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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.)

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Abstract
The international trade of live aquatic animals has gained a momentum in recent years. To facilitate this trade disease/pathogen related risks associated with fish movement have to be minimized. Tests done through molecular tools for confirming presence/absence of a pathogen in consignment have been approved most appropriate by International Organization of Epizootics (OIE), WTO, FAO and NACA, Bangkok. With the help of Polymerase Chain Reaction (PCR) amplification of RNA/DNA, it has become possible to rapidly detect the pathogen even if the quantity of DNA/RNA is very little. In India, there is a paucity or we can say total lack of work on molecular diagnostics of fish parasites particularly 'Protozoans'. Hence we developed and standardized the DNA/RNA polymerase chain reaction (PCR) amplification technique for detection of Myxozoan (Protozoan) parasites. Further, two exotic species of *Myxobolus* Butschli, 1882 from an Indian catfish *Clarias batrachus* have also been detected and identified with the help of present PCR technique which are reported here.

Keywords: Polymerase Chain Reaction, *Myxobolus*, *Clarias batrachus*, Exotic, Myxozoan.

1. Introduction
Aquatic animals trans-boundary movement always has an associated risk of transfer of pathogens along with the aquatic animals. The serious socio-economic, environmental and international trade consequences arising from these pathogens are quite evident [1, 2]. According to Sanitary and Phyto-sanitary (SPS) agreement of World Trade Organization, the import risks (of pathogens) should be proved through scientific evidence/s and uniform health control measures should be adopted globally which are consistent with international standards [3].

In India parasitic diseases are most prevalent among freshwater fishes (about 78%) and encountered more frequently than microbial diseases [4]. The protozoan and monogenean parasites are responsible for huge losses of eggs, fries and fingerlings of freshwater fishes [5, 7]. These parasites have enormous genetic plasticity and can easily alter their genetic makeup to develop resistance to the immune system, environment and chemicals aimed at eradicating them [8].

Since the discovery of "double helix structure of DNA" by Watson and Crick in 1953, no single event has had greater impact upon the field of molecular biology as the description of Polymerase Chain Reaction by Kary Mullis in early 1980s [9-12]. This elegant technology with its apparent simple theory revolutionized almost every aspect of classical molecular biology, especially the 'Molecular Diagnostics'. By using the Polymerase Chain Reaction (PCR) amplification of RNA/DNA, molecular diagnosis has developed into rapid, simple and yet sensitive assays for the detection of fish pathogens. The molecular techniques available today are being continuously refined in order to make them applicable to a diversity of pathogens and conditions, to increase their sensitivity and to reduce the time and steps required in the analytical process [13]. This refinement, standardization and validation of protocols is considered a very important requirement for the implementation of molecular techniques in the pathological, clinical or the environmental fields [14-18]. Present paper describes standardization

of polymerase chain reaction (PCR) technique for detection of myxozoan parasites and identification of two exotic species of *Myxobolus* Butschli, 1882 from an Indian freshwater fish *Clarias batrachus* Linnaeus, 1758.

2. Materials & Methods

The freshwater catfish *Clarias batrachus*, locally known as Desi Magur was collected from culture ponds around Lucknow, Unnao, Raebareli, Haidergarh, Barabanki, Sultanpur and Faizabad .Fish were brought to the Parasitology lab. Screening of total 145 fishes was done for isolation of protozoan parasites. All the internal organs and gills of *Clarias batrachus* were examined with the help of stereozoom and compound microscope for myxozoan infection. In 7 out of 145 fishes, minute white cysts of myxosporans were observed embedded in the brain and kidney. The cysts were removed and put on a slide to make smear. The myxosporans fresh squash preparations and methanol fixed smear slides were examined through a Nikon E600 microscope in various magnifications (with 20X, 40X, 60X and 100X objectives + immersion oil). Each cyst contained numerous spores of parasites. Spores in fresh wet mount were treated with 8% KOH solution for the extrusion of polar filaments. The parasites were identified through microscopy and morphometry. The *Myxobolus* species isolated from brain of *Clarias batrachus* is named '*Myxobolus* A' and from its kidney is called '*Myxobolus* B'.

For DNA extraction from the *Myxobolus* species, cysts were fixed in 85% ethanol. DNA was isolated through phenol: chloroform method [19]. The DNA size was checked by agarose gel electrophoresis and its concentration was measured through Nano-drop spectrophotometer. The optical density of DNA was 1.80nm to 1.90nm.

For polymerase chain reaction amplification of DNA, specific primers were selected from the 18 small subunit ribosomal DNA sequences through NCBI, designed with the help of software 'Primer 3' and were got synthesized by Sigma Aldrich. Primers were selected on the basis of GC content and variable temperatures. The selected primers for different species of family Myxobolidae are given in Table-I.

