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Review Article

Molecular Methods for Detection of Bacterial Pathogens in Finfish and Shellfish with Special Consideration to Public Health Significance: A Review

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Abstract

Fish and shell fish are known as carriers of food borne pathogens and therefore, the hygienic quality of these products must satisfy the International quality regulations, failure of which may cause the rejection of fish and shellfish consignments by the importing countries. Bacterial pathogens related to human health significance are *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Salmonella* sp., *Shigella* sp., *Listeria monocytogens, Aeromonas* sp., *Plesiomonas shigelloides* etc. Numerous DNA molecular markers are now available for use in surveillance and investigation of food borne outbreaks from seafood which was previously difficult to detect. Moreover, molecular approach to identify pathogens is potentially faster, more sensitive than conventional culture techniques, serology and histology. This review describes various microbial pathogens of fish and fishery products and different DNA and RNA based methods which are well known in identification of food borne pathogens.

Key words: Fin Fish and shell Fish; Molecular Techniques; Pathogens; PCR; Rapid Detection.

Introduction

A common problem encountered with exposed fish and fishery products is contamination with bacterial pathogens. In fact, there have been numerous cases of rejection of consignments at International markets due to contamination of fish and fishery products with bacterial pathogens like toxigenic *Escherichia.coli, Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Salmonella* sp., *Shigella* sp., *Listeria monocytogens, Aeromonas* sp., *Plesiomonas shigelloides* etc. In India during June 1995 to December 1997, 31 fish consignments exported to European Union (EU) countries were found to be of poor hygienic quality. This led to a ban of fish export from India to EUcountries (Anonymous, 1998; Anonymous, 1999). In addition, during the same period, many consignments exported to USA and Japan was also rejected for the same reason (Pandian *et al.*, 2000). Moreover microbial pathogens pose a significant health hazards to human health through ingestion of raw, uncooked and improperly cooked seafood. The contamination can occur during different stages of processing of seafood for instance, harvesting, handling, processing, distribution and storage. Several cases of outbreaks of bacterial disease in human are reported from water and seafood throughout the world (Table 1).

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There are some inherent drawbacks associated with conventional microbiological techniques like more time is required for analysis and miss-identification of non pathogenic strains. In order to identify food borne pathogens, conventional culture method includes homogenization, enrichment in non-selective and selective medium followed by plating in differential agar medium for pure culture isolation. At the end of phenotypic and genotypic characterization normally takes 3-4 days for result confirmation. A substantial volume of pure culture is needed for conducting biochemical and immunological method whereas mixed culture or community Deoxyribonucleic acid (DNA) can be used in DNA based method. Identification of bacterial pathogens in conventional microbiological method is somewhat time consuming and labor intensive. A conventional method lacks to differentiate bacterial pathogenesis but can determine genus and species of the micro organism. It led to a search for more faster and sensitive method to detect microbial pathogens in food.

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Table 1: Outbreaks of bacterial disease from water and seafood.

Sl.No	Incidents and Year	Country	Bacteria	Reference
1	62 cases of Septicemia,1981-1987	Florida , USA	*V. vulnificus*	Klontz *et al.* (1988)
2	472 cases of gastroenteritis 1986	USA	*A. hydrophila.*	Abeyta *et al.* (1986)
3	Outbreak of Cholera October,1992	Madras, India	*V.cholerae* BengalO139	Ramamurthy *et al.*(1993)
4	Outbreak of cholera June to October 2003	Nagpur, India	*V.cholerae*Biotype E1Tor	Mishra *et al.* (2003)
5	61-71% outbreak of gastroenteritis 1996-1999	Taiwan	*V. parahaemolyticus*	Wong *et al.* (2000)
6	40 outbreaks of *V. parahaemolyticus* 1973-1998	USA	Do	Daniel *et al.* (2000)

Molecular techniques can be used to solve that type of problems and increase sensitivity to target pathogens such as PCR, multiplex PCR, real time PCR, RAPD, RFLP, AFLP, PFGE, 16S rRNA PCR, hybridization of DNA probes etc.

The different microbial hazards associated with fish food and advancements made for their detection using molecular techniques will be elaborated in the present review.

