Histopathology of head kidney tissues in challenged rohu, *Labeo rohita* Hamilton after vaccinating with *Aeromonas hydrophila* antigens

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A B S T R A C T

The study was conducted to evaluate the vaccination effects on rohu, *Labeo rohita* head kidney tissues while assessing the vaccine efficacy of *Aeromonas hydrophila* antigens. Six acclimatized rohu groups were immunized with three antigenic formulations (outer membrane proteins, somatic and whole-cell antigen) @ 200 µg/fish and also with equal volume of Freund’s incomplete adjuvant (FIA), separately. Simultaneously, two non-vaccinated groups, i.e., injected with FIA (100 µl), normal saline solutions (0.85%) and one control without injection were maintained for 28 days. All rohu were challenged with median lethal dose of *A. hydrophila* (2.85 × 10^6 cells/rohu) intraperitoneally. After 7 days, highest cumulative mortality (%) of >88% was found for all non-vaccinated groups. During histopathological observations in head kidney tissues of all treatment and control groups, numerous histopathological changes in the nephritic cells like mild loss of typical tubular epithelial lining, necrosis, thickening of renal epithelial lining, haemorrhages, inflammation, distorted and widening of the lumen with vacuolated surrounding and the constricted lumen of nephritic tubules were noticed for vaccinated rohu in contrast to non vaccinated groups before *A. hydrophila* challenge. In case of all non-vaccinated fish, including control, extensive degenerated and necrotized head kidney tissues were observed, whereas it was least observed in vaccinated rohu after 7 days *A. hydrophila* challenge. Results suggest that OMPs antigen along with FIA was the premier vaccine approach for improving resistance to *Aeromonas* disease and reduce mortality in rohu. Similarly, vaccination with all three antigenic formulations, preferably when applied along with FIA, can effectively protect the head kidney against *A. hydrophila* infection.

1. Introduction

Aquaculture not only yields high-quality protein but also contributes for secure earnings, employment, and foreign exchange worldwide. According to FAO [1], total world fishing and aquaculture production has hit an all-time high of around 178.5 million tons, with aquaculture contributing 82.1 million tons of total output. Freshwater fish culture activities in India are primarily the culture of Indian major carps (IMC). More than 80 percent of India’s total aquaculture output is carp culture [2]. Satisfactory growth, customer tastes and high nutritional value makes *Labeo rohita*, commonly known as rohu, one of the most popular species in India and other neighboring countries. Like other teleosts, there are pathogen challenges for rohu culture. High degree mortality in wild and cultivated fish is causes due to bacterial infections. *Aeromonas hydrophila*, a gram-negative, rod-shaped, opportunistic bacterium, is known to cause the most common bacterial infection in IMC and is reported to be the causative agent of many different pathological conditions, particularly tissue necrosis, tissue swelling and ulceration [3-5].

Efforts have been made over the past few decades to immunize fish with different vaccines against various infectious diseases [6-10]. Fish have immunological properties, provides adequate protection against pathogens. In particular, an appropriate understanding of the specific immune response in fish is urgently required to develop protective strategies to tackle fish pathogens in the aquafarming sector. Previous works on rohu vaccines against *Aeromonas* infection showed mixed results on successful outputs [11-16]. To date, numerous studies have been
conducted worldwide to improve vaccines, ranging from inactivated and live attenuated species to the introduction of advanced vaccines against *A. hydrophila* in various fish types [17]. Head kidney is considered the most important hematopoietic organ in teleost [18,19], but so far, very little research has been carried out on the histopathological effect on rohu head kidney after vaccination against any bacterial disease. The fish kidney is a compound organ consisting of four structures comprising the endocrine, reticuloendothelial, hematopoietic and secretory systems that are morphologically and functionally distinct. The head kidney is bifurcated into two lobes, without nephrons, and therefore has no renal function. In contrast, the posterior kidney processes a mixture, including renal and immune tissue [20,21]. A few histopathological studies on head kidney in rohu [22,23] and other fish [24] have been performed so far while evaluated clinical signs and internal lesions after *A. hydrophila* injection. Histopathology is a tool of the immune system that can be used to assist in the detection of lymphoid lesions for immunomodulatory investigation because lymphoid organs support specific immune functions and should be evaluated individually [25]. Therefore, the current research was carried out as a prior work to determine the histopathological changes in rohu kidneys during vaccination with *A. hydrophila* antigens.

