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Investigations on Blood Physiology, Tissues Histology and Gene Expression Profile of *Fusarium oxysporum* Challenged Fish

(Penyelidikan terhadap Fisiologi Darah, Histologi Tisu dan Profil Ekspresi Gen Ikan Tercabar *Fusarium oxysporum*)

SUMAIRA PERVAIZ[1], ZAKIA KANWAL[1,*], FARKHANDA MANZOOR[1,2], ASIMA TAYYEB[2] & QAISER AKRAM[3]

[1]*Department of Zoology, Faculty of Natural Sciences, Lahore College for Women University, Lahore, Pakistan*
[2]*School of Biological Sciences, University of the Punjab, Lahore, Pakistan*
[3]*Department of Pathobiology, University of Veterinary and Animal Sciences, Lahore, Pakistan*

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ABSTRACT

Labeo rohita was challenged with *Fusarium oxysporum* to demonstrate a thorough immune response. Significant alterations in hematological parameters viz; erythrocyte, hemoglobin, hematocrit, mean corpuscular hemoglobin concentration, mean corpuscular volume, packed cell volume, platelets, and leukocytes were observed. Total serum protein, albumin, globulin, A/G ratio, aspartate aminotransferase, alkaline aminotransferase, alkaline phosphatase activity, and cholesterol were also altered in the infected fish. Marked histopathological lesions in gills, liver, and kidney (fusion of gill lamella, necrosis, edema, vacuolization, glomerulus congestion, dilated bowman's space and renal tubules alterations) were recorded. Tissue specific gene expression data showed that interleukin was upregulated in infected fish gills, liver, and kidney but was downregulated in blood. Tumor necrosis factor-α expression was upregulated in blood, liver, and kidney and was downregulated in gills. Upregulation of interferon-γ was observed in blood, gills, and kidney but downregulated in liver. All the cellular and molecular findings from this study can be taken as critical biomarkers in the advancement of diagnostic techniques against fungal infections as a part of wide-ranging programs of systematic and comprehensive monitoring of fish health and immune system.

Keywords: *Fusarium oxysporum*; histomorhological index; immune response; *Labeo rohita*; proinflammatory cytokines

ABSTRAK

Labeo rohita telah dicabar dengan *Fusarium oxysporum* untuk menunjukkan tindak balas imun yang menyeluruh. Perubahan ketara dalam parameter hematologi iaitu; eritrosit, hemoglobin, hematokrit, purata kepekatan hemoglobin korpuskul, min isi padu korpuskul, isi padu sel pembungkus, platelet dan leukosit diperhatikan. Jumlah protein serum, albumin, globulin, nisbah A/G, aspartat aminotransferase, alkali aminotransferase, aktiviti alkali fosfatase dan kolesterol juga telah diubah dalam ikan yang dijangkiti. Lesi histopatologi yang ketara dalam insang, hati dan buah pinggang (gabungan lamella insang, nekrosis, edema, vakuolisasi, kesesakan glomerulus, ruang bowman yang diluaskan dan perubahan tubul renal) telah direkodkan. Data ekspresi gen khusus tisu menunjukkan bahawa interleukin dikawal selia dalam insang ikan, hati dan buah pinggang ikan yang dijangkiti tetapi dikurangkan dalam darah. Ekspresi faktor nekrosis tumor-α telah dikawal selia dalam darah, hati dan buah pinggang dan dikurangkan dalam insang. Peningkatan interferon-γ diperhatikan dalam darah, insang dan buah pinggang tetapi dikurangkan dalam hati. Semua penemuan sel dan molekul daripada kajian ini boleh diambil sebagai biopenanda kritikal dalam kemajuan teknik diagnostik terhadap jangkitan kulat sebagai sebahagian daripada program meluas pemantauan sistematik dan komprehensif terhadap kesihatan ikan dan sistem imun.

Kata kunci: *Fusarium oxysporum*; indeks histomorfologi; *Labeo rohita;* sitokin proinflamasi; tindak balas imun

INTRODUCTION

The growing interest in expanding aquaculture industry has motivated scientists to explore and understand the pathogenicity of fish infections and defense mechanism of the fish immune system against these infections. Fish pathogens directly or indirectly affect fish health and limit the physiological response of fish. Waterborne pathogens viz., bacteria, fungi, virus, and parasites are a huge

challenge to the fish industry. Carp culture also faces an alarming situation due to pathogenic challenges resulting in increased infection rate and mass mortalities owing to loss of immune function. Fungal infections lead to staid damages in aquaculture. *Fussarium oxysporum* has been known as an emerging fish pathogen. *F. oxysporum* caused acute infection in zebrafish (Kulatunga et al. 2017). Serious sub-cutaneous infection in tilapia fish has been reported when co-infected with *Aeromonas hydrophila* and *F. oxysporum* (Cutuli et al. 2015). Fungal infections of genus Saprolegnia and Aphanomyces have been well studied with respect to their virulence in major carps but there remains a lot to be discovered about pathogenecity of genus Fusarium.

