



Haematological responses of the Indian major carp *Labeo rohita* to saprolegniasis

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ABSTRACT

Saprolegniasis (cotton wool disease), due to infestation by *Saprolegnia parasitica*, is a major fungal disease in fish. Present study was conducted in the Indian major carps, rohu, *Labeo rohita* (Hamilton, 1822). Fingerlings of rohu (10.5±2.2 g) were artificially infected with the fungal parasite *S. parasitica* via injection (T1), cohabitation (T2) as well as immersion (T3) and compared with a control group (T0) with no infection. Blood samples were collected at 15 days interval over a period of 45 days and analysed for haematological parameters. Haemoglobin level in infected fish was lower ($p \leq 0.05$) than non-infected fish on 15, 30 and 45 days post-infection (dpi) except in cohabited fish on 45 dpi. Total erythrocyte count (TEC) in infected fish was lower ($p \leq 0.05$) than non-infected fish on 15, 30 and 45 dpi. Total leucocyte count (TLC) was higher ($p \leq 0.05$) in infected fish on 15 dpi; however no difference ($p \geq 0.05$) was noticed on 30 dpi. TLC in injected group was higher ($p \leq 0.05$) than control group on 45 dpi. Haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leucocrit (Lct) values in infected group were lower ($p \leq 0.05$) than control group. There was no difference ($p \geq 0.05$) in mortality among the groups on 15 dpi; however, on 30 and 45 dpi, the group subjected to immersion exposure recorded highest mortality followed by injected and cohabited groups. Varying degrees of histological alterations were observed in the tissues due to the fungal infestation. The results indicated that *S. parasitica* significantly altered the blood profile of infected rohu leading to 45-50% mortality. Therefore appropriate management measures need to be adopted for preventing infestation by *S. parasitica* in carp culture systems for realising sustainable fish production.

Keywords: Artificial infection, Haematology, *Labeo rohita*, *Saprolegnia parasitica*

Introduction

Saprolegniasis is a major setback in fisheries and aquaculture (Willoughby, 1994). It is the second most important fungal disease in fish after epizootic ulceration. The causative agent of this disease is a water mold, known as *Saprolegnia parasitica*. The disease is also called 'cotton wool disease' due to the appearance of cotton-like white to greyish patches, radiating in circular, crescent or whorl pattern on skin and gills of affected fish. The infection spreads faster once hyphae invades epidermal tissue. All life stages of fish such as spawn, fry and fingerlings suffer due to saprolegniasis. The pathogen is transmitted through infected fish, eggs, water and equipment (Bruno and Wood, 1999). Malachite green is the most effective chemotherapeutant against the fungus; however, its use is banned since 2002 due to toxicological effects. As a result, there is a dramatic re-emergence of the disease in commercial farms. At present, formalin and Pyceze (a pesticide) are recommended against this

disease; however, they are also expected to be prohibited in near future because of environmental issues (van West, 2006). Therefore, alternative strategies are urgently needed to control saprolegniasis in fish culture systems. Understanding the mode of action of *Saprolegnia* through artificial infection will be helpful for developing strategies to control infections (Howe and Stehly, 1998). Blood parameters were used earlier as an indicator of fish physiology during mycosis (Wedemeyer, 1970); however, so far no study has been conducted in Indian major carps. Therefore, the present study was attempted in rohu *Labeo rohita* (Hamilton, 1822) which is a major aqua-crop species in India, to study the sequential changes during infection by *S. parasitica*.

Materials and methods

The study was conducted at the Fish Health Management Division of the ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), Bhubaneswar, Odisha. Fingerlings of rohu (10.5±2.2 g), procured from

ICAR-CIFA farm were used for the experiments. The fish were transported in 500 l capacity tanks with air pump. They were carefully transferred to circular tanks (1000 l) and left undisturbed overnight. To ameliorate handling stress, fish were dipped in a mild salt solution (3% NaCl) on the next day and acclimatised under aerated condition over a period of 15 days before commencement of the challenge experiment. Fish were observed for overall health, behaviour and presence of any diseases. Crowding and handling were kept minimal and pellet feed having 20% crude protein was provided at 3% of body weight, twice daily. Aeration was regularly provided and water quality was maintained at normal levels. Water parameters, estimated following standard methods (APHA, 1998) were: dissolved oxygen 7.6 ± 0.2 ppm, temperature $27\pm 1^\circ\text{C}$, pH 7.4 ± 0.2 , total alkalinity 230 ± 10 ppm, hardness 40 ppm, carbon dioxide 2.5 ppm, chloride 40 ppm, ammonia 0.27 ppm, nitrate 0.02 ppm, nitrite 0.05 ppm and phosphate 0.003 ppm. Fish showing active movement and good health, were used for challenge experiment with one of the strains of the fungus *S. parasitica* isolated and identified at ICAR-CIFA. Sporulation was induced in the fungus as per Dieguez-Uribeondo *et al.* (2007) and spore density was adjusted to 2.5×10^4 ml⁻¹.

