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An experimental analysis of certain potential fish biomarkers following concomitant infection of indigenous and exotic aero monads in *Labeo rohita*

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Abstract
The following is an experimental approach attempting to delineate the possible effects of interaction between two pathogenic Aeromonads when co-infected within a fish model. The host fish *Labeo rohita* was challenged by a native (*Aeromonas hydrophila*) and an exotic (*Aeromonas salmonicida*) pathogenic Aeromonad individually and simultaneously at respective asymptomatic carrier doses (3×10^7 cfu/ml and 2×10^7 cfu/ml respectively). When inoculated individually no symptom was exhibited but during co-infection prominent symptoms of *A. salmonicida* infection (furunculosis) were observed. The haematological, immunological and biochemical parameters along with other general health indices exhibited highest degree of physiological deterioration in the co-infected condition (p & lt; 0.05, df = 3). The observed trend of sensitivity was of the following order: co-infected > *A. hydrophila* infected > *A. salmonicida* infected > control fishes. The results in general indicated the virulence pattern of invasive pathogens, newly adapted to tropical environments and the findings would further help us to decipher the competitive interaction among different bacterial strains leading to co-existence ultimately inducing pathogenicity.

Keywords: Certain potential fish biomarkers, concomitant, indigenous, exotic aero monads, *Labeo rohita*

Introduction
Aeromonads are environmentally transmitted bacterial pathogens causing pathogenicity to a wide range of warm and cold water fishes [1, 2] which may lead to huge mortality eventually causing economic loss [3]. The native ubiquitous bacteria *A. hydrophila* cause haemorrhagic septicemia or Motile Aeromonad Septicemia (MAS) in several culturable species whereas *A. salmonicida*, the invasive Aeromonad in Indian water bodies, cause symptomatic or carrier state liquefactive muscle lesions (furuncles) in Salmonid and Nonsalmonids throughout the world [4]. These bacteria are opportunistic in nature and are likely to cause both single and coinfections within their host organisms leading to changes in virulence depending on the coinfecting bacterial strains [5]. It is important to understand the interaction between various opportunistic pathogens within the host organism, which are often able to persist in and transmit from the environment **[6].** It affects both pathogen transmission and pathogen virulence which in turn influence disease dynamics and pathogenic evolution [7]. During episodes of co-infection, interactions between the infectious agents yield to varied outcomes: the load of one or both pathogens may be increased, one or both may be suppressed or one may be increased and the other suppressed [6]. Generally there are three types of competition that coinfecting pathogens may encounter viz. resource competition, interference competition and apparent competition [8]. Closely related pathogens are likely to cooperate and exploit their hosts economically in order to maximize their transmission, while distantly related pathogens are more likely to compete, leading to increased virulence and decreased transmission due to facilitated host death [9]. Although recent studies have demonstrated that a single host is often infected by a multitude of pathogenic strains or species but empirical investigations of the effects of co-infection on disease dynamics and virulence are still limited, and their importance for many diseases is still unknown. Some studies have addressed the pathogenic effects of coinfection but the consequences of coinfection by native and exotic pathogenic Aeromonads in Indian Major Carps is yet to be explored.

The current study aims to investigate the infection occurring between exotic and indigenous pathogenic Aeromonads during mixed infection in *Labeo rohita* (Rohu) at an asymptomatic level with respect to several haematological, biochemical and immunological parameters along with general health indices.

Materials and Methods

Bacterial Culture

The bacterial strains used in this study were *Aeromonas hydrophila subsp. hydrophila* (MTCC 646) and *Aeromonas salmonicida subsp. Salmonicida* (ATCC 33658) collected from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and American Type Culture Collection (ATCC), USA respectively. These strains were received as lyophilized culture and subsequently revived by adding Nutrient Broth (at 37 ºC for 24 h) in *A. hydrophila* and Tryptone soy broth (at 22 °C for 48 hours) for *A. salmonicida*. Consequently, streak plate method was followed to get isolated bacterial colonies on selective Rimler Shott's (RS) media supplemented with Novobiocin and Coomasie Brilliant Blue Agar (CBB Agar) for *A. hydrophila* and *A. salmonicida* respectively.

