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RESEARCH ARTICLE

THE HISTOPATHOLOGICAL AND ULTRASTRUCTURAL ANALYSIS OF MICROSPORIDIAN INFECTION IN CATLA (*CATLA CATLA)* AND THEIR EFFECTS ON ANTIOXIDANT ENZYMES AND PROTEIN EXPRESSION

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Abstract

The microsporidian infection has been detected in catla and catla fry. The sporophorous vesicles, necrosis and liquefaction of muscles; as well as hypertrophy, hyperplasia and necrosis in gills and liver were observed. In the ultrathin muscle section, ellipsoidal (1.95 × 2.12 μm) spore with anchoring disc, protuberant area and basal vacuole; and oval merronts (3.2 × 3.1 μm), bounded by the membrane stakes with interrupted pores were observed. In the gill epithelia, disrupted organelles, tubular structures, coated vesicles and round spores (1.43 × 1.47 μm) in direct contact with cytoplasm were found in TEM. The microsporidia appear to belong to *Pleistophora* species. The significant changes in antioxidant enzyme activities in liver in infected catla, indicates activation of defense mechanism. The water analysis showed high level of ammonia, COD and alkalinity. The heavy metal contents and the level of indicators microorganisms were found to be low in pond during infection. The protein analysis indicated expression of new proteins in muscle, gill and liver in infected catla. The black spot region of catla fry has shown large number of spores; and the TEM analysis revealed the presence of spores in muscle (0.41 × 0.58 μm) and defense cells (0.28 × 0.39 μm).

Introduction

The Indian Major Carps (IMCs) are fastest growing fishes reared in the tanks fertilized with organic and inorganic manures (Jhingran and Pullin, 1988). The excessive manuring, overstocking, metabolite accumulation and water quality variations have been reported to result into water quality deterioration and spread of pathogenic organisms which are known to affect the growth and to trigger the development of diseases in carp. These diseases include haemorehagic septicemia, saparolegniasis, dectylogyrosis, trichodiniasis, vibriosis, protozoan infections and several other diseases (Toor et al. 1983).

The microsporidia are known to infect fresh water, brackish water and marine fishes; about 156 microsporidian species have been reported to infect fishes, including cypriniforms, turbot, salmonids and many more species; and in aquaculture, microsporidiosis to cultivable fishes has been known to seriously endanger the whole stock and reduce the productivity (Nepszy and Dechtiar, 1972; Lom and Dykova, 1992 ;Estevez et al. 1992; Lom et al. 1995, 1999; Mathis, 2000; Ramsay et al. 2002; Lom and Nilsen, 2003). Microsporidia are obligate intracellular protozoan parasites, unicellular and characterized by production of small spores as well as polar tubes which are involved in transmission of parasites into the host cells (Keohane et al. 1996a; 1996b). The life cycle of mycrosporidia includes merogony, sporogony, sporoblast as well as spore stages; some of them are known to form xenomas; and the developmental stages are always in contact with host cell cytoplasm (Lom et al. 1995, 1999). In fishes, this infection has been observed in reproductive tract (Muriel et al. 1975), gills (Catherine et al. 2007), digestive tract (Rodriguez et al. 2003), migratory cells (Shaw et al. 1998), kidney (Didier et al. 2004) and muscles

(Ruehl-Fehlert et al. 2005). The microsporidia infecting fish mainly belong to genera *Glugea, Pleistophora, Ichthyosporidium, Heterosporis, Nosemoides, Spraguea, Tetramicra,* 73 *Loma, Microgemma, Microfilum, Nucleospora, Neonosemoides* and *Pseudoloma* (Azevedo and Matos, 2002). During the monitoring of hyginic condition of carp culture units in Central Gujarat during September 2008- February 2009, microsporidian infection was detected in adult catla and catla fry. The present study is the results of our investigation on this.

During infection, organisms are known to generate reactive oxygen species (ROS); the superoxides are mainly produced as a part of the immune defense against foreign micro80 organisms; and anti-oxidants provide protection against free radical damage (Downs et al. 2001; Mohankumar and Ramasamy, 2006). The superoxide dismutase (SOD) and catalase are considered as primary antioxidant defense enzymes involved in negating the toxic effects of reactive oxygen species; they are known to be involved in decomposition of O2 and H2O2 to prevent the formation of free radicals; and are also known to protect the cells against environmental pollutants (Kuthan et al. 1986; Giardina et al. 1997; Rameshthangam and Ramasamy, 2006; Velkova-Jordanoska et al. 2008; Verma and Srivastava, 2010). The alkaline phosphatase (ALP) has been considered as a key enzyme for the defense and reported to be involved in phosphorylation, regulation of membrane permeability, transportation of nutrients and degradation of foreign materials (Zhang et al. 2005; Jat and Kothari, 2006; Atli and Canli, 2007; Verma and Srivastava, 2010). The other important enzymes associated with defense, the serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) are associated with gluconeogenesis and energy production as well as defense against stress and tissue damage (Krishnan et al. 2002; Verma and Srivastava, 2010). The information on alteration in the tissue antioxidant defense system during microsporidiosis in fishes is relatively meager. To understand the role of antioxidant defense during microsporidiosis in carp, the enzyme activity pattern associated with antioxidant defense has been investigated in liver in infected catla in the present study.

The physicochemical quality of water has been known to have a strong influence on health and disease development in fishes, mainly on resistance of fishes against pathogens as well as parasitic fauna (Hossain et al. 2008). The various physicochemical parameters like water temperature, pH, hardness, O_2, BOD (biological oxygen demand), ammonia and several other parameters have been reported to have a strong influence on fish resistance against the parasites (Shresta, 1990). Looking to the influence of environmental stress on the development of diseases in cultivable fishes (Narnaware et al. 1994), different water quality parameter including the level of heavy metals have been investigated during present investigations to understand the influence of water quality on the development of microsporidiosis in carp. The aquatic environment of fresh water fishes is known to harbor pathogenic bacteria, particularly coliform group (Ramos and Lyon, 2000). The total enterobacteriocеae, total coliform, fecal coliform and *E. coli* were considered as indicator organisms; and streptococcus, vibrios and coliforms are known to be associated with fish diseases. In the present investigation, microbiological analysis of pond water has also been carried out to understand the influence of indicator bacteria on the development of microsporidian infection in carp.

