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MOLECULAR PREVALENCE OF FISH BORNE ZOONOTIC TREMATODES IN THE SNAIL INTERMEDIATE HOST OF KOLKATA, WEST BENGAL, INDIA

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Abstract: A molecular approach was done to evaluate the prevalence of fish borne zoonotic trematodes in commonly available and consumed snail species of Kolkata, West Bengal. A total 70 fresh water snails of *Bellamya* spp. (*Bellamya bengalensis*) were collected from selected local markets located in and around Kolkata. Hepatopancreas were isolated in postmortem examination of snail tissues and genomic DNA was extracted from hepatopancreas of snails for molecular level identification. PCR amplification of genomic DNA was done using primers OPHET (470bp), ITS2 (380bp) and CS-ITS2 (380bp). Amplification of entire internal transcribed spacer regions of ribosomal DNA using primers conserved in many members of Opisthorchiidae and Heterophyidae confirmed that the parasite belongs to the above mentioned families. The prevalence of fish borne zoonotic trematodes belongs to Opisthorchiidae and Heterophyidae family in snail was found to be 31.43%, 35.71%, 37.14% using OPHET, ITS2 and CS-ITS2 primers, respectively. Snail harbouring such trematode is a cause of concern, as it acts as a 1st intermediate host.
Keywords: *Bellamya* spp., Hepatopancreas, Opisthorchiidae, Heterophyidae.

Introduction

Fish-borne zoonotic trematodes (FZTs) including liver and intestinal flukes are considered to be the most neglected tropical disease agents (WHO, 2011). Globally, they are considered to be a public health hazards, especially in Asian countries (WHO, 2004; Chai *et al*., 2005; Keiser & Utzinger, 2005) and are recognized as an important group of emerging and re-emerging zoonotic diseases especially to populations living in low and middle income group in different countries (Chai *et al*., 2005). The strong cultural preferences in many countries, particularly in Asia, for consumption of raw or insufficiently cooked fish containing metacercariae of the trematode parasite are believed to be the greatest risk factor for human infection (WHO, 2004; Chai *et al*., 2005; Kumchoo *et al*., 2003). Fish-eating animals *viz*. dog, cat and rodent can also be infected and can serve as reservoir host for spreading the horizontal transmission of these diseases (Le *et al*., 2006).

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Most of the trematode parasites have a complex life cycle with two intermediate hosts. First intermediate host is always snail which plays a very important role in development of life cycle stages *viz*. sporocyst, rediae, and cercariae. Fish may act as a second intermediate host in the life cycle of some trematodes. People use to acquire the fish borne zoonotic trematode infection by consumption of raw or undercooked freshwater or brackish water fish containing infective metacercariae (Chai *et al.,* 2005; Keiser & Utzinger, 2009; Lemly & Esch, 1983; Murrell & Fried, 2007). The presence of the snail, fish and mammalian hosts (including man) is essential for completion of parasite life cycle. These contextual determinants explain why the distribution of fish borne trematodosis is focal (Keiser & Utzinger, 2009).

For diagnosis of zoonotic trematode infection in definitive host *viz*. human, detection of egg in faecal sample through microscopical method is considered to be the gold standard test, but for detection of developmental stages of trematodes *viz.* metacercariae in fish and sporocyst, rediae in snail, molecular biological tests are considered to be more reliable than microscopical test. In recent years, many molecular approaches have been developed for specific detection of different parasites species. Specific DNA probes/primers have been developed for identification of metacercariae in fish (Parvatthi *et al.,* 2008). PCR targeting rDNA has been investigated to discriminate *Opisthorchis viverrini*, *Clonorchis sinensis, Haplorchis taichui,* and *H. pumilio* (Sato *et al.,* 2009), Cytochrome c oxidase I (COI) sequence marker has been introduced to separate *O. viverrini* and *H. taichui* (Thaenkham *et al*., 2007), ITS2 sequences to determine life cycle stages of Heterophyid trematode in Vietnam (Skov *et al*., 2009). Multiplex PCR has been successfully developed to discriminate *C. sinensis and O. viverrini* based on mitochondrial genes (Le *et al*., 2006).

