METHODS FOR THE RAPID DIAGNOSIS AND CONTROL OF BACTERIAL DISEASES IN SHELLFISHES AND FINFISHES

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INTRODUCTION

The culture of finfish and shellfish are often threatened by disease causing bacteria. At times it is difficult to distinguish between infection with a pathogen and disease that may result from such infections. In addition, the stressed animals (physiological or environmental) are easily predisposed to encounter bacterial infections as well as diseases.

The diagnostic tests are useful at several stages in the aquaculture production cycle. For example, the first step may be using them in screening of broodstock to prevent the potential spread of a pathogenic bacterial strain from parent to offspring (vertical transmission). Here, a sensitive and non-lethal test is highly desirable if the goal is to establish a Specific Pathogen Free (SPF) breeding population. Likewise, monitoring the very young individual animals periodically in order to assess and prevent potential spread of pathogens (horizontal transmission). Another critical point is at the time of stocking or purchase of stocking material so as to minimise the risk of introduction of diseases into the culture system. In addition to diagnosing diseases, the biotechnological methods are useful in prophylaxis steps, as well as controlling or managing the infections.

BACTERIAL DISEASES OF FISHES

The first step towards prevention and control a disease is to thoroughly understand the infectious agent/pathogen. Retrospective work on bacterial fish pathogens carried out by different authors have indicated that 12 genera of Gram negative and 7 genera of Gram positive, in addition to 2 genera of acid fast bacteria are major bacterial fish pathogens. The list of bacterial fish pathogens and the diseases they cause are given in Table. 1.

Table 1. ***Bacterial pathogens of finfishes and the diseases caused by them***

Bacterial species	Disease
Gram negative pathogens	
Vibrio anguillarum	Vibriosis
Vibrio ordalii	Vibriosis
Vibrio alginolyticus	Vibriosis
Vibrio damsela	Vibriosis
Vibrio cholerae	Vibriosis
Vibrio vulnificus	Vibriosis
Aeromonas salmonicida	Furunculosis
Aeromonas hydrophila	Motile Aeromonad Septicaemia

Bacterial species	Disease
Pasturella piscicida	Pasteurellosis
Providencia rettgeri	Bactrerial Haemorrhagic Septicaemia
Edwardsiella tarda	Edwardsiellosis
Edwardsiella ictaluri	Enteric Septicaemia
Yershinia ruckeri	Enteric Redmouth Disease
Acinetobacter sp.	Acinetobacterosis
Pseudomonas anguilliseptica	Pseudomonas Septicaemia
Pseudomonas chlororaphis	Pseudomonas Septicaemia
Pseudomonas fluorescens	Pseudomonas Septicaemia
Cytophaga psychorophila	Bacterial Coldwater Disease
Cytophaga spp.	Fin Rot, Bacterial Gill Disease
Flexibacter columnaris	Columnaris
Flexibacter maritimus	Flexibacteriosis
Sporocytophaga sp.	Salt water columnaris
Flavobacterium sp.	Bacterial Gill Disease
Gram positive pathogens	
Renibacterium salmoninarum	Bacterial Kidney Disease
Eubacterium tarantellus	Eubacterial Meningitis
Lactobacillus piscicola	Pseudokidney Disease
Staphylococcus epidermidis	Staphylococcosis
Streptococcus spp.	Streptococcal Septicaemia
Clostridium botulinum	Botulism
Myxococcus piscicola	White Mouth
Acid fast pathogens	
Mycobacterium marinum	Mycobacteriosis
Mycobacterium fortuitum	Mycobacteriosis
Mycobacterium chelonei	Mycobacteriosis
Nocardia asteroides	Nocardiosis
Nocardia kampachi	Nocardiosis

Bacterial Diseases of Shellfishes

In the shellfish aquaculture, eight genera of bacteria are known to cause diseases. In the crustacean culture system, chitinovorous bacteria, epibionts and internal pathogens are reported. The list of some of the common bacterial pathogens and diseases they cause among shellfishes are given in Table 2.

