Rapid Detection and Limitations of Molecular Techniques

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Abstract

Polymerase chain reaction (PCR) has become an important diagnostic tool in the detection of foodborne pathogens. Many PCR tests have been validated, harmonized, and commercialized to make PCR a standard tool used by food microbiology laboratories to detect pathogens in foods. Current PCR technology allows for rapid detection of pathogens in real time. Real-time PCR can provide qualitative as well as quantitative information. However, PCR does have its limitations because of false-negative and false-positive results that may be encountered with the daily running of PCR assays by a diagnostic laboratory. The intent of this review is to help the reader identify these problems as they occur, discuss the nature of this interference, and provide solutions. This review also discusses the future of molecular diagnostics, i.e., high throughput nucleic acid sequencing.

PCR: polymerase chain reaction

Pyrosequencing: new nucleic acid sequencing technology that measures incorporation of specific nucleotides by detecting photon emission produced by firefly luciferase

Anneal: hydrogen base-pair binding of oligonucleotide(s) or nucleic acid (DNA or RNA) to the complementary sequences present in the nucleic acid sample

DNA polymerase: an enzyme that is responsible for synthesizing DNA strands using the complementary strand as a template for its synthesis

Primer: an oligonucleotide, between 18–25 bp in length, that primes DNA synthesis of the complementary DNA strand

HISTORY OF PCR AND ITS EVOLUTION AS A DIAGNOSTIC TEST

The ability of any microbe to cause disease is dictated in part by its genetic composition. In some instances, its virulence rests in a single gene (Greenfield et al. 1983, Matsuda & Barsdale 1967). You detect the gene, you detect the pathogen (Mikhailovich et al. 1995). Molecular biology has transformed diagnostic microbiology since the early days of DNA:DNA hybridization technology (Southern 1975) through the advent of polymerase chain reaction (PCR) (Saiki et al. 1985), pyrosequencing (Ronaghi et al. 1996, Marguilies et al. 2005), single multitest microarrays (Schena et al. 1995), and fluorescent-microsphere technology (Fulton et al. 1997, McHugh et al. 1988), which may supplant today's PCR-based molecular tests. No one single molecular technique has transformed research and diagnostics more than PCR. The thermocycler has become not only the standard lab instrument of the research lab, but it is now commonplace in the diagnostic laboratory. PCR has gone from the theoretical to the practical. There are several commercial PCR kits available for *Salmonella*, *Campylobacter*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Enterobacter sakazakii*, *Staphylococcus aureus*, and Norovirus detection (**Table 1**). Considerable resources have been put into designing, implementing, and validating PCR for detection of pathogens in food. Several of these PCR tests (e.g., *Salmonella* BAX PCR) (USDA Food Safety and Inspection Service 2007) are used by U.S. regulatory agencies involved in screening foods for pathogens (USDA Food Safety and Inspection Service 2007, 2008, 2009). However, PCR is a tool and like any tool it has its strengths and weaknesses. PCR may not be practical or economical, depending on the application. This review explores the current molecular tools available for rapid detection of pathogens in foods and their limitations. The emphasis here is placed on PCR, as it has the greatest application and use in detecting foodborne pathogens. Other future technologies are examined, but because of their experimental nature, the scope of their review is limited and introductory.

The PCR was borne out an understanding of the chemistry of DNA replication and requirements for synthesizing the complementary DNA strand: A single-stranded DNA template and a free, 3′ hydroxyl group is needed for the incorporation of the next nucleotide into the nascent, DNA strand. In the bacterial cell, the DNA gyrases and topoisomerases produce the single-stranded template, and the single-stranded binding proteins prevent the premature reannealing of the DNA strands before the complementary DNA can be synthesized by the DNA polymerase. The primase produces an RNA primer needed to synthesize the complementary DNA on the lagging DNA strand. This RNA primer provides the free 3′ hydroxyl group necessary in the nucleophilic attack

Table 1 Examples of commercially available PCR kits for pathogen detection in foods

Pathogen	PCR	Brand name	Manufacturer
Salmonella	TAQMAN	*Salmonella* BAX® PCR	DuPont Qualicon
	RT PCT with IAC	ADIAFOOD rapid pathogen detection system for *Salmonella* spp.	AES Chemunex Canada
	mPCR with IAC	Multipathogen PCR kit - *Salmonella* spp., *Listeria monocytogenes*, and *E. coli* O157	Diatheva
	RT PCR	AnDiaTec® *Salmonella* real time PCR Kit	AnDiaTec
Listeria monocytogenes	TAQMAN	*Listeria* BAX® PCR	DuPont Qualicon
	RT PCR with IAC	ADIAFOOD rapid pathogen detection system for *L. monocytogenes*	AES Chemunex Canada
Escherichia coli O157	TAQMAN	*E. coli* O157 BAX® PCR	DuPont Qualicon
	RT PCR with IAC	ADIAFOOD rapid pathogen detection system for *E. coli* O157 and *E. coli* O157:H7	AES Chemunex Canada

of the incoming nucleotide to the nascent DNA strand, producing the phosphodiester that strings the nucleotides together in the DNA strand (Kornberg 2000). In the test tube, these reactions and events can be recreated using heat (e.g., 94°C) to produce the single-stranded DNA template and an oligonucleotide primer to initiate synthesis of the complementary DNA strand. With the right salts, pH, and a supply of nucleotides, the purified DNA polymerase can synthesize both DNA strands, yielding double-stranded DNA product. The oligonucleotide primer determines if and where DNA synthesis can be initiated. Positioning of these priming oligonucleotides close to one another and oriented to produce overlapping, complementary DNA strands can over time and with repeated cycles of DNA replication produce a single DNA fragment. The size of this DNA fragment is a function of the distance between the two DNA priming oligonucleotides and its appearance dictated by the presence of DNA sequences complementary to both priming oligonucleotides (primers). To produce a new DNA strand in vitro requires three steps: (*a*) denaturation (94°C) to produce single-stranded DNA; (*b*) annealing at a lowered temperature optimal for the binding of the primers to their complementary, target sequence; and (*c*) extension at a temperature optimal for the DNA polymerase to synthesize the new DNA strand. These three steps can be repeated over and over, amplifying the DNA exponentially with each subsequent cycle.

The first PCR used *E. coli* DNA polymerase. Because of the heat denaturation step, new enzymes had to be added to each subsequent cycle (Saiki et al. 1985). The DNA polymerases of thermophilic bacteria such as *Thermophilus aquaticus* (*Taq*) are resistant to denaturation by boiling and enzymatically active when the temperature is reduced to 72°C. At present, a single application of the enzyme *Taq* DNA polymerase to the reaction mix allows repeated cycling of the denaturation, annealing, and extension steps (Saiki et al. 1988). With the development of the thermocycler, an instrument or heating block that could rapidly fluctuate temperatures from 94°C to 4°C (Saiki et al. 1988), PCR was born.

Extension: synthesis of the complementary DNA strand by the DNA polymerase in PCR, using the bound oligonucleotide as the primer for synthesis

***Thermophilus aquaticus* (*Taq*):** the thermophilic organism from which a key thermally stable DNA polymerase for PCR was obtained

Thermocycler: an instrument with a heating/refrigeration block that can be programmed for a wide range of temperatures

Amplicon: PCR product

Real time: detection of amplicon as it is produced with each cycle of PCR

GEL-BASED PCR DETECTION

Early PCR was a qualitative test that could assess presence or absence of a mutation (Saiki et al. 1985) or gene allele (Saiki et al. 1986). In fact, the earliest applications of PCR were for detecting inheritable metabolic disorders (Saiki et al. 1985). Later applications were turned to detection of infectious agents (Kwok et al. 1987, Olive 1989), reasoning that if the sequence targeted by PCR was present, so too was the pathogen. PCR amplified targeted sequences to sufficiently high levels (>25 ng DNA) to be observed in an ethidium-stained agarose gel. Using this gel-based format, PCR would remain a qualitative test until light/fluorescence detectors were incorporated into the thermocycler (Morrison et al. 1998) for detection of amplicons in real time. The thermocyclers have also evolved from the single heating block instruments (Saiki et al. 1988) to hot-air thermocyclers (Wittwer et al. 1989) to 96-well block thermocyclers (Murphy et al. 1993) and thermocyclers with programmable, individual wells (Raja et al. 2002). These advancements decreased the time it took to optimize PCR and later run these PCR tests. PCR reaction times that once took 90 minutes have been reduced to 10–15 minutes (Morrison et al. 1998, Wittwer et al. 1989) and decreased reaction volumes and cost associated with a single PCR test (Morrison et al. 1998, Wittwer et al. 1989).

REAL-TIME PCR ASSAY

With the birth of PCR came an explosion of diagnostic tests for detecting viruses, bacteria, and parasites in patient specimens, foods, and environmental samples. At the inception of PCR, detection was limited to a gel-based format, which required additional equipment (electrophoresis

unit, camera, etc.) on top of the expensive thermocycler already needed for PCR. Also, the additional handling associated with gel detection opened potential sources for carryover contamination of PCR set-up area and reagents sometimes producing false-positive results (see section PCR Carryover Contamination). The greatest advancement in thermocycler technology came in the addition of a fluorometer/photometer for detection of fluorescent amplicons as they are produced in real time (Morrison et al. 1998). The earliest real-time PCR assay incorporated a sensitive fluorescent dye, SYBR-Green, that binds the double-stranded DNA as it is produced (Morrison et al. 1998). The advantage of this dye over ethidium bromide is that SYBR-Green is 1,000-fold more sensitive (Schneeberger et al. 1995). With the detector built into the thermocycler, the amplicon can now be detected once it reaches the threshold range for detection after *x* number of cycles. There is an inverse correlation between cycle number at which the amplicon is initially detected and target copy number, allowing one to estimate cell or viral numbers in positive samples (Richards et al. 2004, Wolffs et al. 2006). The fewer target cells present in a sample, the more cycles are needed before the amplicon is detected. The real-time PCR can therefore be standardized against a set of known target amounts (DNA concentration) or cell concentrations to transform this test from a qualitative test to a quantitative test (Morrison et al. 1998). The added utility of not only detecting pathogens in foods but also determining pathogen loads is an added benefit, as new regulatory decisions are now being developed based on both prevalence and pathogen load (e.g., *Campylobacter* and poultry) (New performance standards for *Salmonella* and *Campylobacter* in young chicken and turkey slaughter establishments; new compliance guides 2010).

