



Analysis of haemopoietic cells, blood variables and histopathological changes in *Channa punctatus* (Bloch) following experimental inoculation of two strains of aeromonads

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ABSTRACT

Studies have been made to analyse the histopathological changes in head kidney and liver, erythropoietic and leucopoietic cells in head kidney and total erythrocytic and leucocytic populations in the peripheral blood of adult *Channa punctatus* (Bloch) following experimental inoculation of sublethal dose of *Aeromonas hydrophila* and *Aeromonas salmonicida* respectively during various exposure periods. Histopathological examinations showed initiation of necrosis, periglomerular degeneration in head kidney and formation of necrotic hepatocytes in liver compared to sham-injected control (SIC). Significant changes in total erythrocytic count (TEC) in aeromonad-treated groups were less pronounced over the most exposure periods. Similar observations were also recorded from the study among erythropoietic cell lineage from the head kidney tissue imprints. On the contrary, neutrophils, lymphocytes and macrophages among the leucocytes elicited significant variations in aeromonad-injected fish throughout the exposure periods with little fluctuations in the head kidney. An initial steep increase in the total leucocytic count (TLC) was also found in the peripheral blood of aeromonad-treated groups.

Key words: Bacterial strain, *Channa punctatus*, Fish disease, Haematology, Histopathology

Aeromonas salmonicida and *Aeromonas hydrophila* are the most common and troublesome pathogens responsible for diseases of fish raised in ponds and recirculating systems (Foda 1973, Koppang *et al.* 2000, Azad *et al.* 2001, Madetoja *et al.* 2003, Xia *et al.* 2004). Changes in the ecological conditions in water body due to pollution or intense commercial fish breeding promote the appearance of virulent forms of several bacterial species including aeromonads. This impends the need for sensitive bio-indicator tools in pathogen impact assessment that reflect either activation in the initial stages or suppression in response to a chronic challenge so that further spread of the disease and eradication of the pathogen can be controlled. In this regard, studies of haemopoiesis and histopathological changes can serve as important parameters for indication of bacterial pathogenicity (Bruno and Munro 1986, Magnadóttir *et al.* 2002, Kobayashi *et al.* 2004, Altinok *et al.* 2007, Martins *et al.* 2008, Hagiwara *et al.* 2009).

The present work is aimed to study and analyze stress

potentiation in *Channa punctatus* following experimental inoculation of 2 strains of aeromonads, viz. *Aeromonas salmonicida* and *Aeromonas hydrophila*. Adult *C. punctatus* were injected intramuscularly (im) with sublethal dose (4×10^7 cfu/ml) of aeromonads @ 0.5 ml/100 g body weight of fish and haematological observations from peripheral blood and histopathological observations from liver and head kidney were recorded on 3, 7, 14, 21 and 28 days after injection and compared with sham-injected control groups at each interval. The tissue imprints from head kidney were also made and changes in erythropoietic and leucopoietic cell counts among two aeromonad-treated groups and sham-injected controls have been analyzed during the exposure periods.

MATERIALS AND METHODS

Adult spotted murels, *Channa punctatus* (Family: *Channidae*) were obtained from a local point sources in and around the wetland areas near Kolkata, West Bengal, India. The live and healthy fish specimens (200) of both sexes were transported to the Department of Zoology, University of Calcutta and acclimatized in the laboratory for 15 days in 40 glass aquaria (size: 0.6 m $\times$ 0.3 m $\times$ 0.3 m, 5 fish/aquarium) under controlled laboratory conditions with continuous

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aeration. The specimens selected for study were 81.73 ± 0.49 g in weight and 19.93 ± 0.17 cm in length. The fishes were fed *ad libitum* with *Tubifex* sp. and larvae of *Culex* sp. during the acclimatization period. Daily water removal further minimized any other stress factors from the experimental set up.