Alterations in fish innate as well as adaptive immune responses against various infectious pathogens have been described previously (Mohanty & Sahoo 2010). Haemotological, biochemical, and histopathological biomarkers are important end points to study physiological changes following infection. Moreover, up and down-regulation of cytokines in fish due to infection is highly critical and advanced criteria for monitoring the fish's health status. Previous studies explained the up-regulation of various pro-inflammatory cytokines in rainbow trout exposed to pathogens (Chettri et al. 2012; Wiens & Vallejo 2010). Tumor necrosis factor-α (TNF-α), interleukin1-β (il-1β), interleukin-6 (il-6) and interleukin-10 (il-10) were up-regulated in pathogen-associated molecular patterns (PAMPS) challenged *Oncorhynchus mykiss* (Chettri et al. 2012). il-1β has additional role of regulating immune signaling pathways during Saprolegnia infection as it causes up-regulation of other genes by a downstream signaling pathway (de Bruijn et al. 2012). Similarly, expression of il-1β, TNF-α, il-10, interferon gamma (IFN-γ) and inducible nitric oxide synthase (iNOS) were gradually changed that gives the indication of activation of host's immune-regulatory functions of cytokine genes against *Candida albicans* infection (Chao et al. 2010). Therefore, proinflammatory cytokines have important roles in immune system activation against microbial infections. To the best of our knowledge, there is no study available relating to immune functioning of *L. rohita* against *F. oxysporum* infection. In the present study, we challenged *L. rohita* with *F. oxysporum* and investigated the changes in fish's blood physiology, biochemical response, histopathology and inflammatory gene expression. Thus, the present study is novel in that it will help to introduce the advance prophylactic measures and efficient strategies to resistor infections in aquaculture.

Materials and Methods

Experimental specimen

Juveniles of *L. rohita* (weight 18 ± 3 g) were attained from Manawan Fish Farm, Tulspura, Lahore, Punjab, Pakistan. Before performing the experimental trials, fish were acclimatized to the laboratory condition for 14-days. A basal floating diet was offered to the fish (2% of its body weight) twice a day. Feed composition was: crude lipid (15%), crude protein (38%), moisture (10%), ash (11%), and crude fiber (6%). Water was changed daily to remove any waste. Fish was handled according to the guidelines of Local Animal Welfare Regulations of the host University.

Fusarium oxysporum culture preparation

F. oxysporum hyphae were inoculated onto malt extract agar (MEA CM059B, HiMedia) plates in triplicates and were incubated at 28 °C for 9 days. Purified fungal culture was obtained by three round sub-culturing in MEA plates. To make the spore suspension, 1 mL sterilized distilled water was added in *F. oxysporum* culture plates, surfaces of plates were gently scrapped off with sterile spatula. An aliquot of this suspension was taken in a 50 mL falcon tube and vortexed to stir well. Spore suspension was made approximately 10 mL with sterilized distilled water. Ten µL of homogenized spore suspension solution was taken on hemocytometer and a coverslip was placed over it. Following this, spores were counted under the microscope at 40X. Number of spores were determined by using following formula (Kamaruzzaman et al. 2016).

Number of spores per cubic mm suspension = Number of spores counted × dilution factor/ Number of smallest square counted × 4000

Experimental design

Fish was placed randomly into two groups n = 10 fish/ group (experiments were performed in triplicates)

1. Control group: PBS treated (Con)
2. Infected group: *F. oxysporum* challenged (*F. oxysporum*)

Infected group was intramuscularly injected with *F. oxysporum* @ 1200-spores/0.5 mL/fish. Con group was injected with an equal volume of 1% sterilized PBS. 50% of the tank's water was refreshed daily to remove waste material. The fish were closely monitored for gross pathological changes and survival rate was determined. Whole blood and tissue samples were collected at 7 days

post infection (dpi). 100 µg/L of clove oil was used to anesthetize fish for sampling.

HEMATOLOGICAL ASSAYS

Hematological parameters Total erythrocyte count (TEC), Hemoglobin (Hb), Hematocrit (Hct%), Mean corpuscular hemoglobin concentration (MCHC), Mean corpuscular volume (MCV), Packed cell volume (PCV), Platelets (Plt) and Total leukocytes count (TLC) were measured by using an automated blood analyzer (Sysmex-KX-21, Japan).

BIOCHEMICAL ANALYSIS

Blood was centrifuged at 4 ℃ for 10 min at a speed of 4000 × g. Resultant serum was transferred to new eppendorf tubes. Total protein (TP) was analyzed by using Crescent diagnostic, Cat. No. CS.610. UAE kit which employs Biuret method. Albumin (Alb) level was assayed by bromocresol green (BCG) dye binding technique using an Albumin kit (ANMOL-LAB Pvt. Ltd., LOT. DR379E249). Globulin (Glo) was estimated by deducting the albumin from TP. A/G ratio was obtained by dividing Alb from Glo. Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP) were quantified by specific kit protocols devised according to International Federation of Clinical Chemistry and Laboratory Medicine method (IFCC). Plasma total cholesterol (Chol) was analyzed by cholesterol oxidase method (ANMOL-LAB Pvt. Ltd., LOT. HR146619).

HISTOPATHOLOGICAL ANALYSIS

Liver, kidney, and gill soft tissue were taken for histopathological evaluation. Collected tissues were fixed for at least 24 hours in 10% buffered formalin to prevent cellular autolysis. Dehydration of fixed tissues was done through differential grades of alcohol in ascending order for 15-20 min each and in the end were kept in absolute ethanol. Tissues were then put in two rounds of pure xylene (100%) and then were embedded in paraffin wax. Tissue sections of 6-7 µm thickness were cut on microtome (ERM-2301). Later on, sections were deparaffinized by washing with xylene and alcohol to get rid of excess wax. Mounting and staining of the slides were done through DPX (Distyrene-plasticizer: xylene) and eosin-hematoxylin stains (H&E), respectively. Microphotograph of tissues was taken under digital camera fitted optical microscope (Trinocular E-200, Nikon Japan Eil-12).

SEMI QUANTITATIVE HISTOMATRIX SCHEME

For assessment of histological alteration index (HAI), semi quantitative histomatrix scheme was applied (Bernet et al. 1999). The histological alterations of four of following four categorized were included: ‘circulatory’, ‘regressive’, ‘progressive’ and ‘inflammatory’.