Nonspecific amplicon: an erroneous PCR product of size or sequence composition different from that of the intended PCR target

Given that the PCR reaction and subsequent amplicon detection are self-contained, never requiring the reaction vessel to be opened following setup, the potential for carryover contamination is eliminated. However, the earlier real-time PCR tests were fraught with problems with false-positive results associated with sometime production of nonspecific amplicons (see What Is Considered a PCR Positive Result?).

TAQMAN PCR AND MOLECULAR BEACONS

The problems with false-positive results were later resolved with inclusion of internal probes in the real-time PCR assay (Livak et al. 1995, Tyagi & Kramer 1996). The internal probes central to 5′-nuclease PCR (also known as TAQMAN PCR) (Livak et al. 1995) and molecular beacons (Tyagi & Kramer 1996) reduced false-positive results by requiring annealing of these internal oligonucleotides to the true amplicon as it is produced. Rather than using the fluorescent dye SYBR-Green floating freely in the reaction mix, a fluorescent dye is physically incorporated into the internal probe along with a chemical quencher that interferes with fluorescence when the probe is unbound in solution (Livak et al. 1995). Only when the internal probe is bound to the true amplicon can the fluorescent marker be detected. In 5′-nuclease PCR, an exonuclease associated with the thermophilic DNA polymerase liberates the fluorescent chemical group from the chemical quencher, as the enzyme synthesizes the complementary DNA strand (Livak et al. 1995). For the molecular beacon, the internal probe consists of an internal nucleotide sequence complementary to the amplicon generated during PCR, flanked by sequences that are complementary to each other, allowing the 5′ and 3′ ends to anneal with one another and form a stem and loop structure. The fluorescent dye is situated on one end of the oligonucleotide probe and the chemical quencher is placed at the opposite end. The 3′ end of the probe also contains a dideoxy nucleotide, which prevents its incorporation into the nascent DNA strand or degradation by exonuclease activity of the DNA polymerase (Tyagi & Kramer 1996). When this internal probe is allowed to anneal with itself and form this stem and loop structure, the chemical quencher is now brought into close proximity with the fluorescent dye, blocking light emission when excited with ultraviolet (UV)

radiation (Tyagi & Kramer 1996). If the true amplicon is present, the molecular beacon hybridizes or anneals to it, separating the fluorescent dye from the quencher and thus allowing the bound probe to fluoresce upon excitation with UV radiation. As a real-time PCR assay, 5′-nuclease PCR lends itself well as both a qualitative and quantitative test (Bassler et al. 1995). Several of today's commercially available PCR tests have been developed as a TAQMAN PCR (**Table 1**).

Multiplex PCR assay: a single PCR test for detecting multiple genes or pathogens, based on the detection of unique size amplicon

Genome: the complete genetic composition of an organism or virus. For a bacterium like *Escherichia coli* O157:H7, the genome includes plasmid, prophages, and chromosome

MULTIPLEX PCR ASSAY

The PCR assay was originally developed to detect a single target sequence or pathogen. As more unique target gene(s) or sequences were identified for detecting pathogens by PCR, the number of PCR tests a diagnostic laboratory could conduct quickly grew. Depending on the food tested, pathogen prevalence and cell numbers, and clientele's requirements, a laboratory may be required to run a minimum of three separate PCR tests. The natural progression in the evolution of the diagnostic PCR assay was toward a single test for the detection of multiple pathogens (Beuret 2004, O'Leary et al. 2009, Omiccioli et al. 2009), pathotypes (Lopez-Saucedo et al. 2003), serovars (Doumith et al. 2004, Hong et al. 2008, Paton & Paton 1998), phage types (Hu et al. 2002), and strain types (Cooke et al. 2007). Multiplex PCR assay was the logical next step, a single PCR assay that combines multiple primers for detecting multiple pathogens or strains (Beuret 2004, Cooke et al. 2007, Doumith et al. 2004, Hong et al. 2008, Hu et al. 2002, Lopez-Saucedo et al. 2003, O'Leary et al. 2009, Omiccioli et al. 2009, Paton & Paton 1998). Each primer set was designed to produce an amplicon with a defined signature size for the pathogen or strain being tested. The PCR primers were designed to produce a unique amplicon size specific to the gene or sequence being targeted (**Figure 1**). Therefore, based on the size of the amplicon produced by PCR, one can identify the pathogen or strain present in sample *x*. This tool has become useful not only in detecting specific pathogens, but in typing bacterial (Doumith et al. 2004, Hong et al. 2008, Paton & Paton 1998) and viral (Iturriza-Gomara et al. 2004) isolates as well. The multiplex PCR assay has even been adapted to the real-time PCR assay, where in place of detecting specific-sized amplicons, the test is now developed to detect multiple-colored fluorescent dyes associated with the distinct, internal probes for pathogens X, Y, and Z (Woods et al. 2008).

PCR, PYROSEQUENCING, AND GENOMICS

Nucleic acid sequencing technology has made incredible leaps forward in the past five years with the development and commercialization of the open microfabricated high density picoliter sequencing reactors for rapid, high-throughput sequencing, also known as 454 pyrosequencing (Margulies et al. 2005). With the new high-throughput nucleic acid sequencing technologies, genomes can be completely sequenced in weeks rather than years because this methodology does not require DNA libraries and clones (Schloss 2008), only isolated nucleic acid. This new high-throughput sequencing has expanded the repertoire of bacterial genomes sequenced (Maze et al. 2010), including multiple pathogenic strains (Clawson et al. 2009, Gilmour et al. 2010, Hofreuter et al. 2006, Kotewicz et al. 2008, Poly et al. 2008). This powerful sequencing tool has helped identify potential bacterial or viral agents associated with diseases of previously unknown etiology (Coetzee et al. 2010, Cox-Foster et al. 2007, McKenna et al. 2008). As one-third of foodborne illnesses in the United States is of unknown etiology [Surveillance for foodborne disease outbreaks—United States, 2007. (2010)], 454 pyrosequencing and other high-throughput nucleic acid sequencing technologies (Schloss 2008) are likely to identify new bacterial, protozoal, or viral pathogens. The bottlenecks now for this new technology are data storage, processing, and analysis (Schloss 2008).

bp: base pair (or nucleotide)

PPi: pyrophosphate

CCD: charge-coupled device

454 pyrosequencing works as follows: The intact DNA (~4×10^6 bp; *E. coli* chromosome) is fractionated by sonication into smaller, 300–500-bp DNA fragments, to which oligonucleotide adapters are subsequently added for PCR amplification later in the procedure (**Figure 2**). Using limiting dilution, a single DNA fragment is bound to a single bead. Millions of these beads, where each bead contains an individual DNA fragment, are immersed in an oil emulsion. In this emulsion, a single bead is present within an oil droplet. PCR is performed using primers that bind to the adaptors. That single DNA fragment is amplified to coat each bead with ten million copies of a unique template. The beads are subsequently removed from the emulsion, and bound DNA is denatured to produce a single-stranded template for sequencing and deposited into the wells of a fiber optic slide (**Figure 2**). A single slide contains a million wells, and each well contains a single bead with a unique template for sequencing. Sequencing enzymes are delivered to each well, bound to smaller beads that fill each chamber/well. Microfluidics administer to each well a cycle of dATP, dTTP, dCTP, and dGTP. Pyrophosphate (PPi) is produced upon incorporation of each nucleotide into the template DNA strand by the DNA polymerase and detected by luciferase, which produces a photon of light for every PPi molecule that is detected by a million pixel charge-coupled device (CCD) camera. Microfluids wash the wells of the unincorporated nucleotide and administer the next one to start the process anew. A single fiber optic slide can produce a million sequence reads, for which each read may have a 100–500-bp sequence.

Today, there is a plethora of PCR tests available for detecting the many viral, bacterial, and protozoal agents associated with foodborne illnesses. Several of these PCRs have been combined into a single test for detecting multiple pathogens (Beuret 2004, Cooke et al. 2007, Doumith et al. 2004, Hong et al. 2008, Hu et al. 2002, Lopez-Saucedo et al. 2003, O'Leary et al. 2009, Omiccioli et al. 2009, Paton & Paton 1998). It is tempting to abandon current standardized microbiological methods for these newer molecular tests. However, as with any diagnostic test, the PCR assay has its strengths and weaknesses. To bring this new technology into the diagnostic or food microbiology laboratory, one needs to become acquainted with nuances of PCR, including the details of sample preparation, interpretation of test results, the expense and practicality of using a PCR test, and identifying and troubleshooting problems as they arise.

INTERPRETATION OF PCR RESULTS

PCR Results, Statistics, and Validation

Research in the development of molecular diagnostic tests has made major advancements since their infancy, when reporting on the molecular specificity (differentiation of genus, species, serovar, or strain from evolutionarily/genetically related members) and sensitivity (the fewest cells detected) was sufficient for publication (Maurer et al. 1999, Thomas et al. 1991). Now, there are greater requirements placed on authors to validate their molecular test and demonstrate that the new test is comparable to, if not better than, the one currently adopted by diagnostic laboratories (Scope 2010). Authors must address the congruence of the new diagnostic test with an existing test(s), reporting on the statistical specificity (the proportion of true negative results) and sensitivity (the proportion of true positive results), compared with the currently used test that is considered the gold standard (Liu et al. 2002; Hong et al. 2003, 2008, 2009). With regards to validation of PCR tests for detecting foodborne bacterial pathogens, the gold standard is a culture method. A false-positive result occurs when the PCR assay indicates a positive result and the culture method fails to isolate and identify the pathogen of interest, and a false-negative result is the opposite, i.e., the failure of the PCR test to detect the pathogen of interest. What accounts for these false-positive and false-negative results, and what can be done to optimize the PCR test to improve the specificity

and sensitivity of the test? These topics are the primary focus of this review and are discussed in the following sections.

Long PCR: specific PCR that refers to amplification of DNA fragments of 10–30 kb in size by PCR

Kb: kilobase (1 Kb = 1000 bp)

What Is Considered a PCR Positive Result?

