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IMMUNE RESPONSES AGAINST OXIDATIVE STRESS IN THE SKIN OF CYPRINID FISH *CIRRHINUS MRIGALA* CHALLENGED WITH ASYMPTOMATIC DOSES OF *AEROMONAS HYDROPHILA*

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Bacterial fish pathogens are well-resourced to perceive, hold on to and commence infection in their potential hosts. In the present study, we investigated the pathogenic effect of a virulent *Aeromonas hydrophila* in skin of *Cirrhinus mrigala* (Bloch, 1795) challenged with carrier state doses and the nature of immune responses that it triggers. Fishes were artificially inoculated with three sublethal doses of *A. hydrophila* injected intraperitoneally (i.p.) @ 0.5ml/100g body weight.There was a dose dependent pattern of change observed in the potential fish biomarkers that were chosen for the study. The histopathology, oxidative stress and assessment of reported cell death have been characterized. Histopathology revealed changes in stratified epidermis containing fibrous Malphigian cell and mucous cells. Bacterial infestation was observed in skin in treated samples. Cell damage and cell death was reported in conjunction with increase in the melanomacrophage centres. Flowcytometric analysis performed after 7 days revealed an increase in ROS production with a concomitant rise in apoptosis in skin. After 30 days, ROS generation showed an approximate 1, 1.02 and 1.03 fold increases in the DCF mean peak in fishes injected with Dose 1, 2 and 3 respectively in comparison to 4.78, 5.67 and 7.16 fold increases after 7 days in single cell suspensions prepared from skin. Apoptotic cells (early and late) in skin was 4.21 % with respect to the highest dose (Dose 3) after 7 days while after 30 days it was reduced to a meager amount of only 0.27 % with respect to the same dose. Duplex PCR was also performed targeting 16S rRNA and aerolysin genes of *A. hydrophila* from clinical isolates to further establish the hypothesis that the oxidative stress generated was due to the artificial inoculation of the aforesaid pathogen. The current study presents an insight into the effective immune response elicited by skin in innate immunity in *C. mrigala* artificially challenged with *A. hydrophila*.

Key words: Fish, Histopathology, Oxidative stress, Reactive oxygen species, Apoptosis

INTRODUCTION

Population growth in the subcontinent of India over the last few decades and a concomitant need for food to support this populace has made India one of the sixteen Low-Income Food Deficit Countries (LIFDC) in this world (FAO, 2016). With an aim to provide food in the form of cheap animal protein, aquaculture has become an increasingly important source of sustainable food production in India. The recent expansion of intensive aquaculture practices thus has led to several fish diseases. As a result, aquaculture industry suffers heavy financial losses, mainly due to uncontrolled microbial diseases resulting into mass mortalities (Shao, 2001; Wahli *et al*., 2002; Saksida *et al*., 2006; Almeida *et al*., 2009). Several factors are responsible for such disease outbreaks, viz. environmental conditions, excess feeding, improper rearing methods, water temperature, bacterial growth, irregular water renewal and improper removal of wounded and dead fish from the farming area.

One of the major bacterial diseases causing problem in Indian major carps is Aeromoniasis caused by *A. hydrophila*, a ubiquitous gram negative motile rod

bacterium responsible for primary or stress associated pathogenicity in warm and cold water fishes (Harikrishnan *et al*., 2005; Daskalov, 2006). Dermal ulceration, tail rot, erythrodermatitis, hemorrhagic septicemia, red sore, scale protrusion are important pathological conditions accredited to members of *A. hydrophila* (Saha and Pal, 2000; Dash *et al*., 2008). Aeromonads are also known as potent causative agent of a wide range of diseases in man and other animals (Ghenghesh *et al*., 2008). It is already enlisted in the Contaminant Candidate List and the Environmental Protection Agency has validated its detection and enumeration in drinking water system (USEPA, 2001).

Studies on innate immune response mechanisms against *A. hydrophila* has been carried out extensively in several fish species (Sahu *et al*., 2007; Rodríguez *et al*., 2008; Reyes *et al*., 2011). It is evident that immune defense mechanisms play a critical role in preventing the bacterial disease in fishes (Mohanty *et al*., 2007). Several authors have documented such changes in immune parameters in fish exposed to an array of pathogens (Raida and Buchmann, 2008, 2009; Mohanty and Sahoo, 2010). The host pathogen interaction with special emphasis to *A. hydrophila* has been documented in fishes from various parts of India (Saha and Pal, 2000; Vivekanadhan *et al*., 2005; Dash *et al*., 2008).