Experimental Design

Labeo rohita fingerlings (body weight 30 ± 5 gm and body length 15 ± 3 cm) were collected from a local fish farm. In the laboratory, fishes were kept in glass aquaria (2 ft x 1ft x 1ft) and acclimatized for 7 days. The water supply was maintained in flow through circulatory system attached with an iron filter. Water temperature was maintained at 25 ± 2 °C and continuously aerated by an air compressor. The fishes were fed with tubificid worms and water quality was regularly monitored. Dissolved oxygen (DO_2) and ammonia were monitoredevery week, ranging from 5.5 to 7.6 mg O_2/ L and 0.5 to 1 ppm respectively, while pH rangedfrom 7.5 to 8.5 throughout the experimental period. Two-third of the water was renewed everyday to avoid accumulation of unutilized food or metabolic waste products which were siphoned out daily.

Artificial inoculation of fishes with *Aeromonads*

The bacterial strains MTCC 646 and ATCC 33658 were harvested by centrifugation at 5000 x g for 5 min and washed in physiological saline, PS (0.85% NaCl). The strains were enumerated by correlating the OD value measured at 600 nm of the growing culture with the corresponding colony forming units (cfu) obtained by spread plate dilution method [10] (Ref: OD 600nm 1 = 2 x 10^9cfu/ml). For this experiment, fishes were injected intraperitoneally (i.p.) with asymptomatic dose of 3 x 10^7 cfu/ml for A. hydrophila and 2 x 10^7 cfu/ml of A. salmonicida (working volume: 0.5 ml/100 gm body weight of fish) [11]. These asymptomatic carrier doses were determined through changes in several haematological and serum biochemical parameters on the 7th day of exposure [12].

Fishes were randomly divided in 4 sets i.e SHAM operated control, *A. hydrophila* treated, *A. salmonicida* treated and co-infectedsets (infected with both *A. hydrophila* and *A. salmonicida*). There were six replicates for each set containing 10 fishes. Fishes were sacrificed after 7 days of exposure to analyze various parameters. The growth parameters viz. Specific Growth Rate (SGR), Hepato Somatic Index (HSI) and Weight Gain Percentage were measured following Ude *et al*., 2018 [13]. All the haematological parameters were determined following standard techniques [14]. Fishes were anaesthetized by dip treatment and serum was collected and stored at – 20 °C for the further assays. Serum Glucose, Bilirubin, Calcium, Cholesterol and Total protein were measures following Roy *et al*., 2018 [11] using kits from Precision Biomed Pvt. Ltd. *In vitro* quantification of antioxidant enzymes viz. Superoxide dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione Reductase (GR) and Glutathione (GSH) were done using respective fish specific ELISA Kits from Bioassay Technology Laboratory, Korain Biotech Co., Ltd. [17,18,19].

Nitro Blue Tetrazolium (NBT) reduction assay

Nitro blue tetrazolium (NBT) assay was performed following Gómez *et al*., (2012) [20]. Blood smears were drawn on glass slides and stained with Wright stain for 2 minute and subsequently with Giemsa for 1 minute and rinsed with tap water. Finally, the slides were air dried and mounted with DPX and observed under light microscope (45X) to measure the percentage of cells containing black formazan deposits.

Statistical analysis

Means and standard error (S.E.) of the means were calculated from whole range data [21]. 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 (version 17.0).

