Vaccine potentiality of different antigenic preparations of *Aeromonas hydrophila* in Rohu, *Labeo rohita* Ham

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**Abstract**

To develop vaccine for rohu (*Labeo rohita*), efficacy of three antigenic preparations from *Aeromonas hydrophila* were evaluated. Thirty six tanks with ten rohu were divided in quadruplets (R1 to R3) with nine tanks (G1 to G9). Rohu of G1 to G9 tanks were given intraperitoneal vaccine with outer membrane protein, somatic protein and formalin-inactivated whole cell itself and along with Incomplete Freund’s Adjuvant @ 200 µg/fish, G7 and G8 tanks were injected with Incomplete Freund’s Adjuvant (100 µl/fish) and normal saline (100 µl/fish) respectively and G9 tanks were kept as control. After 28 d, rohu of R1 and R4 were subjected to intramuscular *A. hydrophila* challenge (LD50) @ 2.85×10^6 cells/fish for 7 d and RPS (%) was calculated. Specific cellular and humoral immune responses were determined for rohu of R1 and R4. Results showed that rohu immunized with outer membrane protein along with adjuvant could offer an appropriate vaccine strategy.

**Keywords:** *Aeromonas hydrophila*, rohu, vaccine, specific immune response

1. **Introduction**

Aquaculture apart from being a most promising sector, it provides high quality protein, generates income, employment and foreign exchange around the globe. Global fish production has reached to about 178.5 million tons with inland aquaculture representing 28.73% of total production ([FAO](https://www.fao.org)) ([10]). In India, freshwater fish culture practices mainly constitute the culture of Indian major carps namely, catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*). Carp culture constitutes more than 80% of total aquaculture production of India ([Jayasankar](https://www.fao.org)) ([24]), out of which *Labeo rohita* commonly known as rohu, is most prominent among others due to its high growth potential coupled with high consumer preferences and high nutritive value. Like other freshwater fishes rohu can be infected by different pathogens, microorganisms or parasites. The bacterial infections are considered the major factors of mass mortality in farmed and wild fish. *Aeromonas hydrophila* is considered as most common bacterial pathogen in rohu, and has been considered as causative agent of several pathogenic situations including swelling of tissues, necrosis, ulceration, tail/fin rot, motile aeromonas septicemia or haemorrhagic septicemia as a primary pathogen ([Hu et al.](https://www.fao.org)) ([13]), ([Rasmussen et al.](https://www.fao.org)) ([22]), ([Sen et al.](https://www.fao.org)) ([28]), ([Dash et al.](https://www.fao.org)) ([7]), ([Shoemaker et al.](https://www.fao.org)) ([29]), ([Dash et al.](https://www.fao.org)) ([8]), ([Bharadwaj et al.](https://www.fao.org)) ([3]), ([Sen et al.](https://www.fao.org)) ([28]), ([Dash et al.](https://www.fao.org)) ([7]), ([Dubey et al.](https://www.fao.org)) ([9]), (Till date many efforts have been made to develop vaccines throughout world, from inactivated products and live attenuated organisms to advancement high tech vaccines against *A. hydrophila* in different fish species ([Mzula et al.](https://www.fao.org)) ([19]) but till now suitable
immunoprophylactic tool(s) are not commercially available to prevent *Aeromonas hydrophila* infection of rohu. Hence, in the present study rohu (*Labeo rohita*) has been taken as the species of concern to assess the efficacy and vaccine effectiveness among outer membrane protein antigen, somatic protein antigen and formalin killed whole-cell protein antigen preparations of *Aeromonas hydrophila*.

2. Materials and Methods

2.1. Collection of *A. hydrophila* strain: The bacterial strain *Aeromonas hydrophila* N10P (NCBI accession number KC914628) was obtained from the Department of Aquatic Animal Health, Faculty of Fishery Sciences, WBU-AFS, Kolkata. The cell suspension of *A. hydrophila* N10P was prepared and checked by spread plating on Tryptone soya agar after incubation at 30°C for 24 h. The LD$_{50}$ value on 7 d of *A. hydrophila* N10P was calculated following the method of Reed and Muench [23] to determine the pathogenicity.