The criteria like SDS-PAGE profile of spore proteins, western blot analysis of antigenic proteins and use of RAPD markers are considered important for the characterization of some of the protozoan species (Metenier and Vivares, 2001). Study of change in the expression of proteins by SDS-PAGE has proved to be useful in classification and identification of organisms (Kersters and De Ley, 1980). The SDS-PAGE and western blot analysis have also been successfully used for the identification of proteins of spores of myxobolus and microsporidia (Keohane et al. 1996a; Soliman et al. 2003). Looking to the importance of SDS-PAGE method in the analysis of proteins in infected tissues in understanding the disease development, in the present investigations, protein analysis by SDS-PAGE has been performed.

Material and Methods

Sample Collection and Preservation

Catla catla (catla) (500 g), with morphological symptoms of infection and without infection, were collected from Lingda (Anand, Gujarat, India) in December 2008- February 2009. The pond has been manured with cow dung before the stocking of fingerlings. During the culture, the fishes were fed with rice bran and oilcake (1:1) at the rate of 5% of body weight. The fishes were collected with drag net and transported to the laboratory in live conditions for further investigations. From both infected and control fishes, liver, muscle and gill tissues were excised and stored at -20 ^{0}C for enzymological and histopathological analysis. The water samples were collected from the middle of the pond from the surface for physico-chemical and microbiological analysis.

The catla fry (10 to15 mm), with black spots on the body surface, were collected (September 2008) from Bhadrania (Anand, Gujarat, India). Before releasing fry, the rearing pond was manured with cow dung. The fry were fed with finely powdered rice bran and ground nut oil cake at the rate of 5 to 7% of the body weight. The fry were

collected with fry collection net and transported to the laboratory in live conditions and stored at -20 ^{0}C for histopathological analysis at light microscopic and ultra microscopic level.

Wet Mounting and Histopathology

The wet mount preparation of gills from infected catla was prepared, observed under Axioplan Image Analyzer (MPM-400 from Carl Zeiss, Germany); and photographs were taken at 45X and 100X. For histo-pathological analysis, from adult catla, gills, liver as well as muscles, and from catla fry, the black spot region were fixed in Bouin's, sections were cut at 5-8 µm, stained with standard hematoxylene-eosin (H & E) staining method and photographs were taken with Axioplan Image Analyzer (MPM-400 from Carl Zeiss, Germany) at 40X and 100X.

Transmission Electron Microscopy

The gills as well as muscles from adult catla and black spot region from infected fry were fixed in 2.5% cold glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 24 h at 4 ^{0}C. After several rinses with buffer, the tissues were post-fixed in 1% osmium tetroxide (O_SO_4) for 1 h, dehydrated in acetone series and embedded in Spurr's resin. The semi thin sections were cut (Ultracut, Leica) and stained with 1% methylene blue. Next, the ultra-thin sections were double stained with uranyl acetate as well as lead citrate and observed under TEM (Technai, 20; Philips series, 200 KV).

Antioxidant Enzyme Analysis

The liver was homogenized in phosphate buffer (pH 7), centrifuged at 4 ^{0}C for 10 min at 8000 *g* and supernatant was subjected for enzyme assay as follows: The catalase and SOD were estimated by the methods of Aebi (1984) and Kakkar et al. (1984) respectively. The SGPT, SGOT and ALP were estimated using test kit (Eve's Diagnostics, Baroda). Total protein from liver homogenate was estimated by the method of Lowry et al. (1951).

Water Analysis

In the water samples, the temperature and pH were measured using thermometer and electrode respectively. The dissolved oxygen (DO), residual chlorine, biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), phosphate, sulphate, nitrate, nitrite, ammonia, alkalinity, acidity and chlorides have been estimated by the method of APHA (1999); and heavy metals arsenic (As), cadmium (Cd), chromium (Cr), copper (Co), lead (Pb), manganese (Mn), mercury (Mg), zinc (Zn) and nickel (Ni) were measured using atomic absorption spectrophotometer (AAS, Nova 400, Analytik Tcna, Germany). The microbiological analysis of pond water, total viable count and total vibrios count have been performed by spread plate method using TSA (Tryptone soya agar, HiMedia, Mumbai) and TCBS (Thiosulfate-citrate-bile salts-sucrose agar, HiMedia, Mumbai) respectively; and total streptococci count and total fecal coliform count have been carried out by MPN (Most probable number) method using Azide dextrose broth and MacConkey broth respectively, media obtain from HiMedia, Mumbai, India. (APHA 1999).

SDS-PAGE

The gills, muscle and liver tissues, each of 0.5 g, were homogenized in 10 ml of phosphate buffer (pH 7.0), centrifuged at 8000 *g* for 10 min and supernatant was subjected to polyacrylamide gel electrophoresis using 10% resolving gel and 5% stacking gel (Sambrook and Russell 2001). The 300 µl of 10% ammonium peroxodisulphate (APS), 70 µl of N, N, N, N-Tetramethylenediamine (TEMED), 50 µl APS and 20 µl TEMED has been used for the polymerization of resolving gel and stacking gel. Gels were run using a Dual midi vertical gel electrophoresis system at 100V constant voltage in 1X buffer (25mM Tris, 25mM glycine, pH 8.3, 0.1% SDS). Proteins were visualized by silver staining kit (Genie, Bangalore).

Statistical Analysis

The data were subjected to ANOVA, and the significance of the difference between means was determined by Tukey's multiple range test using the SPSS version 17.0 (Chicago, Illinois, USA). The minimum significance levels $P < 0.05$ were considered. Values are expressed as means ± S.E.

Results

Morphological

The adult catla with infection were found to be lethargic and crowding near water surface. On the body, hemorrhage as bluish red marks, several necrotic patches and patches without scales can be seen (Fig. 1a). The gills were observed to be covered with heavy coat of slime; and gill filaments were detected with yellowish marks and necrosis (Fig. 1b).

