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STUDIES ON IMMUNE RESPONSE OF *LABEO ROHITA* VACCINATED WITH FORMALIN KILLED *AEROMONAS HYDROPHILA*

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ABSTRACT

Vaccination studies on Haemorrhagic septicaemia in *Labeo rohita* by injecting formalin killed cells of *Aeromonas hydrophila* and immunogenic response were examined. *A. hydrophila* was harvested from phosphate buffer saline which was incubated on nutrient agar for 24 hrs at 37°C. To the sterile cells, formalin was added at a concentration of 1% to kill the bacteria (*A. hydrophila*) and the cell suspension was adjusted to 1 OD at 450nm which is equivalent to $1x10^8$ cfu/ml. Experimental work was carried out to study various cell-mediated and humoral immune response parameters with two batches of fish 15 in each group (one control and one exposed) for 60 days, booster dose was given on the 14th and 21st day of the experiment. Blood samples were collected through cardiac puncture at 24 hrs after each booster dose on the 15th and 22nd day and also on the 30th, 45th, and at the end of the experiment 60th day. Data recorded on various parameters like cell viability, neutrophil activity, phagocytic assay (cellular immune response), agglutination, myeloperoxidase, lysozyme, and antiprotease enzyme activity (humoral immune response), and statistical analysis was done. Serum protein fractionation on SDS -PAGE was done.

Keywords: *Immune response, formalin killed, SDS-PAGE, Labeo rohita*

INTRODUCTION

Aquaculture continues to be the fastest growing animal food producing sector accounting for about 46% of total food fish supply to meet the protein need of the increasing world population (Food and Agriculture Organization, 2018). The three Indian Major Carp (IMC) species are the important species contributing maximum share i.e., more than 80% of the total aquaculture production in India (CIFA 2004). Disease outbreak can be attributed to several factors such as intense aquacultural practice with high stocking densities, intricate balance between host, pathogen and the environment (Nayak, *et al.,* 1999, Yesmin *et al.,* 2004). In addition, the indiscriminate use of antibiotics has led to the development of drug-resistant bacteria, which are becoming increasingly difficult to control and eradicate (Giri *et al.,* 2013). The most commonly encountered bacterial pathogen in fresh water aquaculture is *A. hydrophila* (Cipriano *et al.,* 1984; Biswajith Maiti *et al.,* 2012). *A. hydrophila* is responsible for diseases such a hemorrhagic septicaemia, dropsy, ulceration, asymptomatic septicaemia and exophthalmos (Karunasagar *et al.,* 1989).

Over the year's disease prophylaxis employing vaccination or immunostimulants are found to be extremely effective and will continue to play a major role in fish disease management, as to avoid pollution associated with chemotherapy and emergence of multidrug resistant bacterial strains. Most of the bacterial infections caused by gram-negative bacteria particularly those belonging to *Vibrio, Aeromonas* and *Yersinia* species were reported to be effectively controlled by vaccination (Gudding *et al.,* 1999). Among these, *Aeromonas hydrophila,* one of the most opportunistic pathogens has been commonly associated to mass mortality and heavy loss to the aquaculture industry. Different forms of infection due to *A. hydrophila* have been recorded in Indian major carps viz. *Labeo rohita, Catla catla*

and *Cirrihinus mrigala*, which are the most important cultured fresh water fishes. Despite the severe economic loss, a limited research has been made to develop a vaccine for *A. hydrophila* (Das *et al.*, 2011). Immunisation procedures like injection, immersion and orally killed and live cells of *Aeromonas hydrophila* produced antibodies in serum, bile, skin and gut mucus and muscle (Loghothetis and Austin 1994). Vaccination of channel catfish with extracellular products of *A. hydrophila* resulted in 100% of relative percent survival when challenged two weeks post vaccination (D. Zhang *et al.*, 2014). Karunasagar *et al.*, 1997 successfully vaccinated Indian Major Carps using both injection and immersion techniques.

Vaccines can be of different kinds, though they generally fall under three categories i.e., dead vaccines, live purified vaccines and purified antigens (Press and Lillehaug 1995; Moral *et al.*, 1998). Dead vaccines are composed of whole cell inactivated pathogens or their extracts like outer membrane proteins (OMP's), extra-cellular proteins (ECP's), lipopolysaccharide (LPS) and biofilms (Santos *et al.*, 1996; Moral *et al.*, 1998; Anbarasu *et al.*, 1998; Fang *et al.*, 2008).

Vaccines have been developed; including live, formalised and heat- inactivated whole cell and sub cellular products, which may be administered by immersion, injection or orally (Loghothetis & Austin 1994). The antigenic diversity of motile aeromondas, many have tried different vaccination strategies using various types of bacterial preparations against *A. hydrophila* with varying success rate (Yin *et al.*, 1996; Newman 1993). Formalin killed *A. hydrophila* cells are inducing cell mediated immune response and humoral immune response and well protect red tilapia (Prasad and Areechon 2010; Bharadwaj *et al.*, 2013). In one of the studies, the PLGA microparticle-encapsulated formalin-killed cell vaccine (PLGA vaccine) protected both cyprinid loach (*Misgurnus anguillicaudatus*) and common carp (*Cyprinus carpio*) from *A. hydrophila* infection (Yun *et al*., 2017). Single booster dose of formalin killed *A. hydrophila* are injected intraperitoneally, is sufficient to elicit immunisation response and offers a high degree of protection in Indian Major Carps (Chandran *et. al*., 2002; Dash *et al.*, 2011). A study is performed to assess the efficacy of the PLGA vaccine in protecting olive flounders from Streptococcus infection by comparing it to the formalin-killed cell (FKC) vaccine (J.W. Jun, *et al*. 2019). Serological data and relative level of protection (RLP) confirm the role of the humoral antibodies in protecting fish against *A. hydrophila* infection. Presence of antibodies in eggs strengthens the possibility of maternal transfer of immunity and supports the results of the agglutination test (Ibrahem *et. al*., 2008).