2.2. Preparation of bacterial antigens

2.2.1. *A. hydrophila* N10P Whole-cell antigen: The whole-cell antigen of *A. hydrophila* N10P strain was prepared as described by Kamlaya et al. [15] with some modifications. The cell suspension of *A. hydrophila* was treated with formalin to a final concentration of 0.5% (v/v) and left overnight at 4°C. Washed with sterile phosphate buffer saline (PBS, HiMedia, pH 7.2) and checked for sterility by streaking on to TSA plates. The washed formalin-killed bacterial cells were resuspended in 5 ml PBS and stored at 4°C until used.

2.2.2. *A. hydrophila* N10P somatic antigen: *A. hydrophila* N10P culture in 10 ml tryptic soy broth (HiMedia, India) were subjected to heat killing by incubating at hot water bath at 60°C for 1 h after addition of 25 mM Phenylmethylsulfonyl fluoride (Sigma) and 24 mM Ethylenediaminetetra-acetic acid (Sigma). The sonication of said culture was conducted on the ice at 60 W with repeating duty cycle of 0.5 μ for 10 times 1 m each with 1 m interval using an ultrasonicator (Labsonic® U, Biotech International). After that the soluble sonicated extracts were centrifuged at 3500 g for 30 m at 4°C. The soluble supernatant was filter-sterilized (0.22 μ) and the filtrates were stored at −20°C as somatic antigens.

2.2.3. *A. hydrophila* N10P outer membrane protein antigen: Bacterial outer membrane proteins were obtained by the method of Mali et al. [18] with some modifications. The pellets, obtained from the centrifugation after sonication, were washed and re-suspended in 20 ml sterile phosphate buffer saline (PBS, HiMedia, pH 7.2). This suspension was treated with 2% sodium dodecyl sulphate and 2% mercaptoethanol for 20 m at 60 °C for solubilization. The extracts were centrifuged at 3500 g for 30 m at 4°C and the supernatant was filtered through 0.22 μ membrane filter and stored at −20 °C until use.

2.3. Acclimatization of experimental fish: Clinically healthy, 80-100 g rohu were obtained from a commercial fish farm in Sonarpur, West Bengal, India. The fishes were disinfected by 5 ppm KMnO$_4$ for 15 m and were shifted to ten circular fiberglass tanks of 500 l capacity @ 50 rohu/ tank. During acclimatization they were fed twice daily at the rate of 1% of the body weight by commercial floating dry pellet diet containing of 30% protein and 2% vitamins minerals mixture. Continuous aeration was provided and the fishes were maintained for 3 weeks before to the experiment.

