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IMPACT OF *Thelohanellus mrigalae* TRIPATHI, 1952 ON *Cirrhinus mrigala*: PREVALENCE, HISTOPATHOLOGICAL AND HAEMATOLOGICAL ALTERATIONS

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Author SM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SN managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Thelohanellus mrigalae Tripathi, 1952 is the important myxosporean parasites causing hemorrhagic thelohanellosis disease in *Cirrhinus mrigala*, a fresh water teleost. Infected fishes show profound swelling, formation of pus, cyst on gill filament and infiltration of pathogen into connective tissue. About 238 live specimens of *Cirrhinus mrigala* were examined and myxozoan parasite *T. mrigalae* were identified which implicates host specificity. Any significant infection was not observed in the sampling period of March-July, whereas prevalence of *T. mrigalae* was much higher in the period of November to February which is post-monsoon period in West Bengal. During monsoon, moderate infection was observed as rain water is the main factor for spreading spore. The plasmodia of *T. mrigalae* were located in the intrafilamental epithelial site of gill. The plasmodia were cylindrical and creamy-white patch and contained ellipsoidal spores as valvular shape. The spores were 20.4 – 22.1 (±6.34) µm in length and 8.5 – 10.2 (±0.87) µm in width. An oblong, irregularly shaped mass of protoplasm was observed between the polar capsule and spore capsule. The polar capsule was 10.2 – 13.5 (±0.05) µm in length and 3.4 – 4.25 (±0.5) µm in width containing a polar filament coiled perpendicular to the longitudinal axis of the spore body. Histology of gill filaments showed a series of nodules of various sizes distorting the normal architecture of the gill cartilage. Distention of the gill filament was pronounced and lamellae adjacent to the cyst were no longer present. Necrotic lesions and hyperplasia were extensively observed in the gill filaments. Infected fish had lower RBC counts, higher WBC counts and more fragile erythrocytes than control fish. Haemorrhaging was distinctly visible. The increase in number of mucous goblet cells on the gill lamellae of infected fish enhance the diffusion distance between water and blood haemoglobin and rapidly impair Oxygen (O_2) and Carbon dioxide (CO_2) exchange.

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Keywords: Myxosporean; plasmodia; polar capsule; polar filament; gill lamella; goblet cells.

1. INTRODUCTION

Myxosporean pathogens are continually emerging and threatening the development of pisciculture. They cause production losses and some fish have to be discarded because they are unsightly and not considered to be fit for human consumption [1]. Different diseases are caused by this type of pathogen such as proliferative kidney disease, whirling disease, ulcerative disease etc in major carp. Therefore, the contribution to the knowledge of fish parasites is a prerequisite for healthy, rapid fish production and correct diagnosis of the disease agent of epizootics [2].

All myxozoans achieve transmission to new hosts by multicellular spores comprised of external valve cells that enclose infectious amoeboid cells (sporoplasms) and cells bearing polar capsules. The fish that attacked by myxozoan parasite showed in difficulties to breathe because it found the existence of a nodule or cyst on the gill filaments [3]. Upto now more than 108 species belonging to the genus *Thelohanellus* Kudo, 1933 [4] have been reported in the literature as parasites of freshwater and marine fishes in several geographical areas [5]. Members of this genus are highly host-specific and generally histozoic in various organs. Pathogenic *Thelohanellus* spp. causes "thelohanellosis", a disease characterized by severe hemorrhaging and first described by Yokoyama et al. [6] for *Thelohanellus hovorkai* Akhmerov, 1960 [7] in carp (*Cyprinus carpio*). Basu et al. [8] listed 32 Indian species of the genus *Thelohanellus* Kudo, 1933 [4]. In India, *Thelohanellus* spp. have been reported predominantly in three states namely West Bengal [9-11], Punjab [12,13] and Andhra Pradesh [14,15]. The presence of plasmodia of *T. mrigalae* was revealed as whitish cysts with microscopic spores. They were characterized by pyriform or broadly ellipsoidal spores (valvular view), which look slimmer in sutural view [16].

Since very scanty and infrequent information are available from *C. mrigala* infected by myxozoan parasites worldwide. Here we describe the genus *T. mrigalae* inhabiting *C. mrigala* in detail including its seasonal prevalence, morphometry, histopathological alterations of host tissue, histochemical analysis of mucous cells in gill filament and secondary lamellae and haematological alterations of fish to help farmers in taking appropriate measures to prevent heavy losses of Mrigal fish, an important carp.