PCR amplification of myxozoan parasite's DNA was done with above specific primers using four different concentrations of each primer. PCR reaction mixtures concentrations were used as mentioned by Jayde and his co-workers [20] with modifications. For standardization of protocol different concentrations of reaction mixture and variable temperature gradients and number of annealing cycles were applied for the amplification program. Each amplification

Table 1: Selected Primers used to amplify species of family Myxobolidae

S. N.	Primer	Code/Sequences
1.	Myx1Forward Myx1Reverse	CTAATCCCGGTAACGAACGA CGTCCTCGCAACAAACTGTA
2.	Myx2Forward Myx2Reverse	TAATCCCGGTAACGAACGAG CGTCCTCGCAACAAACTGTA
3.	Myx3Forward Myx3Reverse	TCGGTTACGGGGAGAGTATG TCGTTCGTTACCGGGATTAG
4.	Mcer1Forward Mcer1Reverse	CCCGTCGCTACTACCGAGT GATCCTTCCGCAGGTTCAC
5.	Mcer2Forward Mcer2Reverse	AGACACTGGGAGGTGGTGAC CACTGCGTGATCCAACTACG

reaction was performed in a final volume of 50 µl using 1x PCR buffer, 0.2mM dNTPs, 1.5mM MgCl2, 2.0U taq polymerase, 0.25µM of each primer set and 2 µl of the DNA. PCR conditions were as follows: initial denaturation at 95 °C for 2 minutes, followed by 30 cycles at 94 °C for 1 minute, at 52°C for 30 seconds & at 72 °C for 2 minutes and final extension at 72 °C for 1 minute to amplify the product. The amplified product was used for electrophoresis separation and gel was stained with ethidium bromide. For sequencing and sequences analysis, Sanger sequencing method [21] and Basic Local alignment Sequence tool (BLAST) were used.

3. Results

The two *Myxobolus* species isolated from brain and kidney of *Clarias batrachus* were named '*Myxobolus* A' and '*Myxobolus* B' for standardization of the PCR technique. The positive control used in gel electrophoresis was *Myxobolus cerebralis* from Norway. The amplified PCR products size of both DNA samples A and B were of 200 base pairs (Fig.1).

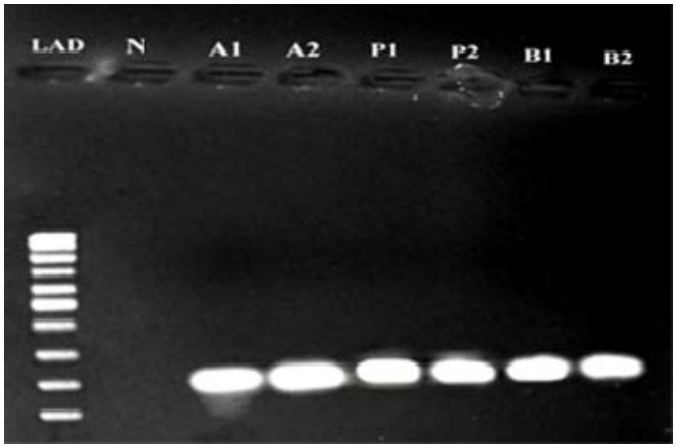

Fig 1: Lad -Ladder; N -Negative;

A1 - PCR product of *Myxobolus* sp. DNA sample 'A' at the annealing temp. 490C;
A2 - PCR product of the *Myxobolus sp.* DNA sample A at the annealing temp. 520C;
P1, P2 - Positive control of *Myxobolus cerebralis* at the annealing temp. 490C & 520C.
B1, B2 - PCR product of *Myxobolus sp*. DNA sample B at the annealing temp. 490C & 520C.

The amplified product of *Myxobolus* sp. sample A was showing 100% similarity with *M. cerebralis* 18s ribosomal RNA gene partial sequence and complete sequence (Accession no. EF370478.1, U96493.1, U96492.1, AF115254.1) and amplified product of *Myxobolus* sp. B was showing 99% similarity with *Myxobolus arcticus* from 18s ribosomal RNA gene, partial sequence (Accession no. JN003830.1), and *Myxobolus arcticus* from Canada 18S ribosomal RNA gene, partial sequence (Accession no. JN0038291.) Soon after confirmation of the species these sequence were submitted to the NCBI. The accession numbers assigned to the partial sequences of *M. cerebralis* and *M. arcticus* by NCBI are KJ701267 and KF662475 partial sequences of *M. cerebralis* and *M. arcticus* by NCBI are KJ701267 and KF662475

4. Discussion

Nucleic acid amplification techniques provide sensitive and rapid tools to study specific pathogens, including new emergent strains and indicators [13]. Present work is one small step towards achieving this goal. Prior to this, no work has been done for molecular detection of Fish protozoans in India although in other countries it started long back.

