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RESEARCH PAPER

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Aeromonas hydrophila Induced Mitochondrial Dysfunction and Apoptosis in Liver and Spleen of *Labeo rohita* Mediated by Calcium and Reactive Oxygen Species

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Abstract

The study shows effect of oxidative stress on liver and spleen of *L. rohita* (Hamilton 1822) challenged with an asymptomatic dose (3×10^7 CFU/ mL) of *Aeromonas hydrophila*. Upon termination of the experiment, intracellular Reactive Oxygen Species (ROS), anti-oxidant enzyme activities of Super Oxide Dismutase (SOD) and Catalase (CAT), anti-oxidative enzyme concentrations of Glutathione Peroxidase (GPx), Glutathione Reductase (GR), Glutathione (GSH) were recorded where an organ specific pattern of significant changes were observed between control and treated samples. Study revealed apoptotic cell death in both organs where early and late apoptotic cells increased significantly. Mitochondrial membrane potential assay reflected higher percentage of depolarized cells in treated samples. Cytosolic free Ca^{2+} level was significantly higher in liver and spleen post infection and was also associated with significant increase in Caspase 3 gene expression. Evidences of severe oxidative stress in fishes resulting from an asymptomatic dose eventually causing apoptotic cell death were reported. It was observed to be mediated by effector Caspase 3 which was triggered by a complex interplay between mitochondrial dysfunction and high level of cytosolic Ca^{2+}. Further, it indicated an intriguing observation that liver is more susceptible towards the pathogen and shows more significant changes in comparison to spleen.

Introduction

Aquaculture industries frequently suffer heavy financial losses that threaten their growth and sustainability, sometimes due to uncontrolled microbial diseases resulting into mass mortalities (Almeida et al., 2009; Shao, 2001; Wahli et al., 2002). One of the major bacterial diseases, causing problems in *Labeo rohita* (Hamilton 1822) farming is Aeromoniasis caused by *Aeromonas hydrophila*, a ubiquitous gram negative bacterium responsible for primary or stress associated pathogenicity in warm and cold water fishes (Daskalov, 2006; Harikrishnan, Balasundaram, & Bhuvaneswari, 2005; Tellez-Bañuelos, Santerre, Casas-Solis, & Zaitseva, 2010). Aeromonas species are known as causative agents of a wide spectrum of diseases in man and other animals (Ghenghesh, Ahmed, El-Khalek, Al-Gendy, & Klena, 2008). It is enlisted in the Contaminant Candidate List and the Environmental Protection Agency (EPA) has validated its detection and enumeration in drinking water system (USEPA, 2001).

The host pathogen interaction with special emphasis to *Aeromonas hydrophila* has been documented in various fishes (Dash et al., 2008; Datta Ray & Homechaudhuri, 2016; Mohanty & Sahoo, 2010; Raida & Buchmann, 2008, 2009; Vivekanandhan, Hatha, & Lakshmanaperumalsamy, 2005) and in humans (Galindo, Sha, Fadl, Pillai, & Chopra, 2006) from various parts of India.

However, the exact mechanism by which *Aeromonas hydrophila* modulates the host immune response in fish to their advantage and the specific factors that contribute to the pathogenesis are poorly understood. The knowledge of fish immune systems are meagre 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 sizes. 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 multiple biomarker approach for early detection at sub-clinical stage or at carrier state i.e. where, there is successful establishment of the bacterial infection without any external morphological lesion is very important.

The aim of the study was to understand the intricate interrelationship between pathogen induced oxidative stress and the cascade of immune response it elicits. An evaluation of the pathogenic effect of *Aeromonas hydrophila* infection on *Labeo rohita* at a carrier state was aimed by quantification of intracellular reactive oxygen species (ROS), study of activity of important antioxidant/detoxifying enzymes in both liver, the primary target organ and spleen, one of the primary immune-competent organ in fishes. Flow cytometric study was performed to characterize the cascade of events taking place at the cellular level. Finally, semi-quantitative gene expression of effector Caspase 3 with reference to the house keeping β-actin was studied from the selected organs to understand the cell death pattern.

Materials and Methods

Bacterial Culture Collection and Dosage Preparation

Bacterial strain, *Aeromonas hydrophila* subsp. *hydrophila*, MTCC 646 was collected from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The lyophilized culture was revived in Nutrient Broth (at 37ºC for 24 h) and transferred to a Nutrient Agar medium. Consequently, streak plate method was followed to get desired single isolated colonies on selective Rimler Shott's (RS) media supplemented with Novobiocin. For dosage preparation, the bacterial strain was inoculated in nutrient broth (NB) and incubated at 37°C for 24 h followed by centrifugation at 5000 x *g* for 5 min and washing 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).

Fish Acclimatization and Maintenance

Labeo rohita fingerlings (body weight±30 gm and total length±15 cm) were collected from local fish farm in Kolkata, West Bengal, India. A number of 10 fishes were kept in each glass aquarium (2 ft x 1ft x 1ft) in the Animal House and were acclimatized for seven days. *Tubifex* and *Artemia* were given as live feed. Various water quality parameters were regularly supervised throughout the experiment. Dissolved oxygen (DO_2) and ammonia were monitored every week, ranging from 5.5 to 7.6 mg O_2/ 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±2°C.

