MOLECULAR METHODS FOR THE DETECTION OF FOOD-BORNE PATHOGENS AN OVERVIEW

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Abstract

With various food-borne pathogens that are liable for human illnesses and in some occasions even deaths, a rapid method for detecting these pathogens has become critical, not only in the food industry for hygienic and monitoring purposes, but also to ensure the safety of consumers. Traditional methods for the detection of food-borne pathogens are cumbersome and time consuming and various rapid methods to detected food-borne pathogens have been established. Among these rapid methods described in the literature, DNA-based methods that purify pathogen DNA from food samples by phenol-chloroform extraction methods or the extraction of pathogen DNA by commercially available DNA extraction kits are commonly used. The DNA-based methods are also more sensitive and selective than the traditional methods, although many of these DNA-based methods are inhibited by food components that play a fundamental role in the sensitivity of the DNA amplification reaction. This article review methods used to extract DNA from food samples, as well as the methods used to separate and/or concentrate bacteria found in food samples.

1. INTRODUCTION

It is believed that over 40 different food-borne pathogens are responsible for human illnesses (Buzby and Roberts, 1996) and in the United Kingdom an average of 9.4 million food-borne illness cases are reported annually (Walker et al., 2003). It is estimated, by the U.S. Centers for Disease Control and Prevention that food-borne diseases cause between six - and 81 million illnesses, 325,000 hospitalisations, and 5,000 deaths in the United States every year (Mead et al., 1999; Lampel et al., 2000; CDC, 2003) and the people mostly affected by the food-borne pathogens are children, the elderly and immunocompromised individuals.

The traditional or conventional methods used to detect food-borne bacteria predominantly rely on enrichment and/or growth on selective media, followed by isolation, biochemical identification, and/or immunological characterization (Ramesh et al., 2002; Volokhov et al., 2002; Wu et al., 2003; Li and Mustapha, 2004). Enrichment of samples for a 24-48h period is usually the first step in this identification process of microorganisms (Gasanov et al., 2004), and a variety of methods can be used for the identification. Commercially identification kits for the validation and identification of some of the pathogens are available, for example the API system, Enterotube, Minitek, Crystal ID system, MicroID, RapID systems, Biolog and Vitek (Fung, 2002) but are expensive. These methods are mostly found to be time consuming (Agersborg et al., 1997; Ramesh et al., 2002; Volokhov et al., 2002; Li and Mustapha, 2004), as well as labour-intensive (Hudson et al., 2001). The rapid detection of the food-borne pathogens is critical and essential for ensuring the safety of consumers and the development of a rapid and accurate procedure for detecting and characterizing food-borne and other micro-organisms is required in many areas of research, as well as in the industry especially in areas were biological safety is a major issue (Scheu et al., 1998). In the light of the serious health-related and economic implications caused by food-borne pathogens, the development

of rapid detection techniques that are highly specific became essential to supplement conventional methods.

The major advantage of molecular techniques is that it is based on differences within the genetic information of an organism and can be used to amplify and characterise deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and do not rely on the expression of certain phenotypic characteristics or enzymes to facilitate identification (Ramesh et al., 2002; Gasanov et al., 2004). These nucleic acid-based techniques pose an additional advantage in that it does not depend on the growth state of the food-borne pathogens or environmental influences (Scheu et al., 1998). It is also found that these techniques are accurate, repeatable and some can be performed in the same time as an immunoassay method (Meyer, 1999; Gasanov et al., 2004).

This article summarizes developments and methods used for extracting DNA from food-borne pathogens present in food samples, as well as refers to other techniques that aided the removal of the food matrix from the food samples before DNA extraction and DNA amplification by the polymerase chain reaction (PCR).

2. MOLECULAR TESTS

Tree steps achieve the principle of identifying microorganisms with nucleic acid-based methods in any sample: 1) nucleic acid extraction 2) nucleic acid amplification by PCR and 3) the verification of the PCR products. It is stated that the molecular tests employing nucleic acid (DNA/RNA) amplification have been the most successful molecular methods used (Van Belkum, 2003) and it has been found that all studies or experiments, regarding nucleic acids required a reliable source of DNA or RNA and thus a dependable method for extraction is required and the quality and quantity of the nucleic acids is thus very important (Nicholl, 1994).

