International Journal of Fisheries and Aquatic Studies 2017; 5(4): 413-417

E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2017; 5(4): 413-417
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www.fisheriesjournal.com
Received: 01-05-2017
Accepted: 02-06-2017

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Isolation and identification of *Myxobolus cerebralis* from brain of *Heteropneustes fossilis* in the beels of South 24-Parganas, West Bengal, India

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Abstract
Myxobolus cerebralis infecting the brain of *Heteropneustes fossilis* (singhi) was identified morphologically, histopathologically and by molecular method captured from the beels of Raidighi, South 24 Parganas, West Bengal, India. Infection of *M. cerebralis* was uncommon in *Heteropneustes fossilis* brain. The infection rate was low to moderate. Microscopically *Myxobolus* parasites were detected in brain of *Heteropneustes fossilis* (singhi). Large plasmodia were localized within brain, causing severe necrotic changes and vacuolization. Inflammatory infiltrates agglomeration, nuclear hypertrophy, vacuolization, infiltration of blood cell and haemorrhages were extensively observed in the brain. Polymerase chain reaction (PCR) with primers specific for the family Myxobolidae was used to amplify an approximately 1600 base pairs (bp) long fragment of the 18S ribosomal RNA gene.

Keywords: *Heteropneustes fossilis,* histopathology, morphometry, *Myxobolus cerebralis,* PCR

1. Introduction
Fisheries have always played a crucial role in food and nutrition all over the world [1]. Aquaculture continues to be the fastest growing animal food producing sector. Health management in aquaculture is of major concern from production and security point of view. Diseases are one of the most important constraints on aquaculture production. Several bacterial, viral, parasitic and fungal diseases have been documented. Among parasitic diseases myxozoans, monogeneans, digeneans, larval cestodes and ectoparasitic crustaceans are of great importance [2]. The myxosporea are common parasites of fishes; and some of them are reported to be serious pathogens associated with epizootics and causing heavy losses to the aquaculture [1]. One of the richest genera of the Myxosporea is the genus *Myxobolus*. To date as many as 500 *Myxobolus* species infecting fish are known, of which 444 valid species were recorded by Lom *et.al* (1992) [3]. Until recently, differentiations of the species were based on a morphological characterization of spores. The molecular method has been adopted for confirmation of myxosporean species by using specific sets of primers. The myxozoan parasite, *Myxobolus cerebralis* is the causative agent of whirling disease is a widely known lethal infection of freshwater salmonid fish that has vast economic and ecological impacts [4]. This parasite has a complex life cycle involving two hosts: it first resides in the digestive tract of *Tubifex tubifex*, an oligochaete worm, and once expelled it infects salmonid fish [4]. Whirling disease is mostly prevalent in North America, throughout Europe, South Africa, North-east Asia and New Zealand [5]. Salmonids are not native freshwater fish of India, and are unlikely to be susceptible to whirling disease. Nevertheless, several reports on *Myxobolus* spp. are available from India, especially from West Bengal [5]. The occurrence of *Myxobolus cerebralis* in India has been reported by Abidi (2015) [6] from Indian catfish *Clarias batrachus*. In this study, we identify the presence of *Myxobolus cerebralis* in the brain of *Heteropneustes fossilis* (singhi) by morphological, histopathological study and by using molecular method.

2. Materials and methods
2.1 Sample Collection
Live *Heteropneustes fossilis* (singhi) captured from the beels of Raidighi (22.0012° N, 88.4354° E), South 24 Parganas district, West Bengal, India were brought to the laboratory

within 50 min of collection in oxygen filled polythene bags during early morning. In the laboratory, the length, weight, external symptoms and general health conditions of the fishes were recorded immediately. Brains were dissected out, placed in separate Petri-dishes and thoroughly examined. The severity of infection was determined by the following scale proposed by Lightner (1993) [7] with slight modification as follows:

Table 1

The severity of infection	Denoted by
No signs of parasite	0
A very few scattered signs of parasitic infection	0.5
Low parasitic infection	1
Low to moderate parasitic infection	2
Moderate parasitic infection	3
Severe parasitic infection	4

2.2 Light microscopy
The parasite identification was performed in the laboratory according to Lom *et al*. (1989) [8]. For detailed study, fresh brains were first taken on clean grease free glass slides. Then it was slightly ruptured and smeared on clean slides with a few drops of distilled water, covered with cover slips and sealed with DPX for examination under oil immersion (100X) lens [Motic BA400]. Permanent mounting of myxosporean parasites were done by staining with Giemsa solution (HiMedia, Mumbai). Air dried smears were treated with acetone free absolute methyl alcohol for about 8 min to fix the parasites. The stock solution of Giemsa was diluted with phosphate buffer (pH 7.2) in the ratio of 1:2. The slides were then placed on a staining rack and covered with Giemsa working solution for 40 min. The slides were then washed by distilled water and air dried. The slides containing myxosporean spores were observed under oil immersion (100X) lens [Motic BA400]. The morphometric measurements were done by Motic Image Plus Version2 software.

2.3 Histopathology
The brain samples of *H. fossilis* were fixed in alcoholic Bouin's fixative for48-72 h. After fixation the tissues were transferred to 70% Ethyl alcohol and kept overnight. Histopathological analysis was made as described by Roberts (2001) [9].

2.4 Molecular identification
2.4.1 DNA extraction
The DNA was extracted using DNA, RNA and Protein purification Kit of Machery-Nagel GmbH & Co. KG, Germany. After extraction, the DNA was collected and stored at -20° C for further use.

2.4.2 PCR amplification
The approximately 1600 base pairs (bp) long fragment of the 18S smaller subunit of ribosomal RNA (18S rRNA) was amplified by PCR using a set of primers (MX5-F, 5´-CTGCGGACGGCTCAGTAAATCAGT-3´ and MX3-R, 5´CCAGGACATCTTAGGGCATCACAGA-3´) [10]. The reaction mixture of 25 µl consisted of 1µl of genomic DNA (10 to 50 ng), 12.5µl of 2x PCR Taq Mixture (HIMEDIA), 10 p moles each of the two primers and 9.5 µl of molecular biology grade water (HIMEDIA). Amplification was done by initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing temperature of primers was 46°C for 30 sec and extension at 72°C for 45 sec. Final extension was made at 72°C for 5 min.

2.4.3 Agarose gel electrophoresis
The PCR products were analysed on a 1.2% agarose (HIMEDIA) gels containing 0.5 µg/ml ethidium bromide in 1× tris-acetate- EDTA (TAE) buffer.

3. Results
In the present study *Myxobolus cerebralis* was detected microscopically in the brain of *H. fossilis*. In frontal view (perpendicular to the sutural plane) the spores were mostly circular in shape. Mature spores (Fig. 1) from the plasmodia were round to oval in frontal view, measuring 9.31µm (9.25 to 9.37 µm) in length, 9.24µm (9.19 to 9.28µm) in width. The polar capsules were pyriform and of equal size, measuring 5.19µm (5.16 to 5.23µm) in length, 3.21µm (3.19 to 3.26µm) in width. The filament inside the capsule was not very distinct in this species (Table 2). The severity of infection was low to moderate.

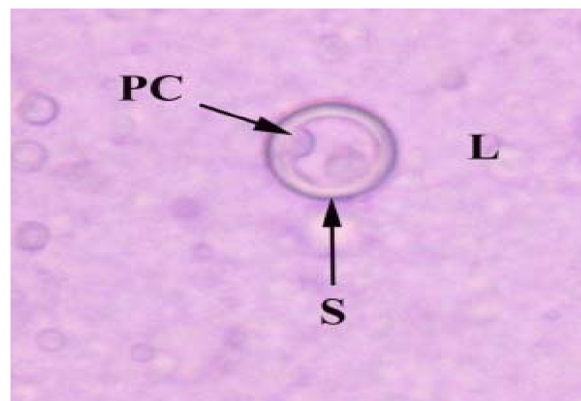

Fig. 1: Spore of *Myxobolus* sp. showing polar capsule (PC), spore (S), collected from brain of *Heteropneustes fossilis* (400x) (wet mount).