Results

Assessing general health parameters, Hepatosomatic Index (H.S.I) was highest in the coinfected group whereas an increasing trend was observed in the infected groups compared to SHAM operated control. Specific Growth Rate (SGR) and weight gain percentage both were lowest in coinfected group compared to other groups (Fig. 1). Duncan's post hoc test revealed that SGR value of *A. hydrophila* treated groups showed no significant difference from both SHAM operated and *A. salmonicida* treated groups. In case of Weight gain percentage four different subsets were found by the post hoc test. The haematological parameters *viz.* haemoglobin content (Hb %), haematocrit value (Hct %) and mean cell haemoglobin concentration (MCHC) value decreased significantly ($p<0.05$) among infected groups. Presence of four different subsets, after performing Duncan's post hoc test indicated that there was a significant difference with respect to all the experimental groups when they were compared pair-wise (Fig. 1 & 2). The lowest value was observed in coinfected group followed by *A. salmonicida*; *A. hydrophila* infected and control groups. Leucocrit (Lct %) value, Mean Corpuscular Volume (MCV) and Mean Cell Haemoglobin (MCH) tend to increase significantly ($p<0.05$) in fishes challenged with pathogenscompared to SHAM operated control groups (Fig. 1 & 2). The coinfected group of fishes showed the highest value followed by *A. hydrophila*, *A. salmonicida* infected and SHAM operated groups. Significant differences ($p<0.05$, df = 3) were observed in neutrophil %, eosinophil %, basophil %, large lymphocyte %, small lymphocyte % and monocyte % among four different experimental groups of *L. rohita*. Study of dWBC % revealed that neutrophil, large lymphocyte, eosinophil, basophil and monocyte percentage increased among the infected groups in a significant manner ($p<0.05$), while small lymphocyte

percentage decreased (p<0.05) in *A. hydrophila*, *A. salmonicida* and co-infected *L. rohita* compared with SHAM operated control fishes after 7 days of exposure period (Fig.2).Fishes treated with *A. hydrophila* and *A. salmonicida* belonged to single subset which indicated that there were no significant difference in terms of neutrophil, basophil and large lymphocyte percentages when compared pair-wise with the third group of fishes *i.e.* the SHAM operated control. In case of eosinophil *A. hydrophila* treated and coinfected groups belonged to the same subset and showed the highest value followed by *A. salmonicida* and control fishes. For small lymphocyte, the value was highest in *A. hydrophila* infected group and decreased in coinfected, *A. salmonicida* infected and SHAM operated control groups respectively. The values of serum biochemical parameters obtained in SHAM operated control, *A. hydrophila* infected; *A. salmonicida* infected and coinfected *L. rohita* are summarized in Fig. 3. Fishes infected with pathogens revealed significant changes (p<0.05) in the biochemical parameters of the serum such as serum glucose, serum bilirubin, serum calcium, total cholesterol and total serum protein. Duncan's post hoc test revealed the presence of three different subsets in case of serum bilirubin and serum calcium levels where no significant difference was observed among *A. hydrophila* and coinfected groups. The values of bilirubin and calcium were highest in these two groups followed by *A. salmonicida* and control groups. For serum glucose and serum cholesterol levels four different subsets were observed in Duncan's post hoc test and the values increased in infected groups. The levels were highest in coinfected group followed by *A. salmonicida*, *A. hydrophila* infected and SHAM operated control groups. Two different subsets were observed in total protein concentration where the value was lowest in SHAM operated control group and no significant difference was observed among the three infected groups. Percentage of NBT positive phagocytic cells was highest in coinfected fishes followed by *A. salmonicida*, showed the alteration in Super Oxide Dismutase (SOD) and Catalase (CAT) activities and concentrations of detoxifying enzymes viz. Glutathione (GSH), Glutathione Peroxidase (GPx), Glutathione Reductase (GR) and in control; *A. hydrophila* infected; *A. salmonicida* infected and coinfected *L. rohita*. For all the enzymes, four different subsets were observed in Duncan's post hoc test. A similar pattern i.e significant increase (p<0.05) in the activity of SOD; CAT as well as in the concentration of GPx, GR and GSH was also observed in *A. hydrophila* treated; *A. salmonicida* treated and coinfected *L. rohita* in comparison to SHAM operated control fishes. The highest values of all enzymes were observed in the coinfected group. Percentage of phagocytic cells with formazan deposition among *A. hydrophila*, *A. salmonicida* infected and coinfected fishes after 7 days of exposure, have shown significant increase (p<0.05, df = 2) compared to SHAM operated control fishes (Table 1; Fig. 5). Duncan's post hoc test revealed the presence of three subsets in percentage of phagocytic cells with formazan deposition for the four different sample groups of fishes. Since, for all the cases, the bacterial dose was asymptomatic, no external leisons were expected but in case of coinfected fishes prominent symptoms of *A. salmonicida* infection viz. dermal ulcerations with whitish, fluid filled, raised furuncles, signs of internal bleeding and haemorrhagic signs around the anal orifice and abdominal region were observed (Fig. 6).

