SPECIAL GUEST EDITOR SECTION

Molecular Methods for Identification and Detection of Bacterial Food Pathogens

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The polymerase chain reaction (PCR) shortens conventional microbiological methods for the detection of food pathogens either by replacing the conventional biochemical and serological identification or by its direct use on pre-enrichment media or food products. PCR allows fast and highly reliable identification of bacterial taxa, particularly phenotypically atypical bacterial strains. For reliablity, PCR primers and reaction conditions must be thoroughly optimized and evaluated, appropriate sample preparations must be developed, and a stringent laboratory protocol must be followed. Positive control systems are used to monitor possible inhibition of the reaction and negative controls are needed to monitor for contamination. The most recent developments involve messenger RNA-based (mRNA-based) detection of viable bacterial pathogens and real-time PCR quantitation of pathogens.

In the early 1990s, the polymerase chain reaction (PCR) was used for simple identification of pure bacterial cultures or colonies on agar plates. Since then, the development of sample preparations suitable for PCR detection of bacteria in food or pre-enrichment media has expanded enormously. PCR-based techniques such as nested-PCR, reverse transcription-PCR (RT-PCR), PCR-based fingerprinting, quantitative PCR, and alternative amplification techniques such as nucleic acid sequence-based amplification (NASBA) were introduced over the years. A few PCR-based tests for the most important food pathogens are now commercially available (Table 1). The most recent developments involve messenger RNA (mRNA)-based detection of viable bacterial pathogens, real-time PCR, and advanced typing techniques such as amplified fragment length polymorphism (AFLP).

Although molecular techniques have improved food microbiology to a great extent, they are not wonder techniques. There is a big difference between the theoretical possibilities of PCR (e.g., PCR has a sensitivity of one target copy) and its practical applications. Food microbiologists and molecular biologists agree that PCR has its limits. Certain techniques and methods look good and work well if used in research laboratories by skillful technicians, but are not useful for routine testing of food pathogens. In general, many problems related to food microbiology are not yet solved. This review discusses the potential use of molecular methods in food control microbiology and describes their possibilities and limitations.

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Nucleic Acid-Based Methods

A promising development in microbial diagnosis was the introduction of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) probes (1–3). The specificity problems inherent to the use of antibodies can be overcome by using RNA or DNA probes, which directly target the nucleic acid of an organism. Therefore, a positive hybridization signal is correlated with the presence of the organism. Although cloned probes can be used, most probes presently used are synthetic oligonucleotides. Several reviews have been published about the use of probes in food microbiology (4–11).

One of the main disadvantages of hybridization-based assays is that a relatively large number (typically 10^4–10^5) of target cells must be present to yield unambiguous results in a background containing large numbers of nontarget microorganisms (7). Therefore, hybridization with bacterial colonies on agar plates was often used in food microbiology. Several methods for the detection of food pathogens based on colony hybridization were published (12–14). This early DNA method relied on the use of radioactive labels, which was undesirable from a laboratory-safety point of view. Homogeneous in-solution hybridization can now be performed by using chemiluminiscent or colorimetric-based detection.

In the late 1980s, the PCR (15) was introduced as a promising in vitro technique for enzymatic amplification of target nucleic acid sequences using a specific pair of primers and a heat-stable DNA polymerase. PCR was presented as an extremely sensitive technique, theoretically able to perform successful amplification starting from a single copy of target DNA. Around 1990, the first PCR-based detection methods for bacterial food pathogens were described (16, 17).

Other DNA amplification techniques such as the ligase chain reaction (LCR; 18) were also described. LCR is based on the principle of ligation of 2 adjacent synthetic oligonucleotide primers, which hybridize uniquely to one strand of the target DNA. The junction of the 2 primers is positioned so that the nucleotide at the 3′ end of the upstream

Table 1. Commercially available PCR kits

System	Manufacturer	Identified or detected bacteria
	Hybridization-based	
Accuprobe	Gen-Probe, San Diego, CA	*L. monocytogenes*
		S. aureus
		C. jejuni, *C. coli*, *C. lari*
Gene-Trak	Gene-Trak, Hopkinton, MA	*Listeria* spp.
		Salmonella
		E. coli
		S. aureus
		Campylobacter
	PCR-based	
TaqMan	Applied Biosystems, Foster City, CA	*Salmonella*
		E. coli O157:H7
		E. coli STX1 en STX2
Probelia	Bio-Rad, Hercules, CA	*Salmonella*
		L. monocytogenes
		E. coli O157:H7
		C. jejuni, *C. coli*
BAX	Qualicon, Inc., Wilmington, DE	*Salmonella*
		L. monocytogenes
		Listeria spp.
		E. coli O157:H7

primer coincides with a potential single base-pair (bp) difference in the targeted sequence. The 2 adjoining primers can be covalently joined by the ligase only if the bp at that site matches the nucleotide at the 3′ end of the upstream primer. A second pair of primers complementary to the first pair is present. In a cycling reaction with a thermostable *Thermus aquaticus* DNA ligase, the ligated product can serve as a template for the next reaction cycle, leading to an exponential amplification process analogous to PCR amplification.

For the amplification of RNA, techniques such as RT-PCR, NASBA (19), self-sustained sequence replication (3SR; 20) and the transcription amplification system (TAS; 21) were developed. These techniques are all based on the initial conversion of RNA to copy DNA (cDNA) using a reverse transcriptase. In the case of RT-PCR, cDNA is amplified by a standard PCR reaction, whereas the other techniques are based on the in vitro transcription of cDNA using an RNA polymerase. In the NASBA and 3SR reaction, RNaseH is used for degradation of the RNA in the RNA:DNA duplex; the duplex is heat-denatured in the TAS reaction. A promotor for RNA polymerase is ligated to one (NASBA) or both (3SR) of the reaction primers.

The most widely used techniques for amplification of DNA and RNA remain PCR and RT-PCR, respectively.

Targets for PCR-Based Identification of Foodborne Pathogens

The choice of the best target for taxon-specific PCR identification of bacteria depends strongly on the heterogeneity within the taxon and the phylogenetic distance to other taxa. Although the highly conserved areas of the 16S and 23S ribosomal RNA (rRNA) genes have been used to study the relationships among distant bacterial taxa, the more variable regions of these genes are useful for differentiation of genera and species and, therefore, are used as targets for genus and sometimes species-specific PCR (16, 22–24). For the development of species-specific probes for highly related organisms, the intergenic rRNA spacer regions may be more preferred targets than the 16S and 23S rRNA genes themselves, because these noncoding regions are under minimal selective pressure during evolution and therefore vary more extensively than sequences within genes that have functional roles (25). Over the last years, the 16S–23S rRNA spacer region has been successfully used as a source for specific DNA probes (25–31).

For species such as *Escherichia coli* or *Yersinia enterocolitica*, where only virulence gene-harboring strains of the species are considered pathogenic, the use of virulence gene-based PCR is the only option for detection of pathogenic strains. PCR assays were described targeting the heat-labile (LT) or heat-stable (ST) toxin genes of enterotoxigenic *E. coli* (ETEC; 32, 33). Enterohemorrhagic *E. coli* (EHEC) and verotoxin or Shiga toxin-producing *E. coli* (VTEC/STEC) can be identified by using the verotoxin (VT) or Shiga toxin genes (*Stx*), the attaching and effacing gene (*eae*), or the hemolysin gene (*hly*; 32, 34–36). The enterotoxin gene *yst* (37), the attachment-invasion locus *ail* (38), and the invasin gene *inv* (39) were used to detect pathogenic bioserovars of *Y. enterocolitica*. Sometimes, however, certain bacterial strains contain nonfunctional virulence genes, example, the *inv* gene that is present in some nonpathogenic isolates of *Y. enterocolitica* (40). For species such as *Listeria monocytogenes*, in which all strains are considered potentially pathogenic, virulence gene-based PCR is only one of the options. Virulence gene-based PCR was described for *L. monocytogenes* using the listeriolysin O-gene (*hlyA*) or the invasion-associated protein gene (*iap*) as a target [*see* review by Scheu et al. (41)].