2.4. Experimental design: The experiments were carried out in the wet laboratory of Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences facilitated with 24 h water supply, drainage system, ventilation and transparent sheet roof for providing adequate light. The fibreglass tanks were scrubbed and cleaned with chlorinated water (200 ppm). Overhead tank water was used throughout the experiment and the basic physicochemical water parameters were maintained at the optimal throughout the experiment as Sahoo et al. [25]. The ambient temperature of the wet laboratory during the trial was in the range of 25-30°C. Fibreglass rectangular tanks (n=36) of 300 l capacity were filled with clean water up to volume 250 l and conditioned for 3 d. Tanks were stocked with experimental fishes from the acclimatized stocks. The rohu were stocked @ 10 numbers in each tank and were acclimatized for 7 d. After acclimatization, the tanks were divided in quadruplicates viz. R$_1$, R$_2$, R$_3$ and R$_4$ having nine tanks (G$_1$, G$_2$, G$_3$, G$_4$, G$_5$, G$_6$, G$_7$, G$_8$ and G$_9$) in each group. Among R$_1$ to R$_4$, fishes of each six tanks (G$_1$ to G$_6$) were vaccinated intraperitoneally with different bacterial antigens @ 200 μg/fish, fishes of all G$_7$ and G$_8$ tanks each were injected intraperitoneally with Incomplete Freund’s Adjuvant (100 μl/fish) and normal saline solution (100 μl/fish), respectively and remaining G$_9$ tanks were kept without injection as control. Fishes of all G$_1$ and G$_2$ tanks each were injected with outer membrane protein antigen itself and along with equal volume of Incomplete Freund’s Adjuvant respectively. Similarly, fishes of G$_3$ and G$_4$ tanks were injected with somatic antigen itself and along with Incomplete Freund’s Adjuvant, whereas whole-cell protein antigen was injected only and also with Incomplete Freund’s Adjuvant into the rohu of G$_5$ and G$_6$ tanks respectively. The immunized fishes of all four groups R$_1$ to R$_4$ were maintained in their respective tank for 28 d.

After 28th d, fishes of R$_1$ and R$_2$ groups (from all G$_1$ to G$_3$ tanks) were taken out for assessment of post vaccination humoral and cellular immune responses. Fish sera were collected from the half of immunized fishes of both R$_1$ and R$_2$ groups and were pooled for different treatment groups before storing at -20°C. Remaining halves of fishes were dissected for isolation of head kidney leukocytes for *in vitro* cellular assay. While the fishes of R$_3$ and R$_4$ group were challenged with *A. hydrophila* N10P for 7 d. Each fish of R$_3$ and R$_4$ were injected intramuscularly with 0.1 ml of *A. hydrophila* N10P cell suspension (2.85×10$^6$ cells/fish). Mortality, external signs of infection and behavioural changes were recorded daily and Relative Percentage of Survival (RPS%) was estimated after 7 d.

$$\text{RPS} = \left(1 - \frac{\% \text{ mortality in vaccinated rohu}}{\% \text{ mortality in control rohu}}\right) \times 100$$

2.5. Isolation of rohu immunoglobulin from sera: Blood was collected aseptically from 25 live rohu and sera were separated. Sera from all the rohu were pooled, as they were homologous in nature. Five millilitres of sera were taken for the isolation of immunoglobulin using 40% saturated ammonium sulphate. Three millilitre of immunoglobulin was harvested and stored at -20°C in aliquots for further use. The protein content of the isolated rohu immunoglobulin was estimated using the standard protocol of Lowry et al. [16] with some modification.
2.6. Raising of hyper-immune sera against rohu immunoglobulin: Hyperimmunisation of New Zealand white rabbit for preparation of anti-rohu rabbit immunoglobulin was carried out (Sahoo and Joardar) [24]. Four doses of rohu immunoglobulin and Freund’s adjuvant (Sigma, USA) (1:1) were injected intramuscularly. The hyper-immune sera was collected after 5 d of the last injection and stored at –20 °C. Specificity between rohu-immunoglobulin and anti-rohu rabbit immunoglobulin and the titre of anti-rohu rabbit immunoglobulin was assessed by an immunodiffusion test (Ouchterlony) [29] with some modifications and contemporaneous immunoelectrophoresis as per Sardar et al. [27].

2.7.1. Coupling of horseradish peroxidase enzyme with anti-rohu rabbit immunoglobulin: Glutaraldehyde method (Sahoo and Joardar) [24] was performed for linking of horseradish peroxidase (HRPO) enzyme with anti-rohu rabbit immunoglobulin. Specificity and titre of the anti-rohu rabbit enzyme immunoconjugate were determined by direct ELISA as per Sahoo and Joardar [24].