2. MATERIALS AND METHODS

2.1 Sampling

238 *Cirrhinus mrigala* ranging from 750 to 850 g in weight and 30-35cm in length were purchased from Taltala market, near Maulana Azad College, Kolkata-13 throughout one year (2019). A parasitological survey was conducted on several organs and tissues, especially the gill filaments. Parasitized gill filaments from the infected host specimens were examined and analyzed using standard protocols [17-19].

2.2 Seasonal Variation and Prevalence

We have studied and examined the protozoan parasite *T.mrigalae* (Myxozoa: Myxosporea) and its prevalence throughout the year of 2019. Number of highly infected, moderately infected and noninfected (non-significant) fishes were identified and counted and infestation period categorized into March-June (Summar), July-October (Rainy) and November-February (Winter). Prevalence frequency index (PFI) was estimated following the formula given by Margolis et al. [20].

$$\text{Prevalence (\%)} = \frac{\text{Total number of infected X 100}}{\text{Total number of fish host examined}}$$

2.3 Morphometry

Macroscopically visible lesions or myxosporean cysts as plasmodia on gills were examined by naked eyes. For morphological and taxonomic measurements, at least one fresh plasmodium was taken from infected gill on slide and ruptured to release their spores. Spores were placed on a slide with normal saline and examined by using a high-resolving oil immersion objective with adjusted illumination of Trinocular Microscope, ZEISS and photographed with the Sony cyber shot DSC-T10 camera and calibrated by ocular micrometer. Smears of parasite spore were prepared, air-dried for 1h, fixed in absolute methanol and stained with Giemsa stain in phosphate buffer (pH 7.2) for 1 h. Presumptive *T. mrigalae* plasmodia were measured, cysts were crushed to obtain spores, and their total length, body length and width (in µm) were determined with ZEISS, Trinocular Microscope. Parasite identification was done according to Hoffman [19] and Feist & Longshaw [21]. Mean and standard deviations of each plasmodia and spore dimension were obtained from fresh mature spores.

2.4 Histopathological Examination

Tissue samples from highly infected, moderately infected and noninfected gills were fixed in 10 % formalin, then dehydrated in a series of alcohols, cleared in xylol, embedded in paraffin wax, and sectioned by a microtome at 6µm thick [22]. Tissue sections were stained with hematoxylin and eosin. The stained sections of gills were examined and photographed. This technique demonstrated the location of cysts within the gills.

2.5 Histochemical Analysis

Gill tissue sections were taken from the middle portion of the gill arches of highly infected, moderately infected and noninfected fishes. Tissue samples were fixed in 10 % formalin, then dehydrated in a series of alcohols, cleared in xylol, embedded in paraffin wax, and sectioned by a microtome at 5µm thickness. Sections were stained with combinations of Alcian Blue- Periodic acid-Schiff (AB-PAS) reagent at pH 2.6 for visualizing mucous goblet cells [23]. Mucous goblet cell counts were made under oil immersion (field size 0.20 mm) and the number of mucous cells containing glycoprotein was expressed as a percentage of the total cell population against pathogenic response.

2.6 Haematological Study

Blood samples were taken by caudal venipuncture after anaesthesizing both the highly infected, moderately infected and noninfected fishes. Total RBC and WBC counts were determined manually with the Neubauer counting chamber after the blood was diluted with Daice diluting fluid solution. Blood smears were prepared immediately by air dried, fixed in 95% methanol for 5mins then stained with Giemsa and observed through light microscope. Giemsa-stained blood smears were used for the measurement and assessment of any morphological alterations of blood cells. Blood cells were identified on the basis of morphology and dimensions using a stage and ocular micrometer.

3. RESULTS AND DISCUSSION

3.1 Seasonal Variation

During the study period a total of 238 numbers of live *Cirrhinus mrigala* were examined.

