Chapter 8
Molecular Strategies: Detection of Foodborne Bacterial Pathogens

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Abstract Conventional methods of pathogen identification have often depended on the identification of disease symptoms, isolation, and culturing of the organisms, and identification by morphology and biochemical tests. The major limitations of these culture-based morphological approaches, however, are the reliance on the ability of the organism to be cultured, the time-consuming nature, and requirement of extensive taxonomic expertise. The use of molecular methods can circumvent many of these shortcomings. Accordingly, there have been significant developments in the area of molecular detection of bacterial pathogens in the last 3 decades. We report here a brief overview of the molecular detection methods applicable to microbes from food.

8.1 Introduction

Diseases caused by contaminated food constitute one of the most widespread public health problems and are an important cause of reduced economic productivity in both developed and developing countries (Anon 2005). Every year approximately 76 million foodborne illnesses are reported in United States of which 325,000 become hospitalized and approximately 5,000 die. The costs in terms of medical care and lost productivity are estimated at between $6.5 and $34.9 billion (Buzby and Roberts 1997; Mead et al. 1999). The number of people in Canada who contract foodborne illness is estimated as 2.2 million annually (Anon 2005).

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I. Ahmad et al. (eds.), *Microbes and Microbial Technology: Agricultural and Environmental Applications*, DOI 10.1007/978-1-4419-7931-5_8,

Generally, due to the presence of very low numbers (<100 CFU g^{-1}) in the midst of millions of other bacteria it is difficult to detect specific bacterial food pathogens. These microbes seem hidden among a background of indigenous microflora, and substances within the foods themselves may hinder detection. There is also the difficulty of demonstrating that the strains recovered from a food sample are, indeed, pathogenic to humans (Sockett 1991). Rapid and simple detection of pathogenic organisms facilitate precautionary measures to maintain healthy food (Feng 1992).

One of the major limitations to research in microbial communities, and consequently the detection of bacteria in the environment, is the inability to isolate and grow in culture the vast majority of bacteria. There continues to remain a discrepancy between cell numbers obtained from direct and viable counts to the numbers actually occurring in vivo (Keer and Birch 2003). Furthermore, some bacteria have been shown to be unculturable but retain their viability after exposure to the environment and have thus been termed "non-culturable but viable" (NCBV) (Oliver 2005). This phenomenon complicates both the detection and enumeration of key pathogenic organisms. A number of species are described as entering the VBNC state and include a large number of human pathogens, including *Campylobacter* spp., *Escherichia coli* (including EHEC strains), *Listeria monocytogenes*, *Salmonella* and *Shigella* spp. and *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Oliver 2005). The genera of *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *E. coli*, *L. monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Shigella* spp., and *Yersinia enterocolitica* comprise primarily foodborne bacterial pathogens (Kumar et al. 2002).

The characterization and detection of foodborne pathogens continue to rely on conventional culturing techniques, which include homogenization, enrichment in nonselective and selective media followed by plating in differential agar media to isolate pure cultures. Finally, phenotypic and genotypic characterization takes 3–4 days to confirm the results. Biochemical and immunological methods for the detection require substantial amounts of pure culture, whereas DNA-based methods can be performed with mixed cultures or community DNA. The final detection stage requires gel electrophoresis after polymerase chain reaction (PCR) steps and further sequencing of the amplified product, thus increasing the time and complexity of detection (Prasad and Vidyarthi 2009).

Currently, diagnostic laboratories are adapting molecular methods for routine detection of pathogens. With advances in molecular biology and biosystematics, the techniques available have evolved significantly over the past decade. In addition to conventional PCR, other technologically advanced methodologies, such as second generation PCR (real-time PCR) and microarrays which allow unlimited multiplexing capability, have the potential to bring pathogen detection to a new and improved level of efficiency and reliability (Mumford et al. 2006).

The rapid methods employed for the identification of foodborne microorganisms are discussed below.

