Molecular Differentiation of Schistosoma japonicum and Schistosoma mekongi by Real-Time PCR with High Resolution Melting Analysis

Amornmas Kongkieng\textsuperscript{1,2}, Worasak Kaewkong\textsuperscript{2,3}, Pewpan M. Intapan\textsuperscript{1,2}, Oranuch Sanpoof\textsuperscript{1,2}, Penchom Janwan\textsuperscript{1,2}, Tongjit Thanchomnan\textsuperscript{2,4}, Viraphong Lulitanond\textsuperscript{2,5}, Pusadee Sri-Aroon\textsuperscript{6}, Yanin Limpanont\textsuperscript{6} and Wanchai Maleewong\textsuperscript{1,2,*}

\begin{itemize}
\item\textsuperscript{1}Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand;
\item\textsuperscript{2}Research and Diagnostic Center for Emerging Infectious Diseases, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand;
\item\textsuperscript{3}Department of Biochemistry, Faculty of Medical Science, Naesuan University, Phitsanulok, 65000 Thailand;
\item\textsuperscript{4}Faculty of Medicine, Mahasarakham University, Mahasarakham 44000, Thailand;
\item\textsuperscript{5}Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand;
\item\textsuperscript{6}Applied Malacology Center, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
\end{itemize}

Abstract: Human schistosomiasis caused by Schistosoma japonicum and Schistosoma mekongi is a chronic and debilitating helminthic disease still prevalent in several countries of Asia. Due to morphological similarities of cercariae and eggs of these 2 species, microscopic differentiation is difficult. High resolution melting (HRM) real-time PCR is developed as an alternative tool for the detection and differentiation of these 2 species. A primer pair was designed for targeting the 18S ribosomal RNA gene to generate PCR products of 156 base pairs for both species. The melting points of S. japonicum and S. mekongi PCR products were 84.5± 0.07˚C and 85.7± 0.07˚C, respectively. The method permits amplification from a single cercaria or an egg. The HRM real-time PCR is a rapid and simple tool for differentiation of S. japonicum and S. mekongi in the intermediate and final hosts.

Key words: Schistosoma japonicum, Schistosoma mekongi, differentiation, high resolution melting analysis, real-time PCR

INTRODUCTION

Schistosomiasis is a neglected tropical disease caused by blood flukes of the genus Schistosoma, which remains prevalent in several nations. About 200 million people are infected worldwide, and more than 600 million reside in endemic zones [1]. Although the most important causative species for human diseases are Schistosoma mansoni, Schistosoma haematobium, and Schistosoma japonicum, a further species, Schistosoma mekongi, found along the Mekong River in Cambodia and Lao People’s Democratic Republic (Lao PDR), also infects humans. Mortality rates are high in all species infections [2]. Schistosomiasis japonica is widespread in China, Indonesia, and the Philippines [3]. Recent increases in the movements of foreign workers, grants, and travelers have meant that infected individuals might seek medical help and diagnosis far from the endemic source of their infection [4-7].

Microscopic methods to detect Schistosoma eggs in stools of final hosts or cercariae shed from snail intermediate hosts are time-consuming. The stool examination has certain problems; it is difficult to differentiate between eggs of S. japonicum and S. mekongi, eggs cannot be detected during the pre-patent period, and it has low sensitivity in cases of light intensity of infection. Moreover, morphological identification of Schistosoma cercariae from snail intermediate hosts is also difficult.

There are several reports dealing with molecular-based methods for the diagnosis of schistosomiasis. Most have focused on finding parasite DNA in samples such as feces [8-10], sera [11, 12], urine [13], and in intermediate snail hosts [14]. Identification and differentiation of major human schistosomes by real-time PCR has been reported for the detection of S. japonicum [15-19], S. mekongi [20], S. mansoni [21,22], and S. haematobium [22,23]. However, simultaneous differentiation and detection of S. japonicum and S. mekongi eggs or cercariae in a
single real-time PCR assay has not been reported yet. Here, we report that the high resolution melting (HRM) real-time PCR can be a useful method for differential identification of *S. japonicum* and *S. mekongi* cercariae from infected snails, and also eggs in fecal samples from infected mice and rats.

