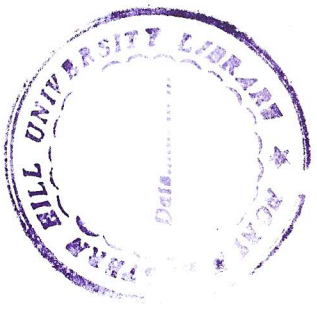
STUDIES ON THE STATUS AND MOLECULAR CHARACTERIZATION OF PARAGONIMIASIS AND OTHER CRUSTACEA-BORNE TREMATODE ZOONOSES IN NORTHEAST INDIA

ABSTRACT



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ABSTRACT

The present work incorporates a study on the status and molecular characterization of paragonimiasis and other Crustacea-borne trematode zoonoses in Northeast India. The study aimed at examining the snail and crustacean species (the first and second intermediate hosts of the causative agent of infection) prevalent in Northeast India to recover the larval stages, if any, of the lung fluke *Paragonimus* and to identify the exact species prevailing and responsible for the disease. By using PCR-based molecular techniques the study aimed to identify the species of the parasite collected and to generate specific molecular markers with vital usage towards correct diagnostics.

- **Status and prevalence of paragonimiasis in the region**

  - The crab hosts were surveyed from rural localities and countryside where eating of crabs is a common food practice among the natives of the region. Emphasis was given to procure those species which are commonly used in local traditional cuisine. Naturally infected freshwater crabs were mostly collected from mountain streams of the suspected foci of infection. Metacercariae were isolated from the muscles of the crustacean host by artificial gastric juice digestion technique. Of the 3 genera of crabs surveyed from various localities in Arunachal Pradesh, Assam, Manipur and Mizoram, only one, i.e., *Barytelphusa* was found to be harboring metacercarial cysts. The crabs collected from all the localities excepting those in Arunachal Pradesh, did not harbor any metacercarial infection; both the collection sites in Arunachal Pradesh were revealed to be positive for this infection. In the Kharshang site *Barytelphusa* (*M*) *lugubris lugubris* was found to be positive with a prevalence of 26%, the intensity of infection being in the range of 9-68 (mean intensity=36). In Miao region, the other sub species namely *B.* (*M.*) *l. mansoniana* revealed a much higher prevalence (87%) and intensity of metacercarial infection (ranging between 3-175, mean intensity=38).

- Snail species were also collected from selected localities wherever the crab hosts were suspected to be positive for metacercarial infections. Hundreds of specimens of 4 snail species (representing 4 genera with one species each) were examined for the recovery of intramolluscan stages, if any, of *Paragonimus*. Of the snail species surveyed, only one species viz. *Brotia costula* was found harbouring the sporocyst, redia and cercaria larval stages.

- Freshly recovered metacercariae from crab hosts and intramolluscan larval stages- sporocyst, redia and cercariae, were processed using standard protocols for light and electron microscopy. The newly excysted metacercaria has an elongate body (815.91μm x 492.79μm) in size; the ventral sucker situated somewhat pre-equitorally, is larger than the oral; the intestinal caeca are long and extend up to the posterior end of the body; the conspicuous excretory bladder extends medially in the intercaecal space. The SEM observations revealed that the encysted metacercaria is oval in shape and has smooth surface. The whole body surface of the excysted metacercaria is covered with numerous single-pointed and thorn-like tegumentary spines; those covering the anterior part of the body are bigger in size and show a gradual reduction in size towards the posterior end. On the basis of morphology and surface fine topography features of the excysted larval stage, the metacercariae were identified as those of *Paragonimus* species.

The cercariae recovered from the snail hosts were always of amphistome, leptocercous type. Microcercous type of cercariae (i.e., having a small stumpy tail) that are typically characteristic of *Paragonimus* spp were never encountered in the collections made during the present study. The larval stages recovered, not being representative of *Paragonimus,* were, therefore, not processed for further study towards molecular characterization.

- **Molecular characterization of *Paragonimus* and other trematodes**

  - The identification of closely related species based on morphological characters can be difficult, particularly so in the case of soft-bodied digenean trematodes. For most of the trematode-borne zoonoses the parasite eggs voided in exudates of the host are the only stages available for diagnostic purpose. Besides *Paragonimus,* infection of *Fasciola gigantica* and *Fasciolopsis buski,* both of which are putatively zoonotic species, have been reported in the northeastern region. The main objective of the present study was to provide molecular characterization of the parasite so as to supplement morphological criteria and develop species-specific molecular markers.