Microbial cultures of *Aeromonas salmonicida* (MTCC 1522) and *Aeromonas hydrophila* (MTCC 646) were collected from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, IMTECH, Chandigarh, India. Lyophilized strains were revived by adding nutrient broth (NB) and transferring the rehydrated culture to a fresh nutrient agar (NA) medium. Consequently, streak plate method was followed to get isolated bacterial colonies on a large part of the agar surface.

Both strains of aeromonads (*A. hydrophila* and *A. salmonicida*) were cultured in nutrient broth (NB) and incubated at 37°C and 25°C respectively for 24 h prior to infectivity testing. Bacterial cells were harvested by centrifugation at 5000 g for 5 min and washed in physiological saline, PS (0.85% NaCl). The strains were enumerated by correlating the OD values (600 nm) of the growing culture with the corresponding colony forming units (cfu) obtained by spread plate dilution method ($OD_{600\text{ nm}} 1 = 2 \times 10^9$ cfu/ml). For this experiment, a sublethal concentration (4×10^7 cfu/ml) from each strain of aeromonads was made up in PS (working volume: 0.5 ml/100 g body weight of fish) and injected i/m into 5 replicates of adult *C. punctatus* per strain (each replicate containing 10 fish). Erythrocytic and leucocytic cell populations from both peripheral blood and head-kidney imprints as well as the histopathological observations of MTCC 1522- treated and MTCC 646-treated fish groups were recorded on 3, 7, 14, 21 and 28 days of exposure respectively and compared with corresponding sham-injected control groups at each interval (5 replicates, each containing 10 fish injected with sterile PS only). For this study, a total of 8 fish samples were collected from each replicate of the two aeromonad-treated groups as well as from the sham-injected control group in each of the five exposure periods unless prevented by mortality.

Aeromonad-treated fish specimens were instantly dissected out at respective exposure periods along with sham-injected control groups and the head kidney and liver were lifted carefully for histopathological examination. The tissues were fixed in aqueous Bouin's fluid for 24 h. Then, tissues were dehydrated in upgraded alcoholic series, immersed in cedar wood oil followed by embedding in molten paraffin (melting point: 56°- 58°C). Serial sections were cut at 6–7 µm. Sections were stained in eosin-haematoxylin. Finally, photomicrographs were taken in Zeiss-Axiostar plus compound microscope under oil immersion.

Erythropoietic and leucopoietic cell populations from the haemopoietic tissue of *C. punctatus* were studied by stained

