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Popular Article

Advances in Molecular Tools for the Detection of Food-Borne Pathogens

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Fish and shellfish are perhaps one of the earliest recognized food commodities in the history of mankind. Fish, with its high protein content and being the sole source of omega-3 fatty acids, are considered nutritionally rich and generally regarded as healthy foods. Also, aquatic foods are the cheapest sources of proteins for a vast and ever-increasing population worldwide. Apart from these, seafood is the most traded commodity with the annual turnover of over 86 billion U.S. dollars. The increased international trade has resulted in world-wide movement of seafood with the consumer having little or no knowledge of the sources, modes of preservation, or the quality of the food. The increased movements of food, changing eating habits and changing ways of growing, harvesting and preparing foods have raised concerns on the safety all foods including the seafood. The concern is consolidated by the global incidences of seafood-borne outbreaks. In the United States, where a strict record keeping system exists, 1,034 foodborne disease outbreaks have occurred in 2008 in which more than 23,000 people fell ill, and 22 people died (CDC 2011). The prominent foods that caused the outbreaks were poultry (15%), beef (14%), and fish (14%). However, the global incidences of food-borne diseases remain largely unknown but are presumed to be in an increasing trend. The most common pathogenic bacteria responsible for seafood-borne illnesses are *Vibrio parahemolyticus*, *V. cholerae*, *V. vulnificus*, *Salmonella*, *Escherichia coli*, *Listeria monocytogenes*. While Vibrios are natural inhabitants of coastal-marine waters, enterobacteria such as *Salmonella* and *E. coli* are introduced *via* fecal contamination of the environment or secondary contamination of seafood during handling, processing or storage. Apart from bacterial pathogens, a number of enteric viruses and parasites such as trematodes are also involved in significant morbidity and mortality. However, little is known about these infectious agents owing to poor understanding of their etiology, lack of surveillance and absence of proper methods of detection.

Ever since the involvement of microorganisms in food-borne illness is realized, their detection in foods has posed a tremendous challenge. The traditional approach has been to try to grow them in laboratory media and identify the target bacterium by performing a number of phenotypical tests which, by far, are qualitative. Further, these methods fail to identify the pathogens present in small numbers co-existing with a large number of other natural bacteria and

also those injured metabolically during processing operations such as freezing, blanching, or smoking. The process of isolation and identification is not only long requiring more than a week to complete and laborious, but also fails to distinguish between pathogenic and non-pathogenic strains of the same species. For example, among *V. parahemolyticus* only those producing a thermostable direct hemolysin (TDH) or a TDH-related hemolysin (TRH) are considered pathogenic. By conventional biochemical tests, it is not possible to distinguish between pathogenic and non-pathogenic strains of *V. parahemolyticus* unless specialized media such as Wagatsuma agar (a special blood agar medium) is used, which again, can only detect TDH^+ but not TRH^+ *V. parahemolyticus*. Thus, in the last two decades, emphasis has been on the development of rapid, sensitive detection methods that are also quantitative. Nucleic acid-based methods fulfill these criteria and have become inevitable tools in food safety management, disease surveillance and outbreak investigations.

Polymerase Chain Reaction detection of food pathogens

During 1980s, testing of foods for microbial hazards was based on conventional microbiology involving the isolation of the pathogen followed by its confirmation using a series of biochemical tests and serology. The discovery of polymerase chain reaction (PCR) contributed enormously to the field of food microbiology and phenomenally changed the perspective of pathogen detection by enabling the development of rapid, sensitive, and specific detection methods based on nucleic acid amplification techniques. However, this does not preclude the requirement for microbiological techniques since selective enrichment or isolation is required in many cases to increase the number of pathogens to a detectable level. The essential steps in the detection of a pathogen in food by PCR include:

1. Pre-enrichment/enrichment of food
2. DNA extraction
3. Target DNA amplification and detection

For pathogenic bacteria such as *Salmonella* and *E. coli* O157:H7, a zero tolerance is advocated in foods. Though principally a PCR technique is able to detect and amplify a single copy of the target gene to detectable level, essentially meaning that a single bacterium can be detected, it is not possible to achieve this level of sensitivity in practice for many reasons such as the presence of inhibitors, interference by non-target templates and the nature of the food matrix itself. Therefore, it is necessary to increase the initial number of target bacteria in the sample by selective enrichment. Several studies have shown that enrichment of the sample tremendously enhance the sensitivity of detection of a pathogen in foods by PCR.

In addition, several concentration techniques are used to enhance the sensitivity of detection of pathogens. Immunomagnetic separation (IMS), in which paramagnetic or polystyrene beads are employed, is extensively used to capture and concentrate target pathogens directly foods or in enrichment broths followed by DNA extraction and PCR detection (Olsvik et al. 1994; Fu et al. 2005).