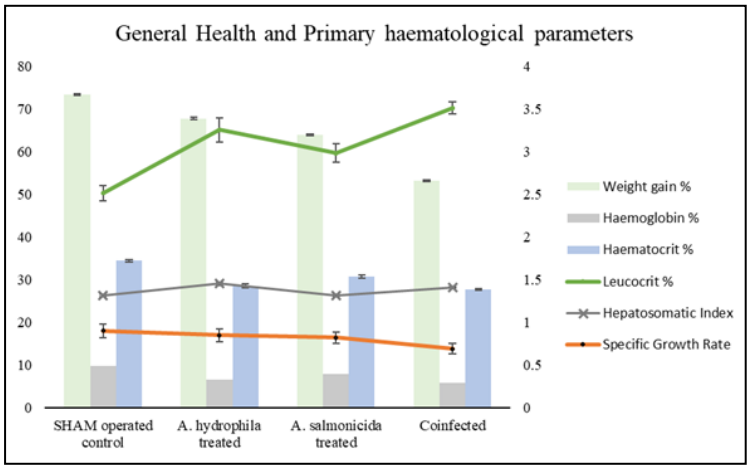

Fig 1: General health parameters and primary haematological profile of SHAM operated control; *A. hydrophila* treated; *A. salmonicida* treated and coinfected fish samples after 7 days of exposure.

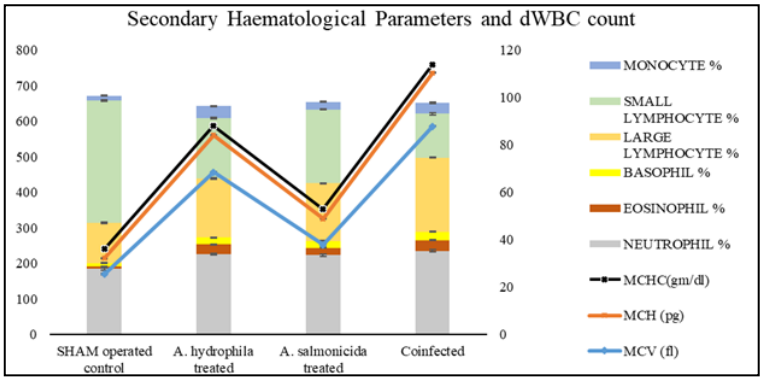

Fig 2: Evaluation of secondary haematological profile and Differential White Blood Cell count of SHAM operated control; *A. hydrophila* treated; *A. salmonicida* treated and coinfected fish samples after 7 days of exposure.

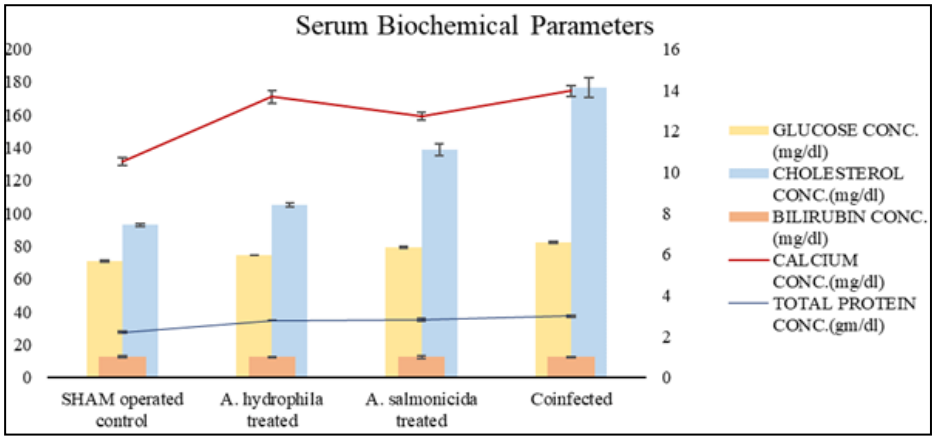

Fig 3: Evaluation of serum biochemical parameters of SHAM operated control; *A. hydrophila* treated; *A. salmonicida* treated and coinfected fish samples after 7 days of exposure.