T. mrigalae is the most prevalent protozoa parasite of *C. mrigala* and were host specific because these were less infecting other major carp. From July to February (both monsoon and winter) plasmodia were detected on the gills of 650 to 750 g adult *C.mrigala* collected from Taltala Market, Entally, Kolkata-13. These protozoans were found mostly on gills. In winter season, the prevalence of *T.mrigalae* reached at 87.62% and gradually decreased in summer (17.64%) and monsoon (41.5%) (Table.1). This indicates that the winter was the most vulnerable period to get parasitic infestation. The water quality gets deteriorated and dissolved oxygen level become decreased during this period and the fishes were in stressed condition which favours the spreading several parasitic diseases. Mukherjee et al. [24], reported that low pH and low temperature were the major factor for spreading protozoan infection in fishes. Monsoon is the onset of myxosporean infection due to the availability of their intermediate host *Tubifex* sp, an oligochaete.

3.2 Light Microscopic Observations

Primary symptoms were mucus laden gills due to the presence of plasmodia within the entire length of the gill filament. Large, creamish milky white, elongated plasmodia present on the gill filament, 15-20 in number, containing1000-1500 microscopic *T.mrigalae* spores (Fig. 1b). The spores were ellipsoidal capsular structure with pointed anterior end and broad rounded posterior end with single valve. Spores (n=6) measured 20.4 – 22.1 (±6.34) µm in length and 8.5 – 10.2 (±0.87) µm in width. Valves were joined along a thickened straight suture line. A single polar capsule was situated anteriorly and occupied three fourth of the spore body cavity. It measured about 10.2–13.5 (±0.05) µm in length and 3.4 – 4.25 (±0.5) µm in width. Coiled polar filament arranged perpendicular to the polar capsule axis. The uninucleated crescentic sporoplasm was confined to a small portion behind the capsule. A medium sized vacuole occupied the vacant spaces of sporoplasm (Fig. 2).

3.3 Histopathological Changes

Histological studies showed that the noninfected gill lamellae have normal architecture (Fig. 3a) but in highly infected gill section the plasmodia were embedded partly or completely in the gill tissues (Fig. 3b). The growing curved plasmodia caused distortion of gill at their site of infection and also in the adjacent layers. The plasmodia produced the cysts by joining numerous small plasmodia (many- to-one type). The cysts containing plasmodia located in the central sinus of the gill filaments immediately adjacent to the gill filament cartilage. The cysts were spherical to elliptical in shape each containing more than 1000 parasites and surrounded by flattened epithelial cells.

Necrotic changes and hyperplasia were extensively observed in the gill arches in severe infection (Fig. 3c). Some of the cysts were also infiltrated by epithelial cells and macrophages (Fig. 3d). Epithelial desquamation and lamellar fusions were noticed. Swelling at the tips of secondary lamellae, curling, degeneration of secondary lamellae observed and oedema increased vacuolation and substituted by numerous growing plasmodia reducing the functional area of gill (Fig. 3e, f, g, h).

Table 1. Seasonal variations in PFI % age of *T. mrigalae* on *C. mrigala*

Parasite	**Season's variations in PFI% age**		
T.mrigalae	Summer (March-June)	Monsoon (July- October)	Winter (November-February)
	17.64	41.5	87.62

