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Review

Categorization and diagnosing of food borne bacterial pathogens with special consideration to DNA based methods

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Detection of food borne pathogen is of outmost importance in the food industries and related agencies. For the last few decades conventional methods were used to detect food borne pathogens based on phenotypic characters. At the advent of complementary base pairing and amplification of DNA, the diagnosis of food pathogen became rapid and target specific. RNA based methods such as NASBA and Real Time RT-PCR are even more sensitive as they reflect pathogen viability in food system. This review describes various DNA based methods that are used widely in detection of food pathogens. Recent rapid methods become more robust with the incorporation of PCR in assays. Simultaneously, the advances in nano-biotechnology have allowed miniature devices permitting development of portable hand held biosensor for the detection of food pathogens.

Key words: Food borne pathogens, molecular techniques, PCR, rapid identification techniques.

INTRODUCTION

Food pathogens are mainly bacteria that comprise the genera of *Clostridium botulinum, Escherichia coli, Salmonella* spp., *Listeria monocytogenes, Yersinia enterocolitica, Staphylococcus aureus, Shigella* spp., *Bacillus cereus,* and *Campylobacter jejuni*. About 98% of microbes found in food commodities are non-pathogenic (SanathKumar et al., 2002) e.g. *L. monocytogenes* is pathogenic but *L. innocua* is non-pathogenic. Therefore it is required to develop diagnostic tests that can specifically detect the target pathogen. The characterization and detection of food borne pathogens continue to rely on conventional culturing techniques. Conventional culture methods for the detection of food borne pathogen in food include homogenization, enrichment in nonselective and selective medium followed by plating in differential agar medium to isolate pure culture. Finally phenotypic and genotypic characterization takes 3-4 days to confirm the result. Biochemical and immunological methods for the detection require substantial amount of pure culture whereas DNA based methods can be performed with mix culture or community DNA. The final detection stage requires gel electrophoresis and further sequencing of the amplified product in addition to the normal step of PCR, thus increasing the time and complexity of detection.

Since the recognition of food borne pathogen, there is rapid advance in the development of suitable methods for their isolation and identification. The universal media were developed for enrichment of range of pathogens from food samples (Bhaduri and Cottrell, 2001). Identification methods that evolved initially are time consuming and labor intensive. It led to a search for more rapid and sensitive methods particularly in the food industries to identify the food borne pathogens at short time frame.

There are several traditional microbiological methods for testing foods for the presence of pathogen, which rely on growth in culture media, followed by isolation, morpho-

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logical, biochemical and serological identification. Recognition of surface antigens by antibodies resulted in the grouping of organisms based upon similarities of lipopolysaccharides (LPS), capsule or flagella. Detecting conserved virulence traits such as fimbriae, toxins or invasion gene were often the first method of pathogen identification. Traditional biochemical and immunochemical methods for the detection of microorganisms in food have been supplemented by a number of DNA-based methods during the last decade (Olsen, 1995). Conventional methods that determine genus and species of the microorganisms are unable to differentiate their pathogenesis especially those caused by *E. coli, Clostridium* spp. and *Listeria* spp.

The recent sequencing of a number of bacterial genome and the development of new analytical tools will have a profound impact on present ability to understand, manipulate, detect, exploit or combat bacteria (Pennisi et al., 1999) . The exponentially increasing DNA sequence database leads to better probe design for better detection and quantification of bacteria. Even a minute amount of target DNA can be amplified with the help of PCR technique. The amplified fragments are sequenced and the primer designed to target conserved DNA sequence can be used to analyze a wide verity of food born microorganisms. The target DNA may be housekeeping genes, genes coding for virulence and strain specific genes, which can be amplified and sequenced.

In order to get the best performance and reproducibility of the results by various method of identification, it is mandatory to follow the standard protocols with utmost care even for sample preparations, DNA isolation, propagation, manipulation and preservation as the quality of template DNA decides the fate of experiments (Weimer et al., 2000). The rapid methods employed for identification of food borne microorganisms are grouped and discussed in this study below.

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLP)

RFLP is very simple method that uses only a restriction enzyme digestion of the genomic DNA. It is used for the comparison of the number and size (mass) of fragments produced by digestion of DNA with a restriction endonuclease cutting at a specific recognition site (Lichtenfels et al., 1997; Ueda et al., 2005) . Usually these restriction sites composed of 4 or 6 base pairs. The resulting DNA fragments are examined by electrophoretic separation. Presence, absence, or change in the mass of the resulting DNA fragments is evidence of altered DNA sequences. RFLP in combination of PCR has been used for the accurate detection of *Staphylococcus* and *Listeria* species (Atanassova et al., 2001; Dubois et al., 2003). This method requires pure culture for the discrimination of bacteria at the species level.