In fishes, the innate immunity is an indispensable component in fighting pathogens. Although several studies on immune response in fishes have been performed, information regarding innate immune responses of skin in the Indian major carp, *C. mrigala* (Bloch, 1795) is lacking and hence the present study has been taken up. *C. mrigala* (Bloch, 1795) is one of the commercially important fishes among the Indian Major Carps. These fishes were challenged with three asymptomatic doses of *A. hydrophila* and the impact of oxidative stress in skin was observed. Gross changes were studied at the histopathology level. Reactive oxygen species (ROS) were originally thought to be released only by phagocytic cells during their role in host defense. It is now clear that ROS have a cell signaling role in many biological systems, both in plants and animals. ROS induces programmed cell death, or necrosis, induce or suppress the expression of many genes, and activate cell signaling molecules and cascades, such as those involving mitogen – activated protein kinase (Hancock, 1997).

Hence, the ROS was quantified and we investigated the extent of damage caused by ROS in fish skin using high throughput techniques like flowcytometry. The innate immune response generated in these cells was also studied.

MATERIALS AND METHODS

Experimental Design

Bacterial culture

The bacterial strain, *A. hydrophila*, MTCC 646 has been used in the present was received as a lyophilized culture from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. It was revived by addition of Nutrient Broth and then it was transferred to a Nutrient Agar medium. Consequently, streak plate method was followed to get isolated bacterial colonies.

Fish and aquarium

The fish model chosen for the study was *C. mrigala* (Bloch, 1795) and it was collected from a local fish farm, Matsyajibi Samabyay Samity, Anandapur, Ruby, Kolkata, West Bengal, India. Average body weight and body length was 30 + 2g and 15 $\pm$ 2 cm respectively. They were acclimatized in the laboratory for 7 days. The fishes were fed with Tubifex and the water quality parameters were regularly monitored. Dissolved oxygen (DO) and ammonia were monitored every week, ranging from 5.5 to 7.6 mg/L and 0.5 to 1 ppm respectively, while pH ranged from 7.5 to 8.5 throughout the experimental period. Water temperature was maintained at 26 $\pm$ 2 °C. Water was renewed every day to avoid accumulation of unutilized food or metabolic waste products.

There were four groups of fishes. Each group contained 10 fishes at a time. There were 6 replicates for each group. The first group was the Control and the remaining three groups contained fishes artificially injected with three sub lethal doses of pathogen, (2×10^5 cfu/ml, 2×10^6 cfu/ml and 2×10^7 cfu/ml) individually. The biomarkers chosen for the present study were evaluated after a duration of 7 days and 30 days independently. Altogether, there were 480 fish samples that were used in this study,

240 samples for 7 days and 30 days duration respectively.

Artificial inoculation of fishes with *A. hydrophila*

The bacterial strain was cultured in nutrient broth (NB) and incubated at 37 °C for 24 hours 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 (Ref: OD 600 nm 1 = 2 X 10^9 cfu/ml), following (Pal and Pradhan, 1990). Three sub lethal concentrations (2 X 10^5 cfu/ml, 2 X 10^6 cfu/ml and 2 X 10^7 cfu/ml) were prepared in PS (working volume: 0.5 ml/100gm body weight of fish) and injected intraperitoneally (i.p.) into six replicates of test fish (each replicate containing 10 fishes).

Sampling

Potential fish biomarkers viz. histopathology, quantification of ROS, activity profile of stress enzymes {Super oxide dismutase (SOD) and Catalase (CAT)} and screening of cell death were done on the 7th and 30th day of exposure and were compared with corresponding sham-operated control groups. Clinical isolates were collected from skin of all fishes for presumptive isolation of *A. hydrophila*. There were six replicates for each set and each set contained 10 fishes. The sham operated fishes were injected with sterile PS only.

Histopathological Study

Fish specimens were taken and the skin after removal of the underlying muscles was fixed in aqueous Bouin's fluid for 24 hours. The tissue was then 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 5-7ìm. Sections were stained in Haematoxylin – Eosin. Photomicrographs were taken in Olympus BX 51 compound microscope under oil immersion for further study.

Estimation of Intracellular Reactive Oxygen Species (ROS)

Skin was treated with Collagenase I (2mg/ml) and single cell suspensions were prepared from it at 37 ºC by constant shaking. ROS generation was recorded in these cells following (Datta Ray and Homechaudhuri, 2014). The mean values were considered. Data were analysed using Cell Quest from Becton Dickinson.

Oxidative Stress Enzymes

Superoxide dismutase (SOD) and Catalase (CAT) activity was estimated following (Datta Ray and Homechaudhuri, 2014).

Annexin V/PI study

Flowcytometric analysis was used to distinguish the pattern of cell death between necrosis and apoptosis. Annexin V/PI study was performed with the single cell suspensions prepared from skin following (Datta Ray and Homechaudhuri, 2014) to interpret the nature of cell death that had occurred in them.