2. Materials and methods

2.1. *A. hydrophila* strain collection

*A. hydrophila* N10P (NCBI accession number KC914628) bacterial strain has been acquired from the Department of Aquatic Animal Health, West Bengal University of Animal and Fishery Sciences, Kolkata. After preparation of *A. hydrophila* cell suspension, it was verified by spread plate technique on Tryptone soya agar (TSA, HiMedia) incubating at 30 ± 2°C for 24 h. The median lethal dose (LD₅₀) value of the bacterium at 7 days was estimated using the Reed and Muench [26] method for pathogenicity determination.

2.2. Preparation of *A. hydrophila* N10P bacterial antigens

2.2.1. Whole-cell antigen (WCA)

With some alterations in Kamilya et al. [27] method, the whole-cell bacterial antigen was prepared. The bacterial cell suspension was treated with 1% formalin to a final concentration of 0.5% (V/V) and allowed at 4°C overnight. It was then centrifuged for 10 min at 10000 x g and washed thrice with sterile phosphate buffer saline (PBS, pH 7.2). Further, the sterility was tested by streaking plate technique on Tryptic soya agar (TSA, HiMedia). Finally, the washed bacterial suspension was suspended in 5 mL PBS and maintained at 4°C till further use.

2.2.2. Somatic antigen

With some changes in Melamed et al. [28] method, the somatic antigen preparation was carried out. The culture of the bacterium in 10 mL Tryptic soy broth (TSB, HiMedia) was exposed to heat killing by incubating for 1 h in a hot water bath at 60°C after introduced of 25 mM Phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 24 mM Ethylene diaminetetraacetic acid (Sigma-Aldrich). The said cell suspension was sonicated on ice at 60 W with a repeating duty cycle of 0.5 μ for ten times 1 min each with 1 min interval using an ultrasonicator (Labsonic® U, Biotech International). Then the soluble sonicated extracts were subsequently centrifuged at 3500 x g at 4°C for 30 min. Soluble supernatant was filter-sterilized (0.22 μ) and the filtrates were maintained as somatic antigen at −20°C till further use.

2.2.3. Outer membrane proteins (OMPs) antigen

With some amendments in Maji et al. [29] method, bacterial outer membrane proteins (OMPs) were prepared. The membrane fractions, derived after sonication from centrifugation, were washed and suspended again in 20 mL sterile PBS (pH 7.2). For solubilization, the suspension was allowed to be processed with 2% sodium dodecyl sulfate (SDS) and 2% mercaptoethanol at 60°C for 20 min. The extracts were centrifuged at 3500 x g for 30 min at 4°C and then purified using a 0.22 μ membrane filter. The membrane supernatant was finally kept at -20°C till further use.

Fig. 1. Experimental design for vaccination to different rohu groups.
2.4. Acclimatization of experimental fish

Rohu (size 18-26 cm and weight 80-100 gm) were acquired from Sonarpur fish farm (Lat 22°26′27.15"N and Long 88°25′28.69"E), West Bengal state, India. The circular fibre-reinforced plastic (FRP) tanks (500 L capacity) were filled with bore well water and allowed 3-4 days for ageing. Upon delivery fish were treated for external infecting organism with 5 ppm KMnO₄ for 15 min and then transferred to 10 circular tanks @ 50 nos/ tank. The fish were fed daily twice with a commercial floating dry pellet diet @ 1% of body weight. All fish were maintained with proper aeration and 20% water exchange daily for 3 weeks prior to experiment.