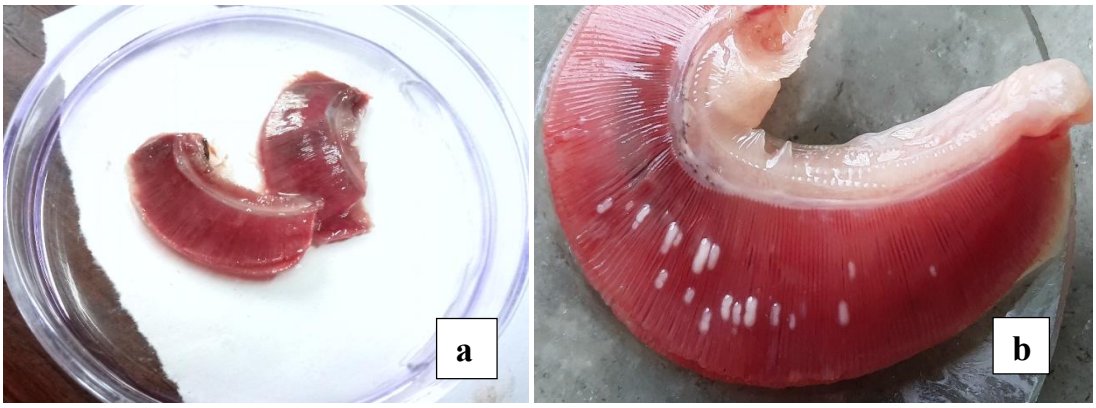


Fig 1(a) Noninfected gill and (b) Infected gill of *C. mrigala* with cyst

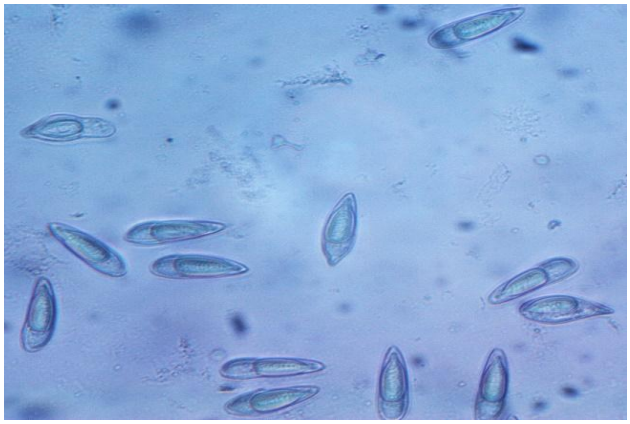
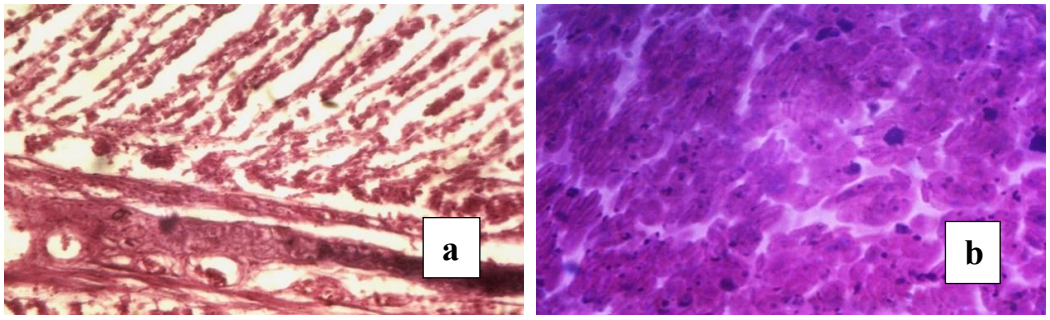