Re-isolation and detection of *Aeromonas hydrophila*

Presumptive diagnosis of *A. hydrophila* was done by selective Rimler Shotts (RS) media supplemented with Novobiocin and it was followed by isolation of Genomic DNA from bacteria. Two pairs of primers viz. primer for conserved regions 16S rRNA (Graf, 1999) and aerolysin gene (Santos, 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. The sequences of the chosen primers are given below

Gene	Sequence (5' – 3')	Amplicon size (bp)
Aerolysin	F- GCAGAACCCATCTATCCAG	252
	R- TTTCTCCGGTAACAGGATTG	
16S rRNA	F- TCATGGCTCAGATTGAACGCT	599
	R- CGGGGCTTTCACATCTAACTTATC	

Statistical Analysis

Mean ± SE values have been calculated from the whole data. One way Univariate Analysis of Variance (ANOVA) at 5% level of significance ($p < 0.05$ at df = 3, 36) and Duncan's Post Hoc test was also performed to identify the homogenous means, if any, using SPSS Statistics 24. Paired t – test was performed between observations made after 7 days and 30 days in fishes exposed to three different doses of *A. hydrophila* ($p < 0.05$ at df = 9), (Zar, 1999).

RESULT AND DISCUSSION

Histopathological changes in Skin

The skin is the primary barrier against the environment, allowing normal internal physiological function, so it's condition is important in many disease processes. The layers of teleost skin comprises of cuticle, epidermis, basement membrane, dermis and hypodermis. It is formed largely from epithelial surface cells. The external layer, the cuticle or glycocalyx, was first described by Whitear (1970) as a mucopolysaccharide layer. It contains specific immunoglobulins and lysozyme and free fatty acids which possess anti-pathogen activity, as part of the mucosal defense system of the skin, working in conjunction with cellular proliferation kinetics to continuously remove microorganisms from the surface (Speare and Mirasalimi, 1992).

The structure of the skin among the control samples appears to be the same in Haematoxylin - Eosin staining. However, small number of bacteria was seen on such surfaces among the affected fishes. Section (Figure 1A) shows stratified epidermis containing fibrous Malpighian cell (long arrow) and mucous cells (short arrow). The surface of the outermost layer is arranged in a whirling pattern of micro-ridges. The Malpighian cells are rounded; very similar in structure at all levels except the outermost. Here they are flattened horizontally. Mucus secreting cells are found in the epidermis of all the fishes. The basement membrane is shown in the image.

Section (Figure 1 B) of fish skin showing the striated muscles in longitudinal sections. It also depicts the presence of mucus-secreting cells (small arrow). These cells are active members of the defense system of the body and help to remove pathogens from the surface. In comparison to control samples, bacterial infestation was observed in skin among the affected fishes.

When physiological, histological and morphological parameters in fishes are affected, this generally indicates irreversible damage or disease. These parameters are therefore not suitable as early warning signals in monitoring programs. Histopathological parameters are, however, relatively easily determined and valuable to evaluate the ecotoxicological significance of other biomarkers. The feasibility of using histopathological parameters in fish as a biomarker for aquatic pollution has been reviewed by Hinton (1994).

Ventura and Grizzle (1988) observed changes in skin of *Ictalurus punctatus* infected with *A. hydrophila*. They reported cellular debris, vacuolation in cells, lymphocytes, neutrophils in hypodermis and in between muscle fibres alongwith edema and focal necrosis. Similar reports of alterations in skin were made by Miyazaki *et al*., (2001) in *A. hydrophila* infected *Cyprinus carpio*. Donta and Haddow (1978) have demonstrated that *A. hydrophila* toxins are cytotoxic. Release of bacterial toxins cause acute hemorrhages and necrosis of vital organs (primarily liver and kidney), leading to rapid death due to organ failure (Huizinga *et al*., 1979). They also studied the histopathological alterations in skin of *Micropterus salmoides* infected with *A. hydrophila*. They observed focal hemorrhage, edema, dermal necrosis, mononuclear and granulocytic inflammatory cells, epidermal necrosis, hyperplasia, proliferation of fibrocytes, infiltrating inflammatory cells of dermis, epidermis. The ability of *Aeromonas* species to produce EUS-like lesions following direct inoculation of healthy fish has also been documented.

The skin, with its scales and surface mucus, provides a protective physical barrier that is important in terms of both osmoregulation and pathogen defense. But fish skin is susceptible to damage from handling, fighting, physical trauma, predation, environmental irritants and pathogens and the damage can lead to opportunistic microbial infections. At that stage the stress response may further compromise the host's defenses, via corticosteroid-mediated immunosuppression or other stress-related immunosuppressive factors (Harris *et al*., 2000). Although fish skin has not been reported extensively

as a stress response target, dermal ulceration was the chief finding in a series of studies in which striped bass, *Morone saxatilis* and striped bass hybrids were exposed to acute confinement stress (Noga *et al.*, 1998; Udomkusonsri *et al.*, 2004). Associated histopathologic lesions, in addition to rapidly occurring epithelial erosions and ulcers that primarily affected the fins, included epithelial cell swelling, edema of the dermis and hypodermis, melanophore aggregation, and stromal tissue necrosis.