**MATERIALS AND METHODS**

Parasites and DNA samples

* S. japonicum* (Japanese Yamanashi strain) cercariae were obtained from experimentally infected *Oncomelania nosophora* snails and adult worms from experimentally infected mice. Similarly, *S. mekongi* (Loatian strain) cercariae were obtained from experimentally infected *Neotricula aperta* (beta race) snails, and adult worms were from experimentally infected rats. All those infected snails were obtained from the Applied Malacology Center, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Thailand. All animal experiments 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).

DNAs extracted from individual *S. japonicum* and *S. mekongi* adults and from experimentally infected snails were prepared using the Nucleospin Tissue kit (Macherey-Nagel GmbH & Co, Duren, Germany). Copro-DNAs were extracted from 100 mg each of *S. japonicum*-infected mouse feces and *S. mekongi*-infected rat feces using the QIAamp® DNA stool mini kit (Qiagen, Hilden, Germany). DNA was eluted in 50 μl of distilled water, 5 μl of which was used for each HRM real-time PCR reaction. The DNA samples were kept at -70°C until use.

The number of *S. japonicum* eggs in infected mice feces (n = 9) was determined and expressed as eggs per gram (EPG) of feces (ranging from 100-1,100 EPG; geometric mean = 367 EPG). Similarly, numbers of *S. mekongi* eggs in infected rats feces (n = 12) were determined (ranging from 1,100-22,000 EPG; geometric mean = 3,805 EPG).

**Determination of analytical sensitivity and specificity**

To determine analytical sensitivity, non-infected *N. aperta* or *O. nosophora* snails were crushed separately. Subsequently, individual aliquots of 1, 5 (pooled), and 10 (pooled) non-infected *N. aperta* and *O. nosophora* snail samples were each separately inoculated with 1, 5, and 10 *S. mekongi* and *S. japonicum* cercariae. To determine detection limits for fluke eggs in fecal samples, 1, 2, 4, or 8 *S. mekongi* eggs were added to 100 mg aliquots of non-infected rat feces. Likewise, to 100 mg aliquots of non-infected mouse feces were added 1, 2, 4, or 8 *S. japonicum* eggs. Genomic DNA was then extracted from these samples (see above) and used for PCRs.

For evaluation of specificity, genomic DNAs from parasites other than *S. mekongi* and *S. japonicum* were used, e.g., human hookworms, intestinal lecithodendriid flukes, *Taenia* spp., *Trichuris trichiura*, *Trichosontrangulus* spp., *Strongyloides stercoralis*, *Stellantchasmus* spp., *Paragonimus heterotremus*, *Opisthorchis viverrini*, *Haplorchoides* spp., *Haplorchis taichui*, *Isospora belli*, *Giardia duodenalis*, *Echinostoma malayanum*, *Capillaria philippinensis*, *Clonorchis sinensis*, and *Ascaris lumbricoides*. DNAs extracted from human leukocytes, feces of non-infected mice or rats, and non-infected snails were also used as controls.

**Primer design and positive control plasmids**

The 18S ribosomal RNA sequence (18S rRNA) of *S. japonicum* (FJ176682) and *S. mekongi* (U89871) were selected and used to differentiate the 2 species. The PCR primers (Schis_F; 5’-GAG TTT CGG GTT GCC TGA TC -3’ and Schis_R; 5’- ACC GGA TCG CTT CAA CAG T-3’) were designed to amplify a particularly variable region [24]. For the positive controls, plasmids were constructed by ligation of amplified products from each species into pGEM-T easy vectors (Promega, Madison, Wisconsin, USA), according to the manufacturer’s instructions. The PCR products were obtained by conventional PCR using the Schis_F and Schis_R primers and control plasmids as template. Each recombinant plasmid was produced in *Escherichia coli* JM109. Each inserted amplicon was sequenced in both directions to confirm its identity.

**HRM real-time PCR assay**

For differential detection, a LightCycler 480 High Resolution Melting Master Kit (Roche Applied Science, Mannheim, Germany) was used. The reaction mixture contained 1 × LightCycler 480 HRM Master Mix, which comprises HRM dye (Roche Applied Science), 2.25 mM MgCl₂, and each of 0.4 μM Schis_F and Schis_R primers. The total reaction volume was 20 μl. The PCR cycling for HRM curve presentation was done under the following conditions: 1 hold at 95°C for 10 min; 45 cycles of 95°C for 10 sec, 55°C for 8 sec, and 72°C for 15 sec; then, the mixture was held at 95°C for 10 sec and 60°C for 30 sec. The reaction products were then melted by increasing the temperature from 60°C to 95°C, with an increment of 0.11°C/sec, to
obtain melting profiles. Amplified product was then cooled to 40°C for 30 sec. All samples were examined in duplicate in 96-well plates.

