MORPHOLOGICAL AND MOLECULAR CHARACTERIZATIONS OF *PARAGONIMUS HETEROTREMUS*, THE CAUSATIVE AGENT OF HUMAN PARAGONIMIASIS IN INDIA

T Shantikumar Singh[1], Hiromu Sugiyama[2], Achariya Rangsiruji[3] and K Ranjana Devi[4]

[1]Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Sikkim, India; [2]Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; [3]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok Thailand; Department of Microbiology, Regional Institute of Medical Sciences, Imphal, Manipur, India

Abstract. In order to identify the causative species of human paragonimiasis, we performed a combined morphological and molecular investigation on the metacercariae and *Paragonimus* eggs isolated from the freshwater crab host, *Potamiscus manipurensis*, and sputum specimens of a patient, respectively. Experimental infection of laboratory animals with the metacercariae resulted in the isolation of adult worms that were morphologically identified as *P. heterotremus*. Molecular characterization based on polymerase chain reaction and DNA sequencing of the metacercariae and *Paragonimus* eggs from the sputum specimens yielded identical ITS2 sequences. Results of phylogenetic analyses of the ITS2 region suggested that Indian *P. heterotremus* is nested within the *P. heterotremus* clade; the Indian population is less closely related to other members within the clade.

INTRODUCTION

Paragonimus species hitherto reported in Asia number 17, of which *P. westermani* is the most common cause of human paragonimiasis (Miyazaki, 1974). *Paragonimus heterotremus* was first described in rats in Guangxi, China (Chen and Hsia, 1964). The first human paragonimiasis due to *P. heterotremus* in the world was reported by Miyazaki and Harinasuta (1964). This species is considered medically more important than other species in Thailand, Lao PDR, Vietnam, and some parts of China where man and mammals serve as naturally infected final hosts (Miyazaki and Harinasuta, 1964; Doanh *et al*, 2005). In Manipur in India, a recently recognized endemic area, *P. westermani* was presumed to be the etiological agent of human paragonimiasis (Singh *et al*, 1982;1993). However, no scientific study supported this speculation nor was able to determine which lung fluke species occurred in Manipur until recently. A joint Indo-Japan research on *Paragonimus* and paragonimiasis in Manipur resulted in the identification of *Potamiscus manipurensis*, a freshwater crab species, as the second intermediate host of at least three *Paragonimus* species, including *P. heterotremus*.

In this study, further investigation on the determination of etiological agents of human paragonimiasis was performed by nucleotide sequencing of the ITS2 region on *Paragonimus* (Sugiyama *et al*, 2002). The study also aimed to determine the phylogenetic relationships of the Indian species with other *Paragonimus* found in various geographical areas in Asia.

Corresponence: T Shantikumas Singh, Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Sikkim, India.
E-mail: shantikumar_singh@rediffmail.com

MATERIALS AND METHODS

Parasite material

Metacercariae harvested from freshwater crab host, *Potamiscus manipurensis*, which were collected from Luwangsangbam Matai in Imphal East and Motbung in Senapati Districts both in Manipur State were used for morphological study, laboratory animal infections, and molecular study. Adult worms as well as immature worms that were recovered from the experimentally infected puppies and albino rats were used for morphological identification. *Paragonimus* eggs were collected from sputum specimens of a patient in Senapati District. All materials, metacercariae, adult worms, and eggs were preserved in equal

proportions in 70% ethanol and 10% formalin until utilized. Morphological features of both fresh and preserved metacercariae and borax-carmine-stained worms were examined under microscope.

DNA isolation, amplification and sequencing

DNA samples were prepared from individual metacercariae and eggs. The ITS2 region of the nuclear ribosomal DNA was amplified by PCR and sequenced as described previously (Sugiyama *et al*, 2002). The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward: Bowels *et al*, 1995) and A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (reverse: Blair *et al*, 1997).

Sequence and phylogenetic analyses

The Indian *Paragonimus* ITS2 sequences were aligned with other *Paragonimus* sequences obtained from the GenBank database and an outgroup (*Fasciola hepatica*; Table 1), using the Clustal X program (Jeanmougin *et al*, 1998). Maximum parsimony analysis was conducted with the branch-and-bound algorithm using PAUP* (version 4.0b) (Swofford, 1998). The robustness of tree(s) inferred from the analysis was evaluated using bootstrap analyses with heuristic searching (Felsenstein, 1985).

