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Morphological Identification of Microsporidian Parasite in *Channa punctatus* from River Gomti, Lucknow, Uttar Pradesh, India

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Abstract: *Channa punctatus* from Gomti river, Lucknow, India were randomly collected and investigated for microsporidian parasites, 89 (64.96%) fishes were found infected. The infection was present in both symptomatic as well as in asymptomatic fishes. Symptomatic fish showed discoloration of body surface externally and internally whitish vesicles have been observed in kidney and liver with liquefied gills and gonads. Microsporidia infection has been detected in different organs of *Channa punctatus* by light microscopic techniques. Observation of fresh wet mounted smears showed microsporidian spores which appeared oval in shape illuminating green florescent with slight Brownian motion with different developmental stages. Giemsa stained smears also showed merogonic and sporogonic stages of the parasite with polar filament extrusion under pressure. Ultrastructural analysis through Scanning electron microscope further confirms the microsporidian infection based on spores' size and morphological appearance. Spores were oval to pyriform-shaped cell body having spongy external appearance and were found of two size ranges measuring 15.62 µm and 12.83 µm which might be non-xenomic *Pliestophora* species.

Keywords: *Channa punctatus*, Microsporidia, Merogonic, Sporogonic, Non-xenomic, Polar filament

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Introduction

Microsporidia are the smallest known unicellular eukaryotic obligate intracellular parasites. Initially they have placed into the protozoa phylum (Spargue, 1977) but later based on molecular data and phylogenetic analysis of tubulin proteins (Keeling and Fast, 2002; Keeling, 2003) and large subunit ribosomal RNA sequence (Peer *et al*., 1998), they were considered as a parasite with fungal affinities (Gaber *et al*., 2017; Park and Poulin, 2021). Microsporidia infects a broad range of host including both vertebrates and invertebrate animals (Canning, 1976; Lom and Dykovi, 1992; Didier *et al*., 2004; Johny *et al*., 2006; Phelps, 2015; Sokolova and Overstreet, 2018). Presently 1,500 species in about 200 genera have been reported from which about 160

species from 21 genera have been identified in fish (Lom and Nilsen, 2003; Stentiford *et al*., 2013; Azevedo *et al*., 2016; Phelps, 2018; Quraishy *et al*., 2019) and new species are continuously discovered in different host groups (Vávra and Lukeš, 2013; Garhy *et al*., 2017). Microsporidia are known to infect fresh, marine, and brackish water fish in their natural environment, as well as in experimental models which impart in decorative, games, and food purposes resulting significant economic losses due to liquified muscles, growth retardation, unhealthy flesh which renders it unfit for human consumptions (Lom *et al*.,1995; Lom and Nilsen, 2003; Joh *et al*., 2007; Sanders *et al*., 2012). Microsporidian infection has been detected in different organs of fish like muscles (Fehlert *et al*., 2005; McGourty *et al*., 2007; Garhy *et al*., 2017), gills (Kummari, 2018), kidney (Didier *et al*., 2004), liver (Matos *et al*., 2003), ovary (Abidi *et al*., 2016), and peritoneal cavity (Casal *et al*., 2008; Ghaffar, 2012; Morsy *et al*., 2013), etc.

Microsporidia are intracellular parasites that contain a characteristic polar filament that helps to invade the host cell. After the invasion, the parasite multiplies inside the host cell through different developmental stages like merogy, sporogony, and sporoblast resulting in hypertrophy and finally bursting of the host cell releasing the spores and infecting new host and cells through vertical and horizontal modes of transmission (Dyova and Woo, 1995; Lom, 1995, 1999; Kent and Stewart, 2003). Microsporidia are highly specialized for their intracellular mode of parasitic nature because they contain genes that are required for glycolysis, trehalose metabolism, and pentose-phosphate pathway while they do not contain genes for an enzyme of tricarboxylic acid and oxidative pathways so glycolysis might provide ATP molecules and other metabolites that are required for its diverse metabolic pathways to conduct different physiological processes of this organism (Keeling and Corradi, 2011). Based on the formation of cysts xenoma, Microsporidia has been broadly divided into two broad groups-xenomic, and non-xenomic (Lom and Dyková, 2005; Kent, 2014).

The spotted snakehead murrel, *Channa punctatus* (Bloch, 1793) is an air-breathing fish distributed throughout South-East Asian countries including India. It is the least concern species in the IUCN data list and can be easily transported, maintained, and acclimatized in the laboratories and also available throughout the year, which makes it an excellent experimental model and is currently used for various pollutant and toxicological as well as mutagenic study. (Kuppu *et al*., 2020). *Channa punctatus* belongs to the family Channidae which contains approximately more than 35 species, among them 26 species identified from Asia (Rainboth, 1994; c; Vishwanath and Geetakumari, 2009). This fish contains high protein with delicious taste and less intramuscular spines. It also contains some medicinal qualities and is recommended during recuperation (Arockiaraj *et al*., 2004). The population of this fish is steadily declining due to overexploitation, parasitic infestation, loss of habitat, the introduction of foreign species, disease, pollution, siltation, poisoning, dynamite, and destructive methods of fishing from the last 10 years. Every year the production of snakehead fish is decreasing and aquatic environment pollution is one of the major causes behind it (Ansari *et al*., 2009).

In the present study, Microsporidian infection was detected in *Channa punctatus* collected from the river Gomti, Lucknow, Uttar Pradesh, India. Previously microsporidia infection has been reported in fish in different organs like gills, liver, kidney, skin, and reproductive organs except for the brain in different regions of India (Dhiraj and Sujata, 2014; Kummari *et al*., 2018). Microsporidian infection in fish from river Gomti Lucknow has also been reported by Abidi *et al*., (2016) in *Cyprinus carpio* fish. This study aims to report the presence of microsporidian infection *Channa punctatus* collected from Gomti river of Lucknow by morphological analysis through light and scanning electron microscopy.

Materials and Methods

Sample collection:

A total of 137 *Channa punctatus* (4-30 g; 8.2-13.5 cm) were randomly collected from the Gomti river Lucknow through a net by the fisherman and transported to the lab in live condition for infection investigation. Fish were sacrificed and organs were collected after dissection.

Visual inspection:

Fish were examined externally and whole visceral organs by naked eyes for infection symptoms.

Wet mounting:

To confirm the infection, samples were taken from different organs of both symptomatic and asymptomatic fishes, homogenization was done with the pestle and mortar. Homogenized samples were observed under the inverted digital microscope (EVOS XL live imaging system) at 400X magnification.

Giemsa staining:

Infected organs were homogenized and thin smears were prepared. Fixation was done with methanol for 2-3 dips. After fixation, the smears were stained with 10% Giemsa stain (1 ml stock blended with 9 ml of phosphate buffer) for 30 min (Moursy, 2013). Slides were rinsed with running tap water to remove excess stain. Prepared slides were observed under the inverted digital microscope (EVOS XL live imaging system) at 400X magnification.