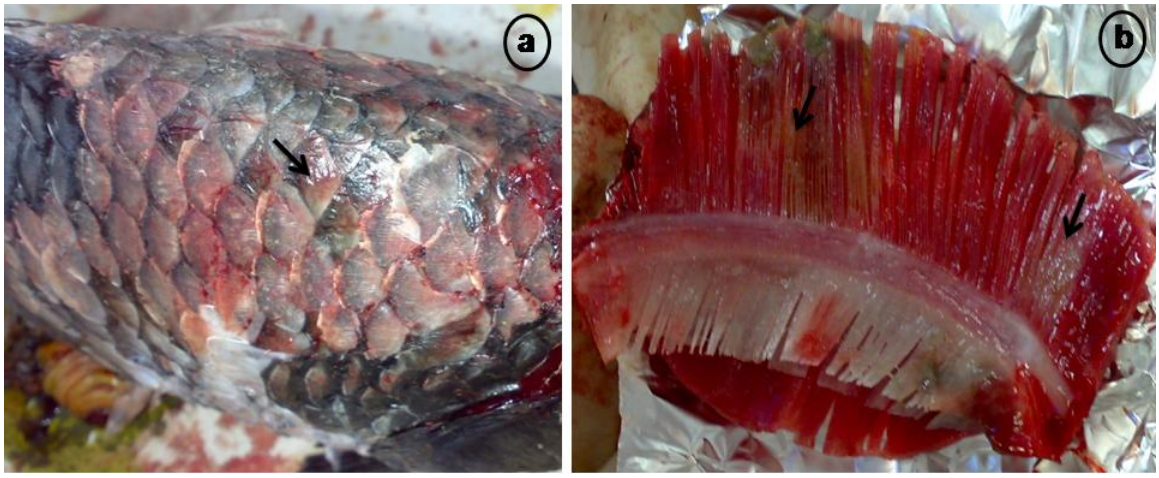

Figure 1a. The arrow shows hemorrhage on the body surface as reddish blue marks and patches without scales.
Figure 1b. The arrow shows gill with small pale yellow marks and hemorrhagic areas.

Wet Mount and Histopathology

The round sporophorous vesicles can be clearly seen in the wet mount preparation of gill from infected catla (Fig. 2a). The hypertrophy, hyperplasia and necrosis in primary and secondary gill lamella are seen in the gill section of infected fishes (Fig. 2b). In the muscle sections of infected fishes, sporophorous vesicles in degenerated fibers, necrosis, complete disorganization of myofibrils, separation of myofibrils and liquefaction of muscles is evident (Fig. 2c). In the liver also, hypertrophy and hyperplasia of hepatocytes as well as necrosis are observed (Fig. 2d).

Transmission Electron Microscopy

In the ultra-thin sections of muscles from infected fishes, different developmental stages of microsporidia, sporogonic and merogsonic stages along with spores in close association of defense cells are observed (Fig. 3b). The spores were found to be round to ellipsoidal in shape, measuring 1.95 × 2.12 µm (n=10), with polar tubes, anchoring disc, protuberant area and basal vacuole (Fig. 3a). The meronts are in oval shape (3.2 × 3.1 µm, n=14) and are found to be isolated from closely encircling muscle tissue (Figs. 3b, 3c). The meronts are found to be bounded by a unit stack of membranes with interrupted pores; and these are likely to represent endospore and exospore in the process of formation. In the ultra-thin muscle section (Fig. 3b), round spore (1.95 × 2.12 µm, n=5) was found to be surrounded by phagocytic cells. The phagocytic cells are marked with a single irregularly shaped nuclei, dense chromatin material, irregular and thick plasma lemma; and with clear cytoplasm, without typical organization of cytoplasmic organelles (Fig. 3c).

In the ultra-thin section of gill, round spores measuring 1.43 × 1.47 µm (n=18) are observed in epithelial cells (Figs. 4a, 4b). The spores are found to be intra-cellular, directly in contact with the cytoplasm. The parasitic stage does not seem to be eliciting formation of parasitophorous vacuole (Figs. 4a, 4b). In the host cell, spores are found to be the main parasitic stage and arranged very close to nucleus; the nucleus is observed to be round with clear nuclear membrane; in the cytoplasm, endoplasmic reticulum, mitochondria and Golgi appear to be in disrupted state; however, tubular structures and coated vesicles are seen clearly in the host cell cytoplasm (Fig. 4b).

The Hepatic Antioxidant Enzyme Activities

The activity of hepatic enzymes associated with free radical scavenging, mainly catalase, SOD, ALP, SGOT and SGPT in catla during microsporidian infection is shown in Table 1. In the liver homogenate of infected fishes, significant increase ($p<0.05$) in catalase and ALP activities; and significant reduction ($p<0.05$) in SGOT and SOD activities have been detected as compared to control. The SGPT activity was also found to be significantly lower ($p<0.05$) in the first infected specimen (infected group 1) as compared to control; however, in the second

infected specimen (infected group 2), the enzyme activity was found to be more or less similar as compared to control.