Table 2. ***Bacterial pathogens of shellfishes and the diseases caused by them.***

Species	Disease	Host
Molluscs		
Vibrio sp.	Vibriosis	*Crassostrea virginica*
	Bacillary necrosis	(Oyster larvae)
V. anguillaram	Bacillary necrosis	*C. gigas* larvae
	Bacillary necrosis	& Bay scallop
V. parahaemolyticus	Bacillary necrosis	*C. gigas* larvae
	Bacillary necrosis	& Bay scallop
Pseudomonas sp.	Bacillary necrosis	*Ostrea edulis* larvae C. gigas
P. enalia	Bacillary necrosis	*Ostrea edulis* larvae C. gigas
Achromobacter		*C. gigas*
Vibrio sp.	Ulcerative necrosis	Cephalopods (*Octopus joubini*)
V. alginolyticus		*O. briareus* Gastropods *Haliotis rufescens*
Crustacea		
a. Chitinovorous bacteria		
Vibrio sp.	Shell disease/	Shrimps
Beneckea sp.	Reust disease/	Lobsters
Pseudomonas sp.	Black spot/	
Photobacterium sp.	Brown spot	
b. Epibionts		
Leucothrix mucor	Fouler	Crustacea In culture system
c. Internal pathogens		
Vibrio alginolyticus *V. anguillarum* *V. Parahaemolyticus* *Vibrio* sp.	Hemocochic infection	Shrimps in culture system
Citrobactor freundii	Enteric	Shrimp
d. Gram positive bacteria		
Aerococcus viridis	Gaffkemia	Lobsters

Rapid Diagnositic Tools

It is always desirable to prevent disease outbreaks and minimise the presence of pathogens rather than to treat the epizootics, once they occur. This approach is particularly important and less expensive since it can result in reduced dependence on antibiotics and other antibacterial compounds.

In order to determine the cause of disease outbreak, a variety of standardized procedures are available to identify and isolate the bacterial pathogens. The methods are labour-intensive, expensive and time consuming. Considering the potential pathogenic dimensions, the following criteria are to be fulfilled before taking up further management aspects:

1. What is the sensitivity, accuracy, speed and cost compared to other procedures?
2. Can the test be used in presumptive and/or confirmatory application?
3. Can the test be adapted to simple field surveillance application or does it require complex laboratory facilities and equipments?
4. Can the test be micro-modified or automated and inexpensive handling of large number of individuals and small volume samples?
5. Does the test require a destructive or a non destructive sample ?
6. Does the test require qualitative and /or quantitative results?
7. How well do the results obtained correlate with the clinical status of the fish?

Immunoassay diagnostic methods

Polyclonal versus monoclonal antibodies

Immunology provided some of the greatest strides in the field of diagnostics. Immunoassays take advantage of the natural specificity of antibodies toward foreign objects. The immunoassays can utilize polyclonal or monoclonal antibodies in a variety of formats to provide rapid detection of infectious agents. Among these, monoclonal antibodies are the reagents of choice because of high degree of specificity.

The production of monoclonal antibodies was accomplished during 1973 by Milstein and Cotton by fusion of mouse myeloma cells rat myeloma cells. Later during 1977 Galfre and Williams used polyethylene glycol for inducing the fusion of myeloma cells with spleen cells and also with thymus cells. They hybrids so farmed (Hybridomas) continuously produced antibodies in a large proportion . The method of production of monoclonal antibodies are given below:

1. Selection of cell lines to be fused
2. Somatic cell fusion
3. Selection of hybrid cells
4. Selection desired clones
5. Production of antibodies from selected clones

Direct fluorescent antibody test (D-FAT)

The D-FAT procedure has gained widespread use in finfish culture. It uses antibody prepared against the pathogen of interest and then conjugate it with a fluorescing dye (Fluorochorme). Either an impression smear, bacterial culture, tissue culture showing cytopathic effect (CPE) or specially prepared tissue sections on a microscope slide can be examined for specific pathogens using fluorescently labelled antibodies. Wherever, the antibodies are attaching to its target, the target glows when viewd through the fluorescence microscope. This particular technique has the advantage of visually pin pointing the pathogen,

and its location within the tissue. However, it requires the use of an expensive fluorescence microscope and also suffers from expertise / subjective interpretation of the results.

