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Application of Immunohistochemistry for Pathogen Detection in Fishes

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ABSTRACT
Fishes, namely,Catlacatla (150 ± 20 g) were reared in laboratory conditions, and were fed with the artificial diet. After 4 weeks of acclimatization, fishes were immunized intraperitoneally with bovine serum albumin (BSA),a protein antigen. After antigen administration, spleen samples were dissected out aseptically from the fish on weakly intervals, i.e., on days-7, 14, 21 and 28. The spleen samples were diagnosed by immuno-electron microscopy for the presence of BSA antigen. From the results it was observed that the antigen/pathogen can be definitely located from the fish tissue at early stages of infection.
Keywords:*Fishes;Pathogen; Antigen; Immunohistochemistry.*

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INTRODUCTION

Fishes have been usually cultured under captive conditions in enclosed spaces to meet the population demand. When fish are cultured in captive conditions with high densities,they are stressed by adverse environmental conditions, and their ability to defend against pathogens is severely impaired.Hence, pathogens may have the advantage and there will be risk of disease outbreak [1]. Culture conditions promoting disease outbreaks include adverse environmental conditions, such as handling, overcrowding, water pollution, accumulation of wastes, ambient flora and fauna, low dissolved oxygen levels. 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 sudden and unexpected death of whole stock. Early diagnosis of the infection is needed to overcome the above situation. Diagnosis is generally made by looking at the physical condition of the fish. Normally, the disease symptoms do not appear until the disease is well progressed. By the time the disease symptoms appear in the fish, wholefish population will get the disease, and it will be very difficult to protect the fish at that advanced stage. So, accurate methods must be developed to inspect the fish at all stages of fish. In this study we have determined the antigen presence in a fish species by immune-electron microscopy at different stages after infection.

MATERIAL AND METHODS

Fish

Fish, namely *Catlacatla* (weighing about 150 ± 20 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). During the experiments, temperature and pH were ranged between 29-33°C and 7.4 - 8.0, respectively. Aerators were used throughout the experiment to maintain the dissolved oxygen level above 5 mg/L.

Artificial diet and feeding strategy

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 in the morning, and continued till the end of the experiment.

Antigen and immunization

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

Sampling

After immunization, spleen samples were aseptically dissected out on days 7, 14, 21 and 28,and then processed for immune-electron-microscopy as described hereunder (Fig. 1).

Diagnosis of spleen for Antigen presence by Immuno-Electron Microscopy

a)Fixation: Tissue samples were fixed in 1% glutaraldehide and 2% paraformaldehide in 0.1M phosphate buffer (PB) for overnight. After fixation tissues were washed twice with 0.1M phosphate buffer.

b)Dehydration: After washing tissues were dehydrated with gradient ethanol (30-100%) at 4°C 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 4°C. 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 55°C 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.

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 PB for 2 h.

h)First antibody: After blocking, the sections were labeled by incubating with rabbit anti-BSA antibodies 1:500 in PB 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 anti-rabbit-IgG conjugated with 15 nm gold particles (TAAB), diluted 1:100 in PB containing 1% fish gelatin and incubated for 2 h. After labeling the grids were washed with PB containing 1% fish gelatin, and finally washed with double distilled water.

j)Staining:

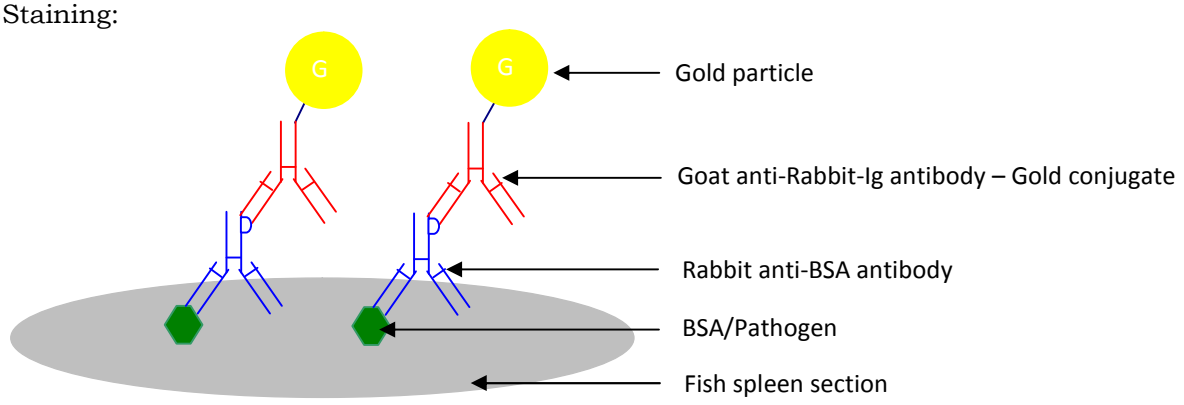

Fig. 1. Schematic diagram for detection of pathogen in fish tissue by Immuno-electron-microscopy

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, Digital TEM with image analysis system.

RESULTS AND DISCUSSION

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. The high susceptibility of fish to stress and the rapid spread of diseases in water have forced the researchers to concentrate their efforts on maintaining their fish in good health in order to achieve sustainable economic performances. It is therefore an ultimate goal to develop methods for establishing microbial control at all stages of the cultivation process [2]. Different ingredients that work as immune boosters have been added to the fish diets to boost the immunity of the fishes to prevent the losses due to diseases [3,4].