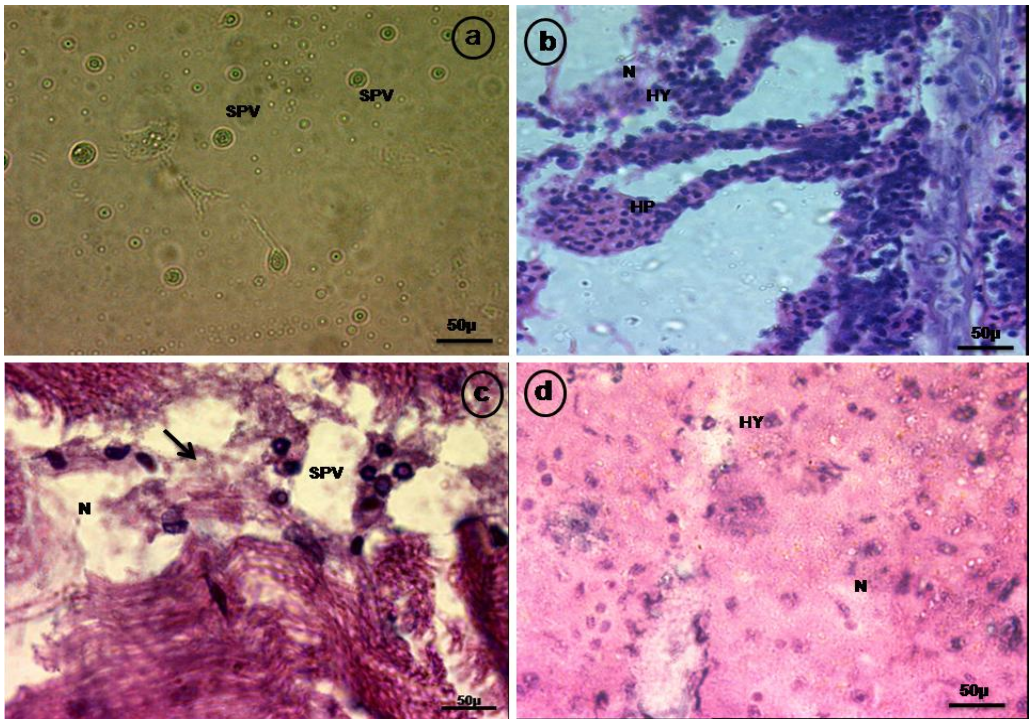

Figure 2a. The wet mount preparation of gill from infected catla showing sporophorous vesicles.
Figure 2b. Showing hyperplasia, hypertrophy and necrosis in primary and secondary gill lamella of infected fishes.
Figure 2c. Showing the muscle section with sporophorous vesicles. The black arrow indicates degenerated muscle fibers, heavy necrosis and liquefaction of myofibrils.
Figure 2d. Showing hypertrophy of hepatocytes and necrosis in the liver tissue in infected fishes.
HY-hepertrophy; HP-hyperplasia; N-Necrosis; SPV- sporophorous vesicles (Figure 3a, b and c scale bar =50µm)

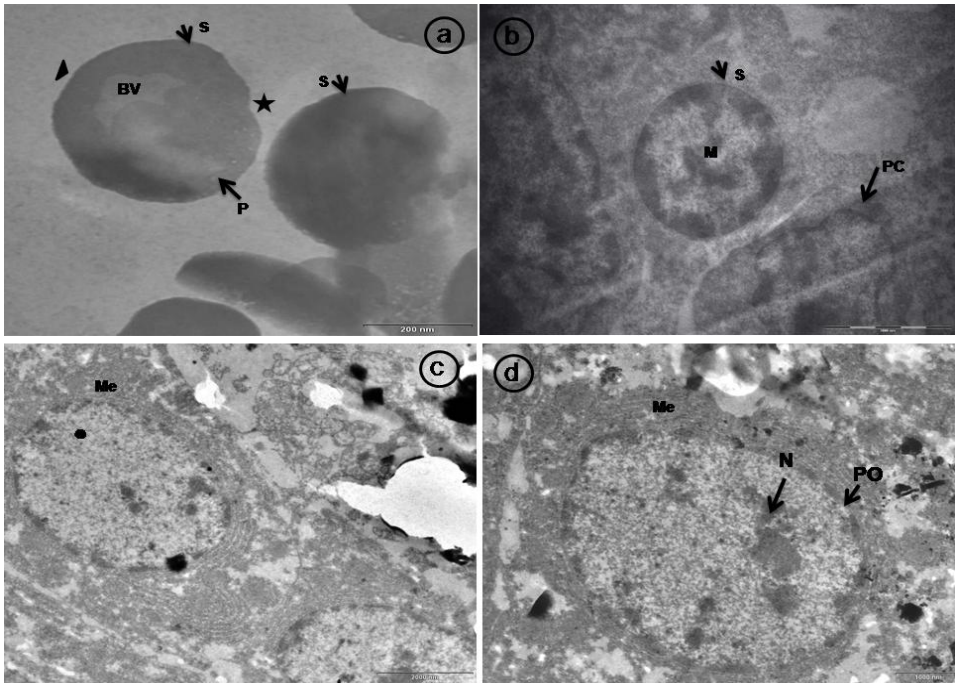

Figure 3a. Oval spores with polar tubes, anchoring disc, protuberant area and basal vacuole
Figure 3b. Spore surrounded by phagocytic cells
Figure 3c, 3d. Meronts in oval shape surrounded by unit stakes of membranes
★ -anchoring disc; ►-protuberant area; P-polar tube; BV-basal vacuole; PC- phagocytic cell; Me- stakes of membranes; N-nucleus; S-spore; ●-meront stage; Po-pores.

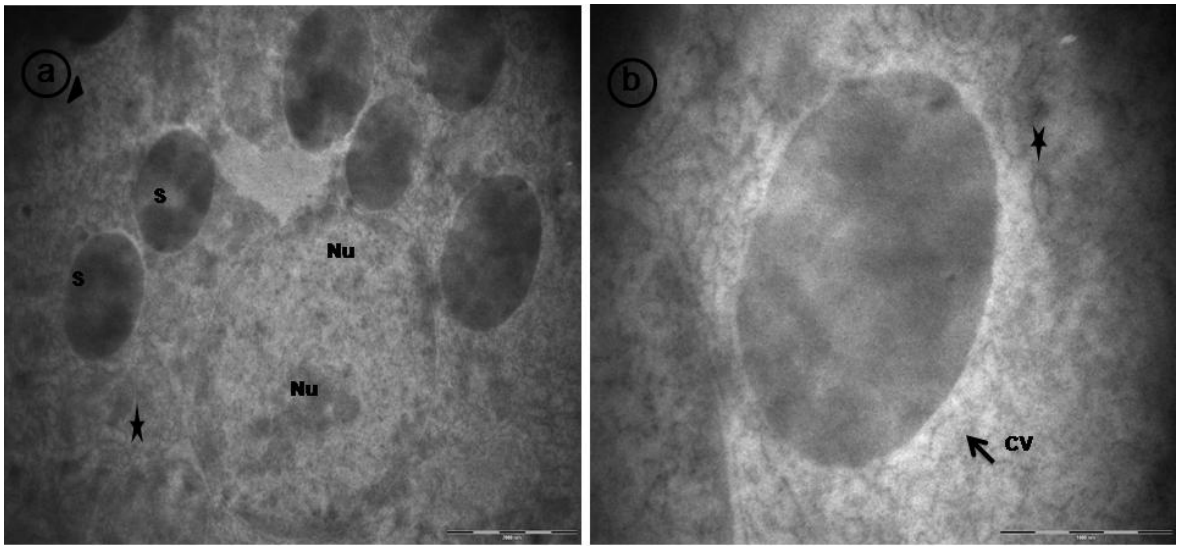

Figure 4a, 4b. Gill epithelial cells showing intracellular spores, nucleus with nucleolus, tubular structures and coated vesicles.
Nu- nucleus; N-nucleus; M- microsporidia; CV- coated vesicles; ★-tubular structure.