Administration of formalin-killed whole-cell vaccines are considered to be most favourable strategy to control and prevent bacterial disease outbreak in culture ponds. In contrast, to other vaccine types, these treatments enable the delivery of highly immunogenic and protective antigens with greater convenience and economy. For these reasons, such vaccines are frequently used by many aqua culturists.

MATERIALS AND METHODS

Study area

Kolleru Lake is a wet land ecosystem of international repute and is largest fresh water lake in India and the main water source for most of the culture ponds (Nagabhatla *et. al*., 2009). The study area covered fish ponds situated in and around the Kolleru Lake area.

Sampling of fish

Fish sampling was done during early hours for a period of 4 to 5 days, particularly when there was disease outbreak. Fish showing symptoms of disease were collected and transported to the nearby fisheries laboratory to conduct the clinical and microbial studies. Fish were examined thoroughly for external symptoms like haemorrhages on the body, loss of scales, pigmentation, protrusion of scales and excessive mucus secretion and details were noted. For microbiological studies, affected areas on the fish body surface was first cleaned with a cotton swab dipped in 70% ethyl alcohol and smears from this region were picked up with a sterile loop and aseptically placed in nutrient broth.

Analysis of cellular and humoral immune response vaccinated with formalin killed A. hydrophila

Preparation of inoculums

Bacterial culture of *A. hydrophila* was inoculated on to agar and incubated for 24hrs at37^{o}C. After 24 hrs of incubation, the culture is harvested in PBS centrifuged at 3000 rpm for 15 mins and washed twice; the pallet was resuspended in sterile PBS. Formalin was added at a concentration of 1% to kill the bacteria and the suspension was left overnight at 4^{o}C. The culture was then washed twice with PBS and again resuspended in sterile PBS. The cell suspension was then adjusted to a turbidity of 1 O.D. at 540nm, which is equivalent to 1x10^{8}cfu/ml and then used as inoculum to study the immune response. Sterility of the prepared antigen was checked by inoculating the culture on nutrient agar plates for growth.

Experimental design

The experiment was set up for a period of 60 days. 250-300 fish of same size and weighing 30gms were selected for experimental studies, and collected in batches whenever required from the culture ponds and carried to laboratory with oxygen filled polythene bags. Fish were maintained in well aerated tanks of 200 litres capacity and acclimatized to lab conditions for about 10 days prior to experimental studies. A batch of 30 fish weighing 30 gms each were selected and divided into two groups with 15 fish in each group. First group of fish were treated as experimental and vaccinated with single intraperitoneal injection of formalin killed bacteria mixed with adjuvant FCA at 1:1 ratio. The second group of fish was treated as control and was given 200µl of PBS. A boosted dose was given on 14th and 21st day of the experiment. Normal feed was provided to both control and experimental fish ones daily throughout the experimental period. Blood samples were collected through cardiac puncture using a sterile 24-gauge needle and 2ml syringe on 24hrs after each booster dose on 15th and 22nd day also on 30th, 45th and 60th day. All blood samples were centrifuged immediately after the collection and the serum was stored at -4^{o}C until further use.

Cellular immune response

Cell viability

Viability test was performed to determine the number of viable cells in diseased fish when compared to healthy ones. It was used here to see the influence of inoculation.

NBT

This test was carried out to determine the number of activated neutrophil cells in immunized and control fishes.

Phagocytic ratio

It was performed by enumerating the number of activated macrophages in infected and control fish to know the percentage of cells with engulfed bacteria.

Phagocytic index

Performed to determine the number of engulfed bacteria per cell in infected and control fish

Humoral immune response parameters

Myeloperoxidase activity

Carried out to determine the activity of myeloperoxidase enzyme in the serum of the inoculated and control fish. Myeloperoxidase is an important enzyme with microbiocidal properties and utilises one of the oxidative radicals to produce hypochlorous acid, important in killing the microorganisms.

Lysozyme activity

Test is carried out to determine the lysozyme levels in serum of inoculated and control fish. Lysozyme enzyme plays an important role in host defence mechanisms against infectious diseases by splitting the beta (1-4) linkages between N-acetylmuramic acid and N-acetylglucosamine of bacterial cells thus causing lysis.

Antiprotease activity

Plays a vital role in regulating and inhibiting the activities of potentially destructive proteases. It inhibits the action of proteases either by binding to their active sites or by trapping the protease to prevent protein hydrolysis and therefore, restrict the ability of the bacteria to invade or to grow in fish.

RESULTS AND DISCUSSION

Cellular immune response

Cell viability

Significant increase in the number of viable cells was noticed in the experimental fishes when compared to control fish. The total number of viable cells at 0th day was lowest recording mean value 6.66 +0.52 and on 60th day was 16.28+0.66 (Table 1 and Fig.1).

Table 1: Range, mean and SD of various Cellular immune response parameters of *L. rohita* vaccinated with formalin killed *A. hydrophila* during different post-immunization intervals.