In choosing an appropriate target for bacterial identification, one must consider that many virulence factors are located on plasmids, which are often unstable and easily lost during laboratory manipulation. Hence, PCR detection of plasmid-encoded genes may lead to false-negative results. For example, many PCR assays for pathogenic *Y. enterocolitica* targeted virulence genes situated on the unstable pYV plasmid (42, 43). Repetitive genetic elements were described as targets for specific PCR by different authors: IS711 in *Brucella* species (44) and IS200 in *Salmonella* species (45). Flagellin genes were described as PCR targets for the detection of *Campylobacter* species (46). Unidentified DNA fragments are sometimes used as a target for PCR or hybridization. This is the case for the *Salmonella*-specific PCR

described by Aabo et al. (47), in which the primers were deduced from a cryptic, 2.3 kilobase DNA fragment.

Not all of the primers described in the international literature have been adequately evaluated for their specificity. Many of them should be tested against a suitable panel of relevant bacteria before being used in routine laboratories. A thoroughly evaluated specific PCR, however, may be a very valuable identification tool, especially for phenotypically atypical bacterial strains. Probes and primers, used for the identification and detection of different foodborne bacterial pathogens, were extensively reviewed by Olsen et al. (48), Feng (49), Hill (50), and Scheu et al. (41).

PCR-Based Detection of Bacterial Food Pathogens

PCR-based methods can be used to characterize strains that are isolated and purified by traditional culture methods. They replace the final step of the conventional method, i.e., the biochemical or serological confirmation. In this context, PCR is used as an identification tool for culture confirmation. On the other hand, it can also be used to shorten conventional methods for the detection of bacteria in foods by being applied either directly on the food product, on the pre-enrichment medium, or on selective agar media containing mixed cultures.

Detection of the PCR products is performed mostly by agarose gel electrophoresis or by nonradioactive hybridization with probes. The latter approach is often adopted by commercially available systems (Table 1) because hybridization is easier to automate; however, many research laboratories still apply standard agarose gel electrophoresis. In many cases, detection by hybridization increases PCR sensitivity 10^2–10^3-fold (50). When PCR is used to identify pure cultures (fluid or on agar media), problems are rarely encountered. PCR can be applied after a short sample preparation involving concentration, and lysis of the bacteria. When PCR is applied on pre-enrichment media, its possible inhibition by media or food components must be considered. In general, a short sample preparation, based on centrifugation, filtration, and washing steps, is sufficient to avoid such inhibition. A minimum of about 10^4 bacteria/mL enrichment medium is necessary for detection by a 30-cycle PCR. The length of the enrichment period needed to reach those numbers is strongly dependent on the pathogen and the food product. Bacteria are often present in foods in an injured state and require a period of recovery before they regain their full growth potential. Many of the agents used in selective enrichment media interfere with the process of repair in sublethally injured cells (51), leading to a significant extension of the lag/recovery phase or complete suppression of such cells. Therefore, for some bacteria, the isolation rate from food products is higher with a short nonselective enrichment than with a longer selective enrichment. PCR allows the detection of certain bacteria after a short nonselective enrichment (52). The level of competition between naturally occurring microorganisms and the test organism depends upon the selectivity of the medium (53, 54). For bacteria such as *Salmonella*, an incubation time of 16–20 h is sufficient to grow to a PCR-detectable amount (52), whereas for *L. monocytogenes*, shortening of the incubation time as described in conventional methods is not recommended for all types of samples, especially not for soft cheeses (55).

Direct detection of bacteria in food products by PCR is also possible but implies the use of a very sensitive PCR to meet the criteria set for the control of bacterial pathogens in food. The strictest criterion is absence of the pathogen in a certain amount of food product (often 25 g or 25 mL). This means that a nested-PCR (28, 30, 56–58) or a PCR with comparable sensitivity (40-cycle PCR) is necessary. However, such great sensitivity could lead to problems from contamination, especially in routine laboratories. Direct detection also requires the development of complex sample preparations to quantitatively recover the bacteria and efficiently remove any components that may inhibit the enzymatic reaction.

In summary, several problems that can be encountered when PCR-based methods are used to detect food pathogens are inhibition of the reaction, the presence of false-positive reactions caused by contamination, detection of dead bacteria, and results that are not quantitative. However, these problems can be overcome. Inhibition of the reaction can be avoided by using an adequate sample preparation and by optimizing the PCR reaction. Nevertheless, it is mandatory to monitor possible reaction inhibition by using positive controls. In order to avoid false-positive reactions, strict anticontamination precautions must be taken in PCR laboratories. The use of mRNA-based detection methods such as RT-PCR or NASBA rather than DNA-based methods such as PCR might allow detection of viable bacteria only. Semiquantitative results can be obtained by using one of the many quantitative PCR methods.

Sample Preparation

PCR is an enzymatic reaction with very high sensitivity when applied on pure DNA. In a PCR system, assuming a sensitivity of 1 cell per reaction tube, approximately 10^3 bacteria per mL sample are required to ensure a reliable and repeatable amplification (48, 59). However, components of the food sample, growth medium, or DNA extraction solutions may dramatically decrease the PCR sensitivity (60–62). A good overview of PCR-inhibiting components and their respective concentrations was published by Rossen et al. (60). It is often difficult to identify individual PCR-inhibitory substances in complex samples such as foods but, in some instances, inhibitors have been recognized. Powell et al. (61) found that a proteinase present in milk inhibits the PCR by degrading *Taq* polymerase. Bickley et al. (63) reported that calcium ions in milk are a major source of PCR inhibition and that the inhibitory effect can be partially reversed by increasing the magnesium concentration in the reaction above standard. Lantz et al. (64) found that the high $MgCl_2$ concentration in irgasan ticarcillin chlorate (ITC) medium was the major PCR inhibitory factor.

The level of PCR inhibition depends on the type of DNA polymerase used. Katcher and Schwartz (65) found that *Tth* DNA polymerase (isolated from *Thermus thermophilus*) maintained its polymerase activity in the presence of 2–5%

phenol-saturated buffer, whereas *Taq* DNA polymerase (isolated from *T. aquaticus*) was inactive under these conditions. Wiedbrauk et al. (66) found that *Tth* and *Tfl* (isolated from *T. flavus*) DNA polymerases were resistant to intraocular fluids, whereas *Taq* DNA polymerase was very sensitive to them. Recently the capacity of 9 thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples was evaluated (67). It was found that the PCR-inhibiting effect of various components in biological samples was somewhat eliminated by the use of the appropriate thermostable DNA polymerase.

Appropriate sample preparation methods are needed for the PCR-based detection of bacteria in foods. A suitable sample preparation concentrates the bacteria, extracts their DNA, and efficiently removes any component that may inhibit the enzymatic reaction. Because food samples vary in consistency, composition, and level and diversity of indigenous microbial competitors, each food/pathogen combination may require a different approach. Over the years, various sample preparation methods have been developed and evaluated.

Sample dilution is probably the easiest way to overcome inhibition; it also reduces PCR sensitivity. A short enrichment is also often used before PCR to enrich the bacteria and simultaneously dilute PCR inhibitors. However, some enrichment media can also inhibit amplification (60, 64).

Centrifugation or filtration or a combination of both can be used to remove large food particles from the sample or to concentrate bacteria (23, 52, 68). Washing steps (with water, phosphate-buffered saline, Triton X-100) in the protocol may increase PCR sensitivity. Disadvantages are that some bacteria have high affinity for food components (69) or may become entrapped by food particles. The choice of filter is also important, because food particles tend to block certain types of filters. Another disadvantage is that many bacteria partition at interphases during centrifugation.

Bacteria can be concentrated from the food product or enrichment medium by using their affinity for a solid phase. With the immunomagnetic separation (IMS) technique, bacteria are specifically bound to antibody-coated magnetic particles (52, 70–72). Rijpens et al. (52) reported that magnetic beads have a very high affinity for bacteria, in general, and even when 100% species- or genus-specific antibodies are used, these magnetic beads will never be specific for one species or genus. Lectin-coated magnetic microspheres can be used to bind bacteria aspecifically, but the type of food product strongly affects the efficiency of binding bacteria to lectins (73), which are proteins or glycoproteins with specific carbohydrate binding activities that can be isolated from a wide range of biological materials.

