Int. J. Adv. Res. Biol. Sci. (2016). 3(1): 134-144

International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
Coden: IJARQG(USA)

Research Article

SOI: http://s-o-i.org/1.15/ijarbs-2016-3-1-19

Serum bactericidal activity as indicator of innate immunity in *Labeo rohita* (Hamilton, 1822) challenged with *Aeromonas hydrophila* as biomarker for clinical monitoring

Subharthi Pal[1], Sriparna Datta Ray[1,2] and Sumit Homechaudhuri[1*]

[1]Aquatic Bioresource Research Laboratory, Department of Zoology, University of Calcutta
35, Ballygunge Circular Road, Kolkata – 700019, West Bengal, India
[2] Department of Zoology, Sreegopal Banerjee College, University of Burdwan, Hooghly,
West Bengal – 712148, India
*Corresponding author: *sumithomechaudhuri@gmail.com*

Abstract

Serum bactericidal activity was studied and analyzed after experimentally challenging Indian major carp *Labeo rohita* (Hamilton,1822), by infecting (i.p.) them with a asymptomatic carrier state dose of *Aeromonas hydrophila* (3×10^7 cfu ml^{-1}). Considerable differences ($p > 0.05$) were observed in the bactericidal activity level of the serum collected at 3 days and 7 days of exposure period respectively. Significant differences were also observed in haematological parameters like total leucocyte count and differential leucocyte percentage in the fishes, post bacterial challenge, compared to control fishes. Also, significant changes in the biochemical parameters such as total serum protein, serum albumin, serum globulin and albumin : globulin ration were noted in the fishes following the infection indicating adequate functioning of the non-specific fish immune system after experimental bacterial challenge. Lastly re-isolation and detection of *A. hydrophila* was carried out by Duplex PCR using primers targeted against 16S rRNA and the aerolysin gene. Previous exposure to *A. hydrophila* infection in fishes from natural habitat was also noticed in this study. These results suggest that these parameters could be screened as multiple potential exposure biomarkers for the development of rapid diagnostic technique of *A. hydrophila* infection as part of comprehensive schemes of fish health, immunity and clinical monitoring system.

Keywords: Serum bactericidal activity; *Labeo rohita*; *Aeromonas hydrophila*; non-specific fish immune system.

Introduction

Aquaculture is considered one of the most rapidly growing food production sectors in the world since the closure of capture fishery and also due to the ever growing demand for food and employment. But at the same time heavy losses corresponding to mass mortalities due to infectious diseases are major obstacles in the growth and feasibility of aquaculture practices throughout the world. The occurrence of diseases in fish farm is due to several factors concerned with the rearing methods, environmental conditions and variations (Kaleeswaran, 2012). The most significant factor affecting aquaculture is the incidence of microbial pathologies, mainly bacterial in origin (Zorrilla et al., 2003). One of the major bacterial diseases is caused by the Gram-negative bacterium *Aeromonas hydrophila*, which produces diseases known as "Motile Aeromonas Septicemia" (MAS), "Hemorrhagic Septicemia", "Ulcer Disease" or "Red-Sore Disease" (Sahoo et al., 2008) affecting a wide variety of freshwater fish species and, occasionally, marine fish (Sahu et al., 2007; Reyes et al., 2010). This bacterium can also behave as a secondary opportunistic pathogen, by assailing already compromised or stressed hosts (Tellez et al., 2010).

Several extracellular toxins and enzymes have been described as responsible for the virulence of *A. hydrophila*, including cytotoxins and enterotoxins (Yadav et al., 1992) and a repertoire of enzymes that digest cellular components, mostly proteases and haemolysins (Leung and Stevenson, 1988). *A. hydrophila* is listed in the Contaminant Candidate List and Environmental Protection Agency has validated its detection and enumeration in drinking water system.

