

# Diagnosis of antigen/pathogen presence in fishes by ELISA and Immuno-Electron Microscopy

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**Abstract:** *Labeo rohita*, rohu fishes  $(150 \pm 25 \text{ g})$  were reared in laboratory conditions, and were fed with the artificial diet. After 4 weeks of acclimatization, fishes were immunized intra peritoneally with a protein antigen, bovine serum albumin (BSA). Fish were sampled on days-7, 14, 21 and 28 after BSA administration. The samples were diagnosed by ELISA and immuno-electron microscopy for the presence of specific-antibody and Antigen, respectively.

Keywords: Labeo rohita, Immunization, Bovine serum albumin, ELISA, Immuno-Electron Microscopy.

#### Introduction

To fulfill the rising population demand, fishes have been usually cultured in enclosed spaces such as ponds or net cages, and efforts have been made to increase the productivity per unit area. When fish are cultured in high densities and are stressed by adverse environmental factors, their ability to generate effective immune responses against pathogens is severely impaired and pathogens may have the advantage and the risk of disease outbreak increases in fish population<sup>1-3</sup>. Culture conditions promoting chronic, acute or subacute disease outbreaks include adverse environmenta conditions<sup>4,5</sup> or other factors such as handling<sup>6,7</sup> environmental overcrowding<sup>8</sup>, water pollution<sup>9</sup>, accumulation of wastes, ambient flora and fauna, low dissolved oxygen levels<sup>10</sup>, exposure to sunlight and water temperature<sup>11</sup>.

Heavy losses of cultured fish continue to occur due to the disease till today. Several preventive and treatment methods have been practiced to reduce the losses due to the disease outbreak. Often it is not possible to detect the diseases early in the aquatic system, particularly with bacterial and viral pathogens. The disease spreads rapidly in aquatic medium and all stock of fish will be infected rapidly and this results in the death of whole stock. The high susceptibility of fish to stress and the rapid spread of diseases in water have forced aquaculturists to concentrate their efforts on maintaining their fish in good health in achieve sustainable order to economic performances. It is therefore an ultimate goal to develop methods for establishing microbial control at all stages of the cultivation process<sup>12</sup>.

To overcome the above situations, early diagnosis of the infection is very important. Diagnosis is often made by looking at the physical condition of the fish. The disease symptoms are often would not appear until the disease is progressed well. By the time the disease symptoms

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appear in the fish, all stock will get the disease, and it would be very difficult or impossible to protect the fish stock. Accurate methods must be developed to inspect the fish at all stages of fish is necessary. In this study we have diagnosed the antigen presence in a fish species by immune-electron microscopy.

### **Materials and Methods**

#### Animals

*Labeo rohita, Rohu* fishes (weighing about  $150 \pm 25$  g) were procured and reared in laboratory conditions. Fish were fed with the artificial diet and acclimatized for 4 weeks. Fish were cultured in outdoor-cemented tanks (170 L). Temperature and pH ranged between 27-350C and 7.4-8.0, respectively, during the experiments. Dissolved oxygen level was maintained above 5 mg/L with the help of aerators throughout the experiment.

#### Artificial diet and feeding

Fish artificial diet was prepared using wheat flour, fishmeal, cod-liver oil and vitamin-mineral premix. Feeding was started four weeks prior to immunization at the rate of 1% of body weight/day, once at 9:00 AM, and continued till the end of the experiment.

#### Antigen and immunization

After four weeks (28 days) of feeding, L. rohita fish were anaesthetized with MS-222 and injected intraperitoneally with 500  $\mu$ I of Bovine serum albumin (BSA, Fraction-V, Merck) dissolved in phosphate buffered saline (i.e. 10 mg of BSA/fish).

#### Sampling

Blood was collected from four immunized fishes on days 7, 14, 21 and 28 after immunization and allowed to clot at room temperature. Serum was obtained by centrifugation. After blood sampling, spleen samples were aseptically dissected out for immuno-electron microscopy.

#### Diagnosis of serum for Antigen-Specific Antibody by ELISA a) Coating

The wells of the microtiter plate (greiner bioone, Germany, ELISA plate, Microlon, 96W, Flatbott, High binding) were coated with 100  $\mu$ l of fish serum (2-fold serial dilutions) diluted in phosphate buffered saline (pH 7.4). The plates were incubated for 12 h at 40C. After incubation the wells were washed three times with PBS containing tween-20 (0.05%).

### b) Blocking

The free binding sites of the wells were blocked by adding 300  $\mu$ l of 5% gelatin diluted in PBS per well. The plates were incubated for 12 h at 40C. After incubation, the wells were washed three times with PBS-tween.

### c) Antigen

100  $\mu$ I of bovine serum albumin (BSA) dissolved in PBS was added to each well (100 ng BSA/well). The plates were incubated for 2 h at 40C. After incubation, the wells were washed three times with BPS-tween.

### d) First antibody

100  $\mu$ l of rabbit anti-BSA serum diluted to 1:500 in PBS was added to each well. Plate was incubated for 1 h at 370C. After incubation, the wells were washed three times with PBS-tween.

### e) Second antibody

Goat anti-Rabbit Ig-G antibodies conjugated to horseradish peroxidase was diluted 1:1000 in PBS and added to all wells (100  $\mu$ I/ well). The plate was incubated for 1 h at 370C. After incubation, the wells were washed three times with PBS-tween.