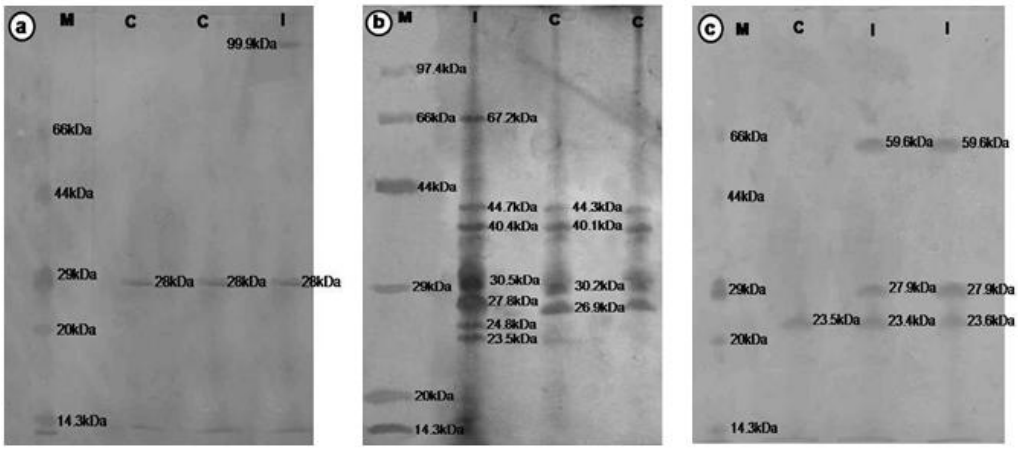

Figure 5a, 5b and **5c** showing expression of proteins in gill, muscle and liver respectively during microsporidian infection in catla by SDS-PAGE.
C-control; M-marker; I-infected

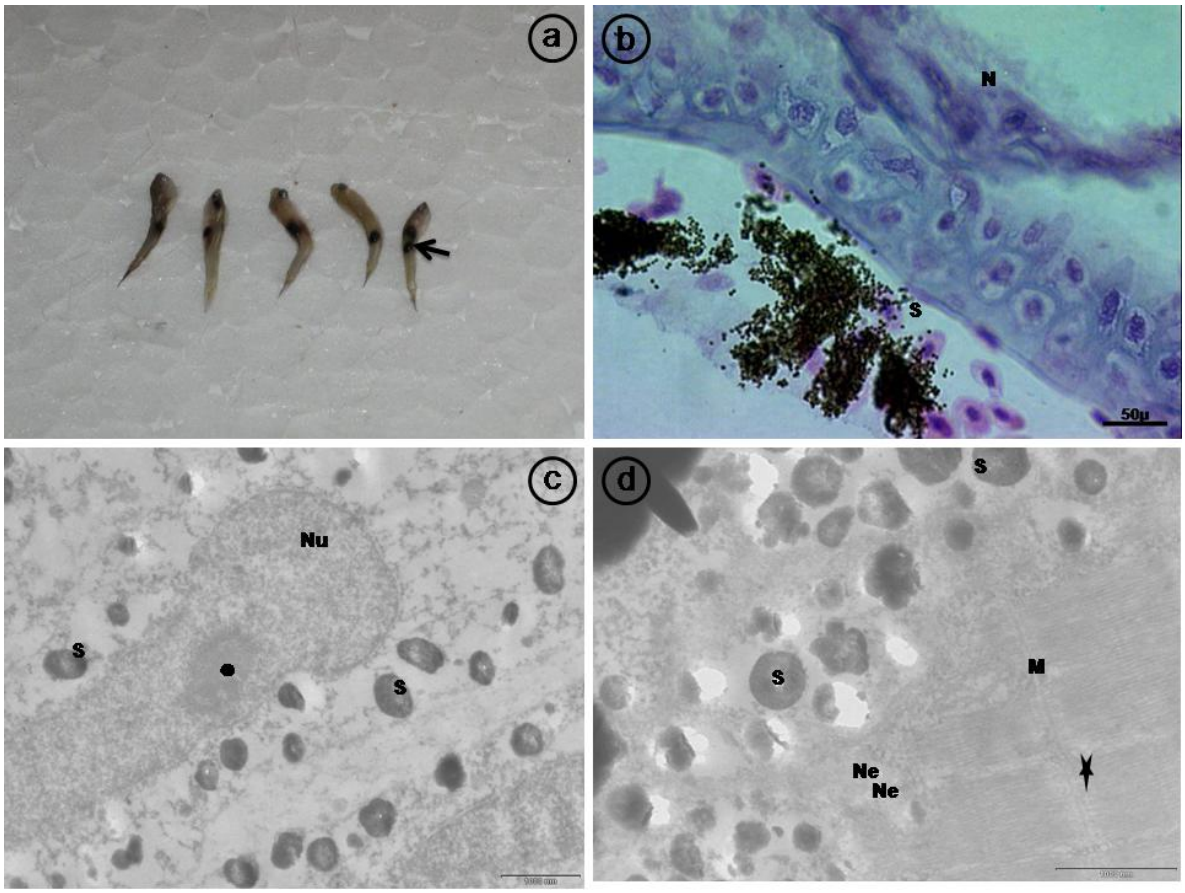

Figure 6a -Catla fry with black spot marked with arrow.
Figure 6b-Black spot region showing spores.
Figure 6c- Ultrathin section showing microsporidian spores in phagocyte cells.
Figure 6d- Ultrathin section of fry muscle showing presence of spores in degenerated muscles.
●-nucleuolus; Nu- nucleus; S- microsporidia; Ne-necrosis; M-mitocondris; ★-Z line

The Physico-Chemical, Heavy Metal and Microbiological Analysis of Pond Water (Lingada Fish Farm)

The detail of physico-chemical analysis of water sample from Lingada fish farm, with microsporidian infection, is presented in Table 2. Except the level of ammonia (2.4 mg/L), total alkalinity (638 mg/L) and COD (16.2 mg/L) (Table 2), the level of all other physicochemical parameters as well as the concentration of heavy metals (Table 3) are found to be acceptable as per the guidelines for aquaculture (Garg 2010).