GENE EXPRESSION ANALYSIS

The gene expression analysis was performed in the Molecular Biology Lab, School of Biological Sciences, University of the Punjab, Lahore, Pakistan. Extraction of total RNA was done by GeneJETTM RNA-Purification Kit (Catalog number: K0732). RNA concentration was quanitified by measuring the absorbacne ratio at 260 nm. Purity of the samples was analyzed on nanodrop ND-1000 (Thermo Scientific, USA) taking OD ratio at 260 nm/280 nm. cDNA was synthsized by using Thermo Scientific RevertAid First Strand cDNA synthesis kit (Thermo Fischer Scientific Inc., USA, Catalog number: K1622). Briefly, 2 µg mRNA and 1 µL random hexamer primer were added into nuclease free water to reach a final volume of 12 µL. Afterward, 4 µL of 5× reaction buffer, 1 µL of Ribolock RNase inhibitor, 2 µL of 10 mM dNTP mix and 1 µL of 200 U/µL RevertAid M-MuLV RT were added in the solution. The reaction mixture was then incubated at 25 °C and 42 °C for 5 and 60 min, respectively. The reaction was stopped by heating to 70 °C for 5 min. The resultant cDNA was stored at -20 °C until further analysis. RT-qPCR (PikoReal™ Real Time PCR-System) was performed by using 1 µL cDNA, 7 µL Maxima-SYBR Green and 1 µL gene specific forward and reverse primers (Table 1). β-actin was used as the housekeeping gene for reference. Variations in gene expression (fold changes) were calculated by ΔΔCt method (Livak & Schmittgen 2001).

STATISTICAL ANALYSIS

Data is expressed as Mean ± SEM. Graph-pad Prism software (Version: 4.0) was used for statistical testing on data. Unpaired student t-test and ANOVA (Analysis of variance) followed by Turkey’s Multiple Comparison analysis as post hoc test was used to compare the difference between groups. Results with $P<0.05$ or lower were designated statistically significant (* indicates $p<0.05$, ** indicates $p<0.01$ or *** indicates $p<0.001$).

TABLE 1. Primer sequences of the genes

Gene Name	Primer Sequences (5'-3')	Reference
IL-1β	F-GTGACACTGACTGGAGGAA R-AGTTTGGGCAAGGAAGA	(Kar et al. 2016)
TNF-α	F-CCAGGCTTTCACTTCAGG R-GCCATAGGAATCGGAGTA	(Mohanty & Sahoo 2010)
INF-γ	F-TTCACTCGCATGGAAAATGA R-GACCGGATCATCAGCCTTTA	(Bedekar et al. 2018)
B-actin	F-GACTTCGAGCAGGAGATGG R-CAAGAAGGATGGCTGGAACA	(Mohanty & Sahoo 2010)

RESULTS

PATHOLOGY PROGRESSION

All fish were examined for gross pathological changes. In Con group, no pathological signs were observed in whole body (Figure 1). In *F. oxysporum* challenged group fish showed pathological symptoms starting from 3dpi, characterized by eye redness and gill damage, trunk bleeding, hemorrhage, ulcerative lesions, swelling, scale loss, tail rupturing and red ulceration (Figure 1).

HEMATOLOGICAL INDICES

Hematological parameters were evaluated from Con and *F. oxysporum* challenged groups after 7dpi. Significant decrease ($p<0.05$, $p<0.01$ or $p<0.001$) in TEC (Figure 2(a)), Hb (Figure 2(b)), Hct (Figure 2(c)), MCHC (Figure 2(d)), MCV (Figure 2(e)), PCV (Figure 2(f)) and Plt (Figure 2(g)) were found. However, TLC was significantly ($p<0.05$) higher in *F. oxysporum* challenged group as compared to Con group (Figure 2(h)).

BIOCHEMICAL ASSAY

TP and Alb were decreased significantly ($P<0.05$) in *F. oxysporum* challenged group (Figure 3(a), 3(b)). Non-significant decrease was observed in Glo (Figure 3(c)). A/G ratio turned to be higher in *F. oxysporum* group relative to Con group (Figure 3(d)). Enzymes, AST, ALT and ALP showed a significant increase ($p<0.05$, $p<0.01$ or $p<0.001$) in their induction after *F. oxysporum* challenge (Figure 3(e), 3(f), 3(g)). A decrease in Cho level was observed in *F. oxysporum* challenged group as compared to the Con group (Figure 3(h)).

HISTOPATHOLOGICAL INDICES

The morphology of the gills of the Con group exhibited typical structure of epithelial cells lining the lamellae, a rod like axis placed at the center of primary gill lamellae around which at both sides a row of secondary gill lamellae was arranged (Figure 4(a)). Secondary gill lamella was free at their distal end with thin epithelial cell layer. The alterations of gill histology in *F. oxysporum* challenged group showed hemorrhage, intracellular edema, blood congestion, cyto-architectural distortion of gills with marked hypertrophy, hyperplasia, loss of central axis with proliferation of mucus cells, fusion of lamellae, curling and shortening of lamellae, vasodilation, cellular necrosis, exfoliation of respiratory epithelium, reduction or complete loss of inter-lamellar region, exudation, aneurisms and cloudy swellings of epithelium of lamellae, hemorrhage due to the rupture of the lamellar epithelium (Figure 4(b)).

Con group liver was surrounded with a connective tissue and serous membrane that extended inward into the parenchymal layer. Cells in polygonal shape with spherical nucleus were identified as hepatocytes arranged in distinct tubules (Figure 4(c)). These hepatocytes were arranged outward with their apices surrounding a biliary lumen. Sinusoids were found in interstitial spaces between hepatic tubules. *F. oxysporum* infection caused severe pathological alterations in liver tissues (Figure 4(d)) including necrosis, pyknotic nuclei, hyperemia, hemosiderin, hemorrhage, intravascular, hemolysis, infiltration of edematous fluid, alteration in tissue structure such as rupturing of central vein, vasodilation in sinusoids, cytoplasmic degeneration, and cellular necrosis. Hemorrhage was also observed in the degenerated and atrophied tissue. Moreover, coagulate type cellular necrosis, hypertrophy, hyperplasia, vacuolization of hepatocytes, degeneration of pancreatic area, congestion of sinusoid and vacuolization were also observed. Melano-macrophages aggregation, leukocytes infiltration, fatty changes and diffuse exudation of erythrocytes were also observed.