Fig. 2. *T. mrigalae* spores



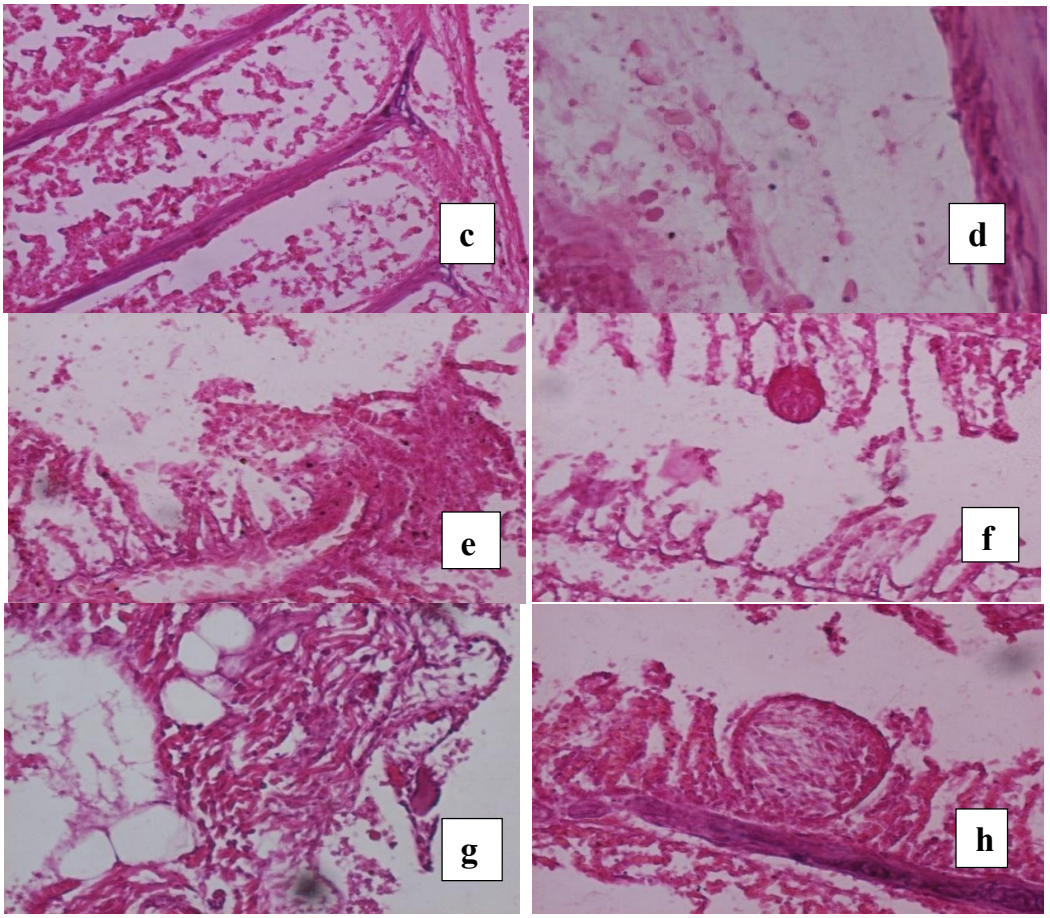


Fig. 3. Histopathology of gills of *C. mrigala* infected with *T.mrigalae* (a)Noninfected gill filament, (b)Intrafilamental plasmodia, (c)Necrotic changes, (d)Infiltration of macrophage cells, (e) Curling, (f)Degeneration of secondary lamellae, (g)Vacuolation, (h)Growing plasmodia

3.4 Histochemistry

Mucous goblet cells detected in the epithelium of gill filaments and their secretion may be a mechanism for adaptation to different stressed conditions of the aquatic environment. Mucous goblet cells counts were made under oil immersion (field diameter 124mm) and the number of goblet cells containing glycoprotein was expressed as a percentage of the total mucous cell population on 10 random fields in each section of gill lamella (Fig.4). With the combined Alcian Blue-Periodic Acid Schiff technique mucous goblet cells were stained magenta colour and demonstrated the presence of neutral and acid mucopolysaccharide groups. *T.mrigalae* infection resulted in hyperplasia and hypertrophy of these cells. During winter season infected fish gills showed higher proportion of magenta coloured secreted mucous goblet cells (Fig. 5b) in areas with lamellar epithelial hypertrophy compared to gill areas with healthy lamellar and filament morphology in moderately infected as well as with gills from uninfected groups (Fig. 5a). The mucosal barriers of the gill are the first line of defense for teleosts and contain a number of immunologically important factors, including immunoglobins, cytokines, proteases, lysozyme, antimicrobial peptides and complement factors [25]. The mucins, present in secreted mucous are mainly comprised of carbohydrates, which themselves can a□ect pathogen growth, virulence and adhesion to epithelia [26]. Jones et al. [27] and Powell et al. [28] observed that the production of the high amounts of glycoprotein within mucous cell populations had been shown in response to parasitic infection and deter the proliferation of pathogenic micro-organisms in freshwater fishes. A single type of mucous cell secretes the different acid and neutral glycoproteins which engaged in the prevention of epithelium damages and increase the water blood barrier for respiratory gases diffusion and consequently reduces O_2 uptake [29] and CO_2 excretion [30].

