Advanced Detection Methods for Seafood-Borne Pathogens

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A major problem in ensuring food safety is the delay in detecting food – borne pathogens and their toxic products. Rapid detection of these pathogens can, to a great extent, prevent massive food poisoning outbreaks and save human populations. This is especially true in recent times because of the large-scale distribution of perishable foods. Standard culture techniques require days to weeks for getting the results. Identification is further complicated by the low numbers of pathogens against the highly competing background flora. In many cases the food matrix can also confuse the analyst. These problems are to a certain extent nullified by the latest developments in pathogen detection and their toxins. While traditional methods are time consuming and laborious, recent advanced methods make pathogen detection and identification faster, more sensitive and more specific. They are referred to as rapid methods because results are available in less than 4 hours in most cases. Advances in instrumentation and biotechnology have enabled automation of some of the techniques to give in-situ results. Rapid methods are generally used as screening tests with negative result accepted as it is, but positive results need to be confirmed by other official techniques.

Seafood borne pathogenic bacteria of importance

The detection methods used in food microbiology are applicable to different foods though minor modifications have to be made depending on the nature of food substrate and the pathogens expected. According to the ecology and origin of pathogen, seafood borne pathogenic bacteria may be grouped into 3 categories.

- Pathogens indigenous to aquatic environment
- Pathogens associated with terrestrial environment
- Pathogens having animal or human reservoir

Pathogens indigenous to aquatic environment

The pathogens in seafood that are naturally present are members of the family *Vibrionaceae, Aeromonadaceae* and *Clostridium botulinum* types, B, E and F. Among the members of the family *Vibrionaceae*, genera *Vibrio, Aeromonas and Plessiomonas* are important. In the genus *Vibrio*, the important species that are considered as inherent pathogens are *Vibrio parahaemolyticus, V. vulnificus* and to a lesser extent *V. hollisae* and *V. mimicus*. The most recognized member of this group, namely *V. cholerae*, is considered as having aquatic origin, but they can also contaminate seafood at later stages of human handling.

Pathogens indigenous to general environment, but frequently present in fish

The proteolytic types of Clostridium botulinum types A,B and F ,*Bacillus cereus, Listeria monocytogenes* and *Clostridium perfringens* belong to this group. They are heat resistant mesophilic, salt –tolerant and have general environment particularly soil as the main habitat.

Pathogenic bacteria in animal / human reservoir

Salmonella Shigella, Escherichia coli, Campylobacter jejuni and other mesophilic Campylobacter and *Staphylococcus aureus* are the important genera that belong this category.

Methods for detection of pathogens and toxins

Methods for the detection of pathogens and toxins are often categorized into two groups, namely the conventional methods and the rapid methods. The conventional methods involve a series of steps including homogenization, enrichment, selective plating, isolation and identification followed by confirmation. Recently an array of advanced methods has come up as alternate way to traditional methods. Few of these methods are adopted as AOAC Official methods after validation and evaluation. Eventhough FDA has listed many rapid methods or kit in Bacteriological Analytical Manual, they need further confirmation by appropriate official method, which, in many instances are cultural only. This is because these methods continue to be modified or innovated so that published information need not be the most recent method. Major improvements in methodology have been in the following areas

- Sample separation
- Separation and concentration of target microbes or toxin
- End detection

Improvements in sample preparation

The agar slide technique in which agar slides of selective or non selective media are pressed on the surface to be examined and incubated relieves the analyst of sampling. Similarly the automation in counting techniques, dilution and homogenization have lessened the burden on analyst.

Separation and concentration of target microbes or toxin

Separation and concentration of target microbes, toxin or virus from the rest of the flora can shorten the detection time and improve the specificity of test procedure. Common methods of this category include,

1) Immunomagnetic separation - IMS

The immunomagnetic separation uses super paramagnetic particles coated with the antibody against target organisms. The target organisms are captured in presence of a mixed population due to antigen-antibody specificity. This eliminates the need for enrichment procedure. Examples are commercially available kit for Salmonella spp., E.coli O157:H7, *Listeria monocytogenes*.

2) Direct Epifluorescent Filter technique – DEFT

The membrane filtration procedure helps to concentrate the target organism present in large volume of sample and thus increases the sensitivity. The membranes are made of cellulose acetate, nitrocellulose, nylon or polyvinyl chloride. The membranes after

filtration are stained with suitable fluorescent dye like acridine orange. Because the membranes are very thin (about 10μm) they can be directly observed in a microscope after suitable staining with fluorescent dyes or with labeled antibodies and pathogens can be detected microscopically.