Table 2: Morphometry of *Myxobolus cerebralis* spores (on the basis of 20 observations) isolated from the brain of *H. fossilis.*

Measurement	Data given by Hofer, 1903 (µm)	Data obtained in the present study (µm)	Mean	SD	CV (%)
LS	7.4 - 9.7	9.25-9.37	9.312	0.037077	0.398
BS	7 -10	9.19-9.28	9.2435	0.029249	0.316
LPC	5 - 6	5.16-5.23	5.1965	0.020844	0.401
BPC	3 - 3.5	3.19-3.26	3.217	0.021788	0.677

LS: Length of Spore, BS: Breadth/width of spore, LPC: Length of polar capsule, BPC: Breadth of polar capsule, SD – standard deviation, CV – coefficient of variation.

By the histopathological analysis we can confirm that infected by protozoan parasites with binucleated nuclei representing *Myxobolus cerebralis* in the brain of *Heteropneustes fossilis* (Fig.2). The brain section also showed inflammatory infiltrate

agglomeration, meningoencephalitis, lesions and enlarged neural cells (Fig.3). Histopathological analysis of the brain of *H. fossilis*, based on histological sections stained with Hematoxylin-Eosin, allowed us to analyze degenerative changes and vacuolization in tissue. There was a marked nuclear hypertrophy, infiltration of blood cells and haemorrhages were also observed in examined fish brain (Fig.4 & 5).

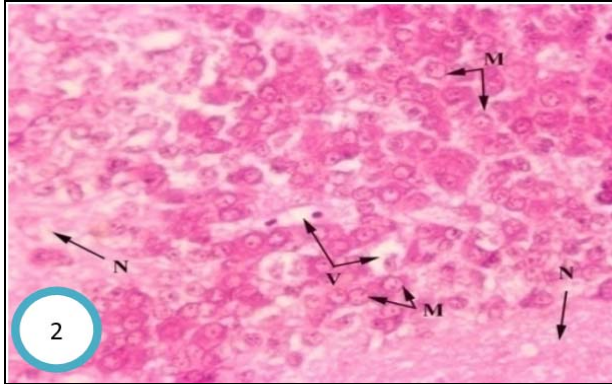

Fig 2: Brain cells of *H. fossilis* showing the presence of huge number of Myxobolus spores (M). Cells were necrotised (N), vacuolization (V) were also present. H&E, 400 X.

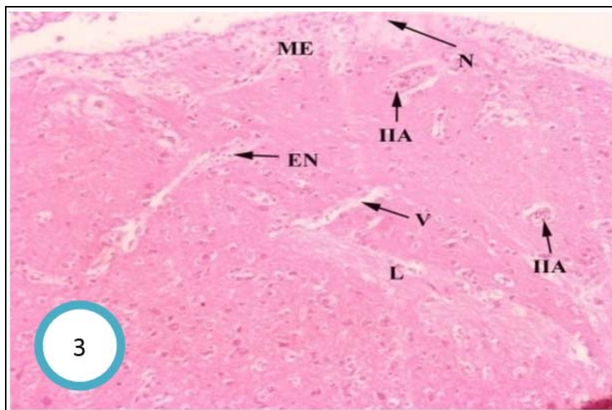

Fig 3: The brain section of *H. fossilis* showing inflammatory infiltrate agglomeration (IIA), meninges indicating meningoencephalitis (ME), lesions (L) with vacuolization (V), necrosis (N), enlarged neural cells (EN). H&E, 100 X.