8.2 Molecular Typing Methods for the Detection of Bacterial Pathogens

Conventional methods of pathogen identification have often depended on the identification of disease symptoms isolation and culturing of organisms, and identification by morphology and biochemical tests. The major limitations of these culture-based morphological approaches are the reliance on the ability of the organism to be cultured, the time-consuming nature of the lab analyses, and the requirement of extensive taxonomic expertise. The use of molecular methods can circumvent many of these shortcomings. DNA-based technologies such as the PCR have revolutionized molecular diagnostics and microbiological investigations.

8.2.1 PCR-Based Detection Methods

The PCR is a technique for in vitro amplification of specific segments of DNA by using a pair of primers (Nguyen et al. 1994). A million-fold amplification of a particular region can often be realized, allowing, among numerous other uses, the detection of specific genes within samples. PCR can be used to amplify genes specific to taxonomic groups of bacteria and also to detect genes involved in the virulence of foodborne bacteria (Finlay and Falkow 1988; Bej et al. 1994).

In our laboratory, for the amplification of *hly* gene (234 bp) a PCR technique was standardized (unpublished data). The reaction mixture was optimized with master mix as follows: 2.5 µl of 10× PCR buffer (20 mM Tris–HCl, pH 8.0 at 25°C, 100 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) 50% glycerol, 0.5% Tween 20, and 0.5% Nonidet-P40), 1.0 µl of dNTP mix (25 mM), 1.0 µl of both forward and reverse primers (15 pmol), 0.2 µl of Taq DNA polymerase enzyme (5 U/µl), and 2 µl of DNA as template. Nuclease-free water was added to make the final volume 25 µl. PCR tubes containing reaction mixture were centrifuged and placed in a thermocycler. Cycling conditions included an initial denaturation step at 95°C for 5 min followed by 40 subsequent cycles consisting of heat denaturation at 95°C for 30 s, primer annealing examined at 53°C, 54°C, and 55°C, respectively for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min to ensure synthesis of all strands. The PCR products were electrophoresed on 1.5% agarose gel which showed a clear band at 234 bp (Fig. 8.1)

Several variations of the standard PCR have recently appeared and have contributed to the development of more sensitive detection methods. These are discussed below.

8.2.1.1 Multiplex PCR and Real-Time PCR

Multiplex PCR (mPCR) and real-time PCR (rPCR) are proving to be the most popular methods for microbial identification. The mPCR allows several targets to

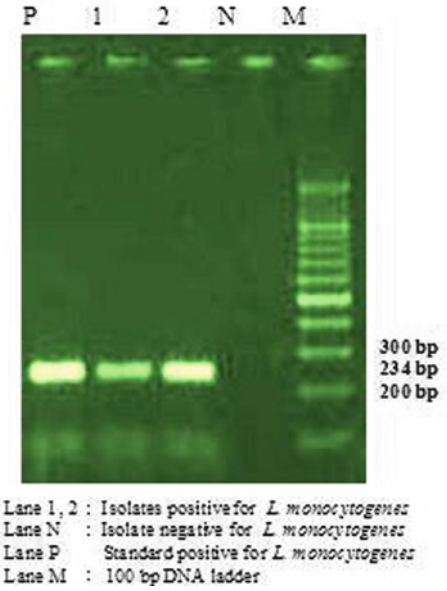

Fig. 8.1 *LL0* gene based detected of *L.* Monocytogenes using PCR

be co-amplified in one PCR by combining or "multiplexing" primer pairs (Newton and Graham 1997). Duffy et al. (2001) described PCR-based detection of foodborne pathogens including *L. monocytogenes*, *Salmonella* sp., *C.jejuni*, and *E. coli* O157:H7. A multiplex PCR protocol was reported for 13 species of foodborne pathogens (Cerniglia et al. 1997). Another protocol was reported by Park et al. (2006) for the simultaneous detection of *E. coli* O157:H7, *Salmonella* spp., *S. aureus*, and *L. monocytogenes* from kimchi (a Korean food preparation).

rPCR allows reactions to be characterized by the time amplification of the PCR product, which can be first detected by the use of a fluorogenic probe (Livak 2000). In recent years, there has been significant progress in the development of rPCR aimed at the quantitation of bacterial load in various food matrices. Its principle is based on the detection of a fluorescent signal, which is proportional to the number of amplicons in the tested sample (Higuchi et al. 1992, 1993; Lee et al. 1993; Livak et al. 1995). Nowadays, rPCR-based detection is frequently used for foodborne bacterial pathogen detection (Malorny et al. 2004; Poltronieri et al. 2009; Life Technologies 2010).