Experimental Setup

Fishes were injected intraperitoneally (i.p.) with a carrier state dose (3 x 10^7 CFU/ mL) of *Aeromonas hydrophila*, made up in PS (working volume: 0.5 mL 100 gm/body weight of fish) following Roy, Pal, Datta Ray, and Homechaudhuri (2018). Asymptomatic carrier dose was determined through changes in several haematological and serum biochemical parameters on the seventh day of exposure (Pal, Datta Ray, & Homechaudhuri, 2015). A total number of 180 fishes were used in the course of the overall experiment to study the different parameters. Fishes were divided into three groups as follows: Standard control (without any injection), sham operated control (intraperitoneal injection of sterile culture media) and *Aeromonas hydrophila* treated. For every group there were 6 replicates, each containing 10 fishes.

Flow Cytometric Analysis

Single cell suspensions were prepared from liver and spleen separately, following Datta Ray and Homechaudhuri (2014) with slight modifications and the following assays were performed. For each set, a total of 10,000 even counts were taken. All flow cytometric data were analyzed using BD Accuri C6 Software.

Reactive Oxygen Species (ROS): Quantification of ROS was done using 2′-7′ - Dicholoro dihydro fluorescein diacetate (H_2-DCFDA) following Datta Ray and Homechaudhuri (2014) with slight modifications.

Antioxidant enzymes activity and concentration: Superoxide dismutase (SOD) and Catalase (CAT) were estimated following Aebi (1984) and Den Besten, Munawar, and Suter (2007) respectively. In-vitro quantitative measurement of Glutathione peroxidase (GPx), Glutathione reductase (GR) and Glutathione (GSH) in the target organs of both control and treated fishes were performed following Lukaszewicz-Hussain and Moniuszko-Jakoniuk (2004).

Annexin V-FITC / PI assay: Nature of cell death in liver and spleen was ascertained using Annexin V and Propidium iodide following Datta Ray and Homechaudhuri (2014) with slight modifications.

Mitochondrial Membrane Potential

Mitochondrial depolarization in liver and spleen cells was done using JC – 1 dye following Zeng, Cheng, Jiang, and Han (2008) with some modifications.

Intracellular (Cytosolic free Ca^{2+}) levels: Ca^{2+} level $[Ca^{2+}]_c$ in liver and spleen cells was measured by Fluo-3 AM, following Mironov, Ivannikov, and Johansson (2005) with some modifications.

RNA extraction: RNA was extracted from 50-100 mg tissue samples were taken from sham injected control and *A. hydrophila* treated fishes using Takara RNA – Iso Plus following standard protocol (Sambrook, Russell, & Russell, 2001). Total RNA concentration and purity was observed by measuring OD at 260 and 280 nm using Microdrop (Multi Skan Go, Thermoscientific, USA).

cDNA Synthesis

Total RNA (5 µg) was used for first strand cDNA synthesis using thermocyler (Applied Biosystems). RNA primer reaction mixture was prepared in a microtube using Random Hexamers (50 µM), dNTP mixture (10 mM each), template RNA and RNase free dH_2O following protocol as provided in the PrimeScript c-DNA Synthesis Kit – 6110A (Takara Bio Inc). The cDNA samples were kept at 0^oC for further use.

Primer Details and Product Size

Multiple primers were designed for the gene of interest using Fast PCR software (Informer Technologies, Inc.). Standard desalted primers were custom made from Integrated DNA Technologies (IDT). The sequences of the chosen primers are given below in Table 1.

Gene expression Analysis of Immune Related Genes

The relative levels of expression of each gene were analyzed by densiometry using ImageJ software (US-NIH, USA). The ratios of immune related gene/β-Actin product were subsequently calculated after subtraction of the background pixel intensity of each gene of interest and used to assess the differences in expression levels between control and the infected samples.

Re-isolation and Detection of *A. hydrophila*

The pathogen was reisolated from clinical isolates of liver and spleen of both treated and control specimens following Datta Ray and Homechaudhuri (2014). The sequences of the chosen primers are given in Table 2.

PCR conditions for different genes: The initial denaturing temperature, PCR cycle conditions

Table 1: Primer sequences for selected genes.

Gene	Sequence (5' – 3')	Amplicon size (bp)
β-Actin	F- GACTTCGAGCAGGAGATGG R- CAAGAAGGATGGCTGGAACA	138
Caspase 3	F- ATGAACGGAGACTGCGTGGA R- TGGTGAGCATCGAGACAATGCAGG	285

Table 2: Primer sequences of selected genes for identification of *A. Hydrophila*

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

Table 3: PCR conditions of selected genes for detection of *A. hydrophila*

Gene	PCR CONDITIONS				
	Initial denaturation	Denaturation	Annealing	Strand Elongation	Final extension
			PCR cycles *		
Aerolysin	94^oC, 5 minutes	94^oC, 1 minute	55^oC, 1 minute	72^oC, 1 minute	72^oC, 10 minutes
16S rRNA	94^oC, 5 minutes	94^oC, 1 minute	55^oC, 1 minute	72^oC, 1 minute	72^oC, 10 minutes

* Number of PCR cycles for each reaction: 30 cycles

(denaturing temperature, annealing temperature, temperature during strand elongation) and final elongation, for screening the different genes, were determined by trial and error methods after multiple trials using a range of temperatures from 90°C - 95°C (for denaturation), 45°C - 60°C (for annealing) and 65°C to 75°C (for elongation). The best results were obtained at the specific temperatures and timings mentioned in Table 3.