The aim of the present study was to broadly discriminate the trematodes of Opisthorchiidae and Hetrophyidae from other fish borne trematodes in intermediate host (snail) by PCR amplification of rDNA. In the eastern region of India, fish consumption is very high but prevalence of these zoonotic trematodes is very less. It may be due to good cooking habits of people of this region. But these trematodes can complete their life cycle in the other reservoir hosts, so they remain in the environment for longer duration. So, it is better to screen the trematodes in the reservoir and intermediate hosts. So, in the study, we tried molecular approach to check the prevalence of trematodes in the commonly available and consumed snail species *Bellamya bengalensis* from markets located in and around Kolkata

Materials and Methods

Study area and Snail sampling: The snails of *Bellamya* spp. are commonly consumed by the peoples of Kolkata and are easily available in the local markets of Kolkata. A total 70 fresh water snails of *Bellamya* spp. (Viviparidae family) were collected from these markets. After sampling, snails were kept in the box, transported to the laboratory and kept alive in glass jar before processing.

Isolation of hepatopancreas: The processing of fresh water snail samples was done to isolate hepatopancreas from snail tissues. Hepatopancreas were isolated in post-mortem examination of snails as the intermediate stages; sporocyst and rediae of trematodes develop in the organ. Isolation was done by crushing method. Individual snails were washed properly and shell was crushed in the mortar pestle. Soft tissues were pulled out and observed for targeted organs. Hepatopancreas were isolated and stored in 70% ethanol at -20°C until DNA extraction.

Total genomic DNA extraction: Genomic DNA from hepatopancreas was extracted using DNeasy tissue kit (Qiagen, USA) according to the manufacturer's instructions. For extraction, hepatopancreas were triturated in the mortar after adding liquid nitrogen to facilitate triturition of tissues. Briefly, tissues were enzymatically digested by proteinase K and final elution was done in the 70ul of elution buffer. After extraction, genomic DNA was checked in 1% agarose gel electrophoresis and visualized in the gel documentation system. A 2µl of genomic DNA was loaded in the gel after proper mixing with 6X loading dye (Thermo Scientific) and gel was run at 70V for 1hr (Fig. 1).

Amplification of regions of ribosomal DNA: The entire second internal transcribed spacer regions were amplified using genomic DNA of hepatopancreas for identification of parasites belonging to the family Opisthorchiidae and Heterophyidae using published primers (OPHET-F: CTCGG CTCGT GTCGA TGA, R: GCATG CARTT CAGCG GGTA (Scov *et al.,* 2009); ITS2- F: CTTGA ACGCA CATTG CGGCC ATGGG, R: GCGGG TAATC ACGTC TGAGC CGAGG (Sato *et al.*, 2009); CS-ITS2 F: CTTGA ACGCA CATTG CGGCC, R: CACGT TTGAG CCGAG GTCAG (Traub *et al.,* 2009). The amplified PCR products were separated and analyzed by 1% agarose gel electrophoresis and visualized in the gel documentation system and photographs were recorded for further use.

Results

Optimization of PCR reaction and conditions: The PCR reaction was carried out in 25µl volume with 8pmol to 10 pmol of each primers either from Xcelris genomics (India), 1unit of

Taq DNA polymerase (Thermo Scientific, USA), 10X Taq buffer with KCl (Thermo Scientific, USA), dNTPs mix (10mM, Thermo Scientific, USA). For amplification using OPHET, ITS2 and CS-ITS2 primers, PCR conditions were 94°C×2m / 94°C×30s-52°C×30s-72°C ×2m (35cycles) / 72°C×7m, 94°C×2m / 94°C ×30s-59°C×30s-72°C×2m (35cycles) / 72°C × 7m and 95°C ×2 m / 94°C×30s-58°C×30s-72°C×2m (35cycles) / 72°C×7m, respectively. The PCR amplified products were analyzed by Agarose gel electrophoresis and visualized in gel documentation system (Fig. 2, 3, 4).

