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Genetic techniques: PCR, NASBA, hybridisation and microarrays

K. Sanderson and D. Nichols, University of Tasmania, Australia

12.1 Introduction: the polymerase chain reaction (PCR)

In principle, complex nucleic acids (i.e. DNA or RNA) are ideal molecules for the specific detection of pathogens in foods. The sequence of the target molecule provides information that can be used to identify the type of microorganism it came from and methods are readily available for the rapid amplification and detection of these molecules. In practice, while genetic techniques have been adopted quickly by the research community, there has been a slower uptake in diagnostic laboratories despite their potential for providing practical, rapid and sensitive detection of microorganisms in foods.

The PCR technique can be used to amplify the number of copies of a specific region of DNA. The region to be amplified is specified by the choice of primers used. Primers are short oligonucleotides usually 20–30 nucleotides in length whose sequence matches the ends of the region of interest. Amplification takes place over a number of cycles. During each cycle the double-stranded DNA template is denatured by heating to produce single strands. The reaction mixture is then cooled allowing the primers to bind to the single-stranded DNA. This provides an active site for DNA polymerase which synthesises the complementary strand, producing double-stranded DNA. In subsequent cycles primers will bind to both the original DNA and the newly synthesised DNA resulting in an exponential increase in the number of copies. The use of a thermostable polymerase allows the cycling to continue with minimal loss of enzymatic activity. Using PCR a section of DNA can be amplified about a million-fold. Usually the region amplified is shorter than 3 kb although larger fragments can be amplified with less efficiency.

The results of PCR are traditionally detected by agarose gel electrophoresis and staining. This enables the amplified DNA to be visualised as bands of differing size. The size of the bands can be determined by comparison with standard molecular weight markers. Presence of a band or bands of the expected size indicates a positive result. Since this method of detection adds significantly to the length of time required for the assay, single tube methods incorporating a colorimetric detection step have been developed.

12.1.1 Nested PCR

Nested PCR is an extension of the PCR technique that uses two sets of primers. The target sequence of one set of primers ('inner' primers) is located within the region amplified by the second set of primers ('outer' primers). A sample is first amplified with the outer primers, then that product is amplified with the inner primers. This reamplification process can improve the sensitivity of an assay.

12.1.2 RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) is a variation of PCR that enables the amplification of RNA. Reverse transcriptase in the reaction mixture transcribes RNA into DNA which is then amplified by PCR. RNA-based assays can have a slightly higher sensitivity due to the presence of multiple copies of RNA per microbial cell compared to usually only a single copy of a gene.

12.1.3 Real-time PCR

Real-time PCR enables both the detection and quantification of a nucleotide signal by continuously measuring a fluorescent reporter during a PCR reaction. The simplest type of reporter is a fluorescent dye that binds specifically to double-stranded DNA (e.g. ethidium bromide, SYBR® Green, Molecular Probes). When bound the dye emits light upon excitation, so fluorescence increases in proportion to the accumulation of PCR product. Dyes will bind to any double-stranded DNA in the reaction such as primer dimers and non-specific reaction products so they are most suitable for well characterised and highly specific assays. The two most widely used alternatives to simple dyes are TaqMan® (Applied Biosystems http://www.appliedbiosystems.com/) and molecular beacon hybridisation probes (http://www.molecular-beacons.org/) which rely on fluorescence resonance energy transfer (FRET) for quantitation.

TaqMan® probes are labelled with two different fluorescent dyes. Usually a 5′ terminus reporter dye and a 3′ terminus quenching dye. In intact probes FRET occurs between these two fluorophors and the fluorescent emission is quenched. Probe bound to a PCR product is cleaved by the 5′ nuclease activity of Taq polymerase during the extension phase of PCR. This removes the reporter dye

from the proximity of the quencher dye and the increase in fluorescence is measured. Increase in fluorescence is proportional to the rate of probe cleavage.

Molecular beacon probes also contain fluorescent and quenching dyes. However, FRET only occurs when the quenching dye is directly adjacent to the fluorescent dye. Probes are therefore designed to have a hairpin structure that brings the fluorescent dye and quencher into close proximity. When the probe hybridises to a target, the fluorescent dye and quencher are separated, FRET no longer occurs and the fluorescent dye emits light upon excitation. Unlike TaqMan® probes, molecular beacon probes remain intact during the amplification reaction and must rebind to target in every cycle for signal measurement.