Histomorphological analysis of kidney of Con group showed that it contains accurate functional part termed as nephron with glomerulus, Bowman's capsule, distal tubules, proximal, and collecting tubules with intact structure. Occasionally, melanomacrophage centers located in hematopoietic tissue (Figure 4(e)). *F. oxysporrum* caused interstitial hemorrhage, hemosiderin accumulation and cellular necrosis, degenerated glomerular, constricted glomeruli, increased bowman's space, constricted renal tubule, occlusion of the tubular lumen, hyperplasia, hypertrophied renal tubule, vacuolization, granuloma formation, massive atrophy of renal tubule, loss of cellular integrity of renal tubules, aggregation of inflammatory cells, infiltration of edematous fluid and diffuse exudation of massive erythrocyte (Figure 4(f)).

Overall, the histological data suggests that the order of histological deformities in infected fish were liver > gills > kidney. The cumulative HAI analysis (of all four alterations; circulatory, regressive, progressive and inflammatory) showed that the extent of liver damage was greater with a mean organ index of 435 as compared to gills (377) and kidney (286). Thus, liver was counted as a highly affected organ due to *F. oxysporum* infection (Figure 5).

THE QUANTITATIVE RELATIVE ABUNDANCE OF il-1β, TNF-α AND inf-γ

Modulation in differential expression of pro-inflammatory cytokines (il-1β, TNF-α and inf-γ) in blood, gills, liver and kidney of control and *F. oxysporum* challenged fish was checked by qRT-PCR after 7dpi. il-1β gene transcript level was upregulated ≈3.4-fold in gills, ≈9.3-fold in liver and ≈5.5-fold in kidney in *F. oxysporum* challenged fish relative to the Con group (Figure 6(a)). However, expression of il-1β was downregulated in blood (Figure 5(a)). TNF-α expression was not much changed in blood, undetected in gills and was upregulated in liver (≈5.35-fold) and kidney (≈9-fold) (Figure 6(b)). IFN-γ expression was upregulated ≈4.9 fold in blood, ≈1.3 fold in gills, ≈3.0-fold in kidney and was downregulated ≈0.20-fold in the liver (Figure 6(c)).

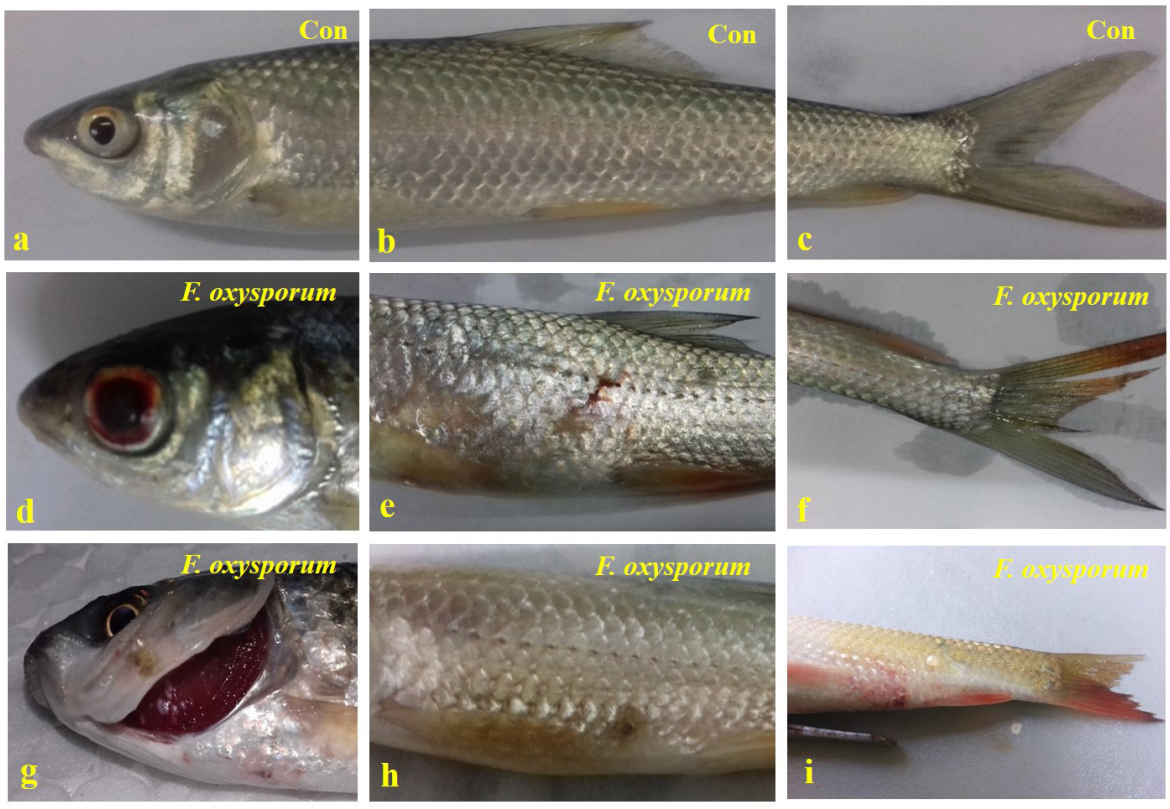

FIGURE 1. Propagation of *F. oxysporum* infection in *L. rohita*. (a-c) Control (Con) group head, trunk region and tail region. (d-i) *F. oxysporum* challenged fish showing redness of eye, gills lesions, ulceration, surface bleeding, scale loss, body swelling, tail rupturing, dark spots and hemorrhage