2.7.2. Assessment of anti-Aeromonas hydrophila antibodies in rohu by indirect plate ELISA: Enzyme-linked immunosorbent assay was performed as per Sahoo and Joardar [24] with some modifications to assess the anti-Aeromonas hydrophila antibodies in all G1 to G9 tanks of R1 and R2 rohu groups. In brief, different antigens i.e., outer membrane protein antigen, somatic protein antigen and whole cell protein antigen were coated in triplicate into 96 wells ELISA plate (Tarsons, India) at a concentration of 2 µg/ well along with carbonate-bicarbonate coating buffer (pH 9.6). The pooled diluted sera (1:200) of vaccinated rohu (G1 to G9), Incomplete Freund’s Adjuvant and normal saline solution injected rohu (G2 and G8) and control rohu (G9) were added @ 100 µl/ well and incubated for 2 h at 37°C. Diluted (1:500 in PBS) anti-rohu rabbit HRPO immune conjugate (Laboratory prepared) was applied and incubated for 2 h at 37°C. After washing, a substrate solution (5 µl H2O2, 0.025 mg o-phenylene diamine dihydrochloride in 25 ml citrate buffer) was added (100 µl/ well). The colour development was noticed after 30 m and reading was taken at 492 nm using an ELISA reader (ECIL, India) after addition of stopper solution (3N H2SO4).

2.7.3. MTT dye assay: MTT dye assay was performed (Maji et al.) [17] to quantify the proliferative responses of rohu kidney lymphocytes in all rohu (G1 to G9) of R1 and R2 groups. First, the head kidney leukocytes of experimental fish was isolated (Kamila et al.) [15].

2.7.4. Mitogens and Antigens: Initially, the stock solution of concanavalin A (Sigma) was prepared at the concentration of 20 µg/ml of the proliferation medium (RPMI-1640, growth medium), membrane filtered (0.22 µm) and stored at –20 °C for use. Stock solution (80 µg/100 µl) of outer membrane protein antigen, somatic protein antigen and whole cell protein antigen of A. hydrophila were prepared in phosphate buffer saline, membrane filtered (0.22 µm) and stored at –20 °C for use.

2.7.5. In-vitro lymphocyte proliferation assay: Cell separation of head kidney leukocytes (5x10^5 cells/ml) of all rohu (G1 to G9) of R1 and R2 groups were pooled and suspended in RPMI-1640. Hundred µl of said cell suspension were seeded into every well of 96-well tissue culture plates in triplicate. The ultimate volume of the wells was ended up to 200 µl with outer membrane protein antigen, somatic protein antigen and whole cell protein antigen at a concentration of 40 µg/100 µl and Con-A at a concentration of 10 µg/100 µl. In control well, only 100 µl RPMI-1640 were added to make the ultimate volume 200 µl. The plate was incubated at 28°C for 48 h containing 5% CO2 tension.

The colourimetric 3 - [4, 5 - dimethylthiazol-2-yl] - 2, 5 - diphenyltetrazolium bromide (MTT) assay (Maji et al.) [17] was used to verify the head kidney leukocytes proliferation. After 48 h of culture, the plate was again incubated at 28°C for 4 h after addition of 20 µl of MTT (5mg/ ml in PBS) to each well. The formazan production was estimated by the method of Plumb et al. [21] with minor modifications. The plate was centrifuged at 50 g for 10 m followed by removal of the supernatant fluids without disturbing the cell pellet or formazan precipitate. The formazan crystals were dissolved by the consecutive addition of 150 µl of Dimethyl sulfoxide (Sigma) and 25 µl of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The plate was incubated at room temperature for 10 m. The formazan development was measured at 595 nm (Maji et al.) [17] using a plate reader (ECIL, India). Stimulation index was calculated by the following formula-

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SI = \left(\frac{\text{Mean optical density of sensitised lymphocyte wells with Con} - \text{A or antigen}}{\text{Mean optical density of control lymphocyte wells}}\right) - 1
\]

2.8. Statistical analysis: The results of each experiment are expressed as the mean ± standard deviation and analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (IBM-SPSS); Version 22.0 to test the significance of the difference between the control and experimental groups.