Statistical analysis

Means and Standard Error (S.E.) of the means were calculated from the whole range of data following Zar (1999). All data obtained were analysed using Student's t-test (P<0.05 as significant level). Test of homogeneity of variances by Levene statistics was performed for all the data with respect to different parameters observed (P<0.05 as significant level). Statistical calculations were performed using SPSS Statistics 17.0

Results and Discussion

Quantification of the Reactive Oxygen Species

Pathogenic effect of *A. hydrophila* on the production of Reactive oxygen species (ROS) in liver and spleen in both sham operated and experimental *L. rohita* is summarized in Figure 1. An approximate 4.78 fold increase is observed in the oxidized DCF mean peak in hepatocytes of fishes injected with the pathogen in comparison to sham operated control. However, in spleen, an approximate 1.46 fold increase is observed between sham operated control and treated samples. A significant difference in the ROS production between sham operated control and treated samples was observed both in liver and spleen. The higher rate of ROS production in liver of infected fishes clearly suggests that it is at a greater risk of undergoing oxidative stress in comparison to spleen.

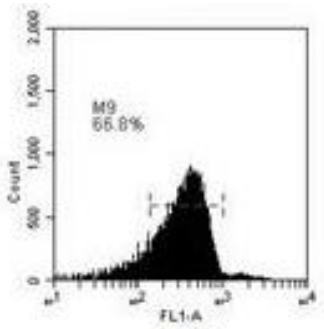

Fig 1a

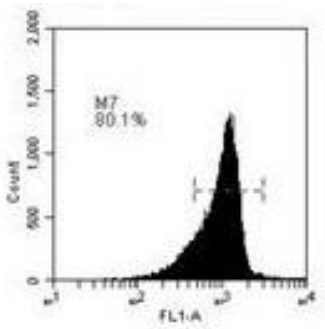

Fig 1b

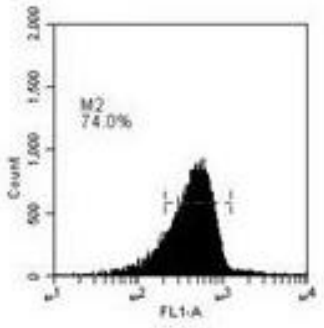

Fig 1c

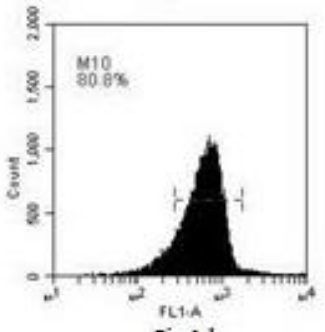

Fig 1d

Reactive Oxygen Species (ROS) production	Sample groups	Liver (Mean ± SE)	Spleen (Mean ± SE)	*t* - value
Mean FL1-A	Control	341.8 ± 0.25	621.8 ± 0.26	793.72 *
	Treated	1631.6 ± 0.27	906.8 ± 0.25	1984.95 *
	t - value	3532.26 *	807.90 *	

* Significant (p < 0.05)

Figure 1: Estimation of Intracellular ROS, where oxidized mean peak has been plotted along the FL1 channel. Fig 1a depicting the mean FL1-A value to be 341.8 ± 0.25 in the control liver sample. Figure 1b showing a higher mean FL1-A value of 1631.6 ± 0.27 i.e. a 4.77 fold increase of the oxidized DCF mean peak in the liver of infected fishes. Figure 1c depicting the mean FL1-A value to be 621.8 ± 0.26 in the control spleen sample. Figure 1d showing a higher mean FL1-A value of 906.8 ± 0.25 i.e. a 1.46 fold increase of the oxidized DCF mean peak in the spleen of infected fishes.

Table 4a: Comparison of stress enzyme activities between liver and spleen in control and *A. hydrophila* infected *L. rohita* after 7 days exposure

Anti-oxidative enzyme activities		Liver (Mean ± SE)	Spleen (Mean ± SE)	*t* - value
SOD (U/mg protein/min)	Control	112.68 ± 0.71	94.09 ± 0.66	19.2 *
	Treated	151.57 ± 0.41	129.47 ± 0.47	35.41` *
	t - value	47.45 *	43.57 *	
CAT (K/mg protein/min)	Control	3.55 ± 0.09	2.80 ± 0.08	6.25 *
	Treated	7.02 ± 0.05	5.86 ± 0.08	12.69 *
	t - value	34.73 *	27.12 *	

*** Significant (*P*< 0.05); SOD:** Super oxide dismutase, **CAT:** Catalase

Table 4b: Comparison of stress enzyme concentration between liver and spleen in control and *A. hydrophila* infected *L. rohita* after 7 days exposure

Anti-oxidative enzyme concentration		Liver (Mean ± SE)	Spleen (Mean ± SE)	*t* - value
GPx (U/ml)	Control	46.13 ± 0.69	47. 21 ± 0.59	1.20
	Treated	88.91 ± 0.15	61.26 ± 3.38	7.90 *
	t - value	76.51 *	6.16 *	
GR (ng/ml)	Control	47.30 ± 0.65	23.76 ± 0.20	29.94 *
	Treated	62.64 ± 3.51	27.89 ± 0.44	8.35 *
	t - value	5.73 *	8.78 *	
GSH (ng/ml)	Control	4.41 ± 0.11	4.30 ± 0.05	0.92
	Treated	7.21 ± 0.06	5.12 ± 0.03	33.63 *
	t - value	30.36 *	10.97 *	

*** Significant (*P*< 0.05); GPx:** Glutathione Peroxidase; **GR:** Glutathione Reductase; **GSH:** Reduced Glutathione

Antioxidant Enzyme Activity

In Table 4 the alteration of super oxide dismutase (SOD) and catalase (CAT) activities in sham operated control and *A. hydrophila* infected *L. rohita* has been depicted. SOD activity was observed to be significantly increased in liver (151.57±0.41) and spleen (129.47±0.47) of infected fish in comparison to sham operated control samples of liver (112.68±0.71) and spleen (94.09±0.66). The increased CAT activity in liver (7.02±0.05) and spleen (5.86±0.08) is also recorded in the infected fishes in comparison to sham operated control samples of liver (3.55±0.09) and spleen (2.80±0.08) upon termination of the experiment. A similar pattern *i.e.* significant increase in the concentration level of other detoxifying enzymes *viz.* glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione (GSH) are also observed in liver and spleen of *A. hydrophila* treated *L. rohita* in comparison to sham operated control fishes.