### f) Substrate

13 mg of o-phenylenediamine dihydrochloride was dissolved in 10 ml of citrate-phosphate buffer (pH 5.0). 10 µl of hydrogen peroxide was added to the above solution before use. The above substrate solution was added in all wells (100 µl/well), and incubated for 10 min. After the development of color, the reaction was terminated by adding 50µl of 1M oxalic acid per well. The optical density was measured at 492 nm in an automatic microplate MS5605A, Electronic reader (Microscan -Corporation of India Ltd.). The highest dilution of the serum that gave the OD>0.1 was taken as the titer.

### Diagnosis of spleen for Antigen presence by Immuno-Electron Microscopy

Since the spleen of *L. rohita* was diffused in to small pieces, these pieces were directly fixed, but in *C. carpio* the spleen is intact one, and it was cut into small pieces of minimum 1 mm size before fixation.

### a) Fixation

Tissues were fixed in 1% glutaraldehide and 2% paraformaldehide in 0.1M phosphate buffer for overnight. After fixation tissues were washed with 0.1M phosphate buffer for 2 times.

# b) Dehydration

After washing tissues were dehydrated with gradient ethanol (30-100%) at 40C with a duration of 30 min. in each.

#### c) Infiltration

Tissues were first infiltrated with ethanol and LR White (1:1) for 2 h and then with pure LR White (TAAB) for overnight at 40C. In the subsequent day tissues were changed into pure LR white and kept for 2 h at room temperature.

### d) Embedding

Tissues were embedded in beam capsule; these capsules were filled with LR White up to the brim and covered to enable the polymerization in oxygen free condition. For polymerization the beam capsules were kept at 550C for 12 h. After polymerization the blocks were ready to cut sections.

### e) Section cutting

Ultra thin sections (70 nm) were cut using glass knives in a microtome (Reichert Jung Ultracut-E).

### f) Grid preparation

After cutting, the sections were slowly lifted onto the nickel grids with the help of a fine-edged forceps.

### g) Blocking the section

The sections on the grid were blocked with 2% fish gelatin in 0.1 M phosphate buffer for 2 h.

### h) First antibody

After blocking, the sections were labeled by incubating with rabbit anti-BSA antibodies (polyclonal) (ICN Biochemicals, USA) 1:500 in phosphate buffer containing 1% fish gelatin, for 12 h. After incubation, the grids were washed thoroughly with 1% fish gelatin in phosphate buffer.

### i) Second antibody

After treating with primary antibody, the grids were labeled with secondary antibody, i.e. goat antirabbit-lgG conjugated with 15 nm gold particles (TAAB), diluted 1:100 in phosphate buffer containing 1% fish gelatin and incubated for 2 h. After labeling the grids were washed with phosphate buffer containing 1% fish gelatin, and finally washed with double distilled water.

#### j) Staining

After labeling the sections were stained, by incubating with uranyl acetate for 10 min at room temperature, and washed with distilled water; and later the sections were incubated with lead citrate for 8 min at room temperature, and washed with distilled water.

### k) Viewing

Stained sections were viewed for labeled particles under electron microscope, Fei-Philips Morgagni 268D Digital TEM with image analysis system.

#### **Results and Discussion**

As any foreign particle/pathogen invades the body, the immune system of the host recognize it, mounts immune response against it, and eliminates it from the body as quickly as possible to confer safety of the host and to protect the host from the onset of infection. Antibody is the major humoral component of the specific immune system, which plays an adaptive role in neutralizing, killing and clearing of invaded pathogens with the help of other humoral and cellular components of the immune system.

After administration of BSA *L. rohita*, BSAspecific antibody level was peaked on day-7 and then gradually decreased up to day-28 (Fig. 1). The titers reduced by 59% on day-28, compared with the titers of day-7. The average antibody titer was 800x103, 640x103, 485x103 and 330x103 on days 7, 14, 21 and 28, respectively. This indicates that the antibody response was already peaked on day-7 after administration of antigen.



**Fig.1**: Antigen-specific antibody level at different weeks of sampling after antigen administration in *L. rohita*.



**Fig.2:** Transmission Electron micrograph of the spleen section of *L. rohita* on day-7 after antigen administration (7100x).



**Fig.3:** Transmission Electron micrograph of the spleen section of *L. rohita* on day-14 after antigen administration (5600x).

Immuno-labeling was performed to locate the antigen molecules/pathogen particles in the spleen under electron microscope, which were shown in Figs. 2-5. The presence of gold particles locates the BSA in the sections. On day-7, heavily crowded particles appeared in all spleen samples (Fig. 2) and found abundantly throughout the sections, which the plentiful presence of antigen indicates molecules/pathogen particles. The density of particles reduced gradually from day-7 to day-28 (Figs.2, 3, 4, 5). The antigen-presence on days 7, 14, 21 and 28 were shone in Figs 2, 3, 4 and 5 respectively. The particles were found rarely in the sections of day-28 and their presence was very low compared to the samples of days 7, 14 and 21, which was evident from Fig. 5. This indicates that the immune response mounted that was against the injected antigen/pathogen and effectively cleared the antigen from the host.



**Fig.4.** Transmission Electron micrograph of the spleen section of *L. rohita* on day-21 after antigen administration (7100x).



**Fig.5.** Transmission Electron micrograph of the spleen section of *L. rohita* on day-28 after antigen administration (5600x).

With these results it may be concluded that both the specific antibodies against any particular pathogen and the presence of the pathogen in the hose may be detected easily and early during the onset of infection, which would help in early and accurate treatment to prevent rapid spread of disease to whole stock and thereby prevent huge losses in aquaculture. Further there is no need to wait for the external disease symptoms. Diagnoses of diseases using these molecular methods are accurate. For diagnosis of various bacterial and viral diseases specific antibodies against each pathogen is required. These pathogen-specific antibodies either polyclonal or monoclonal may be easily produced, which may be used in these molecular diagnostic techniques.

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