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▣ ORIGINAL ARTICLE

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

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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℃ and 85.7±0.07℃, 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, migrants, 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

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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℃ 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*, *Trichostrongylus* 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′-GAC 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 $MgCl_2$, 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℃ for 10 min; 45 cycles of 95℃ for 10 sec, 55℃ for 8 sec, and 72℃ for 15 sec; then, the mixture was held at 95℃ for 10 sec and 60℃ for 30 sec. The reaction products were then melted by increasing the temperature from 60℃ to 95℃, with an increment of 0.11℃/sec, to

obtain melting profiles. Amplified product was then cooled to 40℃ 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-

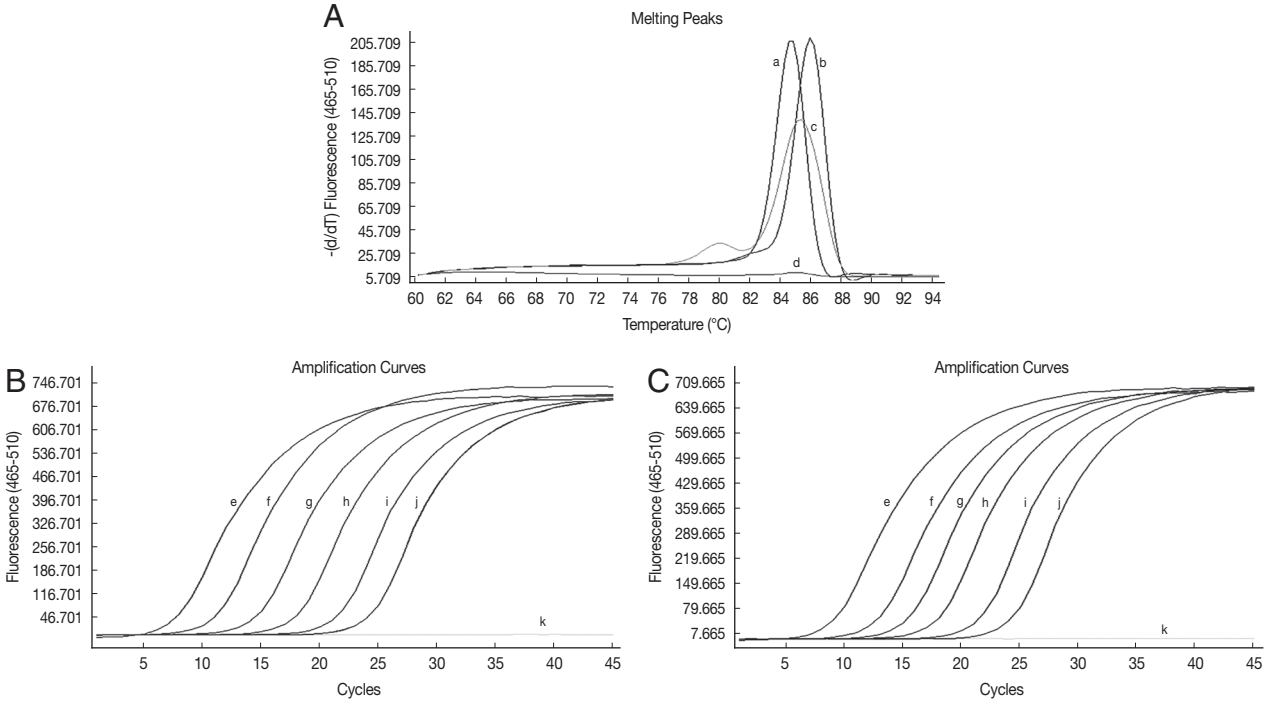

Fig. 1. (A) Representative melting peaks (℃) for *Schistosoma japonicum* (a), *Schistosoma mekongi* (b), mixed-plasmids (c), and distilled water (d). Amplification plot of fluorescence vs cycle number showing analytical sensitivity of HRM real-time PCR for detection of *S. japonicum* (B) and *S. mekongi* (C) plasmids: e-j; 10-fold serial dilutions of *S. japonicum* or *S. mekongi* plasmids, from 4.3×10^7 to 4.3×10^2 copies per reaction. k; distilled water (negative control).