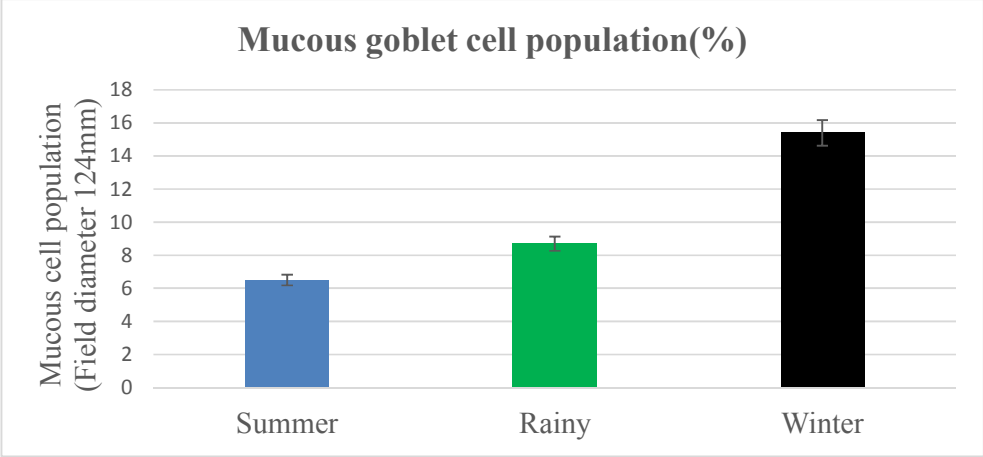


Fig. 4. Relative abundance ofsecreted mucous goblet cells with respect to season

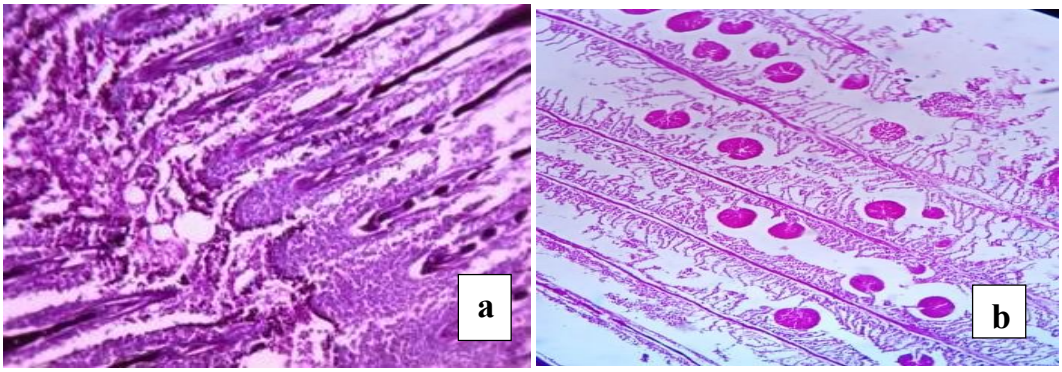


Fig. 5.(a)Noninfected healthy lamellae structure, (b) Infected lamellae with mucous goblet cells

3.5 Haematological Changes

In the Giemsa stained blood smear, deformed RBCs were evident. The deformities were mostly elongated erythrocytes measured 12.5±0.05µm in comparison to noninfected erythrocytes which measured 9.5±0.05 µm in length (Fig. 6a, b), chromatin condensation, hemolysis, membrane wrinkling, vacuolation of erythrocytes and basophilic stippling (Fig.6b). Erythrocyte counts in healthy noninfected *C.mrigala* were 3.90±0.5 × 10^6/mm^3. The highly infected *C.mrigala* during winter season revealed very low erythrocyte count (0.89±0.5 × 10^6/mm^3). Under diseased condition fish erythrocytes undergo amitotic divisions (Fig.6c). Large number of enucleated and ruptured RBCs as well as damaged leukocytes were evident. Swelling and amitotic division of circulating erythrocytes were found to have tendency to cause haemolytic anaemia in teleost fish.White blood cells significantly increased in highly infected fish (20±0.5 x10^6/mm^3) (Table.2) and differentiation into lymphocyte, eosinophil and basophil (Fig. 6d) were evident. However, no significant differences in RBC count occurred between the males and females of *C.mrigala* in case of both noninfected and infected fishes. Decreased RBC and increased WBC were reported due to epizootic ulcerative syndrome by heavy infestation of *T.mrigalae* Increased WBC was suggesting their importance in fish cell mediated immune response as they were the main component of inflammatorty exudate [31].