Previous studies indicate that the immune system of teleost fish has mechanisms responsible for the defence against pathogenic bacteria, through humoral and cell-mediated pathways, which act in a multifactorial approach so as to prevent bacterial colonization after invasion within the body. Disease resistance in fish species is correlated with innate immune parameters, which probably affect the inherent capacity of fish to resist pathogens before a specific immune response (Sahoo et al. 2008; Reyes et al., 2008; Rodríguez et al., 2008). Innate mechanisms against bacterial invasion include the production of antibacterial compounds, such as proteins of the complement system activated by alternative pathway, acute phase proteins, cytokines, phagocytosis and inflammation (Ellis, 2001; Garcia et al., 2012). Innate immune response mechanisms against *A. hydrophila* have been studied in several fish species (Sahu et al., 2007; Reyes et al., 2010; Rodríguez et al., 2008). It is evident that immune defence mechanisms play a critical role in preventing bacterial disease in fish (Mohanty et al., 2007). Several authors have documented such changes in innate as well as in adaptive immune parameters in fish exposed to different pathogens (Rodríguez et al., 2008; Raida and Buchmann, 2008, 2009; Mohanty and Sahoo, 2010). However, the immunomodulation mechanism induced by *A. hydrophila* in fish is still poorly understood. Although, the commercial farming of IMCs are ever increasing and earlier works have shown significant correlation exists between the bacterial disease resistance in fish and non-specific immune parameters, the exact mechanism by which these bacteria modulates the host immune response to their advantage, the factors that contribute to the pathogenesis have been poorly understood. As a whole the knowledge of their immune systems are meager and new information can improve reliability of vaccination against bacterial fish pathogens, which stays potentially the most convenient way for mass administration to fish of all size. Based on these findings it can be hypothesized that with proper knowledge of the immune system functioning in response to the artificial inoculation of *A. hydrophila* in fish, it would be possible to propose appropriate prophylaxis to overcome this disease in aquaculture in near future.

The main objectives of the current study were to demonstrate the increase in the amount of various protective proteins in *L. rohita* blood and serum after bacterial infection by biochemical assays and to evaluate the serum bactericidal activity as a tool for analyzing the innate immune system of the fish at asymptomatic level. The total and differential leucocyte count was also observed to further study the immunological reaction in *L. rohita*, challenged with *A. hydrophila* at sub-lethal carrier state. The present work contributes to the understanding of the immune defence mechanisms of fish against *A. hydrophila*, which is important in terms of control and prevention, as it could provide the basis for the development of improved vaccines (Mohanty et al., 2007). Fishes that survive disease outbreaks are recognized as carriers of the disease and may continue to infect the remaining population without exhibiting signs of infection. So early detection at sub-clinical state or at carrier state i.e. where, there is successful establishment of bacterial infection, without any external morphological lesion is very much important. The parameters determined in this current work might also be screened as multiple potential exposure biomarkers for the development of effective and rapid diagnostic technique of *A. hydrophila* infection as part of comprehensive schemes of fish health, immunity and clinical monitoring system.

Materials and Methods

Bacterial Culture

The bacterial strain used in this study, *A. hydrophila* subsp. *hydrophila*, MTCC 646, was collected from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. This strain was received as lyophilized culture and subsequently revived by adding Nutrient Broth and transferring the rehydrated culture to a Nutrient Agar medium. Consequently, streak plate method was followed to get isolated bacterial colonies.

Fish and experimental condition

L. rohita an Indian major carp having average body weight ± 30 gm and body length ± 15 cm were collected from a local fish farm. Fishes were kept in

glass aquarium (2 ft X 1ft X 1ft) in the fish holding facilities within the animal house and acclimatized for 7 days. The fishes were fed with tubificid worms and the water quality was regularly monitored. Water temperature was maintained at 26 ± 2°C. Two-third of the water was renewed every day to avoid accumulation of unutilized food or metabolic waste products.

Artificial inoculation of fishes with *A. hydrophila*

The bacterial strain MTCC 646 was cultured in nutrient broth (NB) and incubated at 37°C for 24 h prior to artificial inoculation of fishes. Bacterial cells were harvested by centrifugation at 5000 x g for 5 min and washed in physiological saline, PS (0.85% NaCl). The strain was enumerated by correlating the OD value taken at 600 nm of the growing culture with the corresponding colony forming units (cfu) obtained by spread plate dilution method (Pal and Pradhan, 1990) (Ref: OD 600nm 1 = 2 X 10^9 cfu ml-1). For this experiment, fishes were injected intraperitoneally (i.p.) with a sublethal dose, 3x10^7cfu ml-1 of *A. hydrophila*, made up in PS (working volume: 0.5 ml 100 gm-1 body weight of fish). The aim was to produce asymptomatic carrier state. Fishes were divided in 2 sets i.e SHAM operated control set and *A. hydrophila* treated set. The potential fish biomarkers chosen for the study were recorded on the 3rd and 7th day of exposure and compared with corresponding sham-injected control groups (Six replicates, each containing 10 fish injected with sterile PS only).