12.2 Nucleic acid sequence-based amplification (NASBA), hybridisation and microarrays

Nucleic acid sequence-based amplification (NASBA) is an isothermal nucleic acid amplification technique. The reaction involves the simultaneous activity of three enzymes, Avian Myoblastosis Virus-Reverse Transcriptase (AMV-RT), ribonuclease H and T7 RNA polymerase. Their concerted action enables amplification of the nucleic acid sequence of interest to $>10^9$ copies in approximately 90 minutes. The reaction occurs isothermally at 41ºC so thermocycling equipment is not required. NASBA can be used to selectively amplify RNA sequences in the presence of DNA since DNA strands are not melted out, unlike RT-PCR.

12.2.1 Hybridisation

Hybridisation is the process whereby single stranded nucleotides anneal together. The similarity of the two sequences determines their degree of hybridisation. Membrane-based hybridisation methods were developed in the late 1980s and remain a standard method for the detection and quantification of nucleic acids. Two different but similar protocols, Northern blotting and Southern blotting, are used depending on whether DNA or RNA is being analysed. Both procedures involve separating the nucleic acid by electrophoresis. This is then transferred to and immobilised on a support membrane (e.g. nitrocellulose or nylon). The membrane is probed with a labelled probe to detect the sequences of interest. After detection, the nucleotides that have hybridised to the labelled probe are revealed as bands.

Hybridisation can also be assayed in solution, thereby avoiding the time-consuming steps of gel electrophoresis and membrane transfer and probing. Solution hybridisation colorimetric endpoint detection (PCR ELISA) facilitates the specific and sensitive detection of PCR amplification products.

12.2.2 Microarrays

Microarrays or gene chips provide a miniaturised system for the simultaneous analysis of hybridisation to an array of oligonucleotide probes immobilised on a support such as glass or a synthetic membrane. The format enables the analysis of a large number of genetic features in a single hybridisation experiment. Microarrays are a powerful tool for the quantitative study of gene expression and are increasingly being considered as a tool for detecting and describing complex microbial communities.

12.3 Key principles

12.3.1 Probe and primer design

The primary requirement for a probe or primer pair is that it is specific for the organism(s) of interest. That is that the gene sequence is only found in the target, and under the assay conditions the probe or primer pair does not give non-specific reactions. It is difficult to generalise about the choice of target sequence since it depends so much on the target organism. In general the choice of a nucleotide sequence present on extrachromosomal DNA or mobile elements would not be recommended due to the increased possibility of lateral gene transfer to other microorganisms (false positive results) and the possibility of false negative results from isolates that had lost the target sequence. Pathogenicity determinants e.g. toxin genes are an obvious choice as targets since they are often well characterised and are a diagnostic feature of the pathogen. However, not all pathogenic determinants are restricted to a particular genus or species. A large number of PCR assays have been developed for the detection of *E. coli* O157:H7 based on conserved regions of shiga toxin (*stx1* and *stx2*) and intimin (*eaeA*) genes, yet these genes are not unique to serotype O157:H7 (Chizhikov *et al*., 2001). Indeed there have been some reports of shiga toxin-like genes in non-enterics such as *Aeromonas* (Haque *et al*., 1996) and that not all serotype O157:H7 isolates contain these genes (Galland *et al*., 2001). Genes used as molecular taxonomic markers (see Chapter 13) such as ribosomal DNA genes (Tsen *et al*., 2000) and DNA gyrase (Venkateswaran *et al*., 1998) have also been used as targets for probe and primer pair design. These genes are particularly suitable for detecting pathogens were pathogenicity is species specific.

While some pathogens can be detected on the basis of a single nucleotide sequence, detection of pathogens with a complex pathotype or that are genetically similar to other pathogens, such as the pathotypes of *E. coli* or serovars of *Salmonella,* require complex assay procedures such as multiplex PCR or microarray analysis of both general pathogenic determinants and serotype or strain specific determinants (Chizhikov *et al*., 2001). Increasingly, comparison of genomic data or detailed subtractive hybridisation studies are being used to identify novel genes for the detection of pathogens. In addition to providing new targets, such as the single locus that appears to be restricted to

Salmonella enterica Serovar Enteriditis (Agron *et al*., 2001), they also offer a more rational strategy for the choice of target sequences.