Aqueous 2-phase systems (62, 74) composed of 2 polymers with a different chemical structure allow the partitioning of PCR inhibitors and bacteria to separate phases. Lantz et al. (64) found that, in some cases, the PCR sensitivity is negatively affected because the target bacteria partition to the interphase of the aqueous 2-phase system or to the phase containing the PCR inhibitors.

Buoyant density centrifugation was described for the separation of bacteria from food particles based on differences in their buoyant densities in a gradient medium (64, 75, 76). The optimal concentration of the gradient medium and the centrifugation conditions are dependent on the buoyant densities of the food and the bacteria and on other bacterial characteristics (76). Attachment of the bacteria to the food sample, as seen in minced meat, may lead to a reduced PCR sensitivity (76).

More complicated sample preparations requiring handling by experienced personnel are described for the direct detection of certain bacteria in the food product. Herman et al. (28, 56, 57) described a sample preparation based on the chemical extraction of milk components for the detection of *L. monocytogenes* in raw milk and of *Clostridium tyrobutyricum* spores in raw milk and cheese. Rijpens et al. (69) enzymatically broke down fats and proteins present in raw milk to allow direct detection of *Brucella*.

Bacterial cells are efficiently lysed using enzymes such as lysozyme and/or proteinase K or by boiling or heating in water or denaturing solutions. Herman et al. (28) lysed *C. tyrobutyricum* spores by microwave treatment.

Various methods have been described to extract DNA from food products or enrichment media. DNA that has been released from bacterial cells can be separated from PCR-inhibitory substances by using glass beads, affinity columns, and other commercially available matrixes such as Glassmilk. Chelating resins such as Chelex 100 have a high affinity for polyvalent metal ions and can capture some inhibiting substances. Chelex 100 also has a protective effect against DNA degradation by chelating metal ions which catalyze the breakdown of DNA (77) and improves the lysis of Gram-positive bacteria (78). In some samples, inhibition is relieved by addition of bovine serum albumin (BSA) or certain enzymes such as trypsin inhibitor. BSA was reported to bind to the phenolic groups of certain PCR-inhibiting substances (79) and haem (80), thereby preventing their binding to DNA polymerase. Some detergents such as Tween-20 reverse the inhibiting effect of ionic detergents (e.g., sodium dodecyl sulfate) used in lysing solutions (81).

Positive Controls

False-negative results, caused by inhibition of the PCR reaction, are often encountered in PCR-based assays. Unless this phenomenon is adequately monitored, the PCR assay will lack reliability. Therefore, a positive control should be integrated to monitor the efficiency of the PCR reaction, especially when the technique is applied in routine laboratories. Most commercially available PCR kits (Table 1) include some sort of positive control system. The positive control reaction can be performed in the same reaction tube as the test reaction (internal control or internal standard) or in a separate reaction vessel (external control or external standard). In the former case, the efficiency of the internal control PCR must not be higher than the efficiency of the target PCR.

For an internal control, a precisely known amount of either circular or linearized plasmid DNA (82) or an RNA or DNA

fragment is added to each reaction. This control fragment or a region of the plasmid will be co-amplified along with the target DNA of the test organism. Inhibition will be recognized if no signal or only a weak amplification signal, originating from the control DNA, is present. Control fragments consist of either heterologous (also called mimics) or homologous DNA fragments flanked by either unique target sequences for primer annealing or primer sequences identical to the target sequence of the test organism. In the latter case, a competitive PCR is created, with the disadvantage that, in addition to competition for other components, competition for primers between control DNA and target DNA will also occur, resulting in decreased sensitivity. Thus, it is important to use an optimal number of internal standard copies. The advantage is that this approach resembles the actual conditions within the target PCR.

The control amplicon is often distinguished from the target amplicon by a difference in length detectable by agarose gel electrophoresis. The internal control amplicon can be either shorter (83, 84) or longer (85) than the target amplicon. A longer internal control amplicon may be advantageous because a shorter mimic may be preferentially amplified (85). Alternatively, a unique probe-binding region can be introduced in the internal control fragment, allowing differentiation from the target amplicon (82).

Different methods have been described for the construction of DNA fragments bearing the same primer sequences as the target DNA (83, 84, 86). PCR mimics (standards with a random sequence) can also be constructed with commercially available kits (PCR MIMIC Construction Kit, Clontech Laboratories, Inc., Palo Alto, CA). These kits include a DNA fragment with random sequence. The size of the mimic DNA is adjusted by choosing the appropriate sequence along the neutral DNA fragment as the primer template. Composite primers containing target-specific sequences and up to 20 nucleotides that will hybridize to the neutral fragment in the kit are used to generate the mimic.

Rijpens et al. (52) developed an internal control system for PCR confirmation of presumptive *Salmonella* colonies on agar plates, but did not develop a general internal control system for PCR detection of *Salmonella* in pre-enrichment media. Sample-to-sample variation in the number of *Salmonella* and background flora influenced the system, and competition between target DNA and internal control DNA for reaction components, including primers, may have led to false-negative results in low-contaminated samples.

A general solution for the competition problem encountered with internal standards would be to create an internal control reaction with an efficiency lower than that of the test reaction, so that the internal control fragment would be visible only in negative samples where no target DNA is present. Only in the negative samples are questions asked about possible inhibition of the reaction.

Anti-Contamination Precautions

Because of its very high sensitivity, PCR is especially susceptible to contamination. Therefore extreme care must be taken to avoid false-positive reactions. False positives can result from sample-to-sample contamination, but a more serious source is the carryover of DNA from a previous amplification of the same target.

In the first approach, which should be the central part of any contamination control strategy, the PCR is physically separated into sample preparation, pre-PCR, and post-PCR locations (87). All reagents used in the PCR must be prepared, divided into aliquots, and stored in an area free of PCR-amplified product. Similarly, oligonucleotides used for amplification are synthesized and purified in a PCR-product-free environment.

Other approaches are based on pre- and post-amplification sterilization processes. Longo et al. (88) described the use of uracil DNA-glycosilase (UNG) and deoxyuridine triphosphate (dUTP) rather than deoxythymidine triphosphate (dTTP). UNG catalyzes the removal of uracil from single- and double-stranded DNA that has been synthesized in the presence of dUTP; apyrimidinic polynucleotides are subsequently cleaved at elevated temperatures under alkaline conditions (during the initial denaturation phase of PCR). Shortwave UV irradiation procedures have been described as pre-amplification sterilization processes (89, 90), but UV light can also be used to sterilize laboratory surfaces, racks, and pipets. Sarkar and Sommer (91) reported that UV light is less effective if the DNA fragment is <300 bp. Cimino et al. (92) described the use of a photochemical process that destroys the template activity of PCR amplicons but permits the modified amplicon to be probed in hybridization reactions.

Centrifugation is a big source of sample-to-sample contamination but can be avoided with aerosol-tight centrifuge capsules (Eppendorf, Hamburg, Germany). The use of aerosol-tight pipet tips and sterile plastic and glassware are also recommended. For highly sensitive PCR assays, a layer of mineral oil on top of the PCR reaction, even in PCR machines with heat covers, decreases contamination problems. Further, sodium hypochlorite effectively destroys both nucleic acids and bacteria, whereas ethanol, Dettol, and most other disinfectants are only active against the bacteria themselves and not against their nucleic acids.

Finally, by following strict precautions, contamination problems can be avoided with 30-cycle PCR methods. When more sensitive PCR systems such as 35–40-cycle PCR or nested-PCR are applied, it is almost impossible to avoid occasional contamination, especially in routine laboratories. We feel that the use of such extremely sensitive PCR-based detection systems should be restricted to bacteria and foods for which no alternative is available.

Messenger RNA-Based Detection

A disadvantage of conventional PCR is that both viable and nonviable cells may be detected. Because PCR is based on the detection of intact nucleic acids rather than intact viable cells, positive reactions may arise from either dead cells or viable but nonculturable cells (93–95). Because the bacteriological quality of food products is determined by the presence of

living pathogenic or spoiler bacteria at the time of analysis, this poses a problem, which may be overcome by an enrichment step to dilute out nonviable cells. An alternative is to use an RNA-based rather than a DNA-based detection. RT-PCR (96–102) and NASBA (103–105) are convenient techniques for the RNA-based detection of bacteria.