The fish were infected with the fungal spores *via* injection (T1), cohabitation (T2) and immersion (T3). In control group (T0), 30 fish were kept with no spore inoculation. The injected and cohabited groups comprised 40 fish, of which 20 were intramuscularly injected with spore at 0.2 ml per fish and remaining 20 were allowed to co-habit in the same tank with injected fish after amputating one of the caudal fin lobe to mark they are non-injected. In the group exposed to immersion challenge, 40 fish were kept and spores inoculated at 1 ml per liter of water. Triplicate tanks were maintained for each group. Relative percent survival and behaviour of the challenged fish were monitored regularly. At 15, 30 and 45 days post-infection (dpi), six fish were randomly sampled from each group and bled through cardiac vein for analysis of haematological parameters. During each sampling, one fish from each group was dissected and tissue samples collected for histological study and re-isolation of fungus (Chauhan *et al.*, 2014). Moribund specimens were analysed for the presence of fungus and fishes from which fungus was reisolated, were considered affected by saprolegniasis. The experiment lasted for 45 days.

The blood samples were analysed for haemoglobin (Hb) by cyanmethaemoglobin method (Van Kampen and Zijlstra, 1961). Total leucocytes count (TLC) and total erythrocytes count (TEC) were estimated using haemocytometer (Russia and Sood, 1992). Haematocrit

(Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leucocrit (Lct) were analysed as per Shah (2010). Relative percentage survival (RPS) of fish in different groups was determined at 15 days interval. The data were analysed using one-way analysis of variance (ANOVA) in SPSS 21 to find out difference at 5% level of significance.

For histological examination, tissues were fixed in 10% neutral buffered formalin for 48 h and processed routinely for the preparation of paraffin blocks as per the methods of Chauhan *et al.* (2014). The paraffin blocks were then sectioned at 5 μm thickness, stained with haematoxylin and eosin (H&E) and the slides were observed under microscope to see for any change in tissue architecture.

Results and discussion

Results of the haematological investigations in fish due to *Saprolegniasis* are presented in Table 1 and Fig. 1. The haemoglobin content was decreased significantly ($p\leq 0.05$) in infected fish compared with non-infected fish. On 15 dpi, haemoglobin level in injected group (T1) was lower ($p\leq 0.05$) than groups challenged by cohabitation (T2) and immersion (T3). However no difference ($p\geq 0.05$) was observed between fishes in T2 and T3 groups. On 30 dpi, haemoglobin in group T1 was lower ($p\leq 0.05$) than fishes in groups T2 and T3 and on 45 dpi, haemoglobin content in group T1 and T2 was lower ($p\leq 0.05$) than T3. The same was confirmed through blood smearing. This down fall could be attributed to impairment in erythropoiesis and haemoglobin synthesis in fish. Gardner (1974) as well as Hatai and Hoshiai (1994) observed similar condition in salmon's dying due to saprolegniasis.

Table 1. Effect of *Saprolegnia parasitica* infection on selected blood parameters (Hb, TLC and TEC) of *L. rohita*

Parameters	Groups	15 dpi	30 dpi	45 dpi
Haemoglobin (g%)	T0	4.47 \pm 0.07 ^a	4.27 \pm 0.07 ^a	4.00 \pm 0.12 ^a
	T1	3.20 \pm 0.12 ^c	2.73 \pm 0.07 ^d	2.27 \pm 0.07 ^b
	T2	3.73 \pm 0.07 ^b	3.93 \pm 0.07 ^b	3.87 \pm 0.07 ^a
	T3	3.80 \pm 0.12 ^b	3.20 \pm 0.12 ^c	2.33 \pm 0.07 ^b
TEC (x10 ⁶ mm ⁻³)	T0	0.68 \pm 0.04 ^a	0.85 \pm 0.06 ^a	0.91 \pm 0.02 ^a
	T1	0.36 \pm 0.04 ^b	0.28 \pm 0.03 ^c	0.18 \pm 0.02 ^b
	T2	0.23 \pm 0.03 ^b	0.12 \pm 0.03 ^c	0.20 \pm 0.003 ^b
	T3	0.28 \pm 0.07 ^b	0.45 \pm 0.07 ^b	0.13 \pm 0.006 ^c
TLC (x10 ³ mm ⁻³)	T0	2.5 \pm 0.06 ^c	26.00 \pm 5.77 ^a	7.7 \pm 1.74 ^{bc}
	T1	12.0 \pm 0.58 ^a	30.33 \pm 2.03 ^a	27.33 \pm 2.33 ^a
	T2	9.33 \pm 0.38 ^b	19.67 \pm 4.18 ^a	4.7 \pm 1.04 ^c
	T3	8.97 \pm 0.15 ^b	29.67 \pm 4.80 ^a	11.6 \pm 2.09 ^b

