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Development and standardization of PCR technique for detection of ciliate parasite *Ichthyophthirius multifiliis* from freshwater exotic carp *Cyprinus carpio* and Indian major carp *Labeo rohita*

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ABSTRACT

Recent growth in aquaculture and fisheries has reshaped the methodology of treatment and diagnosis of diseases. Molecular techniques, mainly nucleic acid-based testing has become a crucial diagnostic tool for genetic disease and infectious processes. Their application in monitoring several pathogens related risks and identification of emerging pathogens is quite useful. Polymerase chain reaction (PCR) is an efficient and rapid method to increase a small quantity of DNA into ample quantity, for the detection of pathogens. The applications of these technologies are limitless and improving progressively. Validations of molecular tools provide essential underpinnings for a successful application in biological studies. Therefore, we have developed and standardized the PCR based assay for the detection of *Ichthyophthrius multifilliis* which is a fatal parasite of freshwater fishes, distributed worldwide. The present article reports some new sets of primers for the ciliate parasite *I. multifilliis*.

Introduction

In our country India, parasitic diseases are the most prevalent among freshwater fishes (about 78%) and encountered more frequently than microbial diseases (Abidi et.al 2015). Protozoan parasites are serious and fatal pathogens in comparison of metazoans. Different groups of protozoan parasites are present in freshwater fishes. Among these ciliates are known to be highly challenging as fish ecto-parasites that are more dangerous and common group which causes severe mortalities (Shalaby and Ibrahim, 1988). *Ichthyophthrius milltifiliis* is a very common ecto-parasite in freshwater environments worldwide. It is a fatal parasite to fish of all sizes and most of freshwater fishes are susceptible to *I. multifiliis*. (Wong and Leong, 1987). This is a ciliate possessing a large, reniform macronucleus and at least one small, round micronucleus. There are up to four micronuclei per theront, varying from one to four depending on temperature. Tomont is the free living reproductive stage whereas theront is the infective stage of the parasites The micronuclei of ciliates are transcriptionally inactive and play a role in genetic exchange. (Dickerson, 2006). A simple change in the genetic make-up of the parasite may lead its resistance to the immune system of the fish which cause virulence (Molnar and Székely 2003).

Since the early 90's, polymerase chain reaction (PCR) has been used in science for numerous applications. The observations of PCR analyses have been extensively explored and known to have been used for recreating the taxonomic tables, disease diagnostics and disease susceptibility (Hawk 2004). In parasitological studies, the use of PCR as a molecular biological method has been progressively refined and has increased its importance in several disease diagnostic studies. Additionally, the advancement in these techniques has improved its applicability to a diversity of pathogens and conditions with reduction in the time and steps required in the analytical process (Girones et.al. 2010). The modification, standardization and corroboration of protocols are considered as– an essential requirement for the implementation of molecular techniques in the biological and environmental arenas (Senet.al. 2007; Bustin 2010). The present study deals with the development and normalization of PCR technique for the detection of ciliate parasite *I. multifiliis* from freshwater exotic carp *Cyprinus carpio* and Indian major carp *Labeo rohita*.

Material and Methods

The freshwater exotic carp *Cyprinus carpio* and Indian major carp *Labeo rohita* locally known as Common carp and Rohu, respectively. Both of the fishes were collected from culture ponds in and around Lucknow, Unnao, and Barabanki. Screening of both fishes were done for the isolation of the ciliate parasite. Body surface, fins and gill lamellae of both fishes were examined carefully with the help of stereozoom and compound microscope for *Ichthyophthirius multifiliis* infestation. Total 104 *C. carpio* and 85 *L. rohita* were screened, out of which 2 numbers of each fish were infested with *I. multifilis*. In case of *C. carpio*, gills were infested, while among *L. rohita* both gills and fins were found infested with *I. multifilis*. Several tomonts were removed and kept on the slides with few drops of water and mounted with coverslip. The isolated parasites were examined under a Nikon E600 microscope at various magnifications (with 4X, 10X, 20X, 40X and 60X objectives) and further processed for permanent slide preparations and staining in Geimsa. The isolated parasites were identified through microscopy and morphometric analysis Morphometric analysis was done through the software NIS-E- Br.

I. multifiliis isolated from *C. carpio* is named as 'Ic1' and the one isolated from *L. rohita* is named as 'Ic2'. Numerous tomonts were isolated, numbered and preserved in 85% ethanol. DNA was extracted from the preserved parasite, using DNA easy Tissue Kit (Qiagen). DNA concentration was measured through Nano-drop 2000 spectrophotometer (Thermo Scientific, USA). Primers were designed for 18SSU rDNA gene through Primer3 and were synthesized by Sigma Aldrich. Primers were selected on the basis of GC content and different temperatures. The following primers were selected for *I. multifiliis*.