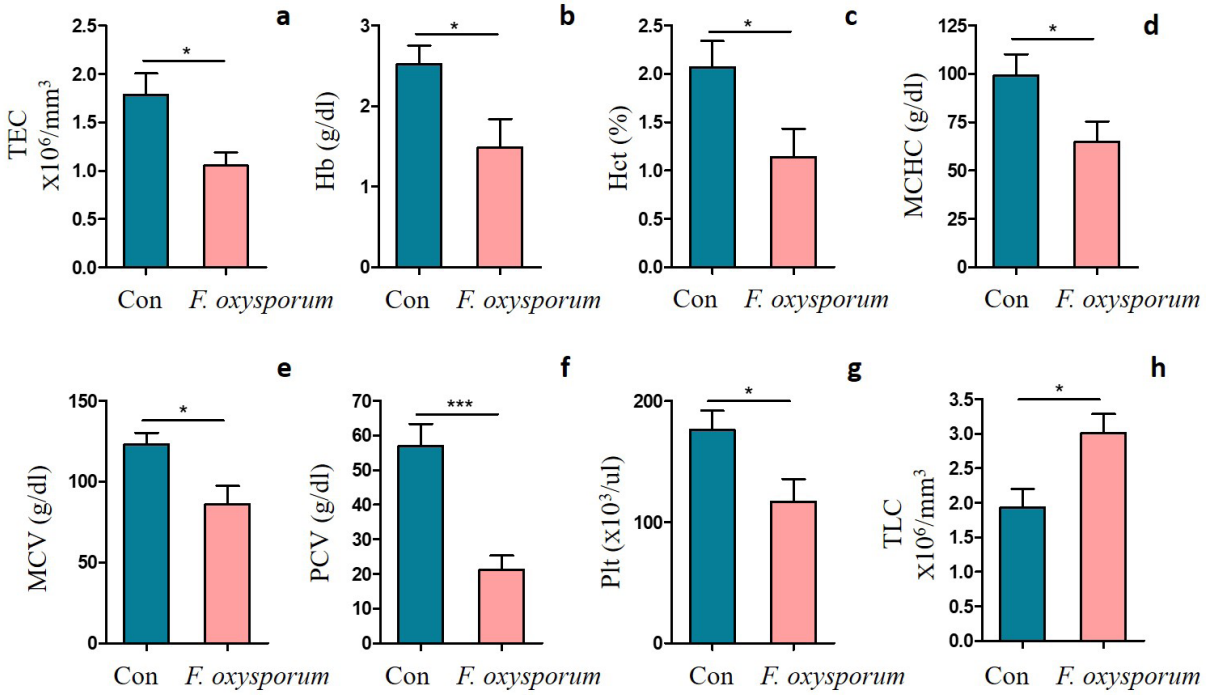

FIGURE 2. Hematological indices in control (Con) and *F. oxysporum* challenged groups at 7dpi. (a) Total erythrocyte count (TEC), (b) Hemoglobin (Hb), (c) hematocrit (Hct %), (d) Mean corpuscular hemoglobin concentration (MCHC), (e) Mean corpuscular volume (MCV), (f) Packed cell volume (PCV), (g) Platelets (Plt), (h) Total leukocyte count (TLC). Data are presented as mean±SEM (*p < 0.05; **p < 0.01; ***p < 0.001)

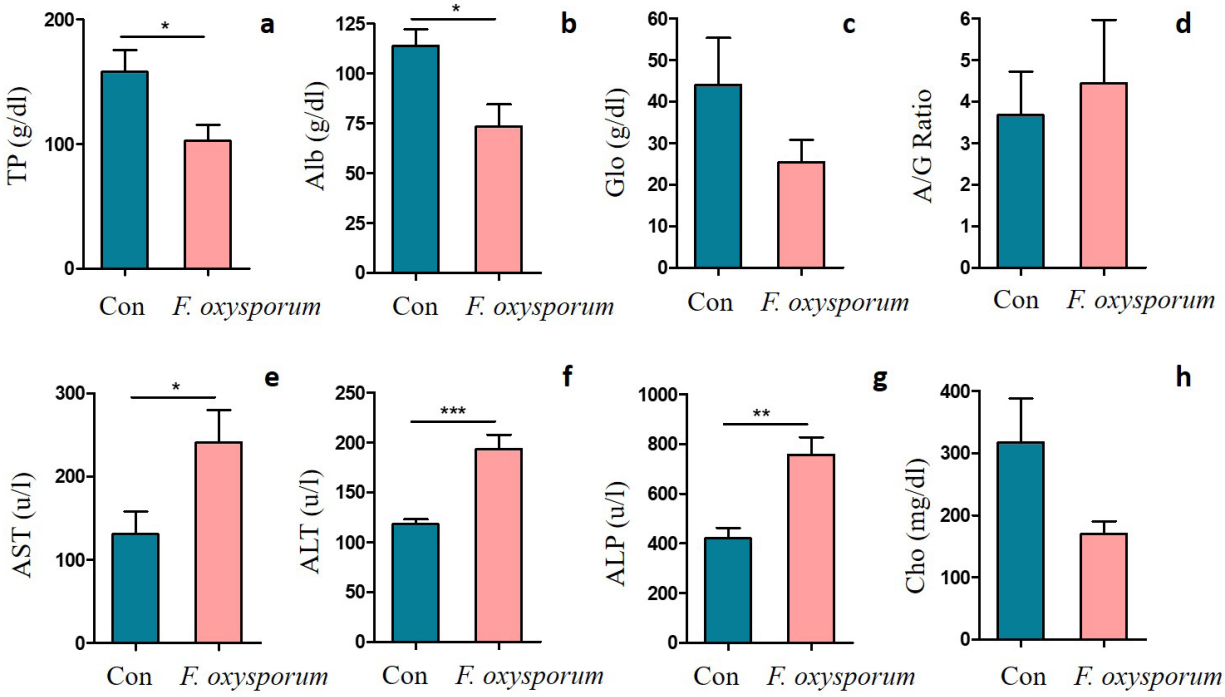

FIGURE 3. Biochemical parameters in control (Con) and *F. oxysporum* challenged groups at 7dpi. (a) Total Protein (TP), (b) Albumin (Alb), (c) Globulin (Glo), (d) A/G ratio, (e) Aspartate aminotransferase (AST), (f) Alanine aminotransferase (ALT) (g) Alkaline phosphatase (ALP), (h) Cholesterol (Cho) Data are presented as mean±SEM (*p < 0.05; **p < 0.01; ***p < 0.001)