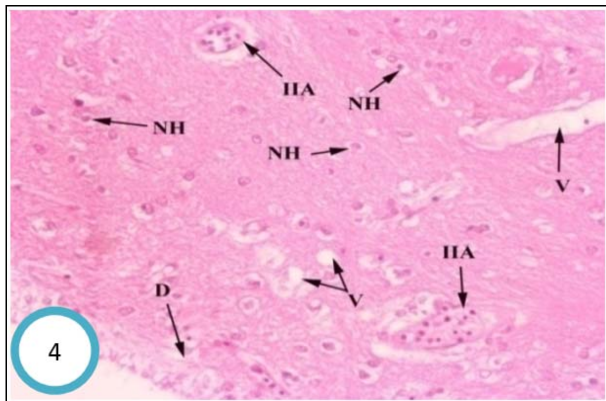

Fig 4: Histopathological changes in the brain of *Heteropneustes fossilis* showing inflammatory infiltrates agglomeration (IIA), nuclear hypertrophy (NH), vacuolization (V) in the tissue and degenerative changes (D). H&E, 200 X.

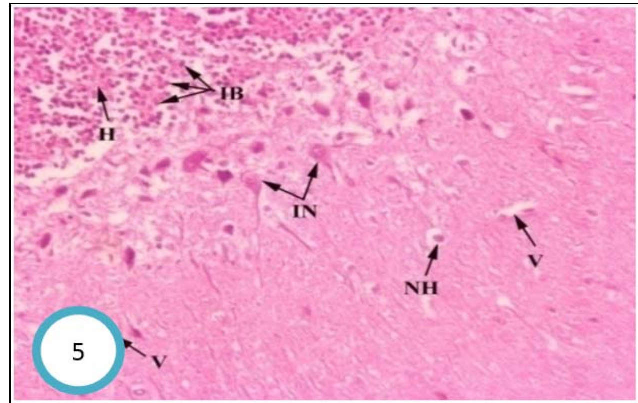

Fig 5: The brain cell infected with *Myxobolus* sp. showing the presence of infiltration of blood cells (IB), haemorrhage (H), inflamed neurones (IN), nuclear hypertrophy (NH), vacuolization (V). H&E, 200 X.

By molecular method we can confirm that the primers MX5 and MX3, according to Eszterbauer *et al.* (2001) [10], successfully amplified approx. 1600 bp fragments of the 18S rRNA gene from every sample of the *Myxobolus* species. These primers are specific for the family Myxobolidae (Fig. 6).

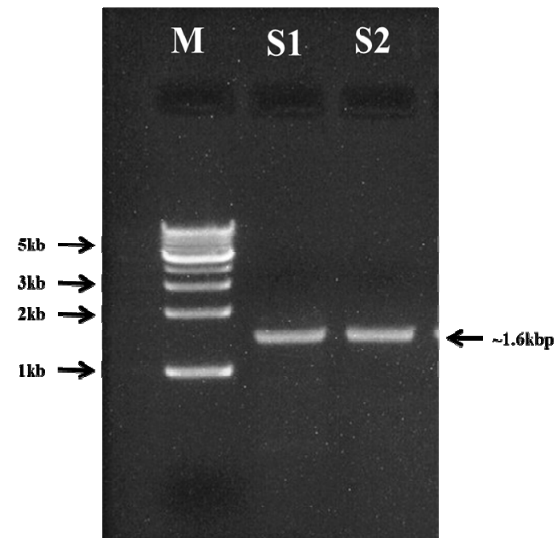

Fig 6: Agarose gel (1.2%) showing amplification of a part of 18S rRNA gene of *Myxobolus cerebralis* from the brain of *H. fossilis.* Lane M: 1kb DNA Ladder (Takara Bio Inc., Japan); Lane S1: Positive control; Lane S2: *Myxobolus cerebralis*.