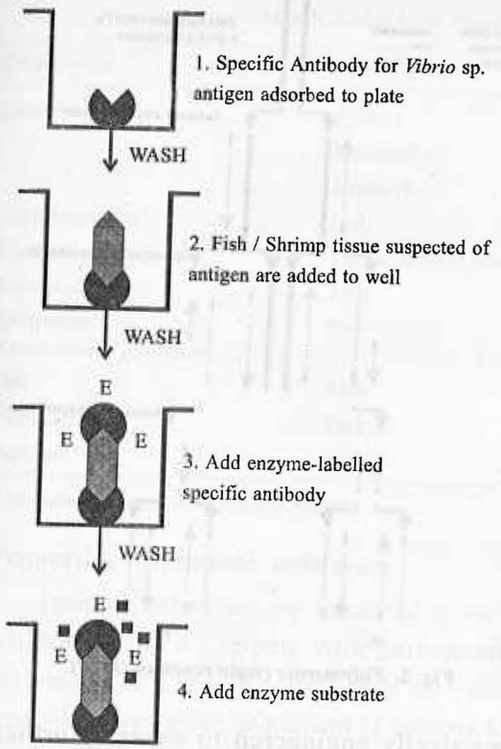
Enzyme-linked immunosorbant assay (ELISA)

The most widely used antibody-based diagnostic technique or enzyme immuno assays (EIAs). In these techniques, the antibody molecules are linked to enzymes either directly or indirectly. In the direct method, the enzyme is conjugated (linked) to a portion of the antibody molecule that does not bind to the antigen. This is referred as "back end". In the indirect method, a second step is required. Here, a carrier or second antibody is linked with the enzyme. The amount of enzyme is important in producing measurable signal when the primary antibody binds to its target. One of the important uses of this technology in the field of diagnostics involves application of antibodies to tissue sections on microscope slides. This permits the antigen for pinpointing within a tissue using a normal light microscope. In addition to its diagnostic applications, the technique also helps in studying how the pathogen spreads within the organism and causes disease (Reddington and Lightner,1994).

The Enzyme Linked Immuno Sorbant Assay (ELISA) detects specific substances in a complex mixture by binding them to antigen or antibody coated substances. It is also capable of detecting viruses, bacteria, drugs , hormones, toxins, carcinogens, depending on the nature of ELISA. Once binding has occurred, other reagents are added that allow the captured substances to be linked to indicators or enzymes, which can be quantified.

An example of the working principle of ELISA in the case of shrimp pathogenic *Vibrio* species is summarized below:

1. Raise antibody against pathogenic *Vibrio* in goat or rabbit (Inject the purified antigen fraction of bacteria into rabbits).
2. Take hepatopancreas / body tissue sample - mix with phosphate-buffered saline (PBS) pH 7.4 + 0.05% tween 20 (PBS - T 20). Heat at 100° C for 15 minutes.
3. Purified rabbit immunoglobulin containing antibodies against antigens of vibrio is attached to the surface of 96-well microtiter plate. (see also Fig 1.)
4. Attachment is accomplished by diluting the antibody to the prescribed concentration in carbonate-bicarbonate buffer (pH 9.6) and incubating the plate overnight adding 200ml to each plate (16 hours at 4°C).
5. Coating buffer or any unadsorbed antibody are removed by washing the wells with buffer.
6. Supernatants of centrifuged test samples are then applied in duplicate to the antibody-sensitized wells. Each well will receive 0.2ml so that the final test sample can be within 0.5 ml.
7. Labelling the plate is important. Usually the following controls are needed:
   a. A blank (B: Test for background with reagent only- no sample)
   b. Conjugate Control - (CC: For conjugate striking directly to plate)
   c. Substrate chromogen control (SC: Test for non-specific color development with substrate and chromogen only)
   d. Positive Control (1:100, 1:1000, 1:2000 and 1:5000 K&P)
   e. Negative Control: Appropriate tissue from control or uninfected fish / shellfish
8. Plates to be read at 405 nm.