Mean values bearing different superscripts within a column for a parameter are significantly different ($p\leq 0.05$)

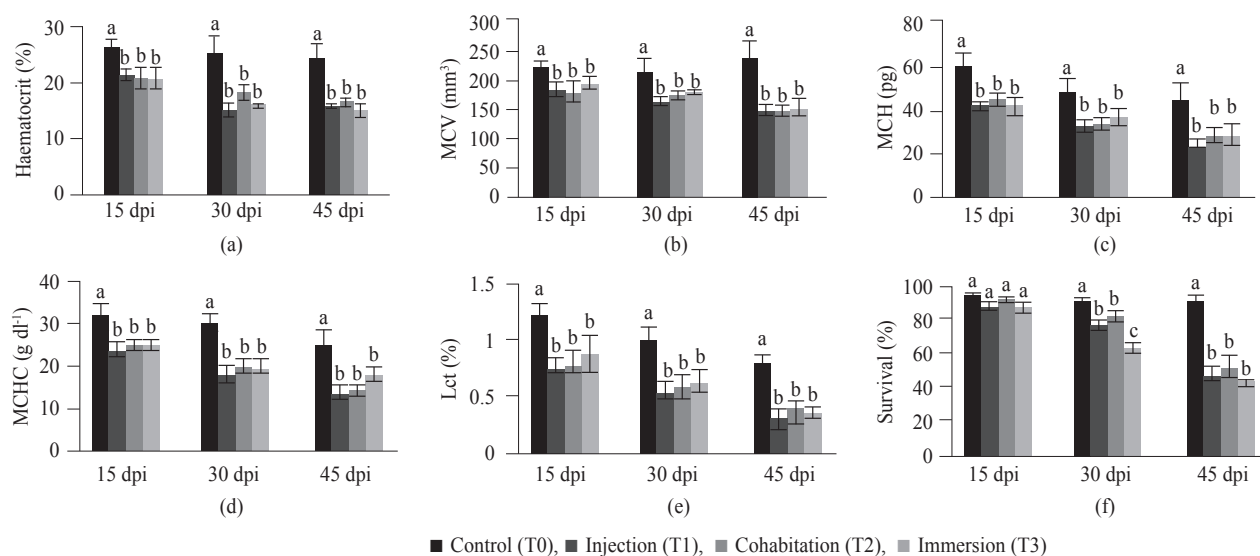


Fig. 1. Haematocrit (a), MCV (b), MCH (c), MCHC (d) Lct (e) values and (f) survival rate in *Labeo rohita* fingerlings exposed to *Saprolegnia parasitica* via different challenge routes

TEC in all the three groups on 15 dpi was lower ($p \leq 0.05$) than control group (T0); however, no difference ($p \geq 0.05$) was noticed among the infected groups. On 30 dpi, TEC in groups T1 and T2 was lower ($p \leq 0.05$) than that of fishes in group T3; however, no difference ($p \geq 0.05$) was observed between groups T1 and T3. On 45 dpi, the TEC was found decreased ($p \leq 0.05$) in group T1 as compared with T2 and T3; however, no difference ($p \geq 0.05$) was found between T1 and T2. This was a strong indicator of stress in fish (McLeay and Gordon, 1977). Destruction of haematopoietic tissue in kidney and spleen would have led to such decrease in blood cell production and subsequent reduction in their count. Similar results have been reported in tench by Shah (2010).