Blood and serum collection

On the sampling days, fishes were anaesthetized by dipping for 30 sec in 0.1 ppm of MS 222 suspension in water. After anaesthetizing fish, the blood samples were collected from the caudal vein into plastic Eppendorf tubes with a 2 ml pre-heparinised syringe and 24 gauge needle from six fish of each sample group. For serum, blood samples were withdrawn from caudal veins in the remaining anaesthetized fish into blood collecting tubes or Eppendorf tubes without anticoagulant in the syringe. Blood samples in Eppendorf tubes were allowed to clot for 2 h at room temperature in a slanting position. The clot was then cut with a glass rod and care was taken not to haemolyse the clot. The tubes were kept at 4 °C for an hour and were then centrifuged at 3000 × g for 10 min and the supernatant serum was collected. The serum was stored at -20 °C in Eppendorf tubes till use within two days (Pal et al., 2015). During sampling the fish were handled very carefully and aseptically to avoid mortality of fish during handling.

Haemato-biochemical studies

Whole blood examination included total leukocyte count (WBC) and leukocytes differential count (dWBC%). Biochemical studies including total serum protein, albumin, globulin, albumin: globulin ratio, were carried out with the serum isolated form fish blood. Total Leucocyte count (TLC) was done by Neubauer's improved double haemocytometer using Hayem solution as diluting fluid. The relative abundance of different leucocytes was determined by counting a total of 200 blood cells after staining the blood smear with Leishman's stain. All the haematological parameters were determined following techniques of Dacie and Lewis (1984). Total serum protein (gm %) and Serum Albumin (gm %) was measured from serum spectrophotometrically following Biuret method and Bromocresol green method respectively. Serum Globulin (gm %) was calculated by simply subtracting Serum albumin from Total protein. Albumin: Globulin ratio (A: G ratio) was also calculated later. Diagnostic Kits obtained from Nice (Nice Chemicals Pvt. Ltd., Cochin) were used for the determination of all indices. Colorimetric measurement was done in UV-VIS Spectrophotometer (UV 1700 Pharmaspec, Shimadzu).

Serum bactericidal activity

Serum was collected from both control and *A. hydrophila* treated fish after 3 and 7 days respectively. 200 µl of bacterial suspension (3.92 x10^9 cfu/ ml) was mixed with 5 ml soft agar (0.8 % agar) and was poured onto nutrient agar plates (soft agar over-lay method). After 3 minutes, 4 drops of serum (50 µl vol.) were added onto the soft agar overlay at 4 different quadrants of the plate. After solidification, the plates were incubated overnight at 37ºC and the zones of inhibition around each drop were observed the following day.

On the other hand, serum was applied to standardize the methodology of bactericidal activity, adapted following Kajita et al., (1990); Rao et al., (2006) and Aly et al., (2008) and the challenge was promoted in order to increase antibacterial proteins. Serum (500 µl) from both control and *A. hydrophila* treated fish after 3 and 7 days of exposure period was added in 1:1 proportion to bacterial suspension (3.92 x10^9 cfu/ml) and incubated at shaking condition for 1 h at 37ºC. Blank control was also prepared by replacing serum

with sterile PBS. The absorbance (O.D.) of the bacterial suspension with PBS and serum from control and treated fish were observed separately at 600 nm using a spectrophotometer and the colony forming unit (CFU) was calculated following Pal and Pradhan, 1990 (Ref: $OD_{600\ nm}$ 1 = 2 X 10^9 cfu ml^{-1}). The fidelity of this method was cross checked by the procedure followed by Das et. al.,(2009).

Re-isolation and detection of pathogen

Presumptive diagnosis of *A. hydrophila* was done by selective Rimler Shotts (R-S) media supplemented with Novobiocin and isolation of Genomic DNA from bacteria was done. Two pairs of primers *viz.* primer for conserved regions 16S rRNA (Graf, 1999) and aerolysin gene (Santos et. al., 1999) were used in the duplex PCR. After gel electrophoresis the PCR products were observed under an UV transilluminator and the results obtained were analyzed by Gel Doc, Bio-Rad Quality One Software, Version 4.6.5.

Statistical analysis

Means and Standard Error (S.E.) of the means were calculated from whole range data following Zar (1999). Student's t-test was done to distinguish between significant differences using Treatments were taken to be differing significantly where ($p < 0.01$). One way Univariate Analysis of variance (ANOVA) at 5% level of significance was used and Duncan's Post Hoc test was also done to identify the homogenous means, if any using SPSS Statistics 17.0.