For the purpose of molecular characterization metacercariae of *Paragonimus* sp collected from the crab host *Barytelphusa lugubris* from Miao region in Arunachal Pradesh were used; live adult *F. buski* and *F. gigantica* were obtained from bovine hosts at local abattoirs. Eggs were obtained from mature adult flukes by squeezing between two glass slides; eggs recovered from each single specimen were also processed separately. DNA was extracted from metacercaria and eggs in Whatman's FTA card and from lysed individual adult worms by standard ethanol precipitation technique. The rDNA region spanning the ITS1 and ITS2 was amplified from the metacercarial, egg, larval and/or adult DNA by PCR. The primers generally used were designed based on the conserved sequences of *Schistosoma* spp, which are considered to be the universal primers for trematode species. The PCR amplification was performed following the standard protocol with minor modifications. For DNA sequencing, the PCR products were purified using Genei Quick PCR purification Kit, and sequenced in both directions using PCR primers on an automated sequencer by DNA sequencing services of Bangalore Genei, Bangalore and The Centre for Genomic Applications (TCGA), New Delhi, India and submitted to GenBank. No intra-specific variations in length or composition of the sequence were

observed, and the ITS sequences of egg, metacercaria and adult origin were found to be identical in length as well as composition. Sequence analysis was carried out using various bioinformatics tool e.g., BLAST, ClustalW, MEGA, mFOLD, Bayesian analysis phylogeny etc.

- ***Paragonimus* sp.:** The PCR amplified products of ITS2 of rDNA were successfully obtained and were compared with other sequences of trematode species from Genbank. The fragments of amplified DNA were estimated to be ~500bp long. Sequence analysis of the ITS2 PCR products revealed that the alignments of the rDNA region spanning the ITS2 were 496bp for forward primer and 494bp for reverse primer, respectively. The actual length of ITS2 was estimated to be 287bp. The Blast hit results showed that the query ITS2 *Paragonimus* metacercariae sequence is more similar to the sequence of the species *Paragonimus westerrmani*, *Paragonimus mexicanus*, *Paragonimus siamensis*, *Paragonimus sismensis*, *Paragonimus miyazakii*, *Euparagonimus cenocopiosus*. Comparing with the known sequences of the other lung fluke species, the present study revealed that the sequence of ITS2 (plus flanking regions) shows close resemblance with *Paragonimus westermani*, the expectation value (e-value) being most significant revealing absolute match. Phylogenetic analyses using the various distance methods and character state method like Maximum Parsimony show that the topology is similar among the trees obtained. A bootstrap value of > 70% among the trees obtained. Sequence analysis of ITS2 region of rDNA of metacercariae isolates of Miao showed that the species prevailing in the said location is *Paragonimus westermani* and not *P. heterotremus* as reported by earlier workers.

Several patterns of predicted secondary structures of RNA were constructed from unique ITS sequences from different geographical isolates of *Paragonimus*, so as to provide additional information for correct identification of the species prevalent in the region. The secondary structure analysis of the

same data also confirmed the results mentioned for primary sequence analysis. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Three similar topologies for seven species of the genus *Paragonimus* were obtained on the basis of traditional primary sequence analyses using MEGA and a Bayesian analysis of the combined data. The latter approach allowed to include both primary sequence and RNA molecular morphometrics; each data partition was allowed to have a different evolution rate. *Paragonimus westermani* was found to group with *P. siamensis* of Thailand; this was best supported by both the molecular morphometrics and combined analyses. *P. heterotremus*, *P. proliferus*, *P. skrjabini*, *P. bangkokensis* and *P. harinasutai* formed a separate clade in the molecular phylogenies, and were reciprocally monophyletic with respect to other species. The observed similarities at the secondary structural level are further reflected at the energy level. Only difference in their topology is due to differences in nucleotide sequences. These secondary structure predictions indicate that the domains basepair to form a core region central to several stem features implying that conservedness is more important for the proper rRNA folding pattern. Moreover the observed phylogenetic trend was identified with respect to the target accessibility sites for different isolates. The orders of preference were interior loop, bulge loop, multiple branch loop, hairpin loop and exterior loop in all the isolates.