Table 1: Sequences of reverse and forward primers for 18SSU rDNA amplification

S.N.	Primer	Sequence
1.	Im1 Forward	GGTTCTGGGGGAAGTATGGT
	Im1 Reverse	GCAGGTTAAGGTCTCGTTCG
2.	Im2 Forward	GGGGATCAAAGACGATCAGA
	Im2 Reverse	AGGTTCCACTTCTGGTGTGC
3.	Im3 Forward	GGGGATCAAAGACGATCAGA
	Im3 Reverse	CTACGTGAGTTTCCCCGTGT
4.	ImR1Forward	AGTGACAAGAAATAGCAAGCCAGGAG
	Im1 Reverse	GCAGGTTAAGGTCTCGTTCG

The standard reaction volume was 50 µl (25 µl of master mix and 3 µl of DNA template (50ng- 100ng) and 40 pmol of each primer). The PCR master mix was comprised of 2X Taq buffer, 3.2 mM $MgCl_2$, 0.4 mM dNTPs, 1U Taq polymerase and 0.02% bromophenol blue (G-Biosciences). Amplifications were performed using Eppendorf thermal cycler (Germany). Parameters of PCR cycling consisted of an initial denaturation step of 94 °C for 5 min, followed by 35 cycles, denaturation at 94 °C for 15 sec, annealing at 58°C for 30 sec, elongation at 72 °C for 30 sec and finished with final extension at 72 °C for 7 min, then rested at 4 °C. The amplified PCR product was electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualized under UV trans-illuminator (Spectroline, USA). For the estimation of DNA size, 3 µl of standard ladder were used (100 bp DNA-Ladder, Promega). PCR product was sequenced in both directions with the same primers used for amplification, through the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1, using the ABI3730xl Genetic Analyzer (Applied Biosystems, Inc.). Homology search was performed using BLAST NCBI database on Genebank (Altschul et.al. 1997) for determination of the sequence.

Results

By using specific primer set ImR1F/ Im1R under optimized conditions, the size of the amplified product was estimated to be approximately 850 bp of *I. multifiliis* DNA (Fig. 1). No amplification products were observed from negative control containing no DNA. Two replicate of each *I. multifiliis* isolated from *C. carpio* 'Ic1' and from *L. rohita* 'Ic2' were used on gel with blank.

The sequences were analysed by BLAST and identity of parasites was confirmed as *I. multifiliis* which displayed 100% similarity with *I. multifiliis* (U17354) complete sequence in case of *C. carpio* but *I. multifiliis* from *L. rohita* showed 99% similarity with *I. multifiliis* (U17354) complete sequence. Both amplified and sequenced samples of *I. multifiliis* from different fishes, namely *C. carpio* and *L. rohita* were submitted to NCBI database on Genebank with 860 bp of *I. multifiliis* from *C. carpio* and 853 bp of *I. multifiliis* from *L. rohita*, respectively. The accession numbers assigned by NCBI were KM822612 for Ic1 and KM870913 for Ic2.

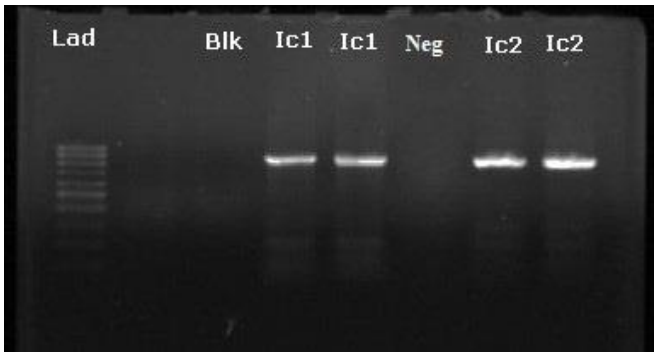

Fig. 1 Agarose gel electrophoresis of PCR products amplified

Discussion

From few past years, noteworthy advancement has been made in molecular techniques for the diagnosis of infectious diseases, taxonomic and systematic studies (Colombo et.al. 2005; Hillis 1987). Girones et.al. 2010, stated that molecular methods associated to nucleic acids are sensitive and rapid tools to study specific pathogens, including new emergent strains and indicators. Molecular methods including study of ribosomal RNA/DNA are useful to understand the relationship among the lower eukaryotes (Mayr and Ashlock, 1991). Field et al, 1988 reported that comparison of 18S ribosomal RNA introduced a provisional reclassification of the invertebrate phyla with significant result which led substantial modifications. A number of studies have proved the utility of PCR and a main tool for risk assessment. The molecular technique provides sequencing of nucleic acids, which has numerous information of the genetic material. The present work has been carried out in view of an effort to obtain a standard protocol in case of isolation of the ciliate parasite. Phylogenetic study showed the parasite of both the hosts are

closely related and has genetic similarity as *I. multifiliis*.

Chen and colleagues (2008) developed a PCR assay for direct, rapid and specific detection of a marine ciliate parasite *C. irritans* which poses a significant problem to marine aquaculture. They reported the importance of the PCR assays for the identification and detection of marine parasite as well as reliability of the tool for prevention and epidemiological investigation of the infection. Real-time PCR assay (using SYBR Green™ fluorescent dye) for rapid detection and quantification of *I. multifiliis* was developed by Jousson et.al. (2005), they designed non-invasive assay for the quantification of *I. multifiliis* and amplification of 18S rDNA gene of *I. multifiliis* with specific primers and related species of the ciliate order Hymenostomatida. In addition, they determined the time intervals of the concentration for highest theront stages for optimization of the treatment procedures. Moreover, the reliability and application of preliminary diagnostic test for adequate treatment to prevent serious outbreaks was also reported (Jousson et.al. 2005).

Conclusion

The present study demonstrates the efficacy of PCR assay for identification, detection and quantification of the ciliate parasite *I. multifiliis*. The present article also reports the first molecular study of freshwater ciliate parasite *I. multifiliis* in India. Additionally, further study is still continued for larger size of the DNA fragments and geographical variations in the strains.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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