3. DNA EXTRACTION METHODS

Many DNA extraction methods exist and are found for a wide range of materials, for example: the CTAB extraction method of Doyle and Doyle (1987) and extractions for fresh plant material (Alary et al., 2002; Busconi et al., 2003); the phenol-chloroform, silica based, InstaGene MatrixTM (BioTest), glass fibre filter and the Chelex based methods for forensic cases (Hoff-Olsen et al. 1999). DNA isolation for genetically modified organisms (GMO) in foodstuffs or in the food production chain has also been successful (Meyer, 1999; Alary et al., 2002; Miraglia et al., 2004; Peano et al., 2004). Extracted microbial DNA from processed food was successfully amplified (Agarwal et al., 2002) and DNA from olive oil was also extracted (Busconi et al., 2003). Bacterial DNA has furthermore been extracted from water samples (Higgins et al., 2001) and soil samples (Henne et al., 1999).

The DNA extraction methods normally involve the following steps:

1) Firstly the starting material must be disrupted. Cell wall disruption can be done enzymatically or mechanically and the lyses of the cell membranes can by done by the use of a detergent such as sodium dodecylsufate (SDS) (Açik et al., 2004).
2) Cell lyses are then followed by the removal of proteins by phenol or phenol-chloroform mixtures (Bhaduri and Cottrell, 1998; Hudson et al., 2001). Proteins accumulate at the

inter phase, while the nucleic acids remain dissolved in the aqueous phase.

3) The aqueous phase is removed and the nucleic acids are precipitated with isopropanol or ethanol.
4) For DNA studies, RNA is removed by the addition of RNase (Nicholl, 1994).

It is important to remember that the DNA extraction from food-borne microorganisms has some complications due to the chemical composition and many different ingredients such as the food matrix (von Blankenfeld-Enkvist and Brännback, 2002). Among the methods described for the extraction of DNA from food-borne pathogens, the phenol-chloroform extraction method seems to be the method of choice, but lately commercially available DNA extraction kits (Table 1) are more frequently used.Table 1. Partial list of commercially available DNA extraction kits used in the extraction of DNA from food-borne pathogens.

Manufacturer	Sample Kit	Principle	Reference
Bio-Rad	InstaGene matrix kit	Extraction of bacterial DNA	Perelle et al., 2004
Macherey-Nagel	NucleoSpin Food	Genomic DNA from food	www.mn-net.com
Promega	WizardMagnetic DNA purification System for food	Purification of DNA from vegetable oils, lecithin and chocolate	www.promega.com
	Wizard Genomic DNA purification Kit	Isolation of gDNA from gram positive and gram negative bacteria	Micka et al., 1996
Qiagen	QIAamp DNA stool mini kit	DNA purification from stool samples	www.qiagen.com/ literature
	DNeasy Tissue Kit	Isolation of gDNA from gram negative bacteria	Perelle et al., 2002
Roche Diagnostics	High Pure PCR template preparation kit	Extraction of DNA from beads	Mercanoğlu and Griffiths, 2005

The phenol:chloroform extraction method was used by, among others Duffy et al. (2001) to detect bacterial pathogens such as Listeria monocytogenes, Salmonella sp. Campylobacter jejuni and E. coli O157:H7. Bacterial DNA from milk samples was extracted by using diethyl ether and chloroform (Ramesh et al., 2002). Other DNA extraction methods without phenol and/or chloroform do exist, and are preferred for the safer handling of the samples. One such a method entails bacterial cells collected by centrifugation and re-suspended in sterile water. The cells are boiled to lyse and the lysate is cooled on ice and used directly as a DNA template (Denis et al., 2001; Agarwal et al., 2002; Martín et al., 2003; Moon et al., 2004; Kawasaki et al., 2005). To enhance lyses, the lysate could furthermore be treated with proteinase K (Bhaduri and Cottrell, 1998) and Agersborg and co-workers (1997) also treated bacterial cells with lysozyme, proteinase K and Triton X-100 respectively and Gouws and Liedemann (2005) resuspend colonies of L. monocytogenes in PCR buffer with added Triton (2%) to obtain effective lyses.

DNA extraction methods including alkali lysis (Ryu, 1940), guanidine isothiocyanate (GuSCN) (Kimura et al., 2001), immunomagnetic beads-GuSCN (Hsih and Tsen, 2001; Kimura et al., 2001) and lysis-GuSCN (Kawasaki et al., 2005) are all described in literature and have been

utilised successfully for pathogens such as Clostridium botulinum, Salmonella spp. Listeria monocytogenes and Escherichia coli. Lampel and co-workers (2000) used FTA filter membranes to prepare bacterial DNA templates derived from pure cultures and from artificially contaminated foods without arduous processing, pre-enrichment and purification steps from bacteria; Shigella, Salmonella and Listeria spp. The FTA filter membranes were also used by Tilsala-Timisjärvi and Alatossava (2004) in there study on dairy products and enhanced detection sensitivities have been observed with this filter-based technology compared to the detection sensitivities obtained with conventional template preparations.