4. Discussion

In the present study, mature spores (Fig. 1) from the plasmodia were round to oval in frontal view, measuring 9.31µm (9.25-9.37 µm) in length, 9.24µm (9.19 to 9.28µm) in width. The polar capsules were pyriform and of equal size, measuring 5.19µm (5.16 to 5.23µm) in length, 3.21µm (3.19 to 3.26µm) in width. According to Hofer (1903) [11] in *Myxobolus cerebralis,* the spores were mostly broadly oval, sometimes more elongated, rarely completely circular, and exceptionally broader than long. The dimensions of spores were: length 8.7 (7.4 to 9.7) µm, width 8.2 (7 to 10) µm, thickness 6.3 (6.2 to 7.4) µm. The spore wall could not be measured accurately but appeared to be about 0.25 µm thick. The oviform polar capsules measured 5.1 (5 to 6) by 3.2 (3 to

3.5) µm. The comparison of spore morphology shows that the present species corresponded to *M. cerebralis*. The spores of other *Myxobolus* species, similar to *M. cerebralis* in shape, mainly, *Myxobolus arcticus, Myxobolus insidiosus* but only the spores of *M. kisutchi* and the microspores of *M. squamalis* were similar in size [12]. However, *M. kisutchi* was related in a host of Cohosalman and Chinook salmon and their spores differed from those of *M. cerebralis* by appear uniform in shape, and contain an iodinophilous vacuole. In contrast to *M. cerebralis*, the plasmodia of *M. arcticus* had larger spores (14.3-16.5x7.6-7.7 µm), with large, elongated polar capsules. However, plasmodia of *M. insidiosus* were found in the muscle of cut throat trout, Chinook salmon and Coho salmon from the western United State [12].

By the histopathological analysis we can confirm that infected by protozoan parasites with binucleated nuclei representing *Myxobolus cerebralis* in the brain of *Heteropneustes fossilis*. We found a correlation between the fish cells and parasitic spores via the hematoxylin-eosin (HE) histological technique, which was used to confirm the presence of *M. cerebralis* in tissue of fish brain [13]. In the present study the brain of *H. fossilis* showed inflammatory infiltrate agglomeration, lesions, necrosis and enlarged neural cells. Elwell *et al.* (2010) [14] showed that cartilage tissue of salmonid fish infected with *M. cerebralis,* was visibly eroded and granulomatous lesions were found in association with the cartilage destruction (host inflammatory response). This result is in agreement with the present findings. Uspenskaya 1982 [15] reported that destruction of cartilage is accomplished both through extracellular digestion (lysis) of the cartilage matrix and by phagocytosis of cartilage cells by *M. cerebralis* [16]. Using histopathological analysis,[17] noted diffuse oedema in brain tissues, and congestion, degeneration, and focal necrosis of the cerebral cortex. We also observed a pronounced quantity of *Myxobolus* sp. spores dispersed throughout the brain of *H. fossilis*. There was a marked nuclear hypertrophy, infiltration of blood cells and haemorrhages was also observed in examined fish brain (Fig.4&5). Severe cases of whirling disease can lead to death in young fish. Early reports of whirling disease frequently referred to (sometimes heavy) mortalities associated with the disease [14].

According to Eszterbauer *et al.* (2001) [10], the 18S rRNA gene and oligonucleotide primers were used to amplify an approximately 1600 bp long fragment from the 18S rRNA gene. The primers were specific for the family Myxobolidae. This result is in agreement with the present study. The primers MX5 and MX3 specific for the family Myxobolidae successfully amplified approx. 1600 bp fragments of the 18S rRNA gene from every sample of the *Myxobolus* species (Fig. 6).

5. Conclusion

Existing knowledge on the phylum Myxozoan enigmatic group of organisms is fragmentary, inadequate and incomplete in many parts of India, especially in the state of West Bengal having rich fish biodiversity. There is great need to identify, describe and satisfactorily classify these parasites both morphologically and at the molecular level. It is evident that study on this group is also important in identification of the pathogenic species which can pose serious threat to fisheries in state of West Bengal. This knowledge will help in diagnostics, management and treatment of the diseases caused by these parasites in freshwater fishes.