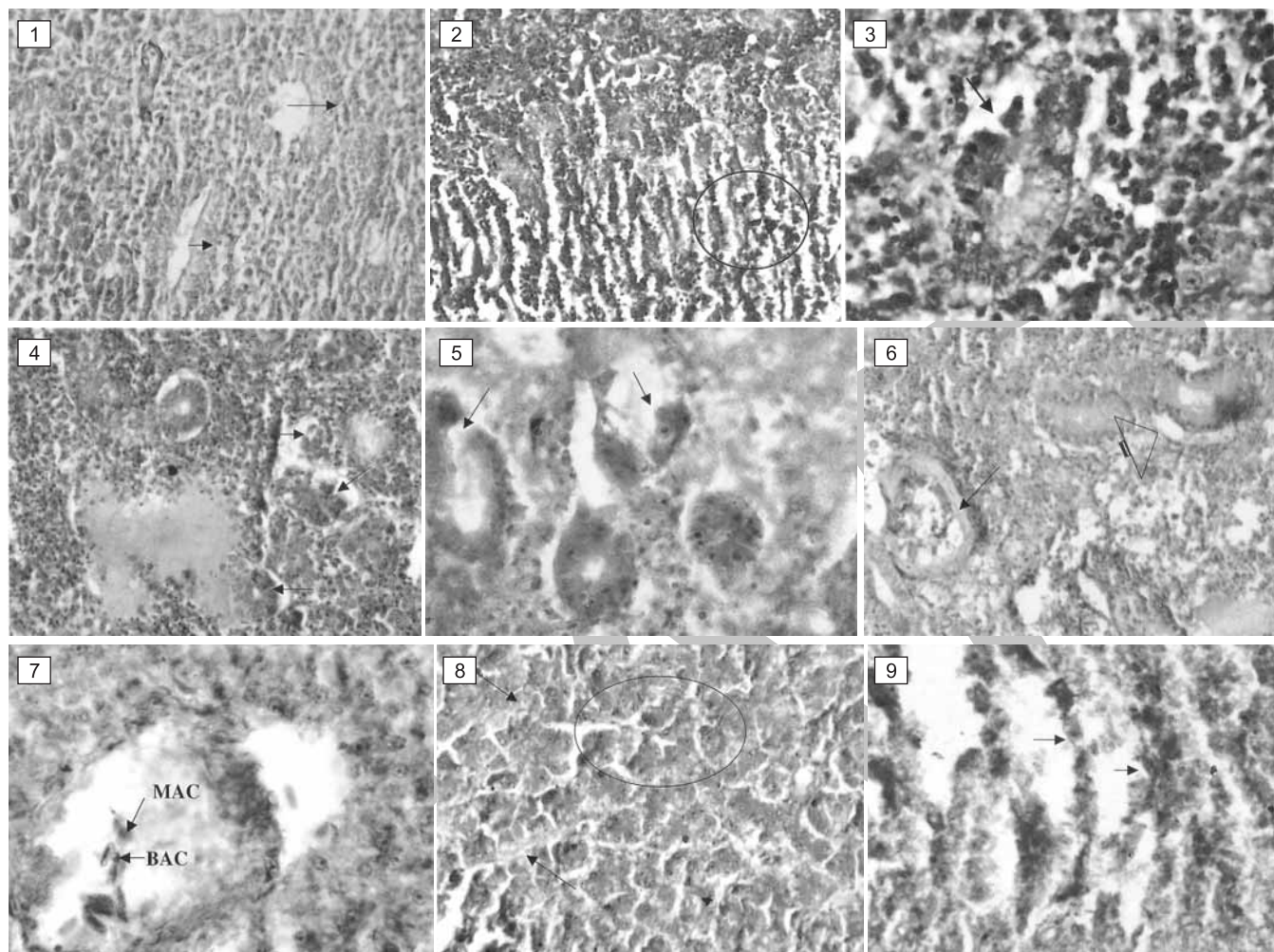
imprints on glass slides for light microscopy. Before collection of haemopoietic tissue samples, each of the fish sampled was anaesthetized by MS 222. The ventral body wall of fish was cut and head kidney was dissected out after exposing the viscera. For light microscopy, air-dried head kidney tissue was imprinted on glass slides using 3–4 pieces from each fish. Following the method of Ashley and Smith (1963), the tissue imprints were stained in Benzidine and counterstained in Giemsa (Mahajan and Dheer 1979a) to differentiate haemoglobin in developing blast cell stages, viz. small lymphoid haemoblast (SLH), basophilic erythroblast (BE), polychromatophilic erythroblast (PE), acidophilic erythroblast (AE), young reticulocyte (YR), mature reticulocyte (MR), young erythrocyte (YE) and mature erythrocyte (ME). The colour atlas of Dominguez and Villalobos (1947) was used to assess the colours of the stained cells. The leucocytes studied in the head kidney tissue imprint were neutrophil, lymphocyte and macrophage. The stained imprints were examined under Zeiss-Axiostar plus compound microscope.

For determination of total erythrocyte count (TEC) and total leucocyte count (TLC), free flowing blood was collected by severing the caudal peduncle of fish, stored in glass vial rinsed with 3.8% sodium citrate (anticoagulant) and the measurements were made within 30 min of sampling following standard methods with some modifications (Blaxhall and Daisley 1973, Dacie and Lewis 1984). Finally, TEC and TLC were made by standard clinical method with the help of improved haemocytometer using Hayem's solution as diluting fluid.

The results were presented as mean and standard error (mean±SE). Independent sample t-test was used to distinguish between means of significant differences. Treatments were taken to be significantly different where $P < 0.05$ and highly significant where $P < 0.01$.