The efficiency of *Taq*-polymerase-mediate amplification relies on the quality of the target DNA

free from inhibitors. To accomplish this, it is important to and use a validated DNA extraction protocol for foods. Several DNA template preparation techniques for PCR have been described for foods such as simple boiling, using chemicals such as cetyl trimethyloammonium bromide (CTAB), guanidinium thiocyanate, urea, chelex 100 etc followed by phenol chloroform extraction and alcohol precipitation. Several kit-based methods which make use of silica-based spin columns do not require solvent extractions and such methods are often preferred owing to a smaller number of steps involved in extraction which drastically reduce the chances of cross contamination.

The amplification of target gene of a pathogen requires two primers which are short single stranded oligonucletodies of about 18-22 bases which bind specifically to the target DNA, a buffer for the *Taq* polymerase, individual deoxyribonucleotide triphosphates (dNTPs) and a thermo stable polymerase or *Taq* polymerase. The amplification reaction essentially has three steps- a denaturation step at 94^{o}C, a primer annealing at 50-60^{o}C and primer extension step at 72^{o}C . These steps are repeated 30-40 times to get required amplification of the target DNA. To perform these steps, a thermocylcer is required which is an integral part of PCR detection system. The products of PCR amplification are generally visualized by performing agarose gel electrophoresis followed by staining with a DNA intercalating dye, ethidium bromide. The stained DNA fragments brightly fluoresce under ultraviolet light and enable the detection of the products of a successful amplification reaction.

Several PCR methods have been described for pathogens in seafood. It is always advantageous to apply PCR directly to the enrichment broth by direct DNA extraction using a suitable technique. Table 1 shows target genes for PCR detection of some common seafood-borne pathogens. As shown in many studies (Parvathi et al. 2005, Deepanjali et al. 2005, Kumar et al. 2006), PCR can be directly applied to the DNA extracted from the seafood sample. However, the disadvantage with this technique is that DNA from dead bacteria may get amplified which can be overcome by enriching sample for a short duration (2-4 h) so that only the bacteria actively multiplying in the medium will be detected.

Real-Time polymerase chain reaction (RT-PCR)

Real-Time PCR has evolved from the conventional PCR to overcome the problem of quantification of the target and post-PCR handling of the products. The latter process invariably leads to the contamination in PCR environment leading to false positive results which can be a persistent problem in a diagnostic or food testing laboratory. Unlike in the conventional PCR, the RT-PCR enables the quantification of the starting material (template DNA) and the real time monitoring of the amplification during the exponential phase of amplification (Higuchi et al. 1993). The number of cycles required to reach detectable level of amplified products is a function of the quantity of the starting material and comparison with a reference standard allows direct quantification of the target. One of the simplest ways of achieving this is by using a DNA intercalating fluorescent dye SYBR GREEN. The quantity of dye binding to the amplified product increases exponentially with the exponential increase in the copy number of the PCR product. One disadvantage with the SYBR GREEN chemistry is that the binding of the dye is non-specific and

does not discriminate between specific and non-specific amplicons. This is overcome by the TaqMan chemistry or hydrolysis probe assay which makes use of the 5'-3'exonuclease activity of polymerase to hydrolyze a probe that has bound to the amplicon (Holland et al. 1991). TaqMan assay makes use of two primers for PCR and a hybridization probe specific to the sequences within the amplicon being generated by real-time PCR. The probe is labeled with two dyes, a fluorophore reporter and a quencher. The fluorophore, by virtue of it being close to the quencher dye, does not fluoresce. During primer annealing stage, the probe also binds to the target gene ahead of the primer. When *Taq* polymerase starts extending the primer from 3'-end, it will degrade the oligonucleotide ahead of it by its exonuclease activity resulting in separation of fluorophore dye from the quencher dye resulting in the fluorescence which can then be measured quantitatively. The use of a amplicon specific probe ensures that only the specific product is quantified and ruling out any intereference from non-specific amplifications. Several recent studies have described or used TaqMan assay for quantification of seafood-borne pathogens such as *V. parahemolyticus* (Liu et al. 2012), *V. vulnificus* (Panicker G and Bej (2005), *Salmonella* and *L. monocytogenes* (Amagliani et al. 2010), pathogenic *E. coli*. In other foods, TaqMan assay has been widely used for detection and quantification of *Campylobacter* in milk, *Salmonella* and *Listeria monocytogenes* in meat sausages (Wang et al. 2004). Apart from TaqMan method, several other variants of real-time PCR make use of probe hybridization with fluorophore-quencher combination such as the molecular beacons, hybridization probes, scorpions etc. With its speed, ability to quantify the target and closed nature, real-time PCR has been widely used for food-borne pathogen detection, genotyping of pathogens and gene expression analyses.