TLC in infected fish on 15 dpi was higher ($p \leq 0.05$) than control fish. In group T1, it was higher ($p \leq 0.05$) than groups T2 and T3. However, no significant difference ($p \geq 0.05$) was observed between T2 and T3 groups. On 30 dpi, no difference ($p \geq 0.05$) was noticed in the three treatment groups. In group T1, on 45 dpi, TLC was higher ($p \leq 0.05$) than control group. In T3, it was higher ($p \leq 0.05$) than T2, but no significant difference ($p \geq 0.05$) was found in T2 and T3 compared with control group. Increase in levels of TLC could be due to increase in production of lymphocytes, thrombocytes and lymphocytosis or squeezing of leucocytes into peripheral blood (Das and Mukherjee, 2000) and decreased levels of TLC could be attributed to increase in secretion of corticosteroids following stress (Tort *et al.*, 1987).

Hct, Lct, MCV, MCH and MCHC in all the three treatment groups were lower than ($p \leq 0.05$) control group throughout the study period. The rate of fall in the levels

of these parameters was drastic with the progress of time; however, no difference ($p \geq 0.05$) was noticed among them over a period of 45 days. Involvement of non-leucocyte cells in the defence against fungus might be the cause of such drop in Hct, Hb and RBC in infected groups of fish (Shah, 2010).

Saprolegnia infection caused significant fish mortality (Fig. 1f) in all the challenged groups. There was no difference ($p \geq 0.05$) in survival of fish among injected, cohabitated and immersed groups compared with control group on 15 dpi. On 30 dpi, survival was reduced ($p \leq 0.05$) in infected groups compared with control fish. In group T3, survival was lower ($p \leq 0.05$) than T1 and T2 groups; however, no difference ($p \geq 0.05$) was noticed between groups T1 and T2. On 45 dpi, survival in infected groups fell significantly ($p \leq 0.05$) compared with non-infected control fish; however, no significant difference ($p \geq 0.05$) was observed among the infected groups. Highest mortality (45%) was recorded in group T3 challenged by immersion exposure which could be attributed to ingestion of infective spores which might have multiplied faster in the tanks under congenial water conditions. The injury due to caudal fin amputation was responsible for infection and fish mortality in cohabitated group (Bruno and Wood, 1999).

Deep penetration of hyphae in the muscle (Fig. 2) and gill tissues, would have caused haemodilution and breakdown of osmoregulation and as a result fish suffered mortality. Shah and Altindag, (2004) observed erythrocyte fragility and haemorrhage leading to anaemia in fish. Damage of haematopoietic tissue and haemodilution

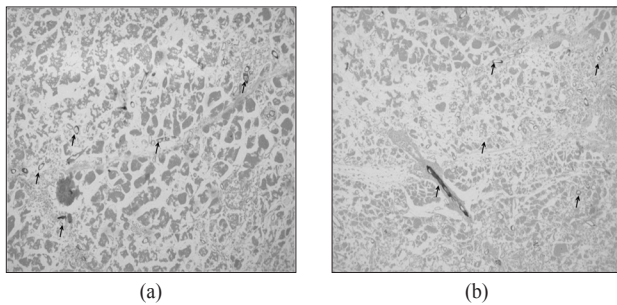


Fig. 2. Histological sections showing deep penetration of fungal hyphae into the muscle tissue of infected fish (Scale bar = 100 μ m)

hampered erythrocyte production (Wepener *et al.*, 1992), whereas, alteration in membrane permeability and mechanical fragility (Gill and Epple, 1993) and haemolysis (Tort *et al.*, 1987) accelerated erythroclasis. The infected fish showed symptoms of lethargy which could be attributed to excessive energy exerted to overcome stress and there was excess mucus secretion in gills which caused increase in the diffusion distance between water and haemoglobin. As a result, gas exchange in the body was found to be rapidly hampered and fish became exhausted. Impairment in gas exchange might have triggered erythropoiesis to maintain haemoglobin at normal level; however, continuous exposure to fungus has suppressed this capacity leading to decreased levels of TEC. In this study, fungal infection was confirmed histologically at tissue level by presence of fungal hyphae in tissue sections and also samples from challenged groups were found positive for *S. parasitica* during re-isolation of the fungus on PDA. Loss of epidermis, necrotised hypodermis with hyphal growth and destroyed musculature were observed at tissue level (Fig. 2) similar to the condition reported by Chauhan *et al.* (2014).

Results of the study clearly indicated that, rohu suffered haematological aberrations during *S. parasitica* infection leading to 45-55% mortality in infected fish. However, this is a preliminary attempt on examination of physiological impairment in a stock of Indian major carp due to saprolegniasis under controlled conditions. Further investigations are needed at population level with other strains of the fungus.

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