3. Results and Discussion

3.1. Anti-rohu rabbit enzyme immunoconjugate assessment: The calculated protein value of the rohu immunoglobulin and anti-rohu rabbit immunoglobulin was found 1.35 mg/ml and 2.08 mg/ml, respectively. Immunodiffusion test and Countercurrent immunoelectrophoresis results confirmed the specificity of anti-rohu rabbit immunoglobulin with the rohu immunoglobulin, which corroborates earlier observation (Das et al.) [6]. The titre of anti-rohu rabbit immunoglobulin was found four. During assessment of specificity of anti-rohu rabbit enzyme immunoconjugate to rohu-lg by direct ELISA, the highest optical density value of 0.507 was found in diluted (1:500) anti-rohu rabbit HRPO immunoconjugate which was more or less similar to Sahoo and Joardar [24] and was found as an effective serodiagnostic tool in rohu.

3.2. Virulence study of Aeromonas hydrophila: The Aeromonas hydrophila N10P strain was found to be virulent as the intramuscular challenge in rohu (100±10 g) at 2.2x10^6, 2.2x10^8 and 2.2x10^10 cells/fish recorded 100% mortality within 12 h. 1 d and 4 d of challenge respectively. Mortality started at 5 d of post-challenge in 2.2x10^6 cells/fish. The LD50 value of A. hydrophila N10P (NCBI accession number KC914628) of 7 d was found 2.85x10^6 cells/fish. The biochemical test results of the bacterium isolated from the moribund fishes confirmed the A. hydrophila infection.
3.3. Gross clinical signs, mortality and relative percentage of survival (%) during experiment: No mortality was observed in all tanks (G1 to G6) of R1 to R4 groups during 28 days of the experiment except few clinical signs. Few pinpoint hemorrhages were noticed in only thirteen rohu out of all groups of quadruplicates tank injected with outer membrane protein antigen, somatic protein antigen, whole cell protein antigen itself and whole cell protein antigen along with Freund’s incomplete adjuvant. Those gross clinical changes observed during 28-days vaccination might be due to the pathogenic effects of virulent A. hydrophila N10P. During 7 d A. hydrophila challenge for R1 and R4 groups, 81.81% RPS (Fig.1) was found in case of fishes vaccinated with both outer membrane protein antigen and somatic protein antigen mixed with equal volume of Incomplete Freund’s Adjuvant. In both cases, less than 25% of rohu showed minute clinical signs like ulceration patches on body. Fishes immunized with outer membrane protein antigen, somatic protein antigen itself and whole-cell protein antigen along with Incomplete Freund’s Adjuvant showed 63.63% RPS (Fig.1) with clinical signs like pinpoint hemorrhages and ulceration patches on body for less than 30% rohu. Whereas, 45.45% RPS (Fig.1) were noticed in case of fishes injected with whole-cell protein antigen itself but number of fishes clinically infected was 45% of population. Relative percentage of survival was found nil for the Incomplete Freund’s Adjuvant injected, normal saline solution injected and control rohu where 70-90% of fishes showed extensive clinical signs with head lesion, fin rot and tail rot, ulceration and hemorrhages on skin with 55-60% mortality. Our results differed from the observations of Sen et al. [28] where they observed highest relative percentage survival in formalin killed whole-cell A. hydrophila along with adjuvant vaccinated rohu after 60 days post-challenge. Sun et al. [39] observed highest relative percent survival in A. hydrophila immunized grass carp (Ctenopharyngodon idella) in the Lipopolysaccharide and outer membrane protein injected groups (83.3% and 72.2%, respectively) but 90% fish died after challenge in phosphate buffer saline injected control group.