Not all 3 RNA groups [rRNA, transfer RNA (tRNA), and mRNA] are suitable for discriminating viable from dead bacteria. Bacterial rRNA and tRNA are much more stable than bacterial mRNA, of which the half-lives are very short and directly proportional to their steady state concentration (106, 107). In most cases, the presence of 16S rRNA and 23S rRNA cannot be used as an indicator of bacterial viability (99, 104, 108, 109). mRNA, on the other hand, may be suitable for monitoring bacterial viability. The success of such an approach depends on the choice of the messenger (55, 97, 110) and the choice of the location of the amplicon within that messenger (55, 111). The expression of several genes is dramatically influenced by temperature and by the composition and pH of the growth medium (112, 113); nonexpression of the target gene in living bacteria may lead to false-negative results. Blais et al. (105) and Simpkins et al. (114) avoided this problem by inducing transcription of the target gene before RNA extraction. Other researchers have used highly abundant genes as a target for mRNA-based detection (98, 99, 102). The stability of mRNA in dead bacteria is also strongly influenced by the killing conditions (55, 99) and by the post mortem storing temperature of the samples (55, 102).

It is mandatory to study each mRNA-based detection system extensively before drawing conclusions about its ability to differentiate viable from dead bacteria. The safest way to detect viable bacteria is the use of a short enrichment step to dilute out dead cells, combined with a DNA-based detection method. Such an approach guarantees exclusive detection of living cells independent of the target, growth circumstances, or the killing procedure. If no enrichment is included, an mRNA-based detection must be adopted. NASBA and RT-PCR were applied for detection of foodborne pathogens in food samples to a very limited extent by Uyttendaele et al. (104, 115, 116), Vaitilingom et al. (98), Szabo and Mackey (100), McKillip et al. (109), and Berry (117).

Quantitative PCR

Because the amount of PCR product increases exponentially during the early cycles of the PCR reaction but levels off in the final cycles, it does not reflect the amount of initial template DNA. Thus, most PCR assays only allow qualitative statements, limiting their use to applications in which only the presence or absence of a specific DNA or RNA molecule must be determined. To compensate for limitations of end point measurements, researchers have developed a variety of quantitative PCR techniques based on indirect most probable number (MPN)-PCR or direct PCR quantitation. Using these techniques, a semiquantitation of the initial amount of target present in a sample is possible. Indirect PCR quantitation is comparable to the MPN procedure applied in conventional bacteriological analyses. PCR is applied on a dilution series of the target DNA. This technique, however, tends to underestimate the amount of bacterial cells present, possibly because of incomplete lysis of bacterial cells or low sensitivity of the PCR (unpublished results). The kinetics of an individual amplification reaction depend not only on the amount of target DNA initially present, but also on the length of the product, the primer sequence, the potential inhibitors in the sample, and the variations in the reaction mix. Therefore, an internal (in quantitative competitive PCR) or an external (in quantitative kinetic PCR) standard is used for direct PCR quantitation.

In quantitative competitive PCR, an internal standard or competitor is co-amplified with the target sequence in each reaction tube. A competitor amplicon is constructed containing the same primer binding sites and the same amplification efficiency as the target, but which is somehow distinguishable from it. Often the target and competitor amplicons have a different size (84), so that gel electrophoresis can be used to discriminate between the 2 products. A known amount of competitor is spiked into the sample, and then the target and competitor are amplified in the same reaction. If the amplification efficiency of target and competitor are, in fact, identical, then the ratio of target to competitor will remain constant throughout the PCR process. Thus, by determining the ratio of target to competitor at the end of the reaction and knowing the starting amount of spiked competitor, the starting amount of target can be calculated. The dynamic range of competitive PCR is limited to a target-to-competitor ratio of about 1:10 to 10:1. The best accuracy is obtained by finding the equivalence point at which the ratio of target to competitor is 1:1. To accomplish this, several dilutions must be tested to achieve a suitable ratio of target to competitor. Several methods for the construction of a competitor amplicon or internal standard have been described (83, 84, 86). Alternatively, PCR mimics (standards with a random sequence) can be constructed with commercially available kits (PCR MIMIC Construction Kit, Clontech).

In quantitative kinetic PCR, an external standard is used. The accumulation of PCR products is followed during several cycles of the PCR. The efficiency of both reactions is determined by looking at the slopes of the increase in PCR product. The slopes of both reactions should be parallel. An automated form of quantitative kinetic PCR (real-time PCR) has been developed.

Quantitative PCR may provide useful information for detecting food pathogens when PCR is applied directly on the sample, that is, without an enrichment step. However, direct detection requires a laborious and lengthy sample preparation method combined with a very sensitive PCR and is highly vulnerable to contamination. Few sample preparations and compatible PCR-systems that allow such sensitive detection of bacteria in foods are described (28, 56, 57, 69). A useful application of direct quantitative PCR detection would be the detection of very slow-growing bacteria such as *Mycobacterium paratuberculosis*.

Real-Time PCR

In real-time PCR, the PCR products are detected as they accumulate. The amount of generated PCR product is proportional to the increase in signal. This cycle-to-cycle increase in signal can be monitored. In contrast to end point analysis in which only the plateau phase of the PCR can be detected, real-time PCR allows monitoring of the exponential phase. The quantitative information in PCR comes only from those few cycles in which the amount of DNA grows logarithmically from barely above the background to the plateau. Often only 4–5 cycles out of 30–40 will fall in this log-linear portion of the curve. Because the complete PCR is monitored during real-time PCR, the log-linear region can be easily identified in each single reaction.

Higushi et al. (118, 119) pioneered the analysis of PCR kinetics by constructing a system based on the use of the intercalator ethidium bromide. In this system, ethidium bromide was included in the PCR reaction, the thermal cycler was irradiated with UV light, and fluorescence was detected with a charge-coupled device (CCD) camera. Amplification produces increasing amounts of double stranded DNA (dsDNA), which binds ethidium bromide, resulting in an increase of fluorescence. This system has been improved, and now other choices of chemistries are available to perform real-time detection: 5′ nuclease chemistry, SYBR^R Green I chemistry, LightCycler chemistry, and molecular beacons.

The SYBR Green system is based on the sequence-aspecific binding of the SYBR Green I dye in the minor groove of dsDNA. The dye is only fluorescent when bound, and this binding characteristic is used to monitor the process of amplification as PCR product is generated. Sequence confirmation of the amplified product is performed by a melting curve analysis after PCR. At the end of the PCR run, the temperature in the thermal chamber is slowly raised and the fluorescence in each tube is measured. As soon as the dsDNA starts to denature, the SYBR Green I dye is released, resulting in a decrease in fluorescence. Because each dsDNA product has its own characteristic melting temperature (T_m), depending on its length and guanosine-cytosine (GC) content, melting curve analysis can be compared with analyzing a PCR product by length in gel electrophoresis. This system is commercially available from Applied Biosystems (Foster City, CA) and Roche Diagnostics (Mannheim, Germany).

The 5′ nuclease chemistry, the LightCycler chemistry, and the molecular beacon system are based on the phenomenon of fluorescence resonance energy transfer (FRET; 120), in which the energy from an excited fluorophore is transferred to an acceptor moiety at distances up to 70–100 Å. As a result, the emission of the fluorophore is quenched.

The 5′ nuclease assay (121) uses the 5′→3′ nuclease activity of *Taq* DNA polymerase to digest a probe, which is labeled with both a fluorescent reporter dye and a nonfluorescent quencher dye. Fluorescence from the reporter dye is efficiently quenched by the quencher dye on the same probe molecule. Because *Taq* DNA polymerase extends from the primer, it displaces and cleaves the probe, separating the reporter dye from the quencher dye. As a result of the probe hydrolysis and consequential separation of the 2 dyes, fluorescence intensity increases. This increase in fluorescence intensity of the reporter dye is due to its lack of proximity to the quencher dye. PCR thermal cycling results in exponential amplification of PCR product and of fluorescence intensity. This system is commercially available as the TaqMan system (Applied Biosystems; Table 1). The TaqMan system was used for the detection of *L. monocytogenes* (122), *E. coli* O157:H7 (123), Shiga-like toxin I-producing *E. coli* (SLTIEC; 124), and *Salmonella* in foods or pure cultures (125–127).