To determine the analytical specificity of the HRM real-time PCR, DNAs extracted from specificity control samples (see above) were evaluated separately. Each run included one distilled water sample as a negative control and *S. japonicum* or *S. mekongi* plasmids in water (10^7 copies) as positive controls.

The melting temperatures (Tm) of each PCR product was determined by melting curve analysis using LightCycler 480 gene scanning software (version 1.5) (Roche Applied Science). The cycle number (Cn), representing the target sequence copy number, was taken to be the number of PCR cycles needed for the change in fluorescence signal of the amplicons to exceed the detection threshold value. The sensitivity and specificity values were calculated and expressed using the method described previously [25].

**RESULTS**

**Standardization of the HRM real-time PCR**

The analytical sensitivity of HRM real-time PCR was determined using 10-fold serial dilutions (4.3 × 10^7–4.3 × 10^2 copies) of the equal concentration mixture of *S. japonicum* and *S. mekongi* positive control plasmids in distilled water. The lowest detection was equal to or less than 4.3 × 10^2 copies of each positive control plasmid (Fig. 1) which is equivalent to 4 × 10^7 ng of each genomic DNA of *S. japonicum* and *S. mekongi*, when considering 40 cycles as the cut-off detection limit. As little as a single *S. japonicum* or *S. mekongi* egg (Fig. 2) mixed artificially in 100 mg of uninfected mouse or rat feces could be clearly detected. Similarly, a single *S. japonicum* or *S. mekongi* cercaria inoculated into an aliquot derived from 10 pooled non-infected *N. aperta* or *O. nosophora* snail samples could be detected.

No fluorescence signal was detected when evaluated with the defined DNA controls (1 µg) other than *S. japonicum* and *S. mekongi* (see Materials and Methods).

**HRM real-time PCR for detection of *S. japonicum* and *S. mekongi* in fecal and snail samples**

The HRM real-time PCR yielded positive results for all fecal samples from *S. japonicum*-infected mice and *S. mekongi*-infected rats (Table 1). Under the conditions described here, the HRM real-time PCR successfully amplified a predicted 156 bp prod.
uct from the DNA of the *S. japonicum* and *S. mekongi*-infected fecal and snail samples (Fig. 3). The analytical sensitivity and specificity were both 100% for differential detection of *S. japonicum* and *S. mekongi*.

To ensure the accuracy of the method, the amplified products from *S. japonicum* and *S. mekongi*-infected fecal and snail samples were sequenced in both directions. The results showed that all sequences were completely identical (data not shown) with the corresponding gene sequences from the relevant species.

**DISCUSSION**

Since Wittwer et al. [26] revealed that the HRM real-time PCR assay can identify sequence variants, the method has been applied for rapid detection and identification of *Brugia malayi*, *Brugia pahangi*, *Dirofilaria immitis* [27], and human hookworms [28]. This allows closed-tube, homogeneous genotyping without fluorescence-labeled probes, consequently...
decreasing the expense on a cost per-sample. Different sequenc-
es are represented by a change in the shape of the different
melting curve plotted.

We have developed the HRM real-time PCR for differential
detection of S. japonicum and S. mekongi in fecal samples of fi-
nal hosts and in tissues of snail intermediate hosts. A single
Schistosoma egg in a 100 mg fecal sample (equivalent to 10 EPG)
or a single cercaria in tissues from 10 pooled snails can be de-
tected. These detection are quite similar with single-species de-
tection limits for S. japonicum [19] or S. mekongi [20] using a
real-time PCR assay with fluorescence resonance energy trans-
fer (FRET) hybridization probes. Similar levels of sensitivity
have been found using SYBR green based real-time PCR; 10 EPG
of S. japonicum in fecal samples could be reliably detected [15].
However, Zhou et al. [29] showed that the TaqMan real-time
PCR assay can detect 1 S. japonicum egg in 500 mg fecal sam-
ple (equivalent to 2 EPG) [29].