| Mortality (%) | RPS (%) |
|---------------|---------|
| Outer membrane protein with IFA | 63.63 |
| Outer membrane protein | 81.81 |
| Outer membrane protein with IFA | 81.81 |
| Somatic protein with IFA | 45.45 |
| Somatic protein | 63.63 |
| Whole cell protein with IFA | 0 |
| Whole cell protein | 0 |
| Incomplete Freund’s Adjuvant | 0 |
| Normal saline solution | 0 |
| Control | 0 |

**Fig 1:** Mortality (%) & RPS (%) after 7 d A. hydrophila challenge of vaccinated rohu

3.4. Assessment of specific humoral immune responses: While Assessing the specific humoral immunities in rohu by indirect ELISA after 28th-d post-vaccination, the sera collected from all vaccinated rohu showed reactivity to the laboratory prepared enzyme immune conjugate (1:500 dilution), i.e., anti-rohu rabbit hyperimmune sera coupled with HRPO. Among fish sera of all vaccinated, Incomplete Freund’s Adjuvant injected, normal saline solution injected and control rohu groups after 28th d of post-vaccination, it was observed that there were significant differences ($P<0.05$) of humoral responses in all six vaccinated fish groups compared to normal saline solution, Incomplete Freund’s Adjuvant injected rohu and control rohu. The highest mean ($\pm$ S.D.) antibody level in terms of optical density value of 0.712±0.012 and 0.722±0.019 was recorded for outer membrane protein antigen along with Incomplete Freund’s Adjuvant and whole cell antigen mixed with Incomplete Freund’s Adjuvant vaccinated rohu, respectively after 28th d post-vaccination. Incomplete Freund’s Adjuvant injected rohu (G7) also showed higher significant difference ($P<0.05$) in humoral responses than control (G6). That confirmed boosting of specific humoral immune responses in all six vaccinated rohu as a result of successful vaccination. The results of the present studies were supported by the observations of Swain et al. [31] where they found enhancement of antibody production level in rohu by A. hydrophila mixed with other two bacterial whole-cell protein. Sen et al. [28] also found the similar results where the antibody production of fish groups vaccinated with three different antigenic preparations like formalin inactivated A. hydrophila along with Incomplete Freund’s Adjuvant, A. hydrophila itself and extracellular products enhanced the antibody production level ($P<0.05$) compared with control. As per Bastardo et al. [1] the degree of immune responses varies depending upon the type of vaccine used. We found that the antibody production in all vaccinated rohu significantly evoked antibody response ($P<0.05$) than Incomplete Freund’s Adjuvant injected, normal saline solution injected and control. Increased antibody production after vaccination against A. hydrophila was also observed in different Indian major carps (Chandran et al.) [9], Kamila et al. [15], Saikia and Kamila [20]. Now it is the time to say that the variation in responses of fish to A. hydrophila and its different antigenic preparation need to be evaluated thoroughly for the successful development of a vaccine for A. hydrophila in rohu.
leucocytes where mushroom glucan and bovine lactoferrin were used as an adjuvant in combination with formalin-killed *A. hydrophila* for 30 days vaccination studies. Our experiments also supported by Das et al. [6], who studied the immune-effector activities of rohu against *A. hydrophila*, using intraperitoneal injection (0.2 ml/fish) with live *A. hydrophila* at a concentration of 1×10⁶ CFU/ml. In this experiment, the higher proliferative responses of all antigen immunized rohu groups than control possibly due to the clonal development of antigen sensitized leucocytes, demonstrated the efficacy of different antigenic preparations of *A. hydrophila*. Similarly, in some cases, antigens induced a higher response but it was not found statistically significant (P<0.05) that indicated lower degree of memory induction compared with other vaccinated groups. Our results were quite similar to the observations of Bharadwaj et al. [3], who studied the antigen specific lymphocyte proliferation in rohu, challenged with *A. hydrophila* after vaccination with somatic and outer membrane protein antigens of *A. hydrophila*. They found all the vaccinated groups showed higher proliferation (P<0.01) after 10⁶ d of vaccination than the control. Same antigen-specific proliferative responses have also been noticed in other fish species (Bera et al.) [2], (Kamilya et al.) [15], (Maji et al.) [17]. We got significantly higher (P<0.05) lymphocyte proliferation in vaccinated rohu which was also partially supported by Fang et al. [10], who performed the MTT assay to measure the head kidney leucocytes proliferation in blue gourami after 5th week of immunization with adhesin from *A. hydrophila*.