12.3.2 Extraction and isolation of DNA from food

The usefulness of molecular methods may be limited by the presence of substances that inhibit the reactions (Lantz *et al*., 1994). A wide variety of foods and compounds present in foods have been reported to inhibit PCR reactions including collagen, heme, acidic polysaccharides, humic substances, cheese and bean sprouts (Lantz *et al*., 1994; Kim *et al*., 2000). Some of these compounds co-purify with the DNA so extensive DNA purification may be required before samples can be assayed. Separation of the pathogens from the food matrix prior to DNA isolation should prevent the carry-over of inhibitors into the reaction since these compounds are associated with the food matrix. Many methods of physical separation have been described including centrifugation, filtration, ion exchange resins and immunomagnetic separation. While these methods improve the sensitivity of detection, none has broad applicability for all foods and the methods need to be optimised for each product or product/pathogen combination (Lantz *et al*., 1994; Lucore *et al*., 2000).

Simple physical separation or direct DNA extraction often lacks the sensitivity required to detect low numbers of pathogens in food samples. A brief cultural enrichment, followed by physical separation of the organisms from the culture has the benefits of separating the pathogen from the food matrix, diluting the concentration of inhibitory compounds and increasing the number of the target organisms (Lucke and ten Bosch, 1998). This approach has been almost universally adopted, where it is applicable, due to the marked increase in sensitivity (from *ca*. 10^2–10^3 cells to 10^0–10^1) given by even a brief (6–12 h) enrichment. Clearly this is not possible for microorganisms that are difficult to cultivate or are nonculturable.

12.4 Applications for particular pathogens and foods

12.4.1 Viruses in seafood

Contamination of shellfish growing/harvesting waters with sewage can result in shellfish accumulating human enteric viruses. As shellfish are often consumed raw or after minimal heat treatment, consumption of virus contaminated shellfish poses a hazard to human health. Viruses of particular concern are members of the family Caliciviridae (Norwalk-like or small round structured viruses and Caliciviruses) and hepatitis A virus. These viral enteric pathogens cannot be cultivated *in vitro*. Some other enteric viruses (rotavirus, enteric adenoviruses and certain astroviruses) can be cultured with some difficulty; however, diagnostic technology is insufficiently developed to permit their routine isolation (CDC, 1990). Molecular methods of detection are therefore the only available technique for detecting the presence of these viruses.

Reported levels of shellfish contamination are low, ranging from 0.3 to 200 plaque forming units (pfu) per 100 grams of shellfish (Sobsey *et al*., 1991; Rose and Sobsey, 1993). Early attempts to detect these RNA viruses were based on hybridisation with virus specific probes. However, the sensitivity of that approach (limit of detection 10^3–10^4 pfu) is not always able to detect the low concentration of virus particles typically found in shellfish (Jean *et al*., 2001; Romalde *et al*., 2002). Recent attention has focused on the development of RT-PCR and NASBA-based assays. Detection is made difficult by the low concentration of virus and the presence of glycogen and acidic polysaccharides in shellfish that inhibit amplification. Detection protocols therefore rely on recovery of intact virus (e.g. by immunocapture) or virus RNA prior to amplification. In a comparison of seven methods for the extraction of hepatitis A virus from shellfish it was found that methods based on the extraction of RNA were the most effective and suitable for routine diagnostic testing (Arnal *et al*., 1999). Studies of the bioaccumulation of viruses by shellfish have shown that virus is accumulated in the digestive tract (stomach and hepatopancreas) of shellfish. Dissection and extraction of these tissues results in increased sensitivity of detection compared with extraction of whole shellfish (Romalde *et al*., 2002).

After more than a decade of development current protocols provide rapid, robust, specific and sensitive detection of enteric viruses in shellfish. Two possible limitations of the techniques are that they cannot distinguish between infective and inactivated viruses and they are not quantitative. Although the infectious dose of these viruses is not known, it is thought to be as low as 10–100 virions. Therefore detection of these viruses is of public health concern whether the amount can be quantified or not.

12.4.2 *Listeria monocytogenes* in food

Listeria are widespread in nature and are commonly found in soil, vegetation and sewage. One species, *Listeria monocytogenes*, is a potentially lethal foodborne bacterial pathogen. It is often present in milk, meats, soft cheese, vegetables and seafood and has the potential to grow on such products even under proper refrigerated storage. Serotypes 1/2a, 1/2b and 4b constitute more than 95 per cent of human clinical isolates although a definitive pathotype has yet to be identified. Detection of *L. monocytogenes* in foods is complicated by the presence of the closely-related but non-pathogenic species *L. innocua*. Conventional species specific identification procedures are labour intensive and time-consuming. In addition isolates with major differences in the key biochemical criteria have been reported, making the utility of these tests uncertain (Bubert *et al*., 1999). Thus there is clear potential for the development of rapid molecular methods for the specific detection of *L. monocytogenes*.