3) Hydrophobic Grid membrane - HGM

This technique is also linked to membrane filter method but special grids are used. After a pre-filtration to remove particulate matter, sample is filtered through a membrane grid, which carry 1600 compartments. The organisms are trapped in grid due to hydrophobic effect. The membranes are either placed in agar plates and incubated or visualized by other methods. Hydrophobic Grid Membrane Filter Methods for Rapid Enumeration of Total Coliforms, Fecal Coliforms, and *E. coli* are described in the APHA *Compendium of Methods for the Microbiological Examination of Foods.*

Advanced End detection methods

Improvements in end detection include,

- Better media design by using chromogenic and fluorogenic substrates, dip slides, petrifilm etc
- Immunoassays like ELISA, RPLA
- Nucleic acid based assays such as PCR ,gene probe etc
- Biological assays

Improved media design

The incorporation of chromogenic and fluorogenic substrates into common media allows improved and specific detection of target microorganisms. Example: 4-methyl umbelliferyl β-d glucuronide added to violet red bile glucose agar, a medium for *E.coli.* Petrifilm is an alternative to conventional plating method. The system uses dehydrated mixture of nutrients and gelling agents on a film. The rehydration with 1 ml of sample inoculum facilitates colony formation and counting can be done as plating method.

Motility

The motile property of bacteria is made use for detection of pathogens.For example, in Rapport medium motile strains of salmonella migrate through medium ahead of other competing organisms. But non-motile strains will not be detected.

Immunological assays

They are much simpler and cheaper than biological assays and have therefore been widely adopted.

1. Gel diffusion assays.
2. Haemagglutination
3. Coagglutination
4. Reverse Passive Latex Agglutination (RPLA)
5. Enzyme linked Immunosorbent Assay (ELISA)
6. enzyme linked Immunofiltration Assay (ELIFA)
7. Radio Immuno Assay (RIA)

Gel diffusion assays

One of the earliest immunological tests to be developed was the double gel immunodiffusion. The precipitations formed when antibodies and antigens are mixed is a major tool. For these gel diffusion methods, antigen must be in a precipitable form. When polyclonal antibodies and an antigen with multiple epitopes are mixed in solution, they interact with each other to

form complexes. The degree to which insoluble antigen-antibody complexes form, resulting in a flocculent precipitate that settles to the bottom of the tube, depends on the extent of lattice formation. If there is too little or too much antigen relative to the amount of antibody present, insoluble complexes cannot form lattices and little or no precipitate results. However, as the ratio of antibody to antigen mixed together approach an ideal equivalence point, the precipitate becomes so extensive that nearly all of the antigen and antibody drop out of solution. Some disadvantages of the method are that atypical or non-specific reactions that occur. More over it is semi quantitative only.

Immunofluorescence

Some antigen mixtures are too complex to be resolved by simple diffusion and precipitation. Greater resolution is obtained by the technique of immunoelectrophoresis. In this technique, antigen is first separated based on their electrical charge and then visualized by the precipitation reaction. In immunoelectrophoresis, antigen diffusing from a well in a agarose gel diffuses towards antibody in a trough. As the diffusion zones of antigen and antibody overlap, a visible precipitate forms a band in the gel in the zone of equivalence.

Haemagglutination

This method is more sensitive than immunodiffusion based assays and do not require the antigen to be in a precipitable form.

Reversed Passive Haemagglutination (RPHA)

Soluble antigen is often coated onto a carrier particle, thus rendering it particulate. Soluble antigens can be passively adsorbed or chemically coupled to red blood cells in which case the test is called an indirect / passive haemagglutination test. A modification of this test involves coupling of antibody onto red blood cells so as to detect antigen in the patient's sample. Since the reactants are reversed from routine, the test is called RPHA.

Reverse Passive Latex Agglutination

In conventional agglutination test, soluble antibody is reacted with the antigen (eg. bacterial cells) whereas in RPLA test, the antibody is attached to latex particles and reacts with the soluble antigen (eg. bacterial toxin). In the assay procedure, 2 fold dilutions of the sample are prepared across 2 rows on a 96 –well microtitre plate. 25 microtitre volumes of sensitized (anti-enterotoxin) latex and control latex are added to the first and second rows, respectively. Positive and negative controls are included using a purified enterotoxin preparation with the control and latex beads. Plate is covered, shaken on a plate shaker at room temperature for 20-24h If the antigen (bacterial toxin) is present agglutination occurs due to the formation of a molecular lattice and a diffuse layer is formed at the base of the well. If no antigen is present, latex particles sediment and form a tight button in the bottom of the well. Results are recorded visually and classified from negative (-) to strongly positive (+ + +) depending on the degree of agglutination. Assay is simple, rapid to perform, but relatively expensive, gives only semiquantitative results. A range of RPLA kits for the detection of bacterial toxins is available from oxoid Ltd. U.K.