Histological examination:

Visceral organs from the infected fish were collected, blotted free of blood. Tissues were cut into 1 mm thick pieces and fixed in 10% neutral buffered formalin. After fixation samples were washed with the tap water. Fixed tissues were processed through routine paraffin method and sections were cut at 4 µm. Tissue sectionsw stained with hematoxylin and eosin (HE) and observed under the inverted digital microscope at 10X, 20X, and 40X for histological examination.

Spore germination:

For the extrusion of polar filament, microsporidian spores were suspended in NaCl, glycine NaOH buffer for 15 min and observed under the light microscope (Undeen and Cockburn, 1989).

Scanning Electron Microscopy:

For SEM the infected organs were homogenized and the homogenate was centrifuged at 6000 rpm for 10 min. After centrifugation, the pellet was collected and fixed in 2.5% glutaraldehyde for 6 h at 4˚C. After fixation washing was done with PBS buffer three times and post-fixation was done in osmium tetraoxide for 2 h and washing was done three times with the same buffer at 4˚C. Sample was dehydrated in acetone with 30%, 50%, 70%, 90%, 95% 100% and finally air drying and critical point dryer and mounted on the aluminum stubs with carbon tape. The coating was done with the sputter coater and samples were observed under Scanning Electron Microscopy (Jeol, Japan; JSM 6490 LV).

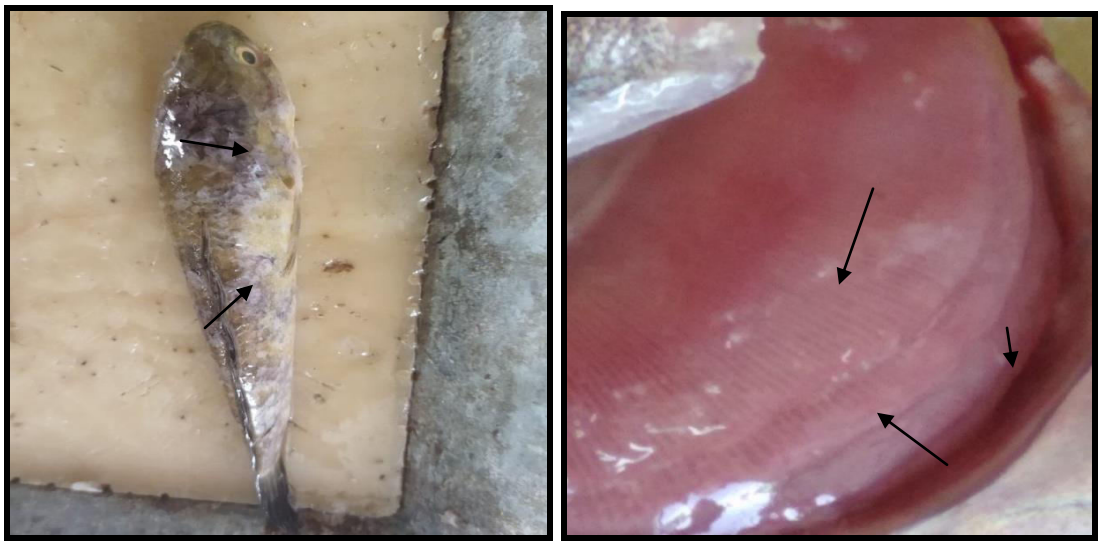
Results

Visual examination:

The present study reported that 64.96% of examined fishes were infected with microsporidian parasites. Both symptomatic and asymptomatic fishes were found to be infected with the parasite with differences in their infection intensity. Symptomatic fish with heavy infection showed discoloration of the external body surface (Fig. 1a) and internally white oval foci on the kidney (Fig. 2a) and liver and liquefied gonads (Figs. 2a, b) and gills with white marks on gill lamellae (Fig. 1b) were present. Infection was observed in all organs including all age groups of fish.

Wet mounting:

Oval to pyriform spores with different developmental stages like meronts (uninucleated, binucleated, and multinucleated meronts with 3-8 nuclei) and sporophorous vesicles containing sporoblast and single spore were seen in wet



(a) **(b)**

Fig. 1: (a) Infected *Channa punctatus* fish. The infection is seen as discoloration of the body surface having a patchy (arrows) appearance; (b) Liquidified gills with white patches.

(a) **(b)**

Fig. 2: (a) Infected kidney having white foci and dissolved gonads; (b) Infected liver with white foci.

mount preparation of different organs of *Channa punctatus*. Infection was found comparatively heavy in kidney, liver, gills, and gonads of symptomatic fish but the infection was also present in asymptomatic fishes. Wet mounted preparation of all organs showed the same pattern of developmental stages. The spores were found oval to pyriform shaped shining florescent green with slight Brownian motion (Fig. 3). Microsporidial spores divide by simple binary fission in the merogonic stage having 2 celled stages (binucleate meronts BM) (Fig. 3) and 4 celled stages (tetranucleate meronts TM), sporophorous vesicle (Fig. 4) and single mature spore (S) were observed in wet mounted smears of all organs (Fig. 3). Microsporidian spore were oval shaped surrounded by two protective membranes, exospore, and endospore. Spore has a

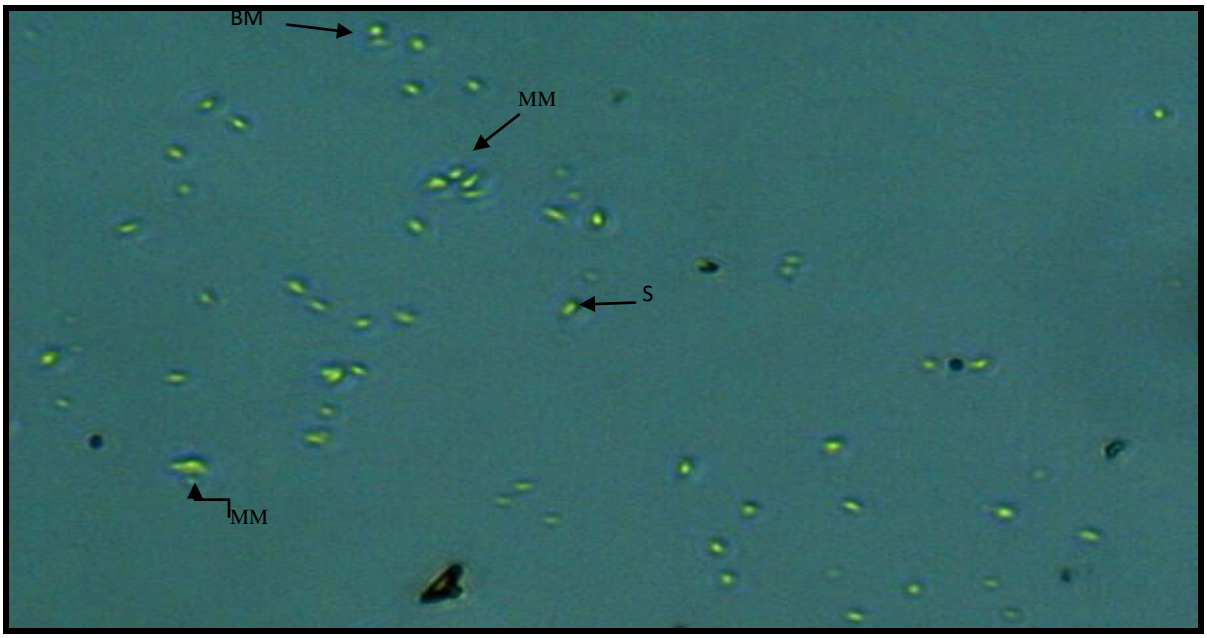


Fig. 3: Wet mount preparation of gill of *Channa punctatus* showing microsporidial infection binucleated (BM) and multinucleated meronts (MM) and single spore (S).