In a PCR assay, the primers are important in setting the sensitivity and specificity of the test. The length of the individual oligonucleotide primer is important in setting the specificity as the inverse of the primer length (in bp) is equal to the probability that the primer sequence appears randomly within any given bacterial, protozoal, or viral genome. For example, any 10-bp oligonucleotide primer might appear in a four-million-bp bacterial genome [assume 50% guanocine/cytosine (GC) content] four times, whereas a primer twice that length is expected to appear within any bacterial genome 0.00004 times. Therefore, the length of most primers is set between 18–25 bp. The distance between the forward and reverse PCR primers dictates the size of the PCR product or amplicon. Most commercially available *Taq* DNA polymerases are best for amplifying DNA less than 5,000 bp. However, the newer thermophilic DNA polymerases, especially those with proofreading activity, can amplify larger DNA fragments in 5–25 Kb range (Ohler & Rose 1992). Referred to as "long PCR," this has become an important tool in closing sequencing gaps in bacterial genomes (Tettelin et al. 1999) and in characterizing variable gene(s) or sequences wedged between evolutionarily conserved genes in certain bacterial families, genera, species, serovars, or strains (Herbelin et al. 2000, Wang et al. 1998, Wang & Reeves 1998). However, with the targeted amplification of larger DNA fragments, one loses sensitivity with the PCR assay. Therefore, with conventional PCR and real-time PCR assays, the recommended spacing of primers is between 500–100 bp and 200–75 bp, respectively. Combined, both primers within the primer pair set the specificity for the PCR assay by their length and spacing between the forward and reverse primer. Although there may be some annealing of a primer to sequences other than the complementary target, it's the distance between it and the second primer that may preclude amplification of larger, nonspecific amplicons with *Taq* DNA polymerase.

A PCR result is considered positive if and only if it produces an amplicon of the expected size, based on the spacing of the forward and reverse primers. Most sequences targeted in the PCR assay are to genes, which are invariant in size within a bacterial population, species, or genera. Therefore, one expects to observe the same size amplicon produced for every individual within the population, species, or genera that is targeted for detection by that PCR assay. Any PCR assay that produces a product that does not correspond in size to the placement of the primers within the targeted genome is ignored and negative only if the expected size amplicon is not observed.

Amplicons produced in a PCR reaction, with size contrary to expected size for the primer pair used, are referred to as nonspecific amplicons. For PCRs where size is critical, % agarose and molecular weight standards selected are essential in providing the resolution, DNA separation, and molecular weight range needed to interpret a gel-based PCR test. Where gel electrophoresis is essential to PCR detection, it's important for the voltage used to allow sufficient time to elapse for adequate separation and resolution of DNA fragments within the size range of the molecular weight standard. Generally, one can use the dye front generated during electrophoresis as a guide to determine when to stop (dye front is approximately 1 cm from the bottom of the gel). Expediency (i.e., time) to generate results can impede accuracy in interpreting gel results and erroneously identify nonspecific amplicons as PCR-positive.

In real-time PCR, the dye SYBR-Green binds to the amplicons as they are synthesized and fluoresces once exposed to UV light. The signal intensifies with each successive round of the PCR until it plateaus, producing a sigmoidal curve for fluorescence versus PCR cycle number. The more target cells present, the sooner the PCR product is detected by the thermocyclers'

fluorometer. If sample and reaction conditions should produce a nonspecific amplicon, this PCR cannot distinguish between the true versus nonspecific amplicons as they are detected in real time. However, one can distinguish nonspecific from true amplicons by the unique melting curve associated with targeted sequences versus the spurious amplification of unrelated gene sequences (Eyigor et al. 2002). Therefore, inclusion of melting curve analysis of each sample and amplicon obtained at the end of each PCR run is a necessary quality control measure for successfully running real-time PCR in the diagnostic laboratory. PCR-negative samples that produce these nonspecific amplicons are useful in further optimizing the PCR. The PCR reaction conditions can be tweaked by increasing the annealing temperature or decreasing the magnesium chloride concentration to eliminate these nonspecific amplicons. The newer, real-time PCR assays use oligonucleotide probes to sequences internal to the amplicon and therefore improve the specificity of the PCR as it eliminates the detection of nonspecific amplicons (Livak et al. 1995, Tyagi & Kramer 1996).

Viable but nonculturable (VBNC): a physiological state in which bacteria are still metabolically active but unable to grow using conventional culture methodology

What Does a Positive PCR Result Mean?

The most conservative conclusion one can draw from any PCR test is that the targeted gene sequence is present in sample *x*, and by inference, the targeted pathogen as well. As DNA is often the template employed in most PCR reactions, this test cannot distinguish between live or dead cells or whether or not the agent present in sample *x* is infectious. Depending on the PCR test in question, one may not be able to infer more than the presence of a specific genus (*Salmonella* and *invA* PCR) (Rahn et al. 1992), species (*L. monocytogenes* and listeriolysin O PCR) (Thomas et al. 1991), or serovar (*E. coli* O157:H7 and *wba/fliC* PCR) (Paton & Paton 1998). Coupled with sample type, finding of generic *Salmonella*, for example, may or may not have any significance depending on whether one were examining the poultry house environment versus ready-to-eat deli turkey meat. Not being able to distinguish *Salmonella* further with the *invA* PCR, finding *Salmonella enterica* subspecies IIIa Arizona (0.0025% human cases for 2006) (CDC PHLIS Surveillance Data 2007) may not be as significant as *S. enterica* subspecies I Enteritidis (16.6% human cases for 2006) (CDC PHLIS Surveillance Data 2007) in, for example, a table egg-laying operation (Mumma et al. 2004). A few PCRs have been identified that can discern strain level differences directly (Hu et al. 2002) or with added sequencing of the amplicons (Grissa et al. 2008). However, until pyrosequencing becomes cost effective and significant advances are made in bioinformatics, epidemiologic investigations are going to continue to require a culture procedure and the isolation of suspect pathogen(s) from foods (Schloss 2008).

With any new test, one needs to be aware of the false-positive and false-negative results associated with it. Because of the sensitivity of the PCR assay and its ability to amplify millions of copies with time, the test can generate false-positive results from carryover PCR contamination of reagents, pipettors, etc. (Erlich et al. 1991). Also, most PCR assays cannot distinguish dead from live cells (Wolffs et al. 2005) and traditional culture may not be able to distinguish dead from damaged or viable but nonculturable (VBNC) cells (Oliver 2010, Reissbrodt et al. 2002).

Although there are many limitations and disadvantages associated with the PCR assay, which are discussed in more detail below, this assay does have the advantage over traditional culture-based approaches to pathogen detection as a qualitative and quantitative test. Real-time PCR can not only detect a specific pathogen but also determine its load in a sample (Richards et al. 2004, Wolffs et al. 2006) in less time than the most probable number (MPN) methods that have been developed to estimate pathogen cell numbers (Pavic et al. 2009, Wolffs et al. 2006). Being able to detect and quantify pathogen populations at different points within the food processing system can provide added validation information that inactivation treatments are working to reduce pathogen cell numbers.

Is a Negative PCR Result Really Negative?

A negative PCR result may be due to (*a*) absence of the targeted pathogen; (*b*) the pathogen is present at cell numbers below the threshold of detection; or (*c*) something in the sample is interfering with the PCR reaction, leading to an erroneous negative result. However, even with traditional culture-based testing, a negative result is not necessarily confirmatory of the pathogen's absence if its prevalence is less than the sample size needed for detection.

DIAGNOSTIC PCR CHALLENGES AND SOLUTIONS

False-Negative Results

Sometimes there is incongruence between PCR and culture, where the PCR is negative but culture yields a positive result. This is refered to as a false negative and has several causes, including (*a*) physical limits of detection associated with PCR and (*b*) sample inhibitors that interfere with PCR. Both topics are covered in the following sections.

Limits of PCR detection. The sensitivity of the PCR to detect the fewest target cells or virions possible varies depending on the food pathogen, food matrix, target sequence, and primers. Therefore, one may need to consider a number of factors in developing and optimizing a PCR method(s) for detecting the target organism in the food matrix in question. One starting point is to identify a procedure optimal for releasing total DNA or RNA from the microbial cell (Lalonde & Gajadhar 2008, Selma et al. 2008) or viral particle (Jean et al. 2004) in question. The physiochemical nature of the microbe, e.g., norovirus, can be exploited in the sample preparation, effectively concentrating and purifying the PCR template (Haramoto et al. 2004, Leggitt & Jaykus 2000). The PCR's sensitivity is also limited by volume constraints of the test. Most traditional culture-based tests involve processing gram and milliliter amounts and working with large culture volumes (e.g., *Salmonella* enrichment in tetrathionate: 100–250 ml), whereas most molecular-based methods involve final volumes that vary from 10 μl to at most 100 μl. The most sensitive PCR test detects a single cell or virion in 1-to 10-μl template samples. Therefore, for that one cell detected in a single μl, the limit of detection is 1000 cells ml^{-1}.

The sensitivity of the PCR assay can be improved by first concentrating the pathogen present in the food matrix (Comelli et al. 2008, Frazar & Orlandi 2007, Tian et al. 2008), capitalizing on the microbe's size and physiochemical properties, as is the case for noroviruses (Comelli et al. 2008), or using magnetic capture beads conjugated with the antibody (Frazar & Orlandi 2007, Tian & Mandrell 2006) or other substrate that specifically binds to the target pathogen (Tian et al. 2008). The magnetic capture beads have been adapted to the PCR detection of *E. coli* O157:H7 (Fu et al. 2005), *Campylobacter* spp. (Rudi et al. 2004), *L. monocytogenes* (Yang et al. 2007), *S. enterica* (Hagren et al. 2008), *Yersinia enterocolitica* (Kapperud et al. 1993), norovirus (Tian et al. 2008), hepatitis A virus (Jothikumar et al. 1998, Shan et al. 2005), and *Cryptosporidium parvum* (Frazar & Orlandi 2007) for samples where pathogen loads are at or below the threshold of PCR detection.

As alternatives to concentrating microbes present in a sample, several protocols have included an enrichment culture step to increase cell numbers to those above the threshold for PCR detection (Lund et al. 2004, Oberst et al. 1998, Stone et al. 1994). These enrichment cultures employ general, all-purpose media (Malorny et al. 2003a, Oberst et al. 1998) or media that are specific to the pathogen being screened (Lund et al. 2004, Oberst et al. 1998, Stone et al. 1994). Even enrichment cultures targeted to specific pathogens vary not only in their sensitivity and specificity in culture isolation of select pathogens (e.g., *Salmonella*) (Iveson & Kovacs 1967, Feder et al. 2001, Soumet et al. 1999) but interference with PCRs (Liu et al. 2002, Stone et al. 1994).