For analytical specificity, DNA samples of the parasites other
than the Schistosoma species tested did not give rise to an iden-
tifiable melting temperature peak, and the primers used did
not amplify a 156 bp product, indicating 100% specificity.

As a result of the increase of outbound tourism from Asia
and the increase of migrants within Asia due to One Asian
Economic Community policy, there is an increasing poten-
tial for overlapping infections of the 2 Schistosoma species, S. japoni-
cum and S. mekongi. In the laboratory setting, the assay system
reported here gave high sensitivity and specificity and will be
most valuable for diagnosis of infection by either species, or to
demonstrate co-infection.

In conclusion, the method established in the present study
has enabled rapid, sensitive, and specific differential identifica-
tion of S. japonicum and S. mekongi cercariae in infected snails
and eggs in fecal samples of infected mice and rats. Its cost-effec-
tiveness is much better than other probe-based real-time
PCR methods.

**ACKNOWLEDGMENTS**

This research was funded by grants from the National Sci-
ence and Technology Development Agency (Discovery Based
Development Grant); the Higher Education Research Promo-
tion and National Research University Project of Thailand,
Office of the Higher Education Commission through the Health
Cluster (SHep-GMS), Thailand; the Faculty of Medicine, Khon
Kaen University; Wanchai Maleewong was supported by TRF
Senior Research Scholar Grant, Thailand Research Fund grant
no. RIA5580004. We wish to acknowledge the support of the
Khon Kaen University Publication Clinic, Research, and Tech-
nology Transfer Affairs, Khon Kaen University, for their assist-
ance.

**REFERENCES**

1. World Health Organization. Report of the Scientific Working
Group Meeting on Schistosomiasis. Geneva, Switzerland. 2005,
p 1.
2. Muth S, Sayasone S, Odermatt-Biays S, Phompida S, Duong S,
Odermatt P. Schistosoma mekongi in Cambodia and Lao People’s
Democratic Republic. Adv Parasitol 2010; 72: 179-203.
3. Zhou XN, Bergquist R, Leonardo I, Yang GJ, Yang K, Sudomo M,
Olveda R. Schistosomiasis japonica control and research needs.
Adv Parasitol 2010; 72: 145-178.
4. Les kem H, Meltzer E, Marva E, Schwartz E. Travel-related schisto-
omiasis acquired in Laos. Emerg Infect Dis 2009; 15: 1823-1826.
5. Gryseels B. Schistosomiasis. Infect Dis Clin North Am 2012; 26:
383-397.
6. Qiu DC, Hubbard AE, Zhong B, Zhang Y, Spear RC. A matched,
case-control study of the association between Schistosoma japoni-
cum and liver and colon cancers, in rural China. Ann Trop Med
Parasitol 2005; 99: 47-52.
7. Sinuon M, Tsuyuoka R, Socheat D, Odermatt P, Ohmoe H, Mat-
suda H, Montresor A, Palmer K. Control of Schistosoma mekongi
in Cambodia: results of eight years of control activities in the two
endemic provinces. Trans R Soc Trop Med Hyg 2007; 101: 34-39.
8. Pontes LA, Dias-Neto E, Rabellio A. Detection by polymerase
chain reaction of Schistosoma mansoni DNA in human serum
and feces. Am J Trop Med Hyg 2002; 66: 157-162.
9. Pontes LA, Oliveira MC, Katz N, Dias-Neto E, Rabellio A. Com-
parison of a polymerase chain reaction and the Kato-Katz tech-
nique for diagnosing infection with Schistosoma mansoni. Am J
Trop Med Hyg 2003; 68: 652-656.
10. Gobert GN, Chai M, Duke M, McManus DP. Copro-PCR based
detection of Schistosoma eggs using mitochondrial DNA markers.
Mol Cell Probes 2005; 19: 250-254.
11. Xu J, Liu AP, Guo J, Wang B, Qiu SJ, Sun H, Guan W, Zhu XQ,
Xia CM, Wu ZD. The sources and metabolic dynamics of Schisto-
soma japonicum DNA in serum of the host. Parasitol Res 2013;
112: 129-133.
12. Hussein HM, El-Ton sy MM, Tawfik RA, Ahmed SA. Experimental
study for early diagnosis of prepatent schistosomiasis mansoni
by detection of free circulating DNA in serum. Parasitol Res 2012;
111: 475-478.
13. Sandoval N, Siles-Lucas M, Perez-Arellano JL, Carranza G, Puente
S, Lopez-Aban J, Muro A. A new PCR-based approach for the
specific amplification of DNA from different Schistosoma species
applicable to human urine samples. Parasitol 2006; 133: 581-587.
14. Hamburger J, Weil M, Pollack Y. Detection of Schistosoma manso-
14. ni DNA in extracts of whole individual snails by dot hybridization. Parasitol Res 1987; 74: 97-100.