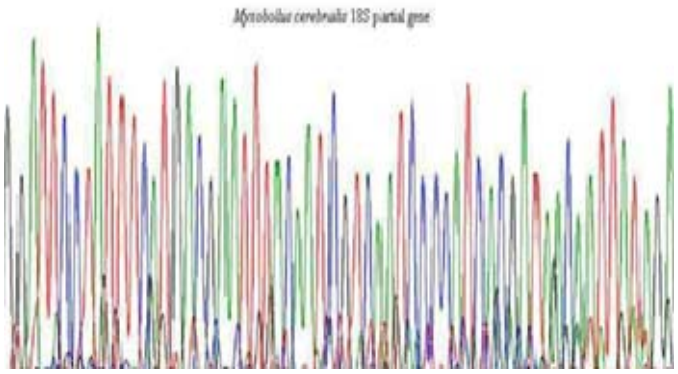

Fig 2: Partial Sequence of *Myxobolus cerebralis* 18S Gene

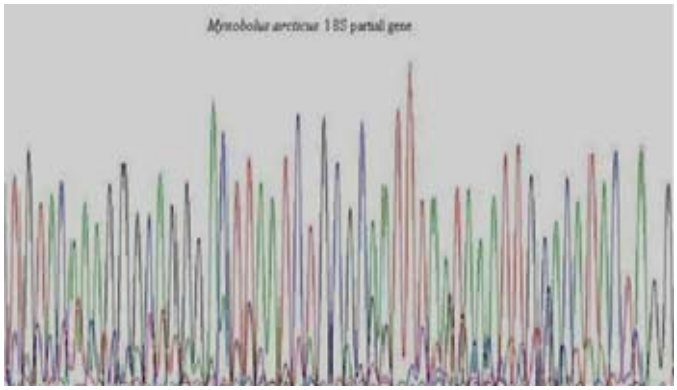

Fig 3: Partial Sequence of *Myxobolus arcticus* 18S Gene

Andree and his co- workers in 1997 [22] provided molecular evidence for the life cycle of *M. cerebralis* and revealed that *M. cerebralis* placed in two different taxonomic classes Myxosporea and Actinosporea are indeed forms of the same organism. Again in the year 1999, Andree with his research team compared selected geographic isolates of this parasite from Europe and North America through molecular study [23]. They used the 18S and ITS-1 ribosomal DNA sequences to identify and compare potential strain differences between two geographically isolated *M. cerebralis* species. The PCR-based procedures are increasingly being used in quality control [24]. These new methods can be utilized with improved sensitivity proposed by U.S. Department of Agriculture, USDA [25]. The validation studies for most of the pathogens molecular diagnostic assays are still required [26, 27]. Although present technique has not been validated in field but reproducibility of results in the laboratory has been ascertained.

Myxobolus cerebralis Hofer, 1898 is a fatal parasite of fish cartilage and bones of trouts in USA, Europe and USSR but its first record was from brain of rainbow trout in Germany reported by Hofer who named it *M. cerebralis* in the year 1898. Its occurrence in India has not been reported by any researcher previously. Present record is the first report of *M. cerebralis* in a new host and new place. The reason of *M. cerebralis* presence in native fish of India, may be legal and illegal entry of exotic fishes in India with whom the associated pathogens also get entered into our aquatic systems. With the help of PCR techniques, it is clear now that *M. cerebralis* responsible for salmonid whirling disease was introduced to the USA in 1958 as a new infection from Alaska [28]. Morris and his co-workers in 1999 developed and evaluated some nucleic acid based methods for identification and detection of genetic variations within same species & among various species of parasites [29]. The Japanese and Canadian *M. cerebralis* isolates' sequences of 4560 base pairs of ribosomal RNA gene, including small subunit (SSu) and internal transcribed spacer (ITS) regions, showed a high similarity of 99.9%, suggesting that they may be conspecific.

The *Myxobolus arcticus* Pugachev and Khokhlov, 1979 infects brain, nerves and spinal cord of salmonids found in North Pacific region of Far East Asia and North America. The principal fish host is sockeye salmon *Oncorhynchus nerka* in North America and masu salmon *O. masou* and Arctic char *Salvelinus alpinus* in Japan. *M. arcticus* presence in kidney of *C. batrachus* from river Gomti in U.P. is strange and new and may be due to trans boundary movement of fishes. *M. arcticus* may be geographically isolated due to the specific homing migration of the anadromous fish hosts and has specialized its morphology selection for its local environment in the ongoing process of differentiation, potentially leading to speciation [30].

5. Conclusion

The present technique is particularly developed for detection of myxozoan parasites. It will provide researchers a highly sensitive and specific assay for detection and quantification of myxozoans. This paper also includes the first reports of the occurrence of *M. cerebralis* and *M. arcticus* in India. Besides the host *Clarias batrachus* is also new in both cases and site of infection (kidney) is new in case of *M. arcticus.* Further work is going on to revalidate the results with larger size of DNA segments.

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