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Avijit Biswas
Assistant Professor, School of Fisheries, Centurion University of Technology and Management, Odisha, India

Dr. Gadadhar Dash
Professor, Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Chakgaria, Panchasayar, Kolkata, West Bengal, India

Prasenjit Mali
Assistant Professor, Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Chakgaria, Panchasayar, Kolkata, West Bengal, India

Dr. Siddhartha Narayan Joardar
Professor, Department of Veterinary Microbiology, Faculty of Veterinary and Animal Sciences West Bengal University of Animal and Fishery Sciences, Kshudiram Bose Sarani, Belgachia, Kolkata, West Bengal, India

Dr. T Jawahar Abraham
Professor, Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Chakgaria, Panchasayar, Kolkata, West Bengal, India

Sutanu Karmakar
Assistant Professor, Department of Aquatic Environment Management, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Chakgaria, Panchasayar, Kolkata, West Bengal, India

Debapriyo Mukherjee
Senior Research Fellow, National Surveillance Programme for Aquatic Animal Diseases, Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Chakgaria, Panchasayar, Kolkata, West Bengal, India

Dr. Koel Bhattacharya Sanyal
Senior Research Fellow, National Surveillance Programme for Aquatic Animal Diseases, Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India

Corresponding Author:
Prasenjit Mali
Assistant Professor, Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Chakgaria, Panchasayar, Kolkata, West Bengal, India

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

Avijit Biswas, Dr. Gadadhar Dash, Prasenjit Mali, Dr. Siddhartha Narayan Joardar, Dr. T Jawahar Abraham, Sutanu Karmakar, Debapriyo Mukherjee and Dr. Koel Bhattacharya Sanyal

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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 quadruplicates (R_1 to R_4) with nine tanks (G_1 to G_9). Rohu of G_1 to G_6 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, G_7 and G_8 tanks were injected with Incomplete Freund's Adjuvant (100 µl/fish) and normal saline (100 µl/fish) respectively and G_9 tanks were kept as control. After 28 d, rohu of R_3 and R_4 were subjected to intramuscular *A. hydrophila* challenge (LD_{50}) @ 2.85×10^6 cells/fish for 7 d and RPS (%) was calculated. Specific cellular and humoral immune responses were determined for rohu of R_1 and R_2. 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) [11]. 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 (Jaysankar) [14] 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 distinct pathological situations including swelling of tissues, necrosis, ulceration, tail/fin rot, motile aeromonas septicemia or haemorrhagic septicemia as a primary pathogen (Hu *et al.*) [13], (Rasmussen *et al.*) [22].
During the past decades, a lot of efforts have been given for immunization of fish with the vaccines. It has been already established that the improvement of a suitable vaccine approach have successfully given protection to the teleost against different infectious diseases caused by pathogen (Uribe *et al.*) [32], (Gudding and Muiswinkel) [12], (Bøgwald and Dalmo) [4]. It is well known that fish are equipped with immunological properties and are quite able to raise competent protection against invading pathogens. Particularly a proper knowledge of acquired immune response in fish is urgently needed for the betterment of defensive strategies to combat against fish diseases in the aquaculture sector. Several attempts had been made by different scientist for immunization of rohu against *Aeromonas hydrophila* infection (Shoemaker *et al.*) [29], (Dash *et al.*) [8], (Bharadwaj *et al.*) [3], (Sen *et al.*) [28], (Dash *et al.*) [7], (Dubey *et al.*) [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.*) [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, WBUAFS, 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 Kamilya *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 re-suspended 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 60^0C 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 25-30^0C. 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 28^{th} d, fishes of R_1 and R_2 groups (from all G_1 to G_9 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 -20ºC. 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 milliliter 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 ºC. Specificity between rohu-immunoglobulin and anti-rohu rabbit immunoglobulin and the titre of anti-rohu rabbit immunoglobulin was assessed by an immunodiffusion test (Ouchterlony) [20] with some modifications and counter-current 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 G_1 to G_9 tanks of R_1 and R_2 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 (G_1 to G_6), Incomplete Freund's Adjuvant and normal saline solution injected rohu (G_7 and G_8) and control rohu (G_9) 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 H_2O_2, 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 H_2SO_4).