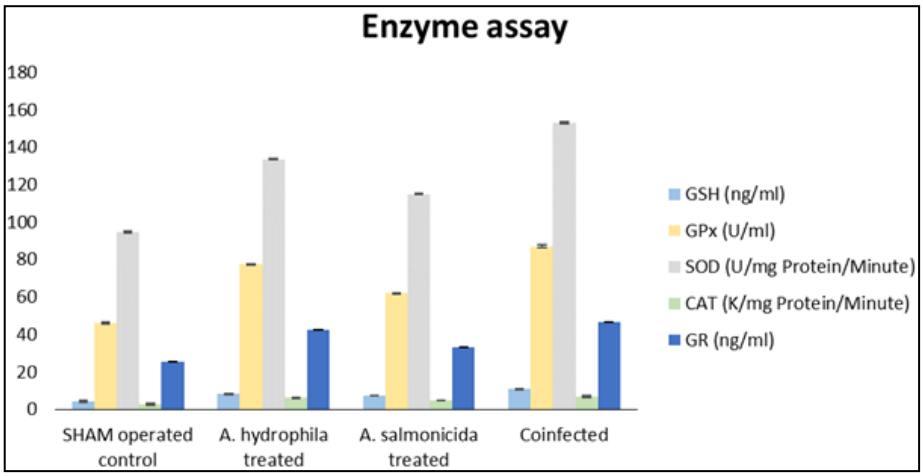

Fig 4: Antioxidant enzyme activity (Super Oxide Dismutase and Catalase) and concentration (glutathione, glutathione peroxidase and glutathione reductase) of SHAM operated control; *A. hydrophila* treated; *A. salmonicida* treated and coinfected fish samples after 7 days of exposure.

Table 1: Percentage of phagocytic cells with formazan deposition in SHAM operated control; *A. hydrophila* treated; *A. salmonicida* treated and coinfected fish samples after 7 days of exposure. Similar alphabets within rows denote homogenous means due to Duncan's post hoc test at 5% level of significance

	Sham Operated Control	*A. hydrophila* treated	*A. salmonicida* treated	Coinfected
Number of cells without Formazan Deposition	156.89 ± 7.91	139.33 ± 4.03	129 ± 2.55	110.89 ± 2.46
Number of Cells with Formazan Deposition	7.67 ± 0.74	28.11 ± 1.44	26.67 ± 1.77	71.22 ± 1.49
Percentage of cells with deposition	4.70 ± 1.43 [a]	16.88 ± 0.96 [b]	17.12 ± 1.09 [b]	39.12 ± 0.54 [c]

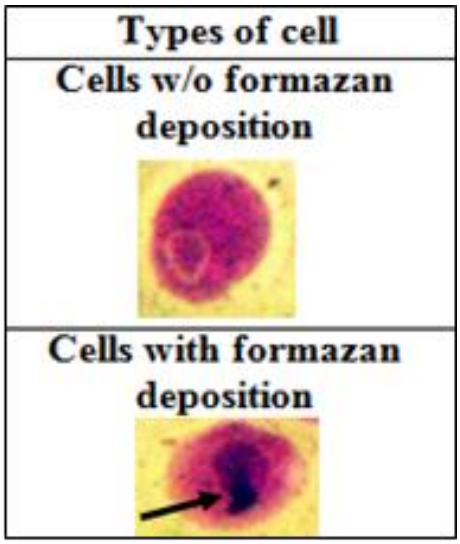

Fig 5: Neutrophils of *L. rohita* showing normal structure (without formazan deposition) and with deposition of blue-black, insoluble formazan deposit within the cytoplasm.

Fig 6: The pathological symptoms of coinfection in *Labeo rohita*. a. dermal ulcerations and haemorrhagic signs at the abdominal region; b. whitish, fluid filled, raised furuncles at the external layer of the body; c. haemorrhagic signs at anal orifice; d. Internal bleeding and haemorrhagic symptoms at the head region.