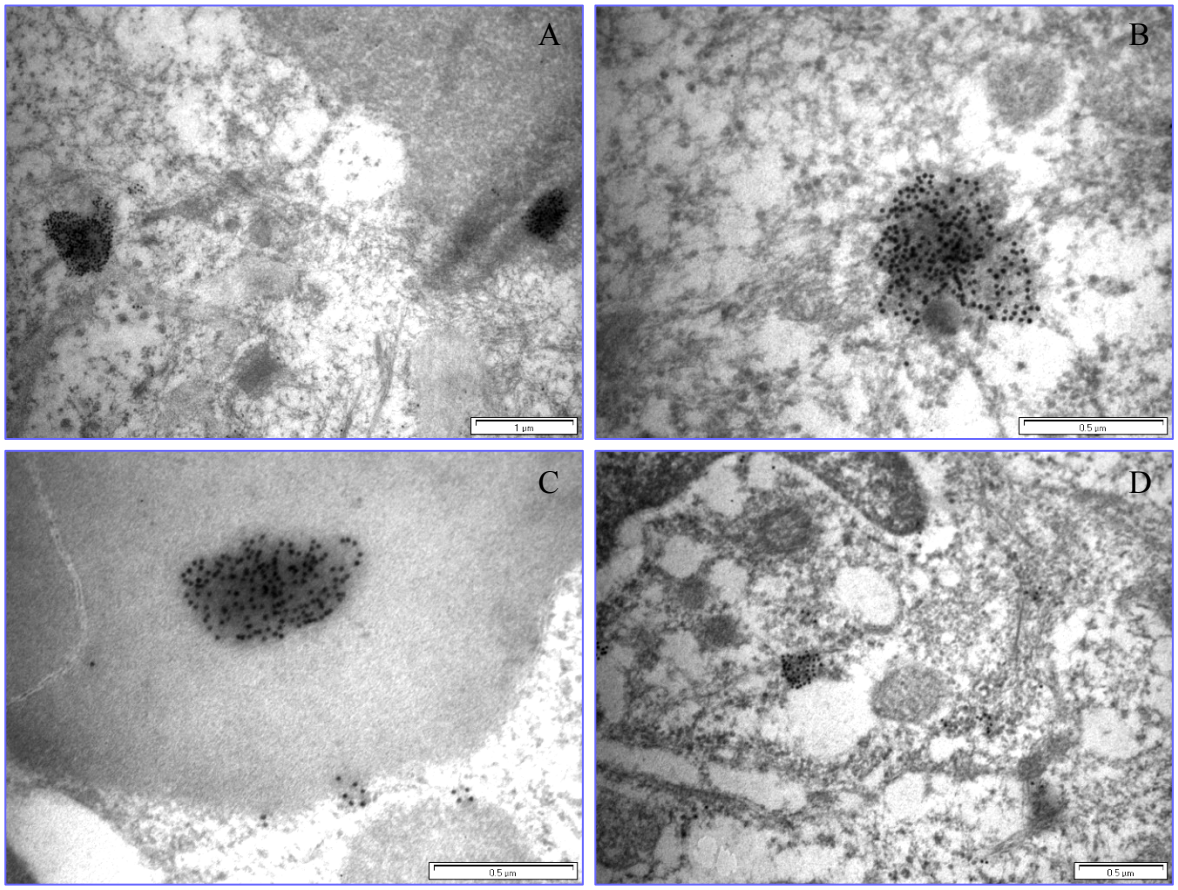

Fig. 2. Transmission Electron micrograph of the spleen section of *L. rohita* on day-7 (A, 3500x), day-14 (B, 8900x), day-21 (C, 8900x) & day-28 (D, 5600x) after antigen administration.

Immuno-labeling was performed to detect the injected antigen in the spleen sections under electron microscope (Fig. 2). The presence of gold particles locates the BSA in the sections. On day-7, heavily crowded particles appeared in the spleen sample (Fig. 2A) and found abundantly throughout the section, which indicates the plentiful presence of antigen molecules/particles. The antigen-presence on days 7, 14, 21 and 28 were shone in Figs 2-A, B, C and D, respectively. The density of particles reduced gradually from day-7 to day-28 due to the mounting of the immune response of the fish.
The presence of the pathogen in the host may be detected easily and early during the onset of infection.This would help in early and accurate treatment to prevent rapid spread of disease to whole fish population, and thereby prevent huge losses in aquaculture. Further

there is no need to wait for the external disease symptoms to appear, which would generally appear at later stages or at acute infection stages. Using these molecular methods diseases can be detected early and accurately. This same procedure can be used to detect any disease-causing pathogens from the tissues of the fishes. For diagnosis of various bacterial and viral diseases specific antibodies against each pathogen is required. These pathogen-specific antibodies either poly-clonal or mono-clonal may be easily produced, which may be used in these molecular diagnostic techniques. Early and accurate diagnosis of pathogensusing molecular methods would benefit the aquaculture industry by preventing severe losses due to rapid infections.

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