Cell Viability		Control	Vaccinated		PR	Control	Vaccinated
0	Range	5.8-7.4	5.8-7.4		0	52.6-64.7	52.6-64.7
	Mean	6.66	6.66			57.85	57.85
	SD	0.52	0.52			3.70	3.70
14	Range	8.6-12.4	14.4-18.2		14	55.5-59.4	81.1-109.4
	Mean	10.32	15.68			58.04	90.48
	SD	1.28	1.26			1.48	10.68
21	Range	11-13.2	15-17.4		21	55.5-78.1	81.6-108.8
	Mean	12.02	15.7			61.12	91.93
	SD	0.73	0.88			6.79	10.06
30	Range	11-13.2	14.4-16.4		30	54-78.1	81.6-107.5
	Mean	12.22	15.38			63.12	93.54
	SD	0.66	0.57			7.13	9.04
45	Range	11-12.8	14.4-17.4		45	53.8-69.4	82.3-107.5
	Mean	11.96	15.72			62.07	93.03
	SD	0.62	0.87			4.90	8.65
60	Range	11.8-13	15.4-17.2		60	58.3-75	77-108.3
	Mean	12.36	16. 28			63.06	91.04
	SD	0.45	0.66			5.10	9.04
NBT					**PI**		
0	Range	51-66	51-66		0	1.37-1.66	1.37-1.66
	Mean	58.9	58.9			1.50	1.50
	SD	4.88	4.88			0.09	0.09
14	Range	44-64	47-67		14	1.42-1.66	2.3-3.2
	Mean	55.4	57.9			1.51	2.60
	SD	7.12	7.30			0.07	0.21
21	Range	44-67	52-72		21	1.33-1.77	2.3-3.1
	Mean	57.6	64			1.57	2.67
	SD	7.69	7.21			0.13	0.21
30	Range	44-68	52-74		30	1.38-1.8	2.37-3.05
	Mean	57.9	66.1			1.57	2.76
	SD	7.50	7.38			0.11	0.19
45	Range	54-68	52-72		45	1.38-1.67	2.37-3.05
	Mean	60.6	64.3			1.55	2.78
	SD	5.05	7.49			0.09	0.18
60	Range	45-65	47-70		60	1.47-1.68	2.39-3.19
	Mean	57.8	61.9			1.56	2.79
	SD	7.66	8.37			0.06	0.29

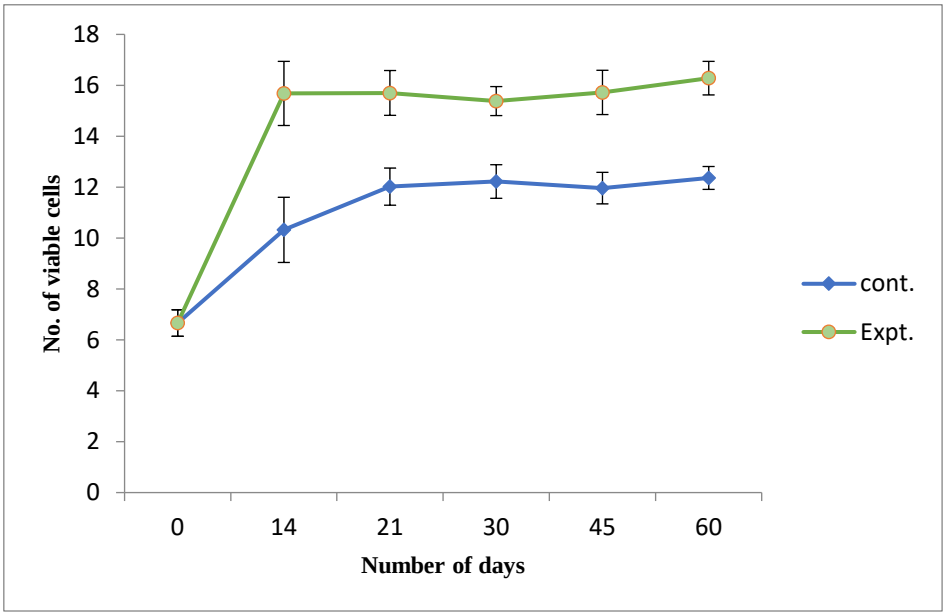


Figure 1: Number of viable cells in vaccinated and unvaccinated *L. rohita*

NBT

A gradual increase of neutrophil cells was observed in vaccinated group on 21st and 30th day followed by a decrease on 45th and 60th day. The results of activated neutrophil cells in control and experimental groups are represented in Table 1 and Fig 2.

Phagocytic ratio

Enhanced phagocytic ration was observed in vaccinated fishes, particularly on 30th and 45th day of the experiment. The phagocytic ratio of *L. rohita* in control and experimental groups was presented in Table 1 and Fig.3.

Phagocytic index

A gradual increase in phagocytic index was noted at all intervals especially on 45th and 60th day of the experiment. The phagocytic index of *L. rohita* in control and experimental groups were presented in Table 1 and Fig 4.

Humoral immune response

Agglutination

Experimental fish showed enhanced antibody response over control fish, during all post exposure days, more specifically on day 21st and 30th day of post immunisation shown the Table 2 and Fig.5.

Myeloperoxidase activity

A considerable increase was noted in the myeloperoxidase activity of vaccinated fish over unvaccinated fish. A significant elevation of enzyme activity was noted on 21st day of post immunisation, followed by a slight reduction in the activity after 30th day of the experiment. The activity of myeloperoxidase in control and vaccinated group are presented in Table 2 and Fig.6.

Lysozyme activity

Not much variation was recorded in the lysozyme activity of fishes belonging to vaccinated and unvaccinated groups. Nevertheless, the peak of enzyme activity remained high at all post exposure intervals with maximum peak on day 14 of post immunisation. The data on lysozyme enzyme activity levels in control and experimental groups is presented in the Table 2 and Fig.7.

Antiprotease activity

The results obtained during the present study indicated increased level of anti-protease activity in vaccinated fishes, which showed gradual increase throughout the experimental period with maximum peak on day 60. The results are shown the Table 2 and Fig.8.