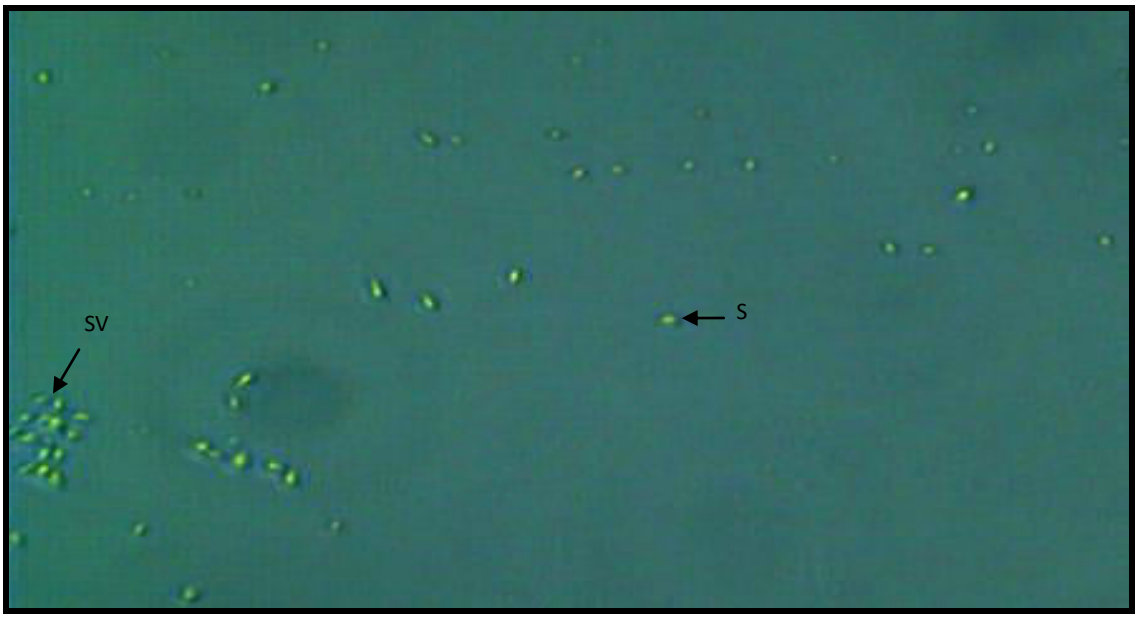


Fig. 4: Wet mounted smear of gill showing sporophorous vesicle (SV) and single spore (S).

nucleus and a posterior vacuole (Fig. 7) and a characteristic polar tubule (Fig. 8). Infection was recorded in all the organs examined including the brain.

Staining:

Giemsa stained samples also showed different stages of the life cycle which include single spore (S), binucleated meront stages (BM) and tetranucleated meront stages (TM), and sporophorous vesicle (SV) containing sporoblast and single mature spores enclosed by plasmalemma and a single polar capsule containing polar filament stained dark blue and

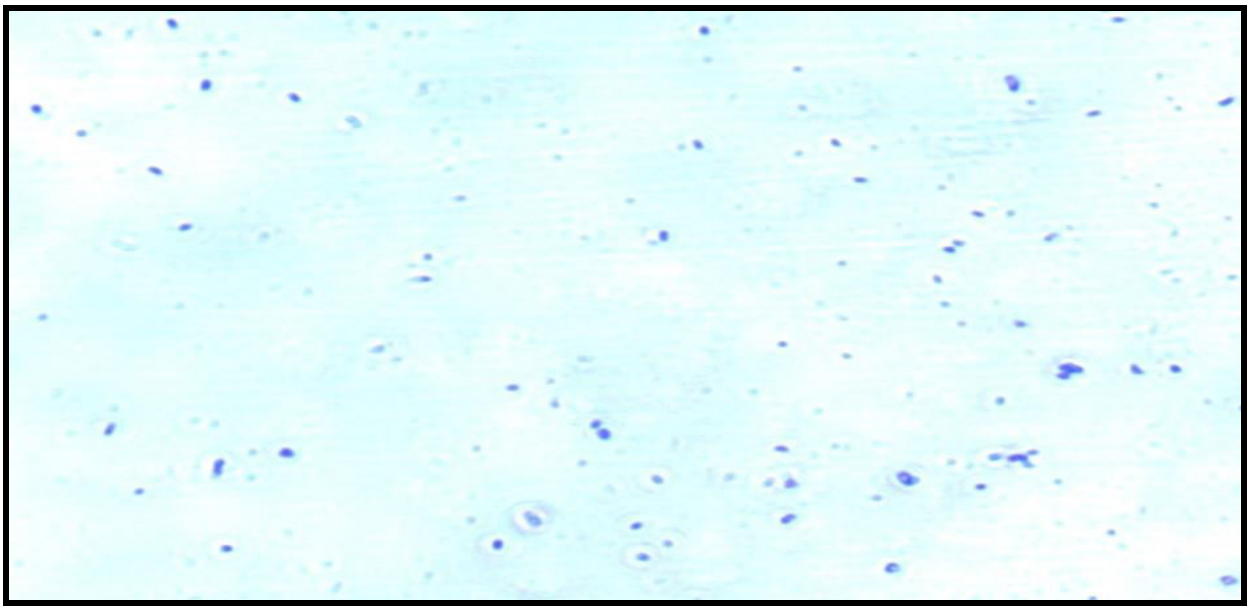


Fig. 5: Giemsa stained smear of gill showing single spore (S), binucleated meront (BM), and tetranucleated meront (TM).