2.5. Experimental design and challenge with A. hydrophila N10P antigens

Rectangular FRP tanks (n=36) with a capacity of 300 L were filled with clean bore-well ageing water with a volume of 250 L. Experimental fish from the adapted stocks were placed in tanks. Every tank was stocked with 10 nos of rohu and acclimatized for 7 days. After adaptation, tanks were divided into quadruplicates, namely TR1, TR2, TR3 and TR4, have nine series (TG1, TG2, TG3, TG4, TG5, TG6, TG7, TG8 and TG9) each (Fig. 1). Among TR1 to TR4, fishes of every six series (TG1, TG2, TG3, TG4, TG5, and TG6) were vaccinated with bacterial antigens @ 200 µg/ rohu. All individual fish of series TG7 and TG8 were injected with FIA (Sigma-Aldrich) @ 100 µL/ rohu and normal saline solution (NSS) @ 100 µL/ rohu respectively. The remaining TG9 was retained as control (without injection). Individual fishes of two tanks of TG1 and TG2 series were administered with OMPs antigen separately and remaining two tanks with OMPs mixed with equal volume of FIA (1:1 ratio). Similarly, fishers of TG3, TG4 and TG5, TG6 series were administered likewise with somatic antigen and WCA, respectively. The immunized and non-immunized rohu groups were maintained in tanks for 28 days with proper aeration and 20% water exchange daily. 10th and 20th days post-vaccination, two fishes of TR1 and TR2 groups (from all TG1 to TG9 tanks) were randomly selected for histopathological examination of head kidney tissue. Simultaneously, the TR3 and TR4 group’s stocked rohu were challenged with A. hydrophila N10P for 7 days. Each fish of the remaining TR3 and TR4 was administered intraperitoneally with 0.1 mL of bacterial cell suspension (2.85 × 10⁵ cells/fish). After 7 days post-challenged, two rohu of TR3 and TR4 groups were randomly selected for similar histopathological examination.

2.6. Histopathology

The primary lymphoid organ head kidney (pronephros) of rohu were fixed in Bouin’s fixative (Sigma-Aldrich) for 48-72 h after collection from each control and experimental sub-groups of both sensitized (TR1 and TR2) groups and also challenged (TR3 and TR4) groups. The tissues
were immediately fixed in 10% formalin, dehydrated in acetone, cleared in xylene, embedded in paraffin wax, and sectioned at 3-5 µM with the help of a rotary microtome. Slides were stained with Harris hematoxylin stain and counterstained with eosin, dipped in xylene followed by mounted in DPX (a mixture of distyrene, a plasticizer, and xylene). Histopathological observation was carried out with an advanced Trinocular Research Microscope (Olympus, Japan, Model: BX51) using SCO-LUX camera 16 MP attached to the microscope.

3. Results and discussion

*A. hydrophila* N10P strain was confirmed to be deadly as the challenged rohu that showed 100% mortality within 4 days. The median lethal dose (LD$_{50}$) value of the bacterium was found $2.85 \times 10^6$ cells/rohu. Biochemical findings of the isolated bacterium from dying fish indicated the infection with *A. hydrophila*.

Deaths began in rohu at 4 days of post-challenged with the bacterium. OMPs antigen and somatic antigen along with FIA were associated with lower cumulative (%) mortality, i.e., 20.17% and 33.33% (Fig. 2), respectively. Whereas non-immunized groups, i.e., FIA injected, NSS injected and control (without injection) groups were showed 100%, 88.82% & 95.29% death rates (Fig. 2), respectively, with different clinical signs and pathology like pinpoint haemorrhages, head lesion, fin rot, tail rot and diluted kidney (Fig. 3A, 3B and 3C). In all immunized populations, no clinical symptoms or pathological alterations were detected (3D). Kamila et al. [27] remarked on very similar findings.

Histopathological samples were taken from the rohu head kidney due to its significance in the fish immune response [3,20,21,30]. The kidney of freshwater fish has reticulo-endothelial and antibody-producing cell mechanism also possesses the functional resemblance to the mammals’ lymph nodes [31]. All the damage, deformation and expansion size of nephritic cells of vaccinated rohu groups were observed and compared with the nephritic cells of three non-vaccinated rohu groups.