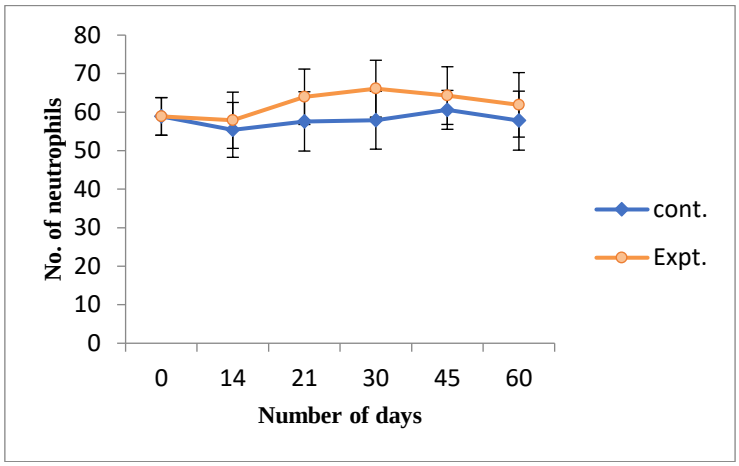


Figure 2: Number of activated neutrophils in vaccinated and unvaccinated *L. rohita*

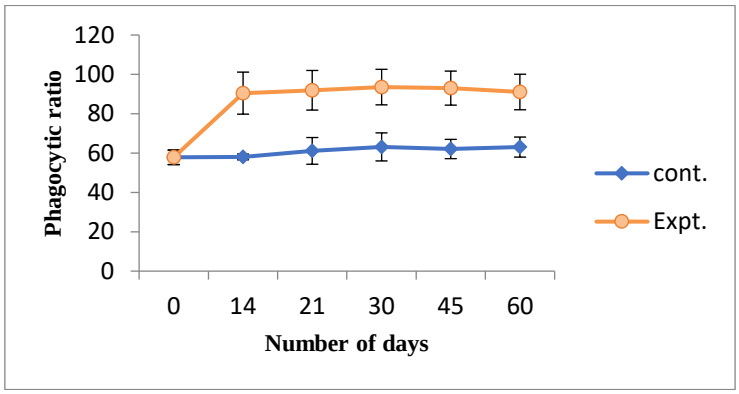


Figure 3: Phagocytic ratio in vaccinated and unvaccinated *L. rohita*

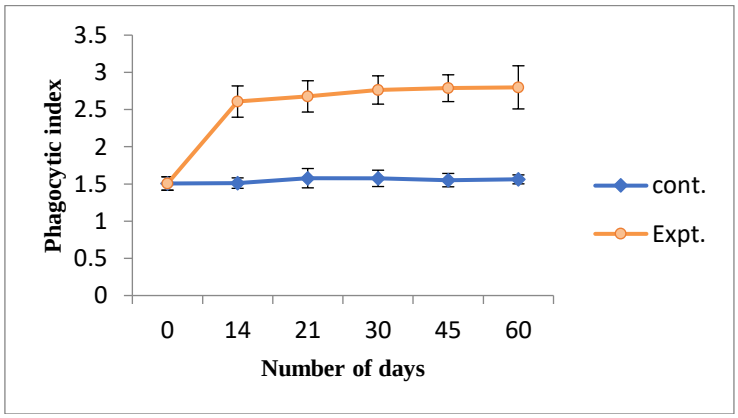


Figure 4: Phagocytic index in vaccinated and unvaccinated *L. rohita*

Table 2: Range, mean and SD of various humoral immune response parameters of *L. rohita* vaccinated with formalin killed *A. hydrophila* during different post-immunisation intervals.

Agglutination		**Control**	**Vaccinated**		**Lysozyme**	**Control**	**Vaccinated**
0	Range	-	-		0	0.54-0.968	0.54-0.968
	Mean	8	8			0.77	0.77
	SD	0	0			0.13	0.13
14	Range	-	-		14	0.885-0.985	0.936-1.001
	Mean	16	256			0.94	0.97
	SD	0	0			0.02	0.02
21	Range	-	-		21	0.891-0.989	0.923-1.006
	Mean	16	512			0.93	0.95
	SD	0	0			0.03	0.02
30	Range	-	-		30	0.946-0.992	0.9-0.999
	Mean	16	512			0.97	0.96
	SD	0	0			0.01	0.02
45	Range	-	-		45	0.962-1.022	0.891-0.983
	Mean	32	256			0.98	0.93
	SD	0	0			0.02	0.02
60	Range	-	-		60	0.935-1.062	0.901-0.981
	Mean	32	256			0.98	0.93
	SD	0	0			0.03	0.02
Myeloperoxidase					**Antiprotease**		
0	Range	0.136-0.159	0.136- 0.159		0	0.557-0.708	0.557-0.708
	Mean	0.14	0.14			0.61	0.61
	SD	0.01	0.01			0.04	0.04
14	Range	0.156-0.196	0.302-0.592		14	0.471-0.52	0.509-0.635
	Mean	0.16	0.36			0.49	0.55
	SD	0.01	0.08			0.01	0.04
21	Range	0.139-0.251	0.378-0.449		21	0.456-0.578	0.46-0.537
	Mean	0.18	0.40			0.49	0.50
	SD	0.03	0.02			0.04	0.02
30	Range	0.101-0.202	0.19-0.279		30	0.469-0.552	0.489-0.549
	Mean	0.15	0.24			0.50	0.51
	SD	0.04	0.03			0.02	0.01
45	Range	0.222-0.282	0.247-0.468		45	0.494-0.57	0.48-0.633
	Mean	0.25	0.35			0.51	0.54
	SD	0.01	0.07			0.02	0.05
60	Range	0.143-0.244	0.243-0.356		60	0.518-0.574	0.553-0.618
	Mean	0.17	0.30			0.54	0.58
	SD	0.03	0.04			0.01	0.02