In recent years, PCR has become important as a technique for the detection and identification of bacteria. The main reason for its popularity is that DNA from a single bacterial cell can be amplified in about 1 h, which is significantly more rapid than times necessary for the methods described previously. However, the method can also amplify dead cells, and this makes data interpretation complex and is an issue that must be addressed, as it has long-term implications from legal perspectives. So care must be taken in designing experiments. Some investigators have detailed the PCR-based detection protocol for some foodborne bacterial pathogens (Islam et al. 1993; Keer and Birch 2003).

Diagnostic PCR has been greatly improved by the introduction of second-generation PCR, known as real-time PCR, where closed-tube fluorescence detection and quantification during PCR amplification (in real time) occurs, eliminating the need for laborious post-PCR sample processing steps which greatly reduces the risk of carryover contamination. Using real-time PCR it is possible not only to detect the presence or absence of the target pathogen, but also to quantify the amount present in the sample. Enumerating the pathogen upon detection is crucial for estimating the potential risks with respect to disease development and provides a useful basis for disease management decisions.

In another PCR assay targeting the 3′-prime end of the eae gene (*E. coli* attaching and effacing) of *E. coli* O157:H7 (no RT-PCR) was found to be specific, with sensitivity being 1 pg DNA or 10^3 CFU PCR per reaction (Uyttendaele et al. 1999). Furthermore, studies were carried out to determine the effect of the food matrix and sample preparation method on PCR detection of nonviable cells using heat-killed bacteria in ground beef. Sample preparation methods included centrifugation, buoyant density centrifugation (BDC), immunomagnetic separation (IMS), chelex extraction, and swabbing. It was found that IMS was the only method which did not produce false positive results, provided the number of cells was below 10^8 CFU g^{-1}. Above this number, IMS produced a false positive, which is a severe limitation of this approach.

8.2.1.2 Random Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD) technique was first employed by Williams et al. (1990) to examine human DNA samples from anonymous individuals. Earlier, several authors reported on the application of the RAPD technique for the analysis of microbial DNA (Wagner et al. 1996; Byun et al. 2001). The method uses random primers (Williams et al. 1990) and can be applied to any species without requiring any information about the nucleotide sequence. The amplified products from this analysis exhibit polymorphism and thus can be used as genetic markers. The RAPD band, however, does not allow distinction between hetero- and homozygous states. The fragments are scored as dominant Mendelian elements, and the protocols are relatively simple.

Hamza et al. (2009) described a RAPD protocol for lactic acid bacteria identification from traditional Sudanese sour milk. The band pattern generated in the analysis represents genome characterization of a specific bacterial strain (Welsh and McClelland 1990). In addition, the method has the potential for analyzing phylogenetic relationships among closely related species (Williams et al. 1990) and can distinguish between strains within a species.

8.2.1.3 Restriction Fragment Length Polymorphism

The restriction fragment length polymorphism (RFLP) procedure involves isolation of DNA, digestion with restriction endonucleases, size fractionation of the resulting

DNA fragments by electrophoresis, DNA transfer from the electrophoresis gel matrix to a nylon membrane, preparation of radiolabeled and chemiluminescent probes, and hybridization to membrane-bound DNA (Olive and Bean 1999).