Discussion

In the present study, we have examined how the native (*A. hydrophila*) and invasive strain (*A. salmonicida*) interact during coinfection and what are the cumulative effects on virulence (measured as physiological and biochemical changes) in Indian major Carp, *L. rohita*. Disease virulence was found to be significantly influenced during coinfection,as specific growth ratedecreased significantly in the fishes coinfected with the pathogens. Whereas decreased weight gain percentage and the high hepatosomatic index indicated that *A. salmonicida* has higher virulence compared to the native *A. hydrophila*. These findings are in accordance with the findings of Datta Ray *et al*., 2016 [22], where a deterioration of health parameters was observed in fishes infected with Aeromonads (*A. hydrophila* and *A. salmonicida* separately). Moreover a significant decrease in haemoglobin concentration among the co-infected fishes can be attributed to progressive reduction in the haemoglobin content due to depression/exhaustion of haemopoietic potential of the fish [23] or due to increased removal of dysfunctional red blood cells [24]. This decreased Hb% may lead to insufficient oxygen supply to the tissues which resulted in decreased physical [25, 26, 27]. In addition to this, a decrease in the Haematocrit (measurement of packed erythrocytes) value among the infected groupsalso deteriorate oxygen supply to vital organs [28]. In both the cases maximum effect was found in the coinfected group which indicated a greater virulence compared to single infection. In case of White blood cells (WBCs), the leucocrit% got increased among infected fishes by producing huge amount of leucocytes (leucocytosis) to protect the fish by phagocytosis and through producing antibacterial chemicals to stop the disease causing agents from getting spread in the system. The calculated secondary haematological indices viz. Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC) are other important indicators in the diagnosis of anaemia in most animals [29]. The MCV value wassignificantly higher (p<0.05) in *A. hydrophila* infected fishes compared to *A. salmonicida* infected fishes but the highest value was observed in the coinfected group. This rise could be because ofintense damage or cirrhosis of liver or due to increased number of immature RBC [30]. Significant increase (p<0.05) of MCH value among coinfected fishes compared to control and other infected fishes could be attributed to macrocytosis, which was probably an adaptive response through the influx of immature erythrocytes from the hematopoietic tissues to the peripheral blood to make up for the reduced number of erythrocytes and decreased hemoglobin concentration [31].The MCHC value decreased significantly (p<0.05) in the infected fishes compared to the control fishes but no significant difference was found among *A. hydrophila* and *A. salmonicida* infected groups. This was an indication of erythrocytes swelling [32] due to a decrease in hemoglobin synthesis leading to severe hypochromic anemia [33] upon coinfection. Result of differential WBC count revealed that except small lymphocytes, percentage of all types of cells viz. neutrophil, basophil, eosinophil large lymphocyte and monocyteincreased significantly (p<0.05) among the infected groups that the innate immunity of the fish was stimulated to fight against the bacterial pathogen as the primary line of defense [34]. According to Burtis and Ashwood (1994) [34], presence of toxicants in aquatic ecosystem exerts its effect at cellular and / or molecular levels which leads to changes in biochemical compositions of the organisms. Thus, elevated glucose level among the infected groups might be due to gluconeogenesis to supplement additional energy needed to meet the increased metabolic demands [35, 36] under physiological stress. The Significant increase in serum bilirubin value (p<0.05) amongcoinfected as well as *A. hydrophila* and *A. salmonicida* infected fishes compared to control fishes also indicated liver dysfunction as it was the organ responsible for the elimination, via the bile, of the products of heme breakdown, primarily bilirubin. Impairment of this process caused a condition of jaundice and is reflected in a rise in bilirubin in the plasma [37]. Moreover, an elevated cholesterol level indicated disorders of lipid and lipoprotein metabolism, especially liver dysfunction [38]. Alteration in liver [34] and destruction of RBCs with concomitant release of cell contents into the blood stream might lead to increased concentrations of serum total protein among all the infected groups compared to the control fishes. Since serum proteins include various humoral elements, the increased level could also be an indication of antibody production in moribund fish with infectious diseases [39]. Rehulka, 1998 [40] found that *Aeromonas* induced ulcerous dermatitis in rainbow trout