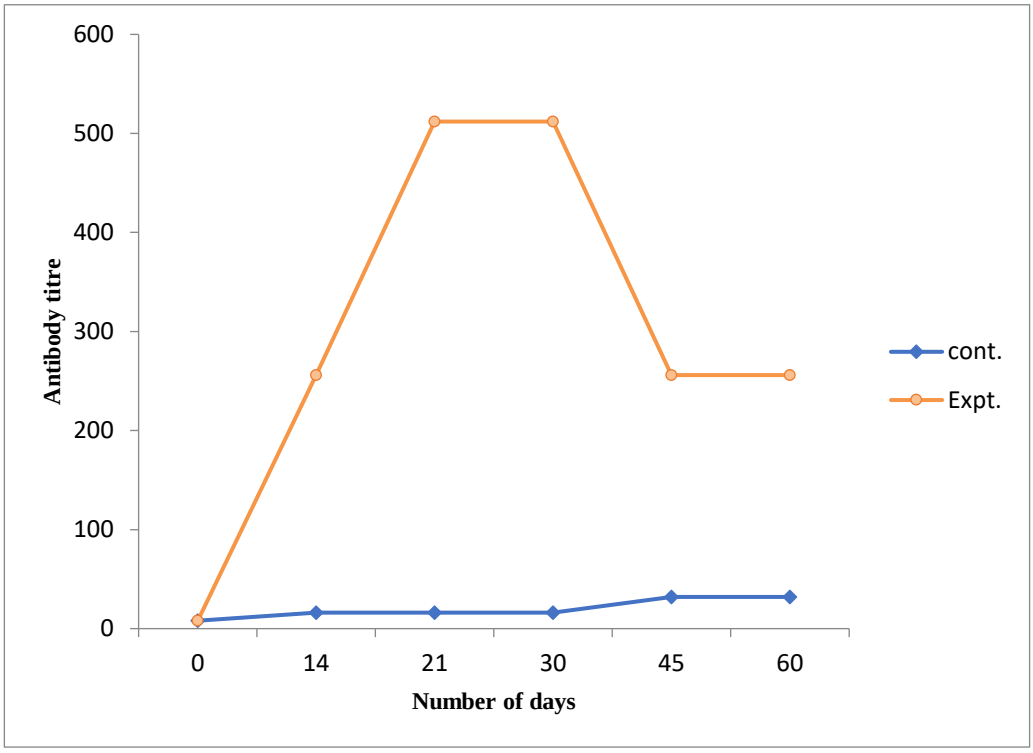


Figure 5: Antibody titres in vaccinated and unvaccinated *L. rohita*

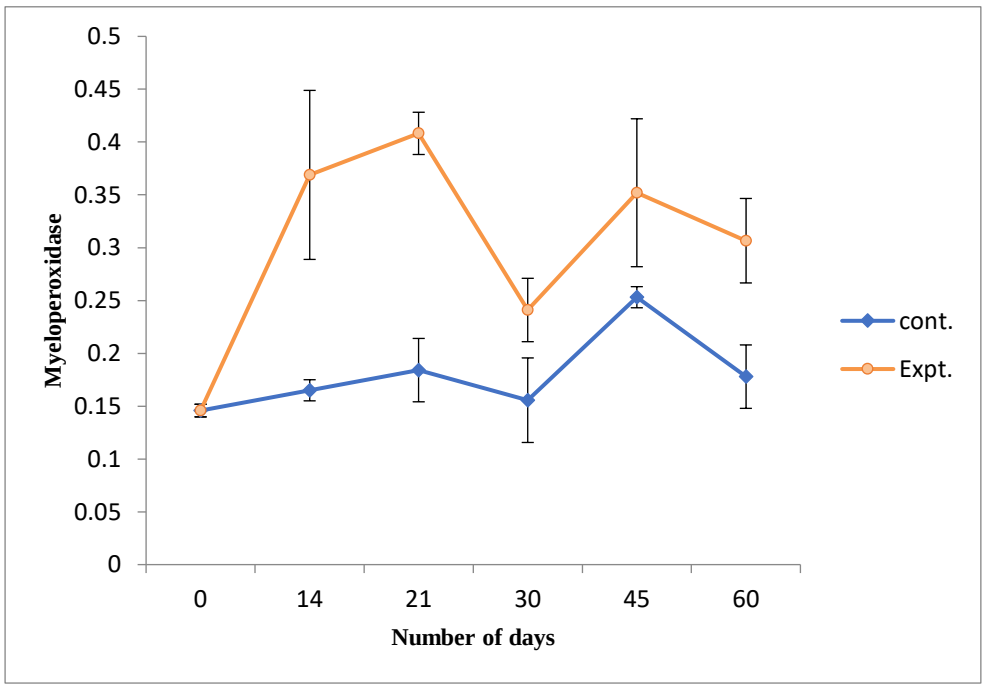


Figure 6: Myeloperoxidase activity in vaccinated and unvaccinated *L. rohita*

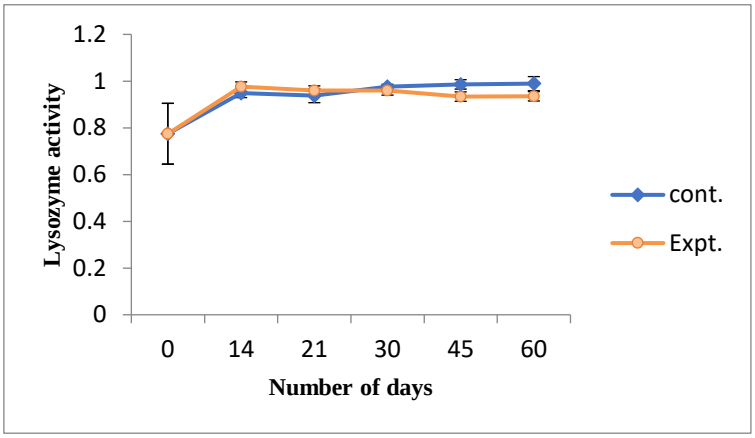


Figure 7: Lysozyme activity in vaccinated and unvaccinated *L. rohita*

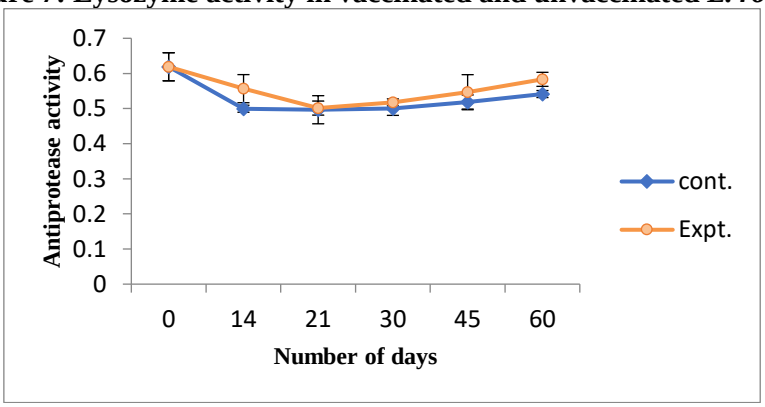


Figure 8: Antiprotease activity in vaccinated and unvaccinated *L. rohita*

Protein profiling (SDS-PAGE)

Serum protein profiling of control and vaccinated fish was carried out by employing SDS-PAGE electrophoresis. The range of the molecular marker used in the present study is from 15-150 KD. The protein bands obtained during the present study can be categorised into two major groups - high molecular weight proteins (150-50 KD) and low molecular weight proteins (50-15 KD). Lane 1 represents 0th day, lane 2-6 were loaded with sera samples collected from control group and lane 8-11 loaded with sera samples collected from vaccinated group.

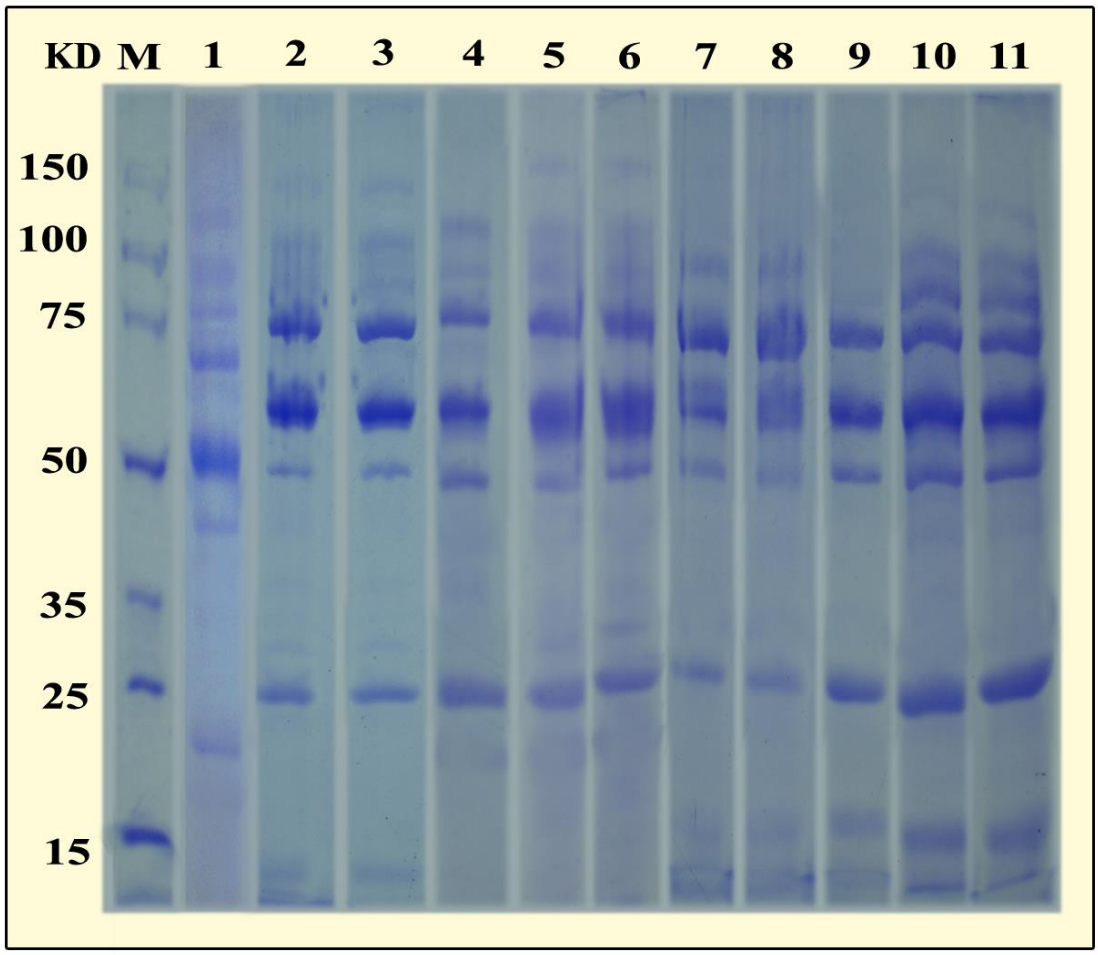
Six proteins bands with apparent molecular weight of 91, 83, 69, 50, 42 and 20 KD were distinguishable in the protein profile of the serum on 0th day. In control fish, four protein bands ranging in their molecular weight from 77-25 KD on 14th day, six bands with their molecular weights ranging from 104-25 KD and 109-25 KD on 21st and 30th day respectively and four protein bands with their molecular weight ranging from 75-25 KD and 75-25 KD on 45th and 60th day respectively were visualized in the gel documentation picture. On the other hand, in the vaccinated fish, five protein bands ranging in their molecular weight from 95-26 KD, 95-25 KD and 75-15 KD were noticed on 14th, 21st and 30th day of the experiment respectively. Seven bands with their molecular weight ranging from 100-14 KD and 100-14 KD were noticed on 45th and 60th day of the experiment respectively. Seven bands with their molecular weight ranging from 100-14 KD and 100-14 KD were noticed on 45th and 60th day of the experiment respectively. It is interesting to note that in both groups, a greater number of HMWP appeared on day 45 and 60 when compared to rest of the days of the experiment. However, very strong and broad bands with molecular weight between 100-25 KD appeared in experimental group on 45th and 60th day. The results depicting protein bands and their molecular weight are present in the Table 3 and Fig.9.