### Table 1: OD values after 28th d post vaccination (R₁ and R₂) as assessed by ELISA

| Different vaccinated rohu group ↓ | Antigens coated for antibody production assessment → | Outer membrane protein coating @ 2 µg/well of ELISA plate | Somatic protein antigen coating @ 2 µg/well of ELISA plate | Whole cell protein antigen coating @ 2 µg/well of ELISA plate | Without Antigen coating well of ELISA plate |
|----------------------------------|-----------------------------------------------------|--------------------------------------------------------|---------------------------------------------------------|--------------------------------------------------------|------------------------------------------|
| Outer membrane protein           | 0.534 ±0.018                                         | 0.341 ±0.011                                           | 0.380 ±0.028                                            | 0.138 ±0.022                                           |
| Outer membrane protein with Incomplete Freund’s Adjuvant (1:1) | 0.712 ±0.012                                         | 0.417 ±0.010                                           | 0.393 ±0.019                                            | 0.112 ±0.010                                           |
| Somatic protein antigen          | 0.408 ±0.010                                         | 0.483 ±0.032                                           | 0.335 ±0.029                                            | 0.121 ±0.013                                           |
| Somatic protein antigen with Incomplete Freund’s Adjuvant (1:1) | 0.490 ±0.009                                         | 0.593 ±0.011                                           | 0.348 ±0.011                                            | 0.119 ±0.011                                           |
| Whole cell protein antigen       | 0.406 ±0.010                                         | 0.338 ±0.015                                           | 0.539 ±0.022                                            | 0.124 ±0.021                                           |
| Whole cell protein antigen with Incomplete Freund’s Adjuvant (1:1) | 0.424 ±0.010                                         | 0.427 ±0.009                                           | 0.722 ±0.019                                            | 0.144 ±0.014                                           |
| Incomplete Freund’s Adjuvant     | 0.351 ±0.007                                         | 0.303 ±0.006                                           | 0.335 ±0.004                                            | 0.119 ±0.005                                           |
| Normal saline solution           | 0.270 ±0.016                                         | 0.259 ±0.021                                           | 0.220 ±0.016                                            | 0.121 ±0.008                                           |
| Control                          | 0.204 ±0.014                                         | 0.183 ±0.026                                           | 0.215 ±0.017                                            | 0.113 ±0.010                                           |

[Data (n=3) are presented as mean±standard deviation. Values with different lower case letter superscripts differ significantly (P<0.05) between the control and different treatment within the same column. Values with different upper case letter superscripts differ significantly (P<0.05) between *in vitro* humoral immunity assessment against homologous and heterologous antigen cross-response within the same vaccinated groups.]

### 3.5. Assessment of specific cellular immune responses:

There were significantly high (P<0.05) cellular immune responses in all the immunized rohu (G₁ to G₆) of R₁ and R₂ groups in contrast to the control (G₀) and Incomplete Freund’s Adjuvant and normal saline solution injected rohu (G₇ and G₈) while assaying *in vitro* proliferation of fish kidney leukocytes stimulated specifically by three different antigenic preparations and also non specifically by Con-A (Table 2). Significantly high (P<0.05) *in vitro* cellular responses in terms of SI values were found in fishes vaccinated with outer membrane protein (0.466±0.013) followed by somatic antigen along with Incomplete Freund’s Adjuvant (0.458±0.016) and outer membrane protein along with Incomplete Freund’s Adjuvant (0.433±0.015), while the homologous antigen was used as a stimulating antigen in fish kidney leucocytes culture in tissue culture plate. In most of the cases, highest *in vitro* cellular response of vaccinated fish kidney leukocytes were found in homologous antigen-stimulated leucocytes as shown in Table 2. Kamilya et al. [15] also found higher *in vitro* antigen-specific responsiveness of catla (*Catla catla* Ham.)