RESULTS

Characteristics of metacercariae, eggs, and adult worms

The metacercariae (Fig 1) were oval to suboval in shape. The inner cyst measured 163 to 215 µm (av = 196 µm) in the long axis and 133 to 188 µm (av = 162 µm) in the transverse axis. The thickness of the inner wall was 4.2 to 10.4 µm (av = 6.3 µm) on the side and 10.4 to 27.1 µm (av = 18.2 µm) at the pole. The oral sucker, provided with a stylet, was smaller than the ventral sucker.

Paragonimus eggs (Fig 2), golden-yellow in color, oval shaped, and operculated, measured 89-100 µm (av = 92 µm) in length and 47-58 µm (av = 50 µm) in width. The eggshell thickness was almost uniform in 22 (63%) and discernible at the nonoperculated end in 13 (37%). The

Table 1
GenBank accession numbers of *Paragonimus* species and *Fasciola hepatica*.

Species	Origin	Accession No.
P. heterotremus	Thailand	AF159603
P. heterotremus	China	AY618758
P. heterotremus	India	AB308377, AB308378
P. skrjabini	China	AY618752
P. miyazakii	China	AY618741
P. westermani	Thailand	AF159604
Fasciola hepatica	Australia	AB207148

Fig 1- *P. heterotremus* metacercariae: average longitudinal diameter 196 µm and average transverse diameter 162 µm.

Fig 2- Morphological characteristics of eggs discharged from a patient. Size of eggs: av length = 92 µm, av width = 50 µm.

widest transverse diameter was located at the middle 28 (80%), at operculated half 6 (17%), and at nonoperculated half 1(3%).

The borax-carmine-stained worms (Fig 3) that were recovered from the experimentally infected puppies showed singly spaced cuticular spines, oral suckers (385-500 µm) that were much larger than the ventral suckers (260-300 µm), and the ovaries and testes that were delicately branched. The vitellaria were not seen in immature worms. The morphological features of metacercariae, eggs, and worms conform to the features of *P. heterotremus*.

Fig 3- *P. heterotremus* adult worm recovered from the experimentally infected puppies showed delicately branched ovary and testes and the oral sucker was much larger than the ventral sucker.

Sequence and phylogenetic analyses

Molecular characterization, which is based on PCR and DNA sequencing of the metacercariae (accession No. AB308377) and eggs (AB308378), yielded identical ITS2 sequences. The alignment of the ITS2 region of six taxa of *Paragonimus* and its outgroup was 378 bp in length. Twenty-four characters (6.3%) were phylogenetically informative. A single most parsimonious tree (Fig 4), with a length of 144 steps, was obtained from a maximum parsimony analysis of the informative characters with 1,000 bootstrap (BS) replicates. Fit measures of the tree were as follows: consistency index (CI) = 0.951, retention index (RI) = 0.811, and rescaled consistency index (RC) = 0.771. The phylogenetic tree revealed that Indian *P. heterotremus* is nested within *P. heterotremus* clade (BS = 99%), which includes *P. heterotremus* from Thailand and China. The Indian population is however, less closely related to other members of the clade.

DISCUSSION

Although India is the first country from whence *P. westermani* was first described by Kerbert in 1878, from a Bengal tiger, very little attention has been given to this parasite because human paragonimiasis was never considered a public health problem. In India, there was no record of an autochthonous human case of paragonimiasis, although *P. westermani* infection was described in many mammals.