PCR inhibitors. A diagnostic microbiology laboratory may have to process a variety of food matrices. In addition to consideration of sample size, volumes, and processing steps needed to test foods, these foods can also contain natural PCR inhibitors (Wilson 1997). For example, unwashed produce may prove challenging to PCR tests because of the presence of soils. In several cases, these inhibitors have been identified (Al-Soud et al. 2000, Al-Soud & Radstrom 2001, Monteiro et al. 1997, Opel et al. 2010, Sutlovic et al. 2008) as well as the mechanism behind their inhibition (Al-Soud et al. 2000, Al-Soud & Radstrom 2001, Opel et al. 2010, Sutlovic et al. 2008). The humic acid present in soil interferes with PCRs by inhibiting *Taq* DNA polymerase (Sutlovic et al. 2008). The blood and fat present in meats can also interfere with PCR (Al-Soud et al. 2000, Al-Soud & Radstrom 2001). Hemoglobin, immunoglobulin, and lactoferrin present in blood inhibit the PCR reaction through their binding to single-stranded DNA or the DNA polymerase and preventing of DNA polymerization (Al-Soud et al. 2000, Al-Soud & Radstrom 2001). PCR inhibition can be circumvented through the addition of PCR facilitators/enhancers to the reaction (Al-Soud et al. 2000, Al-Soud & Radstrom 2001), selection of a thermophilic DNA polymerase that is resistant to PCR inhibitor(s) (Al-Soud & Radstrom 2001), or a template purification protocol that removes the inhibitor(s) (Cremonesi et al. 2006, Kim et al. 2008, Lelonde & Gajadhar 2008, Selma et al. 2008, Tian et al. 2008)

One approach to overcoming PCR inhibitors comes in sample and template preparation methodologies that remove PCR inhibitor(s) (Cremonesi et al. 2006, Kim et al. 2008, Lelonde & Gajadhar 2008, Selma et al. 2008, Tian et al. 2008). These methods have been developed in accordance to food matrices involved and the physiological nature of the pathogen being screened (Butot et al. 2007, Cremonesi et al. 2006, Kim et al. 2008, Lalonde & Gajadhar 2008, Selma et al. 2008, Tian et al. 2008, Wolffs et al. 2007). Adding magnetic capture beads can not only improve the sensitivity of the PCR detection (Frazar & Orlandi 2007, Fu et al. 2005, Hagren et al. 2008, Jothikumar et al. 1998, Kapperud et al. 1993, Shan et al. 2005, Tian et al. 2008), but also frees template of PCR inhibitors (Tian & Mandrell 2006). Several of these protocols have been developed to incorporate a nucleic acid purification step involving a solid support matrix with an affinity for nucleic acid (Hong et al. 2003) or an affinity for select nucleic acid species (polyadenylated mRNA) (Kim et al. 2008) or target sequences (Regan & Margolin 1997). Once bound, the nucleic acid can be washed free of the PCR inhibitors and eluted from the spin columns (Luan & Levin 2008) or beads (Hong et al. 2003, Kim et al. 2008) used to concentrate the PCR template. The physiochemical properties of foods can also be exploited by altering the temperature (Hong et al. 2003), using differential or density gradient centrifugation (Hong et al. 2003, Lindqvist 1997, Maurer et al. 1999, Wolffs et al. 2004, 2007), or using filtration (Butot et al. 2007, Wolffs et al. 2006) to remove fats (Hong et al. 2003, Maurer et al. 1999), colloids (Maurer et al. 1999), blood (Hong et al. 2003), or tissues (Butot et al. 2007). These procedures can effectively concentrate the template as well as remove PCR inhibitors. Density gradient centrifugation has the broadest application toward PCR detection of pathogens across diverse food matrices (Lindqvist 1997, Wolffs et al. 2004, 2007). However, some foods may necessitate extractions or digestions (Butot et al. 2007) to ultimately free the template of these inhibitors. Application of activated charcoal to samples has also proven effective in removing PCR inhibitors (Luan & Levin 2008).

Inclusion of additional reagents to the PCR reaction can counteract these inhibitors and facilitate or enhance PCR amplification of the target amplicon in the process (Al-Soud et al. 2000, Al-Soud & Radstrom 2001). The mechanism behind how these reagents facilitate or enhance these PCRs varies from physically removing the inhibitor to protecting the nucleic acid template from inactivation. In addition, facilitators such as DMSO help remove the secondary DNA structures in the primers, template, or product that interferes with DNA polymerase's ability to synthesize the complementary DNA strand (Choi et al. 1999). Target DNA or RNA sequences with high GC

content, palindromes, or repetitive sequences are likely to form secondary structures, i.e., hairpins, that may interfere with initiation of the PCR or continued replication of the target amplicon (Choi et al. 1999). Also, dimer-pair formation between primers affects the PCR reaction and at least reduces the sensitivity of detection (Rychlik 1995). This is especially problematic for multiplex PCR assays in which multiple primer pairs are involved. DNA analysis programs can identify which primer pair combinations form potential dimers as well as secondary hairpins from the selected nucleotide sequence(s) (e.g., GeneRunner; **http://www.generunner.net/**). Depending on the percent GC content of targeted gene/sequence(s), there may not be any primers available that are free of secondary structures or dimer-pair formation. However, a facilitator such as DMSO melts these secondary structures (Varadaraj & Skinner 1994) and allows the primers to anneal to their complementary target with the specificity required of the PCR test.

IAC: internal amplification control

Internal amplification controls (IAC) have been developed to detect PCR inhibitors (Casas et al. 1997) and have become an important quality control measure for diagnostic PCR assays (Malorny et al. 2003b). Sample or processed templates are spiked with the control DNA (Malorny et al. 2003a). This control DNA or IAC is generally derived through an internal engineered deletion within the cloned amplicon (Abdulmawjood et al. 2002) (**Figure 3**). Spiked with the IAC, the PCR primers would produce an amplicon with a size expected for the engineered deletion. A sample containing the targeted pathogen will produce two amplicons, one corresponding to the size expected for the pathogen and the other smaller amplicon expected for the IAC (Abdulmawjood et al. 2002). Inclusion of an IAC into the diagnostic PCR test has become an important part in harmonization of PCR-based detection protocols for foodborne pathogens (Malorny et al. 2003a). With the advance of real-time PCR, IAC have been adapted to these newer diagnostic tests as part of a TAQMAN PCR (Rodríguez-Lázaro et al. 2005). In this case, the internal oligonucleotide probe is directed to unique sequences obtained by the deletion of internal sequences present in the IAC. With inclusion of different colored fluorescent dyes in the labeling of pathogen-specific oligonucleotide probes and IAC probes, real-time thermocyclers can differentiate between the two signals obtained with either probe (Rodríguez-Lázaro et al. 2005).

False-positive PCR results. In the validation of the PCR assay, this molecular test is compared to another that is considered the standard in diagnostics. Depending on the pathogen, the gold standard for detection may be a culture, method, microscopy, or serology. The best PCR applications have significant congruence with culture-based methods for detecting pathogens, with few false-positive and false-negative results. Controlling for sample contamination or carryover contamination, to be discussed below, false-positive results may be attributed to (*a*) dead cells and a stable template (Wolffs et al. 2005); (*b*) damaged cells requiring resuscitation (Reissbrodt et al. 2002); (*c*) selectivity of the culture enrichment method (Iveson & Kovacs 1967); or (*d*) bacterial cells existing in a VBNC state (Oliver 2010).

LIMITATIONS TO CULTURE-BASED METHODS FOR PATHOGEN DETECTION

The broth enrichment cultures themselves vary in their uniformity in isolating a specific pathogen from a given sample type (Iveson & Kovacs 1967). For example, *Salmonella* isolation frequency varies depending on which enrichment media are used (Iveson & Kovacs 1967) or whether a delayed secondary enrichment is required (Waltman et al. 1991). However as a screen, the PCR assay has proven effective in identifying and improving pathogen isolation for samples requiring a delayed secondary enrichment (Liu et al. 2002). False-positive results may also be attributed to cells within the pathogen population that have an atypical biochemical profile (e.g., H_2S-negative

Salmonella) (Olsen et al. 1992), which is inadvertently missed in the enrichment culture, isolation, and identification process. Detection of pathogenic *E. coli* is especially challenging using culture-based methods for detection, as there are so few biochemical differences to set them apart from commensal *E. coli*. When a biochemical difference is identified (e.g., sorbitol fermentation and *E. coli* O157:H7), biochemically atypical isolates (Karch et al. 1993) may be missed but identified following a PCR-based assay. Even when reliable biochemical tests are available for detecting pathogenic *E. coli*, such as enterohemorrhagic *E. coli* O157, they are the minority relative to the majority of commensal *E. coli*, hence immunomagnetic separation is needed to enrich this minority population (Fu et al. 2005).

The choice of enrichment and differential culture media, additional modifications, and its application to a specific sample type need to be considered in the detection of pathogen *x* in sample *y*. Without confirmatory tests, the enrichment or differential medium may prove inappropriate for identifying pathogen *x* in sample *y* and thus erroneously report more culture-positive results than the PCR test (Blanco-Abad et al. 2009). Therefore, disagreement between culture and PCR methods may be due more to the inappropriateness of the culture method used and therefore obtain erroneous results for detecting the pathogen by culture method (Blanco-Abad et al. 2009).

PCR CARRYOVER CONTAMINATION

The PCR assay is a powerful, sensitive tool for detecting as few as one cell. Its sensitivity is attributed to multiple rounds of DNA replication that amplifies the DNA target exponentially to levels detectable with fluorescent dyes. In 30 cycles, the PCR can produce millions of amplicon molecules. While contained within its capped microfuge tube, simply opening the PCR tube can aerosolize the reaction mix, thereby contaminating surfaces that come in contact with the dispersed amplicon. The amplicon itself can now serve as a template rendering every subsequent PCR assay positive that comes in contact with contaminated pipettes, hands, or reagents.