RESULTS AND DISCUSSION

Histopathological analysis: Kidney tissue from all sham-injected control fish appeared normal (Fig. 1). The renal tubules possessed intact columnar epithelia. The histopathological study of kidney of aeromonad-treated fish revealed a distinct change in the nephric architecture in comparison to control. After 3 days of exposure, peritubular haemorrhages were observed in *A. salmonicida* infected fish (Fig. 2). Necrotic lesions were present in kidney accompanied by dilation of renal tubules after 7 days of exposure of *A. salmonicida* (Fig. 3). The epithelial cells in the tubules of the treated fish were flattened and incomplete in some tubules and rounded in others. The separation of renal tubular epithelia from the basement membrane was also observed after 14 days of exposure of *A. salmonicida* (Fig. 4). On the other hand, haemorrhages in the parenchyma of the kidney and modest tubular necrosis were found in *A. hydrophila* treated fish after 14 days of exposure (Fig. 5). Disseminated



Figs 1–9. **1.** Kidney tissue from sham-injected *C. punctatus* showing intact renal tubules having columnar epithelia with brush borders (arrow). Haematoxylin and Eosin staining [$\times 600$]. **2.** Peritubular haemorrhage in the kidney of *C. punctatus* (circle) after 3 days of exposure with *A. salmonicida*. Haematoxylin and Eosin staining [$\times 480$]. **3.** Necrotic lesions and degeneration of the tubular epithelia (arrow) in the 7day-exposed kidney of *A. salmonicida* treated *C. punctatus*. Haematoxylin and Eosin staining [$\times 1200$]. **4.** 14day - exposed kidney of *A. salmonicida* treated *C. punctatus* showing separation of renal tubular epithelia from the basement membrane (arrows). Haematoxylin and Eosin staining [$\times 600$]. **5.** Modest tubular necrosis (arrows) in the *A. hydrophila* infected kidney of *C. punctatus* after 14days of exposure, Haematoxylin and Eosin staining [$\times 1200$]. **6.** Peritubular haemorrhage (arrowhead) and periglomerular fibrosis (arrow) in the *A. hydrophila* infected kidney of *C. punctatus* after 21days of exposure. Haematoxylin and Eosin staining [$\times 600$]. **7.** *A. salmonicida* infected *C. punctatus* showing an initial host response in the liver after 7days of exposure. Macrophage (MAC) cells starting to organize around bacterial colony (BAC). Haematoxylin and Eosin staining [$\times 1200$]. **8.** Necrotic hepatocytes (arrows) and sinusoidal congestion (circle) in the liver of *A. hydrophila* treated *C. punctatus* after 14days of exposure. Haematoxylin and Eosin staining [$\times 480$]. **9.** Rupture of plasma membranes of liver parenchyma showing distortion of the hepatocytic cords (arrows) in the *A. hydrophila* infected *C. punctatus* after 28days of exposure. Haematoxylin and Eosin staining [$\times 600$].

peritubular haemorrhage and glomerular changes (periglomerular fibrosis) indicating several renal damage were also found in kidney after 21days and 28 days of exposures respectively due to *A. hydrophila* infection (Fig. 6).

An initial host response in the liver, with interlocking epithelioid cells, macrophages (MAC) and lymphocytes starting to organize around bacteria (BAC), was seen in *A. salmonicida* treated fish after 7 days of exposure (Fig. 7). Necrotic hepatocytes and sinusoidal congestions were present in the liver tissue after 14 days of exposure of *A. hydrophila*

(Fig. 8). The plasma membranes of the liver parenchymal cells were ruptured after 28 days of exposure of *A. hydrophila* causing distortions of the hepatocytic cords (Fig. 9).