In the present study, the significant increase in SOD activity in liver and spleen of *A. hydrophila* infected *L. rohita* in comparison to sham operated control fish can be attributed to the fact that SOD being an enzyme whose function is to protect erythrocyte membrane against oxidation by decomposition of superoxide radicals (O_2^-) generated due to the inherent protective mechanism working against the bacterial pathogen both in liver and spleen. Significant increase in GPx level and CAT activity in liver and spleen of *A. hydrophila* infected *L. rohita* in comparison to sham operated control fish can be ascribed to the fact that both GPx and CAT functions by decomposing H_2O_2 (generated by the functioning of SOD) into H_2O and molecular O_2 ($^1/_2\ O_2$) in liver and spleen and in the process maintain H_2O_2 concentration in these tissues. Oxidized glutathione (GSSG) is produced upon reduction of hydrogen peroxide by GPx which is recycled to its reduced state by GR and NADPH. Significant increase in GR level in liver and spleen of *A. hydrophila* infected *L. rohita* in comparison to sham operated control fish can be recognized due to the fact that 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 is clearly understood as there is a significant increase of GSH level in both liver and spleen of *A. hydrophila* infected *L. rohita* in comparison to sham operated control fish. Related study showed a dose dependant increase in hepatic CAT and SOD in Indian major carps under *A. hydrophila* infection in *Cirrhinus mrigala* (Datta Ray & Homechaudhuri, 2014) and in *Catla catla* (Datta Ray & Homechaudhuri, 2013) and such results are in conformity with our present findings.

The outcome of these studies signifies the fact that the activity of different oxidative stress enzymes increase concomitantly with excess production of ROS after bacterial pathogenesis is established. It induces the release of ROS in liver and spleen subsequently causing simultaneous increase in the activity and concentrations of all the stress enzymes *viz.* SOD, CAT, GPx, GR and GSH. These changes are also higher in liver than in spleen indicating liver as more sensitive to be

considered for evaluation of oxidative stress in *L. rohita* under infection with *A. hydrophila* at an asymptomatic carrier state.

Study of Cell Death

In Figure 2 the induction of apoptosis in liver and spleen of sham operated control and *A. hydrophila* infected *L. rohita* by flow cytometric studies involving Annexin V- FITC / PI staining has been presented. Previous study (Pal et al., 2015) reported cell death in liver and spleen through histopathological observation with the same infection. Therefore, the present analysis with the Annexin V – FITC/ PI is attempted to ascertain the nature of cell death. Significant increase in the percentage of early apoptotic cells (15 %) and late apoptotic cells (3 %) was observed in liver of infected fish in comparison to control samples. Similar trend was also observed in spleen of infected fishes where early apoptotic cells (17 %) and late apoptotic cells (0.9 %) increased significantly when compared to the control.

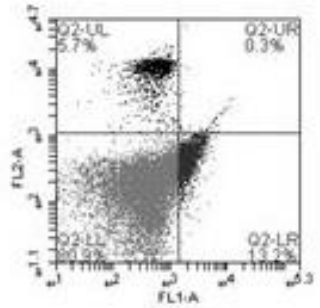

Fig 2a

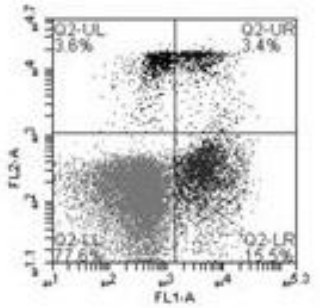

Fig 2b

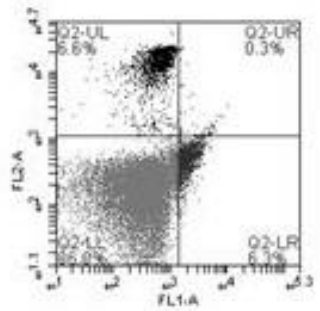

Fig 2c

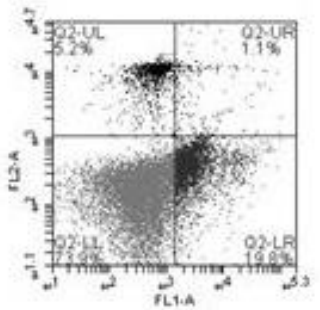

Fig 2d

Types of cells (%)	Sample groups	Liver (Mean ± SE)	Spleen (Mean ± SE)	*t* - value
Normal cells	Control	87.2 ± 0.25	83.00 ± 0.21	12.86 *
	Treated	76.2 ± 0.33	76.98 ± 0.16	0.60
	t - value	26.77 *	26.29 *	
Early apoptotic cells	Control	5.00 ± 0.21	11.00 ± 0.11	25.46 *
	Treated	15.00 ± 0.21	17.68 ± 0.09	11.73 *
	t - value	33.54 *	48.66 *	
Late apoptotic cells	Control	0.025 ± 0.02	0.23 ± 0.02	0.94
	Treated	3.00 ± 0.21	0.90 ± 0.02	9.37 *
	t - value	13.21 *	23.06 *	