The probes can be labeled with detectable moieties, such as radioactive isotopes, enzyme-colorimetric, or enzyme chemiluminescent substrates (Arbeit 1995; Olive and Bean 1999). Due to the species and strain differences in the location of the restriction enzyme sites and with the specificity of the probe, the resulting fingerprint is simplified and therefore easier to analyze. The rRNA probe is more applicable for a wide variety of bacteria than other probes that are more species or strain-specific. The use of this probe for characterization is called ribotyping where restriction enzyme digestion and Southern blot hybridization are used together for analysis. Since the ribosomal operons in bacteria are organized into 16S, 23S, and 5S rRNA and are often separated by noncoding spacer DNA (Towner and Cockayne 1993), the probe can be either one of the rRNA genes or a mixture or parts of the rRNA genes and the spacer sequences. Hybridization patterns differ depending on the probe used (Saunders et al. 1990). Labeled probes containing *E. coli* 23S, 16S, and 5S rRNA sequences are most often used for ribotyping (Bingen et al. 1994).

Ribotyping has been shown to be advantageous in identifying strains, such as *Carnobacterium* species (*C. divergens*, *C. piscicola*, *C. gallinarum*, and *C. mobile*) which are difficult to type with classical phenotypic methods. Kabadjova et al. (2002) established a rapid PCR-RFLP-based identification scheme for four closely related *Carnobacterium* species (C. *divergens*, *C. piscicola*, *C. gallinarum*, and *C. mobile*) that are of interest to the food industry. Using the rapid PCR-RFLP scheme, three isolates previously incorrectly identified as *C. divergens* (INRA 508, INRA 586, and INRA 515) were reclassified as *C. piscicola*. Similarly, four isolates identified as *C. piscicola* (INRA 545, INRA 572, INRA 722, and ENSAIA 13) were reclassified as *C. divergens* based on the patterns obtained by the 16S–23S ISR-RFLP methods.

Manceau and Horvais (1997) used RFLP analysis of rRNA operons to assess phylogenetic diversity among strains of *Pseudomonas syringae pv. tomato*. They successfully established the close relationships existing between *P. syringae* and *P. viridiflava* species.

8.2.1.4 Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) analysis was developed by a team led by Marc Zabeau at Keygene N.V., Wageningen, The Netherlands (Zabeau and Vos 1993; Vos et al. 1995). Vos et al. (1995) described the principle of the AFLP fingerprinting technique. AFLP is a variation of RAPD and is able to detect restriction site polymorphisms without prior sequence knowledge using PCR amplification for the detection of restriction fragments (Zabeau and Vos 1993; Vos et al. 1995; Blears et al. 1998; Mueller and Wolfenbarger 1999).

Basically, AFLP is a genome fingerprinting technique based on the PCR amplification of only certain fragments that have been the result of restriction digestion of the whole genome (Vos et al. 1995; Lin et al. 1996; Olive and Bean 1999). The basic procedure includes enzyme digestion by two restriction enzymes that yield DNA fragments with two different types of sticky ends. To these ends, adapters are ligated to form templates for the PCR. The selective amplification reaction is performed using two different primers containing the same sequence as the adapters, but extended to include one or more selec tive bases adjacent to the restriction site of the primer. Only fragments that are a complete match are amplified. This technique results in about 30–40 DNA fragments, some of which are species-specific while others are strain-specific (Janssen et al. 1996; Koeleman et al. 1998; Jackson et al. 1999; Jureen et al. 2004; Melles et al. 2007).

AFLP analysis is one of the most robust multiple-locus fingerprinting techniques among genetic marker techniques that have been evaluated for genotypic characterization (Koeleman et al. 1997). Restrepo et al. (1999) used AFLP to characterize the genetic relationships between *Xanthomonas axonopodis pv. Manihotis* strains. The study of Janssen et al. (1996) revealed extensive applicability of AFLP in bacterial taxonomy through comparison of newly obtained data with results previously obtained by well-established genotypic and chemotaxonomic methods such as DNA hybridization and cellular fatty acid analysis.

8.2.2 Pulsed-Field Gel Electrophoresis

Schwartz and Cantor (1984) described the pulsed-field gel electrophoresis (PFGE) method to produce a molecular karyotype from the chromosomal DNA of yeast *Saccharomyces cerevisiae*. PFGE is based on the digestion of chromosomal DNA by using rare cutting enzymes. The use of these enzymes minimizes the total amount of DNA fragments.