Table 2. Total counts of red blood cells (RBC), white blood cells (WBC) in noninfected, moderately infected and highly infected *C. mrigala*

	Blood parameters	Non infected (Control)	Moderate infection	High infection
Cirrhinus mrigala	RBC (×10^6)	3.9 ±0.5	1.49±0.5	0.89±0.5
	WBC (×10^6)	4.1± 0.05	16±0.25	20±0.5

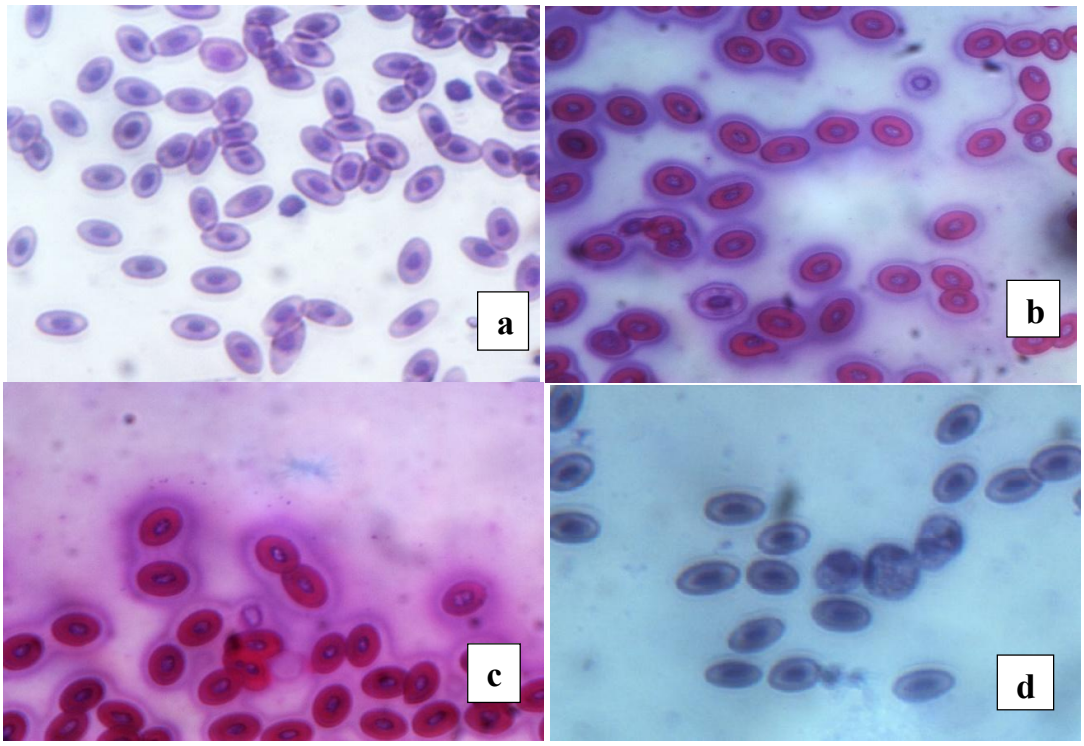


Fig. 6. (a)Blood smear of noninfected fish, (b)Infected fish blood smear with deformed RBC, chromatin condensation, vacuolation, basophilic stippling, (c)Amitotic division in diseased erythrocytes, (d)Leucocyte differentiation

4. CONCLUSION

This investigation concluded that the post monsoon season, i.e. winter season is most favorable for spreading *T.mrigalae* infection to *C.mrigala*. The probable reason for the availability of these parasites more in winter may be due to the unfavourable water condition for fishes.The plasmodia, sporogenic stages of Myxozoan parasite damaged more than 50% of the gills causing respiratory distress and suffocation. The curved plasmodia had tendency to expand the surface area in contact with adjacent gill lamellae facilitating the attainment of nutrients from the host. As the gill lamellae was was ruptured by growing cysts with necrotic lesions and hyperplasia, the normal respiratory function of the gill appeared to be impaired. The present tumour like cyst was studied histologically in an advanced stage of development with only mature spores found, earlier developmental stages awaite further study to evaluate whether the present plasmodia were transmitting from oligochaete. *T.mrigalae* infection gave the opportunity to other pathogenic microorganisms to enter the connective tissue through damaged gill lamellae and responsible for destruction of RBC and greater infiltration of WBC. Changes in the total number of and types of WBC can be used as an indicator of the presence of certain infectious diseases that occur in fish. Our work revealed that greater number of mucous cell population secreted by goblet cells can have roles in protozoan infection by preventing adhesion to gill lamella.

ETHICAL APPROVAL

Animal ethical care guidelines were followed as edible fishes were used in the study and did not require approval.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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