A variety of PCR assays have been developed for *L. monocytogenes* based on a number of genes. Most assays include a brief culture enrichment step to increase sensitivity and to remove compounds inhibitory to amplification. Soft

cheeses in particular pose difficulties because of inhibitors. The assays have demonstrated that *L. monocytogenes* can be detected in the presence of high numbers of other *Listeria* species (Stewart and Gendel, 1998). However, there is a potential problem with culture enrichment prior to PCR as *L. innocua* can suppress the growth of *L. monocytogenes* during co-culture in commonly used enrichment media, which may result in false negative results (Norton *et al*., 2001). The presence of certain foods during enrichment may also inhibit the growth of *L. monocytogenes*. It has been reported that the minimum level at which *L. monocytogenes* spiked onto carrots could be detected was 100-fold higher than for other vegetables, presumably due to the presence, in the carrots, of substances inhibitory to the growth of *Listeria* (Shearer *et al*., 2001). Immunocapture has been used successfully to circumvent the need for culture enrichment but limits the possibilities for improving the sensitivity of the assay (Hudson *et al*., 2001) as the immunocapture step has a detection limit of approximately 10^2 organisms/ml (Oberst *et al*., 1998). In general molecular methods of detection show good correlation with conventional culture techniques and provide results in 1–2 days compared with 4–7 days for conventional methods.

12.5 Advantages and disadvantages

12.5.1 PCR

PCR by itself is a way of amplifying a nucleotide sequence. Following amplification the presence of an amplicon still needs to be detected. The most commonly used research technique for this is gel electrophoresis which reveals the PCR products as bands on a gel. This does not show the specificity of the PCR product as it merely demonstrates whether a band of the expected size is obtained or not. The band could be due to a non-specific reaction product that happened to be the same size as the expected product. Hybridisation with diagnostic probes or sequencing are required to prove that the expected amplicon is obtained. Gel-based systems also lack sensitivity for detecting low levels of PCR product. Their advantages are that they are cheap to set up and run and can readily show the presence of unexpected bands.

12.5.2 Hybridisation

Hybridisation is, in general, less sensitive and more time-consuming than amplification-based assays. An additional limitation of membrane hybridisation is the difficulty associated with multiple probe analysis. To detect more than one message, it is usually necessary to strip the initial probe before hybridising with a second probe. This process can be time-consuming and problematic, since harsh treatment is required to strip conventional probes from blots. The use of microarrays removes the need for reprobing as many targets can be detected at once. Microarrays are also suited for automation although at a higher capital cost.

12.5.3 Contamination

Amplification techniques are susceptible to in-lab contamination with PCR amplicons resulting in false positive results. This is especially true for nested PCR due to the extra handling step between the two PCR reactions. This problem can be minimised by controlling the work flow so that sample preparation areas are physically separated from PCR and PCR product detection areas. An additional level of protection is provided by using dUTP rather than dTTP in the amplification reaction. Samples are pre-treated with uracil *N*-glycosylase (UNG) which specifically degrades nucleotides containing dUTP before amplification thereby removing any contaminants. The use of a heat labile UNG allows this to be done in the same tube as the PCR reaction itself (Schwab *et al*., 2001).

As noted earlier, if an RT-PCR assay is designed to be specific for a RNA target then it is essential that no contaminating DNA is present in the sample as this will be amplified as well. Treatment with DNAase is normally sufficient to ensure samples are free of DNA. All RNA-based methods are susceptible to false negative results due to degradation of RNA during isolation. If RNA samples are even slightly degraded the quality of the data and particularly the ability to quantitate results are severely compromised. RNase-free reagents and techniques are essential and often a dedicated RNA work area is required.

A further consideration for RNA-based microbial assays is that it is important that the target gene is either expressed constitutively or under those conditions present on the food or in the enrichment culture since, if the gene is not expressed, there will be no RNA to detect.