Estimation of Oxidative stress in Skin and study of innate immune response

The cell is armed with a powerful antioxidant defense system to combat excessive production of ROS. Oxidative stress occurs in cells when the generation of ROS overwhelms the cells' natural antioxidant defenses. ROS and the oxidative damage are thought to play an important role in many diseases. Thus, establishing their precise role requires the ability to measure ROS accurately and the oxidative damage that they cause. There are many methods for measuring free radical production in cells. The most straight forward technique uses cell permeable fluorescent and chemiluminescent probe 22 - 72 - Dichlorodihydrofluoresceindiacetate (DCFH-DA). This is one of the most widely used techniques for directly measuring the redox state of a cell. It has several advantages over other techniques. It is very easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive and can be used to follow changes in ROS over time.

The potential of *A. hydrophila* to generate oxidative stress in terms of intracellular ROS production and its consequences following a one month recovery period was analyzed by flow cytometry. Single cell suspensions prepared from skin of *C. mrigala* exposed to four different doses of artificial inoculation of pathogen were labeled with H_2DCFDA. Results show an approximate 4.78, 5.67 and 7.16 fold increase of the oxidized DCF mean peak in case of fishes injected with Dose 1, 2 and 3 respectively, compared to the sham operated control (Dose 0). This is a clear indication of higher H_2O_2 in the treated fishes in comparison to the control.

After a span of 30 days, during which the fishes were maintained in a stress free condition *ad libitum*, the ROS was quantitated again and results showed an approximate 1, 1.02 and 1.03 fold increase in the DCF mean peak in case of fishes injected with Dose 1, 2 and 3 respectively, compared to the control (Dose 0). Thus, it can be inferred that an innate immune response functions in *C. mrigala* which tries to combat the bacterial infection. The amount of ROS generation was almost the same as in the control specimen.

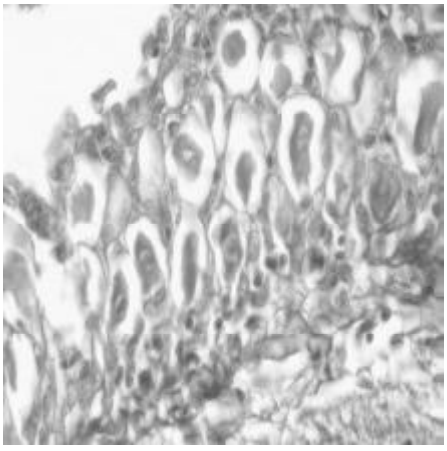

Fig. 1A. Histopathology of Skin in *Cirrhinus mrigala* control sample. Haematoxylin and Eosin staining [x 600].

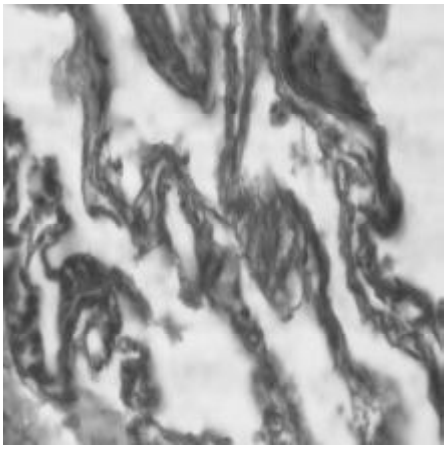

Fig. 1B: Histopathology of Skin in *Cirrhinus mrigala* treated sample. Haematoxylin and Eosin staining [x 600]