PULSE FIELD GEL ELECTROPHORESIS (PFGE)

PFGE is a DNA-based sub-typing method that generates DNA banding patterns after DNA is cut into fragments with rare cutter restriction enzymes. The restriction enzymes cut the DNA yielding 8-25 large bands. After restriction digestion, the fragments are separated electrophoretically by size on an agarose gel. PFGE separates DNA in an agarose gel by using current applied at alternating angles to reduce sheering of large DNA fragment.

The restriction enzymes selected must yield sufficient size fragments for electrophoretic separation (1,000 to 15,000 base pairs) and appropriate number of bands (It should not be too numerous to detect or too few to discriminate between isolates) . PFGE has been used for characterizing *E. coli* (Mitsuda et al., 1998; Philippe et al., 2005), *Salmonella, Listeria* and other food borne pathogens. Databases of these pathogens are stored in PulseNet and Food-Net, for access by Centers for Disease Control and Prevention, Food and Drug Administration, and United States Department of Agriculture.

RIBOTYPING

The ribosomal genes are highly conserved relative to other gene within the different species of bacteria. This property can be used to determine the genetic relationship among the different species. In ribotyping, total genomic DNA is digested with a restriction enzyme similar to RFLP technique and the fragments thus generated are separated (electrophoretically), blotted (to a membrane) and hybridized (to probes specific) for the 16S to 23S rRNA genes (Ryser et al., 1996; Weidmann et al., 1997). Automated ribotyping has been used for the discrimination of *L. monocytogenes* (Gendel et al., 2004) and for the characterization of virulence gene polymorphism lineage (Weidmann et al., 1997). This technique has more impact in the study of outbreaks and epidemiological studies but has low impact in rapid detection of pathogenic microorganisms (Swaminathan et al., 1996).

HYBRIDIZATION OF DNA PROBES

DNA has a unique property to stick to the complementary base pair of itself and this property can be utilized to

identify the specific sequence within the genome. Duplex DNA is denatured into two intact strands followed by immobilization of the two on a membrane or support as a target for the probe. Probes are labeled single stranded complementary DNA that is added in solution. Random collisions of the probe with the target will base pair along complementary regions. The “stringency” of the conditions (temperature, ionic strength, etc.) affects how closely sequences must match for hybridization to occur. Excess unbound probe is washed off and the labeled double stranded DNA is detected. Radio labeled (usually P^{32} or S^{35}) or non-radioactively labeled digoxigenin and biotin (chemiluminescent) probes are detected visually. It can detect as little as 0.1 pico grams of homologous DNA. As many as 384 different ssDNA could be spotted into a single glass slide or nylon membrane to hybridize with homologous labeled sequences by the new techniques like oligonucleotide array and Micro array. Identification of various food pathogens simultaneously was carried out using the former technique by Jiang et al. (2004). Oligonucleotide array is gaining popularity in investigating the clinical samples as it does not require costly reagents and machines.

DNA AMPLIFICATION AND POLYMERASE CHAIN REACTION (PCR)

The PCR, a molecular technique, is used to amplify a specific gene or DNA fragment present in a species within the genome. This method is accurate and reliable and could be performed in short time (Almeida et al., 2000). Use of PCR techniques and DNA probes in DNA hybridization, are getting wide popularities in the food industries recently. The PCR product is a logarithmic increase in the number of copies of the targeted gene(s). They are then detected on EtBr stained gels. The size (mass) or migration of the PCR product (amplicon) suggests an expected target existed in the specimen preparation. This may be confirmed by hybridization to a labeled probe or sequencing of the amplicon. Several primers pairs with similar annealing requirements can be added to a PCR mixture to simultaneously detect several target sequences, a process called ‘multiplex-PCR’ (Khan et al., 2000).

Although the PCR method has advantages (particular specificity) it has also limitations, like it is unable to differentiate viable and non-viable microorganisms. Furthermore, the inhibition of PCR by food-derived inhibitors can lead in false negative results (Radstrom et al., 1998). Besides the development of direct hybridization techniques, there are various non PCR based methods for DNA amplification such as Transcription based Amplification (NASBA) and Strand Displacement Amplification (Schweitzer et al., 2001; Christine et al., 2004).