* Significant ($p < 0.05$)

Figure 2: Annexin V-FITC /PI study in liver and spleen of *A. hydrophila* infected *L. rohita* after 7 days of exposure where Annexin V emitting green fluorescence (530 nm) and Propidium Iodide (PI) emitting red fluorescence (570 nm) have been plotted along FL1 and FL2 channels respectively. Figure 2a showing 80.9 % live, 13.2 % early and 0.3 % late apoptotic cells in lower left (LL), lower right (LR) and upper right (UR) quadrants respectively in liver of control fishes. Figure 2b showing 77.6 % live, 15.5 % early and 3.4 % late apoptotic cells in the LL, LR and UR quadrants respectively in infected fish liver. Figure 2c and Figure 2d depicting proportion of live cells (86.8 % and 73.9 %), early apoptotic cells (6.3 % and 19.8 %) and late apoptotic cells (0.3 % and 1.1 %) respectively in the spleen of control and *A. hydrophila* infected fishes.

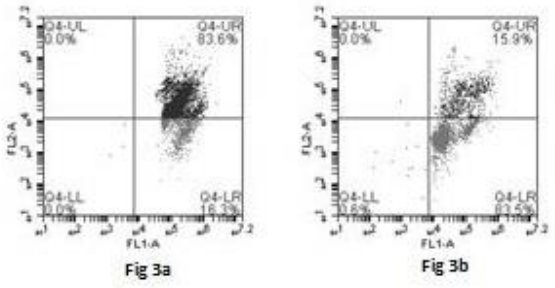

Fig 3a

Fig 3b

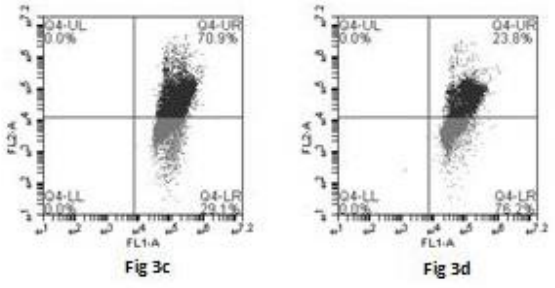

Fig 3c

Fig 3d

Types of cells (%)	Sample groups	Liver (Mean ± SE)	Spleen (Mean ± SE)	*t* - value
Polarized cells	Control	86.49 ± 0.05	75.4 ± 1.15	9.66 *
	Treated	15.83 ± 0.13	34.56 ± 0.77	24.04 *
	t - value	515.64 *	29.58 *	
Depolarized cells	Control	13.51 ± 0.06	24.57 ± 0.56	19.74 *
	Treated	83.96 ± 0.19	65.46 ± 0.49	35.18 *
	t - value	362.84 *	55.0 *	

* Significant ($p < 0.05$)

Figure 3: Study of mitochondrial membrane potential (ΔΨm) in Liver and Spleen of control and *A. hydrophila* infected *L. rohita* where JC-1 Green fluorescence (530 nm) and JC-1 Red fluorescence (570 nm) have been plotted along FL1 and FL2 channels respectively. Figure 3a depicting 83.6 % of polarized cells i.e. live cells and rest 16.3 % to be in depolarized stage i.e. undergoing apoptosis in the control liver sample. Figure 3b showing 15.9 % live cells and an higher proportion of depolarized cells (83.5 %) in the liver of infected fishes. Figure 3c depicting 70.9 % polarized and 29.1 % depolarized cells in the control spleen sample. Figure 3d showing a lower number of polarized cells (23.8 %) and significantly higher percentage of depolarized cells (76.2 %) in the spleen of infected fishes.

The percentage of live cells also decreased significantly in both the liver and spleen of infected fishes.

Although, the cumulative amount of cells undergoing apoptotic cell death (early and late) in both liver and spleen is observed to be almost similar but the percentage of late apoptotic cells was significantly higher in the liver (3 %) in comparison to spleen (0.9 %). However, the percentage of early apoptotic cell in spleen (17.6 %) was higher compared to liver (15 %) as shown in Figure 2. This finding conforms with the results obtained in earlier histopathological study (Pal et al., 2015) and an earlier observation in *Cirrhinus mrigala* (Datta Ray & Homechaudhuri, 2014) thus indicating that production of intracellular ROS (liver > spleen) follows an organ specific pattern.

Evaluation of Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) assay of liver and spleen in sham operated control and infected fish is shown in Figure 3. A distinctive feature of the early stages of apoptosis is the disruption of active mitochondria, including changes in membrane potential and alterations to redox potential. In healthy live cells, JC - 1 selectively accumulates in mitochondria and forms J-aggregates that exhibit red fluorescence whereas in apoptotic cells with reduced mitochondria membrane potential, JC-1 exists as a monomer resulting in green fluorescence (Di Lisa et al., 1995; Reers, Smith, & Chen, 1991; Zeng et al., 2008). The majority of cells in control liver (86 %) and spleen (75 %) maintained normal MMP (ΔΨm) with bright JC - 1 staining detected in both FL1 and FL2 (FL1 bright, FL2 bright; upper right plot) while majority of cells in liver (83 %) and spleen (65 %) of infected fish remained bright in the FL1 channel but had reduced FL2 intensity (FL1 bright, FL2 dim; lower right plot), indicative of compromised ΔΨm.