The best way to address carryover PCR contamination is to introduce measures that avoid or lessen the likelihood of reagent contamination in the initial PCR setup. This can be accomplished by physically separating the three stages of any PCR protocol: (*a*) sample and template preparation; (*b*) PCR setup; and (*c*) analysis of PCR results (e.g., gel electrophoresis). A second, self-contained room for the PCR setup is critical. All reagents, materials, lab coats, disposable gloves, and pipettors should be kept here, including the –20°C freezer for storing templates, controls, primers, and other PCR reagents. Rather than having one tube of reagent (e.g., buffer), it is recommended to dispense aliquots into separate tubes. After finishing with PCR setup, it is best to dispose of the remaining reagent rather than return it to the freezer. This strategy is likely to reduce chances for PCR carryover contamination of reagents or at least help identify the likely reagent (e.g., *Taq* DNA polymerase) that became contaminated.

A set of micropipettors should be designated for this room, never to leave the PCR setup station. Barrier tips are also a necessity for performing PCR assays on a routine basis in the laboratory. These tips prevent backwash of fluids from contaminating the barrel or shaft of the pipettor. However, micropipettors can be disassembled and the component parts cleaned with bleach. Bleach is fairly effective in decontaminating PCR-contaminated surfaces. It is also recommended to set up all PCR reactions in a UV-illuminated PCR workstation or hood. Illuminating the workstation with UV light before and after each PCR setup is recommended for decontaminating surfaces within the hood. In the PCR setup room, it is recommended to have laboratory personnel wear designated lab coats and disposable gloves. A logical workflow is also recommended, with each stage physically separated or confined within specified areas in the laboratory, and assay activities should proceed in the direction from template preparation to PCR setup, thermocycler run, and

final analysis (gel electrophoresis). One should limit back and forth movement especially at the later stages. The handling of PCR tubes and setting up of gel electrophoresis following the thermocycler run can introduce PCR carryover contamination by the technician. Going back into the PCR setup room increases the likelihood of contamination of reagents, especially if laboratory personnel do not change lab coats and dispose of gloves prior to entering the room. If these PCR stages cannot be physically separated due to room constraints, a UV PCR workstation/hood is essential.

In addition to the measures described above, substituting dTTP with dUTP and a pretreatment step with uracil *N*-glycosylase can eliminate carryover PCR contamination (Erlich et al. 1991). Uracil is incorporated into amplicons during the PCR assay, producing products that are susceptible to degradation with uracil *N*-glycosylase. Amplicons contaminating the PCR reaction mix are destroyed with initial preincubation at 37°C with uracil *N*-glycosylase. However, the DNA template itself is resistant to this enzymatic degradation. The initial DNA denaturation step at 94°C inactivates the uracil *N*-glycosylase and allows new amplicons to be produced during the PCR.

Real-time PCR, by design, reduces the likelihood of PCR carryover contamination. The amplicon is detected as it is synthesized in real time. Once the PCR reaction has been set up, the tube never needs to be opened again, as the fluorometer can detect fluorescence within the sealed tube. There is no need for additional handling of the PCR reaction tubes once the samples are set and placed in the real-time PCR thermocycler.

DIFFERENTIATING LIVE FROM DEAD CELLS

One of PCR's greatest weaknesses is its inability to distinguish dead from live cells. DNA serves as a template in most PCR tests. This molecule is quite stable as can be attested by the procedures developed for PCR assays that employ boiling (Madico et al. 1995) or ethanol treatment (Hilton et al. 1997) of cells in the template's preparation. At least two studies have revealed DNA stability following cell death (McKillip et al. 1999, Wolffs et al. 2005). The use of the DNA cross-linking agent ethidium monoazide (Soejima et al. 2008) can be exploited for detecting only live bacterial cells by PCR assay. This chemical agent permeates the cell membrane of dead cells and irreversibly cross-links DNA (Nogva et al. 2003). The cross-linked DNA cannot serve as a template in the PCR reaction. This application was successful in distinguishing between live and dead *L. monocytogenes* cells (Soejima et al. 2008), and dead versus live or VBNC *Campylobacter* (Josefsen et al. 2010).

Alternatively, RNA has been explored as a substitute template in PCR assays (Gonzalez-Escalona et al. 2009, McIngvale et al. 2002, McKillip et al. 1998, Werbrouck et al. 2007), as this molecule, depending on the treatment conditions, is quickly degraded upon death of the bacterial cell (McKillip et al. 1998). RNases are quite prevalent in the environment, requiring the inclusion of inhibitors (e.g., DEPC) (Permutt et al. 1976) to prevent the premature degradation of RNA. In the bacterial cell, mRNA generally has a short half-life. However, depending on the gene, the mRNA half-life can vary from 40 s to 20 min (Pedersen & Reeh 1978). Upon death, RNA is quickly degraded, undetectable by PCR within <2 h (McIngvale et al. 2002). Some RNA molecules within the cell exhibit greater stability and take longer to decay to below the level of PCR detection upon death of the cell (McKillip et al. 1998). Careful consideration, therefore, needs to be given in that the target primers need to be matched well with the nucleic acid template.

Another explanation in the reporting of PCR false-positive results may be attributed to bacteria in the VBNC state. The VBNC state may be attributed to the bacterial cell's adaptation to harsh environmental conditions, starvation, or physical injury to the cell (Oliver 2010) that may necessitate resuscitation (Reissbrodt et al. 2002). Several enrichment culture methods currently used in the isolation and detection of foodborne bacterial pathogens employ temperatures, chemicals

(dyes, iodine, etc.), and antibiotics to suppress the unwanted growth of commensal bacteria and favor the growth of the pathogen. However, these same enrichment conditions may not favor growth of injured cells without a preenrichment culture (Liao & Fett 2003) or resuscitation of bacterial cells (Reissbordt et al. 2002) from the VBNC to a growth state. When *Campylobacter* enters the VBNC state, the normally helical bacterial cell rounds up into a cocci shape (Rollins & Colwell 1986). This type of profound change in cell shape is due to alterations in the cell wall (Costa et al. 1999, Spratt 1975). The media used to isolate *Campylobacter* includes cephalosporins, which are cell wall inhibitors (Bolton et al. 1983) that may prevent *Campylobacter* from exiting its VBNC state. *Campylobacter* and *Vibrio* are quite adept at transitioning into a VBNC state and persisting in the environment, especially an aquatic environment (Brayton et al. 1987, Rollins & Colwell 1986). The VBNC state is a limitation of the current culture methodology in detecting some pathogens.

FUTURE

The PCR has advanced considerably in the past twenty years, from conceptual (Saiki et al. 1988) to validation, harmonization (Malorny et al. 2003a,b), and use in the diagnostic laboratory setting (USDA Animal and Plant Health Inspection Service 2010; USDA Food Safety and Inspection Service 2007, 2008, 2009). In fact, the PCR assay is the only way some foodborne pathogens (e.g., noroviruses) can be detected in foods and the environment because of our current inability to cultivate them in the laboratory (Gentry et al. 2009). What will be the significant technological advances in the next 20 years? With the current rate of advances in high-throughput sequencing, the cost associated with this technology is expected to decrease at most to $1,000 per 1 billion bp genome(s) or one U.S. dollar per bacterial genome (1 million bp genome) (Schloss 2008). This means affordable high-throughput sequencing could replace some of the current antimicrobial susceptibility testing, serotyping, and strain-typing methods presently used by diagnostic laboratories. The new technology is expected to identify new pathogens associated with those 33% of foodborne outbreaks of unknown etiology [Surveillance for foodborne disease outbreaks—United States 2007. (2010)]. There will always be a need for standard culture and isolation of pathogens, except now it will be to assess the microbe's susceptibility to certain manufacturing processes developed to reduce or eliminate them from foods.

SUMMARY POINTS

1. The PCR has become an important diagnostic tool in the detection of foodborne pathogens.
2. Current PCR technology allows for rapid detection of pathogens in real time, because of fluorescence monitors built into the thermocycler. Real-time PCR assays can provide information regarding pathogen cell numbers in a sample.
3. False-negative results sometimes occur with any PCR test because (*a*) pathogen loads are below the limit of detection or (*b*) PCR inhibitors are present.
4. PCR false-negative results can be reduced by (*a*) using sample enrichment cultures, (*b*) adopting protocols that remove inhibitors or concentrate a pathogen template, (*c*) substituting *Taq* DNA polymerase with inhibitor-resistant polymerase, or (*d*) adding PCR enhancers and facilitators. Inclusion of an internal amplification control (IAC) can alert the user of problems with the PCR and template.

5. PCR false-positive results sometimes occur due to (*a*) nonspecific amplicons, (*b*) carryover contamination of PCR reagents or setup equipment, (*c*) presence of an atypical pathogen, (*d*) inability of the PCR assay to distinguish live from dead cells, or (*e*) presence of injured cells or cells existing in a VBNC state. Nonspecific amplicons and carryover contamination can be countered by adopting TAQMAN or molecular beacon real-time PCR assays. A preenrichment culture step may be useful in the recovery of injured cells. Using mRNA as target template or ethidium monoazide allows the user to detect only the viable cells present in the sample.

FUTURE ISSUES

1. As more PCR protocols become validated, harmonized, and commercialized, this molecular technique may become a standard tool of many food microbiology laboratories.
2. With recent advances in high-throughput sequencing, previously unrecognized viruses, bacteria, and protozoans associated with foodborne illnesses will be discovered, which in turn will lead to the development of new diagnostic tests for pathogens in foods.
3. High-throughput sequencing will become the prominent tool in epidemiologic investigations of foodborne outbreaks. With a decrease in cost, nucleic acid sequencing may supplant existing methods for determining antibiotic susceptibility, serotyping, and strain typing.