(*Oncorhynchus mykiss*) resulted in an increase in total protein and cholesterol level in the plasma which were in accordance with the present study. Ultimately increased serum calcium levels of infected fish indicated the production of second messengers for activation of several downstream signaling pathways involved in the defense mechanism against bacterial infection [11]. After the initial screening of the above mentioned parameters in *L. rohita* against Aeromonad infection, it was found that the immune reaction and resultant stress was most prominently exhibited among the coinfected group. To get an insight on the immune system functioning of *L. rohita* in response to Aeromonad infection, a further detailed study on the immune functioning at cellular level was focused. In fish, phagocytosis was recognized as one of the important elements in the host defense against invading micro-organisms [41] and thus the quantification of oxidative radical (primarily Reactive Oxygen Species or ROS) production from neutrophils and monocytes as a defense mechanism was done using nitrobluetetrazolium (NBT) assay. In the present study, a significantly (p<0.05) higher number of NBT-positive cells i.e. cells with dark bluish formazan deposit within the cytoplasm were observed in the blood of *L. rohita* post infection, compared to the sham operated control fishes (Fig. 5) was indicative of the fact that upon pathogenic bacterial infection, the phagocytic activity and respiratory burst activities of neutrophils were significantly induced due to the innate immune system functioning [42]. The release of reactive oxygen species (ROS) from the phagocytic cell population of infected *L. rohita* fishes, subsequently caused simultaneous increase in the functioning of antioxidative stress enzyme pathway leading to increased activity of superoxide dismutase (SOD), catalase (CAT) and significantly higher concentrations of glutathione peroxidase (GPx), glutathione reductase (GR) and reduced glutathione (GSH) [Table. 6]. The significant increase (p<0.05) in SOD activity in ofinfected *L. rohita* in comparison to sham operated control fish attributed to the fact that SOD converts superoxide radical (O^{2-}) into H_2O_2 and O_2 to protect the cell from oxidative damage [43].The significant increase (p<0.05) in GPx level and catalase activity could be ascribed to the fact that they function by decomposing H_2O_2 (generated by the functioning of SOD) into H_2O and molecular O_2 (1/2 O_2) in the process of maintaining H_2O_2 concentration in these tissues [44]. Oxidized glutathione (GSSG) produced upon reduction of hydrogen peroxide by GPx was recycled to its reduced state by GR and NADPH. Thus the Significant increase (p<0.05) in GR level was recognized as excess production of GSSG instigates GR activity to reduce GSSG into GSH to maintain the cellular redox status by increasing the GSH/GSSG ratio. This clearly indicated the reason behind significant increase (p<0.05) of GSH level among the infected fishes in comparison to the control ones. A variety of internal or external pathological factors such as viral and bacterial infections leading to oxidative stress, reported by several workers were also in agreement with the current findings [45, 43, 46, 47,. 48]. In case of the confected fishes, though the bacteria were administered at an asymptomatic level, prominent symptoms of furunculosis viz. hemorrhagic signs at the abdominal muscle and reddening of skin, dermal ulcerations (Fig. 6) were observed which might indicate that in coinfected fishes, Invasive species *A. salmonicida* was capable to exclude the native *A. hydrophila* from the system.

Conclusion

The overall results obtained during the present study indicated that the native, indigenous pathogen i.e. *A. hydrophila* and exotic, invasive pathogen i.e. *A. salmonicida* when challenged at an asymptomatic, carrier state dosage did act as strong physiological stressors for *L. rohita* but coinfection resulted in the activation of alarm–stress response subsequently compromising the health status of the fish. It caused a severe deterioration of general health parameters leading to oxidative stress and ultimately resulted in prominent external symptoms of the exotic *A. salmonicida* infection. Though interaction of pathogens inside host organism very critical to understand but it could be assumed that this kind of multi parametric case study might led to further identification of species specific biomarkers at cellular and molecular levels for better understanding of co-existance mechanism of native and invasive Aeromonad species.

Conflict of interest

The authors declare no conflict of interest.