The analysis of microbiological parameters of pond water, especially indicator pathogenic micro-organisms total viable count, total vibrios count, total fecal coliforms and total fecal streptococci during microsporidian infection indicated that the level of indicator pathogens in carp pond was found to be in permissible limit (Table 4) as per the guidelines of world Health Organization for fish pod water (Garg, 2010).

Protein Analysis By SDS-PAGE During Infection (Adult Catla)

The protein profile of gills, muscle and liver from microsporidia infected and healthy catla, analysed by SDS-PAGE, is shown in Figs. 5a, 5b and 5c respectively. In the infected gills, one higher molecular weight protein, 99kDa, has been detected as newly expressed protein (Fig. 5a); whereas, in infected muscle, three newly expressed proteins were detected, two with low molecular weight, 23kDa and 24kDa, and one with higher molecular weight,

67kDa. In the infected liver also, two newly expressed proteins have been detected with molecular weight 27kDa and 59kDa.

The Light and Ultramicroscopic Analysis of Black Spot Region of Catla Fry

In Fig. 6a shows catla fry with black spot region. In the light microscopic preparation, large no of spores can be seen at the infected site (Fig. 6b). In the ultra-thin sections, spores are found to be located in degenerated myofibrils without special boundaries (Fig. 6d). In the section, the destructed myofibrils along with sarcomere, Z line, mitochondria and ER can be seen. The necrotic regions are also evident in the section. The spores are found to be round in shape, very small in size, 0.41 × 0.58 µm (n=8). In the fry, along with the presence in muscles, the spores are also detected in the cytoplasm of leukocytes (Fig. 6c). The spores in phagocytic cells are round to ellipsoidal in shape, measuring (0.28 × 0.39 µm, n=5). The leukocyte nucleus is elongated with clear nuclear membrane as well as nucleolus; the cytoplasm appears clear without typical organization of cytoplasmic organelles; and the spores are observed to be the main parasitic stage (Fig. 5c).

Table 1. The hepatic antioxidant enzyme activity during microsporidian infection. Values are mean ± S.E. (n=6). Values with different superscript are significantly different ($p < 0.05$).

	Control-1	**Control-2**	**Infected-1**	**Infected-2**
Catalase (µg H_2o_2/min/ml)	4.72^a ±0.19	2.84^a ±0.21	12.25^b ±0.44	28.4333^c ±1.45126
ALP (KA Units)	11.81^a ±0.06	16.87^a ±0.76	68.55^b ±2.52	44.6800^c ±1.81995
SGPT (Unit/ml)	58.04^b ±1.19	48.64^b ±0.25	17.42^a ±5.38	48.75^b ±8.174
SGOT (Unit/ml)	38.53^a ±0.21	28.49^b ±0.15	19.55^c ±0.12	16.82^d ±0.09
SOD (unit activity/ml)	67.07^b ±0.40	73.37^b ±1.76	34.02^a ±0.44	37.10^a ±0.29

Table 2. Physico-chemical parameters of water samples collected from carp pond during microsporidian infection. (*Unit shows as mg/L)

Temperature	pH	DO*	BOD*	COD*	Residual Chlorine*	Phosphate*	Sulphate*	Nitrite*	Nitrate*	Ammonia*	Alkalinity*	Acidity*	Chlorides*	Hardness*
21^0c	8.87	5.6	3.9	16.2	nil	2.82	1.35	0.3	2.8	2.4	638	79	1.76	69.34

Table 3. Heavy metal analysis of water sample during microsporidian infection in pond.

Metal	**mg/L**
Arsenic	BDL
Cadmium	0.03763
Chromium	BDL
Cobalt	BDL
Copper	BDL
Lead	0.03796
Managanese	0.045

Mercury	BDL
Nickel	BDL
Zink	BDL

BDL: Below detectable limit.

Table 4. The microbiological analysis of pond water during microsporidian infection.

Total viable count	4.9×10^{2} CFU/mL
Total vibrios count	1×10^{1} CFU/mL
Total fecal coliforms	33 MPN/100mL
Total fecal streptococci	13 MPN/100mL

CFU = colony forming units; MPN = most probable number.

Discussion

The catla with morphological symptoms like necrotic patches, bluish red marks and patches without scales on body surface; and catla fry with black spot on body surface revealed the microsporidian infection with light and ultramicroscopic analysis during present investigations. In catla, muscles and gills are found to be heavily infected; and in the catla fry, muscles and cells associated with defense are observed to be infected with microsporidia. Presently observed myodegeneration, vacuolization of sarcoplasm, separation of myofibrils and liquefaction of muscles (Fig. 2c) along with the presence of various lifecycle stages of microsporidia, mainly merogonic and sporogonic stages, in disintegrated sarcoplasm clearly indicate the microsporidian infection in muscle of catla. The literature reveals the microsporidia infection in muscles of decapod crustaceans (Larsson, 1999), turbot (Estevez et al. 1992) and eel (Tsui and Wang, 1988). The microsporidia are known to be classified on the bases of their ultra-structure, size and morphology of spores, number of polar tubes, lifecycle stages and host parasitic interactions (Sprague et al. 1992). The presently observed round to ellipsoidal spores measuring 1.95 × 2.12 µm with anchoring disc and protuberant area (Fig. 3a) in the muscle appear to be very similar to *Pleistophora* species as described by Purivirojkul and Khidprasert (2009). The *Pleistophora* species is usually known to infect skeletal muscle in fish; they are reported to be haplokaryotic, remaining in direct contact with host cell cytoplasm; and exhibit the destruction and degeneration of myofibrils as well as liquefaction of muscle (Pulsford and Matthews, 1991; Lom and Nilsen, 2003; Ruehl-Fehlert et al. 2005). In the muscles, they are known to form isolated islets and each parasite cell is known to form minute schizonts to large sporogonial plasmodia; and they are known to migrate to the outer layer of muscle and to get released in the environment without the host being killed (Lom et al. 2000; Lom and Nilsen, 2003). The presently observed liquefaction and pathological changes in muscle further confirm that the causative parasite appear to be from genus *Pleistophora*. The *Pleistophora* species infecting the muscle of toad, reptile, immuno-compromized human and pink shrimp have already been identified (Canning et al. 1964; Kelly, 1979; Canning and Lom 1986; Cali and Takvorian, 2003); and the *Pleistophora hyphessobrycoins* is reported to be responsible for systemic disease in neon tetra and angle fish (Harms, 1996; Schmahl, 1998). The presently detected meronts with unit stake of membranes, interrupted pores and isolated from closely encircling muscles appear to be very similar to the microsporidian developmental stages reported by several investigators (Morrison and Sprague, 1981a; Azevedo et al. 2000; Cheney et al. 2001; Azevedo and Matos, 2002; Lom and Nilsen, 2003; Monaghan et al. 2009). Moreover, the membrane stakes of merogonic stages may represent the endospores and exospores in the process of formation as suggested by Lom and Nilson (2003).