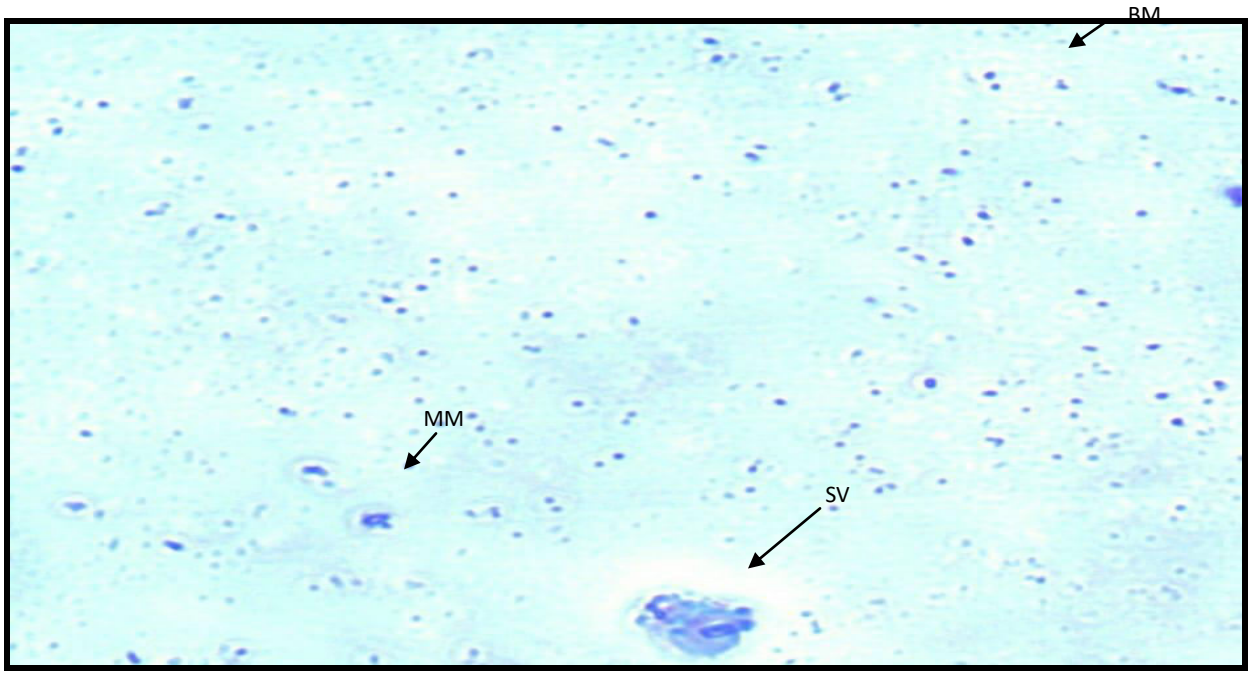


Fig. 6: Giemsa stained smear of gill showing single spore (S), binucleate sporoblast (Sb) and tetranucleated sporont (TS), and sporophorous vesicle(SV).

posterior vacuole were also identified (Fig. 7). Slight changes in alkalinity of wet mounted smear showed polar filament extrusion (Fig.8).

Histological examination:

Light microscopic images of HE stained infected sections of gills showed extensive hypertrophy, hyperplasia and necrosis with curling of the secondary gill lamellae. The parasite destroyed the respiratory epithelium of the secondary gill lamellae, only spool shaped pillar cells appeared and distorted inter-filament

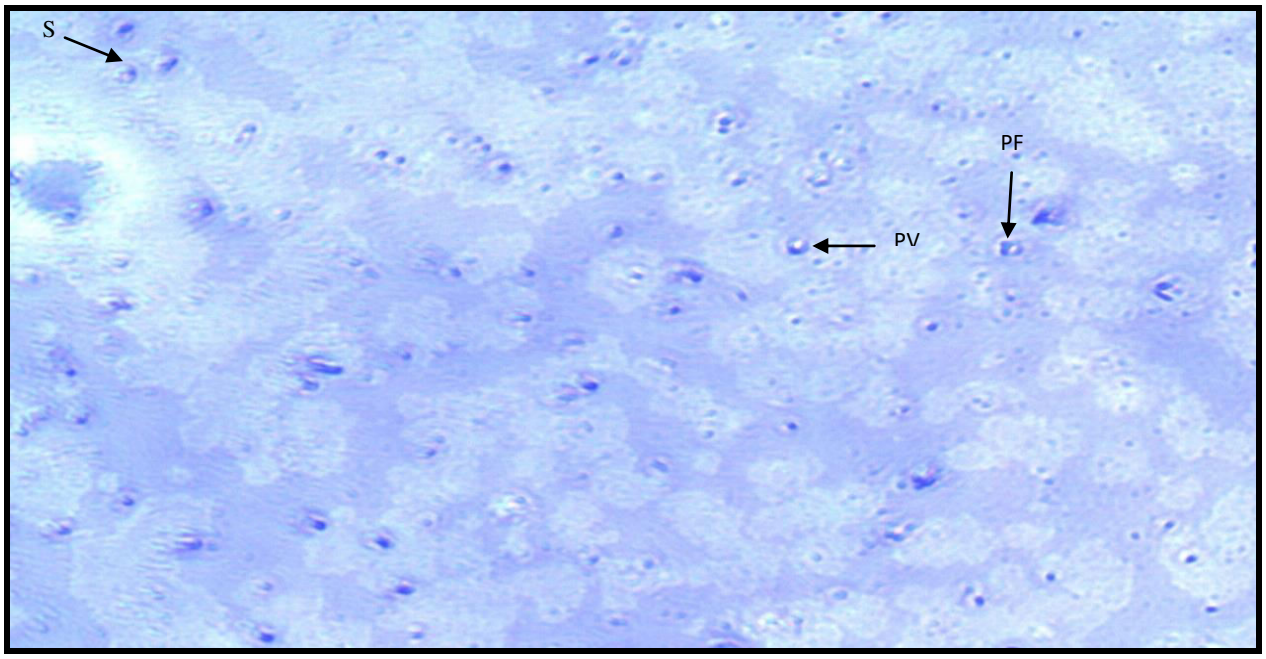


Fig. 7: Giemsa stained smears of gill showing dark stained polar filament and posterior vacuole.

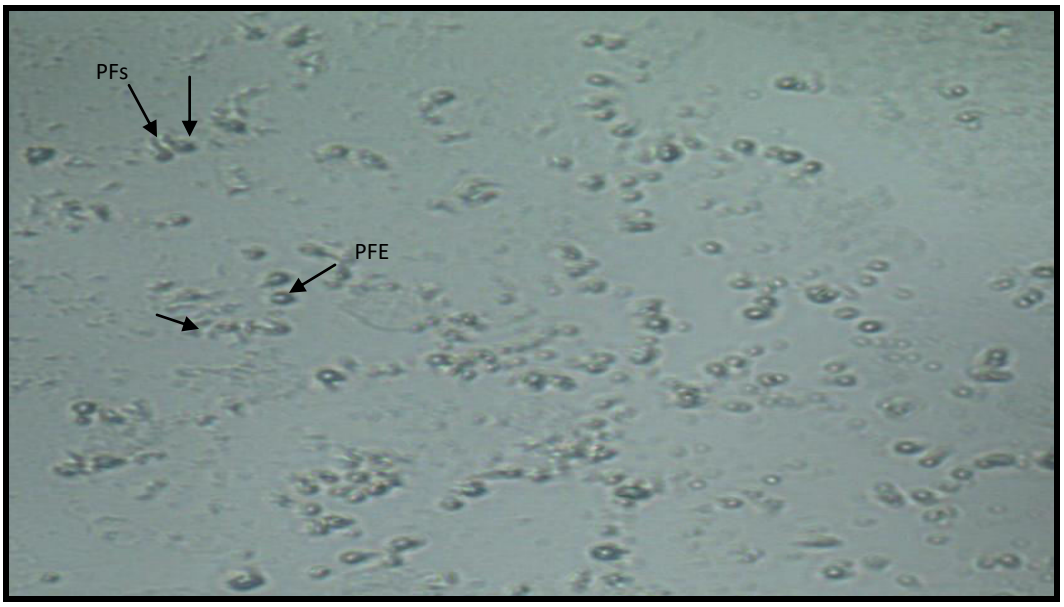


Fig. 8: Image showing polar filament extrusion of spore (PFE).

capillary space were observed (Fig. 1b). Microsporidian spores were present in the epithelial cells of secondary gill lamellae and in the primary gill lamellae instigating hemorrhages and infiltration of the central venous of primary lamellae.