- ***Fasciolopsis buski*:** With regard to *F. buski*, for which only 18S rDNA sequences were available so far, ITS regions were sequenced for the first time in the present study. The nucleotide sequences obtained for ITS1 & ITS2 of rDNA (of both adult and egg origin), were compared with sequences of other trematode species obtained from Genbank. The fragments of amplified DNA were estimated to be 480-550bp long. Sequence analysis of the ITS PCR products revealed that the alignments of the rDNA region spanning ITS2 were 481bp and 498bp; 559bp and 548bp for ITS1, forward and reverse sequences,

respectively in adult and egg. The Blast hit results showed that the query ITS2 *Fasciolopsis buski* sequence is more similar to the sequence of the species *Fasciola gigantica, Echinostoma revolutum, Isthmiophora melis, Echinostoma sp, Paryphostomum radiatum, Echinostoma trivolvis, Echinostoma paraenei, Fasciola sp, Fasciola hepatica* and *Isthmiophora hortensis.* Phylogenetic trees were obtained by comparing the sequences of *F. buski* and available ITS (1&2) sequences for other digenean trematodes including fasciolid species. Boot strapping of the sequences with Neighbour-Joining revealed significant support (100%) for the clade containing *F. buski*, *F. hepatica*, *F. gigantica* and *Fascioloides magna* indicating reliable grouping among different members of fasciolids.

- ***Fasciola gigantica*:** The PCR-amplified products were successfully obtained and were compared with sequences of other fasciolid species. The fragments of amplified DNA were estimated to be 480-550bp long. For comparative purpose, the ITS2 sequences of fasciolids from various geographical regions were obtained from GenBank. The Blast hit results showed that the query ITS sequences were more similar to the sequences of various geographical isolates of *Fasciola* sp., *F. hepatica* and *F. gigantica* besides *Fasciolopsis buski* and *Fascioloides magna* (both belonging to the same family, i.e., Fasciolidae). Primary sequence analysis of *Fasciola* spp, revealed a close relationship between the query sequence (from NE India) and isolates of *F. gigantica* from China, Indonesia, Japan, Egypt and Zambia. ITS2 sequence of the Indian isolate revealed closest similarity with isolate from China with significant bootstrap value revealing that the species prevailing in the region is *Fasciola gigantica.* Sequence of another Indian isolate, designated as *F. gigantica* (Accession-EF198867) from IVRI, Bareilly, showed absolute match with *F. hepatica.* Hence on the basis of molecular similarity this isolate should be identified as *F. hepatica* and not *F. gigantica.*

Secondary structure analysis of data confirmed the results mentioned for primary sequence analysis. Five predicted RNA secondary structures were reconstructed from the unique sequences with highest negative free energy of *F. gigantica* to provide the basic information for phylogenetic analysis. The ITS2 plus flanking regions of nuclear region ranged from 720bp in *F. gigantica* India to a minimum length of 361bp in *F. gigantica* China. *F. gigantica* isolates from India and China show overall similarity in the ITS2 rRNA folding and have identical secondary structure. Secondary structures of remaining species are somewhat variant. The topology based only on the predicted RNA secondary structure of the ITS2 region resolved most relationships among the species studied. Bayesian analysis of the alignment retained the same topology and supported the same branches as the primary sequences.

- **Design of genus/species-specific primers**
  - To establish a more direct PCR procedure for species discrimination and identification, the genus/species-specific primers were designed using Primer3, a widely used program for designing PCR primers to target unique rDNA region spanning ITS2 for all the three trematodes viz. *Paragonimus westermani, Fasciolopsis buski* and *Fasciola gigantica.* Sequence analysis of the ITS2 PCR products revealed no stage-specific or intra-specific variations in length or composition. Multiple sequence alignment was done for all the three sequences using ClustalW programme. The *P. westermani*-specific (PwAR1), *F. buski*-specific (FbMR1) and *F. gigantica*-specific (FgMR1) primers were designed to target the 3'- terminal position of the ITS2 sequences, and the specificity of these primers was evaluated by PCR using primer 3S. As was expected, the primer set 3S-PwAR1 amplified a PCR product only from *P. westermani* DNA, 3S-FbMR1 amplified a PCR product only from *F. buski* DNA and 3S-FgMR1 amplified a PCR product only from *F. gigantica*. Primer set 3S-A28 was used as control for the presence of parasite genomic DNA in each sample. These PCR

products were sequenced using the corresponding specific primer and were confirmed to be the ITS2 region of rDNA from the respective species.

Sequences deposited in GenBank

i) DQ351841- *Fasciolopsis buski* adult 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ii) DQ351842- *Fasciolopsis buski* egg 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

iii) DQ351843- *Fasciolopsis buski* adult internal transcribed spacer 1, partial sequence.

iv) DQ351844- *Fasciolopsis buski* egg internal transcribed spacer 1, partial sequence.

v) DQ351845- *Paragonimus westermani* metacercariae 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

vi) EF027103- *Fasciola gigantica* 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

vii) EF027104- *Fasciola gigantica* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

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