Results and Discussion

The Total Leucocyte Count (TLC) and differential WBC % (*viz.* large lymphocytes, neutrophils, monocytes and eosinophils) in the *A. hydrophila* treated fishes were found to be significantly higher as compared with the control fishes ($p < 0.01$) (Table 1 and Table 2) after 3 and 7 days of exposure. When infectious disease agents such as bacteria enter the fish body the non-specific (cellular) defense system gets stimulated during the first stage of disease manifestation. In this situation, the leucocytes get increased (leucocytosis) initially in order to protect the fish body by phagocytosis and produce antibacterial chemicals to stop the agent from spreading. The significant increase ($p < 0.01$) in the total WBC count and the number of different leucocytes (dWBC%) observed in this study signifies the fact that the innate immunity of the fish was stimulated to fight against the bacterial pathogen as the primary line of defense and these cells would further contribute to higher level of non-specific immunity. It was also observed that the increase in the TLC value was significantly higher after 3 days of exposure and it got reduced after 7 days indicating the fact that immune system functioning is gradually lowered after initial confrontation with bacterial infection in due course of time as the normal condition is re-established.

TABLE 1: Total leucocyte count (TLC) in *A. hydrophila* treated *L. rohita* and SHAM operated control samples after 3 days and 7 days of exposure.

Parameter	3 days exposure		*t* – values (n = 12)	7 days exposure		*t* – values (n = 8)
	Control (Mean ± SE)	Treated (Mean ± SE)		Control (Mean ± SE)	Treated (Mean ± SE)	
Total leucocyte count (10^3 ml^{-1})	5.717 ± 0.513	16.033 ± 1.612	5.951 *	5.819 ± 0.483	9.628 ± 0.518	4.479 *

* *Significant (**p < 0.01**)*

TABLE 2: Differential leucocyte count in *A. hydrophila* treated *L. rohita* and SHAM operated control samples after 7 days of exposure.

Differential leucocyte count %	SHAM operated Control (Mean ± SE)	*A. hydrophila* Treated (Mean ± SE)	*t* – values (n = 12)
Neutrophil (%)	26.76 ± 1.4	34.97 ± 1.09	14.63 *
Eosinophil (%)	0.44 ± 0.1	4.56 ± 0.7	18.42 *
Basophil (%)	1.19 ± 0.3	3.26 ± 0.7	8.59 *
Large lymphocyte (%)	14.84 ± 3.2	25.15 ± 1.08	9.65 *
Small lymphocyte (%)	56.52 ± 4.2	26.31 ± 1.9	20.72 *
Monocyte (%)	0.25 ± 0.17	5.7 ± 1.8	9.62 *

* *Significant (**p < 0.01**)*

The level of total protein is considered to be major indices of the health status of teleosts. Total serum protein, albumin and globulin values were significantly ($p < 0.01$) higher in the *A. hydrophila* infected fishes compared to control fishes (Table 3). Increased level of serum total protein could also be an indication of antibody production in moribund fish with infectious diseases (Rehulka and Minarik, 2005). As the serum proteins include various humoral elements of the non-specific immune system, high concentrations of total serum protein, albumin and globulin might be due to the functioning of non-specific immune response of fishes against the bacterial infection.

Proteins activated by alternative complement pathways are considered the most effective antibacterial compounds due to their lytic activity, pro-inflammatory chemotaxis and opsonizing action that influence the defense cell response. Regarding phagocytes, neutrophils and macrophages are very important because of their large quantities of lysosomal enzymes and the production of reactive oxygen species responsible for the destruction of invading bacteria (Ellis, 1999; Biller et al., 2012). Bacteria growth inhibition factors are essential to prevent tissue damage. Among these factors, the transferrin, a soluble blood protein with high iron affinity is present in high concentration mainly in the acute inflammation phase so as to activate macrophages and decrease the available iron, an essential ion for some bacteria in infection establishment. In addition, antiproteases, such as lectins, are blood proteins which act on the proteolytic compound of bacteria responsible for the lysis of fish tissue. Lectins generate pathogen agglutination due to its high affinity to certain carbohydrates on the bacteria wall (Arason, 1996; Bayne and Gerwick, 2001; Stafford and Belosevic, 2003; Salinas et al., 2011).