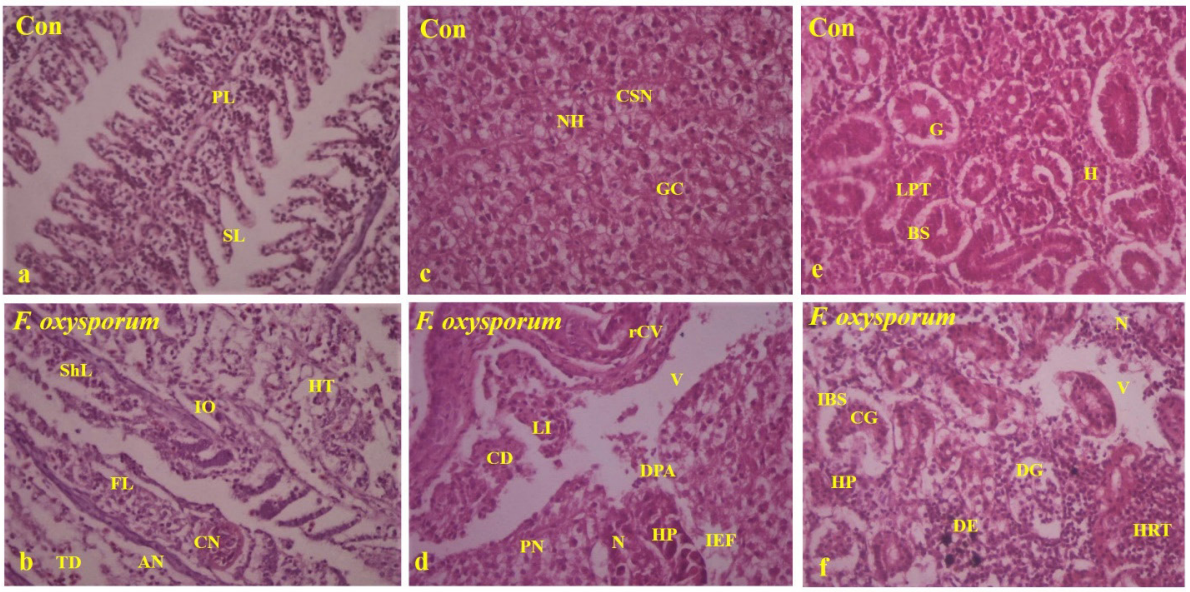

FIGURE 4. Histological evaluation. Gill tissues of control (Con) and *F. oxysporum* challenged fish (a & b, respectively): Primary lamellae (PL), Secondary lamellae (SL), Fusion of lamellae (FL), Cellular necrosis (CN), tissues debris (TD), shortening of lamellae (ShL), Interstitial oedema (IO), aneurism (AN). Liver tissues of Con and *F. oxysporum* challenged fish (c & d, respectively): Normal hepatocytes (NH), Granular cytoplasm (GC), Central spheroidal hepatocyte nucleus (CSN), Cell necrosis (N), Pyknotic nuclei (PN), Cytoplasmic degeneration (CD), Infiltration of oedematous fluid (IEF), Rupturing of the central vein (rCV), Vacuolization of hepatocytes (V), Degeneration of pancreatic area (DPA), Leukocytes infiltration (LI), Hypeplasia (HP). Kidney tissues of Con and *F. oxysporum* challenged fish (e & f, respectively): Glomeruli (G), Bowman's space (BS), Haemopoietic tissue (H), Longitudinal section of proximal tubule (LPT), Increased Bowman's space (IBS), Degenerated glomeruli (DG), Hypertrophied renal tubule (HRT), Vacuolization (V), Constricted glomeruli (CG), Hyperplasia (HP), Necrosis (N), Diffuse exudation of erythrocytes (DE). H&E stained, 40X

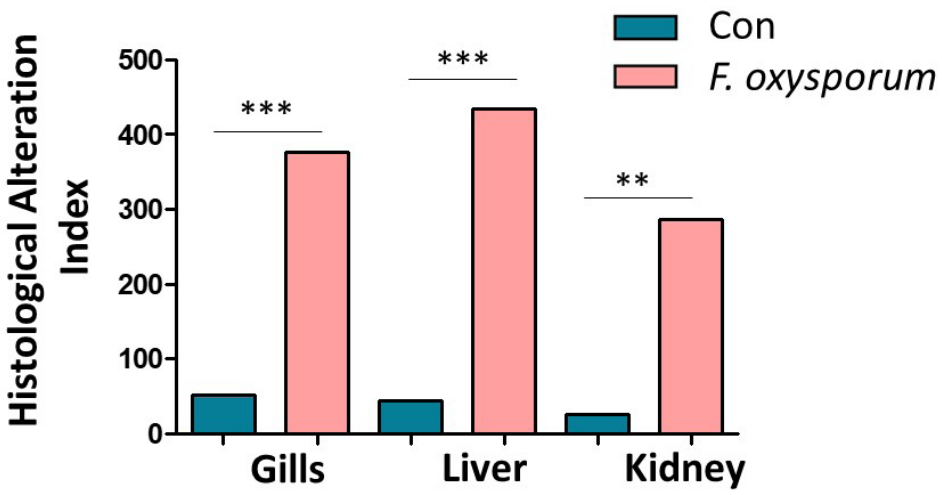

FIGURE 5. Histological alteration index (HAI). Mean HAI for gills, liver and kidney were counted as 377, 435 and 286, respectively. as compared to control (Con). Data are presented as mean±SEM (*p < 0.05; **p < 0.01; ***p < 0.001)