Apoptosis is a cellular process involving a genetically programmed series of events leading to the death of a cell (Gupta, Kass, Szegezdi, & Joseph, 2009)

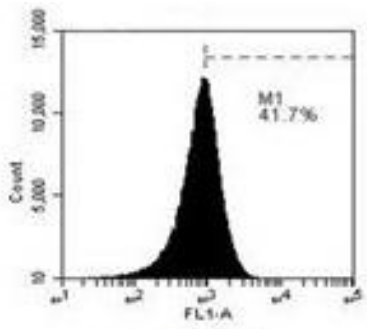

Fig 4a

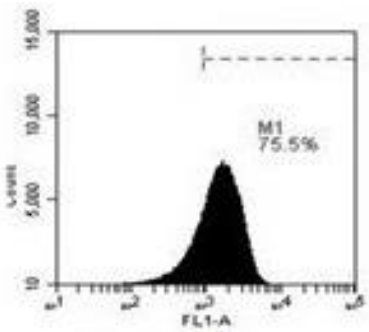

Fig 4b

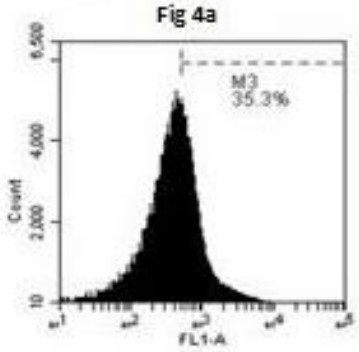

Fig 4c

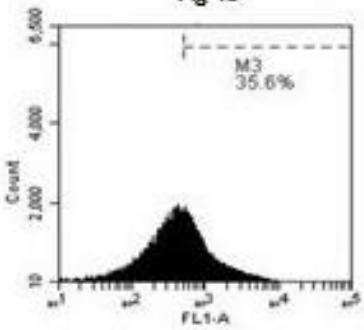

Fig 4d

Cytosolic Ca^{2+} level	Sample groups	Liver (Mean ± SE)	Spleen (Mean ± SE)	*t*-value
Mean FL1 - A	Control	891.34 ± 5.56	536.67 ± 9.03	33.46 *
	Treated	1714.64 ± 7.20	672.94 ± 6.70	105.91 *
	t-value	90.50 *	12.12 *	

* Significant ($p < 0.05$)

Figure 4: Estimation of Intracellular free (Cytosolic) Ca^{2+} in liver and spleen of control and *A. hydrophila* infected *L. rohita*, the free Ca^{2+} level has been plotted along the FL1 channel. Figure 4a depicting the mean FL1-A value to be 891.34 ± 5.56 in the control liver sample. Figure 4b showing a higher mean FL1-A value of 1714.64 ± 7.20 i.e. a 1.92 fold increase in the cylosic Ca^{2+} level (Mean FL-1) in the hepatocytes of Rohu injected with *A. hydrophila.* Figure 4c depicting the mean FL1-A value to be 536.67 ± 9.03 in the control spleen sample. Figure 4d showing a higher mean FL1-A value of 672.94 ± 6.70 i.e. a 1.25 fold increase in the cylosic Ca^{2+} level (Mean FL-1) spleen of infected fishes in comparison to the control.

During this process, several key events occur in mitochondria, including the release of caspase activators such as cytochrome c, changes in electron transport and loss of mitochondrial trans-membrane potential (ΔΨm). Hence, ΔΨm is an important marker of mitochondrial function and has been used as an indicator of healthy cells. Collapse of the ΔΨ results in a depolarised ΔΨ and is often but not always observed to occur early during apoptosis (Green & Reed, 1998). This may be a prerequisite for cytochrome c release and one of the first events occurring during apoptosis (Wang & Youle, 2009).

Estimation of Intracellular free Ca^{2+}: To study the role of Ca^{2+} in induction of apoptotic cell death, estimation of intracellular Ca^{2+} was done by Ca^{2+} binding Fluo 3 AM, which indicated significantly higher amount of intracellular Ca^{2+} ($[Ca^{2+}]_c$) in liver and spleen cells of *A. hydrophila* infected *L. rohita* compared to the sham operated control ones (Figure 4). The value of free cytosolic Ca^{2+} observed in *A. hydrophila* infected (1714.64±7.20) fish liver exhibited a 1.92 fold increase compared to sham operated control fish liver (891.34±5.56). However, in the case of spleen, a 1.25 fold increase in free cytosolic Ca^{2+} level was observed in the *A. hydrophila* infected fishes (672.94±6.70) in comparison to sham operated control fishes (536.67±9.03).