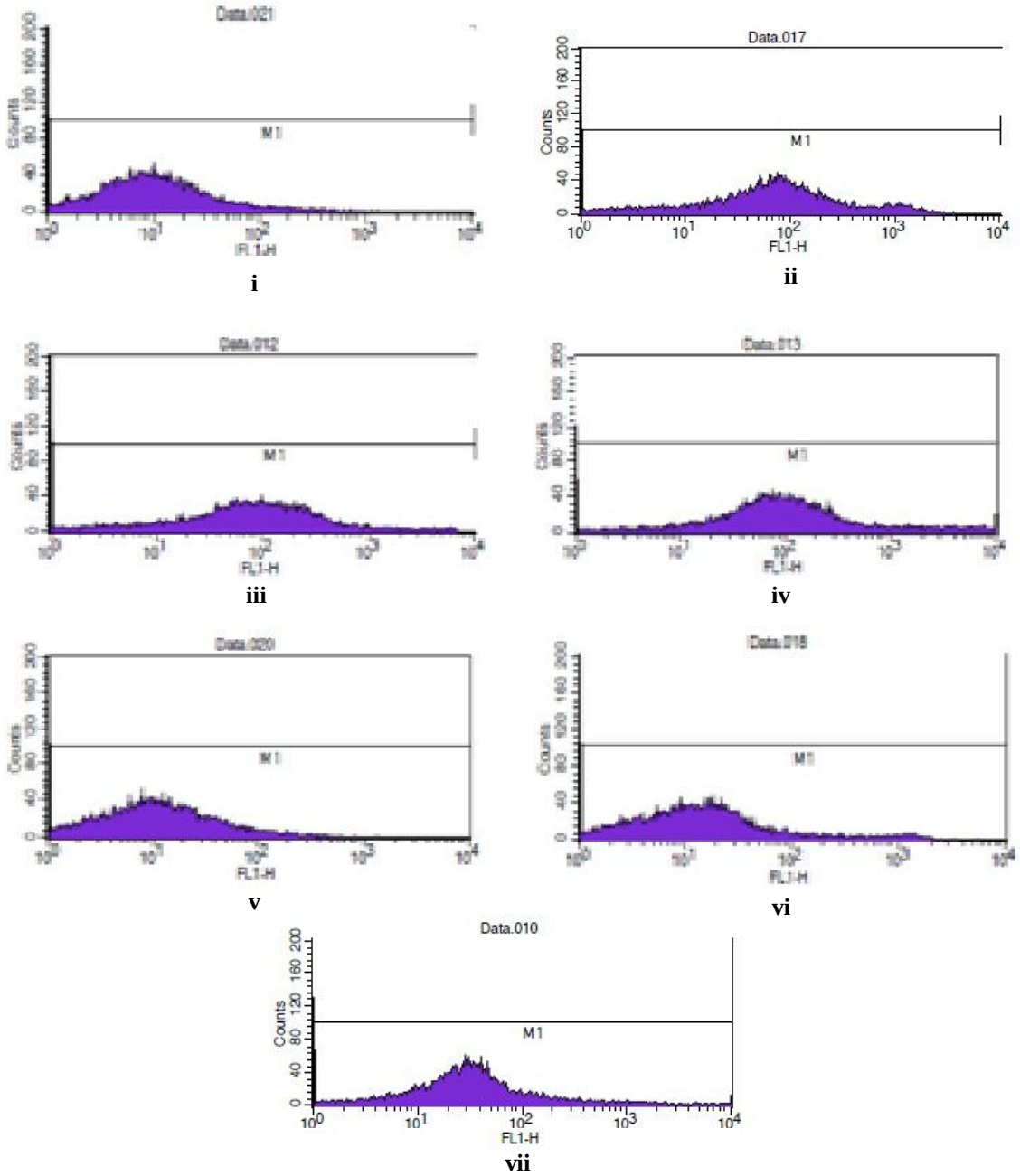

Fig. 2. Effect of Immune Response on *Aeromonas hydrophila* induced ROS production in *Cirrhinus mrigala*. Intracellular accumulation of ROS measured in cells of skin isolated from fishes injected with: (i) dose 0, sham operated control; (ii) dose 1, exposed for 7 days (iii) dose 2, exposed for 7 days; (iv) dose 3, exposed for 7 days; (v) dose 1, exposed for 30 days; (vi) dose 2, exposed for 30 days; (vii) dose 3, exposed for 30 days

Intracellular changes with reference to the generation of reactive oxygen species in the single cell suspensions prepared from fish skin have been analyzed statistically to construe the observations made at the cellular level. The oxidative stress has been quantified in the fishes injected with three different doses of bacteria after 7 days and 30 days respectively. Sham operated control was considered in each case. Significant difference was found in ROS generated among fish populations of *C. mrigala* injected with four different doses (Sham operated control, 2 X 10^5cfu/ml, 2 X 10^6 cfu/ml and 2 X 10^7cfu/ml) ($p < 0.05$ at df = 3, 36).

When results obtained after 7 days was analyzed by Duncan's Post Hoc Test for homogeneity, it showed the presence of 4 subsets. This indicates that there was a significant difference in the generation of ROS with respect to all the four doses when they were compared pair-wise. However, when the same study was performed with results obtained after 30 days, only a single subset was obtained indicating that there is no significant difference in the amount of ROS generation in fishes injected with Dose 0, 1, 2 and 3 respectively.

This study shows that the generation of ROS in skin cells is an act of self defense, as part of the innate immune response generated in fishes when they are subjected to pathogenic infection. The *in vivo* immune response was significantly observed in these cells as homeostasis was restored within a span of 30 days. And thus, when ROS was estimated after 30 days, the amount of ROS generation was meager in contrast to the initial amount of oxidative stress (Table 1A). It is pertinent to mention here that the stress enzyme pathway which results in the increased activity and concentrations of all the oxidative stress enzymes viz. SOD, CAT, GPx, GR and GSH plays a crucial role in combating the increased level of ROS production within the melanocytes to prevent the deleterious effects of the oxidative stress generated due to the pathogenic effect of *A. hydrophila* which was injected at three asymptomatic carrier state dosages.

