

Pyrosequencing for rapid molecular identification of *Schistosoma japonicum* and *S. mekongi* eggs and cercariae



Tongjit Thanchomnang^{a,b}, Chairat Tantrawatpan^{a,c}, Pewpan M. Intapan^{a,d,*}, Pusadee Sri-Aroon^e, Yanin Limpanont^e, Viraphong Lulitanond^{a,f}, Penchom Janwan^{a,d}, Oranuch Sanpool^{a,d}, Somjintana Tourtip^b, Wanchai Maleewong^{a,d}

^a Research and Diagnostic Center for Emerging Infectious Diseases, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

^b Faculty of Medicine, Mahasarakham University, Mahasarakham 44000, Thailand

^c Division of Cell Biology, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Rangsit Campus, Pathum Thani 12121, Thailand

^d Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

^e Applied Malacology Center, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand

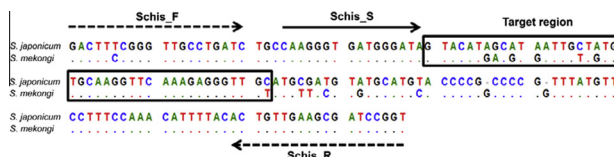
^f Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

HIGHLIGHTS

- Pyrosequencing assay coupled with PCR differential detecting *Schistosoma* spp.
- Method yields rapid, accurate and high throughput of species-level identification.
- Differentiation between *S. japonicum* and *S. mekongi* eggs and/or cercariae was found.
- Providing an exciting alternative tool for molecular surveillance of *Schistosoma*.
- Important implications for epidemiological studies of human Asian blood flukes.

GRAPHICAL ABSTRACT

Alignment of the 18S ribosomal RNA gene derived from *S. japonicum* and *S. mekongi*. The dotted arrows indicate the position of Schis_F (forward primer) and biotinylated Schis_R (reverse primer) for amplification. A solid arrow indicates Schis_S (sequencing primer), and a rectangular box shows the position of the target region used for species level identification. Dots indicate identical nucleotides between lines.



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ABSTRACT

Schistosomiasis, which is caused by *Schistosoma japonicum* and *S. mekongi*, is a chronic and dangerous widespread disease affecting several countries in Asia. Differentiation between *S. japonicum* and *S. mekongi* eggs and/or cercariae via microscopic examination is difficult due to morphological similarities. It is important to identify these etiological agents isolated from animals and humans at the species or genotype level. In this study, a pyrosequencing assay designed to detect *S. japonicum* and *S. mekongi* DNA in fecal samples and infected snails was developed and evaluated as an alternative tool to diagnose schistosomiasis. New primers targeting the 18S ribosomal RNA gene were designated for specific amplification. *S. japonicum* and *S. mekongi* were identified using a 43-nucleotide pattern of the 18S ribosomal RNA gene and were differentiated using 7 nucleotides within this region. *S. japonicum* and *S. mekongi*-infected snails and fecal samples derived from infected mice and rats were differentially detected within a short period of time. The analytical sensitivity of the method enabled the identification of as little as a single cercaria artificially introduced into a pool of 10 non-infected snails and 2 eggs inoculated in 100 mg of non-infected fecal sample. To evaluate the comparative efficacy of the assay, identical samples were also analyzed via microscopy and Sanger sequencing. The pyrosequencing technique was found to

* Corresponding author at: Research and Diagnostic Center for Emerging Infectious Diseases, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. Fax: +66 43 202475.

E-mail addresses: thanchomnang@yahoo.com (T. Thanchomnang), talent3003@yahoo.com (C. Tantrawatpan), pewpan@kku.ac.th (P.M. Intapan), pusadee.sri@mahidol.ac.th (P. Sri-Aroon), yanin.lim@mahidol.ac.th (Y. Limpanont), viraphng@kku.ac.th (V. Lulitanond), pair_wu@yahoo.com (P. Janwan), sanpoolor@yahoo.com (O. Sanpool), stourtip@gmail.com (S. Tourtip), wanch_ma@kku.ac.th (W. Maleewong).

be superior to the microscopy method and more rapid than the Sanger sequencing method. These results suggest that the pyrosequencing assay is rapid, simple, sensitive and accurate in identifying *S. japonicum* and *S. mekongi* in intermediate hosts and fecal samples of the final host.

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1. Introduction

Schistosomiasis is an important parasitic disease, caused by blood flukes of the genus *Schistosoma*, which continues to cause widespread burden in several countries in tropical areas. An estimated 200 million people are infected worldwide, and more than 600 million people live in endemic areas (World Health Organization, 2005). While the main species responsible for human infections are *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*, the *S. mekongi* species is found in communities along the Mekong River in Cambodia and Lao People's Democratic Republic associated with high mortality rates (Muth et al., 2010). Schistosomiasis japonica is prevalent in China, Indonesia and the Philippines (Zhou et al., 2010). Recently, an increase in the number of foreign workers, migrants and travelers has affected the spread of schistosomiasis (Leshem et al., 2009; Gryseels, 2012). The principal pathology of schistosomiasis japonica involves feverish syndrome, intestinal diseases and hepatosplenic inflammation during the acute stage of the infection and liver fibrosis during the chronic stage of the disease. Human infection has been associated with anemia, hepatosplenomegaly, liver fibrosis, liver cancer and colon cancer (Qiu et al., 2005). Schistosomiasis mekongi is associated with cachexia, hepatosplenomegaly, stunting and retardation of puberty, portal hypertension, ascites and rupture of esophageal varices (Sinuon et al., 2007).

