minimum essential medium), than in the nutrient-rich BHI broth. Interestingly, other virulence genes, *prfA*, *plcA*, and *hly*, behaved similarly in BHI (Sokolovic et al. 1996). According to Marr et al. (2006), *L. monocytogenes* growing in the presence of glucose showed that higher PrfA expression resulted in lower carbohydrate intake and slower growth. This study indicates that overexpression of regulatory genes hinders the expression of metabolic genes in *L. monocytogenes*. Potassium nitrate and anaerobiosis has been shown to significantly affect the expression of genes in *Pseudomonas aeruginosa* (Filiatrault et al. 2005; Wu et al. 2005).

6.2.2. Effect of Stress on the Expression of Proteins in Bacteria

Bacteria are exposed to various stress conditions in the host cell, the environment, or during processing and storage. In response to the changes in the environment, the physiological response changes, so that the bacteria are able to survive and cope under the new conditions. These bacteria respond to stress conditions by activating small or large groups of genes under the control of common regulatory proteins. Stress conditions result in the accumulation of these regulatory proteins; and the subsequent transcription of many genes allows cells to cope with specific stress situations, conferring stress tolerance and survival (Chung et al. 2006). Studies have shown that environmental or growth factors such as nutrient concentration, acidity, temperature, carbon sources, and osmotic and oxidative stresses, could down-regulate certain antigen expression and thus their detection (Jaradat and Bhunia 2002; Milohanic et al. 2003; Shetron-Rama et al. 2003; Lemes-Marques and Yano 2004; Sue et al. 2004).

E. coli O157:H7, *Salmonella Enteritidis*, and *L. monocytogenes* were subjected to various stress conditions and were tested by ELISA for their reaction to specific antibodies (Hahm and Bhunia 2006). In general, the study demonstrated that under all stress conditions, including temperature (4° and 45°C), NaCl (5.5 %), oxidative stress (15 $mmol^{-1}$ H_2O_2), acidic pH (5.5), and ethanol (5 %) for 3 h (short-term stress) or for 5 days (long-term stress), the bacteria differentially expressed the antibody reactive antigens. Reaction with *Listeria* PAb showed an up-regulation with most stresses; whereas anti-*E. coli* and anti-*Salmonella* antibodies demonstrated reduced expression levels. Overall, these stress conditions caused an 18–59 % reduction in immunoreaction (Hahm and Bhunia 2006). Likewise, Geng et al. (2003) observed a similar reduced reaction of monoclonal antibodies C11E9 and EM-7G1 to stress-exposed

L. monocytogenes in ELISA and Western immunoblot, due to the reduced surface expression of antibody reactive proteins. Banada et al. (2006) evaluated the virulence protein (InlB and ActA) expression profile in *L. monocytogenes* using specific antibodies after growth in a low conductive growth medium, which was designed for microfluidic biochip applications. This medium, though a minimal medium, did not affect the expression of those proteins, thus suggesting a favorable application on protein biochips for antibody-mediated capture and growth-based sensitive detection of this pathogen (Yang et al. 2006).

A 55 kDa protein of *Salmonella* serovar Typhi was expressed at high intensity when the cells were grown under inorganic acid stress (pH 5.5, 5.0, and 4.5), organic acid stress (2, 4, and 6 % of 100-mM stock of acetate, propionate, and butyrate), and heat stress (42°, 45°, and 50°C for 30 min). However, there was no or reduced expression of the same protein when exposed to the same temperatures for less than 30 min (Chander et al. 2004).

During stress exposure, *E. coli* and *Vibrio cholerae* can convert into a viable but nonculturable (VBNC) state or a dormant state. Generally, it is difficult to culture and detect those cells. Desnues et al. (2003) showed that protein expression in *E. coli* under a VBNC state results in increased and nonreversible oxidative damage, which affects various bacterial compartments and proteins. They further showed that the VBNC state is due to stochastic deterioration, rather than an adaptive program, and they pinpoint oxidation management as the "Achilles' heel" of these cells.

These studies suggest that not only antibodies, but also the bacterial physiological status, their response to stress, the growth media, selective enrichment broths, and media compositions can substantially affect the antibody-based detection of pathogens. Therefore, while designing an immunoassay, it is essential to understand the antibody-reactive protein expression profile in a pathogen during exposure to stress or selective enrichment media in order to achieve an optimal immune reaction. This is particularly important in immunosensor applications in which the system is highly sensitive and a slight decline in the immune reaction may lead to a false-negative result.

7. Conclusions and Future Perspectives

An antibody is one of the most important molecules used in biorecognition and detection. Its use continues to grow as we introduce more novel detection technologies, such as those that are bionanosensor-based. There is always a great demand for highly specific and good quality antibodies for assay development. Conventional animal-based antibody production strategies are still useful and valuable, but recombinant and phage display technologies have been shown to be promising in developing a new class of antibodies. In order to obtain the optimum signal from an antibody reaction, one must understand the microorganism's response to environmental stress, the growth media, and its physiological state while designing an immunoassay. When these conditions are thoroughly understood, one can expect improvement in specificity and sensitivity. As we continue to improve our assay systems, there is great demand for a portable device for on-site application. Furthermore, the assay should be inexpensive, easy to read/interpret, automated, and produce a minimum of false results. With the rapid advancement in microfluidics and nanotechnology, the adaptation of the lab-on-chip concept in immunoassays will soon be a reality for commercial use. Bioanalytical immunosensors for real-time monitoring of pathogenic bacterial presence in the food and pharmaceutical industries will greatly reduce the contamination risks to consumers and the call-back risks to the industries. On-field handheld immunodiagnostic instruments will be best suited for farmers and for soldiers to combat the possible spread of pathogenic and bioterrorism-related microbial agents.

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