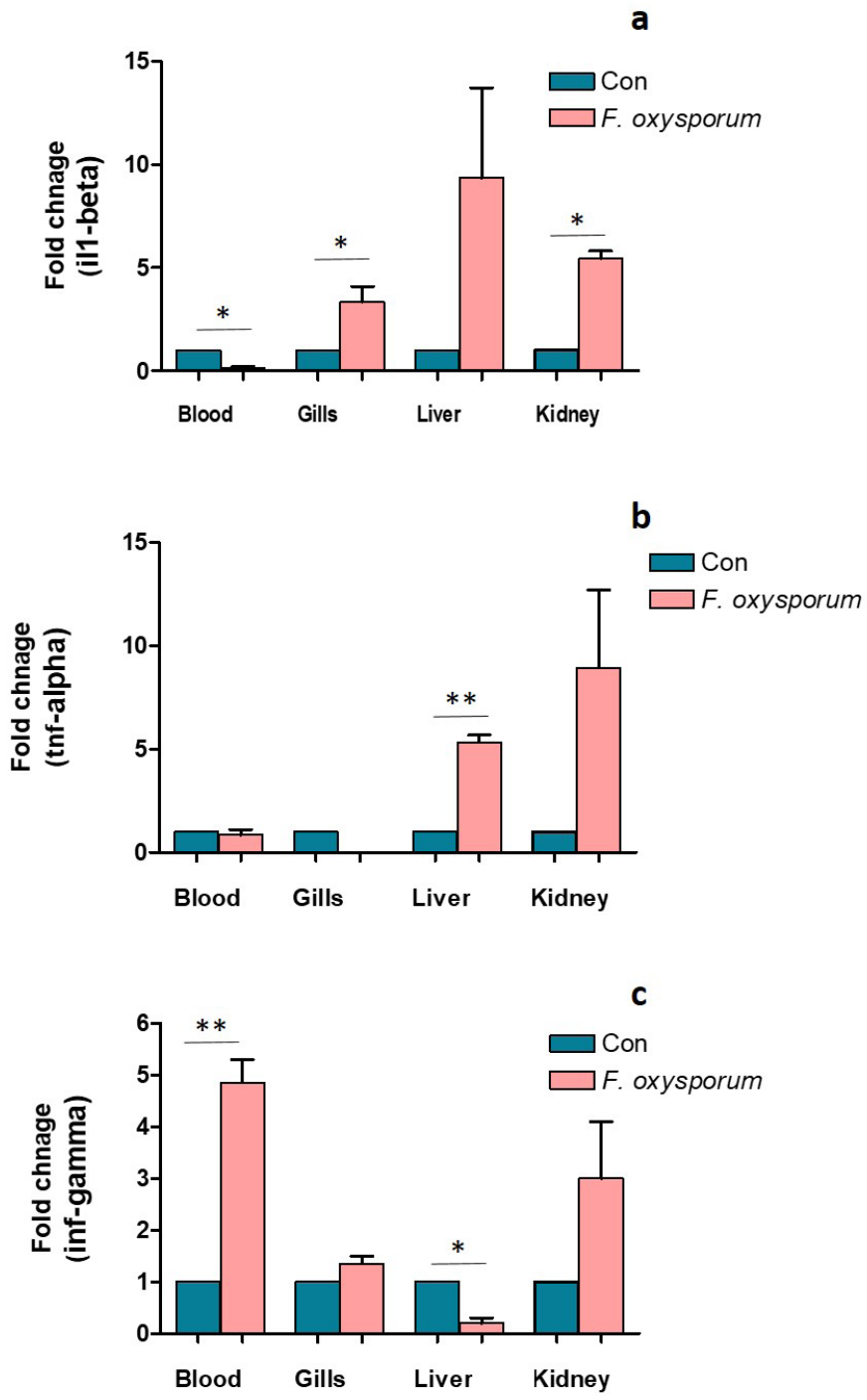

FIGURE 6. Gene expression analysis of IL1-β (a), TNF-α (b) and INF-γ (c). Data are presented as mean±SEM (*p < 0.05; **p < 0.01; ***p < 0.001)

DISCUSSION

Due to the rapid occurrence of *F. oxysporum* infections in aquatic species e.g., zebrafish, tilapia and prawn (Cutuli et al. 2015; Khoa & Hatai 2005; Kulatunga et al. 2017) we established an *in-vivo L. rohita* model to examine the disease progression and immune response of the host.

The challenged fish showed phyenotypic lesions on gills, eyes and tail. Previously, Salter et al. (2012) and Yanong (2003) reported that Fusarium infestation leads to severe infection in the form of epidermal lesions and highly localized skin defects e.g., ulcer.

F. oxysporum infection led to variation in hematological indices of fish. Significant reduction of Hb level and RBCs number may cause erythrocytic anemia in fish that becomes a major reason of infection progression and mortality in fish. Such erythrocytic anemia in a variety of fish spp. has been reported by Shah (2010). Our results are also in accordance with Zaki et al. (2008). They stated that *S. parasitica* infection in Tilapia cause decrease in total number of RBC, Hb and PCV. Increasing trend in TLC counts in *F. oxysporum* challenged fish could probably be due to deep tissues necrosis as was shown by histological data. Shah (2010) proposed that infection and various stress-related factors cause tissue damage and cellular necrosis which led to an increase in granulocytes.