The use of parasitological methods in the detection of eggs in infected stools via microscopic examination and cercarial shedding from infected snails is time-consuming. Stool examination as a standard method has certain weak points: it is difficult to differentiate between *S. japonicum* and *S. mekongi* eggs, it is not possible to use stool samples for detection during the pre-patent period, and this method displays low sensitivity regarding mild infection cases. Moreover, it is difficult to differentiate between different species of shed *Schistosoma* cercariae. Several investigators have reported new molecular-based approaches to detect schistosomiasis: schistosome DNA in samples such as feces (Pontes et al., 2002, 2003; Gobert et al., 2005), sera (Xu et al., 2013; Hussein et al., 2012) and urine (Sandoval et al., 2006) or in intermediate snail hosts (Hamburger et al., 1987). Pyrosequencing, a real-time sequencing technique, facilitates direct sequencing via the synthesis of short nucleotide fragments using a novel enzymatic-cascade system (Ahmadian et al., 2006). This method has been used for the high-throughput genotyping and identification of infectious agents (Cebula et al., 2005), and it allows the detection of nucleotide polymorphisms for species-level identification. In this study, we report PCR amplification with novel pyrosequencing for the differential identification of *S. japonicum* and *S. mekongi* cercariae from infected snails and eggs in fecal samples derived from infected mice and rats.

2. Materials and methods

2.1. Parasite and DNA materials

Schistosoma japonicum (Japanese Yamanashi strain)-experimentally infected *Oncomelania nosophora* snails and mice, *S. japonicum* adults and cercariae as well as *Schistosoma mekongi* (Laotian strain)-experimentally infected *Neotricula aperta* (beta race) snails and infected rats, *S. mekongi* adults and cercariae were obtained

from the Applied Malacology Center, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Thailand. Adult worms of each species were isolated from the mesenteric and portal veins of experimental animals, washed with phosphate buffer saline several times and then stored at -70°C until used. Stool samples derived from experimentally infected animals were collected at 7–8 weeks after infection. *Schistosoma*-infected snails of each species were collected at 16 weeks after infection with miracidium. *Schistosoma* cercariae of each species were emerged via light stimulation at 25°C .

The animals were maintained under standard laboratory conditions in an animal section. Mice were housed in groups of 5, and rats were housed in groups of 3 in plastic box cages and provided with rodent chow and water *ad libitum*. All animal procedures applied in this study were approved by the Animal Ethics Committee of Khon Kaen University based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Reference No. 0514.1.12.2/70).

2.2. Specimen preparation for pyrosequencing

DNA samples were extracted from schistosome adults, experimentally infected snails (including their shells) and cercariae stages. Each specimen was homogenized and extracted using a Nucleospin Tissue kit (Macherey-Nagel GmbH & Co., Duren, Germany). For DNA extraction from feces, 100 mg of each *S. japonicum*-infected mouse feces and *S. mekongi*-infected rat feces were extracted using a QIAamp[®] DNA stool mini kit (Qiagen, Hilden, Germany). The number of *S. japonicum* eggs in the feces derived from infected mice ($n = 5$) is presented as eggs per gram (EPG) of feces (ranging from 200–1100 EPG; geometric mean = 391.86 EPG), and *S. mekongi* eggs present in the feces of infected rats ($n = 5$) is presented as EPG of feces (ranging from 220–2,200 EPG; geometric mean = 780.22 EPG). The parasite eggs were examined via the quantitative formalin ethyl acetate concentration technique (Elkins et al., 1986). The DNA was eluted in 100 μl of distilled water, 2 μl of which was used for PCR assays.

The DNA samples were kept in a DNA bank at -70°C in the Department of Parasitology, Faculty of Medicine, Khon Kaen University until used.

2.3. Analytical sensitivity and specificity determination

To determine analytical sensitivity, tissues from non-infected *N. aperta* or *O. nosophora* snails were ground separately. Next, individual aliquots of 1, 5 and 10 non-infected *N. aperta* and *O. nosophora* snail samples were separately inoculated with 1, 2, 5 and 10 *S. mekongi* and *S. japonicum* cercariae, respectively. To detect parasite eggs in fecal samples, 100-mg fecal aliquots derived from non-infected rats were separately inoculated with a serial dilution of 1, 2, 4 and 8 *S. mekongi* eggs. Similarly, 100-mg fecal aliquots derived from non-infected mice were separately inoculated with a serial dilution of 1, 2, 4 and 8 *S. japonicum* eggs. The samples were also processed for genomic DNA extraction (as described above). The resulting DNA samples were then used for PCR reaction.

For specificity evaluation, genomic DNA isolated from parasites other than *S. mekongi* and *S. japonicum* such as *Ascaris lumbricoides*, hookworm, *Trichuris trichiura*, *Cappillaria philippinensis*, *Strongyloides stercoralis*, *Trichostrongylus* spp., *Taenia* spp., *Opisthorchis*

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