Common bacterial contaminants in fish and fishery products

Vibrio sp.

Vibrios constitute a major portion of the bacterial flora of both fin fish and shell fish in the tropics. More than 37 Vibrio species have been isolated and characterized, out of which 12 nos. of species are considered as human pathogens of zoonotic importance. *V. cholerae, V. parahaemolyticus, Vibrio vulnificus* are important members of this group that are considered as pathogens associated with fish and fishery products.

Vibrio cholerae: Vibrio cholerae is widely distributed in aquatic animals. Contamination of seafood occurs particularly when the seafood is caught from contaminated water bodies. The other source of contamination is un-hygienic condition of fish farmers and workers of the processing plants. Seafood is often taken from waters or stored under conditions that induce the viable not culturable state in *V. cholera* (Oliver *et al.*,1989). It has been realized that the viable but not culturable (VBNC) state exhibited by *V. cholerae* can explain the seasonality and distribution of the organism in regions where cholera is endemic. According to Koch *et al.* (1993) and Karunasagar *et al.*(1995) *Vibrio cholerae* can be detected in seafood by application of PCR based method. Karunasagar *et al.*(1995; 1997) studied that the *ctx* gene based PCR could identify both *V. Cholerae* O1 and O139 contamination in seafood. According to Urakawa *et al.*(1997) who analysed the restriction pattern of 16s RNA of 35 no. of Vibrionaceae species which are found useful for the classification and identification of Vibrionaceae strains.

Vibrio parahaemolyticus: *Vibrio parahaemolyticus* is often associated with seafood like shrimp, prawn, crabs , mollusks etc and as well as finfish. Kaper *et al.*(1984) described that *Vibrio parahaemolyticus* could produce Thermostable Direct Hemolysin (TDH) which was encoded by *tdh* gene. According to Honda *et al.*(1988) non hemolytic (Kanagawa negative) strains of *Vibrio parahaemolyticus* might be associated with gastrointestinal infection. Sakazaki *et al.*(1968) studied that most of the environmental strains were Kanagawa negative. Karunasagar *et al.*(1996) reported a PCR based assay targeting *tdh* gene which could detect *Vibrio parahaemolyticus* contamination in shell fish.

Shigella sp.

Virulent shigella causes human illness known as bacillary dysentery. The bacteria are found in fishes collected from river, estuary and sewage fed beels. Davis *et al.* (1988) stated that the bacteria could be traced in contaminated fish, chicken and fishery products.

Listeria monocytogenes

Fish and shell fish are exposed to potential contamination by *Listeria monocytogenes.* In India there are very few reports on the incidence of Listeria in clinical as well as food samples (Gupta *et al.*,1997). A number of workers investigated the application of PCR for rapid identification of *L. monocytogenes* on various food products.(Hill, 1996). Listeriolysin is a major virulence factor for *L. monocytogens* which is encoded with *hly* gene. The *hly* and *lap* gene can be used as target gene for PCR amplification in order to detect the said bacteria.

Escherichia coli.

E.coli contamination in fishery products occurs during processing through contaminated water or handlers in the plant. (Mishra. *et al.*, 2004). The poor un-hygienic and sanitary condition of fish landing centers and retail markets are also responsible for toxigenic *E. coli* contamination in seafood. This group of *E.coli* is classified into five categories based on their virulence properties, clinical syndromes, epidemiology and distinct O:H groups such as Enteropathogenic *E coli* (EPEC), Enteroinvasive *E coli* (EIEC), Enterotoxigenic *E.coli* (ETEC), Enterohemorrhagic *E.coli* (EHEC) and Enteroaggregative *E.coli* (EAEC). Detection of EIEC by amplification of genes including invasion factors has been reported by Keasler and Hill (1992). *E.coli* O157:H7(EHEC) is responsible for the food borne illness. Since EHEC produce Shiga like toxin (SLT), PCR based assays targeting SLT genes have been useful for detection and identification of this serotype(Gannon *et al.*,1992). Lang *et al.* (1994) described a multiplex PCR for detection of heat liable toxin gene and Shiga like toxin and 11 genes in *E.coli* isolated from natural waters.