NUCLEIC ACID SEQUENCE BASED AMPLIFICATION (NASBA)

NASBA, an isothermal nucleic acid amplification technique designed specifically to detect RNA, requires three enzymes: an AMV reverse transcriptase, RNase-H and T7-RNA polymerase along with a set of primer for the amplification of the target RNA (Kievits et al., 1991). One of the primers have the T7 promoter sequence amplifies cDNA strand using reverse transcriptase enzyme. The DNA/RNA Hybrid formed is digested by RNase-H to produce a single stranded DNA. The second primer having complementary base pair of the cDNA binds it and polymerizes to form dsDNA by reverse transcriptase. The dsDNA then produces multiple copies of single ssRNA with the help of T 7 RNA polymerase. The resulted single-stranded product could be utilized for hybridization without prior denaturation step. Detection sensitivity for the NASBA assay was determined at 1 *cfu*/*ml* (Uyttendaele et al., 1995; Cook et al., 2003). NASBA is a sensitive method, but requires more time to generate a result (Cook et al., 2004). Specific probes like molecular beacons are incorporated for the detection in real time manner (Hope et al., 2003).

LIGASE CHAIN REACTION (LCR)

LCR is a relatively new technique used to detect nucleic acid sequence of microorganisms by amplification. The method of DNA amplification is similar to PCR; however, LCR amplifies the probe molecule rather than producing amplicon through polymerization of nucleotides. Two probes for each DNA strand are ligated together to form a single probe. LCR uses both a DNA polymerase enzyme and a DNA ligase enzyme to drive the reaction. Like PCR, LCR requires a thermal circler to drive the reaction and each cycle results in a doubling of the target nucleic acid molecule. LCR can have greater specificity than PCR (Barany et al., 1991; Khanna et al.,1999; Wu and Wallace, 1989). This technique has one disadvantage over the detection of food pathogen that it can detect DNA from dead organism.

RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS (RAPD)

RAPD, a PCR based technique, generates pattern of DNA bands on gel electrophoresis using amplification of random DNA segments with primers of arbitrary nucleotide

sequence (Williams et al., 1990) . ERIC-PCR, REP-PCR and BOX-PCR are few examples of this technique.

Short length primers bind randomly along the prokaryotic genome resulting number of different DNA fragments. The array of fragments is examined for similarity of genotypes based upon the number and size (mass) of the amplicons. RAPD has been used in detection of *Listeria* species in poultry processing environment and vegetable processing plants to identify the source of contamination and dissemination routes (Gilmour et al., 1995; Garcia-jalon et al., 2004).

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

AFLP has been applied successfully to variety of food pathogenic bacteria (Mclauchlin et al., 2002). The method involves restriction endonuclease digestion of total purified genomic DNA followed by ligation of the resulting fragments to a double-stranded oligonucleotide adapter complementary to the base sequence of the restriction site. The adapters are designed such that the original restriction site is not restored after ligation, thus preventing further restriction digestion. Selective amplification of sets of these fragments in PCR is achieved with primers corresponding to the contiguous base sequences in the adapter, the restriction site plus one or more nucleotides in the original target DNA. The resulting PCR-amplified DNA fragments are then analysed by gel electrophoresis. It was reported previously that AFLP could be applied successfully for epidemiological typing of *B. cereus* and *C. perfringens* cultures from cases of food poisoning (Ripabelli et al., 2000) A problem related to AFLP analysis is the incomplete digestion of chromosomal DNA which may result in aberrant AFLP pattern (Lukinmaa et al., 2004). AFLP can be applied for the determination of source of contamination, the natural reservoir of the strain such as live stocks etc (Siemer et al., 2004, 2005). AFLP in combination with automated laser fluorescence analyzer has been used for rapid and reliable identification at the strain level (Aarts et al., 1999).

MULTIPLEX PCR VS UNIVERSAL PRIMERS

Multiplex PCR is a technique in which more than one pair of a primer is used to amplify the different fragment of target gene simultaneously to save time and minimize the expense on detection of food borne pathogens (Slavik et al., 2003; Bottero et al., 2004). However, great care must be taken to ensure that primers have same melting temperature and they must not interact with each other. The only limitation is that the amplified fragments of same length cannot be detected and less quantity of amplified product may not be visible on agarose gel.