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LITERATURE CITED

Abdulmawjood A, Roth S, Bulte M. 2002. Two methods for construction of internal amplification controls for the detection of *Escherichia coli* O157 by polymerase chain reaction. *Mol. Cell. Probes* 16:335–39

Al-Soud WA, Jonsson LJ, Radstrom P. 2000. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *J. Clin. Microbiol.* 38:345–50

Al-Soud WA, Radstrom P. 2001. Purification and characterization of PCR-inhibitory components in blood cells. *J. Clin. Microbiol.* 39:485–93

Bassler HA, Flood SJ, Livak KJ, Marmaro J, Knorr R, Batt CA. 1995. Use of fluorogenic probe in a PCR-based assay for the detection of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 61:3724–28

Beuret C. 2004. Simultaneous detection of enteric viruses by multiplex real-time RT-PCR. *J. Virol. Methods* 115:1–8

Blanco-Abad V, Ansede-Bermejo J, Rodriguez-Castro A, Martinez-Urtaza J. 2009. Evaluation of different procedures for the optimized detection of *Vibrio parahaemolyticus* in mussels and environmental samples. *Int. J. Food Microbiol.* 129:229–36

Bolton FJ, Coates D, Hinchliffe PM, Robertson L. 1983. Comparison of selective media for isolation of *Campylobacter jejuni/coli*. *J. Clin. Pathol.* 36:78–83

Brayton PR, Tamplin ML, Huq A, Colwell RR. 1987. Enumeration of *Vibrio cholerae* O1 in Bangladesh waters by fluorescent-antibody direct viable count. *Appl. Environ. Microbiol.* 53:2862–65

Butot S, Putallaz T, Sanchez G. 2007. Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. *Appl. Environ. Microbiol.* 73:186–92

Casas I, Tenorio A, Echevarría JM, Klapper PE, Cleator GM. 1997. Detection of enteroviral RNA and specific DNA of herpesviruses by multiplex genome amplification. *J. Virol. Methods* 66:39–50

CDC PHLIS Surveillance Data. 2007. *Salmonella* Annual Survey 2006. **http://www.cdc.gov/ncidod/dbmd/phlisdata/salmonella.htm#2006**

Choi JS, Kim JS, Joe CO, Kim S, Ha KS, Park YM. 1999. Improved cycle sequencing of GC-rich DNA template. *Exp. Mol. Med.* 31:20–24

Clawson ML, Keen JE, Smith TPL, Durso LM, McDaeld TG, et al. 2009. Phylogenetic classification of *Escherichia coli* O157:H7 strains of human and bovine origin using a novel set of nucleotide polymorphisms. *Genome Biol.* 10:R56

Coetzee B, Freeborough MJ, Maree HJ, Celton JM, Rees DJG, Burger JT. 2010. Deep sequencing analysis of viruses infecting grapevines: virome of a vineyard. *Virology* 400:157–63

Comelli HL, Rimstad E, Larsen S, Myrmel M. 2008. Detection of norovirus genotype I.3b and II.4 in bioaccumulated blue mussels using different virus recovery methods. *Int. J. Food Microbiol.* 127:53–59

Cooke FJ, Wain J, Fookes M, Ivens A, Thomson N, et al. 2007. Prophage sequences defining hot spots of genome variation in *Salmonella enterica* serovar Typhimurium can be used to discriminate between field isolates. *J. Clin. Microbiol.* 45:2590–98

Costa K, Bacher G, Allmaier G, Dominguez-Bello MG, Engstrand L, et al. 1999. The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. *J. Bacteriol.* 181:3710–15

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, et al. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* 318:283–87

Cremonesi P, Castiglioni B, Malferrar G, Biunno I, Vimercati C, et al. 2006. Technical note: improved method for rapid DNA extraction of mastitis pathogens directly from milk. *J. Dairy Sci.* 89:163–69

Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P. 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J. Clin. Microbiol.* 42:3819–22

Erlich HA, Gelfand D, Sninsky JJ. 1991. Recent advances in the polymerase chain reaction. *Science* 252:1643–51

Eyigor A, Carli KT, Unal CB. 2002. Implementation of real-time PCR to tetrathionate broth enrichment step of *Salmonella* detection in poultry. *Lett. Appl. Microbiol.* 34:37–41

Feder I, Nietfeld JC, Galland J, Yearly T, Sargeant JM, et al. 2001. Comparison of cultivation and PCR-hybridization for detection of *Salmonella* in porcine fecal and water samples. *J. Clin. Microbiol.* 39:2477–84

Frazar CD, Orlandi PA. 2007. Evaluation of two DNA template preparation methods for post-immunomagnetic separation detection of *Cryptosporidium parvum* in foods and beverages by PCR. *Appl. Environ. Microbiol.* 73:7474–76

Fu Z, Rogelj S, Kieft TL. 2005. Rapid detection of *Escherichia coli* O157:H7 by immunomagnetic separation and real-time PCR. *Int. J. Food Microbiol.* 99:47–57

Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR Jr. 1997. Advanced multiplexed analysis with the FlorMetrix^{TM} system. *Clin. Chem.* 43:1749–56

Gentry J, Vinjé J, Lipp EK. 2009. A rapid and efficient method for quantitation of genogroups I and II norovirus from oysters and application in other complex environmental samples. *J. Virol. Methods* 156:59–65

Gilmour MW, Graham M, Van Domselaar G, Tyler S, Kent H, et al. 2010. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. *BMC Genomics* 11:120

Gonzalez-Escalona N, Hammack TS, Russell M, Jacobson AP, De Jueus AJ, et al. 2009. Detection of live *Salmonella* sp. cells in produce by a TaqMan-based quantitative reverse transcriptase real-time PCR targeting *invA* mRNA. *Appl. Environ. Microbiol.* 75:3714–20

Greenfield L, Bjorn MJ, Horn G, Fong D, Buck GA, et al. 1983. Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage beta. *Proc. Natl. Acad. Sci. USA* 80:6853–57

Grissa I, Bouchon P, Pourcel C, Vergnaud G. 2008. On-line resources for bacterial micro-evolution studies using MLVA or CRISPR typing. *Biochimie* 90:660–68

Hagren V, von Lode P, Syrjala A, Korpimaki T, Tuomola M, et al. 2008. An 8-hour system for *Salmonella* detection with immunomagnetic separation and homogeneous time-resolved fluorescence PCR. *Int. J. Food Microbiol.* 125:158–61

Haramoto E, Katayama H, Ohgaki S. 2004. Detection of noroviruses in tap water in Japan by means of a new method for concentrating enteric viruses in large volumes of freshwater. *Appl. Environ. Microbiol.* 70:2154–60

Herbelin CJ, Chirillo S, Melnick KA, Whittam TS. 2000. Gene conservation and loss in the *mutS-rpoS* genomic regions of pathogenic *Escherichia coli*. *J. Bacteriol.* 182:5381–90

Hilton AC, Banks JG, Penn CW. 1997. Optimization of RAPD for fingerprinting *Salmonella*. *Lett. Appl. Microbiol.* 24:243–48

Hofreuter D, Tsai J, Watson RO, Novik V, Altman B, et al. 2006. Unique features of highly pathogenic *Campylobacter jejuni* strain. *Infect. Immun.* 74:4694–707

Hong Y, Berrang M, Liu T, Hofacre C, Sanchez S, et al. 2003. Rapid detection of *Campylobacter coli*, *C. jejuni* and *Salmonella enterica* on poultry carcasses using PCR-enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* 69:3492–99

Hong Y, Liu T, Lee MD, Hofacre CL, Maier M, et al. 2008. Rapid screening of *Salmonella enterica* serovars Enteritidis, Hadar, Heidelberg and Typhimurium using a serologically-correlative allelotyping PCR targeting the O and H antigen alleles. *BMC Microbiol.* 8:178

Hong Y, Liu T, Lee MD, Hofacre CL, Maier M, et al. 2009. A rapid screen of broth enrichments for *Salmonella enterica* serovars Enteritidis, Hadar, Heidelberg, and Typhimurium using an allelotyping multiplex PCR that targets O and H antigen alleles. *J. Food Prot.* 72:2198–201

Hu H, Lan R, Reeves PR. 2002. Fluorescent amplified fragment length polymorphism analysis of *Salmonella enterica* serovar Typhimurium reveals phage-type-specific markers and potential for microarray typing. *J. Clin. Microbiol.* 40:3406–15

Iturriza-Gomara M, Kang G, Gray J. 2004. Rotavirus genotyping: keeping up with an evolving population of human rotaviruses. *J. Clin. Virol.* 31:259–65

Iveson JB, Kovacs N. 1967. A comparative trial of Rappaport enrichment medium for the isolation of salmonellae from faeces. *J. Clin. Pathol.* 20:290–93

Jean J, D'Souza DH, Jaykus LA. 2004. Multiplex nucleic acid sequence-based amplification for simultaneous detection of several enteric viruses in model ready-to-eat foods. *Appl. Environ. Microbiol.* 70:6603–10

Josefsen MH, Löfström C, Hansen TB, Christensen LS, Olsen JE, Hoorfar J. 2010. Rapid quantification of viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and propidium monoazide treatment, as a tool for quantitative risk assessment. *Appl. Environ. Microbiol.* 76:5097–104

Jothikumar N, Cliver DO, Mariam TW. 1998. Immunomagnetic capture PCR for rapid concentration and detection of hepatitis A virus from environmental samples. *Appl. Environ. Microbiol.* 64:504–8

Kapperud G, Vardund T, Skjerve E, Hornes E, Michaelsen TE. 1993. Detection of pathogenic *Yersinia enterocolitica* in foods and water by immunomagnetic separation, nested polymerase chain reactions, and colorimetric detection of amplified DNA. *Appl. Environ. Microbiol.* 59:2938–44

Karch H, Bohm H, Schmidt H, Gunzer F, Aleksic S, Heesemann J. 1993. Clonal structure and pathogenicity of Shiga-like toxin-producing, sorbitol-fermenting *Escherichia coli* O157:H-. *J. Clin. Microbiol.* 31:1200–5

Kim D, Kim SR, Kwon KS, Lee JW, Oh MJ. 2008. Detection of hepatitis A virus from oyster by nested PCR using efficient extraction and concentration method. *J. Microbiol.* 46:436–40

Kornberg A. 2000. Ten commandments: lessons from the enzymology of DNA replication. *J. Bacteriol.* 182:3613–18

Kotewicz ML, Mammel MK, LeClerc JE, Cebula TA. 2008. Optical mapping and 454 sequencing of *Escherichia coli* O157:H7 isolates linked to the US 2006 spinach-associated outbreak. *Microbiology* 154:3518–28

Kwok S, Mack DH, Mullis KB, Poiesz B, Ehrlich G, et al. 1987. Identification of human immunodeficiency virus sequences by using in vitro enzymatic amplification and oligomer cleavage detection. *J. Virol.* 61:1690–94