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 (G_1 to G_9) of R_1 and R_2 groups. First, the head kidney leucocyte of experimental fish was isolated (Kamilya *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 leucocytes (5×10^5 cells/ml) of all rohu (G_1 to G_9) of R_1 and R_2 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% CO_2 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 leucocytes 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-

$$\text{S. I.} = \left(\frac{\text{Mean optical density of sensitized lymphocyte wells with Con} - \text{A or antigen}}{\text{Mean optical density of control lymphocyte wells}}\right) - 1$$

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-Ig 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 2.2×10^9, 2.2×10^8 and 2.2×10^7 cells/fish recorded 100% mortality within 12 hr, 1 d and 4 d of challenge respectively. Mortality started at 5 d of post-challenge in 2.2×10^6 cells/fish. The LD_{50} value of *A. hydrophila* N10P (NCBI accession number KC914628) of 7 d was found 2.85×10^6 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 (G_1 to G_9) of R_1 to R_4 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 R_3 and R_4 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.* [30] 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.

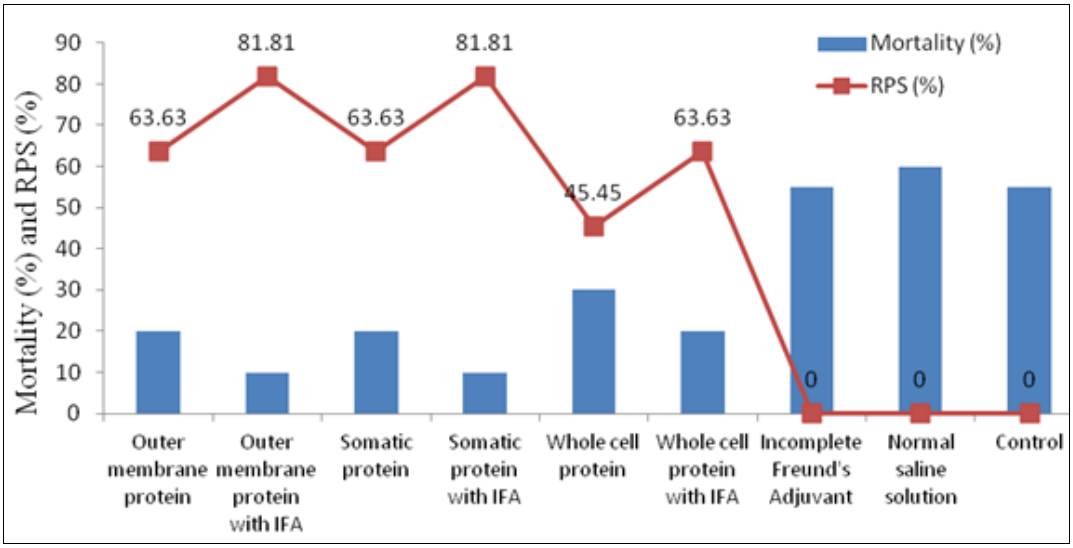

Fig 1: Mortality (%) & RPS (%) after 7 d *A. hydrophila* challenge of vaccinated rohu

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 (± 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 (G_7) also showed higher significant difference ($P< 0.05$) in humoral responses than control (G_9). 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.*) [5], Kamilya *et al.* [15], Saikia and Kamilya [26]. 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.