Decrease in serum proteins was found in *F. oxysporum* challenged fish. Significant reduction in TP and Alb has been reported by Shah et al. (2015) Saprolegnia challenged fish. Decreased level of serum proteins is directly related to the infectious state and impairment of synthesis process along with low absorption rate and high rate of protein loss. The decrease of Glo levels generally cause AG ratio reversal in fish. Mastan et al. (2008) and Rairakhwada et al. (2007) also reported similar alterations in Glo level after exposure to stressors. Thus, propagation of fungal infection in fish induces physiological stress that ultimately causes overall hemostatic imbalance.

Liver is the chief organ for detoxification and metabolic activities of molecules. Induced levels of AST, ALT and ALP might indicate disorders in the liver function. Changes in these enzymes have been observed in other studies. Ahmad et al. (2011) noticed marked fluctuations in ALT and AST level in *A. hydrophila* challenged Nile tilapia. El-Barbary et al. (2017) also observed increase in AST and ALT in *O. niloticus* post intraperitoneal injection with *A. hydrophila*. Xia et al. (2017) reported a significant increase in ALP and acid phosphatase activity in *M. amblycephala* challenged with *A. hydrophila*. The enhanced activity of phosphatases is directly linked with the breakdown of high energy reserves required for fish survival and growth during infection. Chol level was found to be reduced in *F. oxysporum* challenged group which might increase the susceptibility of fish to diseases owing to disturbed metabolic reaction of lipids. Harikrishnan et al. (2003) also observed a decrease in chol levels in *Cyprinus carpio* after *A. hydrophila* challenge. Cnaani et al. (2004) reported that total chol level altered in three species of *Oreochromis* exposed to stressors. In high density situation, sea-bream fish showed decreased level of plasma chol and increased rate of mortality in response to Iridovirus (Tanaka & Inoue 2005).

The histopathological changes were seen to be coincided with hemato-biochemical data. We observed discrete alterations in histomorphology of gills, liver and kidney tissues. Gills are extremely sensitive to waterborne pathogens as they remain in direct contact with external environment (Fernandes & Mazon 2003). Similar histopathological observations of gills, liver and kidney exposed to infections and stressors were also reported by other researchers (Fernandes & Mazon 2003; Maftuch et al. 2018; Oğuz & Oğuz 2019; Özak et al. 2012). Butchiram et al. (2009) reported pathological variations in fish liver exposed to different contaminants. We previously have also reported similar histopathological changes in *Labeo rohita* with *P. aeruginosa* challenge (Kanwal et al. 2019). Blood cells come out of the vessels in stress situation and cause the inflammation in adjoining tissues (Novoa et al. 2010).

Proinflammatory cytokines play important roles in defense against microbial pathogens. IL1-β, TNF-α and IFN-γ show bimodal stimulation patterns and are considered crucial for phagocyte recruitment and onset of immune response at the site of infection (Laing et al. 2001; So et al. 2006; Tanekhy et al. 2010). Very few studies are available showing inflammatory genes' expression in a variety of organs in fish. In present work we have incorporated three major fish organs (gill, liver and kidney) as well as blood for finding universal effect of the infection on physiological and immune responses. We found that IL1-1β was upregulated in gill, liver and kidney of *F. oxysporum* challenged group but was downregulated in blood. Tanekhy et al. (2010) reported that IL1-1β expression was significantly upregulated in head kidney and spleen of Japanese flounder when exposed to *N. seriolae*. Expression of TNF-α was increased significantly in liver and kidney but was negligibly downregulated in blood. TNF-α expression was found to be up-regulated in head kidney leucocytes after triggering with lipopolysaccharide (Laing et al. 2001). TNF-α expression was significantly up-regulated in blood of large yellow croaker after 2 and 4 dpi with *Vibrio parahaemolyticus* (Xie et al. 2008). *F. oxysporum* infection in this study caused down regulation of TNF- in blood. These differences in expression might be associated to different fish species, infection time

and microbe type. Expressions of TNF-α in the gills of infected fish was undetectable. Syahputra et al. (2019) also reported a weak regulation of TNF-α gene in gills of *O. mykiss* fish challenged with *I. multifiliis*. IFN-γ is an important member of the innate immune response which is secreted by immune cells in response to pathogenic challenges. An increased induction in IFN-γ expression was noticed in blood, gills and kidney. High expression of IFN-γ in kidney after LPS challenge has been reported by Zhang et al. (2017). We observed a significant downregulation of INF-γ in liver of infected group. Similar to our findings, Oladiran et al. (2011) also found upregulation of INF-γ in kidney and spleen but downregulation in liver of goldfish at 7dpi infected with *T. carassii*. Thus, we found that IL1-1β, TNF-α, and INF-γ showed highest expression in liver, kidney and blood, respectively. The uniformly induced expression of all the three cytokine markers in kidney showed the foremost importance of kidney in fighting against *F. oxysporum* challenge.

Conclusion

The present study reports that *F. oxysporum* infection in *L. rohita* causes behavioral and phenotypic changes, disruptions in hemato-biochemical parameters and alterations in histomorphology. The proinflammatory marker genes IL1-1β, TNF-α and INF-γ showed differential expression in infected fish blood, gills, liver and kidney as compared to the control. However, infected fish kidney showed a uniformly high expression of all the three markers. The absence or downregulation of these genes in blood (IL1 1β), gills (TNF-α) and liver (INF-γ) may indicate aggressive virulent behavior of *F. oxysporum* which might have suppressed or delayed a ubiquitous immune response to a robustly developing infection. Further molecular analysis of detailed interaction of cytokines will provide a better understanding of the complete defense response of fish to fungal pathogens. The information provided from this work may help to find novel measures for the control and regulation of disease outbreaks in aquaculture.

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*Corresponding author; email: zakia.kanwal@lcwu.edu.pk