Salmonella sp.

The importance of *Salmonella* as food borne pathogens in seafood should not be underestimated. There are numerous examples of rejection of shrimp consignment from South –East Asian Countries due to contamination of *Salmonella.* Bej *et al.* (1994) described a method for identification of *Salmonella* sp. in oyster by PCR techniques. Their method involved amplification of *himA* gene which encodes for a DNA binding protein found in several enteric bacteria. For detection in food samples different procedures screening specific genes of Salmonella as the target have been developed by single PCR (Stone *et al.*, 1994), multiplex –PCR (Way *et al.*, 1993).

Molecular method of Detection

Polymerase chain reaction (PCR)

Polymerase chain reaction is a technique for amplifying a specific region of DNA, defined by a set of two primers at which DNA synthesis is initiated by a thermo-stable DNA polymerase. PCR is a relatively simple technique by which a DNA template is amplified over a million fold quickly and reliably in 3-4 hrs time frames. In PCR methods, the reaction mixture constitute template DNA which may be simple tissue lysate to purified DNA, primers, polymerase enzyme in order to catalyze creation of new DNA strands and nucleotides. During each cycle of the thermo-cycling reaction, the template DNA is denatured, primers anneal to their complementary region of DNA strand and polymerase enzyme catalyses the addition of nucleotide to the end of each primer. Thus new copies of target region are created in each cycle. PCR methods have been described in more detail by Hoelzel and Green(1998). Saiki *et al.* (1985) published the first experimental data on PCR. Incase of pathogenic *E.coli* , the potential targets for amplification include *stx* gene, *eae* gene coding intimin, heat –liable (LT) and heat stable(ST), *bfpa* gene etc. In case of pathogenic *V. cholerae* the targets include *ctx* and for *V. parahaemolyticus tdh, trh* are important. In *L. monocytogenes*, several target genes have been reported like *iap, hly A* and *prfA*. The table 2 depicts bacterial genetic targets and molecular methods.

Multiplex Polymerase chain reaction

According to Field and Wills (1998), multiplex PCR means that several bacterial species can be identified in the single assay method. In multiplex PCR more than one target sequence can be amplified using more than one pair of primer in the reaction mixture. Care should be given that the primers should be same melting temperature and must not interact with each other. French *et al.* (1999) used a universal primer for the detection of multiple pathogens simultaneously. Here attempts are made by introducing single set of primer to amplify conserved stretches of DNA from 16s to 23s r DNA.

Real time PCR

Real time PCR is aimed to detect pathogens in food both in quantitative and qualitative methods. In this technique one can continuously monitor the developments of amplicons in a fluorimeter. SYBR-Green Dye or other fluorescent labeled probes that emits light during amplification are widely used in this technique. The emitted light signals corresponding to DNA amplification recorded at frequent intervals generating a curve which shows product generation. The more targets DNA amplifies in the sample, the earlier amplicons can be detected and the peak curve is recorded. (Tichopad *et al.*, 2003). Baggi *et al.* (2005) described that diarrheagenic *E.coli* could be detected in Real time PCR using SYBR Green Dye.

Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR based technique which generates DNA band pattern on gel electrophoresis using

amplification of random DNA segments with primers of arbitrary nucleotide sequence (Williams *et al.*,1990). Enterobacterial Repetitive Intergenic Consensus PCR (ERIC PCR), Repetitive extragenic palindromic-PCR (REP-PCR) and BOX PCR are few examples of this technique. The presence of a RAPD band, however does not allow distribution between hetero and homozygous state. Several authors have reported on the application of RAPD techniques in microorganisms (Babalola, 2003). Sudesh *et al.* (2002) described that *V. alginolyticus* and *V. parahaemolyticus* have different RAPD profiles. In rep PCR amplification involves intervening sequences located between highly repetitive DNA motifs. Nowrouzian *et al.* (2001) designed a RAPD typing method for identification of *E.coli* strains in the micro flora of human intestine.