15. Lier T, Simonsen GS, Haaneheim H, Hjelmervoll SO, Vennervald Bj, Johansen MV. Novel real-time PCR for detection of *Schistosoma japonicum* in stool. Southeast Asian Trop Med Public Health 2006; 37: 257-264.

16. Lier T, Johansen MV, Hjelmervoll SO, Vennervald Bj, Simonsen GS. Real-time PCR for detection of low intensity *Schistosoma japonicum* infections in a pig model. Acta Trop 2008; 105: 74-80.

17. Lier T, Simonsen GS, Wang T, Lu D, Haukland HH, Vennervald Bj, Hegstad J, Johansen MV. Real-time polymerase chain reaction for detection of low-intensity *Schistosoma japonicum* infections in China. Am J Trop Med Hyg 2009; 81: 428-432.

18. Hung YW, Remais J. Quantitative detection of *Schistosoma japonicum* cercariae in water by real-time PCR. PLoS Negl Trop Dis 2008; 2: e337.

19. Thanchomnang T, Intapan P, Sri-Aroon P, Lulitanond V, Janwan P, Sanpool O, Maleewong W. Molecular detection of *Schistosoma japonicum* in infected snails and mouse faeces using a real-time PCR assay with FRET hybridisation probes. Mem Inst Oswaldo Cruz 2011; 106: 831-836.

20. Sanpool O, Intapan PM, Thanchomnang T, Sri-Aroon P, Lulitanond V, Janwan P, Sanpool O, Maleewong W. Development of a real-time PCR assay with fluorophore-labelled hybridization probes for identification of *Schistosoma mekongi* in infected snails and rat faeces. Parasitol 2012; 139: 1266-1272.

21. Gomes AL, Melo FL, Werkhauser RP, Abath FG. Development of a real-time polymerase chain reaction for quantitation of *Schistosoma mansoni* DNA. Mem Inst Oswaldo Cruz 2006; 101(suppl): 133-136.

22. ten Hove RJ, Verweij JJ, Vereecken K, Polman K, Dieye L, van Lieshout L. Multiplex real-time PCR for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* infection in stool samples collected in northern Senegal. Trans R Soc Trop Med Hyg 2008; 102: 179-185.

23. Kjetland EF, Hove RJ, Gomo E, Midzi N, Gwanzura I, Mason P, Friis H, Verweij JJ, Gundersen SG, Ndhlouvu PD, Mduluza T, Van Lieshout L. Schistosomiasis PCR in vaginal lavage as an indicator of genital *Schistosoma haematobium* infection in rural Zimbabwean women. Am J Trop Med Hyg 2009; 81: 1050-1055.

24. Thanchomnang T, Tantrawatpan C, Intapan PM, Sri-Aroon P, Lulitanond V, Janwan P, Sanpool O, Tourtip S, Maleewong W. Pyrosequencing for rapid molecular identification of *Schistosoma japonicum* and *S. mekongi* eggs and cercariae. Exp Parasitol 2013; 135: 148-152.

25. Galen RS. Predictive value and efficiency of laboratory testing. Pediatr Clin North Am 1980; 27: 861-869.

26. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 2003; 49: 853-860.

27. Wongkamchai S, Monkong N, Mahannel P, Taweethavonsawat P, Loymak S, Foongladda S. Rapid detection and identification of *Brugia malayi*, *B. pahangi*, and *Dirofilaria immitis* by high-resolution melting assay. Vector Borne Zoonotic Dis 2013; 13: 31-36.

28. Ngui R, Lim YA, Chua KH. Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis. PLoS One 2012; 7: e41996.

29. Zhou L, Tang J, Zhao Y, Gong R, Lu X, Gong L, Wang Y. A highly sensitive TaqMan real-time PCR assay for early detection of *Schistosoma* species. Acta Trop 2011; 120: 88-94.