The hybridization probe format (*see* www.biochem.roche.com/lightcycler/lc_principles) was developed for use in the LightCycler (Roche Diagnostics) and is often referred to as LightCycler chemistry. In addition to the reaction components used for conventional PCR, 2 specially designed, sequence-specific probes labeled with 2 different fluorescent dyes are used for this detection method. The detection is based on generation of a fluorescent signal by FRET when the 2 probes bind next to each other to the target sequence.

Molecular beacon probes (128) are single-stranded nucleic acid molecules with a stem-and-loop structure. The loop portion of the molecule is a probe sequence that is complementary to a predetermined sequence in a target nucleic acid. The stem is formed by the annealing of 2 complementary arm sequences on either side of the probe sequence. The arm sequences are unrelated to the target sequence. A fluorescent moiety is attached to the end of one arm and a nonfluorescent moiety is attached to the end of the other arm. The stem keeps these 2 moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by fluorescence resonance energy transfer. The nature of the fluorophore–quencher pair is such that energy received by the fluorophore is transferred to the quencher and dissipated as heat, rather than light. As a result, the fluorophore is unable to fluoresce. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than that formed by the arm sequences. Because nucleic acid double helixes are relatively rigid, formation of a probe–target hybrid precludes the simultaneous existence of a hybrid formed by the arm sequences. Thus, the probe undergoes a spontaneous conformational change that forces the arm sequences apart and causes the fluorophore and quencher to move away from each other. Because the fluorophore is no longer in close proximity to the quencher, it fluoresces when illuminated by UV light. Tyagi and Kramer (128) called these probes molecular beacons because they emit a fluorescent signal only when hybridized to target molecules. Because unhybridized molecular beacons are dark, it is not necessary to remove them to observe hybridized probes. Multiplex detection of pathogenic retroviruses (129), detection of *Salmonella* (130), and genotyping of *M. tuberculosis* (131) using molecular beacons was recently described. Leone et al. (132) described the coupling of RNA amplification by NASBA with amplicon detection by molecular beacon technology to produce a homogeneous RNA assay, which they called AmpliDet RNA.

Nazarenko et al. (133) described a method based on the method of Tyagi and Kramer (128) in which the donor and quencher moieties are both attached to a hairpin structure on the 5′ end of the amplification primer. Oligonucleotide primers are designed so that the fluorescent signal is generated only when the labeled oligonucleotides are incorporated into the double-stranded amplification product. Nazarenko et al. (133) argued that the main advantage of their method is the generation of the fluorescent signal by the product itself, rather than by the hybridized probe. This system was later described as Sunrise system (134) and commercialized under the name Amplifluor™ by Oncor/Intergen (Gaithersburg, MD; www.intergenco.com/pcr.html).

Other variants of the molecular beacon technique such as Scorpions technology (135) have been described. In this case, the hairpin structure is attached to a primer by a linker that prevents copying of the hairpin. The probe element is designed so that it hybridizes to its target only when the target site has been incorporated into the same molecule by extension of the tailed primer.

Applied Biosystems improved its TaqMan system by replacing the fluorescent quencher TAMRA with a nonfluorescent quencher coupled to a minor groove binder. Minor groove binders increase the specificity of DNA probes when bound to them by increasing the melting temperature and provide a more efficient fluorescence quenching (136). The advantage of using a nonfluorescent chromophore as a quencher is that it absorbs the energy of the fluorescein without emitting light itself. As a result, the emission of the fluorescein may be detected more precisely, without interference from the emission of the acceptor (133).

Equipment for real-time detection is available from several firms. The ABI Prism 7700 and 7900 Sequence Detection Systems (Applied Biosystems), the LightCycler and the iCycler Thermal Cycler (Bio-Rad, Hercules, CA) combine PCR and real-time detection. The NucliSensEasyQ Analyser (OrganonTeknika, Boxtel, The Netherlands) allows real-time detection of NASBA with molecular beacons. Kreuzer et al. (137) reported that the TaqMan chemistry can be used in the LightCycler after minor modifications. On the other hand, the LightCycler chemistry was compatible with the ABI Prism 7700 Sequence Detection system (138). The iCycler Thermal Cycler is said to be compatible with all fluorescent technologies. A big advantage of real-time PCR when applied in routine laboratories is that because PCR and detection are performed in a 1-tube system, carryover contamination is no longer a problem.

Commercially Available PCR-Based Detection/Identification Systems

Some hybridization-based [Accuprobe (Gen-Probe, San Diego, CA) and Gene-Trak (Hopkinton, MA)] or PCR-based [Probelia (Bio-Rad, Hercules, CA), BAX (Qualicon, Wilmington, DE), TaqMan] systems are commercially available for identification or detection of certain foodborne bacterial pathogens (Table 1). Both hybridization-based systems do not use an amplification step but use probes directed to the highly expressed rRNA. Nevertheless, they still require at least 10^5–10^6 bacteria/mL (139) and are therefore recommended for use as culture confirmation assays. In the Accuprobe culture identification test (Table 1), the probe is labeled with an acridinium ester and used in a hybridization protection assay. When the DNA-probe is hybridized to its target rRNA, the acridinium is protected from chemical hydrolysis and reacts with peroxide under basic conditions to produce chemiluminescence. If the probe remains unbound, the ester bond undergoes hydrolysis and renders the acridinium permanently nonchemiluminiscent. In the Gene-Trak assays (Table 1), the target rRNA is hybridized with a capture probe that is fixed on a dipstick. The hybridization is then confirmed by sandwich hybridization with a signal-generating probe.

The 3 PCR-based assays are recommended for detection of bacteria in enrichment media, but can also be used for identification purposes.

The TaqMan system is available for the detection of *Salmonella* spp., *E. coli* O157:H7, and Shiga-toxin-containing *E. coli* in food products. DNA is extracted from the enrichment medium with an anionic resin, PCR-amplified and simultaneously detected in the 5′ nuclease assay. An internal PCR amplification control (PAC), implemented within each reaction, consists of a synthetic template and a corresponding probe. The reporter dye of the specific PAC probe is different from that of the test organism-specific probe.

The Probelia system is available for detection of *L. monocytogenes*, *Salmonella* spp., *C. jejuni* and *C. coli*, and *E. coli* O157:H7 (Table 1). DNA is extracted from the enrichment media with an anionic resin, PCR-amplified and subsequently detected by hybridization and colorimetric reaction. An internal control fragment is added to each tube before amplification, and the results depend on a comparison of the optical density obtained on the test organism detection plate with that for the same sample on the internal control microplate. Currently, the Probelia system (Table 1) is being adapted so that, in the future, detection can take place in the iCycler. The detection will then be performed with fluorescent molecular beacons.

The BAX system (Table 1) is available for detection of *Listeria* spp. (111), *L. monocytogenes* (140), *Salmonella* spp., and *E. coli* O157:H7. Detection is accomplished by gel electrophoresis or temperature-dependent fluorescence analysis with SYBR Green I dye.

For some commercial PCR-based assays, the use of certain enrichment media and enrichment times is recommended. For detection of *L. monocytogenes*, some suppliers recommend a 24 h protocol. We found that stressed *L. monocytogenes* do not reach concentrations detectable by a 30-cycle PCR after 24 h enrichment of soft cheese samples (55). This may be the reason that these suppliers recommend the use of a highly sensitive 35–40-cycle PCR. However, the use of such a PCR results in many false-positive samples due to contamination, especially in routine laboratories (141). We therefore recommend a 40–48 h enrichment and a subsequent PCR of 30 cycles to avoid contamination problems (55).

Evaluation of Short Methods

All new short methods developed for identifying or detecting pathogens or their toxins must be compared thoroughly with standard microbiological methods before they can be used in routine food analysis. Several rapid method validation and evaluation programs were started by private laboratories, research institutes, and government agencies. The value of these validations is highly dependent on the organization.