Restriction Fragment Length Polymorphism (RFLP)

RFLP method is very simple which uses a restriction enzyme digestion of the genomic DNA. The procedure are as follows like isolation of DNA, digestion of DNA with restriction endonucleases, size fraction of the resulting DNA fragments by electrophoresis, transfer of DNA from electrophoresis gel matrix to membrane, preparation of radio labeled and chemiluminiscent probes and hybridization to membrane bound DNA.(Babalola, 2003) The PCR-RFLP techniques consist of PCR amplification of certain genes eg. 16S rRNA, gyrB and *rpoD* and subsequent restriction of the PCR products with endonucleases to obtain band pattern.

Amplified Fragment Length Polymorphism (AFLP)

A rapid PCR based technique AFLP can be used for prokaryotes as well as Eukaryotes typing. This method starts with digestion of total purified genomic DNA by restriction endonuclease. Then ligation is formed which results in forming fragments to a double stranded oligonucleotide adapter complementary to the base sequence of the restriction site. The adapters are designed such a way that the original restriction site is not restored after ligation process which can prevent further digestion of restriction site. Selective amplification of sets of these fragments in PCR is achieved with primers corresponding to the adapter. The resulting PCR amplified DNA fragments are analyzed by gel electrophoresis (Prasad *et al.*, 2009). According to Altinok *et al.* (2003) two restriction enzymes are used namely average cutting frequency (EcoRI) and higher cutting frequency (Mse I or TaqI). Babalola (2003) described that the primer contain the restriction enzyme recognition site as well as additional "arbitrary" nucleotides which extend beyond the restriction site. The fixed portion gives the primer stability and random portion detect many loci simultaneously.

Pulse field gel electrophoresis (PFGE)

This method involves DNA in which it is cut into fragments with rare cutter restriction enzymes yielding 8-25 large bands. After restriction digestion process, the fragments are separated electrophoretically by size on an agarose gel by using current at alternating angles. PFGE has been used for characterization of pathogenic bacteria. (Prasad *et al.*, 2009).

16S rRNA PCR

The use of 16S rRNA gene sequence to study bacterial phylogeny because 1. its presence in almost all bacteria, often existing as a multigene family, or operons ii) its sequence is sufficiently conserved, viable and hyper variable sequence iii) size (1500 bases) which is easily sequenced but large enough to contain sufficient information for identification of bacteria. (Spratt *et al.*,2004). The process involves for identification of bacteria by 16S rRNA PCR are as follows:-a) preparation of DNA from pure culture of bacteria. b) PCR amplification of 16S rRNA gene c) checking good PCR product in gel electrophoresis d) cleaning e) sequencing of PCR product f) analysis base sequence online Basic local alignment search tool (BLAST) or Ribosomal database project (RDP) software g) identification closest match in the database. (Spratt *et al.*,2004)

DNA probe hybridization

It is possible to develop probes for specific micro-organism. Probes are short nucleic acid sequence complementary to the target sequence of micro-organism. Generally probes are labeled either with a radio active molecule (P^{32}, S^{35}, C^{14}), ligand (biotin) or antigenic substrate. eg digoxigenin.(Tyagi and Kramer *et al.*, 1996). Probe hybridization analysis requires no sophisticated equipments. So probes are widely acceptable in quality control laboratory in food processing sector.

Conclusion

Microorganisms are known to play the pivotal role for spoilage of fish and fish products as well as food

borne infection outbreaks. Hence it is essential that the pathogens should be absent in seafood in order to ensure quality in relation to human health. Quick and accurate detection methods of microbial pathogens in seafood eliminate the chance of contamination to the consumers. Isolation and identification of food borne pathogens by conventional biochemical and immunological methods are time consuming, laborious and lees sensitive in comparison to molecular approach. Now a day's 16S rRNA gene, strain specific and virulence gene are the targets used for identification of food borne pathogens. Sequencing of target genes of microorganisms gives a better insight towards understanding of species, subspecies and pathogenicity in future. Molecular techniques such as PCR and hybridization are useful for rapid detection of pathogens and specific detection of virulent strains. Since these are rapid, specific and sensitive, they have immense applications in seafood quality control laboratory. It can be suggested that pool of samples of seafood products should be tested by molecular methods at quality control laboratory prior to export to the foreign countries.

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