Histology of muscle showed very minute infection by microsporidian parasite in *Channa punctatus* with no any significant histological changes.

HE stained section of kidney showed xenoma near the renal tubules. The infected kidney showed massive infiltration with increased Bowman's capsule space, necrosis and vacuolization of pseudostratified mucus epithelium and enlargement of renal tubules with

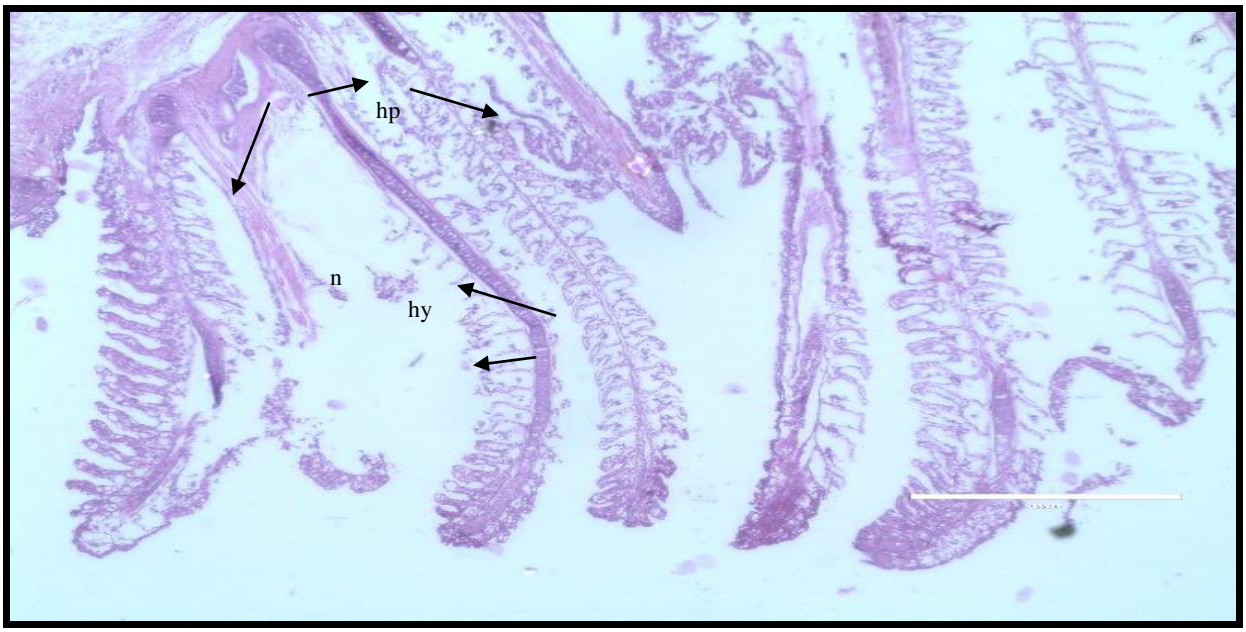


Fig.9: Histology of infected section of gill filaments of *Channa punctatus* showing hypertrophy (hy), hyperplasia (hp) and necrosis (n) in primary and secondary gill lamellae. Scale bar=400µm.

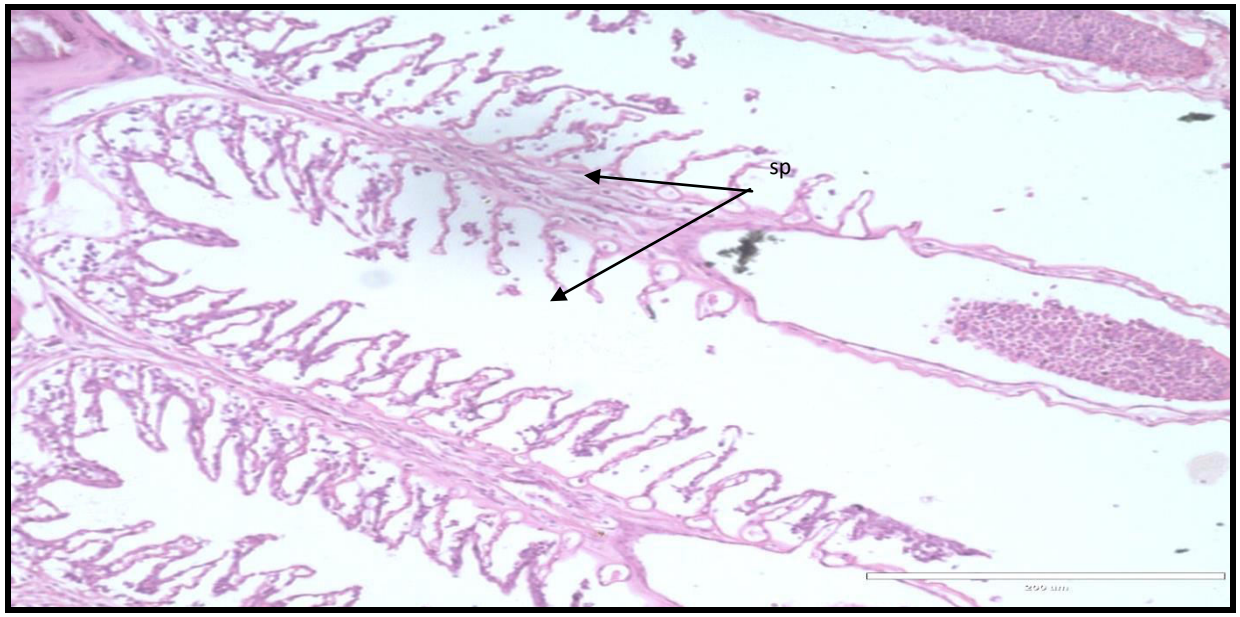


Fig.10: Showing microsporidian spores in the primary and secondary gill lamellae with severe necrosis and distortion of interfilamental lamellae space. Scale bar=200 µm.