Table 3: Protein profiling of vaccinated and unvaccinated *L. rohita* during different post-immunization intervals.

S.No	Mol. Wt. (KD)	Protein groups	0 day	Control					Expt.				
			Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11
1	150	HMWP	-	-	-	-	-	-	-	-	-	-	-
2	<->150-100		-	-	104	109	-	-	-	-	-	-	-
3	100		-	-	-	-	-	-	-	-	-	100	100
4	<->100-75		91 83	77	91 77	95 81	-	-	95	95	-	87	87
5	75		-	-	-	-	75	75	75	-	75	-	75
6	<->75-50		69	60	57	60	56	56	60	74 57	57	74 54	56
7	50	LMWP	50	50	50	-	50	50	50	50	50	-	50
8	<->50-35		42	-	-	47	-	-	-	-	-	47	-
9	35		-	-	-	-	-	-	-	-	-	-	-
10	<->35-25		-	-	-	-	-	-	26	-	-	-	-
11	25		-	25	25	25	25	25	-	25	25	-	25
12	<->25-15		20	-	-	-	-	-	-	-	-	23	-
13	15		-	-	-	-	-	-	-	-	15	15	15

<-> Between; - No bands.

Figure 9: Serum protein profiling of *L. rohita,* vaccinated with formalin killed *A. hydrophila*



Serum protein profiling of *L. rohita,* vaccinated with formalin killed *A. hydrophila*

1. 0th day
2. Control of 14th day
3. Control of 21st day
4. Control of 30th day
5. Control of 45th day
6. Control of 60th day
7. Experimental of 14th day
8. Experimental of 21st day
9. Experimental of 30th day
10. Experimental of 45th day
11. Experimental of 60th day

In view of the negative impact of excessive usage of antibiotics on the pathogen, fish host and also on the human health, alternative therapeutic methods involving vaccination and herbal products are gaining lot of significance in the recent years from researchers in the field of fishery science. India, which occupies one of the top positions in aquaculture production, the attention paid to develop strategies for better fish health management, are not very effective and are neglected.

During the present study, i.e., vaccination with formalin killed *A. hydrophila* tested of the efficiency in eliciting immune response in the host *L. rohita*. The immune response was assessed by employing a panel of 8 cellular and humoral immune response parameters.

During the last 10-20 years vaccination has become established as an important method for prevention of infectious diseases in farmed fish. Vaccination of fish in aquaculture has been particularly successful against several bacterial diseases (Gudding *et al.,* 1997; Press & Lillehaug, 1995). However, so far there is no universal method for the preparation of bacterial inoculum in vaccination and various methods were adopted by various workers. Moreover, Moral *et. al*., (1998) successfully used live as well as attenuated vaccines against specific bacterial pathogens. Baba *et. al*., (1988) noted that vaccination with crude lipopolysaccharide induced better protection than formalin killed vaccine against *A. hydrophila* in the common carp. Chandran *et al.,* (2002) used two different polyvalent antigen preparations namely whole cell and extracellular products for immunisation against *A. hydrophila* in Indian Major Carps, *C. carpio* and *C. mrigala* in field conditions. He noticed 80 – 90% increase in relative in relative percent survival (RPS) upon challenge with virulent strain of the bacteria. Bacterial infections caused by most of the gram-negative bacteria like *Vibrio anguillarum*, *A. hyrophila* and *Yersinia* species have been effectively controlled by vaccination (Gudding *et al.,* 1999). Commercial vaccines are available against rainbow trout fry syndrome (RTFS) and red mouth disease (FRM) either as single component or combination vaccine. But so far most commercial vaccines have been 'inactivated vaccines' administered by infection or immersion. The overall positive effect of vaccination in farmed fish is reduced mortality.

Karunasagar *et. al*., 1991 conducted vaccination experiments in India Major Carps, *C. catla*, *L. rohita, C. mrigala* against *A. hrdrophila* using homologous and heterologous bacterial preparation. Nayak (1993) conducted a similar study using monovalent and polyvalent preparations of *A. hydrophila* strains in live and attenuated conditions. Azad *et al.,* 1997a used biofilm of *A. hydrophila* for oral vaccination of Indian Major Carps. Chandran *et al.,* 2002 successfully carried out immunisation of IMC's by intraperitoneal injection using two different polyvalent antigen preparations.