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Table 2: Supplementary Data

	Sham Operated Control	***A. hydrophilic* treated**	***A. salmonicida* treated**	**Confected**
Hepatosomatic Index	0.41904 ± 0.01^{a}	0.60153 ± 0.02^{c}	0.4928 ± 0.02^{b}	0.72028 ± 0.06^{d}
SGR	0.8987 ± 0.02^{c}	$0.8501 \pm 0.01^{b,c}$	0.8228 ± 0.02^{b}	0.6907 ± 0.02^{d}
Weight gain %	73.434 ± 0.27^{d}	67.829 ± 0.24^{c}	63.935 ± 0.21^{b}	53.164 ± 0.19^{a}
Haemoglobin %	9.715 ± 0.10^{d}	6.553 ± 0.14^{b}	7.917 ± 0.17^{c}	5.819 ± 0.14^{a}
Haematocrit %	34.426 ± 0.27^{c}	28.497 ± 0.45^{a}	30.78 ± 0.34^{b}	27.669 ± 0.22^{a}
Leucocrit %	1.197 ± 0.06^{a}	1.806 ± 0.03^{b}	1.667 ± 0.04^{b}	2.104 ± 0.09^{c}
MCV (fl)	170.12 ± 1.64^{a}	457.234 ± 1.72^{c}	253.002 ± 1.02^{b}	585.811 ± 0.82^{d}
MCH (pg)	44.647 ± 0.56^{a}	104.016 ± 0.75^{c}	73.133 ± 0.69^{b}	150.822 ± 0.79^{d}
MCHC (gm/dl)	28.958 ± 0.68^{b}	23.108 ± 0.34^{a}	23.304 ± 0.35^{a}	22.444 ± 0.17^{a}
Neutrophil %	27.702 ± 0.39^{a}	33.94 ± 0.47^{b}	33.514 ± 0.47^{b}	35.363 ± 1.57^{c}
Basophil %	1.3981 ± 0.14^{a}	2.9548 ± 0.18^{b}	2.8862 ± 0.20^{b}	3.5514 ± 0.18^{c}
Eosinophil %	1.1352 ± 0.10^{a}	4.097 ± 0.22^{c}	3.137 ± 0.19^{b}	4.5272 ± 0.18^{c}
Small Lymphocyte %	51.525 ± 0.31^{d}	25.449 ± 0.35^{b}	31.169 ± 0.23^{c}	18.4842 ± 0.35^{a}
Large Lymphocyte %	16.9524 ± 0.23^{a}	24.864 ± 0.27^{b}	24.313 ± 0.19^{b}	31.2348 ± 0.20^{c}
Monocyte %	2.0737 ± 0.17^{a}	5.189 ± 0.17^{c}	3.239 ± 0.22^{b}	4.73 ± 0.33^{c}
Glucose (mg/dl)	70.916 ± 0.86^{a}	74.64 ± 0.09^{b}	79.16 ± 0.60^{c}	82.42 ± 0.67^{d}

Bilirubin (mg/dl)	0.155 ± 0.01^{a}	0.476 ± 0.01^{c}	0.339 ± 0.02^{b}	0.928 ± 0.03^{c}
Calcium (mg/dl)	8.31 ± 0.19^{a}	10.90 ± 0.32^{c}	9.93 ± 0.20^{b}	10.98 ± 0.25^{c}
Cholesterol (mg/dl)	92.964 ± 0.90^{a}	105.36 ± 1.23^{b}	138.648 ± 3.47^{c}	176.62 ± 6.05^{d}
Total protein (mg/dl)	2.208 ± 0.04^{a}	2.783 ± 0.01^{b}	2.805 ± 0.08^{b}	2.986 ± 0.07^{b}
SOD (U/mg Protein/Minute)	94.574 ± 0.31^{a}	133.541 ± 0.45^{b}	114.812 ± 0.49^{c}	152.989 ± 0.48^{d}
CAT (K/mg Protein/Minute)	2.893 ± 0.14^{a}	$6.071 \pm .20^{c}$	4.724 ± 0.16^{b}	6.852 ± 0.21^{d}
GSH (ng/ml)	4.336 ± 0.24^{a}	8.2 ± 0.16^{c}	7.36 ± 0.17^{b}	10.777 ± 0.28^{d}
GPx (U/ml)	45.871 ± 0.47^{a}	77.493 ± 0.35^{c}	61.995 ± 0.35^{b}	87.075 ± 0.60^{d}
GR (ng/ml)	25.452 ± 0.24^{a}	42.379 ± 0.35^{c}	33.148 ± 0.37^{b}	46.675 ± 0.32^{d}

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