Lysines, found in blood and body tissue, mainly where there are leukocytes (especially monocytes and neutrophils) are antibacterial peptides that attack pathogen membranes. Among lysines, lysozyme lyses peptidoglycan components of the bacterial walls; C-reactive protein binds to phosphorylcholine of the bacterial walls and may increase its concentration after a heat shock, inflammatory agents and during warm periods of the year besides promoting the activation of complement and phagocytosis (Ellis, 1999, 2001; Magnadottir, 2011). *Aeromonas* induced ulcerous dermatitis in rainbow trout, *Oncorhynchus mykiss* (Rehulka, 1998) and *Aeromonas* infected red tilapia (Mastoi et. al., 2011) resulted in an increase in total protein in the plasma which were in accordance with the present study.

TABLE 3: Serum biochemical parameters in *A. hydrophila* treated *L. rohita* and SHAM operated control samples after 3 days and 7 days of exposure.

Parameter	3 days exposure		*t* – values (n = 12)	7 days exposure		*t* – values (n = 12)
	Control (Mean ± SEM)	Treated (Mean ± SEM)		Control (Mean ± SEM)	Treated (Mean ± SEM)	
Serum total protein (gm %)	0.6040 ± 0.005	1.2906 ± 0.003	395.616 *	0.6526 ± 0.0025	0.8489 ± 0.0027	840.838 *
Serum Albumin (gm %)	0.3873 ± 0.0046	0.6923 ± 0.0086	76.233 *	0.4208 ± .0068	0.5367 ± 0.0081	88.828 *
Serum Globulin (gm %)	0.2167 ± 0.0003	0.5984 ± 0.0053	66.681 *	0.2418 ± .0043	0.3122 ± .0054	64.992 *
A:G Ratio	1.7870 ± 0.0184	1.1606 ± 0.0248	97.488 *	1.7881 ± .0523	1.7352 ± .0561	13.827 *

* *Significant (**p < 0.01**)*

The serum biochemical parameters such as total serum protein, albumin, globulin and A:G index were determined to prove the increased protective protein production after bacterial challenge. Among total serum proteins, globulins correspond to proteins present in blood responsible for the organism's defence, such as immunoglobulins, proteins of the complement activated by alternative pathways, acute phase proteins, cytokines, lysozyme, transferrin and lectins. In the present study the increase in total serum

protein and globulin indicate the raise in protective proteins after the challenge and can be correlated to the serum bactericidal activity (Arason, 1996; Ellis 1999, 2001; Magnadottir, 2006; Maqsood et al., 2009).

There was no mortality registered in fish post *A. hydrophila* challenge. After 3 and 7 days, serum was analyzed for bactericidal activity, and therefore protective proteins were satisfactorily evaluated by counting CFU survivors. The results were expressed in two ways: the first one shown in Figure 1, expressed in CFU values, and the second one in Figure 2 expressed in serum bactericidal activity [1-cfu/positive (%)]. In Figure 1, the positive group (serum free) displayed the highest values, indicated by the largest bacterial growth, this result was expected once there was no serum to destroy bacteria, however, in control and treated groups, the bacterial growth was reduced because of bactericidal ability in serum. Duncan's Post Hoc test for homogeneity revealed the presence of 5 subsets in bacterial colony count (CFU ml^{-1}) after adding serum. Bacterial colony count after adding serum from control fishes after 3 days (Group 2) and 7 days exposure (Group 4), serum form treated fish after 3 days exposure (Group 3) and 7 days exposure (Group 5) and without adding serum (Group 1) belonged to single subsets each, indicating there is significant difference between them when compared pair-wise. The reduction was much higher in the serum collected from treated fish after 3 days exposure period in comparison to fish after 7 days which was in accordance with the earlier findings.