Because the incidence of some foodborne bacteria in certain food products is low, it is almost impossible to evaluate detection methods for these bacteria on naturally contaminated samples. Therefore, many PCR-based short methods have been evaluated on artificially contaminated food, using dilutions of pure bacterial cultures. However, bacteria present in food are not in the same physiological condition as they are in pure bacterial cultures (142). Pathogens are often injured by processes such as heating, freezing, drying, and sanitizing (143) and require a period of recovery before they regain their full growth potential. Therefore, the sensitivities of methods evaluated on artificially contaminated samples may be overestimated. Also, different types of food products or even different formulations of a same type of product can differ in competitive flora or composition, which may influence the performance of the method. As such, tests on artificially contaminated food give an indication of the performance of the test but do not guarantee that the method will perform well under all conditions.

A valid alternative that mimics the situation for naturally contaminated samples is the use of stressed bacteria for artificial contamination. Stressed bacteria are commercially available as reference material (noncertified or certified) from the SVM/RIVM (National Institute of Public Health and Environment, Bilthoven, The Netherlands). The SVM/RIVM prepares this reference material by spray-drying a mixture of bacterial suspension and pasteurized full-fat milk, and subsequently mixing the highly contaminated milk powder with sterile milk powder (144, 145). Bacteria treated by this process are considered as stressed. Capsules are available containing fixed numbers of *L. monocytogenes* [(5 or 5000 colony-forming units (CFU)], *Salmonella* (5 CFU), *E. coli* (500 CFU), *Staphylococcus warneri* (500 CFU), *Clostridium perfringens* (5000 CFU), *Enterobacter cloacae* (500 CFU), or *Enterococcus faecium* (500 CFU). These capsules, however, present only one type of stress, which is not always comparable with that in food products. The PCR detection of stressed *Salmonella* in the pre-enrichment medium of different food products has been described by Rijpens et al. (52).

References

(1) Viscidi, R.P., & Yolken, R.G. (1987) *Mol. Cell. Probes* **1**, 3–14

(2) Tenover, F.C. (1988) *Clin. Microbiol. Rev*. **1**, 82–101

(3) Yolken, R.H. (1988) *Mol. Cell. Probes* **2**, 87–96

(4) Lampel, K.A., Feng, P., & Hill, W.E. (1992) in *Molecular Approaches to Improving Food Quality and Safety*, D. Bhatagnar & T.E. Cleveland (Eds), Van Nostrand Reinhold, New York, NY, pp 151–188

(5) Hill, W.E., Datta, A.R., Feng, P., Lampel, K.A., Payne, W.L., & Wentz, B.A. (1992) in *Bacteriological Analytical Manual*, 7th Ed., Association of Official Analytical Chemists, Arlington, VA, Ch. 24

(6) Hartman, P.A., Swaminathan, B., Curiale, M.S., Firstenberg-Eden, R., Sharpe, A.N., Cox, N.A., Fung, D.Y.C., & Goldschmidt, M. (1992) in *Compendium of Methods for the Microbiological Examination of Foods*, American Public Health Association, Washington, DC, pp 665–746

(7) Wernars, K., & Notermans, S. (1990) in *Gene Probes for Bacteria*, A.J.L. Macario & E. Conway de Macario (Eds), Academic Press, San Diego, CA, pp 353–356

(8) McKee, R.A., Gooding, C.M., Garrett, S.D., Powell, H.A., Lund, B.M., & Knox, M. (1991) *Biochem. Soc. Trans.* **19**, 698–701

(9) Wachsmuth, I.K., Kiehlbauch, J.A., Bopp, C.A., Cameron, D.N., Strockbine, N.A., Wells, J.G., & Blake, P.A. (1991) *Int. J. Food Microbiol*. **12**, 77–90

(10) Wolcott, M.J. (1991) *J. Food Prot.* **54**, 387–401

(11) Feng, P., Lampel, K.A., & Hill, W.E. (1996) in *Diagnostic Nucleic Acid Probe Technology*, C. Dangler, & B. Osburn (Eds), Hauser, New York, NY, Ch. 13

(12) Moseley, S.L., Huq, I., Alim, A.R.M.A., So, M., Samadpour-Motalebi, M., & Falkow, S. (1980) *J. Infect. Dis.* **142**, 892–898

(13) Hill, W.E., Madden, J.M., McCardell, B.A., Shah, D.B., Jagow, J.A., Payne, W.L., & Boutin, B.K. (1983) *Appl. Environ. Microbiol*. **45**, 1324–1330

(14) Datta, A.R., Wentz, B.A., & Hill, W.E. (1987) *Appl. Environ. Microbiol*. **53**, 2256–2259

(15) Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., & Erlich, H.A. (1988) *Science* **239**, 487–491

(16) Border, P.M., Howard, J.J., Plastow, G.S., & Siggens, K.W. (1990) *Lett. Appl. Microbiol*. **11**, 158–162

(17) Bessesen, M.T., Luo, Q.L., Rotbart, H.A., Blaser, M.J., & Ellison III, R.T. (1990) *Appl. Environ. Microbiol.* **56**, 2930–2932

(18) Barany, F. (1991) *PCR Methods Applications* **1**, 5–16

(19) Compton, J. (1991) *Nature* **350**, 91–92

(20) Guatelli, J.C., Whitfield, K.M., Kwoh, D.Y., Barringer, K.J., Richman, D.D., & Gingeras, T.R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1874–1878

(21) Kwoh, D.Y., Davis, G.R., Whitfield, K.M., Chapelle, H.L., DiMichelle, L.J., & Gingeras, T.R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1173–1177

(22) Giesendorf, B.A.J., Quint, W.G.V., Henkens, M.H.C., Stegeman, H., Huf, F.A., & Niesters, H.G.M. (1992) *Appl. Environ. Microbiol*. **58**, 3804–3808

(23) Wang, R.-F., Cao, W.-W., & Johnson, M.G. (1992) *Appl. Environ. Microbiol*. **58**, 2827–2831

(24) Wang, R.-F., Cao, W.-W., Franklin, W., Campbell, W., & Cerniglia, C.E. (1994) *Mol. Cell. Probes* **8**, 131–137

(25) Barry, T., Colleran, G., Glennon, M., Dunican, L.K., & Gannon, F. (1991) *PCR Methods Applic.* **1**, 51–56

(26) Rossau, R. (1991) in *Rapid Methods and Automation in Microbiology and Immunology*, A. Vaheri, R.C. Tilton, & A. Balows (Eds), Springer-Verlag, Berlin, Germany, pp 76–83

(27) Rossau, R., Michielsen, A., Jannes, G., Duhamel, M., Kersters, K., & Van Heuverswijn, H. (1992) *Mol. Cell. Probes* **6**, 281–289

(28) Herman, L., De Block, J., & Waes, G. (1995) *Appl. Environ. Microbiol*. **61**, 4141–4146

(29) Rijpens, N.P., Jannes, G., Van Asbroeck, M., Herman, L.M.F., & Rossau, R. (1995) *Mol. Cell. Probes* **9**, 423–432

(30) Rijpens, N.P., Jannes, G., & Herman, L.M.F. (1997) *J. Food Prot*. **60**, 548–550

(31) Gürtler, V., & Stanisich, V.A. (1996) *Microbiology* **142**, 3–16

(32) Lang, A.L., Tsai, Y.-L., Mayer, C.L., Patton, K.C., & Palmer, C.J. (1994) *Appl. Environ. Microbiol*. **60**, 3145–3149

(33) Schultsz, C., Pool, G.J., Van Ketel, R., De Wever, R., Speelman, P., & Dankert, J. (1994) *J. Clin. Microbiol*. **32**, 2393–2397

(34) Thomas, A., Jiggle, B., Smith, H.R., & Rowe, B. (1994) *Lett. Appl. Microbiol*. **19**, 406–409

(35) Witham, P.K., Yamashiro, C.T., Livak, K.J., & Batt, C.A. (1996) *Appl. Environ. Microbiol*. **62**, 1347–1353

(36) Paton, A.W., & Paton, J.C. (1998) *J. Clin. Microbiol*. **36**, 598–602

(37) Ibrahim, A., Liesack, W., & Stackebrandt, E. (1992) *Mol. Cell. Probes* **6**, 163–171