In Multiplex PCR Large number of primer pairs is used. This is impractical in single tube experiment. French et al. (1999) used a universal primer for the detection of multiple pathogens simultaneously. In this method single set of primer is used to amplify conserved stretches of DNA from 16 and 23 s rDNA. By developing the suitable probe the organism can be identified at species level but not the genus.

REAL-TIME PCR

Although PCR is very sensitive method but detection of the amplified fragment using gel electrophoresis is limited to the end point analysis only. Real-time PCR method allows built in product detection (both quantitative and qualitative) during the entire reaction period including exponential phase of the amplification reaction. RT- PCR is so named as one can continuously monitor the development of amplicons in a fluorimeter. SYBR-Green or other fluorescent labeled probes that emit lights during amplification are widely used in Real-time PCR. The emitted light signals corresponding to DNA amplification recorded at frequent intervals generating a curve showing product generation. The more targets DNA amplifies in the sample, the earlier amplicons can be detected and the peak curve is generated. The specificity, however, relies on the use of a specific probe (Tichopad et al., 2003).

Baggi et al. (2005) have used Real Time-PCR for rapid detection of diarrheagenic *E. coli* using SYBR Green Dye and best sensitivity and specificity was observed. However, in order to validate the data or incorporate the data one should be very particular about the similarities of test conditions, test parameters and sequence data of target genes if the experimental set up does not belong to same laboratory.

For the detection of food samples 5' nuclease multiplex PCR can also be employed. The method uses the 5' nuclease activity of Taq Polymerase (Holland et al., 1991; Exner et al., 2002; Fach et al., 2003) and FRET Technology (Tobias and Koo, 2003) in same experimental setup with multiple primers and probes. The method can be optimized by the amount of each primer pair to achieve the maximum amplification in separate reaction condition and the target loci must be checked separately for amplification with same reaction conditions and same PCR program.

Although these methods are highly specific and accurate, utmost care has to be taken to standardize the method to isolate DNA from various microbes in food samples. The DNA of dead microorganisms is also pre-

sent which can amplify and give false positive results. Ethidium monoazide can be used to separate dead and viable bacteria (Bolton and Kearns, 1978; Saiki et al., 1985; Max et al., 1997; Caron et al., 1998; Nogva et al., 2003; Keer et al., 2003; Rudi et al., 2002, 2005) . Buoyant density centrifugation called floatation is used successfully as prior sample treatment to eliminate the free DNA in the sample (Wolffs et al., 2005). This can lower the risk of false positive results. Real-time PCR using RNA as template is more authentic since the RNA is present only in viable microbes. RNA is first reverse transcribed to cDNA and in second step used for amplification. The following things should also be taken into account: search of virulence genes, strain specific genes, appropriate nucleic acid sequence, selection of primer and probes, adequate length of primer and probe, nucleotide composition, melting temperature etc.

LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

For the detection of very low amount of target food pathogen/DNA, amplification of DNA is essential. Next to the PCR, LAMP method of DNA amplification does not require a cyclic process with a specific temperature profile and amplifies DNA with greater specificity, efficiency and rapidity (Notomi et al., 2000). *Bst* DNA polymerase is the largest fragment of *Bacillus stearothermophilus* DNA polymerase protein that contains the 5' 3' polymerase activity, but lacks the 5' 3' exonuclease domain which performs 5' 3'exonuclease activity. *Bst* DNA polyme-rase has strand displacement activity which synthesizes DNA with loop forming primers to yield long stem loop products under isothermal condition. The two set of primers are used that recognize a total of six distinct sequences on target DNA. LAMP assay has been successfully used to detect *Yercenia ruckeri*, causative agent of enteric red mouth disease in fish whit 10 folds higher sensitivity then PCR (Mansour El Matbouli et al., 2008). The limitation of this method is presence of impurities in DNA sample (Wang et al., 2008), though it has been used successfully to detect salmonella (Wang L et al., 2007).