Study of Antioxidant Enzymatic activities

The Superoxide dismutase (SOD) and Catalase (CAT) activity has been recorded in the skin cytosol of fishes injected with four different doses of bacteria. The Mean $\pm$ S.E. values have been shown in Table 1B. Significant difference was found in Superoxide dismutase and Catalase activity among fish populations of *Cirrhinus mrigala* injected with 4 different doses (Sham operated control, 2 X 10^5 cfu/ml, 2 X 10^6 cfu/ml and 2 X 10^7 cfu/ml) ($P < 0.05$ at df = 3) when observed after 7 days.

Duncan's post hoc test revealed the presence of 4 subsets. This indicates that there is a significant difference in the Superoxide dismutase and Catalase activity with respect to all the four doses when they were compared pair - wise.

However, when the same study was performed with respect to both the enzymes after 30 days, no significant difference was observed between control and treated samples. Duncan's post hoc test revealed the presence of only a single subset for both SOD and CAT when observed after 30 days, clearly supporting the findings.

Study of cell death and nature of innate immune response

Histopathology revealed certain cellular damage in skin of treated samples in comparison to sham operated control which appeared normal. Cell damage was also associated with reported cell death, analyzed by Trypan Blue Exclusion Test of Cell Viability. The maximum extent of such damage was observed in fishes exposed to the highest dose of the pathogen (Dose 3: 2 X 10^7cfu/ml) in comparison to Dose 1 and Dose 2. Further study was taken up to ascertain the nature of such cell death that was observed among the cells of skin and it was identified as Apoptosis by flowcytometric analysis.

Flowcytometric study revealed the presence of 3 sub-population of cells in the cytogram viz Normal cells (Annexin V^-/PI^-), Early apoptotic cells (Annexin V^+/PI^-) and Late apoptotic cells (Annexin V^+/PI^+). This analysis was done initially after 7 days and was then repeated after 30 days of recovery period to ascertain the extent of damage that was caused initially and the intensity of innate immune response that was triggered inside the host body to combat the pathogenic infection that was caused by the artificial inoculation of the pathogen. The results obtained have been tabulated in Table 2A.

Single cell suspensions of skin from fishes exposed to different doses of the pathogen MTCC 646 (Dose

Table 1A. Changes in oxidative stress was measured by the quantification of Reactive oxygen species (ROS) in single cell suspensions of skin of *Cirrhinus mrigala* due to experimental inoculation by carrier state doses of *Aeromonas hydrophila* (MTCC 646) exposed for 7 and 30 days. Dose of bacteria are given @ 0.5ml/100gm of body weight of fish; Mean ± SE values have been shown in the Table; there were 10 fishes for each set and six replicates per set up; similar alphabets within column denote homogeneous means due to Duncan's Post Hoc test at 5 % level of significance ($p < 0.05$ at df = 3, 36).

Duration of experiment	Skin samples	Mean ± SE
7 DAYS	Dose 0 Sham operated Control	11.97 ± 0.01^{a}
	Dose 1 2×10^5cfu/ml	57.25 ± 0.01^{b}
	Dose 2 2×10^6cfu/ml	67.93 ± 0.01^{c}
	Dose 3 2×10^7cfu/ml	85.82 ± 0.02^{d}
30 DAYS	Dose 0 Sham operated Control	11.95 ± 0.01^{aa}
	Dose 1 2×10^5cfu/ml	12.06 ± 0.01^{aa}
	Dose 2 2×10^6cfu/ml	12.21 ± 0.01^{aa}
	Dose 3 2×10^7cfu/ml	12.27 ± 0.01^{aa}

Table 1B. Changes in Superoxide dismutase and Catalase activity in skin cytosol in adult *Cirrhinus mrigala* due to experimental inoculation by carrier state doses of *Aeromonas hydrophila* (MTCC 646) exposed for 7 days and 30 days. Dose of bacteria are given @ 0.5ml/100gm of body weight of fish; (Mean ± S.E.); 10 fishes for each set and there were six replicates per set up; similar alphabets within column denote homogeneous means due to Duncan's test at 5 % level of significance.

Experimental groups	SOD (U mg $^{-1}$) Mean ± SE		CAT (U mg $^{-1}$ min $^{-1}$) Mean ± SE	
	7 Days	30 Days	7 Days	30 Days
Dose 0 Sham operated control	111.52 ± 0.03^{d}	110.51 ± 0.03^{a}	3.54 ± 0.04^{d}	3.21 ± 0.02^{a}
Dose 1 2×10^5 cfu/ml	132.55 ± 0.39^{c}	109.55 ± 0.02^{a}	4.88 ± 0.05^{c}	3.19 ± 0.03^{a}
Dose 2 2×10^6 cfu/ml	145.43 ± 0.15^{b}	108.58 ± 0.04^{a}	5.95 ± 0.01^{b}	3.22 ± 0.01^{a}
Dose 3 2×10^7 cfu/ml	156.9 ± 0.10^{a}	110.52 ± 0.02^{a}	7.09 ± 0.05^{a}	3.23 ± 0.01^{a}