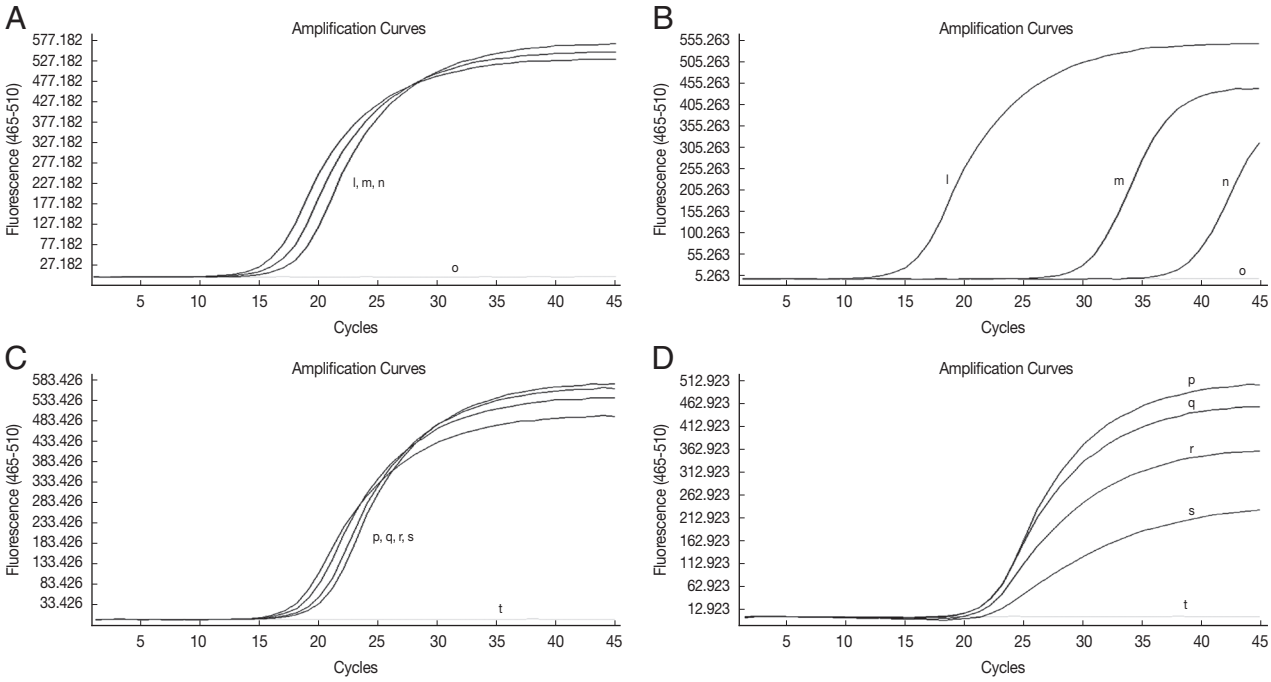

Fig. 2. Analytical sensitivity for detection of cercariae (A, B) and eggs (C, D) of *S. japonicum* (A, C) and *S. mekongi* (B, D). Cycle numbers for detection of 4 cercariae (l), 2 cercariae (m), 1 cercaria (n), and for detection of 8 eggs (p), 4 eggs (q), 2 eggs (r), and 1 egg (s). o and t; distilled water (negative control).

Table 1. The cycle number and melting temperature values of HRM real-time PCR

	Cycle numbers			Melting temperatures		
	Range	Mean±SD	Median	Range	Mean±SD	Median
S. japonicum-infected mice (n=9)	15.8-29.7	22.0±4.2	21.9	84.4-84.6	84.5±0.07	84.5
S. mekongi-infected rats (n=12)	19.5-29.2	23.5±3.4	22.6	85.6-85.7	85.7±0.04	85.7

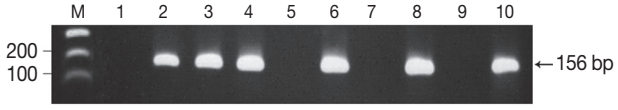

Fig. 3. Ethidium bromide staining patterns of the PCR products on a 1.5% agarose gel. The arrows indicate the 156 bp of *S. mekongi* and *S. japonicum* specific bands. Lane M: DNA size markers (1 kb plus DNA ladder from Invitrogen, Carlsbad, California, USA). Negative control containing no DNA (Lane 1); *S. mekongi* positive control plasmid (Lane 2); *S. japonicum* positive control plasmid (Lane 3); *S. mekongi*-infected *Neotricula aperta* snails (Lane 4); non-infected *N. aperta* snails (Lane 5); *S. japonicum*-infected *Oncomelania nosophora* snails (Lane 6); non-infected *O. nosophora* snails (Lane 7); *S. mekongi*-infected rat feces (Lane 8); negative healthy human feces (Lane 9); and *S. japonicum*-infected mice feces (Lane 10).

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 sequences 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 final 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 detected. These detection are quite similar with single-species detection limits for *S. japonicum* [19] or *S. mekongi* [20] using a real-time PCR assay with fluorescence resonance energy transfer (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 sample (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 identifiable 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 potential for overlapping infections of the 2 *Schistosoma* species, *S. japonicum* 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 identification of *S. japonicum* and *S. mekongi* cercariae in infected snails and eggs in fecal samples of infected mice and rats. Its cost-effectiveness is much better than other probe-based real-time PCR methods.

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