Lalonde LF, Gajadhar AA. 2008. Highly sensitive and specific PCR assay for reliable detection of *Cyclospora cayetanensis* oocysts. *Appl. Environ. Microbiol.* I74:4354–58

Leggitt PR, Jaykus LA. 2000. Detection methods for human enteric viruses in representative foods. *J. Food Prot.* 63:1738–44

Liao CH, Fett WF. 2003. Isolation of *Salmonella* from alfalfa seed and demonstration of impaired growth of heat-injured cells in seed homogenates. *Int. J. Food Microbiol.* 82:245–53

Lindqvist R. 1997. Preparation of PCR samples from food by a rapid and simple centrifugation technique evaluated by detection of *Escherichia coli* O157:H7. *Int. J. Food Microbiol.* 37:72–82

Liu T, Liljebjelke K, Bartlett E, Hofacre CL, Sanchez S, Maurer JJ. 2002. Application of nested PCR to detection of *Salmonella* in poultry environments. *J. Food Prot.* 65:1227–32

Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl.* 4:357–62

Lopez-Saucedo C, Cerna JF, Villegas-Sepulveda N, Thompson R, Velazquez FR, et al. 2003. Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic *Escherichia coli*. *Emerg. Infect. Dis.* 9:127–31

Luan C, Levin RE. 2008. Use of activated carbon coated with bentonite for increasing sensitivity of PCR detection of *Escherichia coli* O157:H7 in Canadian oyster (*Crassostrea gigas*) tissue. *J. Microbiol. Methods* 72:67–72

Lund M, Nordentoft S, Pedersen K, Madsen M. 2004. Detection of *Campylobacter spp.* in chicken fecal samples by real-time PCR. *J. Clin. Microbiol.* 42:5125–32

Madico G, Akopyants NS, Berg DE. 1995. Arbitrarily primed PCR DNA fingerprinting of *Escherichia coli* O157:H7 strains by using templates from boiled cultures. *J. Clin. Microbiol.* 33:1534–36

Malorny B, Hoorfar J, Hugas M, Heuvelink A, Fach P, et al. 2003a. Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method. *Int. J. Food Microbiol.* 89:241–49

Malorny B, Tassios PT, Rådström P, Cook N, Wagner M, Hoorfar J. 2003b. Standardization of diagnostic PCR for the detection of foodborne pathogens. *Int. J. Food Microbiol.* 83:39–48

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, et al. 2005. Genome sequencing in open microfabricated high density picoliter reactors. *Nature* 437:376–80

Matsuda M, Barsdale L. 1967. System for the investigation of the bacteriophage-directed synthesis of diphtheria toxin. *J. Bacteriol.* 93:722–30

Maurer JJ, Schmidt D, Petrosko P, Sanchez S, Bolton LF, Lee MD. 1999. Development of primers to O-antigen biosynthesis genes for specific detection of *Escherichia coli* O157 by PCR. *Appl. Environ. Microbiol.* 65:2954–60

Maze A, Boel G, Zuniga M, Bourand A, Loux V, et al. 2010. Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. *J. Bacteriol.* 192:2647–48

McHugh TM, Miner RC, Logan LH, Stites DP. 1988. Simultaneous detection of antibodies to cytomegalovirus and herpes simplex virus by using flow cytometry and a microsphere-based fluorescence immunoassay. *J. Clin. Microbiol.* 26:1957–61

McIngvale SC, Hlhanai D, Drake MA. 2002. Optimization of reverse transcriptase PCR to detect viable shiga-toxin-producing *Escherichia coli*. *Appl. Environ. Microbiol.* 68:799–806

McKenna P, Hoffmann C, Minkah N, Aye PP, Lackner A, et al. 2008. The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. *PLoS Pathog.* 4:e20

McKillip JL, Jaykus L, Drake M. 1998. rRNA stability in heat-killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 64:4264–68

McKillip JL, Jaykus LA, Drake M. 1999. Nucleic acid persistence in heat-killed *Escherichia coli* O157:H7 from contaminated skim milk. *J. Food Prot.* 62:839–44

Mikhailovich VM, Melnikov VG, Mazurova IK, Wachsmuth IK, Wenger JD, et al. 1995. Application of PCR for detection of toxigenic *Corynebacterium diphtheriae* strains isolated during the Russian diphtheria epidemic, 1990 through 1994. *J. Clin. Microbiol.* 33:3061–63

Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, et al. 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *J. Clin. Microbiol.* 35:995–98

Morrison TB, Weis JJ, Wittwer CT. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* 24:954–58, 960, 962

Mumma GA, Griffin PM, Meltzer MI, Braden CR, Tauxe RV. 2004. Egg quality assurance programs and egg-associated *Salmonella enteritidis* infections, United States. *Emerg. Infect. Dis.* 10:1782–89

Murphy E, Hieny S, Sher A, O'Garra A. 1993. Detection of in vivo expression of interleukin-10 using a semi-quantitative polymerase chain reaction method in *Schistosoma mansoni* infected mice. *J. Immunol. Methods* 162:211–23

New performance standards for *Salmonella* and *Campylobacter* in young chicken and turkey slaughter establishments; new compliance guides. 2010. *Fed. Regist.* 75:27288–94

Nogva HK, Drømtorp SM, Nissen H, Rudi K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease PCR. *Biotechniques* 34:804–8, 810, 812–13

O'Leary J, Corcoran D, Lucey B. 2009. Comparison of the EntericBio multiplex PCR system with routine culture for detection of bacterial enteric pathogens. *J. Clin. Microbiol.* 47:3449–53

Oberst RD, Hays MP, Bohra LK, Phebus RK, Yamashiro CT, et al. 1998. PCR-based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with an internal fluorogenic probe and the 5′ nuclease (TaqMan) assay. *Appl. Environ. Microbiol.* 64:3389–96

Ohler LD, Rose EA. 1992. Optimization of long-distance PCR using a transposon-based model system. *PCR Methods Appl.* 2:51–59

Olive DM. 1989. Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. *J. Clin. Microbiol.* 27:261–65

Oliver JD. 2010. Recent finding on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Lett.* 34:415–25

Olsen JE, Brown DJ, Baggesen DL, Bisgaard M. 1992. Biochemical and molecular characterization of *Salmonella enterica* serovar Berta, and comparison of methods for typing. *Epidemiol. Infect.* 108:243–60

Omiccioli E, Amagliani G, Brandi G, Magnani M. 2009. A new platform for real-time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. *Food Microbiol.* 26:615–22

Opel KL, Chung D, McCord BR. 2010. A study of PCR inhibition mechanisms using real time PCR. *J. Foren. Sci.* 55:25–33

Paton AW, Paton JC. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA*, *rfbO111*, and *rfbO157*. *J. Clin. Microbiol.* 36:598–602

Pavic A, Groves PJ, Bailey G, Cox JM. 2009. A validated miniaturized MPN method, based on ISO 6579:2002, for the enumeration of *Salmonella* from poultry matrices. *J. Appl. Microbiol.* 109:25–34

Pedersen S, Reeh S. 1978. Functional mRNA half lives in *E. coli*. *Mol. Gen. Genet.* 166:329–36

Permutt MA, Biesbroeck J, Chyn R, Boime I, Szczesna E, McWilliams D. 1976. Isolation of a biologically active messenger RNA: preparation from fish pancreatic islets by oligo(2′-deoxythymidylic acid) affinity chromatography. *Ciba Found. Symp.* 41:97–116

Poly F, Read TD, Chen YH, Monteiro MA, Serichantalergs O, et al. 2008. Characterization of two *Campylobacter jejuni* strains for use in volunteer experimental-infection studies. *Infect. Immun.* 76:5655–67

Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galan JE, et al. 1992. Amplification of *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes* 6:271–79

Raja S, El-Hefnawy T, Kelly LA, Chestney ML, Luketich JD, Godfrey TE. 2002. Temperature-controlled primer limit for multiplexing of rapid, quantitative reverse transcription-PCR assays: application to intraoperative cancer diagnostics. *Clin. Chem.* 48:1329–37

Regan PM, Margolin AB. 1997. Development of a nucleic acid capture probe with reverse transcriptase-polymerase chain reaction to detect poliovirus in groundwater. *J. Virol. Methods* 64:65–72

Reissbrodt R, Rienaecker I, Romanova JM, Freestone PPE, Haigh RD, et al. 2002. Resuscitation of *Salmonella enterica* serovar Typhimurium and enterohemorrhagic *Escherichia coli* from the viable but nonculturable state by heat-stable enterobacterial autoinducer. *Appl. Environ. Microbiol.* 68:4788–94

Richards GP, Watson MA, Kingsley DH. 2004. A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. *J. Virol. Methods* 116:63–70

Rodríguez-Lázaro D, Pla M, Scortti M, Monzó HJ, Vázquez-Boland JA. 2005. A novel real-time PCR for *Listeria monocytogenes* that monitors analytical performance via an internal amplification control. *Appl. Environ. Microbiol.* 71:9008–12

Rollins DM, Colwell RR. 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl. Environ. Microbiol.* 52:531–38

Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P. 1996. Real-time DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.* 242:84–89

Rudi K, Høidal HK, Katla T, Johansen BK, Nordal J, Jakobsen KS. 2004. Direct real-time PCR quantification of *Campylobacter jejuni* in chicken fecal and cecal samples by integrated cell concentration and DNA purification. *Appl. Environ. Microbiol.* 70:790–97

Rychlik W. 1995. Selection of primers for polymerase chain reaction. *Mol. Biotechnol.* 3:129–34

Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. 1986. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. *Nature* 324:163–66

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, et al. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–91

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, et al. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–54

Schena M, Shalon D, Davis RW, Brown PO. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467–70

Schloss JA. 2008. How to get genomes at one ten-thousandth the cost. *Nat. Biotechnol.* 26:1113–15

Schneeberger C, Speiser P, Kury F, Zellinger R. 1995. Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain. *PCR Methods Appl.* 4:234–38

Scope. 2010. *J. Clin. Microbiol.* **http://jcm.asm.org/misc/journal-ita_sco.dtl**

Selma MV, Martinez-Culebras PV, Aznar R. 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus caronarius* in wine grapes. *Int. J. Food Microbiol.* 122:126–34

Shan XC, Wolffs P, Griffiths MW. 2005. Rapid and quantitative detection of hepatitis A virus from green onion and strawberry rinses by use of real-time reverse transcription PCR. *Appl. Environ. Microbiol.* 71:5624–26