The present study from the aeromonad-infected fish showed initiation of necrosis and damage in the liver and head kidney tissues probably hampering the process of erythropoiesis. Wood and Yasutake (1956) observed damage to the kidney and spleen in fish infected with Bacterial Kidney Disease (BKD). The destruction of the tissues of kidney and liver could result in decreased blood cell production while

the tubular necrosis in the kidney could result in a haemodilution through a net uptake of water in infected fish in freshwater (Hickman and Trump 1969). Internally, the liver and kidneys are the target organs of an acute septicaemia and these organs are apparently attacked by bacterial toxins and lose their structural integrity (Huizinga *et al.* 1979). Similar histopathological lesions in the gills, liver, spleen, trunk kidney and head kidney of channel catfish, *Ictalurus punctatus* were also reported by Walters and Plumb (1980) where fish were injected ip with a sublethal dose of *A. hydrophila*. Young *et al.* (1994) reported that moribund striped bass, *Morone saxatilis* also displayed various histopathological changes which involved inflammation of the visceral peritoneum, necrosis of hepatocytes, formation of swollen and disorganized hepatic cells in the liver. They also found dilation and necrosis of the kidney tubules as well as changes in the glomerulus. Graham *et al.* (2002) reported an investigation into a disease outbreak of persistent high mortality in Atlantic salmon, *Salmo salar* and included histopathological changes including extensive multifocal haematopoietic necrosis in the kidney and spleen. Chen *et al.* (2004) conducted a comparative study of blood chemistry and histopathology of Nile tilapia (*Oreochromis niloticus*) after infection with *Vibrio vulnificus* or *Streptococcus iniae*. Björnsdóttir *et al.* (2005) induced systemic disease in turbot,

Scophthalmus maximus by experimental infection of *A. salmonicida* subsp. *achromogenes* and observed haemorrhages and necrosis in the kidney among the most prominent histopathological changes which were similar to the present study.

Haemopoietic and haematological analysis: The variations of the number of cell types, both erythropoietic and leucopoietic lineages, in the pronephric kidney on different days of exposure to inoculated bacterial strains are given in Tables 1 and 2. With *A. salmonicida* (Table 1), the progenitor SLH cells did not vary significantly. The transient stages (BE, PE and AE) were significantly varying only after inoculation (3 days) and never beyond. The reticulocytes (YR and MR) as well as erythrocytes (YE and ME) were not significantly deviated from the sham-injected control (SIC). Similar results were obtained with *A. hydrophila* (Table 2), when at the initial phase of infection, the progenitor and transient cell stages were increased significantly. The reticulocytes (YR and MR) and YE stage showed significant variations at later stages of infection. Such erythropoietic response can be correlated with the total erythrocytic count (TEC) in circulating blood, when the values did not indicate any significant response to the infection of any of the strains. However, neutrophil and lymphocytic cell populations were significantly varying in the head kidney following infections

Table 1. Changes in some haemopoietic parameters in head kidney over time of MTCC 1522 (*A. salmonicida*) injected fish groups with corresponding sham-injected control (SIC)

