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Prof (Dr.) Mohan Joseph Modayil
Director, CMFRI, Kochi

Dr. P.C. Thomas
Winter School Director

Co-ordinators:

Dr. R. Paul Raj, Head, PNPD
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Dr. P. Jayasankar, Senior Scientist
Dr. D. Noble, Senior Scientist

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Course Manual

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P.B. No. 1603, Tatapuram P.O.,
Kochi – 682 014

RAPID METHODS FOR DIAGNOSIS OF FISH AND SHELLFISH PATHOGENS

A.P. Lipton

Central Marine Fisheries Research Institute, Cochin

Introduction

In order to prevent disease outbreaks, minimize the presence of pathogens and to reduce the dependence on antimicrobial compounds, rapid diagnostic steps are essential. While considering rapid diagnostic tests, it should be ensured that they fulfill the following criteria:

1. Compared to other procedures, they should be more speedy, sensitive and accurate.
2. The tests should have presumptive and/or confirmatory application.
3. The tests be micro-modified or automated and inexpensive handling of large number of individuals and small volume samples.
4. The tests should require non-destructive samples.
5. The tests must yield qualitative and quantitative results.
6. The results obtained from such tests should correlate with the other clinical symptoms of the fish.

IMMUNOASSAY DIAGNOSTIC METHODS:

Polyclonal versus Monoclonal antibodies:

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 favored due to their high degree of specificity. The method of production of monoclonal antibodies is as follows:

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). 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 labeled antibodies. Wherever, the antibodies are attaching to its target, the target glows when viewed through the fluorescence microscope.

Though this technique has the advantage of visually pin pointing the pathogen, and its location within the tissue, it requires the use of an expensive fluorescence microscope and suffers from expertise (subjective) interpretation of the results.

ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

Enzyme immune assays (EIA s) are most widely used as antibody-based diagnostic technique. 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 recognizes the antigen. This is the "lock and key". 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. The antibody is applied to tissue sections on microscope slides. This permits the antigen for pinpointing within a tissue using a normal light microscope. Apart from the diagnostic applications, this technique also helps in studying how the pathogen spreads within the organism and causes disease.

The 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 and 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 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).

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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. (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 200µl to each plate (16 h at 4°C).

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5. Coating buffer or any unused antibody are removed by washing the wells with buffer.

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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.

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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.

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

A few of the ELISA protocols are given in Table 1.

DNA-BASED DIAGNOSTICS:

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 commercializing 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. A product of

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

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Table 1. ELISA protocols

ELISA Protocol	Uses	Required reagents	Comments
Indirect	Antibody screening; epitope mapping	Antigen, pure or semipure: test solution containing antibody; enzyme conjugate that binds Ig of immunized species	Does not require the use of preexisting specific antibodies; requires relatively large amounts of antigen.
Direct competitive	Antigen screening; detect soluble antigen	Antigen, pure or semipure; test solution containing antigen; enzyme-antibody conjugate specific for antigen	Rapid assay with only two steps; excellent for measuring antigenic cross reactivity
Antibody-sandwich	Antigen screening; detect soluble antigen	Capture antibody (purified or semi-purifeid specific antibody); test solution containing antigen; enzyme-antibody conjugated specific for antigen	Most sensitive antigen assay; requires relatively large amount of pure or semi-pure specific antibody (capture antibody)
Double antibody-sandwich	Antibody-screening epitope mapping	Capture antibody: (specific for Ig of immunized species); test solution containing antigen; enzyme-antibody conjugate specific for antigen	Does not require purified antigen; relatively long assay with five steps
Direct cellular	Screen cells for expression of antigen; measure cellular antigen expression	Cells that express antigen of interest; enzyme-antibody conjugate specific for cellular antigen	Sensitive assay for bulk screening; insensitive to heterogeneity of expression in mixed population of cells
Indirect cellular	Screen for antibodies against cellular antigens	Cells used for immunizing; test solution containing antibodies; enzyme conjugate that binds Ig of immunized species.	May not detect antibodies specific for cellular antigens expressed at a low density