Table 1: OD values after 28th d post vaccination (R_1 and R_2) as assessed by ELISA

Different vaccinated rohu group ↓ / Antigens coated wells 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	$0.534^{g\ C}$±0.018	$0.341^{c\ B}$±0.011	$0.380^{b\ B}$±0.028	$0.138^{a\ A}$±0.022
Outer membrane protein with Incomplete Freund's Adjuvant (1:1)	$0.712^{f\ C}$±0.012	$0.417^{d\ B}$±0.010	$0.393^{b\ B}$±0.019	$0.112^{a\ A}$±0.010
Somatic protein antigen	$0.408^{d\ BC}$±0.010	$0.483^{e\ C}$±0.032	$0.335^{b\ B}$±0.029	$0.121^{a\ A}$±0.013
Somatic protein antigen with Incomplete Freund's Adjuvant (1:1)	$0.490^{e\ C}$±0.009	$0.593^{f\ D}$±0.011	$0.348^{b\ B}$±0.011	$0.119^{a\ A}$±0.011
Whole cell protein antigen	$0.406^{d\ C}$±0.010	$0.338^{c\ B}$±0.015	$0.539^{c\ D}$±0.022	$0.124^{a\ A}$±0.021
Whole cell protein antigen with Incomplete Freund's Adjuvant (1:1)	$0.424^{d\ B}$±0.010	$0.427^{d\ B}$±0.009	$0.722^{d\ C}$±0.019	$0.144^{a\ A}$±0.014
Incomplete Freund's Adjuvant	$0.351^{c\ C}$±0.007	$0.303^{bc\ B}$±0.006	$0.335^{b\ C}$±0.004	$0.119^{a\ A}$±0.005
Normal saline solution	$0.270^{b\ B}$±0.016	$0.259^{b\ B}$±0.021	$0.220^{a\ B}$±0.016	$0.121^{a\ A}$±0.008
Control	$0.204^{a\ B}$±0.014	$0.183^{a\ B}$±0.026	$0.215^{a\ B}$±0.017	$0.113^{a\ A}$±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 heterologus 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 (G_1 to G_6) of R_1 and R_2 groups in contrast to the control (G_9) and Incomplete Freund's Adjuvant and normal saline solution injected rohu (G_7 and G_8) 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 leukocytes as shown in Table 2. Kamilya *et al.* [15] also found higher *in vitro* antigen-specific responsiveness of catla (*Catla catla* Ham.) 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 1×10^6 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 10th 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 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 leukocytes →	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	$0.466^{g\ D}$±0.013	$0.046^{a\ A}$±0.004	$0.161^{d\ B}$±0.012	$0.338^{c\ C}$±0.009
Outer membrane protein with Incomplete Freund's Adjuvant (1:1)	$0.433^{f\ C}$±0.015	$0.091^{b\ A}$±0.003	$0.343^{e\ B}$±0.016	$0.344^{c\ B}$±0.014
Somatic protein antigen	$0.098^{c\ A}$±0.002	$0.380^{d\ D}$±0.008	$0.127^{c\ B}$±0.004	$0.332^{c\ C}$±0.008
Somatic protein antigen with Incomplete Freund's Adjuvant (1:1)	$0.061^{b\ A}$±0.004	$0.458^{e\ D}$±0.016	$0.126^{c\ B}$±0.005	$0.326^{c\ C}$±0.021
Whole cell protein antigen	$0.216^{e\ A}$±0.004	$0.325^{c\ B}$±0.012	$0.383^{f\ C}$±0.013	$0.204^{b\ A}$±0.013
Whole cell protein antigen with Incomplete Freund's Adjuvant (1:1)	$0.133^{d\ A}$±0.005	$0.107^{b\ A}$±0.005	$0.348^{e\ B}$±0.012	$0.126^{a\ A}$±0.007
Incomplete Freund's Adjuvant	$0.066^{b\ B}$±0.004	$0.047^{ab\ A}$±0.004	$0.088^{b\ C}$±0.004	$0.222^{b\ D}$±0.007
Normal saline solution	$0.054^{ab\ AB}$±0.006	$0.041^{a\ A}$±0.003	$0.061^{ab\ B}$±0.003	$0.228^{b\ C}$±0.004
Control	$0.038^{a\ A}$±0.002	$0.037^{a\ A}$±0.003	$0.055^{a\ A}$±0.007	$0.195^{a\ B}$±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 heterologus *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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6. References

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