In the infected gills, necrosis, inflammation and hyperplasia in primary and secondary gill lamella as well as the presence of spores (1.43 × 1.47 µm), intracellular in position, embaded in the cytoplasm of gill lamellar cells without formation of paracytophorous vacuole have confirmed the microsporidian infection. According to Shaw (1999), the gill endothelium is reported to be the preferred site for the development of microsporidia. Presence of microsporidia in host cell cytoplasm suggests that parasites are obtaining essential nutrients from host cell cytoplasm as proposed by Metenier and Vivares (2001). Moreover, the presence of large number of coated vesicles in the cytoplasm appears to be associated with the transportation of nutrients. Similarly, increase in the coated vesicles, possibly associated with transportation of proteins and polysaccharides have been detected in liver cells, oocyte and neurons during microsporidian infection as reported by Muriel et al. (1975). The microsporidian spores observed in the gill of catla are similar with many respects to *Pleistophora*. Microsporidian infections in fish gill and branchial asphyxiation have been observed by several investigators (Kent et al. 1999; Rodriguez-Tovar et al. 2003; Catherine et al. 2007). The microsporidian infection in the gill of brook trout and cod has been reported by Morrison and Sprague (1981a); and *Loma salmonella* is known to cause microsporidian gill disease in Salmonids (Kent et al.

1995a). The microsporidian infection in rainbow trout exhibited presence of spores and meronts in gills in association with capillary channels and lamellar arteries (Rodriguez-Tower et al. 2003).

During pathogenesis in aquaculture, study of change in tissue antioxidant defense system is considered to be important for the improvement of disease management practices (Mathew et al. 2007). During the present study, the significant increase ($p < 0.05$) in the activity of catalase and alkaline phosphatase; and significant decrease ($p < 0.05$) in the activity of SGPT, SGOT and SOD in liver (Table 1) clearly indicates the activation of defence mechanism during microsporidian infection. Organisms are known to form free radicals during infection. The anti-oxidants are involved in providing protection to organisms against free radical damage; and they are considered as potential indicators of oxidative stress (Wayner et al. 1987; Downs et al. 2001; Mohankumar and Ramasamy, 2006). An increase in catalase activity has been observed by Mathew et al. (2007) in shrimp during initial phase of infection by WSSV. The catalase has been considered as primary antioxidant defense enzyme involved in decomposition of H_2O_2 generated from superoxide anions (Rameshthangam and Ramasamy, 2006); and it is known to protect the cell against pollutants (Velkova-Jordanoska et al. 2008). The presently observed significant increase ($p < 0.05$) in hepatic catalase activity (Table 1) appear to be an attempt to neutralize the free radicals, generated during microsporidian infection as first line of defense against oxidative injury. The significant increase ($p < 0.05$) in hepatic ALP (Table 1) during infection probably indicates an increase in phosphorylation, change in membrane permeability and increase in glucose as well as phosphate transfer as suggested by Jat and Kothari (2006). This clearly confirms the role of ALP in increase in the transportation of nutrients for the development of microsporidian stages because microsporidia are reported to be intracellular parasites and depend on host for their requirements (Lom and Dykova, 1993). The ALP is also known to be an indicator of histological damage to tissues; and is suggested to be involved in degradation of foreign materials entering in tissues (Zhang et al. 2005; Atli and Canli, 2007). This also support the damage caused by microsporidia in gills and muscle as well as the role of this enzyme in degradation of microsporidia. The significant decrease ($p < 0.05$) in hepatic SOD, SGPT and SGOT activities (Table 1) indicates that the tissue antioxidant status is probably operating at a lower rate despite the increased requirement of antioxidant defense to counteract the increased free radicals because of heavy microsporidian infection. The SOD is known to be one of the key enzymes involved in protection of cell against the toxic effect of environmental pollutants (Kuthan et al. 1986). A similar trend in drop in SOD level in *P.monodon* during WSSV infection and its challenge have been reported by Rameshthangam and Ramasamy (2006) and Chang et al. (2003) respectively. The decline in SOD level has also been reported in haemocytes and haemolymph in *Bombax mori* during bacterial infection (Krishnan et al. 2002). The superoxide is considered as the major free radical stress produced by the cell; and the SOD is considered as one of the main antioxidant defense pathway negating the direct toxic effects of reactive oxygen species (McCord and Fridovich, 1969; Lin et al. 2004; Mathew et al. 2007). In the present study, significant lowering of SOD activity also suggests the role of abundant availability of reactive oxygen and hydroxyl radicals as these radicals are known to inactivate the chemical structure of SOD resulting into loss of enzyme activity (Rameshthangam and Ramasamy, 2006). The exposure of fishes to different metals is known to elevate the levels of SGPT and SGOT (Verma and Srivastava, 2010). During bacterial infection to *L. rohita*, increase in SGPT and SGOT activities have been reported (Krishnan et al. 2002). However, during microsporidian infection in catla, significant decrease ($p < 0.05$) in hepatic SGPT and SGOT activities indicate the possibility of damage to the liver during infection; since damaged hepatocytes are likely to affect the synthesis of these enzymes as suggested by Asztalos (1990).