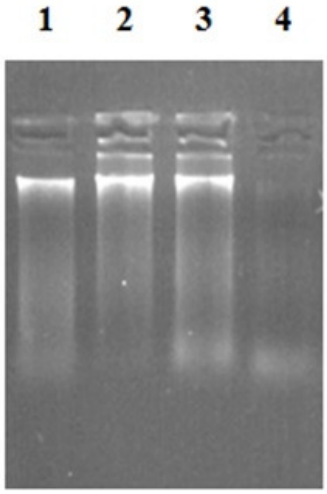

Figure 1. 1% Agarose gel electrophoresis of genomic DNA. Lane1-4: DNA samples of hepatopancreas

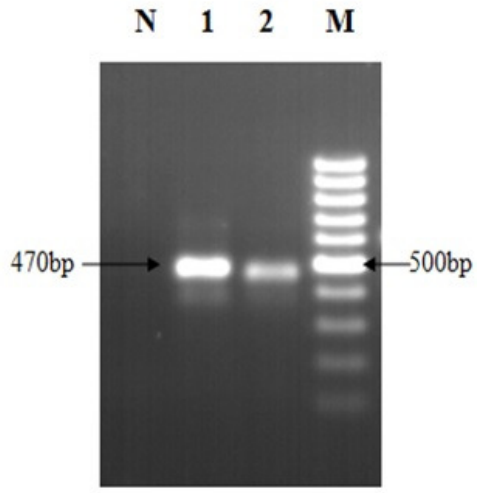

Figure 2. 1% Agarose gel electrophoresis of amplified products of OPHET primer. Lane M: DNA Marker (100bp DNA ladder, Thermo scientific). Lane1-2: DNA sample of hepatopancreas; N: Negative control.

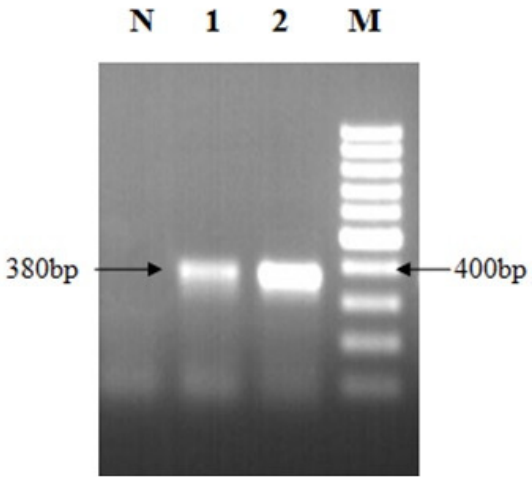

Figure 3. 1% Agarose gel electrophoresis of amplified products of OPHET primer. Lane M: DNA Marker (100bp DNA ladder, Thermo scientific). Lane1-2: DNA sample of hepatopancreas; N: Negative control.

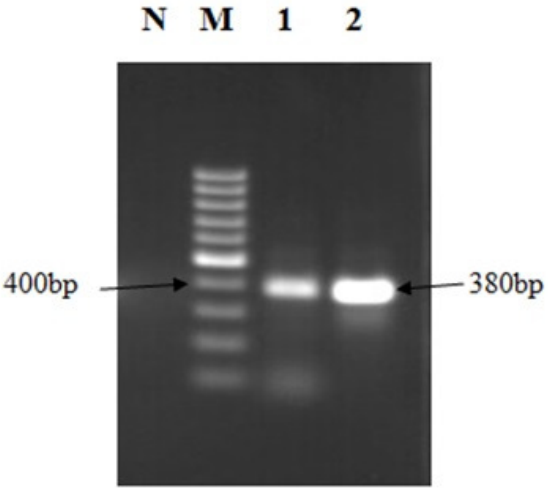

Figure 4. 1% Agarose gel electrophoresis of amplified products of OPHET primer. Lane M: DNA Marker (100bp DNA ladder, Thermo scientific). Lane1-2: DNA sample of hepatopancreas; N: Negative control.