These results indicate higher percentage of cell undergoing cell death (i.e. with more amount of $[Ca^{2+}]_c$) in liver and spleen of infected fishes due to excess ROS production in comparison to sham operated control samples. A tissue specific pattern of induction of apoptotic cell death (liver > spleen) was also observed. Calcium ions play a critical role in the process of

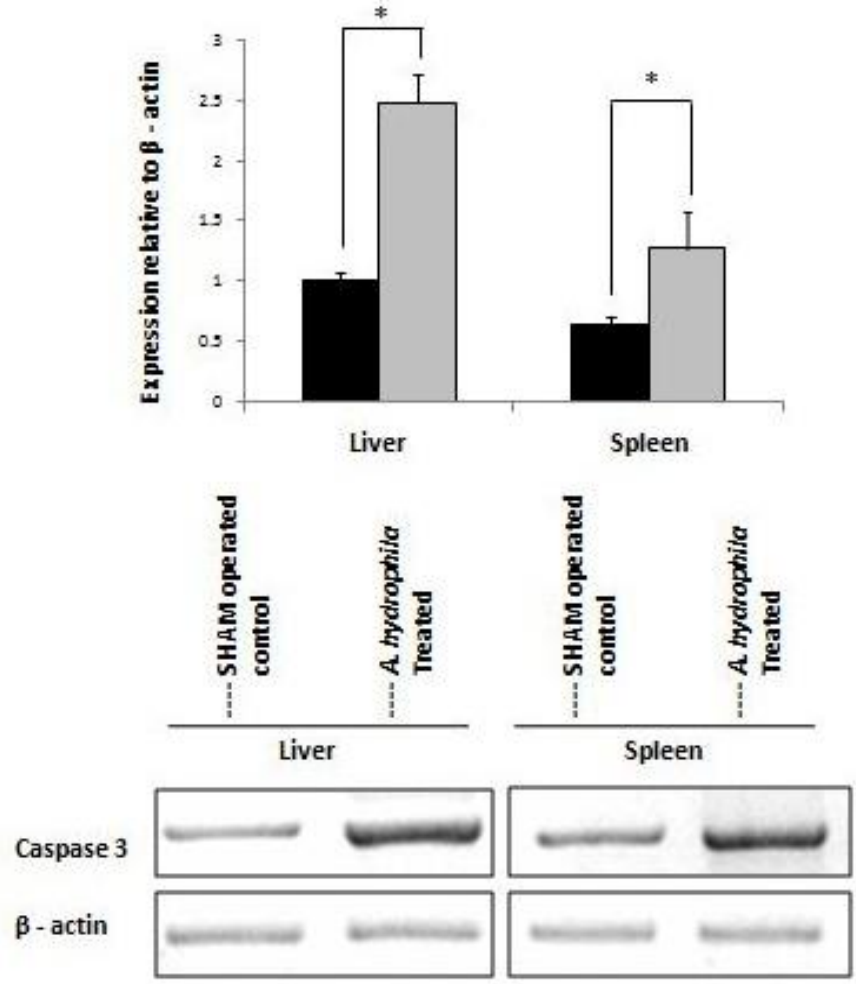

Figure 5: Differential Expression of Caspase 3 in liver and spleen of SHAM operated control and *A. hydrophila* infected *L. rohita.* In case of liver a significant increase was observed in *A. hydrophila* infected fishes (2.48 ± 0.24) compared to control fishes (1.01 ± 0.07). Similar pattern was observed in the spleen of the infected fishes (1.27 ± 0.31) and control fishes (0.64 ± 0.06).

apoptosis, and intracellular Ca^{2+} overload appears to mediate the lethal effects of receptor over activation (Choi, 1992). Ca^{2+} overload has even been suggested to be the final common pathway of all types of cell death. More specifically, it has been suggested that both Ca^{2+} release from the ER and capacitative Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels are apoptogenic (Pinton et al., 2001). Over the last few years, several studies have reported that increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) occur, both at early and late stages of the apoptotic pathway (Kruman, Guo, & Mattson, 1998; Lynch, Fernandez, Pappalardo, & Peluso, 2000) which are in support of the present findings.

Differential Expression of Caspase 3

Relative gene expression analysis of Caspase 3 gene was done by semi quantitative reverse transcriptase PCR after isolation of total RNA from liver and spleen, which exhibited a significant increase in the expression of Caspase 3 in the *A. hydrophila* infected fishes compared to SHAM operated control fishes both in liver and spleen (Figure 5). The expression levels were normalized with β-actin (house keeping gene) as internal control to avoid any errors in the initial RNA concentration. The ratio of Caspase 3 gene / β-Actin product was subsequently calculated by densiometry using ImageJ software. In case of liver a significantly higher value of Caspase 3 expression was observed in *A. hydrophila* infected fishes (2.48±0.24) compared to control fishes (1.01±0.07). Similar trend was also observed in case of spleen, where significantly higher value was found in *A. hydrophila* infected fishes (1.27±0.31) in comparison to control fishes (0.64±0.06).

From the results obtained it was found that in liver and spleen the expression levels showed a significant increase after infection with *A. hydrophila* in comparison to the sham operated control fishes. Activated caspase-3 is a central effector of apoptosis that cleaves and inactivates a number of molecules contributing to the typical morphology of apoptosis (Fischer, Jänicke, & Schulze-Osthoff, 2003). Increased gene expression of caspase-3 has been described in cells under apoptosis (Chiang et al., 2001; Miller et al., 1997). In this case, it was obvious that upon bacterial infection and subsequent ROS production the caspase-3 mRNA levels