The environmental stress is known to be a risk factor in development of diseases in fishes. In intensive culture practice, fishes are subjected to various stress which leads to immuno-suppression and increased infection risk (Narnaware et al. 1994). It is known that disease development in fishes mainly depends on water quality of pond; and various physicochemical factors like temperature, pH, hardness, O_2, BOD, ammonia and several other water quality parameters have influence on the resistance of fishes against the causative agents (Plumb et al. 1988; Shresta, 1996; Bhatnagar and Singh, 2010). In the present investigation, during microsporidian infection in catla, high level of ammonia (2.4 mg/L) and COD (16.2 mg/L) in pond water are likely to have influenced the development of this parasitic infection in catla. The water quality parameters like BOD, COD, chlorides, phosphates and ammonia are reported to be considered as pollution indicators (Bhatnagar and Garg, 2000; Islam et al. 2000; Garg and Bhatnagar, 2003; Bhatnagar and Sangwan, 2009; Bhatnagar and Singh, 2010b;). The maximum limit of ammonia concentration for aquatic organisms has been reported as 0.1 mg/L; and its higher concentration causes osmoregulatory imbalances, kidney failure and damage to gill epithelia leading to suffocation in fishes (Garg et al. 2010; Bhatnagar and Singh, 2010). The presently observed high ammonia level clearly indicates organic pollution in the pond; and the high values of COD indicates the higher pollution level in the pond water as COD has been considered as reliable parameter for judging the extent of pollution in water (Amirkolaie, 2008). However, a higher total alkalinity value (638 mg/L) in this pond indicates that the pond is with a good trophic status and is well

buffered as suggested by Singh (1998). The concentration of heavy metals and the level of other physicochemical parameters of presently investigated pond water were found to be acceptable as per the guidelines from WHO for aquaculture. The presence of aquatic weeds, anthropogenic activities in the pond and runoff from surrounding agriculture land are likely to be the potential sources for the presently observed contamination. The stress generated by the presence of pollutants seems to have played a role in the development of microsporidian infection in catla because environmental stress is known to be involved in development of disease in fishes (Narnaware et al. 1994).

The microorganisms in aquaculture ponds influence the water quality along with the physiology and health of the fishes. In the pond water, use of manure and fertilizers may give rise to pathogenic bacteria, parasites and cause water pollution (Abdelhamid et al. 2007). Some of the normal bacterial flora of water like *Pseudomonas*, *Aeromonas*, *Vibrios* and *Myxobacteria* are known to produce disease epizootics under environmental stress (Sugita et al. 1980). The high load of *E.coli* indicates sewage pollution in pond (Hatha et al. 2008). The opportunistic pathogens present in the rearing environment, mainly vibrios, have been reported to cause black spot necrosis in juvenile and adult *M. rosenbergi* (Jayshree et al. 1999). According to Guzman et al. (2004), when fishes are raised in pond water with higher content of fecal coliforms *E. coli* and *Salmonella*, pathogens are likely to invade fish muscle due to the breakage of immunological barrier. Fresh water fishes are most commonly known to suffer from *Aeromonas* sp. and *Pseudomonas* sp. (Ding et al. 2007). Increase in vibrios population has been associated with mortalities in fin fish and shell fish (Jayprakash et al. 2006). In fresh and marine water, some 25 bacterial genera have been implicated as pathogens; and some of them are known to cause furunculosis, bacterial kidney disease and enteric red mouth disease in fishes (Ewing et al. 1978; Fryer and Sanders 1981). Moreover, Fin Rot and Vibriosis usually develop in polluted water (Ewing et al. 1978). Presently investigated level of indicator pathogens in carp pond (Table 3) during microsporidian infection is found to be in permissible limit as per the guidelines of World Health Organization for fish pond water. The findings have ruled-out any possible link between presence of indicator pathogens and development of microsporidian infection in catla.

Among the different diagnostic methods, analysis of proteins by SDS-PAGE of infected tissues is one of the key methods in understanding disease development in fishes (Catherine et al. 2007). Large numbers of reports have revealed the polar tube proteins and spore wall proteins from several microsporidian species. The 23kDa and 43kDa polar tube proteins have been indentified from microsporidian species, *Amelson michaeris*, infecting crab (Keohane et al. 1996). Dolgikh et al. (2005) have identified 40kDa spore wall protein and 56kDa, 46kDa as well as 34kDa polar tube proteins in microsporidia, *Paranosena grulli*, infecting cricket. A spore wall protein of 51kDa was identified in the human microsporidia, *Encephalitozoon cuniculi* (Dolgikh et al. 2005). During present investigation, newly expressed proteins 99kDa (in gills), 27kDa and 59kDa (in liver) and 23kDa and 67kDa (in muscle) (Figs. 5a to 5c) in infected catla are likely to be a response generated by microsporidian infection in these tissues. This information might be helpful in understanding pathogenesis of microsporidia in carp and for finding appropriate measures to control the infection.

According to Hossain et al. (2008), in fish nurseries, parasitic infection is one of the most important limiting factors for the performance of fry and fingerlings. Several factors are known to influence parasitic fauna of fish including age, diet, abundance of fish and season (Dogiel, 1961). The light microscopic and TEM examination of catla fry with black spot (Fig. 1d) revealed the presence of microsporidian spores in the muscle and phagocyte cell during present investigation. The destruction of myofibrils, liquefaction of muscle (Fig. 5b) and the presence of spores in disintegrated myofibrils without special boundary is similar to the pathological conditions observed during microsporidian infection in fish muscle as observed by Lom and Nilson (2003). The spores detected in muscles (0.41 × 0.58 µm) are found to be very small in size and could not be compared with any of the reported microsporidian species. However, the microsporidia *Sarcocystis* has been known to infect muscle in catla (Lom and Nilsen, 2003). In the fry, along with the presence in muscles, the presence of spores in the cytoplasm of leukocyte (Fig. 5b) indicates active phagocytosis of spores. Several investigators have reported phagocytosis of microsporidian spores by leukocytes and other migratory cells, mainly to control infection (Estevez et al. 1992; Lom and Nilsen, 2003; Rodriguez-Tovar et al. 2003). The phagocytic cells are known to play crucial role in the host defense mechanism and elimination of spores (Dykova and Lom, 1980). Blood cells are known to harbor the parasites and get infected during phagocytosis (Canning and Lom, 1986; Didier and Bessinger, 1999). The present observation of phagocytic cell with large number of spores (0.28 × 0.39 µm) is in agreement with Lom and Nilsen (2003) and Sene et al. (1997). The present findings suggest that probably infected fish seeds are responsible for microsporidian infection in catla.

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