Vaccinated rohu during 10th and 20th days showed numerous histopathological changes in the nephric cells. Randomly selected immunized rohu exhibited degenerative changes with vacuole formation in the head kidney with minutely affected areas. OMPs antigen vaccinated rohu during 10th and 20th days showed almost similar histopathological changes in head kidney like melano-macrophage centre, haemocytic infiltration, necrosis, constricted lumen of nephric tubules, glomerulopathy with diluted bowmen’s space, inflamed nephric tubules with...
vacuolated surrounding and thickening of renal epithelial lining, widen lumen of nephric tubules and vacuolated surrounding (Fig. 4A), but almost recovered after 7 days post-challenge with minute changes like degeneration of outer lining of renal tubule, degeneration of epithelial lining, constricted lumen of nephric tubules and mild necrosis (Fig. 4B).

But in case of OMPs antigen and FIA (1:1) immunized group, less adverse change was observed during 10th and 20th days with minute degeneration of the inner epithelial layer of renal tubule, constricted lumen of nephric tubules etc (Fig. 5A). After 7 days post-challenge, a less melanomacrophage centre, degeneration of inner epithelial layer of renal tubule, constricted lumen of nephric tubules were observed (Fig. 5B). The challenge study exhibited less similar changes, and there was lots of improvement in the kidney tissue structure and systematic arrangement of _A. hydrophila_ N10P challenge.

After 10th days immunized fish groups with somatic antigen showed more extensive damage of nephric tubules, haemocytic infiltration, degeneration of outer lining of renal tubule and inner epithelial layer of renal tubule, necrosis, widen lumen of nephric tubules and thickening of renal epithelial lining (Fig. 6A). Whereas, after 7 days of bacterial challenge, numerous changes in head kidney were observed with little constricted lumen of nephric tubules, haemocytic infiltration, degeneration of epithelial lining with surround necrosis (Fig. 6B). Alike previously stated the somatic antigen and FIA (1:1) vaccinated group fishes kidney also showed better improvement during 7 days post-challenge compared to somatic antigen without FIA immunized fish. Here degeneration of epithelial lining, haemocytic infiltration, glomerulopathy with diluted bowmen’s space, necrosis, vacuole formation, widen lumen of nephric tubules and inflamed nephric tubules were observed in 20th days post-vaccination (Fig. 7A). In contrast, only very less existence of constricted lumen of nephric tubules, Inflamed nephric tubules, degeneration of outer lining of renal tubule, necrosis, glomerulopathy with diluted bowmen’s space were noticed during 7 days bacterial post-challenge (Fig. 7B).

A similar finding was recorded in case of inactivated WCA separately and along with FIA immunized fish groups during 10th and 20th days post-vaccinations. In both cases, very less constricted lumen of nephric
tubules, degeneration of inner epithelial layer of renal tubule, necrosis, glomerulopathy with diluted Bowman’s space, vacuole formation of the nephric cell, widen lumen of nephric tubules and inflamed nephric tubules were found (Fig. 8A and 8B). Stratev et al. [32] noted that the most histopathological damage was seen in the kidneys while identified pathological variations in investigational infection of carps (Cyprinus carpio) with A. hydrophila. Yardimci et al. [33] reported diffuse necrosis in the anterior kidney in Nile tilapia due to experimental A. hydrophila infection. In present study almost well organized nephritic cells along with very minute existence of inflamed nephric tubules were found (Fig. 8A and 8B). Mamun et al. [34] also observed several histopathological observations like destruction of Bowman’s space, inflammatory exudate, glomerular necrosis, tubular necrosis and infiltration of leukocyte cells in the kidney of striped catfish, Pangasianodon hypophthalmus while they fed with A. hydrophila vaccine. Espenes et al. [35] observed a subpopulation of melanomacrophages and some nonpigmented macrophages reactivity in the head kidney of Salmo salar only for A. salmonicida as vaccine antigens. Aly et al. [36] observed histopathological finding in kidney such as vacuolar degeneration of catfish (Clarias gariepinus) treated by levamisole-adjuvanted A. hydrophila vaccine. Our results also corroborate the finding of Julinta et al. [37], where glomerulopathy with dilated Bowman’s space, necrotized areas, inflammation, degeneration and thickening of nephritic tubules was observed in Oreochromis niloticus against A. hydrophila intramuscular challenge. In both pre and post-challenge state, melano-macrophage centres were also found in all immunized fish kidney. Besides those, fibrosis, haemocytic infiltration and proteinaceous cast formation were also observed in vaccinated rohu kidney. The challenge study exhibited less similar changes, and there was lots of improvement in the kidney tissue structure and systematic arrangement after 7 days of the bacterial challenge for immunized fish.