This method is capable of separating large DNA molecules (up to 2,000 kb) by applying alternately pulsed electric fields established perpendicular to each other and of which one is inhomogeneous. The basic principle of PFGE is the use of successive alternating electric fields which allow the DNA molecules to continuously change their direction of migration. The large DNA molecule will uncoil and elongate parallel to an electric field such that it can enter a pore opening in the agarose. When the electric field is turned off and a new electric field is applied perpendicular to the opened DNA, the molecule must re-orient itself to enter a new opening. The pulse time (ramping) and electron force (gradient) are constantly increased to achieve better separation of all sizes of DNA fragments (Towner and Cockayne 1993).

According to Arbeit (1995), PFGE is highly discriminatory and superior to many other microbial typing methods. The method is capable of differentiation between species and strains involved in foodborne outbreaks and therefore has been investigated for use in epidemiological studies such as with *Campylobacter*

coli, *C. jejuni* (Yan et al. 1991), *L. monocytogenes* (Brosch et al. 1991), and *S. aureus* (Schlichting et al. 1993).

In 1996, PFGE became the standard procedure for bacterial foodborne disease outbreak analysis (Swaminathan et al. 2001) due to its discriminatory capabilities (Gerner-Smidt et al. 2006). Uniform guidelines for performing PFGE and interpretation of the data have been established to confirm reproducibility among laboratories (Tenover et al. 1995). Therefore, PFGE is considered the "gold standard" for molecular-based studies. It has become the preferred subtyping method for networks that have been created within the United States (PulseNet) and Europe (PulseNet Europe) for surveillance and for collection of PFGE fingerprints of bacteria related to foodborne infections (Swaminathan et al. 2001; Rodríguez-Lázaro et al. 2007). Currently, PulseNet USA has standardized PFGE protocols for *Shiga toxigenic E. coli* O157, *S. enterica*, *Shigella* spp., *L. monocytogenes*, thermotolerant *Campylobacter* spp., and *V. cholerae* and *S. enterica sv. Braenderup strain H9812* digested with *Xba*I as the universal standard (Gerner-Smidt et al. 2006).

8.2.3 Biosensors

A biosensor is defined as a device or instrument comprising a biological sensing element coupled to a transducer. The biological sensing elements might include enzymes, organelles, antibodies, whole cells, DNA, and tissue. Transducers include electrochemical, calorimetric, optical, acoustical, or mechanical types (Richter 1993).

Microfabrication technology has enabled the development of electrochemical DNA biosensors with the capacity for sensitive and sequence-specific detection of nucleic acids. The ability of electrochemical sensors to directly identify nucleic acids in complex mixtures is a significant advantage over approaches such as PCR that require target purification and amplification. Application of DNA sensor technology to infectious diseases has the potential for recognition of pathogen-specific signature sequences in biological fluids (Liao et al. 2007).

Immobilization of a DNA probe on the desired substrate is the most crucial step in developing the electrochemical biosensor because sensitivity, specificity, and reproducibility are significantly affected by this step. For effective binding of DNA to its substrate, the terminus of the DNA or the surface of the substrate must be functionalized. Affinity binding of streptavidin and biotin has been successfully used for immobilization of DNA probes. Gold substrates are also gathering special attention due to their covalent attachment with thiolated DNA. This technology has a special interest in the search for rapid, portable, and low-cost testing systems. Electrochemical biosensors have been successfully used to detect *E. coli* 0157:H7 DNA combined with PCR (Berganza et al. 2007). A biosensor combined with gold nanoparticles (GNPs) has been used for the rapid detection of food pathogens (Leonard et al. 2003). Nanometer-sized gold particles have been used for the detection of specific DNA sequences (Daniel and Astruc 2004). As functionalized chemistry is not popular because of costs involved, an approach was proposed to use

nonfunctionalized GNP for the detection of dsDNA and ssDNA (Huixiang and Rothberg 2004; Sangchul et al. 2009). In this method, citrate-coated GNPs have a characteristic red color in the colloidal state. The aggregation of GNPs can be readily induced by the addition of salts resulting in a purple color. The difference in color is visualized with the unaided eye. The negatively charged GNP has an electrostatic interaction with ssDNA which can uncoil so that its hydrophilic negatively charged phosphate backbone is exposed to aqueous solution and DNA bases interact with the GNP surface by Vander Waals forces. These interactions add negative charge to GNPs and enhance their repulsion. Such properties have been exploited to design a biosensor which can detect a PCR product directly in the same tube within minutes.