### Table 2: Stimulation index (SI) values after 28 d of immunization

| Different vaccinated rohu group ↓ | Antigens used for assessing *in vitro* cellular response of head kidney leucocytes → | Outer membrane protein @ 40 µg/ well of tissue culture plate | Somatic protein antigen @ 40 µg/ well of tissue culture plate | Whole cell protein antigen @ 40 µg/ well of tissue culture plate | Con-A @ 10 µg/ well of tissue culture plate |
|----------------------------------|---------------------------------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|------------------------------------------|
| Outer membrane protein           | 0.466 ±0.013                                                                          | 0.046 ±0.004                                               | 0.161 ±0.012                                               | 0.338 ±0.009                                               |
| Outer membrane protein with Incomplete Freund’s Adjuvant (1:1) | 0.433 ±0.015                                                                          | 0.091 ±0.003                                               | 0.343 ±0.016                                               | 0.344 ±0.014                                               |
| Somatic protein antigen          | 0.098 ±0.002                                                                          | 0.380 ±0.008                                               | 0.127 ±0.004                                               | 0.332 ±0.008                                               |
| Somatic protein antigen with Incomplete Freund’s Adjuvant (1:1) | 0.061 ±0.004                                                                          | 0.458 ±0.016                                               | 0.126 ±0.005                                               | 0.326 ±0.021                                               |
| Whole cell protein antigen       | 0.216 ±0.004                                                                          | 0.325 ±0.012                                               | 0.383 ±0.013                                               | 0.204 ±0.013                                               |
| Whole cell protein antigen with Incomplete Freund’s Adjuvant (1:1) | 0.133 ±0.005                                                                          | 0.107 ±0.005                                               | 0.348 ±0.012                                               | 0.126 ±0.007                                               |
| Incomplete Freund’s Adjuvant     | 0.066 ±0.004                                                                          | 0.047 ±0.004                                               | 0.088 ±0.004                                               | 0.227 ±0.007                                               |
| Normal saline solution           | 0.054 ±0.006                                                                          | 0.041 ±0.003                                               | 0.061 ±0.003                                               | 0.228 ±0.004                                               |
| Control                          | 0.038 ±0.002                                                                          | 0.037 ±0.003                                               | 0.055 ±0.007                                               | 0.195 ±0.007                                               |
[Data (n=3) are presented as mean±standard error. Values with different lower case letter superscripts differ significantly (P< 0.05) between the control and different treatment within the same column. Values with different upper case letter superscripts differ significantly (P< 0.05) between homologous and heterologous in vitro cellular antigenic cross-response within the same vaccinated groups.]

4. Conclusion
In short, it may be concluded that outer membrane protein antigen of A. hydrophila along with Incomplete Freund’s Adjuvant may be considered best for vaccination strategies among the three said antigenic preparations in rohu because it showed higher significant (P< 0.05) results in both humoral response and cellular responses in compare to control and also gave 81.81% RPS after 7 d of A. hydrophila challenge. Highest humoral immune responses was noticed for rohu immunized with whole cell protein antigen mixed with Incomplete Freund’s Adjuvant and highest cellular immune responses were observed in case of rohu immunized with A. hydrophila outer membrane protein antigen itself. Although, considering the relative percentage of survival, pathological signs during trial period and overall cellular and humoral responses, the outer membrane protein antigen of A. hydrophila along with Incomplete Freund’s Adjuvant was selected most suitable vaccine for rohu (Labeo rohita) against Aeromonas hydrophila infection.

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