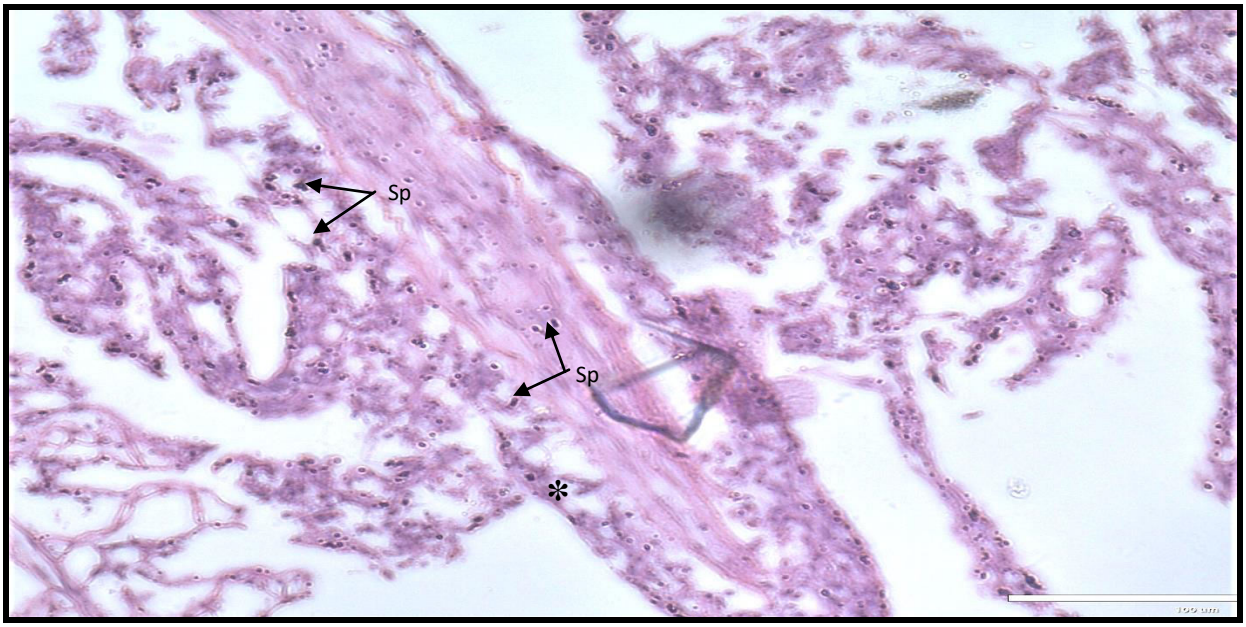


Fig.11: Showing necrotic gill filament invaded with microsporidian spores which can be seen clearly in the primary gill lamella with increased area of capillary lacuna (*) and in the secondary gill lamellae with extensive degeneration. Scale bar=100 µm.

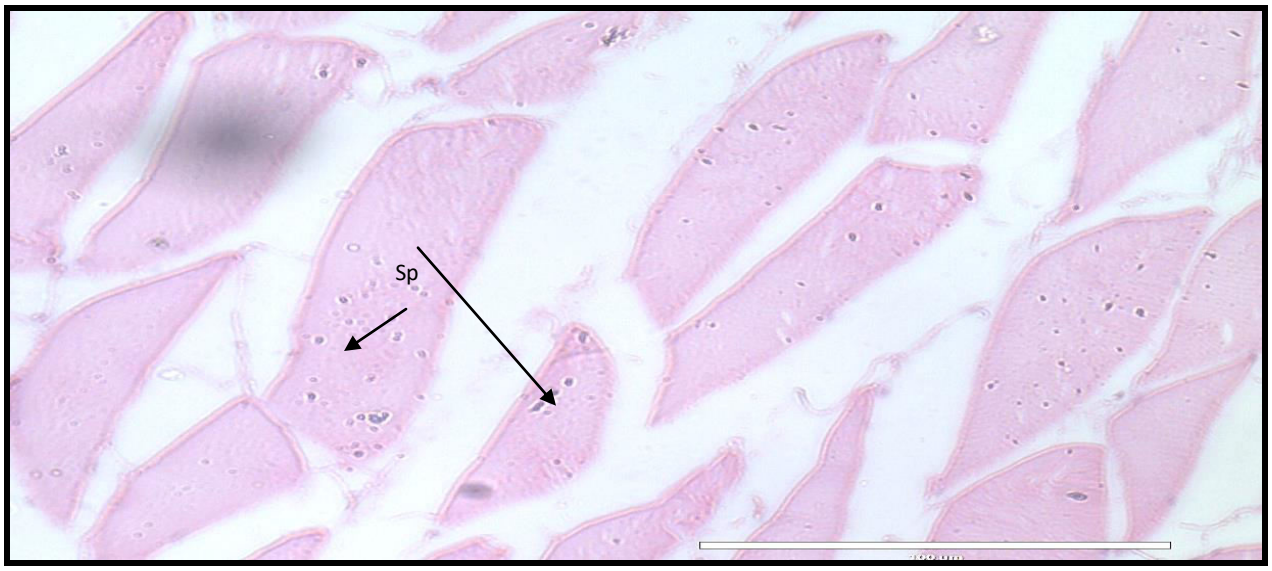


Fig. 12: Showing H and E stained section of muscles of *Channa punctatus* with very minute microsporidian infection. Scale bar=100 µm.

desquamation of epithelial lining. In the mesenchymal cells pyknotic nuclei of hematopoietic cells were clearly seen.

The histology of the liver showed normal polyhedral hepatocytes with centrally placed nucleus, while the infected section showed the microsporidian spores with vacuolization, apoptosis with pyknotic hepatocytes and dilated blood sinusoids.

Scanning electron microscopy:

Ultrastructural analysis through SEM further confirmed the microsporidian infection as fresh ovoid spores having a spongy outer surface with two size ranges measured 15.62 µm and 12.83 µm