Radio Immunoassay (RIA)

Antibody raised in rabbits is used to coat the well. Dilutions of serum are added to

the coated well. Excess serum is washed off and antigen is coupled to antibody. Bound antigen is measured by addition of radioactive labelled antibody raised in another animal. Measurements of radioactivity in the well are then recorded and quantified using a Geiger counter.

Nucleic acid based assays

Polymerase chain reaction (PCR) and DNA probe hybridization are two of such methods used for seafood-borne pathogen detection.

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 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*, *hlyA*, and the regulatory protein, *prf*A. PCR is a DNA amplification technique and therefore, even if there were dead bacteria, they would show up in PCR.

DNA probe

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 re-associate. 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. Pathogenic *V.cholerae* produce 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.

Table. 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

Biological assay

A convenient means of testing the biological activity of toxins is by animal inoculation studies. Thus biological assays and related tests remain the method of choice for some bacterial toxins. Different types of bioassays are

1. Whole animal tests

a. Mouse lethality

b. Rabbit and guinea- pig skin tests

c. Monkey and kitten emesis tests

2. part-animal tests

a. Illeal loop tests

3. cell culture system

Chinese hamster ovary (CHO) cells

Detection of *Clostridium perfringens* enterotoxin, *Clostridium botulinum* neurotoxin is examples of this method.

Other methods

Apart from these methods, several techniques have been developed which make use of the physical or chemical changes of bacteria in a growth medium.

Flow cytometry and the fluorescence activated cell sorter (FACS)

Cells in suspension and already reacted with fluorochrome-conjugated antibodies are passed through the light of one or more lasers that illuminate the stream at a point. Multiple antibodies, each labeled with a fluorochrome that emits a different color may be used to label cells in a complex population. As a cell passes by the laser and is illuminated, a record of a number of parameters related to that cell is stored in a computer. Cells obtained in this way can be kept alive and sterile, and used for many different *in vitro* and *in vivo* assays.

Impedance (Conductance) microbiology

In direct method, the production of ionic end products (organic acids and ammonium

ions) in the growth medium causes changes in the conductivity of the medium. These changes are measured at regular intervals and the time taken for impedance value change is referred to as "time to detection" The greater the number of organisms, the lower will be the detection time. A calibration curve is constructed and equipment automatically reads the cell density. The indirect method is more versatile. In this method, a potassium hydroxide bridge in agar separates the electrodes. The test sample is separated from Potassium Bridge by a headspace. During growth, carbon dioxide accumulates in the headspace, which in turn reacts with KOH to generate potassium carbonate. This compound being less conductive, there is a decrease in conductivity and it is monitored. The indirect technique is used to estimate a wide range of organisms like *Staphylococcus aureus*, *L monocytogenes*, Salmonella, *Enterococcus faecalis* etc.

ATP Bioluminescence technique

The ATP content in bacterial cell is estimated. The limit of detection is around 1pg ATP, which is equivalent to 1000 bacterial cells, based on the assumption that 10^{-15} g ATP is present in one bacterial cell. This method is much faster than any other method. ATP is detected using the luciferin-luciferase reaction.

Rapid Identification kits

Rapid Identification of the bacterial isolates is another area in food microbiology where vast advancement is taking place. Miniaturized biochemical kits for identification of pure isolates of different bacterial types from food are available in market. They consist of disposable devices containing 15-30 media ingredients designed to identify specific groups of bacteria such as enteric bacteria, non-fermentative gram negatives, gram positives etc.

Limitations of rapid methods

A positive result has to be confirmed by standard methods. In such cases, the advantage of rapid procedure is nullified. Rapid method although, theoretically trustworthy, lack sufficient sensitivity to detect low numbers in different food matrix. Inhibitory components present in the food may interfere with the test. Cells that are injured during processing may escape detection. Similarly viable cells cannot be differentiated from non-viable cells. DNA based methods like gene probe only indicate that target sequence such as a toxigenic gene is present in the food sample, but whether the gene is actually expressed is not known. For certain assays like E.coliO157: H7, more than 30 kits are available in the market and such wide options may confuse the user. Finally most of the kits are very expensive, so users particularly in the developing countries may find the test uneconomical. Technology continue to advance in such rapid pace that advancement in the so called 'next generation assays' like biosensors and DNA chips are being developed that are capable of real time and on-line monitoring of multiple pathogens in foods.