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Detection of Food Pathogens by Molecular Biology Techniques

Rakesh Kumar
Central Institute of Fisheries Technology
Cochin 682 029

Conventional microbiological methods for the detection of pathogens is very time consuming and laborious. It takes between 4-5 days to detect pathogens by this method. Further problems arise due to situations such as, among *V.cholerae*, only serotypes O1 and O139 cause cholera. Among environmental isolates of serotype O1, non-toxigenic strains exist. Among *V. parahaemolyticus* only less than 2% of environmental strains are pathogenic. These are characterized by their ability to produce a thermostable direct haemolysin (TDH) or TDH- related haemolysin (TRH). Traditional method of identifying bacteria by biochemical tests can identify them up to species level, but cannot differentiate between strains. Techniques such as serotyping and phage typing also have little discriminatory power. PCR based techniques such as Random Amplification of polymorphic DNA (RAPD) can generate DNA fingerprints of organisms. RAPD patterns are helpful in studying similarity or differences in strains. For example, this technique has been used to differentiate strains of *Listeria monocytogenes* isolated from raw fish, from smoked fish, in processing environs etc. This type of study would help in understanding the source of the strains found in the product, eg cold smoked fish. In this technique single 10-mer oligonucleotide primer is used to perform amplification at low annealing temperature (eg 37°C). The primer is not targeted at any particular region of the genome and therefore, this reaction can be performed even in the case of organisms whose genome sequence is not available.

Molecular methods that are gaining popularity in food safety are (a) DNA probe hybridization and (b) polymerase chain reaction (PCR). PCR is based on nucleic acid amplification and therefore has very high sensitivity. But it requires special laboratory facilities and can also detect dead bacteria in processed foods. On the other hand, DNA probe hybridization, when performed as colony hybridization, does not require expensive equipment, detects only live bacteria and give quantitative data. The objective of this article is to illustrate the application of PCR and DNA probe hybridization in seafood safety assurance.

DNA probe hybridization is based on the principle that DNA is a double stranded molecule, the two strands of DNA can be separated by heating or chemical treatment, and the two separated strands can reassociate. DNA strands from different sources can hybridise, provided there is complementarity of bases (A-T; G-C) between them. Based on this principle, it is possible to make probes specific for different microorganisms. Probes are short stretches of nucleotides that have sequences complementary to the target sequences. To detect probe hybridization, probes are labeled either with a radioactive molecule (p32), enzymes, ligands (eg. biotin) or antigenic substrates (eg. Digoxygenin).

Genes which are chosen as targets are specific for each bacteria. In the case of pathogenic organisms, these are genes that encode virulence factors i.e. factors that make the organism pathogenic. Some examples of these genes are given in Table 1.

Table 1. Examples of pathogens and target genes

Pathogen	Target genes for probe hybridization
Vibrio cholerae	*ctx*
V. parahaemolyticus	tdh, trh
V. vulnificus	Vvh
Salmonella	inv, hns
Enterohemorrhagic *E.coli*	*stx, eae*
Enterotoxigenic *E.coli*	St, Ct
L. monocytogenes	iap, hly

Pathogenic *V.cholerae* produces a toxin, cholera toxin encoded by *ctx* gene. The virulence factors in *V. parahaemolyticus* are encoded by *tdh* and *trh* genes. The virulence factors in *L. monocytogenes* are encoded by *iap* and *hly* genes. In *Salmonella,* there are virulence associated genes such as *inv.* Enterohemorrhagic *E. coli* have virulence genes such as *stx, eae.* Using such specific probes, it is possible to specifically detect the pathogenic bacteria. A flow chart of colony hybridization is given Fig.1

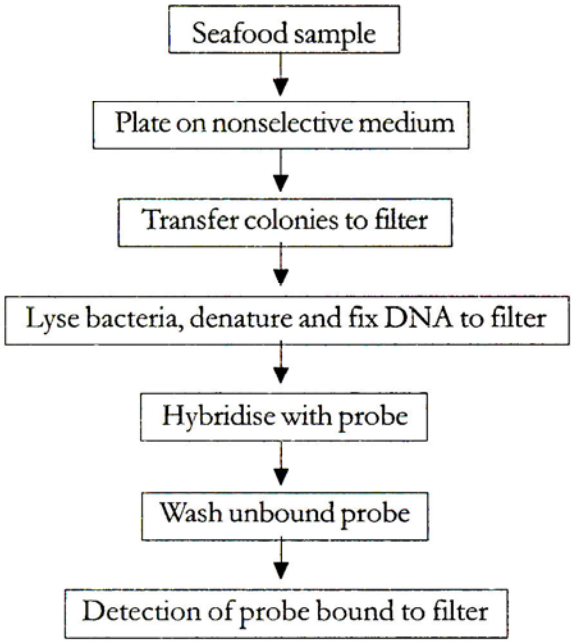

Fig. 1. General protocol for pathogen detection by colony hybridization

The food sample is homogenized (1:1) in a buffer and plated on a non-selective agar. The plates are incubated at 35°C for 18 h. The colonies appearing on the plate are transferred to a suitable filter. The bacterial cells are lysed by immersion in a lysing solution and the DNA released are denatured and fixed to the filter.