Cell types	Fish groups	Exposure periods (days)				
		3	7	14	21	28
SLH	SIC	50.46±1.52	38.97±1.89	35.13±2.2	38.76±2.08	43.39±1.88
	MTCC 1522injected	41.11±1.45	39.48±1.40	32.64±2.28	41.15±2.44	44.52±2.09
BE	SIC	10.8±0.76	13.8±0.49	13.3±1.11	11.4±1.01	14.38±0.84
	MTCC 1522injected	16.58±1.13**	15.57±1.57	17.25±1.75	8.23±1.69	14.85±1.83
PE	SIC	8.98±0.86	16.08±1.91	12.93±1.53	12.23±2.06	8.73±0.69
	MTCC 1522injected	13.99±1.41*	14.73±1.12	13.95±1.20	12.43±1.52	11.51±2.20
AE	SIC	8.92±0.72	6.13±1.15	10.01±0.82	5.31±0.95	6.38±1.03
	MTCC 1522injected	5.29±0.80**	6.98±0.97	8.97±0.88	4.88±0.60	5.80±0.70
YR	SIC	3.56±0.51	5.26±0.98	5.12±0.95	5.2±0.71	5.7±1.21
	MTCC 1522injected	5.90±1.13	4.95±0.89	7.23±0.76	6.89±0.70	6.06±1.15
MR	SIC	6.02±0.21	5.35±1.17	7.83±1.28	7.9±0.88	10.06±0.93
	MTCC 1522injected	7.21±1.12	6.24±1.16	5.29±0.98	8.73±1.54	6.82±1.05*
YE	SIC	7.14±1.17	6.87±0.98	10.35±1.21	11.35±0.88	6.18±0.66
	MTCC 1522injected	4.65±0.83	7.05±1.14	7.87±0.90	9.0±1.37	5.11±0.76
ME	SIC	4.12±0.59	7.54±1.23	5.34±0.81	7.86±1.65	5.17±0.92
	MTCC 1522injected	5.25±1.23	4.95±0.89	6.76±1.34	10.66±1.43	5.31±1.19
NEU	SIC	11.78±1.63	13.02±2.24	14.07±2.41	6.36±0.9	22.97±0.89
	MTCC 1522injected	20.63±1.90**	7.48±0.96	7.34±1.11*	19.99±2.67**	3.91±0.54**
LYM	SIC	56.0±1.09	66.94±1.88	61.39±1.94	74.4±2.96	50.86±2.29
	MTCC 1522injected	49.90±0.76**	73.65±2.08*	63.38±2.72	71.24±2.43	84.31±1.50**
MAC	SIC	32.22±1.11	20.03±1.53	24.55±1.82	19.25±2.37	26.18±1.65
	MTCC 1522injected	29.45±2.18	18.85±1.81	27.36±1.68	19.77±1.36	11.77±1.02**

SLH, Small lymphoid haemoblast; BE, basophilic erythroblast; PE, polychromatophilic erythroblast; AE, acidophilic erythroblast; YR, young reticulocyte; MR, mature reticulocyte; YE, young erythrocyte; ME, mature erythrocyte; NEU, neutrophil; LYM, lymphocytes; MAC, macrophage; values shown are mean±SE, *Significant ($P < 0.05$), ** highly significant ($P < 0.01$).

Table 2. Changes in some haemopoietic parameters in head kidney over time of MTCC 646 (*A. hydrophila*) injected fish groups with corresponding sham-injected control (SIC)

Cell types	Fish groups	Exposure periods (days)				
		3	7	14	21	28
SLH	SIC	50.46±1.52	38.97±1.89	35.13±2.2	38.76±2.08	43.39±1.88
	MTCC 646injected	40.79±2.14**	43.28±1.73	36.78±2.14	42.42±0.64	41.29±1.04
BE	SIC	10.8±0.76	13.8±0.49	13.3±1.11	11.4±1.01	14.38±0.84
	MTCC 646injected	17.63±1.36**	10.91±0.91*	7.3±0.83**	11.13±1.34	12.06±0.99
PE	SIC	8.98±0.86	16.08±1.91	12.93±1.53	12.23±2.06	8.73±0.69
	MTCC 646injected	18.01±2.36**	10.98±0.88*	9.19±1.13	15.85±1.68	6.32±0.7*
AE	SIC	8.92±0.72	6.13±1.15	10.01±0.82	5.31±0.95	6.38±1.03
	MTCC 646injected	6.07±0.65*	5.93±0.98	6.48±0.88*	6.38±0.93	6.47±1.0
YR	SIC	3.56±0.51	5.26±0.98	5.12±0.95	5.2±0.71	5.7±1.21
	MTCC 646injected	4.02±0.56	4.3±0.49	9.62±0.99*	4.02±0.15	7.01±1.08
MR	SIC	6.02±0.21	5.35±1.17	7.83±1.28	7.9±0.88	10.06±0.93
	MTCC 646injected	4.02±0.56*	8.72±0.69*	13.09±0.85**	4.02±0.15**	9.92±1.03
YE	SIC	7.14±1.17	6.87±0.98	10.35±1.21	11.35±0.88	6.18±0.66
	MTCC 646injected	5.42±0.84	10.11±1.01*	9.78±0.97	8.91±1.06	9.67±0.75**
ME	SIC	4.12±0.59	7.54±1.23	5.34±0.81	7.86±1.65	5.17±0.92
	MTCC 646injected	4.04±0.61	5.07±0.81	6.58±1.56	5.66±1.05	6.64±1.22
NEU	SIC	11.78±1.63	13.02±2.24	14.07±2.41	6.36±0.9	22.97±0.89
	MTCC 646injected	16.86±1.65*	6.77±1.12*	18.93±2.07*	9.8±1.28	5.28±1.12**
LYM	SIC	56.0±1.09	66.94±1.88	61.39±1.94	74.4±2.96	50.86±2.29
	MTCC 646injected	58.84±2.51	70.64±2.19	62.5±0.76	65.58±2.09*	77.26±1.49**
MAC	SIC	32.22±1.11	20.03±1.53	24.55±1.82	19.25±2.37	26.18±1.65
	MTCC 646injected	24.3±2.4*	22.58±1.85	18.57±1.95*	19.93±4.48	17.46±0.85**