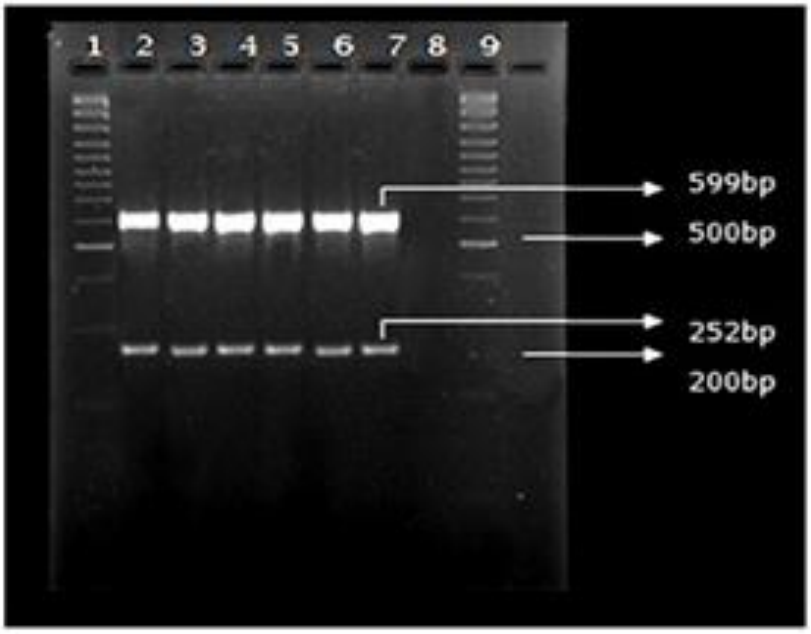

Figure 6: *Aeromonas hydrophila* (MTCC 646) showing product obtained from primer for 16S rRNA (599 bp) & Aerolysin (252 bp). Where, Lane 1 & 9:100 bp ladder (Fermentas); Lane 2 & 3: PCR product from DNA isolated from colonies of *Aeromonas hydrophila* in NB plate; Lane 4 & 5: PCR product from DNA isolated from *Aeromonas hydrophila* colonies recovered from liver tissue of *L. rohita* after challenge; Lane 6 & 7: PCR product from DNA isolated from *Aeromonas hydrophila* colonies recovered from spleen tissue of *L. rohita* after challenge; Lane 8: PCR negative control.

increasein order to meet the increasing demand for the execution of apoptosis.

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 at an asymptomatic level, tissue homogenates were prepared from liver and spleen and was plated onto RS media supplemented with Novobiocin. It resulted in the production of bright yellowish colonies indicating and confirming the presence of *A. hydrophila* in fishes infected with the bacterial pathogen. 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 6). These findings are in accordance with the previous results obtained (González-Rodríguez, Santos, Otero, & García-López, 2002).

The fate of the target cells as observed in liver and spleen of *L. rohita* upon infection with *A. hydrophila* at an asymptomatic dose was ultimately confirmed to be apoptosis. A schematic diagram is provided to explain the results (Figure 7) which underline the pathways and the interplay of various factors essential for the induction and execution of the programmed cell death in liver and spleen of infected fishes. Moreover, the effect of oxidative stress and apoptotic cell death due to bacterial infection was found to be more pronounced in the liver, followed by the spleen.

Conclusion

In conclusion, the findings show that *L. rohita,* an Indian major carp when infected with *A. hydrophila* at an asymptomatic carrier state are physiologically impaired as detected by using various oxidative stress biomarkers. Moreover, the role of *A. hydrophila* as a potent inducer of apoptosis has been analyzed by flow cytometry with the help of MMP assay, intracellular Ca^{2+} ion estimation and Annexin V-FITC / PI study. From the overall findings, it might be concluded that *A. hydrophila* infection, particularly at an asymptomatic carrier state dosage, induced mitochondrial dysfunction which leads to apoptosis in liver and spleen of *L. rohita.* The process was triggered an array of virulence determinants viz. increase in the levels of cytosolic Calcium and Reactive Oxygen Species, which ultimately interact with the key component of the cell death pathway i.e. Caspase 3.

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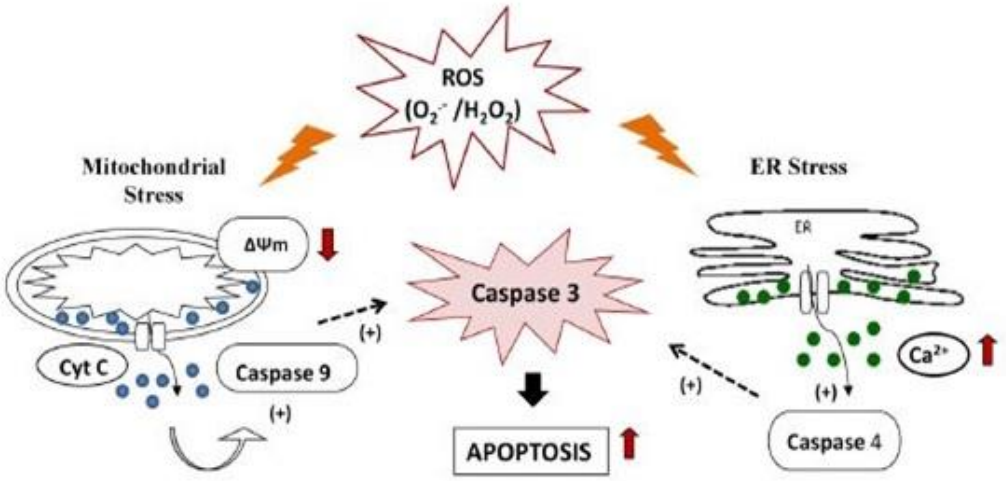

Figure 7: Schematic diagram representing induction of apoptotic cell death in *L. rohita* post *A. hydrophila* infection. ROS: Reactive Oxygen Species; ER: Endoplasmic Reticulum.

Technology (DST), Government of India [Sanction No: SR/SO/AS-067/2013 dated 10.07.2014] to Prof. Sumit Homechaudhuri.

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