GOLD NANOPARTICLE BASED BIOSENSOR

Biosensor combined with goldnanoparticles has been used for the rapid detection of food pathogen (Leonard et al., 2003). The nanometer size gold particles have been used for the detection of specific DNA sequence (Daniel et al., 2004). Thiol modified oligonucleotide covalently bound with gold nanoparticles is being used as a probe in various rapid detection methods (Gaynou et al., 2003). Again the functionalized chemistry is not so popular because of its cost. The approach was taken to use non functionalized GNP for the detection of dsDNA and ssDNA (Huixiang and Rothberg 2004; Sangchul et al., 2009). In this method citrate coated Gold nano particles have a characteristic red color in colloidal state. The aggregation of GNP can be easily induced by addition of salts resulting in purple color; the difference in color was visualized with naked eyes. The negatively charged GNP has electrostatic interaction with ssDNA which can uncoil in such a way so that its hydrophilic negatively charged phosphate backbone is exposed to aqueous solution and DNA bases interact with the GNP surface by van der Waals forces. These interactions add negative charge to GNPs and enhance their repulsion. These properties have been exploited to design a biosensor which can detect a PCR product directly in the same tube in few minutes. The developed biosensor is highly specific and sensitive and could detect low concentration of DNA compared to existing methods.

FIBRE OPTIC BIOSENSOR

Recent developments for rapid detection have become robust with the combined use of PCR. Evanescent wave fiber optic biosensor developed by Centre for Biomolecular science and Engineering Naval Research Laboratory has been used to detect 8.1 CFU/ml in a complex food matrix (Simpson et al., 2005). This time of detection has been tremendously reduced to 2 from 10 h previously done (Tims and Lim, 2003). This was achieved because the enrichment step was omitted. The drawback with this type of biosensor is pretreatment of waveguide optic fiber at low temperature for the attachment of antibodies to capture the cells.

Over the last decade biosensors has been developed for the detection of microorganisms, allowing rapid and "real-time" identification, which include surface plasmon resonance (SPR) (Chinowsky et al., 2003; Campbell et al., 1998) amperometric, potentiometric, and acoustic wave sensors and their applications for the detection of pathogens in food and water. There are certain limitations of applying biosensors for the detection of pathogens, such as sensitivity, cost and the need for sample pre-treatment. Nano-technology is becoming the standard for diagnostic assays and combining monoclonal antibodies to capture target organisms, with rapid DNA detection by PCR resulting in very specific and sensitive results.

ELECTROCHEMICAL / ELECTRIC BIOSENSORS

Electrochemical DNA biosensors exploit the property of

ssDNA to hybridize with its complementary strand and are used in the detection of specific sequence of DNA. Upon addition of fully complementary DNA or mismatched DNA, the time dependent changes in the redox signal were monitored. The most crucial step in developing the electrochemical biosensor is immobilization of DNA probe on the desired substrate since the sensitivity, specificity and reproducibility are significantly affected by this step. For the effective binding of the DNA to its substrate, the terminus of the DNA or the surface of the substrate must be functionalized. Affinity binding of streptavidin and biotin has been successfully used for the effective immobilization of DNA probe. Gold substrates are also gathering special attention due to their covalent attachment with thiolated DNA. This technology has a special interest in the search for rapid, portable and low cost testing systems. The electrochemical biosensors have been successfully used to detect *E. coli* 0157:H7 DNA combined with PCR (Berganza et al., 2007). There are devices that comprise nanoelectrodes separated by a narrow gap having width comparable to the size of nanoparticles. These goldnanoparticles are immobilized with ssDNA. The hybridization reaction with target DNA leads to change in electric conductance (Chil et al., 2006). These nanoelectrode devices are ultrasensitive and can be used repeatedly. The main constrain with these devices are their fabrication and preparation of DNA probe. Similarly goldnanoparticles were replaced with carbon nanotubes and silicon nanotubes were used for the detection of DNA (Andrew et al., 2008).

CONCLUSION

Isolation and identification of food borne pathogen by biochemical and immunological methods are time consuming and have less sensitivity over the molecular methods. DNA polymorphism among the different species of bacteria has been exploited to identify food pathogen. 16S ribosomal RNA gene, strain specific gene, and virulence gene are the targets used for the identifica-tion of food pathogens. The property of complementary base pairing has been used in PCR and Real Time PCR methods. The 5' nuclease Multiplex PCR assay also found applications in screening of microorganism in food commodities and various environmental samples; the method will be also effective for slow growing or non-cultivable microorganism. RNA based method is more reliable as it produces no false positive result.

The huge database on genomic of microorganism need to be exploited in future to identify target genes of various microorganisms, sequencing of these genes leads better understanding of species, subspecies and pathogenicity of microorganism. Recently nanotechnology combined with the molecular methods has profound impact in detection technology. Little change in optical and amperometric properties can be detected with the electronic devices giving rise to very sensitive and rapid results. Besides the detection of food borne pathogen, the molecular methods can also be used in clinical microbiology, identification of trans gene in food, forensic science and agriculture.

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