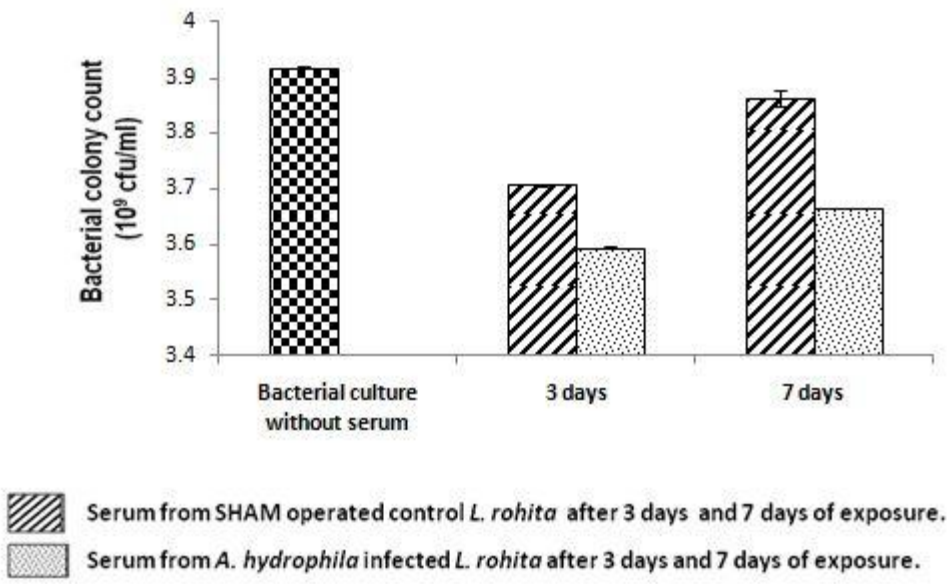

Figure 1: Bacterial colony count (CFU ml^{-1}) (Mean ± SE) after adding serum from *A. hydrophila* challenged *L. rohita* and from SHAM operated control samples after 3 days and 7 days of exposure by spectrophototometric analysis. (Ref: $OD_{600\ nm}$ 1 = 2 X 10^9 cfu ml^{-1}).

These results are in accordance with various studies i.e. evaluation of the effects of vitamin A on serum anti-bacterial activity in the juvenile Japanese flounder, *Paralichthys olivaceus (*Hernandez et al., 2007); assessment of the serum bactericidal activity for determining the effects of *Bacillus subtilis* and *Lactobacillus acidophilus* as potential probiotics on Nile tilapia, *Oreochromis niloticus* (Aly et al., 2008) and observation of the effect of *Achyranthes aspera* on the immunity of *Labeo rohita* infected with *A. hydrophila* through this immune parameter (Rao et al., 2006). The CFU counting values in each study may be different due to their respective modifications and bacteria concentration applied in the challenge trial.

On the other hand, the result in Figure 2 showed the serum bactericidal activity of *L. rohita* after *A. hydrophila* challenge. In that, the positive group (serum free) displayed zero activity once there was no serum, but in the control and treated groups, the bacterial activity increased significantly ($p < 0.05$), indicated by the higher serum bactericidal activity profile. Duncan's Post Hoc test for homogeneity revealed the presence of 5 subsets in bactericidal activity after adding serum. Bacterial colony count after adding serum from control fishes after 3 days (Group 2) and 7 days exposure (Group 3), serum form treated fish after 3 days exposure (Group 4) and 7 days exposure (Group 5) and without adding serum (Group 1) belonged to single subsets each, indicating there is

significant difference between them when compared pair-wise. The bactericidal activity was higher in treated fish after 7 days in comparison to fish after 3 days which indicates higher serum bactericidal activity profile just after bacterial infection and it gets diminished as an when the immune system successfully fight against the infection in due time.

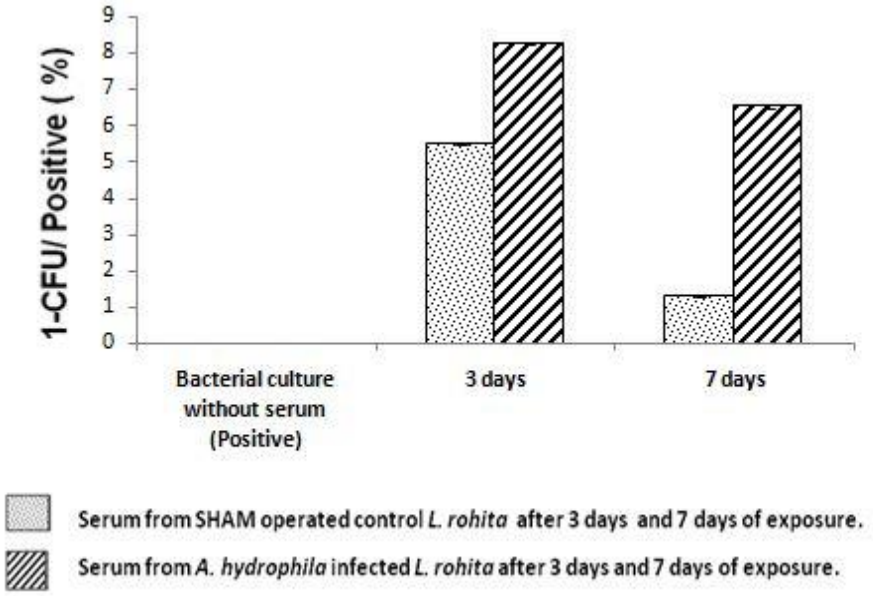

Figure 2: Serum bactericidal activity in *L. rohita* (1 - cfu/positive %) (Mean ± SE) after *A. hydrophila* challenge and in SHAM operated control samples after 3 days and 7 days of exposure.