Commercially available kits are highly suitable for the extraction of target genomic DNA (gDNA) of pathogens from foods (Moon et al., 2004) and some of the kits provided by companies such as Bio-Rad, Macherey-Nagel, Promega, Qiagen and Roche are listed in Table 1. The DNA extraction kits that are commercially available have the advantage that they use small amounts of starting material (± 0.025 g) and the DNA can be isolated in a shorter time period (approximately 30 min, depending on the lyses period). The DNA extraction kits can effectively remove PCR inhibitors and the inhibitor free isolated DNA can be amplified in order to identify microorganisms.

Bhaduri and Cottrell (1998) overcame inhibition, by removing the food matrix from the enrichment medium through swabbing. This sampling procedure has been widely used for the enrichment of a wide variety of food-borne pathogens associated with meat (Dorsa et al., 1997). Several other approaches to prevent inhibition have also been introduced and such methods are firstly the enrichment of the food samples followed by the removal of the sample matrices by biochemical methods, immunological methods, such as immunomagnetic separation (Cudjoe et al., 1995), or physical methods such as buoyant-density centrifugation, filtration or dilution, or isolation procedures using magnetic beads, dipsticks or membranes (Lantz et al., 1994; Par-Gunnar et al., 1994; Powell et al., 1994; Agersborg et al., 1997; Lindqvist et al., 1997; Bhaduri and Cottrell, 1998; Lampel et al., 2000; Li et al., 2000; Hsih and Tsen, 2001; Hudson et al., 2001; Cocolin et al., 2002; Jothikumar et al., 2003; Li and Mustapha, 2004). None of these are, however, ideal and in many cases the optimization for one food matrix or pathogen is not readily adaptable to other pathogens or matrices (Lantz et al., 1994). The DNA extraction method can thus play a cooperative role in preventing inhibition in the amplification reaction.

4. PRINCIPLE OF THE PCR FOR AMPLIFICATION OF DNA

To detect and identify the food-borne pathogens with nucleic acid based methods, DNA fragments need to be amplified by rapid and reliable methods such as the widely applied PCR (Bhaduri and Cottrell, 1998; Gouws et al., 1998; Scheu et al., 1998; Ramesh et al., 2002; Li and Mustapha, 2004). In PCR, the primers are hybridized to a specific DNA template, which is then enzymatically amplified using a thermocycler (Hill, 1996). This is a powerful technique for the detection of pathogens in food and can be used in environmental samples as well and assays based on PCR are now accepted as rapid methods for the confirmation of the presence or absence of specific pathogens in food (Fratamico, 2005)

The PCR reaction cycle consists of the following three steps (Fig. 1):

1) denaturation of the double-strand DNA (dsDNA) into single stranded DNA;

2) annealing of the primers (P1 and P2) to the complementary single-strand DNA;
3) extension of the primers with a thermo stable DNA-polymerase enzyme in the presence of free deoxynucleoside triphosphates (dNTPs) (Li and Grauer, 1991; Meyer, 1999; Duffy et al., 2001).

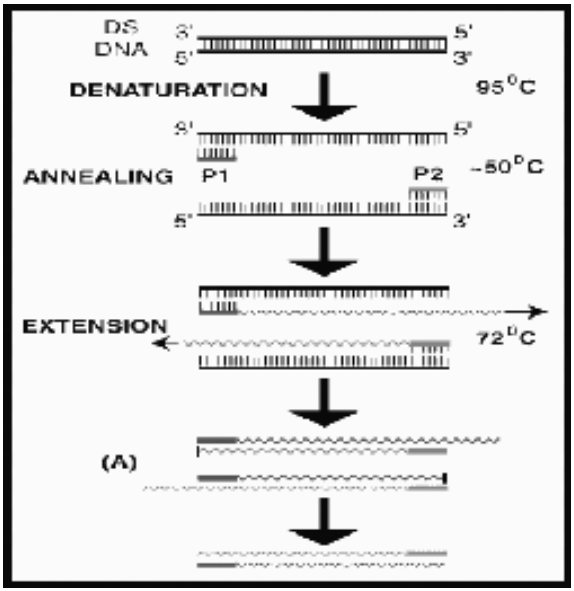

Figure 1. Different steps in PCR.