(38) Kwaga, J., Iversen, J.O., & Misra, V. (1992) *J. Clin. Microbiol*. **30**, 2668–2673

(39) Nakajima, H., Inoue, M., Mori, T., Itoh, K.-I., Arakawa, E., & Watanabe, H. (1992) *J. Clin. Microbiol*. **30**, 2484–2486

(40) Pierson, D.E., & Falkow, S. (1990) *Infect. Immun*. **58**, 1059–1064

(41) Scheu, P.M., Berghof, K., & Stahl, U. (1998) *Food Microbiology* **15**, 13–31

(42) Wren, B.W., & Tabaqchali, S. (1990) *Lancet* **336**, 639

(43) Portnoy, D.A., Moseley, S.L., & Falkow, S. (1981) *Infect. Immun*. **31**, 775–782

(44) Bricker, B.J., & Halling, S.M. (1994) *J. Clin. Microbiol*. **32**, 2660–2666

(45) Cano, R.J., Rasmussen, S.R., Sánchez Fraga, G., & Palomares, J.C. (1993) *J. Appl. Bacteriol*. **75**, 247–253

(46) Kirk, R., & Rowe, M.T. (1994) *Lett. Appl. Microbiol*. **19**, 301–303

(47) Aabo, S., Rasmussen, O.F., Rossen, L., Sørensen, P.D., & Olsen, J.E. (1993) *Mol. Cell. Probes* **7**, 171–178

(48) Olsen, J.E., Aabo, S., Hill, W., Notermans, S., Wernars, K., Granum, P.E., Popovic, T., Rasmussen, H.N., & Olsvik, Ø. (1995) *Int. J. Food Microbiol*. **28**, 1–78

(49) Feng, P. (1996) *J. AOAC Int*. **79**, 809–812

(50) Hill, W.E. (1996) *Crit. Rev. Food Sci. Nutr*. **36**, 123–173

(51) Speck, M.L., & Ray, B. (1977) *J. Food Prot*. **40**, 333–336

(52) Rijpens, N., Herman, L., Vereecken, F., Jannes, G., De Smedt, J., & De Zutter, L. (1999) *Int. J. Food Microbiol*. **46**, 37–44

(53) Petran, R.L., & Swanson, K.M.J. (1993) *J. Food Prot*. **56**, 616–618

(54) MacDonald, F., & Sutherland, A.D. (1994) *J. Dairy Res*. **61**, 433–436

(55) Rijpens, N. (2000) Ph.D. Thesis, *Identification and Detection of Bacterial Food Pathogens Using Molecular Methods*, University of Ghent, Belgium

(56) Herman, L.M.F., De Block, J.H.G.E., & Moermans, R.J.B. (1995) *Appl. Environ. Microbiol*. **61**, 817–819

(57) Herman, L., De Block, J., & Van Rentergem, R. (1997) *J. Dairy Res*. **64**, 311–314

(58) Herman, L.M.F., Vaerewijck, M.J.M., Moermans, R.J.B., & Waes, G.M.A.V.J. (1997) *Appl. Environ. Microbiol*. **63**, 3139–3149

(59) Persing, D.H. (1993) in *Diagnostic Molecular Microbiology Principles and Applications*, D.H. Persing, T.F. Smith, F.C. Tenover, & T.J. White (Eds), American Society for Microbiology, Washington, DC, pp 51–87

(60) Rossen, L., Nørskov, P., Holmstrøm, K., & Rasmussen, O.F. (1992) *Int. J. Food Microbiol*. **17**, 37–45

(61) Powell, H.A., Gooding, C.M., Garret, S.D., Lund, B.M., & McKee, R.A. (1994) *Lett. Appl. Microbiol*. **18**, 59–61

(62) Lantz, P.-G., Tjerneld, F., Borch, E., Hahn-Hägerdal, B., & Rådström, P. (1994) *Appl. Environ. Microbiol*. **60**, 3416–3418

(63) Bickley, J., Short, J.K., McDowell, D.G., & Parkes, H.C. (1996) *Lett. Appl. Microbiol*. **22**, 153–158

(64) Lantz, P.-G., Knutsson, R., Blixt, Y., Al-Soud, W.A., Borch, E., & Rådström, P. (1998) *Int. J. Food Microbiol*. **45**, 93–105

(65) Katcher, H.L., & Schwartz, I. (1994) *BioTechniques* **16**, 84–92

(66) Wiedbrauk, D.L., Werner, J.C., & Drevon, A.M. (1995) *J. Clin. Microbiol*. **33**, 2643–2646

(67) Abu, A.-S., & Rådström, P. (1998) *Appl. Environ. Microbiol*. **64**, 3748–3753

(68) Starbuck, M.A.B., Hill, P.J., & Stewart, G.S.A.B. (1992) *Lett. Appl. Microbiol*. **15**, 248–252

(69) Rijpens, N.P., Jannes, G., Van Asbroeck, M., Rossau, R., & Herman, L.M.F. (1996) *Appl. Environ. Microbiol*. **62**, 1683–1688

(70) Blackburn, C. de W., & Patel, P.D. (1989) *Proceedings of the Society of Applied Bacteriology Conference* ii, 50

(71) Skjerve, E., Rørvik, L.M., & Olsvik, Ø. (1990) *Appl. Environ. Microbiol*. **56**, 3478–3481

(72) Skjerve, E., & Olsvik, Ø. (1991) *Int. J. Food Microbiol*. **14**, 11–17

(73) Payne, M.J., Campbell, S., Patchett, R.A., & Kroll, R.G. (1992) *J. Appl. Bacteriol*. **73**, 41–52

(74) Albertsson, P.-Å. (1986) *Partition of Cell Particles and Macromolecules*, John Wiley and Sons, New York, NY

(75) Lindqvist, R., Norling, B., & Thisted-Lambertz, S. (1997) *Lett. Appl. Microbiol*. **24**, 306–310

(76) Lindqvist, R. (1997) *J. Food Microbiol*. **37**, 73–82

(77) Singer-Sam, J., Tanguay, R.L., & Riggs, A.D. (1989) *Amplifications* **3**, 11

(78) Walsh, P.S., Metzger, D.A., & Higuchi, R. (1991) *BioTechniques* **10**, 506–513

(79) Kreader, C.A. (1996) *Appl. Environ. Microbiol*. **62**, 1102–1106

(80) Holbrook, R., Anderson, J.M., Baird-Parker, A.C., & Stuchbury, S.H. (1989) *Lett. Appl. Microbiol*. **9**, 161–164

(81) Rossen, L., Holmstrøm, K., Olsen, J.E., & Rasmussen, O.F. (1991) *Int. J. Food Microbiol*. **14**, 145–152