The results presented in percentage of activity is commonly used, as in Misra et al., (2006) who assessed the bactericidal activity to observe the effect of β-glucan in *Labeo rohita* fingerlings and in Misra et al., (2009) who also applied the assay to evaluate the bactericidal activity after a long-term administration of levamisole to the same fish. Das et al., (2009) used the same protocol in a study to examine the effect of *Euglena viridis* on the immune response of *L. rohita*.

Serum bactericidal activity was also studied by adding serum drops from control and *A. hydrophila* challenged fish onto soft agar overlay mixed with *A. hydrophila* (3.92 x10^9 cfu/ ml) after incubating at 37^0C overnight and by observing the zones of inhibition (Figure 3) around each drops (one drop per quadrant). Zones created by serum from treated fish (Figure 3c and 3d) appear significantly larger than the zones created by serum from control fish (Figure 3a and 3b). Moreover, the area of the zones decreased in the 7 days (Figure 3b and 3d) in comparison to 3 days exposure set (Figure 3a and 3c) both in control and treated fish indicating lowering of serum bactericidal activity which was noticed earlier. The increase of this parameter in the present study is a result of pathogen detection by innate system, which has prompted acute phase proteins so as to protect fish against invading microorganisms. that usually occurs after a natural infection, such as in disease outbreaks, or in artificial infections, such as after vaccination and challenge. These proteins are known as inflammation acute phase proteins. Increased acute phase proteins can also occur immediately after infection or injury, involving changes in hepatic, neuro-endocrine and immune systems. The presence of injury (damage in cell membrane, with tissue factors and arachidonic acid metabolites release) or pathogen affect the release of inflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), that are proteins synthesized by the liver, encephalon or immune cells which act in tissue reparation, homeostasis maintenance and innate and acquired system modulation (Bayne and Gerwick, 2001; Magnadottir et al., 2011).

Another intriguing fact was that a specific amount of serum bactericidal activity was present in the serum of control fishes in all the three different experiments which suggests that they had previous exposure to *A. hydrophila* in their respective natural habitats. This further validates the need for identifying bio-markers of *A. hydrophila* infection, particularly at sub-clinical state, both in natural habitats and also in aquacultural set-ups.