Major improvements in signal intensity of a biosensor have been achieved, contributing significantly toward our goal of developing a microfluidics-based "lab-on-a-chip" electrochemical sensor assay for the detection of bacterial pathogens, including *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, and *Enterococcus faecalis* (Liao et al. 2007). An evanescent (i.e., quickly fading) wave fiber optic biosensor was used to detect the bacteria in 10 and 25 g ground beef samples. The biosensor uses a 635-nm laser diode to direct light onto optical fiber probes, which generates the evanescent wave. Fluorescent molecules within the evanescent field are excited and a portion of the emission recouples into the fiber probe. A photodiode detects and quantifies the fluorescent signal. A sandwich immunoassay was utilized, which allowed the detection of 9.0×10^3 CFU g^{-1} for 25 g samples and 5.2×10^2 CFU g^{-1} for the 10-g sample. No false positives were obtained with results obtained 25 min after sample processing (Demarco and Lim 2002).

8.2.4 Microarrays

The DNA microarray technology was originally designed to study gene expression and generate single nucleotide polymorphism (SNP) profiles. Currently, it serves as a diagnostic technology for emerging pathogens. Microarray technology offers a platform for unlimited multiplexing capability. Thousands of specific DNA or RNA sequences can be detected simultaneously on a small glass or silica slide measuring about 1–2 cm^2 (Aitman 2001) using microarray technology.

DNA microarrays consist of a solid surface (glass, silicon, nylon substrates) to which a large number of probes, DNA fragments, or oligonucleotides are immobilized that will hybridize to fluorescently labeled target DNA from the sample (Call 2005). The target can be genomic DNA isolated from the sample or an amplified PCR product. The DNA microarray is basically of two types, genomic microarrays and oligonucleotide arrays. In genomic DNA microarrays, the probes are complete genes or their fragments from a strain of a microorganism, while in oligonucleotide microarrays the target DNA hybridizes 18–70

nucleotide-length oligonucleotides. Although both types of microarrays can be used, pathogen detection, oligonucleotide microarrays are commonly chosen for the detection of either genomic DNA directly or the PCR-amplified portion of the genomic DNA, such as rRNA genes or virulence genes (Kostrzynska and Bachand 2006).

Microarrays have been developed for the identification of foodborne bacterial pathogens belonging to *Bacillus* spp., *C. jejuni*, *E. coli*, *L. monocytogenes*, *S. enterica*, *Shigella dysenteriae*, *Staphylococcus* spp., and *Vibrio* spp. (Call et al. 2003; Chiang et al. 2006; Garaizer et al. 2006; Sergeev et al. 2006; Eom et al. 2007) and for the discrimination of multiple pathogens and their virulence factors (Sergeev et al. 2006; Wang et al. 2007) in the case of food poisoning outbreaks and biological warfare (Sergeev et al. 2004; Wang et al. 2007).

In order to design a method for accurate detection and identification of foodborne pathogens, Kim et al. (2008) used comparative genomics to select 70 mer oligonucleotide probes specific for 11 major foodborne pathogens (ten overlapping probes per pathogen) for use in microarray analysis. Researchers analyzed the hybridization pattern of this constructed microarray with the Cy3-labeled genomic DNA of various foodborne pathogens and other bacteria. A highly specific hybridization pattern with the genomic DNA of each pathogen was observed. Microarray data were analyzed and clustered using the GenePix Pro 6.0 and GeneSpring GX 7.3.1 programs. The dendrogram revealed the discriminating power of the constructed microarray. Each foodborne pathogen clustered according to its hybridization specificity and nonpathogenic species were discriminated from pathogenic species. This method can be applied for rapid and accurate detection and identification of foodborne pathogens in the food industry. In addition, genome sequence comparison and DNA microarray analysis have a powerful application in epidemiologic and taxonomic studies as well as in the food safety and biodefence fields.