Multiple repeats of the denaturation, annealing and extension steps result in an exponential increase in the original DNA target sequence and theoretically PCR can amplify a single copy of DNA by a million fold in less than 2hrs. Amplification will continue as long as there are surplus primer and nucleotides available (Scheu et al., 1998; Duffy et al., 2001). The specificity of amplification of the DNA depends on the design of the primer sequences and the primer pair selection is thus critical in any PCR assay to avoid cross-reaction for the simultaneous detection of different pathogens.

The amplified DNA fragment can be detected through ethidium bromide stained gel electrophoresis and visualized using an ultra violet light source (Scheu et al., 1998; Denis et al., 2001). Southern blotting, hybridisation and sequencing can further be applied to confirm the identity of the amplification product (Scheu et al., 1998; Meyer, 1999) and while the PCR method has been most widely accepted, other assay formats that have yet not reached practical applications in food diagnostics, have been developed. For a recent review see Schweitzer and Kingsmore (2001).

5. LIMITS OF PCR AND POSSIBLE SOLUTIONS

The reliability of PCR detection methods depends, in part on the purity of the target template and the presence of sufficient numbers of target molecules and with the complex matrix of

foodstuff, and substances in enrichment and cultivation media, steps surely need to be taken to limit the effects of any potentially inhibitory compounds present that may limit the PCR amplification of the intended target (Bhaduri and Cottrell, 1998; Scheu et al., 1998; Ramesh et al., 2002; Bhaduri, 2003). The amplification of DNA of non-viable organisms can also be a disadvantage in quantitative studies (Ramesh et al., 2002). This problem can be prevented either by the enrichment of the viable organisms or by amplifying RNA by reverse transcriptase PCR (RT-PCR) (Denis et al., 2001). One of the disadvantages of using RNA is that the isolation of RNA is far more difficult than that of DNA and more laborious than DNA isolation and the detection of messenger RNA (mRNA) using PCR-based methods can be less sensitive than that of DNA (Scheu et al., 1998). PCR inhibition occurs thus due to various reasons for example the presence of substances chelating divalent magnesium-cations necessary for PCR as well as the degradation of nucleic acid targets and/or primers due to the presence of nucleases such as RNase and DNase and the direct inhibition of the DNA polymerase activity all play a role. Possible solutions to prevent inhibition include: 1) to dilute food samples, but with dilution of food samples, the target DNA is also diluted; 2) to separate bacteria from the food matrix by culturing prior to DNA extraction, but this have a disadvantage of time (Bhaduri and Cottrell, 1998; Scheu et al., 1998); 3) differential centrifugation or the use of immunomagnetic separation (IMS), where micro-organisms are captured with antibody-coated paramagnetic beads (Bhaduri and Cottrell, 1998; Scheu et al., 1998; Ramesh et al., 2002); 4) gel filtration or anion exchange columns to purify DNA; 5) the use of DNA extraction methods which removes PCR inhibitors.

6. ADVANTAGES AND SHORTCOMINGS

The identification of food-borne pathogens using molecular methods has become increasingly popular in quality and safety aspects of food and food production, because these techniques are extremely accurate, sensitive and specific (Meyer, 1999; Bhaduri and Cottrell, 2001). Although enrichments are experienced as shortcomings, particularly when looked at extra time spend on assays, it provides essential benefits, such as diluting the effects of the inhibitors. The enrichments also allow the separation of viable from non-viable cells and allowing repair of cells that may have been injured during food processing. However, the molecular methods are still not widely incorporated in standardized methods and are unsuitable for on site application and have some disadvantages compared to culture methods such as equipment and reagent costs (Gasanov et al., 2004). These techniques are also relatively complicated, require significant expertise and utilize hazardous chemicals and for this reason make the routine testing of many samples impractical (Bhaduri and Cottrell, 1998). Due to the lack of standard protocols, variable quality of reagents and equipment, the methodology has difficulties to move from expert-to-end-user laboratories. Most of the PCR-based methods published differ in specificity, detection limits and sample treatments. In most studies an internal amplification control (IAC), necessary to indicate false-negative results caused by PCR inhibitors, is rarely included in the final diagnostic test (Malorny et al., 2003). Although these new methods are currently mainly used in research laboratories, their considerable potential for routine testing in the future cannot be overlooked.

Food-borne microorganisms are continuously changing due to their inherent ability to evolve and their amazing capacity to adopt to different forms of stress (von Blankenfeld-Enkvist and Brännback, 2002). Therefore, food safety should be seen as an ongoing process, which is influenced by environmental, socio-economical, political and cultural factors and molecular methods can help in detecting these pathogens and surely save lives.

7. REFERENCES

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