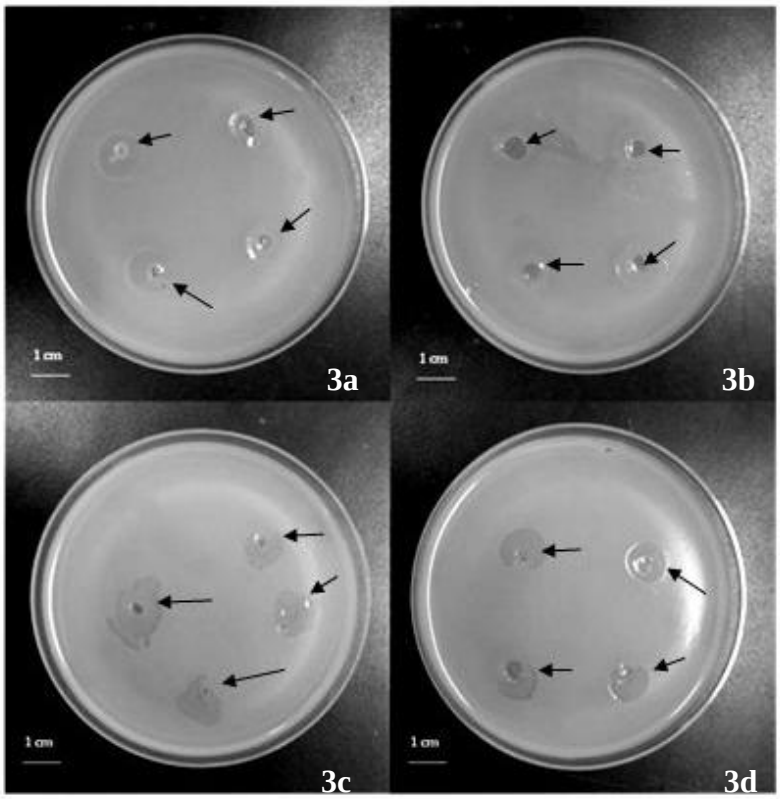

Figure 3: Observation of zones of bacterial inhibition after adding serum drops in soft agar overlay containing bacterial culture (3.92 x10^9 cfu/ ml) after 24 h incubation at 37^0C.
Fig 3a and **3b**: Serum from SHAM operated control samples after 3 days and 7 days of exposure respectively.
Fig 3c and **3d**: Serum from *A. hydrophila* challenged *L. rohita* after 3 days and 7 days of exposure respectively.

Two bands have been obtained at length 599 bp and 252 bp respectively when Duplex PCR was done with DNA isolated from presumptive *A. hydrophila* colonies re-isolated from liver of *A. hydrophila* challenged *L. rohita*. Electrophoretic analysis of the PCR product revealed the specific amplifications of the above mentioned fragments without any spurious product for both the primers targeted against 16S rRNA and the aerolysin gene (Figure 4). These findings are in accordance with the previous results obtained (Jee, 2005). This ensured the fact that changes observed in haematological, biochemical parameters and also in the serum bactericidal activity of *L. rohita* in this current study was due to successful establishment of *A. hydrophila* infection at asymptomatic and sub-clinical level.

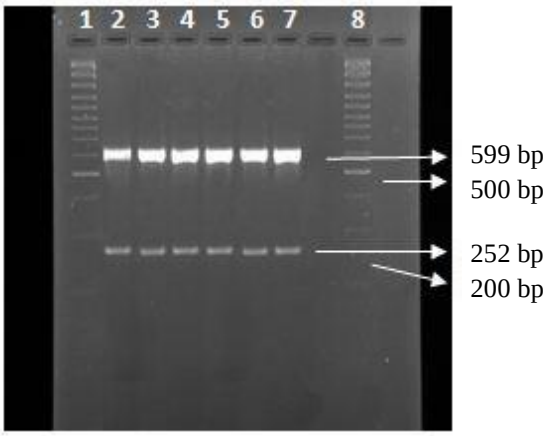

Figure 4: Re-isolation and detection of pathogen by Duplex PCR. *Aeromonas hydrophila* (MTCC 646) showing product obtained from primer for 16S rRNA (599 bp) and primer for aerolysin (252 bp) from bacteria isolated from fishes.

Conclusion

Summarizing the present findings, it can be said that serum bactericidal activity is an important parameter in immunology studies due to its ability to evaluate innate defense systems since the immune system responses may be influenced or manipulated by several substances or processes and it could be analyzed as an indicator of innate immunity in *Labeo rohita* challenged with *Aeromonas hydrophila* as biomarker for clinical monitoring in both aquacultural conditions and natural habitats. Furthermore, hemato-biochemical parameters could be used along with it for development of rapid diagnostic technique in the form of fish biomarkers with regard to diagnosis of *Aeromonas* infection at a sub clinical and/or asymptomatic stage. In conclusion it could be assumed that this kind of multi-parametric bio-marker approach can be highly convenient as rapid diagnostic technique with regard to diagnosis of *Aeromonas* infection at a sub clinical stage or even in an immuno-compromised stage, without obvious symptomatic manifestation of the disease. This approach once validated and standardized for a particular aquatic system, could be a useful management tool because it can lead to frequent cost-effective early detection which would have its importance both in aquaculture/economic sector and public health sector eventually leading to economic growth.

Acknowledgments

The authors are thankful to the Head of the Department of Zoology, University of Calcutta, India for the facilities provided. Financial grant from the University Grant Commission – National Eligibility Test (NET JRF) project is gratefully acknowledged. Technical assistance from Anupam Podder and Dola Roy is also highly acknowledged.

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How to cite this article:

Subharthi Pal, Sriparna Datta Ray and Sumit Homechaudhuri. (2016). Serum bactericidal activity as indicator of innate immunity in *Labeo rohita* (Hamilton, 1822) challenged with *Aeromonas hydrophila* as biomarker for clinical monitoring . Int. J. Adv. Res. Biol. Sci. 3(1): 134-144.