8.2.5 Integrated Systems

In the past few years some integrated systems (i.e., lab-on-a-chip) have grown, and some have been reported for the detection of bacterial pathogens (Kopp et al. 1998; Liao et al. 2007). These systems are popular because they decrease analysis times and increase efficiency of detection.

Recently, Lu et al. (2008) developed an on-chip immunoassay that detects an intracellular antigen of *L. monocytogenes* (Aad) based on polystyrene beads functionalized with the Aad antibody. Polystyrene beads were mixed thoroughly with cell lysate in the microfluidics channel so that beads were bound with the antigen in the lysate. The beads were exposed to fluorescently labeled Aad and the detected bacterial concentration was inversely proportional to the fluorescence intensity from the beads after washing. This chip can be useful for immunoassays based on cell lysates. Woolley et al. (1996) described the integration of PCR and capillary electrophoresis in a microfabricated DNA analysis device. The approach combines

thermal cycling with high-speed DNA separation by the CE chips. This system provided an assay of genomic Salmonella DNA in about 45 min. Andreas Manz's group has used a micromachined chemical amplifier to perform PCR in continuous flow at high speed (Kopp et al. 1998). The authors report that input and output of DNA are continuous, and amplification is independent of input concentration. They have reported that *Neisseria gonorrhoeae* was investigated and a 20-cycle PCR was completed in 90 s to 18.7 min, depending on flow rate.

An advanced nucleic acid analyzer (ANAA) was described by Lawrence Livermore National Laboratory for the detection of bacterial pathogens such as *Erwinia herbicola*, *Bacillus subtilis*, and *B. anthracis* (Belgrader et al. 1998). The instrument was composed of ten silicon reaction chambers with thin-film resistive heaters and solid-state optics. The authors reported that detection times were as short as 16 min and that 10^2–10^4 organisms per ml could be detected. The instrument allows for rapid analysis, low-power consumption, real-time monitoring, and for ruggedness due to lack of moving parts.

8.3 Conclusions and Future Prospectives

It was not the intent for this review article to list all the organisms that have been detected using molecular techniques but to show the range of new methods that are applicable for detecting bacteria in food samples. Foodborne pathogen identification is an important aspect of human health care. Isolation and identification of foodborne pathogens by biochemical and immunological methods are time-consuming and have less sensitivity compared with molecular methods. DNA polymorphism among the different species of bacteria has been exploited to identify food pathogens.

PCR methods have been developed for the identification of these bacterial pathogens. PCR is an effective, rapid, reliable, and sensitive technique for the detection of genes of bacterial pathogens from various foods (Park et al. 2006). The 5′ nuclease multiplex PCR assay has also found applications in simultaneous screening of bacterial pathogens in food commodities and various environmental samples. The method will be also effective for slow-growing or nonculturable microorganisms.

The electrophoresis-based methods described in this chapter (mPCR, RAPD, RFLP, AFLP, and PFGE) are time-consuming and laborious. RFLP requires pure culture for the discrimination of bacteria at the species level. The disadvantages of the RAPD technique are that standardization of concentration of primers and templates are needed to make reproducible amplification products, and most of the RAPD markers are dominant, i.e., it is difficult to distinguish between similar DNA sequences amplified. A problem related to AFLP analysis is the incomplete digestion of chromosomal DNA which may result in an aberrant AFLP pattern (Lukinmaa et al. 2004). PFGE has been considered the "gold standard" in identifying the causative organisms in cases of food poisoning, and water and hospital epidemics. PFGE has become the standard procedure for bacterial foodborne disease outbreak

analysis (Swaminathan et al. 2001) due to its discriminatory capabilities (Gerner-Smidt et al. 2006). Although the method is reliable and accurate, sample preparation and analysis are time-consuming, i.e., the method is slow.