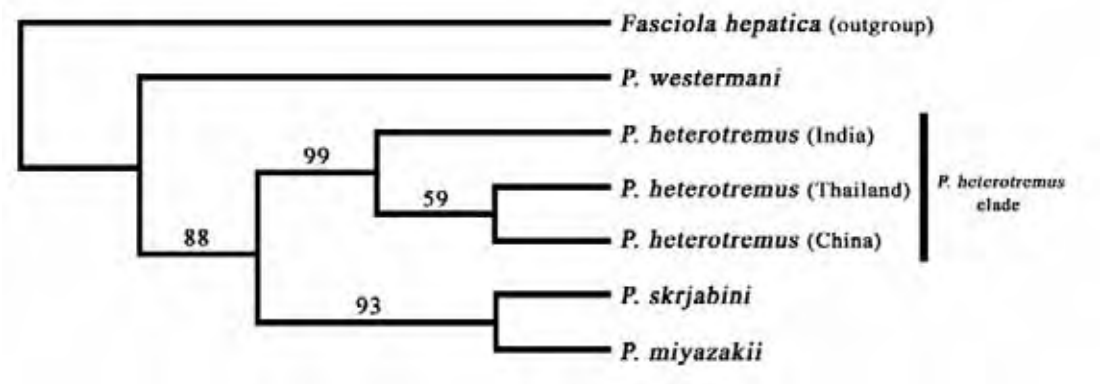

Fig 4- Single most parsimonious tree with a length of length 144 steps, based on parsimony analysis of the informative characters of the ITS2 region. Fit measures of the tree: CI = 0.951, RI = 0.811, RC = 0.771. Numbers above the branches indicate bootstrap values (%).

Evidence of infection with lung flukes of the genus *Paragonimus* in wild mammals has often been reported in India (Gaur *et al*, 1980; Rao, 1935; Srivastava, 1938; Singh and Somvanshi, 1978; Parihar and Shrivastava, 1988; Sano *et al*, 1994). The authors described *P. westermani* as the causative agent, based on the morphology of the eggs in the fecal specimens only or sections of worms and worm cysts in the lungs obtained on autopsy or postmortem examination of the animals. In the absence of detailed morphological descriptions of the adult worms, it was not possible to identify the species by examination of histopathological sections of the worm or worm cyst in the tissue and eggs in the feces. *P. westermani* was also reported to be the causative agent of human paragonimiasis in Manipur, based on the morphology of the eggs seen on microscopy examination of the sputum specimens of the patients (Singh *et al*, 1982). Therefore, doubts prevailed as to whether or not *P. westermani* was actually the only species infecting mammals and humans in India. Singh and Vashum (1994) first described the *P. heterotremus* adult worm from the biopsy specimen of a subcutaneous nodule in a 10-year-old boy in Imphal, Manipur. No other information on the *Paragonimus* species causing human paragonimiasis has been available in India.

The occurrence of *P. heterotremus* in freshwater crab, *Barytelphusa lugubris*, in an endemic area of paragonimiasis in Arunachal Pradesh was reported by Narain *et al* (2003). However, the morphological features of the metacercariae and adult worms, as described by these authors require further confirmation. In addition, it may not be safe to assume that this species is the causative agent of human paragonimiasis without morphological and molecular characterization of the parasite material recovered from the patient.

Recently, molecular analysis of any one of the developmental stages of the parasite has proved to be highly sensitive, and specific techniques are required to confirm the parasite species and its relationship with other species occurring elsewhere in the world. Technique is of importance in the identification of *Paragonimus* species, which can be made from the eggs in clinical specimens. Adult worms are rarely recovered from the patient, and hence not available for morphological identification and molecular characterization. The results of the present study confirmed that *P. heterotremus* was the causative agent of human paragonimiasis in Manipur, India.

Phylogenetic analysis indicated that all *P. heterotremus* species that originate from Vietnam, Thailand, and China form a distinct group (Le *et al*, 2006). However, our study revealed that the Indian species, although situated within the *P. heterotremus* group, is distantly related to the Chinese and Thai species.

This species has been identified as significant cause of human paragonimiasis in Southeast Asia, and endemic in South/Southwest China, Thailand, Lao PDR, and Vietnam (Blair *et al*, 1997; De *et al*, 2000; Doanh *et al*, 2005; Waikagul and Yoonuan, 2005). Morphometric and molecular characterization of the *Paragonimus* species are important for epidemiological, ecological, and taxonomic studies. This knowledge will also help in the control and treatment of paragonimiasis.

Potamiscus manipurensis, the natural second intermediate crustacean host of *P. heterotremus*, was found to contain metacercariae of *P. skrjabini* (Singh *et al*, 2006), and possibly two more species as well. The metacercariae of *P. skrjabini* were most frequently isolated from the freshwater crabs in some localities in Manipur State, where patients of pulmonary as well as cutaneous paragonimiasis have been reported. The possible relationship of *P. skrjabini* with human paragonimiasis in these localities is now under investigation.

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