12.5.4 Viable and non-viable organisms

One often cited disadvantage of molecular methods is their inability to discriminate between viable and non-viable cells. RNA-based methods are believed to be less prone to this disadvantage as RNA is quickly degraded upon the death of microbial cells. However, it has been reported that residual microbial DNA and mRNA could be detected by PCR and NASBA, respectively, for up to 30 h post-thermal death. The authors concluded that a single quantitative measurement based on nucleic acid amplification did not permit unequivocal determination of cell viability (Birch *et al*., 2001). In practice, the magnitude of the problem will depend on how the microbial cells were killed, the physical medium they are in and the number of dead cells present. Greater than 10^6 dead cells/25 g of sample was required to give a false positive result with the Probelia Salmonella kit (Fach *et al*., 1999) due to the enrichment stage which effectively dilutes this to *ca*. five dead cells per assay. Since most microbial assays include a culture enrichment stage the presence of low numbers of non-viable cells is unlikely to result in false positive results.

12.6 Examples of commercial kits

While there are many commercially available options for the basic components of molecular assays there are only a small number of complete molecular-based detection kits available commercially. Table 12.1 lists the most widely used of these kits. Three of the kits are PCR-based (Probelia, BAX and TaqMan®) while the other is a hybridisation assay (Gene-Track). All kits rely on a culture pre-enrichment step. For the PCR based kits this is 16–24 h, while for the Gene-Track a 40–48 h enrichment is used. All the kits use ready-prepared reagents so there is minimal preparation required by the user. For Probelia, detection involves a colorimetric reaction in a microplate format following PCR. TaqMan® and the automated BAX system are a microplate format that incorporates both PCR and detection in the same unit. BAX is also available as a manual system where results are obtained by gel electrophoresis of the PCR reactions. Gene-Track is based on a colorimetric detection system following hybridisation with labelled probes.

There are no reports of comparisons between these systems with literature reports being confined to comparison of these assays with conventional culture (e.g. Oberst *et al*., 1998 TaqMan®; Stewart and Gendel, 1998 BAX; Fach *et al*., 1999 Probelia). These reports show that with minor limitations these assays are comparable in sensitivity to culture-based methods. Although in one report it was noted that both BAX and culture assay were unable consistently to detect low numbers of *E. coli* O157:H7 from alfalfa sprouts (Shearer *et al*., 2001).

Table 12.1 Partial list of commercially available, nucleic acid-based assays used in the detection of foodborne bacterial pathogens

Trade Name	Organism	Format	Manufacturer
BAX	*E. coli* O157:H7 *L. monocytogenes* *Listeria* spp. *Salmonella*	PCR	DuPont Qualicon http://www.qualicon.com/
Gene-Trak	*L. monocytogenes* *Listeria* spp. *Salmonella*	probe	Neogen Corporation http://www.neogen.com
Probelia	*Campylobacter* *Listeria* *E. coli* O157:H7 *Salmonella*	PCR	BioRad http://www.biorad.com
TaqMan®	*E. coli* O157:H7 *Salmonella*	PCR	Applied Biosystems http://www.appliedbiosystems.com

12.7 Future trends

Molecular methods are available that enable the rapid, sensitive and cost effective detection of microbial pathogens in foods. These have been adopted quickly by the research community as the technology can easily be adapted and customised for particular research needs, resulting in a proliferation of different methods for the detection of particular pathogens based on different genes and different chemistries. This proliferation has probably been a detrimental factor slowing the adoption of molecular methods in diagnostic laboratories. Publication of a method in the scientific literature does not automatically make that method usable in a commercial setting. In this increasingly litigious world and in a climate where the presence of microbial pathogens in a product can be used as a barrier to trade, commercial laboratories are obliged to use standard, approved methods for detecting pathogens in food. Proliferation of methodologies does nothing to help standardisation. Lack of standardisation increases the difficulty of gaining approval from national regulatory bodies, the latter being a bottleneck for the adoption of any new technology or methodology. In contrast the availability of commercial molecular-based kits for the detection of microbial pathogens should facilitate the adoption of this methodology in commercial laboratories as the manufacturers have already established them as standard methods and in many cases gained acceptance from some national regulatory bodies.

Future trends in the development of molecular methods will be focused on improving the sensitivity of direct detection without the requirement for culture enrichment and on the simultaneous detection and identification of several pathogens. The latter will rely increasingly on microarray technologies which are becoming more commonplace in laboratories.

12.8 References

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