Molecular identification of fish borne zoonotic trematodes (FZTs) of Opisthorchiidae and Heterophyidae family: The DNA samples extracted from hepatopancreas (n=70) were used for amplification of conserved regions of ITS2 of Opisthorchiidae and Heterophyidae using primers OPHET, ITS2 and CS-ITS2. We found the amplicons 470 bp, 380 bp and 380 bp from genomic DNA isolated from metacercariae and hepatopancreas using primers OPHET, ITS2 and CS-ITS2, respectively. These results were similar to the reports of Scov *et al*. (2009), Sato *et al*. (2009) and Traub *et al*. (2009). PCR products were purified using gel purification kit (Sigma-Aldrich, USA) and sent for sequencing and sequence analysis showed the maximum relationship with the trematodes of Opisthorchiidae and Heterophydae. Based on the amplification, prevalence of fish borne zoonotic trematodes belongs to Opisthorchiidae and Heterophyidae family in snail was found to be 31.43%, 35.71% and 37.14% using OPHET, ITS2 and CS-ITS2 primers, respectively.

Discussion

Fish borne zoonotic trematodoses remain a public health problem worldwide and are recognized as emerging zoonotic disease affecting millions of people. The disease is mostly prevalent in the countries where fish consumption is high especially in south East Asian countries. In the eastern region of India *viz.* Kolkata and surrounding regions, the fish consumption is very high and for completion of life cycle of these trematodes. The street animals are also getting the infection by consuming the residue of fishes available normally in and around the fish markets and these animals can act as a reservoir hosts for FZTs and spreading infection in the environment without involvement of human host (Chai *et al*., 2005).

In this study, we used snail species which are commonly available in the markets and frequently consumed by the peoples of Kolkata, West Bengal. For isolation of cercariae, normally shedding method is done but isolation of cercariae from snail by shedding method is not always give true prevalence because the artificial environmental conditions in laboratory are not always congenial for shedding so, we isolated hepatopancreas from snail tissues as larval stages; sporocyst and redia, develop in these organs.

For further identification of larval stages (sporocyst, rediae and cercariae) by molecular methods, genomic DNA of hepatopancreas were used for amplification of entire segment of ITS2 regions which are conserved for the trematode belonging to family Opisthorchiidae and Heterophyidae. A number of PCR-based molecular approaches have been developed to identify several trematode species in every life stages forms. Sato *et al*. (2009) identified O.

viverrini, *C. sinensis*, *H. taichui* and *H. pumilio* from eggs, adults as well as metacercariae by using ITS2 primer amplifying the ITS2 segment of rDNA. Similarly, Scov *et al*. (2009) successfully amplified the ITS2 region of rDNA belonging to the family Opisthorchiidae and Heterophyidae using OPHET primer and Traub *et al*. (2009) developed the PCR test capable of amplifying the ITS2 segment of rDNA belonging to the family Opisthorchiidae and Heterophyidae using CS-ITS2 primer.

For this study, three published primer sets were selected for identification and evaluation of their sensitivity in terms of detection of parasites of these two families (Scov *et al.,* 2009; Sato *et al*., 2009; Traub *et al.,* 2009). From the results, it was confirmed that larval stages of Heterophyidae and Opisthorchiidae in the intermediate host (snail) were belonging to the Opisthorchiidae and Heterophyidae. It was also confirmed that primer set CS-ITS2 is more sensitive for detection as compared to other two primers (OPHET and ITS2).

As these trematodes are highly zoonotic, a systematic study will be required using experimental infection through metacecariae and demonstration of eggs of trematodes in faecal material and subsequently demonstration of adult parasite in experimental animal. The study provides beneficial applications for demonstrating the epidemiology and detection of larval stages like sporocyst and rediae in snail by molecular means.

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