Fig. 8. A: Photomicrograph of head kidney of 10th days post inactivated whole-cell antigen vaccinated fish. 200X, H&E staining showing constricted lumen of nephric tubules (C), degeneration of inner epithelial layer of renal tubule (DI), necrosis (N), glomerulopathy (G) with diluted bowmen’s space (BS), vacuole formation (V) of the nephric cell, widen lumen of nephric tubules (W) and inflamed nephric tubules (I). B: Photomicrograph of head kidney of 7th days post-challenge A. hydrophila N10P fish vaccinated with inactivated whole-cell antigen. 200X, H&E staining showing inflamed nephric tubules (I), melano-macrophage centre (MMC), necrosis (N), haemocytic infiltration (HI).

Fig. 9. A: Photomicrograph of head kidney of 10th days post inactivated whole-cell antigen along with FIA vaccinated fish. 200X, H&E staining showing necrosis (N), degeneration of outer lining of renal tubule (DO), glomerulopathy (G), haemocytic infiltration (HI), thickening of renal epithelial lining (T), widen lumen of nephric tubules (W), constricted lumen of nephric tubules (C) and inflamed nephric tubules (I). B: Photomicrograph of head kidney of 7th days post-challenge A. hydrophila N10P fish vaccinated with inactivated whole-cell antigen along with FIA. 200X, H&E staining showing haemocytic infiltration (HI), constricted lumen of nephric tubules (C), inflamed nephric tubules (I), degeneration of outer lining of renal tubule (DO), vacuole formation (V) surrounding the nephric cell.
Whereas in FIA injected, NSS injected and control rohu groups, normal structure and systematic arrangement of head kidney tissues with well-defined glomerulus were observed after 10th and 20th days (Fig. 10 A, 11 A and 12 A), but extensive histopathological changes were noticed after the challenge (Fig. 10 B, 11 B and 12 B). Most of the nephritic cells were fully degenerated and necrotized in three non-vaccinated groups, including control. The head kidney’s main changes in the non immunized *A. hydrophila* N10P challenged group were lymphoid cell transformation to macrophage-like cells and further appearance of a meshwork-like parenchyma. Apart from that, loosening of haemopoietic tissue, vacuolated cytoplasm, damaged uriniferous tubules, shrinkage in glomeruli with bowman’s space, widen lumen of nephritic tubules with hydropic swelling, haemocytic infiltration, degeneration of both inner and outer epithelial layer of renal tubule in the kidney were also observed in NSS injected, FIA injected and control rohu groups challenged with the said bacterium. More or less, similar results were noted by Sharma & Tamot [38]. Azad et al. [39] found renal tubular necrosis, depletion of the cell in tubular interstitium and glomerular necrosis in tilapia infected with *A. hydrophila*. Less histopathological changes were observed for the fish groups vaccinated along with FIA instead of antigen alone. But FIA itself was not found suitable to protect head kidney tissues against the bacterium infection. Our findings were also supported by Villumsen et al. [40,41], where whole-cell bacterin or mineral oil adjuvants alone produced several histopathological adverse effects in rainbow trout kidney during vaccination against *Aeromonas salmonicida*.

4. Conclusion

In conclusion, administration of OMPs antigen along with FIA through intraperitoneal route is a most promising approach of rohu and may other fishes for improving the resistance of *Aeromonas* disease and mortality. Similarly, vaccination of any three antigenic formulations itself and with FIA can effectively protect hematopoietic tissue necrosis of head kidney, which is considered a major primary and secondary lymphoid organ of rohu, and trigger the immune response against *Aeromonas* infection.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial
interests or personal relationships that could have appeared to influence
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Supplementary materials

Supplementary material associated with this article can be found, in
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