In the present study formalin killed whole bacteria along with FCA was used for inoculating fingerlings of *L. rohita*. An increase in the immune response was noticed in fished inoculated with *A. hydrophila* when compared to control fish. Loghothetis & Austin (1994) during their studies on rainbow trout observed high immune response with an increase in antibody titre values in fishes inoculated with formalin killed bacteria. Viola (1995) and Yin *et. al*., (1996) noticed an increase in antibody titre values in fishes inoculated with formalin killed bacteria.

The protective role of formalin killed bacteria in eliciting strong immune response was shown by high antibody titre values in carps immunized either intramuscularly or intraperitoneally with *A. hydrophila* (Karunasagar *et al.,* 1991 and Swain *et al.,* 2007). Shome and Shome (2005) noticed high agglutinating antibody titres and strong phagocytic capability of mononuclear cells in Indian Major Carps vaccinated intraperitoneally with *A. hydrophila*. The observations made during the present study fall in line with studies carried out by others on antibody titre values in recording high titre values.

Pre-treatment with glucan (100 – 1000 micrograms glucan/ fish) had an adjuvant effect on antibody production and resulted in the highest antibody titre against *A. hyrophila* following vaccination (Selvaraj *et. al*., 2005). Moreover, the advantage of using adjuvants along with inoculation (formalin killed or heat killed bacteria) to improve the efficiency of vaccine in eliciting strong immune response, was also stressed in studies carried out by several workers. In the present study Freund's complete adjuvant is used to improve the efficacy of the formalin killed inoculum as vaccine.

The cellular immune response of a fish was generally assessed by phagocytic index and the number of neutrophils activated against the bacteria. Rostami *et. al*., (2007) demonstrated increase in the number of activated neutrophils in immunised fish when compared to unimmunized fish. Dash *et. al*., (2011) noticed significant increase in neutrophil count in all immunized groups of fish when compared to controls. They also noticed peak-level of NBT activity in the first week of post immunisation followed by a decreasing trend during subsequent intervals. The neutrophil activities, as measured through superoxide production and myeloperoxidase levels appear to be important contributors against infections with *A. hydrophila* (Kumari and Sahoo, 2006).

Fishes generally get protected from pathogens due to the lytic activity of various enzymes present in the serum. Dash *et. al*., (2011) during their studies, reported elevated levels of lytic enzymes like lysozyme, antiprotease and myeloperoxidase, particularly during initial period of exposure. In the present study, though an increase in MPO enzyme activity was noticed during initial stages, reported elevated levels of lytic enzymes like lysozyme, antiprotease and myeloperoxidase, particularly during initial period of exposure. In the present study, though an increase in MPO enzyme activity was noticed during initial stages, the other two enzymes i.e., lysozyme and antiprotease showed variations in their activity during different intervals of post-immunization.

The present study and other such studies clearly indicate the fact that, vaccination of fish renders protection to the host against the pathogen, which is evident by an increase in cellular and humoral immune response parameters. But, one of the major constrains in the development of vaccination is as new diseases and pathogens emerge from time to time, it is impossible to develop proactive strategies using vaccines. Moreover, the high antigenic variation of some pathogens like *A. hydrophila* markedly limits the development of vaccines against these microorganisms and to date no commercial vaccines are available to protect farmed fish against *A. hydrophila* infection (Anbarasu *et al.,* 1998).

All the vaccinated fish groups showed higher leucocyte proliferation when primed with antigen. This might be due to polyclonal activation of leucocyte already sensitised with the antigen. Similarly, antigen-specific proliferative responses have also been reported in rohu (Das *et. al*., 2009). Fish vaccinated with antigenic preparations conferred higher protection for at least 15 days post-vaccination. The FAH + A group showed highest degree of antigen-specific leucocyte proliferation, nitric oxide production, superoxide anion (-O2) production, lysozyme activity, and antibody production (Shib Sankar Sen *et. al*., 2014). Tu *et. al*., 2010 in their study, the unvaccinated control group registered 6.7% survival, AHG (*Aeromonas hydrophila* ghosts) vaccinated fish were fully protected with 80% survival while those vaccinated with FKC (formalin killed cells) had only 60% survival.

The results observed in this study are very promising since booster of formalin-killed whole-cell vaccine against *A. hydrophila*, administered through intraperitoneal and immersion routes, could result in more effective protection in pacu against this bacteriosis, by increasing innate and adaptive mucosal and systemic immune responses (Thais Heloisa Vaz Farias *et al.,* 2020). The protective role of formalin killed bacteria in electing immune response was shown by high antibody titre values in carps immunised either intramuscularly or intraperitoneally with *A. hydrophila* (Karunasagar *et al.,* 1991 and Swain *et. al*., 2007).

Vaccinated *L. rohita* showed high immune-effector activities in the first 15 days post-vaccination period and subsequently lowered at different post-challenge and the experiment revealed that the highest protection was generated in *A. hydrophila* vaccinated group compared to the other two vaccinated groups and this might be explained by the specific cellular immune responses (Bharadwaj *et. al*., 2013)

CONCLUSION

The present study showed the enhanced cellular and humoral immune responses in fishes exposed with formalin killed cells of *A. hydrophila* when compared to the one in control group at a dose of $1x10^8$cfu/ml. The cellular immune responses including cell viability, NBT, phagocytic ratio and phagocytic index resulted in increased immune response and humoral immune response showed enhanced myeloperoxidase, agglutination and antiprotease activity and not much variation was observed in lysozyme activity in the present study.

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