Loop-mediated Isothermal Amplification (LAMP)

Discovered by Notomi et al. in 2000, the LAMP technique is novel in that the target gene is amplified with high sensitivity, specificity and speed under isothermal conditions. The technique makes use of a DNA polymerase with high strand displacement activity (e.g. *Bst* polymerase) and four sets of primers, two inner and two outer primers. The fact that the amplification takes place at room temperature obviates the need for specialized thermal cyclers required by conventional PCR amplifications. Further, the LAMP technique faster than the conventional PCR and does not require specialized techniques to detect the amplified products. The amplified products can be visualized by naked eye or quantified using UV-spectrophotometer. Further, LAMP reaction less affected by the presence non-target DNA templates in the reaction mixture (Notomi et al. 2000).

The simplicity of performing LAMP and the ease with which the product can be detected have made LAMP technique popular and a number of researchers have published the application of LAMP for the detection of various food-borne pathogens including *Salmonella enterica* (Ye et al. 2011), *E. coli* O157:H7 (Hara-Kudo et al. 2008), *Salmonella* (Ohtsuka et al, 2005; Wang et al. 2008), *Edwardsiella* (Savan et al. 2004), enteroinvasive *E. coli* (Song et al. 2005), *V. parahemolyticus* and *V. vulnficus* have been described. The sensitivity of the LAMP assay is as

high as 7 CFU of *V. vulnificus* after 5 h enrichment, which is 1000-fold higher than the conventional PCR (Han and Ge 2008).

Microarray hybridization

A recently developed technique, microarray has become immensely popular and finds wide applications in pathogen detection, genotyping and transcriptomics. The technique is advantageous in that it can be used for the detection of multiple pathogens simultaneously in a miniature slide (Bryant et al. 2004). In microarray technique for pathogen detection, microliter volumes of oligonucleotide probes of 18-24 bases in length are robotically deposited on microarray slides (or chips) made of silica or quartz and fixed by heat. Probes for more several pathogens or for several genes of a single pathogen, such as in the case of enterohemorrhagic *E. coli*, can be used on a single slide. The templates for hybridization are PCR amplified with simultaneous incorporation of a specific fluorescent dye such as the hexachloro-6-carboxylfluorescein (HEX). The labeled PCR products are hybridized with microarray under standard conditions. The slides are scanned in a microarray scanner to determine the fluorescence signal intensities. A positive hybridization reaction between the probe and the template is indicated by bright fluorescence at appropriate wavelength depending on the type of label used. A flowchart representation of steps involved in microarray detection is shown in Fig. 1.