Table 2A. Evaluation of apoptotic potential of *Aeromonas hydrophila* in Skin of *Cirrhinus mrigala*. Single cell suspensions of skin from fishes exposed to different doses of the pathogen MTCC 646 (Dose 0, 1, 2 and 3) were analysed after 7 days for acute toxic effect and after 30 days for understanding the mode of recovery in these fishes. Similar alphabets within column denote homogeneous means due to Duncan's test at 5 % level of significance ($p < 0.05$). Single alphabet in the columns denotes results analyzed after 7 days while double alphabets depict results analyzed after 30 days. Paired t-test was performed for early apoptotic cells for Doses 1, 2 and 3 respectively for 7 days and 30 days exposure. Mean ± SE values have been shown here. Significant difference was observed in early apoptotic cell samples ($p < 0.05$) at df = 9 collected after 7 days and 30 days duration with respect to all the three doses.

Duration of experiment	Skin samples	Normal Cells (%) (Annexin V^-/PI^-)	Early Apoptotic cells (%) (Annexin V^+/PI^-)	Late Apoptotic & Necrotic cells (%) (Annexin V^+/PI^+)
7 DAYS	Dose 0 Sham operated Control	99.24	0.75^{a}	0.01^{a}
	Dose 1 2 X 10^5cfu/ml	99.04	0.96^{b}	0.00^{a}
	Dose 2 2 X 10^6cfu/ml	97.41	2.56^{c}	0.01^{a}
	Dose 3 2 X 10^7cfu/ml	95.79	3.85^{d}	0.34^{b}
30 DAYS	Dose 0 Sham operated Control	99.25	0.24^{aa}	0.02^{bb}
	Dose 1 2 X 10^5cfu/ml	99.66	0.23^{aa}	0.01^{bb}
	Dose 2 2 X 10^6cfu/ml	99.25	0.23^{aa}	0.05^{bb}
	Dose 3 2 X 10^7cfu/ml	99.03	0.25^{aa}	0.00^{bb}

Table 2B.

Type of Cells	Sample Groups	Dose 1 (Mean ± SE)	Dose 2 (Mean ± SE)	Dose 3 (Mean ± SE)
Early Apoptotic Cells	7 days	0.96 ± 0.01	2.56 ± 0.01	3.85 ± 0.01
	30 days	0.23 ± 0.01	0.23 ± 0.01	0.25 ± 0.01
	***t* value**	* 52.31	* 161.52	* 414.66

0, 1, 2 and 3) were analysed after 7 days for acute toxic effect and after 30 days for understanding the mode of recovery in these fishes. Similar alphabets within column denote homogeneous means due to Duncan's test at 5 % level of significance ($p < 0.05$). Single alphabet in the columns denotes results analyzed after 7 days while double alphabets depict results analyzed after 30 days. Paired t-test was performed for early apoptotic cells for Doses 1, 2 and 3 respectively for 7 days and 30 days exposure. Mean ± SE values have been shown here. Significant difference was observed in early apoptotic cell samples ($p < 0.05$) at df = 9 collected after 7 days and 30 days duration with respect to all the three doses.

The dot plots enumerate the percentage increase of apoptotic cells among treated fishes in comparison to control. The amounts of healthy cells were 99.24%, 99.04%, 97.41% and 95.79% for control, fishes injected with Dose 1, Dose 2 and Dose 3 respectively. The amounts of early apoptotic cells were 0.75%, 0.96%, 2.56% and 3.85% for control, fishes injected with Dose 1, Dose 2 and Dose 3 respectively. And the amounts of late apoptotic cells were 0.01%,

0.00%, 0.01%, and 0.34% for the same doses respectively. At lower doses (Dose 1 and Dose 2), the amounts of early and late apoptotic populations were less significant but upon increment of dose the amount of apoptotic population had increased dramatically.

After 30 days of recovery, the percentage of normal, healthy cells increased to about 99.66%, 99.25% and 99.03% in case of fishes injected with Dose 1, Dose 2 and Dose 3 respectively. The number of early apoptotic cells decreased to 0.23%, 0.23% and 0.25% in comparison to the earlier report after 7 day study. Similarly the percentage of late apoptotic cells also became very low approximately 0.01%, 0.05% and 0.00% for fishes injected with Dose 1, Dose 2 and Dose 3 respectively.

Study shows that *A. hydrophila* is capable of inducing apoptosis in skin of *C. mrigala*. The extent of damage caused in directly proportional to the bacterial dose injected to the fishes. There was a clear dose dependent pattern of damage among the fishes. When results obtained after 7 days and 30 days were analysed statistically by Duncan's post hoc test, study revealed different subsets for acute and chronic studies.

As shown in Table 2A, similar alphabets within column denote homogeneous means due to Duncan's post hoc test at 5 % level of significance ($p < 0.05$). Single alphabet in the columns denotes results analyzed after 7 days while double alphabets depict results analyzed after 30 days. Analysis of data obtained after 7 days shows four subsets for the early apoptotic cells while the late apoptotic cells belonged to two subsets. The highest dose belonged to a single subset indicating that there is a significant difference between this group and others when compared pairwise. While late apoptotic cells with reference to Dose 0, 1 and 2 belonged to a single subset.