Soejima T, Iida KI, Qin T, Taniai H, Seki M, Yoshida SI. 2008. Method to detect only live bacteria during PCR amplification. *J. Clin. Microbiol.* 46:2305–13

Soumet C, Ermel G, Rose V, Rose N, Drouin P, et al. 1999. Identification by a multiplex PCR-based assay of *Salmonella typhimurium* and *Salmonella enteritidis* strains from environmental swabs of poultry houses. *Lett. Appl. Microbiol.* 29:1–6

Southern EM. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–17

Spratt BG. 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. *Proc. Natl. Acad. Sci. USA* 72:2999–3003

Stone GG, Oberst RD, Hays MP, McVey S, Chengappa MM. 1994. Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation-PCR procedure. *J. Clin. Microbiol.* 32:1742–49

Surveillance for foodborne disease outbreaks—United States, 2007. 2010. *MMWR* 59:973–79

Sutlovic D, Gamulin S, Definis-Gojanovic M, Gugic D, Adnjelinovic S. 2008. Interaction of humic acids with human DNA: proposed mechanisms and kinetics. *Electrophoresis* 29:1467–72

Tettelin H, Radune D, Kasif S, Khouri H, Salzberg SL. 1999. Optimized multiplex PCR: efficiently closing a whole-genome shotgun sequencing project. *Genomics* 62:500–7

Thomas EJ, King RK, Burchak J, Gannon VP. 1991. Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. *Appl. Environ. Microbiol.* 57:2576–80

Tian P, Engelbrekston, Mandrell R. 2008. Two-log increase in sensitivity for detection of norovirus in complex samples by concentration with procine gastric mucin conjubated to magnetic beads. *Appl. Environ. Microbiol.* 74:4271–76

Tian P, Mandrell R. 2006. Detection of norovirus capsid proteins in faecal and food samples by a real time immuno-PCR method. *J. Appl. Microbiol.* 100:564–74

Tyagi S, Kramer FR. 1996. Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* 14:303–8

USDA Animal and Plant Health Inspection Service (APHIS). 2010. NPIP approved rapid assays for *Salmonella*. **http://www.aphis.usda.gov/animal_health/animal_dis_spec/poultry/**

USDA Food Safety and Inspection Service (FSIS). 2007. FSIS procedure for the use of a polymerase chain reaction (PCR) assay for screening *Salmonella* in raw meat, carcass sponge samples, whole bird rinses, ready-to-eat meat and poultry products and pasteurized egg products. **http://www.fsis.usda.gov/PDF/MLG_4C_02.pdf**

USDA Food Safety and Inspection Service (FSIS). 2008. FSIS procedure for the use of *Escherichia coli* O157:H7 screening tests. **http://www.fsis.usda.gov/PDF/Mlg_5A_01.pdf**

USDA Food Safety and Inspection Service (FSIS). 2009. FSIS procedure for the use of a *Listeria monocytogenes* polymerase chain reaction (PCR) screening test. **http://www.fsis.usda.gov/PDF/MLG_8A_04.pdf**

Varadaraj K, Skinner DM. 1994. Denaturants or cosolvents improve the specificity of PCR amplification of a G + C-rich DNA using genetically engineered DNA polymerases. *Gene* 140:1–5

Waltman WD, Horne A, Pirkle C, Dickson T. 1991. Use of delayed secondary enrichment for the isolation of *Salmonella* in poultry and poultry environments. *Avian Dis.* 35:88–92

Wang L, Curd H, Qu W, Reeves PR. 1998. Sequencing of *Escherichia coli* O111 O-antigen gene cluster and identification of O111-specific genes. *J. Clin. Microbiol.* 36:3182–87

Wang L, Reeves PR. 1998. Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. *Infect. Immun.* 66:3545–51

Werbrouck H, Botteldoorn N, Uyttendaele M, Herman L, Van Coillie E. 2007. Quantification of gene expression of *Listeria monocytogenes* by real-time reverse transcription PCR: optimization, evaluation and pitfalls. *J. Microbiol. Methods* 69:306–14

Wilson IG. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63:3741–51

Wittwer CT, Fillmore GC, Hillyard DR. 1989. Automated polymerase chain reaction capillary tubes with hot air. *Nucleic Acid Res.* 17:4353–57

Wolffs PFG, Glencross K, Norling B, Griffiths MW. 2007. Simultaneous quantification of pathogenic *Campylobacter* and *Salmonella* in chicken rinse fluid by a flotation and real-time multiplex PCR procedure. *Int. J. Microbiol.* 117:50–54

Wolffs PF, Glencross K, Thibaudeau R, Griffiths MW. 2006. Direct quantitation and detection of salmonellae in biological samples without enrichment, using two-step filtration and real-time PCR. *Appl. Environ. Microbiol.* 72:3896–900

Wolffs P, Knutsson R, Norling B, Radstrom P. 2004. Rapid quantification of *Yersinia enterocolitica* in pork samples by a novel sample preparation method, flotation, prior to real-time PCR. *J. Clin. Microbiol.* 42:1042–47

Wolffs P, Norling B, Radstrom P. 2005. Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *J. Microbiol. Methods* 60:315–23

Woods DF, Reen FJ, Gilroy D, Buckley J, Frye JG, Boyd EF. 2008. Rapid multiplex PCR and real-time TaqMan PCR assays for detection of *Salmonella enterica* and the highly virulent serovars Choleraesuis and Paratyphi C. *J. Clin. Microbiol.* 46:4018–22

Yang H, Qu L, Wimbrow AN, Jiang X, Sun Y. 2007. Rapid detection of *Listeria monocytogenes* by nanoparticle-based immunomagnetic separation and real-time PCR. *Int. J. Food Microbiol.* 118:132–38

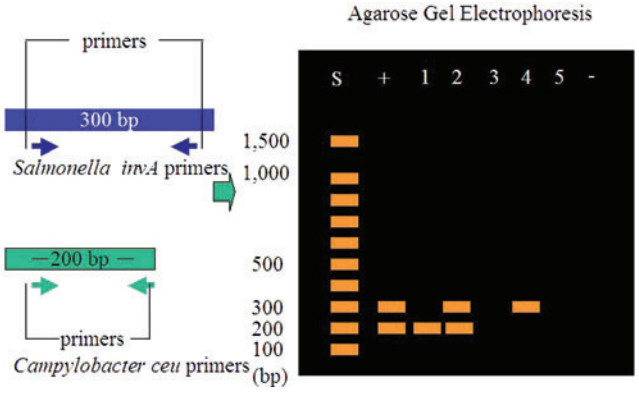

Figure 1

Multiplex PCR assay. PCR primers are designed to target sequences unique to pathogen *Salmonella* (*invA*) and *Campylobacter* (*ceu*) and also produce an amplicon with size that is unique for the pathogen being screened: *Salmonella*, 300-bp amplicon and *Campylobacter*, 200-bp amplicon. The primers are designed and PCR is optimized so that both primer pairs can work in the same reaction: a single test for two pathogens. PCRs for samples 1–5 are loaded into wells for gel electrophoresis. +, positive control and contains both *Salmonella* and *Campylobacter* template; −, negative control, water added in place of template; S, molecular weight standards for sizing PCR amplicons.

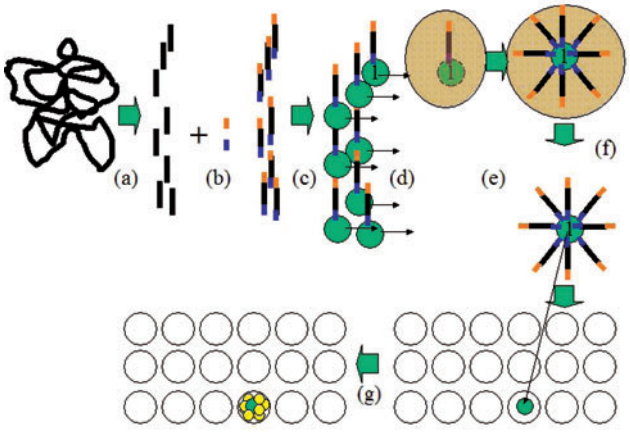

Figure 2

454 pyrosequencing. (*a*) DNA is sheared to produce smaller, 300–500-bp DNA fragments. (*b*) Oligonucleotide adapters are added to DNA fragments. (*c*) Through limiting dilution, individual DNA fragments are attached to a single bead. (*d*) The DNA-bound beads are placed in oil immersion–containing PCR reagents (primers, polymerase, etc.). A single oil droplet contains a single bead with a DNA fragment. (*e*) PCR amplifies the single DNA fragment into millions of copies now covering the surface of the bead. (*f*) Oil immersion is broken to free bead for its deposition into single well of a million-well slide. (*g*) Pyrosequencing reagents and enzymes are added to each well.

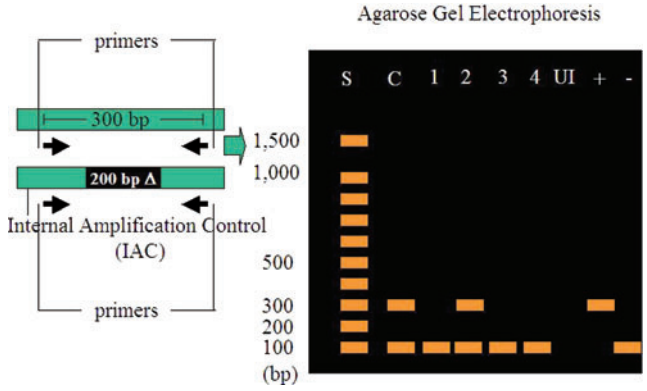

Figure 3

Internal amplification control (IAC). Primers are used to initially amplify targeted sequences. The amplicon is cloned and engineered to contain an internal 200-bp deletion. The plasmid containing the IAC is used to spike samples. Regardless of the pathogen's presence, all samples should be PCR assay-positive and produce the 100-bp amplicon for the IAC. If the pathogen is present, then two DNA fragments should be observed, the 300-bp amplicon for the pathogen and the 100-bp IAC amplicon. PCR reactions for samples 1–4 are loaded into wells for gel electrophoresis. C, positive control with IAC; UI, negative control: no IAC; -, IAC alone; S, molecular weight standards for sizing PCR amplicons.

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Errata

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