Amount of hydrolysis (Color intensity) ° Amount of antigen present

Note:
- All buffers should be made in acid-washed glasswares
- Better to do shrimp individual samplings
- Use -70° C for storing tissues

Fig 1. ***Diagrammatic protocol for ELISA***

The degree of colour change is proportional to the amount of antigen in the sample, i.e., the wells containing samples from uninfected animals will not show any colour whereas the others will show varying amounts of colour change which can provide quantitative analysis.

Indirect fluorescent antibody test (I-FAT)

This test is usually considered as a double layer technique. The first layer is unconjucated purified immunoglobulin or antibody prepared in one animal species (rabbit). The second layer is fluorochorme - conjugated antibody raised in a second animal species (e.g., goat), which is specific for the first one.

DNA-based diagnostics

The most recent advances in diagnostics have come from the field of Molecular Biology. The cloning and manipulating of genetic material has led to the development of extremely sensitive and specific diagnostic systems. For example, DNA based test formats have entered into the area of infectious disease diagnosis for aquatic species. The DiaXotics Inc, Wilton, CT. are pioneers in commercialising DNA-based diagnostics. They produced Shrimp Probe for detecting viral infections of shrimp.

The DNA probe is created by purifying the infectious agent of interest and isolating its nucleic acid. An exact copy of the DNA or a portion of the DNA is made by the cloning process. This copy or probe will bind to the original DNA of the pathogen whenever the two come into contact. In order to accomplish this efficiently, the DNA strands of both the pathogen and the probe must first be separated by heating. After the strands have been separated, one of the strands of the probe can bind to its complementary strand from the pathogen. By attaching a non-radioactive reporter molecule, such as digoxigenin (DIG), the hybrid DNA can be identified and measured (Reddington and Lightner, 1994).

Polymerase chain reaction (PCR)

In the Polymerase Chain Reaction the DNA to be amplified is denatured by heating the sample in the presence of DNA polymerase and excess dNTPs, the oligonucleotides that hybridize specifically to the target sequence can

prime new DNA synthesis. The first cycle is characterized by a product of indeterminate length; however, the second cycle produces the discrete "short product" which accumulates exponentially with each successive round of amplification. This can lead to the many million-fold amplification of the discrete fragment over the course of 20 to 30 cycles. (Fig .2) For example 30 cycles can result in 2 X 28 fold (270 million fold) amplification of the discrete product.

Control of Bacterial Diseases

The important biotechnological methods in controlling bacterial diseases include: development of bacterins / vaccines and also non specific Immunostimulants.

Bacterins/Vaccines

A vaccine could be defined as a substance that causes a specific immune response. Vaccination as a part of standard fish culture programme is relatively new although the impact of vaccination is found to be dramatic. For example, the culture of salmon in brackish water and marine environment was made possible by usage of the Vibriosis vaccine, which led to a great expansion in pen-rearing of Atlantic salmon in Norway and Chile (Leong, 1993).

Considering the importance of vaccination, biotechnologists are trying to develop sub-unit vaccines, i.e., vaccines consisting of the major protective antigens of the pathogen. This type of sub-unit vaccines have evident advantages. The important advantage is that the vaccine contains only a component of the pathogen and is therefore, more chemically defined and likely to be more stable. The other advantage is that the vaccine can be produced by direct synthesis or recombinant DNA technology. Thus, these vaccines may be