(82) Rosenstraus, M., Wang, Z., Chang, S.-Y., Debonville, D., & Spadoro, J.P. (1998) *J. Clin. Microbiol.* **36**, 191–197
(83) Pallen, M.J., Puckey, L.H., & Wren, B.W. (1992) *PCR Methods Applic.* **2**, 91–92
(84) Jin, C.-F., Mata, M., & Fink, D.J. (1994) *PCR Methods Applic.* **3**, 252–255
(85) Thisted-Lambertz, S., Ballagi-Pordány, A., & Lindqvist, R. (1998) *Lett. Appl. Microbiol.* **26**, 9–11
(86) Ballagi-Pordány, A., & Belak, S. (1996) *Mol. Cell. Probes* **10**, 159–164
(87) Kwok, S., & Higushi, R. (1989) *Nature* **339**, 237–238
(88) Longo, M.C., Berninger, M.S., & Hartley, J.L. (1990) *Gene* **93**, 125–128
(89) Sarkar, G., & Sommer, S. (1991) *BioTechniques* **10**, 590–594
(90) Cimino, G.D., Metchette, K., Isaacs, S.T., & Zhu, Y.S. (1990) *Nature* **345**, 773–774
(91) Sarkar, G., & Sommer, S. (1990) *Nature* **343**, 27
(92) Cimino, G.D., Mechette, K.C., Tessman, J.W., Hearst, J.C., & Issacs, S.T. (1991) *Nucleic Acids Res.* **19**, 99–107
(93) Josephson, K.L., Gerba, C.P., & Pepper, I.L. (1993) *Appl. Environ. Microbiol.* **59**, 3513–3515
(94) Masters, C.I., Shallcross, J.A., & Mackey, B.M. (1994) *J. Appl. Bacteriol.* **77**, 73–79
(95) Herman, L. (1997) *Food Microbiol.* **14**, 103–110
(96) Bej, A.K., Mahbubani, M.H., & Atlas, R.M. (1991) *Appl. Environ. Microbiol.* **57**, 597–600
(97) Klein, P.G., & Juneja, V.K. (1997) *Appl. Environ. Microbiol.* **63**, 4441–4448
(98) Vaitilingom, M., Gendre, F., & Brignon, P. (1998) *Appl. Environ. Microbiol.* **64**, 1157–1160
(99) Sheridan, G.E.C., Masters, C.I., Shallcross, J.A., & Mackey, B.M. (1998) *Appl. Environ. Microbiol.* **64**, 1313–1318
(100) Szabo, E.A., & Mackey, B.M. (1999) *Int. J. Food Microbiol.* **51**, 113–122
(101) Norton, D.-M., & Batt, C.A. (1999) *Appl. Environ. Microbiol.* **65**, 2122–2127
(102) Sheridan, G.E.C., Szabo, E.A., & Mackey, B.M. (1999) *Lett. Appl. Microbiol.* **29**, 375–379
(103) Van der Vliet, G.M.E., Schepers, P., Schukkink, R.A.F., Van Gemen, B., & Klatser, P.R. (1994) *Antimicrob. Agents Chemother.* **38**, 1959–1965
(104) Uyttendaele, M., Bastiaansen, A., & Debevere, J. (1997) *Int. J. Food Microbiol.* **37**, 13–20
(105) Blais, B.W., Turner, G., Sooknana, R., & Malek, L.T. (1997) *Appl. Environ. Microbiol.* **63**, 310–313
(106) Nierlich, D.P., & Murakawa, G.J. (1996) *Prog. Nucleic Acid Res. Mol. Biol.* **52**, 153–216
(107) Rauhut, R., & Klug, G. (1999) *FEMS Microbiol. Rev.* **23**, 353–370
(108) Tolker-Nielsen, T., Halberg Larsen, M., Kyed, H., & Molin, S. (1997) *Int. J. Food Microbiol.* **35**, 251–258
(109) McKillip, J.L., Jaykus, L.-A., & Drake, M. (1998) *Appl. Environ. Microbiol.* **64**, 4264–4268
(110) Holmstrøm, K., Tolker-Nielsen, T., & Molin, S. (1999) *J. Bacteriol.* **181**, 1733–1738
(111) Norton, D.M., McCamey, M., Boor, K.J., & Wiedmann, M. (2000) *J. Food Prot.* **63**, 343–346
(112) Park, S.F., Stewart, G.S.A.B., & Kroll, R.G. (1992) *J. Gen. Microbiol.* **138**, 2619–2627
(113) Datta, A.R. (1994) *Food Microbiol.* **11**, 123–129
(114) Simpkins, S.A., Chan, A., Cook, N., Pöpping, B., & Hays, J. (1999) *Proceedings of the International Conference on Rapid Detection Assays for Food and Water*, York, UK, poster P15
(115) Uyttendaele, M., Schukkink, R., van Gemen, B., & Debevere, J. (1995) *Int. J. Food Microbiol.* **27**, 77–89
(116) Uyttendaele, M., Schukkink, R., van Gemen, B., & Debevere, J. (1995) *Appl. Environ. Microbiol.* **61**, 1341–1347
(117) Berry, E.D. (2000) *Lett. Appl. Microbiol.* **31**, 265–269
(118) Higushi, R., Dollinger, G., Walsh, P.S., & Griffith, R. (1992) *Biotechnology* **10**, 413–417
(119) Higushi, R., Fockler, C., Dollinger, G., & Watson, R. (1993) *Biotechnology* **11**, 1026–1030
(120) Stryer, L. (1978) *Annu. Rev. Biochem.* **47**, 819–846
(121) Holland, P.M., Abramson, R.D., Watson, R., & Gelfand, D.H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7276–7280
(122) Bassler, H.A., Flood, S.J.A., Livak, K.J., Marmaro, J., Knorr, R., & Batt, C.A. (1995) *Appl. Environ. Microbiol.* **61**, 3724–3728
(123) Oberst, R.D., Hays, M.P., Bohra, L.K., Phebus, R.K., Yamashiro, C.T., Paszko-Kolva, C., Flood, S.J.A., Sargeant, J.M., & Gillespie, J.R. (1998) *Appl. Environ. Microbiol.* **64**, 3389–3396
(124) Witham, P.K., Yamashiro, C.T., Livak, K.J., & Batt, C.A. (1996) *Appl. Environ. Microbiol.* **62**, 1347–1353
(125) Chen, S., Yee, A., Griffiths, M., Larkin, C., Yamashiro, C.T., Behari, R., Paszko-Kolva, C., Rahn, K., & De Grandis, S.A. (1997) *Int. J. Food Microbiol.* **35**, 239–250
(126) Kimura, B., Kawasaki, S., Fujil, T., Kusunoki, J., Itoh, T., & Flood, S.J.A. (1999) *J. Food Prot.* **62**, 329–335
(127) Nogva, H.K., & Lillehaug, D. (1999) *Int. J. Food Microbiol.* **51**, 191–196
(128) Tyagi, S., & Kramer, F.R. (1996) *Nature Biotechnol.* **14**, 303–308
(129) Vet, J.A.M., Majithia, A.R., Marras, S.A.E., Tyagi, S., Dube, S., Poiesz, B.J., & Kramer, F.R. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 6394–6399
(130) Chen, W., Martinez, G., & Mulchandani, A. (2000) *Anal. Biochem.* **280**, 166–172
(131) Rhee, J.T., Piatek, A.S., Small, P.M., Harris, L.M., Chaparro, S.V., Kramer, F.R., & Alland, D. (1999) *J. Clin. Microbiol.* **37**, 1764–1770
(132) Leone, G., van Schijndel, H., van Gemen, B., Kramer, F.R., & Schoen, C.D. (1998) *Nucleic Acids Res.* **26**, 2150–2155
(133) Nazarenko, I.A., Bhatnagar, S.K., & Hohman, R.J. (1997) *Nucleic Acids Res.* **25**, 2516–2521
(134) Winn-Deen, E.S. (1998) *Mol. Diagn.* **3**, 217–221
(135) Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., & Little, S. (1999) *Nature Biotechnol.* **17**, 804–807
(136) Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempey, R., Reed, M.W., Meyer, R.B., & Hedgpeth, J. (2000) *Nucleic Acids Res.* **28**, 655–661
(137) Kreuzer, K.A., Lass, U., Bohn, A., Landt, O., & Schmidt, C.A. (1999) *Cancer Res.* **59**, 3171–3174
(138) Nitsche, A., Steuer, N., Schmidt, C.A., Landt, O., & Siegert, W. (1999) *Clin. Chem.* **45**, 1932–1937

(139) Mabilat, C., Bukwald, S., Machabert, N., Desvarennes, S., Kurfurst, R., & Cros, P. (1996) *Int. J. Food Microbiol*. **28**, 333–340

(140) Stewart, D., & Gendel, S.M. (1998) *J. AOAC Int*. **81**, 817–822

(141) De Zutter, L., Herman, L., & Daube, G. (1997) *Poster Presented at the Congrès de Microbiologie*, Liège, Belgium

(142) Niederhauser, C., Candrian, U., Höfelein, C., Jermini, M., Bühler, H.-P., & Lüthy, J. (1992) *Appl. Environ. Microbiol*. **58**, 1564–1568

(143) Golden, D.A., Beuchat, L.R., & Brackett, R.E. (1988) *Food Microbiol*. **5**, 17–23

(144) in 't Veld, P.H., Notermans, S.H.W., & van der Berg, M. (1995) *Food Microbiol*. **12**, 125–134

(145) in 't Veld, P.H., van Strijp-Lockefeer, N.G.W.M., Havelaar, A.H., & Maier, E.A. (1996) *J. Appl. Bacteriol*. **80**, 496–504