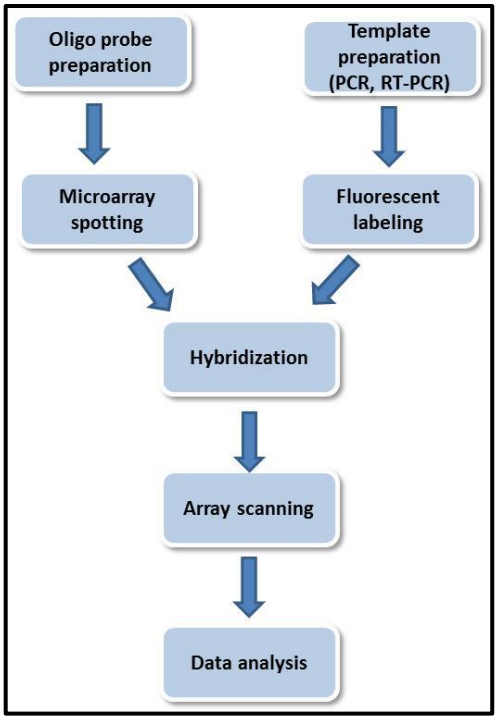


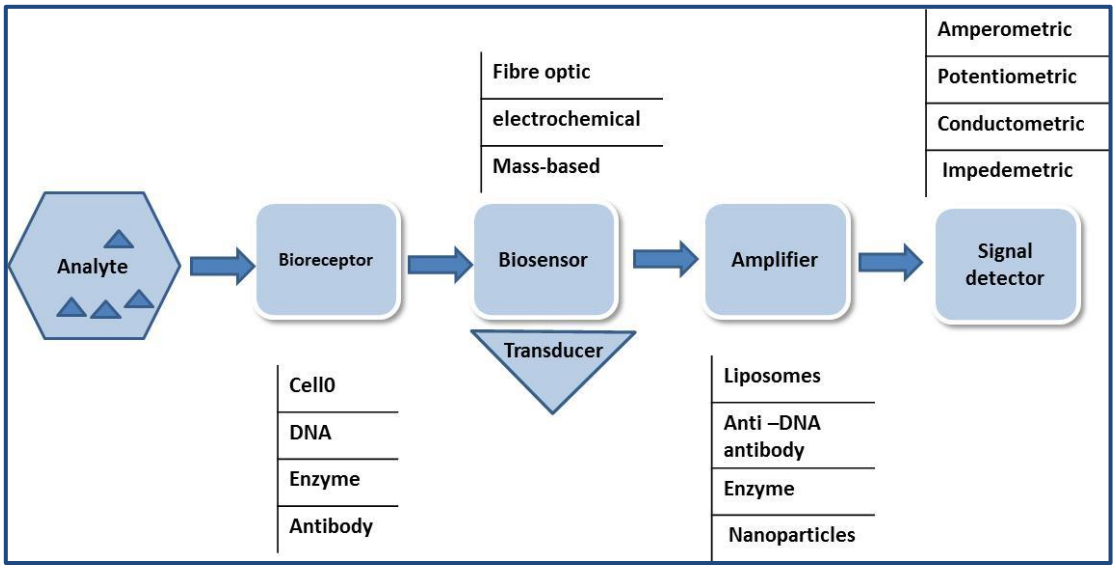
Fig. 3. Steps in an oligonucleotide-based microarray detection of a pathogen.

Though the microarray technique is rapid, sensitive and specific, its forbidding instrumentation and consumables cost prevent the routine use in food testing and diagnostics. Nevertheless, microarray is a powerful nucleic acid-based technique with wide applications with tremendous potential as powerful pathogen detection tool in the future.

Biosensors

A recent advance in the process of pathogen detection in food is the use of biosensors which essentially involves amplification of a biological signal to a detectable level. In simple terms, the system consists of a bioreceptor such as a tissue, nucleic acid, enzyme or an antibody which recognizes the analyte and transmits to the second component, the transducer, which converts the biological event into an electrical signal that can be easily captured and measured (Velusamy et al. 2010). In nucleic acid-based biosensor, the target nucleic acid is detected by hybridization reaction with the bioreceptor which is immobilized on an electrode. Different detection systems have been reported depending on the type of transducer system used (See Fig. 3). Piezoelectric

quartz crystal microbalance (QCM) biosensors have been frequently used as a signal transducer for the detection of food-borne pathogens (Chen et al. 2008; Mao et al. 2006; Wu et al. 2007). Using QCM sensors integrated with lac PCR, Mot et al. (2002) could detect 1-10 *E. coli* in water. The use of nanoparticles as signal amplifiers in a QCM system has resulted in substantial increase in the sensitivity of detection. A QCM biosensor with gold nanoparticles as signal enhancers has been used to detect *E. coli* O157:H7 in foods (Chen et al. 2008). Electrochemical biosensors detect foodborne pathogens based on potentiometry, conductometry and impedimetry (Mahari and Gandhi, 2022). Because of their benefits, such as fast processes, great sensitivity, excellent specificity, affordability, portability, compact design, and on-site detection, electrochemical biosensors are extensively utilized in the field of food, biology, and life sciences (Melo *et al.*, 2016 ; Long *et al.*, 2022). Electrochemical biosensors offer a quick, effective, and alternative approach for identifying foodborne pathogens to safeguard the safety of ready-to-eat (RTE) foods and can function as independent devices for on-site testing. Nanomaterials (NMs) utilized in the creation of nano-biosensors consist of metallic nanoparticles, carbon nanotubes (CNTs), organic nanoparticles, metal oxide nanoparticles, and silica nanoparticles (Kurmendra, 2023). Additionally, these nanomaterials can function as transduction elements, enhancing the sensitivity and detection threshold of electrochemical biosensor techniques (Hussain *et al.*, 2017; Naresh and Lee., 2021). Hence, choosing a highly specific bioreceptor in tandem with a nanomaterial is crucial for developing electrochemical biosensors that can rapidly and effectively identify foodborne pathogens (Awang *et al.*, 2021). While these biosensors demonstrate notable benefits compared to traditional laboratory techniques, each category of biosensor has its own set of limitations that future studies should aim to address for enhancement.



Conclusions

The advances in the fields of physical and chemical sciences have significantly contributed to improvising the existing methods of pathogen detection and discover novel methods such as microarray and biosensors. Though these methods are still in various stages of development and

the technical intricacies and high costs involved forbid the use of some of these techniques, their universal application in food pathogen detection is not far from real. The microarray technique, for example, has enormously enhanced the pathogen detection abilities in clinical samples and is being widely used for gene expression analysis and single nucleotide polymorphism (SNP) detection. The emergence of new, sensitive, and rapid methods is expected to significantly help reduce the risk of food-borne infections and ensure the safety and quality of foods meant for human consumption.

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