After 30 days, the early apoptotic cells belonged to a single subset; similar observation was made with reference to the late apoptotic cells with reference to all the four doses. Thus, this study reveals in vivo immune response in skin of the Indian major carp, *C. mrigala*.

Paired t-test was also performed for the early apoptotic cells for Doses 1, 2 and 3 respectively for 7 days and 30 days exposure. Mean $\pm$ SE values have been shown in Table 2 B. Significant difference was observed in early apoptotic cell samples ($p < 0.05$) at df = 9 collected after 7 days and 30 days duration with respect to all the three doses.

Re-isolation and detection of pathogen by Duplex PCR

To confirm the fact that successful establishment of the infection was caused by experimental challenge with bacterial pathogen *A. hydrophila* and to conclusively state that all the changes have occurred due to the pathogen, re-isolation of the bacterial colonies from clinical isolates was employed. After 7 days of exposure, post bacterial infection by intraperitoneal mode at asymptomatic level, tissue homogenates were prepared from skin and was plated onto RS media supplemented with Novobiocin which resulted in the production of bright yellowish colonies indicating and confirming the presence of *A. hydrophila.* After isolation of bacterial genomic DNA from those colonies PCR was performed. Electrophoretic analysis of the PCR product revealed the specific amplifications of 599 bp and 252 bp fragments without any spurious product for both the primers targeted against 16S rRNA and the aerolysin gene (Figure 3). These findings are in accordance with the previous results obtained (Gonzalez – Rodriguez *et al*., 2002).

Several other studies have been done with different serotypes of *A. hydrophila* which showed the presence of variable band lengths of targeted genes (Porteen *et al*., 2006; Porteen *et al*., 2007). In this study, we have standardized the Duplex PCR using primers targeted at 16 S rRNA and aerolysin gene (Santos *et al*., 1999; Graf *et al*., 1999) for the identification of the strain MTCC 646 (*A. hydrophila*).

CONCLUSION

The apoptotic nature of *A. hydrophila* has been observed earlier in *C. mrigala* with reference to vital organ in the body viz. liver (Datta Ray and Homechaudhuri, 2014) and immunocompetent organs like Head kidney and Spleen (Datta Ray and Homechaudhuri, 2016) but information of such damage in the fish skin is being reported for the first time. *A. hydrophila* deploys a range of virulence determinants, which interact with the key components of the cell death pathway of the host or

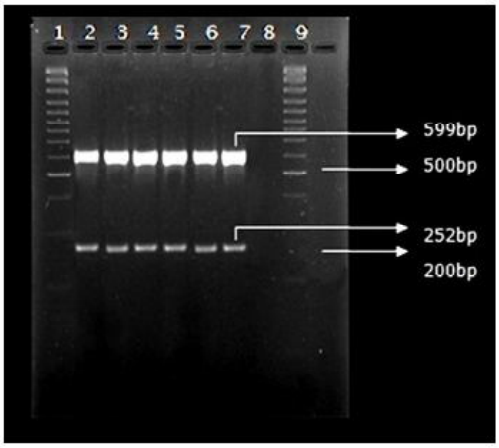

Fig. 3. *Aeromonas hydrophila* (MTCC 646) showing product obtained from primer for 16S rRNA (599 bp) & Aerolysin (252 bp). Where, Lane 1 & 9:100 bp ladder (Fermentas); Lane 2 & 3: PCR product obtained from DNA isolated from *Aeromonas hydrophila* colonies recovered from Skin tissue of *Cirrhinus mrigala* challenged with Dose 1 (7 days and 30 days Exposure); Lane 4 & 5: PCR product obtained from DNA isolated from *Aeromonas hydrophila* colonies recovered from Skin tissue of *Cirrhinus mrigala* challenged with Dose 2 (7 days and 30 days Exposure); Lane 6 & 7: PCR product obtained from DNA isolated from *Aeromonas hydrophila* colonies recovered from Skin tissue of *Cirrhinus mrigala* challenged with Dose 3 (7 days and 30 days Exposure); Lane 8: PCR negative control.

interferes with the regulation of such transcription factors that monitor cell survival. This aims to evade, counteract or restrain host defenses. In conclusion, the findings of this study shows that the cultured fish species, *C. mrigala* that are infected with *A. hydrophila* at an asymptomatic carrier state are physiologically impaired and this could be detected by quantification of the oxidative stress biomarker (ROS) and assessment of the extent of cellular damage in skin can be done using flowcytometry as a molecular tool with high throughput. The pathogenic effects such as oxidative stress generation and resulting apoptotic cell death due to bacterial infection is more pronounced for short term exposure and for the highest bacterial dosage.

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