For abbreviations see Table 1; values shown are mean±SE, *significant ($P < 0.05$), ** highly significant ($P < 0.01$).

with both the bacterial strains (Tables 1, 2). Following initial boosting of neutrophil generation, the cells showed either significant increase or decrease as the infection was pronounced. Lymphocytes were significantly increasing in most of the infective stages. Macrophages counts were decreased, though not varied significantly, except on 28th day of infection when the change was highly significant. These variations were reflected in the significant changes in total leucocyte count (TLC) in the peripheral blood following infection of both the strains.

The teleost head kidney has been considered as major haemopoietic organ from which most of the blood cell types are produced (Bielek 1981, Fänge 1982). Peters and Schwarzer (1989) described the changes in haemopoietic tissues of rainbow trout under the influence of stress. The findings of the present study in regard to the changes in erythrocytic cell populations following experimental inoculation of *A. hydrophila* and *A. salmonicida* in *C. punctatus* showed certain responses to stress but no significant trend in variations could be detected. This may be due to the fact that they are not able to keep pace with other developing stages possibly because of time lag in recruitment from the preceding ones with the pace of the penultimate stages.

Neutrophils are present in large numbers in blood and hemopoietic organ as a reserve and under normal conditions,

are rare in the tissues and body cavities. In contrast, macrophages are present in all body compartments and are the first phagocytes to encounter the invading microorganisms when superficial barriers are breached. Neutrophils from the reserve pools are quickly attracted to the infectious foci by microbial products and by chemotactic agents produced by immune cells including macrophages (Lehrer *et al.* 1988, Appelberg 1992). The present study also showed similar consistency with neutrophils being more in number initially. Later neutrophil dynamics decreased or increased probably due to migration into the site of infection and in turn increased formation to meet the demand. Besides, macrophage populations showed a decrease probably due to migration to point of infection. Mahajan and Dheer (1979b) while studying cell types in the peripheral blood of an air-breathing fish *C. punctatus* stated that neutrophils are the most important leucocytes as they show the greatest sensitivity to changes in the environment. The present data supports the fact that the release of neutrophils into the blood is known to occur as a non-specific response to a variety of stress stimuli in fish. In this experiment, the bacterial inoculation as being the stress factor thus seems to act as stimuli for initiation of innate response in fish. Thus neutrophil populations can be considered as an important tool to assess health of pathogen- exposed fish as well as the quality of the surrounding aquatic media. However,

information are still inadequate with regard to its complete biomarker status and it is hoped that the present contribution will stimulate research with further refinements of techniques. Moreover, the present study showed increase in lymphocyte generation with progressive exposure periods. But their numbers significantly increased in treated specimen at later exposure period. These detections may attribute to the fact that lymphocytes being associated with adaptive immunity develop at a later stage following pathogenic invasion.

Thus the results of the present investigation indicated that experimental bacterial inoculation produced statistically significant effects in certain haemopoietic cell types even at sublethal level of inoculation. This indicates that this particular species is susceptible to pathogenicity by aeromonads.

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