Real-time PCR permits the acquisition of more rapid results with minimal manipulation. It is now possible to follow the amplification in real time, thus eliminating laborious postamplification processing steps such as gel electrophoresis. Real-time PCR offers better multiplexing possibilities; however, due to the availability of dyes emitting fluorescence at different wavelengths, multiplexing is still limited. Thus, detection of more than a few pathogens is currently not possible using these systems.

The microarray technology is currently a new and emerging pathogen diagnostic technology which, in theory, offers a platform for unlimited multiplexing capability. Tens of thousands of such probes can be spotted in a defined and addressable configuration on the glass slide forming the chip. The unlimited capability for simultaneous detection of pathogens offers much promise for microarrays to detect all relevant pathogens within a specific food matrix. In food microbiology, the development of microarrays for diagnostic applications is a recent development in this field, and has been detailed in this chapter. Microarrays have allowed for more rapid analyses; however, there are drawbacks to its use. Microarray instruments are expensive, of limited availability, and require specialized knowledge and training to extract useful information from the huge amount of data generated. This limits the broad application of microarray technology in ordinary laboratories. The effort to add a quantitative aspect to microarrays must continue and more work is needed to address the challenges of studying food samples where contaminants such as organic substances and heavy metals may interfere with DNA hybridization and affect the performance of microarrays.

Thus far, microbial biosensors and bioassays have been applied more for the detection of food additives and food contaminants than in direct monitoring of food pathogens (Table 8.1). Although biosensor research has sporadically appeared in the literature over 2 decades, few biosensors are commercially available. Major drawbacks include the delicate nature of the biological component and the miniaturization of the electrical components. As electronic innovation continues to deliver smaller and more reliable electronic devices and as the biological sciences continue to develop the unique understanding of enzyme and microbial genetics, the future will see reliable biosensors for the detection of biological events on-line. The food industry will significantly benefit from developments in rapid detection of microorganisms.

Although the above-described methods are highly specific and accurate, utmost care must be taken to standardize methods to isolate DNA from microbes in food samples. The DNA of dead microorganisms (VNBC) is also present which can amplify and give false positive results. Ethidium monoazide can be used to separate dead and viable bacteria (Rudi et al. 2002; Nogva et al. 2003; Keer and Birch 2003; Rudi et al. 2005). BDC also termed floatation, may be used successfully as a prior sample treatment to eliminate free DNA in samples (Wolffs et al. 2005). This can lower the risk of false positive results by avoiding DNA from VNBC bacteria.

Table 8.1 Summary of methods used to detect *Escherichia coli* O157:H7

Method	Approx. detection time	Detection limit	Selected references
Plating/culturing	1 day to 1 week	Low CFUs	Silk and Donnelly (1997)
Biochemical tests	1 day to several days	Low CFUs	Adams and Moss (1995)
ELISA	12 h to 2 days	10–100 CFU/ml	Gehring et al. (1999)
PCR	2–24 h depending on enrichment	10^2–10^5 CFU/ml	Uyttendaele et al. (1999)
Multiplex PCR	24 h	1–2 CFU/ml	Hu et al. (1999)
RT-PCR	6–12 h	10^7 CFU/ml	Yaron and Matthews (2002)
Laser-induced fluorescence	Few hours	Single organism	Johnson et al. (2001)
Fiber optic biosensor	ca. 30 min	5.2×10^2 CFU/g	Demarco and Lim (2002)
SPR biosensor	1 h	5×10^7 CFU/ml	Fratamico et al. (1997)
Microarrays	<1 h	55 CFU/ml	Call et al. (2001)
Integrated systems (lab-on-a-chip)	16–45 min	10^2–10^4 organisms/ml	Belgrader et al. (1998)

Due to inherent limitations in the methods developed thus far, it is unlikely that any one detection system will be suitable for monitoring genetically modified microorganisms. Similarly, the detection of recombinant microorganisms in the food microbiology industry may become an issue of interest that will stimulate further investigations into molecular methods for food microbiology.

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