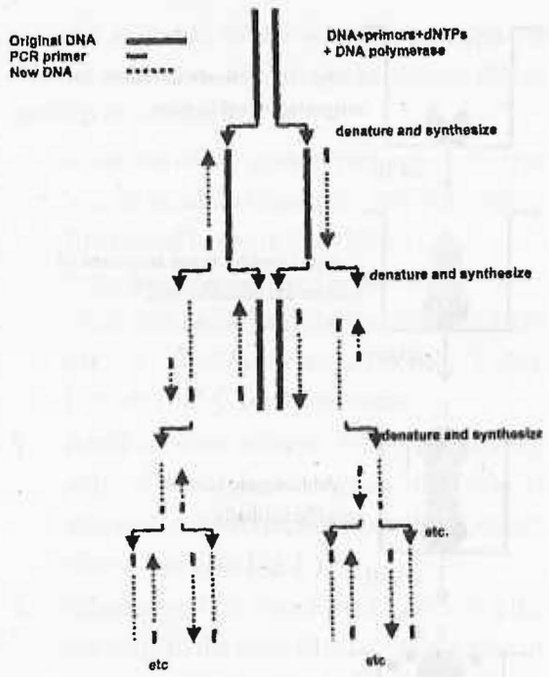


Fig. 2. ***Polymerase chain reaction (PCR).***

genetically engineered to express further protective antigens from other fish pathogens and thus yield multivalent vaccines.

Immunostimulants

Immunostimulants are substances which elicit non-specific defense mechanisms and enhance the barrier of infections against pathogens. They are isolated from natural sources and then synthesised chemically. Cell wall preparations from bacteria, fungi, mushrooms and yeast are rich sources of immunostimulants. They exist in various structural forms. The possible use of immunostimulants in managing diseases on fish and shellfish have been initiated recently. Some of the common immunostimulants include: i. Glucans, ii. Lectins, iii. Lipopolysaccharides (LPS) from bacterial cell wall and iv. Wheat germ agglutinins, which are listed in Table 3.

Table 3. ***List of some common non-specific immunostimulants****

Compound	Animals tested	Results
Glucans	Prawns	Very short term
	Salmonids	Used in conjunction with alum and
	Ictalurids	bacterins. Intermediate term.
Peptidoglycans	Fish	Short term
Lipopolysaccharides	Larval juvenile and Adult shrimp/ fish	45-120 days
Levamisole	Fish	Modest immunostimulatory protection
Quarternary Ammonium Compound (QAC)	Fish	Modest immunostimulatory protection
ISK	Fish	Modest immunostimulatory protection
Henbane	Fish	Modest immunostimulatory protection
Ascogen	Fish	Modest immunostimulatory protection

* Source: Newman and Deupree (1995).

Properties of immune enhancers

Immune enhancers are bacterial growth inhibitors which compete with pathogenic *Aeromonas* or *Vibrio*/for iron. They are also capable of preventing attachment of bacteria to the gut. If the bacteria cannot attach in the gut and multiply, they cannot secrete toxins or enter the blood system. In addition, they make bacteria to clump together which are easily expelled from the digestive system. The enzymes are capable of destroying the bacterial cellwalls and membranes selectively without damaging host cells.

In shrimps, the prophenoloxidase ('Propo'), the defense enzyme system, is activated by immunostimulants. The activation of 'Propo' results in recognising pathogens and providing resistance. In fish, the non - specific defense system is activated by the immunostimulants. The first line of defense - *i.e.,* non specific humoral defense or proteases, lysins and agglutinins in mucous cell secretion; the second line of defense provided by the mucosal lining cells and the third line of defense function (achieved by blood cells, especially granulocytes and monocytes) which destroy microbes present in the circulation are activated. The final defense is taken up by endocytically active cells such as endothelial cells, macrophages and granulocytes in organs and tissues which degrade microbes or microbial products. The final endocytic and degradation process strongly depend on the effectiveness of reticulo endothelial system which consist of endothelial cells and macrophages which line the small blood vessels (sinusoids and ellipsoids). During infection, an inflammatory response such as elevated production of antimicrobial substances is often encountered. The central cells in the production of antimicrobial substances are macrophages and granulocytes which are activated by the immune enhancers

The immunostimulants have several advantages: i. Being natural products, there is no environmental hazard and ii. Unlike vaccines, which gives protection to a specific pathogen, immunostimulants provide a wide range of protection against several pathogens. Most of the immunostimulants can be synthesized and the problem of residual effect on shrimps or fish is not encountered.

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