6. Acknowledgements

The authors gratefully acknowledge the assistance extended by the Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, West Bengal, India for providing necessary facilities for undertaking the work. Special thanks to National Bureau of Fish Genetic Resources (NBFGR), Lucknow, Uttar Pradesh, India and National Fisheries Development Board__(NFDB), Hyderabad, Telangana, India for financial support during the study.

7. References

1. Chavda D, Bhatt S, Sreepada RA, Sheth A. Pathogenicity of Myxobolus infection and its effect on protein expression in *Catla catla* in central Gujarat region. Journal of Cell & Tissue Research. 2010; 10(1):2157-2164.
2. Mondal A, Banerjee S, Patra A, Adikesavalu H, Ramudu KR, Dash G, *et al.* Molecular and morphometric characterization of *Thelohanellus caudatus* Myxosporea: Myxobolidae infecting the caudal fin of *Labeo rohita* Hamilton. Protistology. 2014; 8(2):41-52.
3. Lom J, Dyková I. Protozoan parasites of fishes. Elsevier Science Publishers, 1992.
4. Wolf K, Markiw ME. Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science. 1984; 225:1449-1453.
5. Banerjee S, Patra A, Adikesavalu H, Mondal A, Dash G, Abraham TJ. Spinning (whirling) disease in Indian major carp fingerlings. Proceedings of National Conference on Challenges in Biodiversity Conservation and Resource Management. University of Kalyani, Kalyani. 2014, 50-55.
6. Abidi R, Fariya N, Chauhan UK. Development and standardization of PCR technique to detect myxozoan parasites and its use in identification of two exotic Myxobolus species from Indian catfish *Clarias batrachus* linn. International Journal of Fisheries and Aquatic Studies. 2015; 2(4):374-377.
7. Lightner DV. Diseases of cultured penaeid shrimp. In: CRC Handbook of mariculture: Crustacean aquaculture, Edn 2. CRC Press Inc., Boca Raton, Florida, 1993, 393-486.
8. Lom J, Arthur JR. A guideline for the preparation of species descriptions in Myxosporea. Journal of Fish Diseases.1989; 12(2):151-156.
9. Roberts RJ. The parasitology of teleosts. In: Fish Pathology, Edn. 2001; 3:254-296.
10. Eszterbauer E, Benko M, Dan A, Molnar K. Identification of fish-parasitic Myxobolus Myxosporea species using a combined PCR-RFLP method. Diseases of Aquatic Organisms Journal. 2001; 44:35-39.
11. Hofer B. Uber die drekhrankheit der Regenbogenforelle. Allgemeine Fischerei-Zeitung. 1903; 28(1):7-8.
12. Pugachev ON, Khokhlov PP. Myxosporidia of the genus Myxobolus-parasites of the brain and spinal column of salmonid fishes. Vladivostok. 1979; (1):137-139.
13. MacConnell E, Bartholomew J. Whirling disease of salmonids. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, Edn 5, Fish Health Section, Bethesda, Maryland, 2003.
14. Elwell LCS, Stromberg KE, Ryce EK, Bartholomew JL. Whirling disease in the United States: A summary of progress in research and management. West Yellowstone,

2010, 203.
15. Uspenskaya AV. New data on the life cycle and biology of Myxosporidia. Archivfür Protistenkunde. 1982; 126(3):309-338.
16. Taylor RE, Haber MH. Opercular cyst formation in trout infected with *Myxosoma cerebralis.* Journal of Wildlife Diseases. 1974; 10(4):347-351.
17. Yokoyama H, Meng F, Hirai M, Takagi S, Katagiri T, Endo M, *et al*. Recently emerged myxosporean encephalomyelitis of cultured yellowtail *Seriolaquinqueradiata* in Japan. Journal of Aquaculture Research and Development. 2013; 2:1-4.