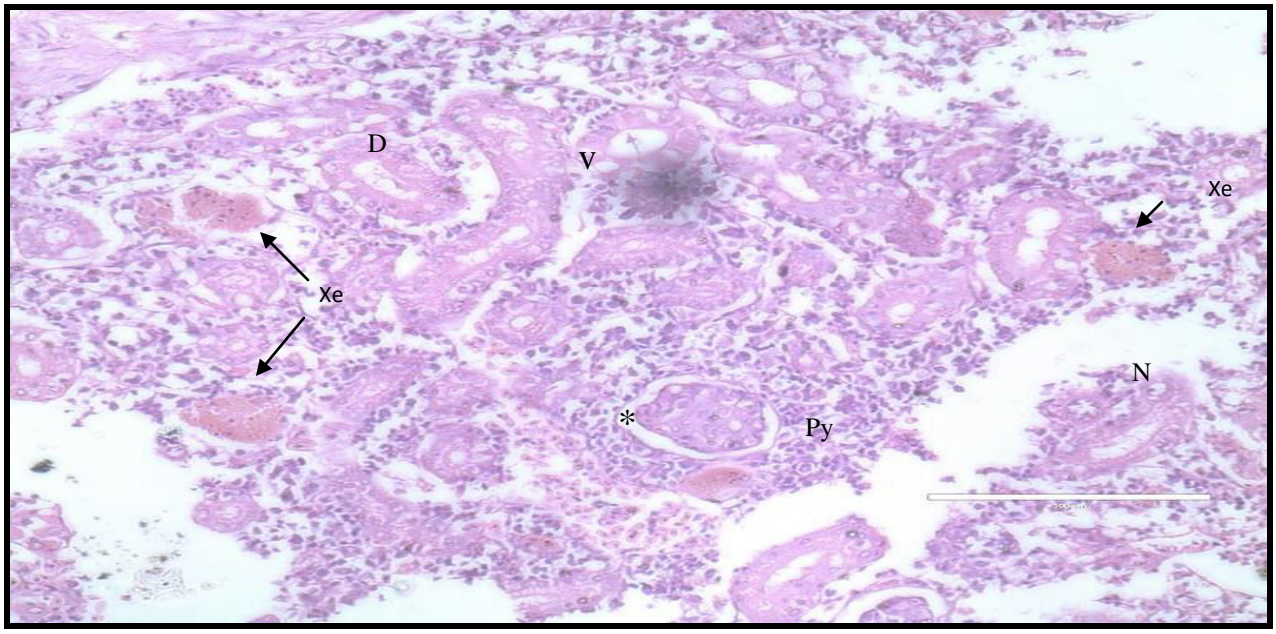


Fig. 13: Histology of kidney of channa punctatus showing xenoma near the renal tubules (Xe). The infected kidney showed massive infiltration with increased bowman's capsule space (*), necrosis (N) and vacuolization (V) of pseudostratified mucus epithelium and enlargement of renal tubules with desequamation (D) of epithelial lining. In the mesenchymal cells pyknotic nucleus (Py) of hematopoietic cells were clearly seen. Scale bar=100 µm.

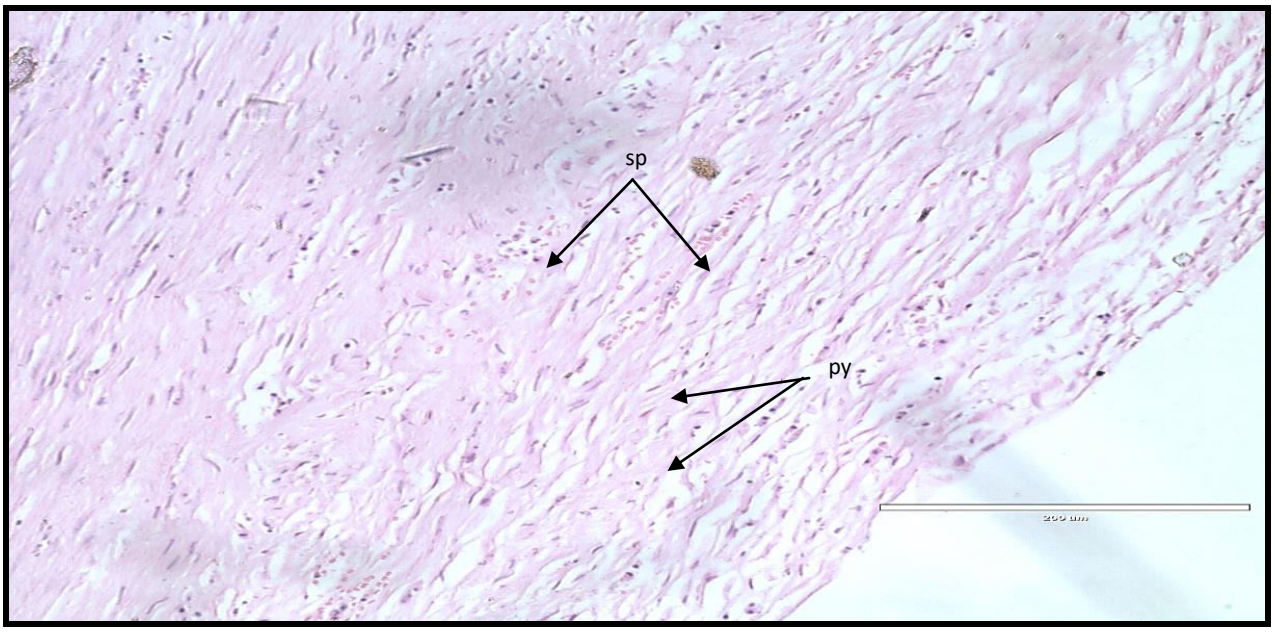


Fig.14: Histology of liver of *Channa punctatus* showing polygonal hepatocytes infected with microsporidian spores causes vacuolization, apoptosis, pyknotic nucleus and dilation of blood sinusoids. Scale bar=200 µm.

were observed (Fig. 15).

Discussion

Microsporidiasis which is caused by microsporidia has been reported in this study in *Channa punctatus* fish collected from the Gomti river, Lucknow. Microsporidia are obligate eukaryotic intracellular parasites with fungal affinities which contain a unique polar filament that imparts to infect a new host. The infected fishes showed

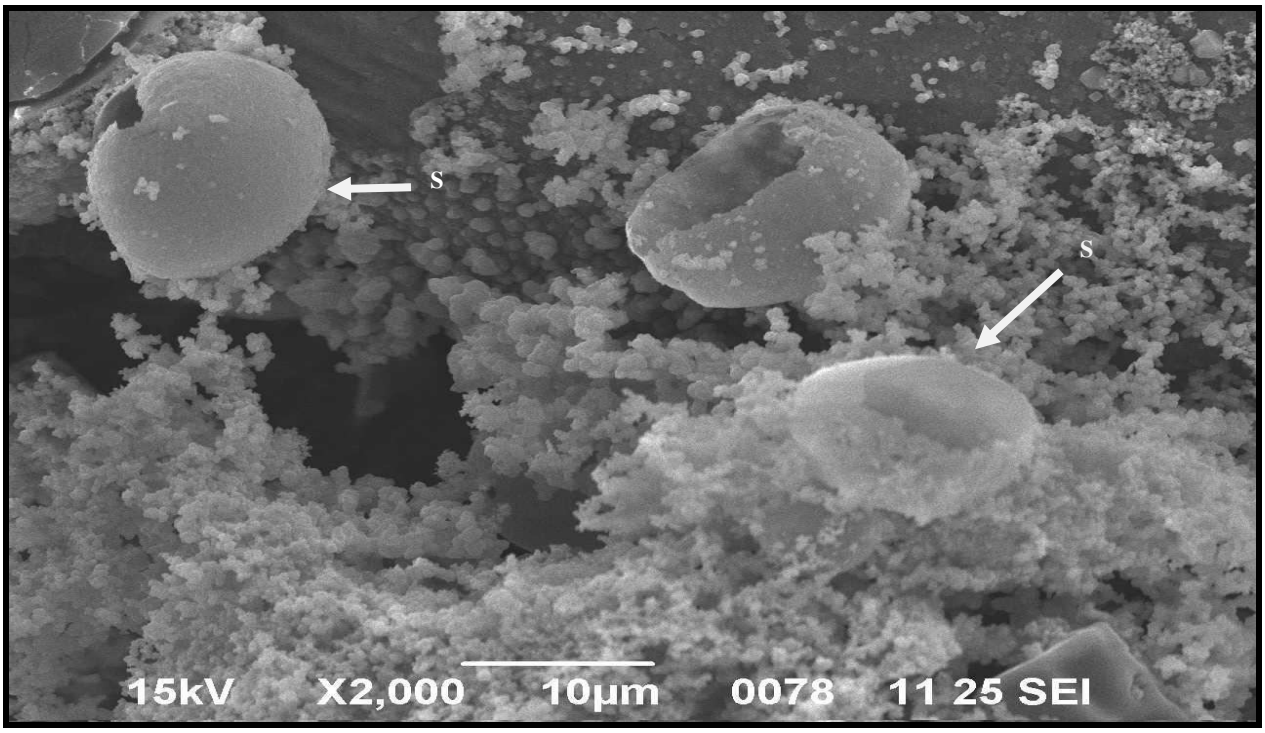


Fig. 15: Microsporidial spore under Scanning Electron Microscope, isolated from the gill of *Channa punctatus*.