The filter is now incubated in a prehybridising solution and then the probe is added. Hybridisation is performed at a temperature appropriate for the probe. After hybridization, the filters are washed at a specified temperature. Probe hybridizing to DNA on the filter is detected depending on the type of label. If radioactive probe is used, detection is by autoradiography, wherein the filter is incubated with a X ray film, one can see spots corresponding to colonies to which probe has hybridized. However, presently a number of non-radioactive probe labels are available. Most convenient are enzyme labels, which can be detected using the appropriate chromogenic substrate.

To detect pathogens that may be present in extremely small numbers, the food samples may be enriched before plating. The filters after hybridization can be preserved as a record of analysis. Probe hybridization analysis requires no sophisticated equipment. A hybridization incubator is all that is needed. Therefore this technique can be most conveniently adopted in seafood quality control laboratories. In some situations, DNA probe based methods are essential to detect pathogenic strains of organisms Eg. *Vibrio parahaemolyticus.* This organism is commonly found in coastal and estuarine areas all over the world. 98% of environmental strains are not pathogenic. Hence mere detection of this organism by conventional microbiology is not sufficient to determine the hazard. DNA probe hybridisation methods are getting wide acceptability in quality control laboratories and the US FDA Bacteriological analytical manual describes this

method indicating acceptance by the regulatory agencies. In the case of bacteria such as *Salmonella* and *Listeria monocytogenes*, DNA probe hybridization methods have undergone multilaboratory evaluations conducted through AOAC and are accepted as official methods.

PCR is a nucleic acid amplification technique wherein a specific portion of nucleic acid from a target organism is amplified in vitro. This specific amplification is achieved using oligonucleotide primers that are specific for the region flanking portion to be amplified. The amplification requires the enzyme DNA polymerase, and the building blocks of DNA, the deoxyribonucleotides (dATP, dTTP, dGTP, dCTP). The reaction is performed in several cycles, each cycle consisting of three steps (a) DNA denaturation : this is the step in which the target DNA strands are separated by heating to about 95°C . (b) Primer annealing: this is the step in which the primer binds to the target region specifically. This step is carried out at 55-65°C (c) primer extension : this is the step in which the new DNA strand is synthesized by the DNA polymerase on the template strand. Normally about 30 cycles of reaction are performed. Since each cycle involves denaturation of DNA at 95°C, the DNA polymerase used in the reaction should be thermostable. The discovery of thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* led to rapid application of PCR in diagnostics.

PCR technique for detection of most pathogenic bacteria associated with seafood has been studied thoroughly. By designing oligonucleotide primers that are specific for an organism, it is possible to design PCR to amplify specifically DNA from any desired organism. In the case of RNA viruses, it is possible to first copy the RNA into DNA using the enzyme reverse transcriptase. PCR used to detect RNA targets is referred to as RT-PCR. In most cases the oligonucleotide primers have been designed to specifically amplify virulence associated genes. For example contamination of seafood with toxigenic *V.cholerae* can be detected using PCR amplifying the *ctx* gene encoding the production of cholera toxin. Contamination of seafood with pathogenic *V. parahaemolyticus* can be detected using PCR amplifying the *tdh* and *trh* genes that encode virulence associated haemolysin. In the case of pathogenic *Escherichia coli*, the potential targets for amplification include *stx* gene encoding the production of shiga-like toxin, *eae* gene encoding intimin, heat-labile (LT) and heat stable toxins (ST) etc. In the case of *Listeria monocytogenes*, several target genes have been reported. These include the gene encoding the production of the invasion-associated protein, *iap*, listeriolysin, *hly*A, and the regulatory protein, *prf*A. PCR is a DNA amplification technique and therefore, even if there are dead bacteria, they would show up in PCR. Therefore for determination of seafood safety, it would be important to ensure that only viable pathogens are detected. Molecular techniques such as PCR and colony 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.