discoloration of the external body surface and dissolved gonads and gills with white patches on gill lamellae and whitish foci on the liver and kidney. Similar findings were reported by Phelps *et al*. (2015) and Jalenques *et al*. (2021) who observed microsporidian infection-causing discoloration of muscle. Abidi *et al*. (2016) also reported infection in the ovary of *Cyprinus carpio* fish collected from Gomti river, Lucknow. In our study infection was present in both symptomatic and asymptomatic fish with differences in infection intensity which was previously reported by Tomamichel *et al*. (2018) who found a 100% prevalence of microsporidia in both infected and uninfected fishes which do not have any clinical signs of infection on visual and microscopic examination.

Very minute microsporidian infection was present in the muscle of *Channa punctatus* while all the other organs like gonads, kidneys, liver, brain, gills, and heart were severely infected from which highest infection was recorded in the kidney, liver and gills of the fish.

Wet mounted and Giemsa stained smears clearly showed different developmental stages of microsporidia like uninucleated, binucleated, and multinucleated meronts having 3-8 nuclei. The results are as par with the reports of Ghaffar *et al.* (2011) and Jithendran *et al*. (2018) who observed microsporidian infection as whitish cyst embedded in the peritoneal cavity of *Saurda tumbil* and infection in the hepatopancreatic and feces sample of shrimp, respectively. Sporogonic stages were also present in wet and Giemsa stained smears as sporophorous vesicles containing 8-16 spores enclosing all life cycle stages, i.e., meronts, sporonts, and mature spores were present. Similar findings have been recorded by Ghaffar *et al*. (2011) who reported the microsporidian infection in three marine species of fishes namely *Saurida tumbil*, *Pagrus pagrus*, and *Epinephelus chlorostigma* containing sporophorous vesicles collected from the Red sea. Giemsa stained smears showed dark blue polar filament and a vacuole at the posterior side. Mladineo and Lovy (2011), Jitendran *et al*. (2011)

and Quraishy *et al*. (2019), also reported the same as spore polar filament stains dark blue and occupied anterior end while the vacuole is present at the posterior end of the spore. Extrusion of polar filament was also recorded with the altered salinity which was previously proposed by Undeen and Cockburn (1989) for the DNA extraction of the spore. Ghaffar *et al*. (2011) recorded the polar filament extruded outside the spore under slight pressure.

In the HE stained sections of the gill microsporidian parasite causes hypertrophy, hyperplasia and necrosis along with the hemorrhage and infiltration of the blood cells in primary and secondary gill lamellae. Similar findings have been reported by Rakesh *et al*. (2018), and Becker and Speare (2007). Steigen *et al*. (2018) also reported the xenomic microsporidian infection in the gills of wrasse from western Norway and Nylund *et al*. (2011) reported the extensive necrosis due to *Paranucleospora theridion* in the gills of Atlantic salmon *Salmo salar*. The gills are the most susceptible organ for microsporidian infection as this organ is in direct contact with the water which favors horizontal transmission of infection as Sanders *et al*. (2012) and Morsy *et al*. (2012) observed the horizontal transmission of microsporidian infection in rearing tanks of zebra fish and in *Pagrus pagrus* fish.

Microsporidian infection was very minute in the muscle of the *Channa punctatus*, while Zhang *et al*. (2010) reported microsporidian infection in the form of cyst in the trunk muscle of Pacific bluefin tuna.

Microsporidian infection was also present as xenomic patches in the HE stained sections of the kidney of *Channa punctatus* and showed massive infiltration in the inter-tubular area and distortion in the Bowman's capsule and alteration in the renal tubules. Similar infection had been reported by Lovy and Friend (2017) as they reported xenoma near the renal tubule of bluegill sunfish collected from the Assunpink Lake U.S.A. Rakesh *et al*. (2018) also reported the microsporidian spores in the kidney of *Cirrhinus mrigala*.

Microsporidian infection was present in the liver of the infected *Channa punctatus*. The liver showed extensive necrosis with cytoplasmic vacuolization with pyknotic nuclei of the hepatocytes. Similar findings had been reported by Dhiraj and Bhatt (2014) and Rakesh *et al.* (2018) who also reported the microsporidian spores in the liver of *Catla catla* and *Cirrhinus mrigala*, respectively. Lovy and Friend (2017) reported the aggregates of *Ovipleistophora diplostomuri n. sp.* in the liver of bluegill sunfish.

In the present study, spores of two size ranges measuring 15.62 µm and 12.83 µm have been identified which were oval to pyriform shaped which might be two different kinds of parasitic spores i.e. macrospore and microspore. The surface of the spores was spongy under scanning electron microscopy, similar to findings reported by Faye and Toguebaye, (2005) and Kashyap *et al*. (2018) who reported the hepatic microsporidiasis in fishes *Selene dorsalis* and *Trachurus. trachurus* and infection in *Apis mellifera*, respectively. Previously the size of the spore was reported as 7.5-12 µm and 6.5-7.5 µm by Putz *et al*. (1965) and Lom and Nilsen, (2003) for *Ovipleistophora mirandellae* and *Pleistophora mirandellae*, respectively but in this study the size of the spore is slightly more so it could be another species as it was already found that all the species of microsporidia which were investigated in a different host had slight variation and they were considered as a new species but for this further molecular-based analysis is required.

Conclusion

In the present study microsporidian infection have been investigated in the *Channa punctatus* fishes collected from the Gomti river, Lucknow and infection was clearly evident in both symptomatic and asymptomatic. Microsporidia damaged their host cell causing hypertrophy and liquification of the gills, gonads, and ulcers in the kidney and liver. Ultrastructural results further confirmed the

presence of non-xenomic microsporidial spores of 15.62 µm and 12.83 µm in size. As the size of the present spore is slightly big so it could be another species or might be due to a different host as some authors reported that microsporidia are host specific so to identify the species we need the molecular analysis. As Tomamichel *et al*. (2018) reported massive mortality (100%) due to microsporidian infection so it is very important to monitor this parasite before its transport from nature to farms to avoid any massive loss of aquaculture productivity due to this parasite.

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