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ORIGINAL PAPER

Rapid detection and identification of four major *Schistosoma* species by high-resolution melt (HRM) analysis

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Abstract Schistosomiasis, caused by blood flukes belonging to several species of the genus *Schistosoma*, is a serious and widespread parasitic disease. Accurate and rapid differentiation of these etiological agents of animal and human schistosomiasis to species level can be difficult. We report a real-time PCR assay coupled with a high-resolution melt (HRM) assay targeting a portion of the nuclear 18S rDNA to detect, identify, and distinguish between four major blood fluke species (*Schistosoma japonicum*, *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma mekongi*). Using this system, the *Schistosoma* spp. was accurately identified and could also be distinguished from all other trematode species with which they were compared. As little as 10^{-5} ng genomic DNA from a *Schistosoma* sp. could be detected. This process is inexpensive, easy, and can be completed within 3 h. Examination of 21 representative *Schistosoma* samples from 15 geographical localities in seven endemic countries validated the value of the HRM detection assay and proved its reliability. The melting curves were characterized by peaks of 83.65 °C for *S. japonicum* and *S. mekongi*, 85.65 °C for *S. mansoni*, and 85.85 °C for *S. haematobium*. The present study developed a real-time PCR coupled with HRM analysis assay for detection and differential identification of *S. mansoni*, *S. haematobium*, *S. japonicum*, and *S. mekongi*. This method is rapid, sensitive, and inexpensive. It has important implications for epidemiological studies of *Schistosoma*.

Keywords *Schistosoma* · Schistosomiasis · High-resolution melt curve (HRM) · Rapid identification · Differentiation · 18S rDNA

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Introduction

Schistosomiasis is a neglected tropical disease caused by blood-dwelling trematodes of the genus *Schistosoma* (Phylum Platyhelminthes, Class Trematoda). It has a high prevalence in subtropical regions of Africa, the Americas, and Asia (Gryseels 2012). This helminthiasis has been ranked second only to malaria among parasitic diseases, affects about 200 million people and causes about 41,000 deaths annually worldwide (Gryseels et al. 2006; WHO 2008). Out of the 21 currently recognized *Schistosoma* species, *Schistosoma japonicum* (China and Southeast Asia; dwells in peri-intestinal venules), *Schistosoma mansoni* (Africa, Arabia, and South America; dwells in peri-intestinal venules), and *Schistosoma haematobium* (Africa and Arabia; dwells in the perivesical plexus) are the main species responsible for human infections and are

hence have public health significance as well as imposing an economic burden (Clerinx and Van Gompel 2011). Additionally, *Schistosoma mekongi* (dwells in peri-intestinal venules) is found in communities along the Mekong River in Cambodia and Lao People's Democratic Republic. Although primarily of local importance, it is associated with high mortality rates (Muth et al. 2010; Meltzer and Schwartz 2013). Two of these species, *S. japonicum* and *S. mekongi*, can also infect some wild and domesticated animals as their reservoir hosts. Their zoonotic nature complicates control efforts (Hotez et al. 2007; Clerinx and Van Gompel 2011).

Nowadays, people are moving around the world more than ever before, responding to the need for social and economic development. Travel to a variety of tropical destinations by expatriates from nonendemic areas exposes them to risk of infection by several *Schistosoma* species (Li et al. 2015). The specific diagnosis of schistosomiasis in humans is central to control of this disease and to understand the intricacies of its epidemiology. Traditionally, microscopic, biochemical, and serological techniques have been applied for diagnosis of *Schistosoma* infections, but most of them have limitations in the specific identification, especially in the case of mixed infections and hybrids (Huyse et al. 2009; Zhao et al. 2012a). Increasing evidence from experimental and human studies has shown that polymerase chain reaction (PCR) and PCR-coupled methods improve the diagnosis of *Schistosoma* infection as well as the identification of *Schistosoma* species (Gobert et al. 2005; Sandoval et al. 2006; Kato-Hayashi et al. 2010; Lv et al. 2015). Although these techniques are useful and effective, the electrophoresis or sequencing analysis can be, in some cases, quite time consuming and expensive, rendering such tools impracticable for large-scale molecular epidemiological studies (Lymbery and Thompson 2012).

The high-resolution melt (HRM) technique uses a single closed tube in which PCR is followed by precise dissociation (melting) behavior determination of double-stranded DNA amplicons. The accuracy of the melting curve is maximized by acquiring fluorescence data over small temperature increments (as low as 0.02 °C) (Price et al. 2007). Samples can be discriminated according to sequence, length, GC content, or strand complementarity, down to single base-pair changes. The intercalating double-stranded DNA-binding dyes used do not inhibit PCR reactions at the concentrations necessary for them to fully saturate the target DNA duplexes and ensures the accuracy of experimental results. Such a probe-free assay is easier and more cost-effective and, unlike conventional methods, it has no manual post-PCR processing, thus avoiding the risk of contamination with PCR products. This method greatly simplifies the operation and reduces the analysis time, making it suitable for broad application. In the area of parasitology, it has been adapted recently for the genotyping of apicomplexan parasites (Al-Mohammed 2011; Costa et al. 2011; Ngui et al. 2012; Zhang et al. 2012; Higuera et al. 2013; Salim et al. 2013).

In the present study, we established a simple and effective real-time PCR assay coupled with HRM analysis, using the 18S ribosomal DNA (rDNA) region as the genetic marker, for the detection and differentiation of the four main *Schistosoma* spp. infecting humans, and discuss the value of such a tool in rapid screening and detection of closely related species in a laboratory.

Materials and methods

Parasites and isolation of genomic DNA

Twenty-five worm samples were originated from endemic areas in Puerto Rico, Cameroon, Egypt, China, Philippines, Japan, and Cambodia (Table 1). No specific permission was required for the collection of intermediate host snails of *Schistosoma* spp. Field studies did not involve any endangered or protected species. No ethical approval was required for the present study because all the parasite samples used in the present study had been prepared and used in previous studies (Sugiyama et al. 1997; Li et al. 2011; Zhao et al. 2010; 2012b). All *S. japonicum* worms in China were collected from rabbits exposed to cercariae from infected snails (*Oncomelania hupensis*) in our laboratory (Li et al. 2011; Zhao et al. 2010; 2012b), while *S. japonicum* from the Philippines and Japan were isolated from experimentally infected mice (Sugiyama et al. 1997). For *S. mekongi*, *S. mansoni*, and *S. haematobium*, worms were collected from rabbits or mice experimentally infected with cercariae from the relevant intermediate snail hosts (*Neotricula aperta*, *Biomphalaria glabrata*, and *Bulinus* spp., respectively). These worms were provided by Dr. Hiroshi Ohmae, Department of Parasitology, National Institute of Infectious Diseases, and by Dr. Mona S. Mahmoud in the Department of Parasitology and Animal Diseases, National Research Center, Dokki, Giza, Egypt. All the *Schistosoma* specimens were fixed in 70 % molecular grade ethanol and stored at −20 °C until used for DNA extraction. Additionally, some adults of common zoonotic parasites, namely *Fasciola hepatica*, *F. gigantica*, *Clonorchis sinensis*, and *Opisthorchis viverrini* were used as "controls" for the evaluation of the specificity of the specific PCR-coupled HRM assay.

Total genomic DNA (gDNA) of each parasite was extracted using SDS/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System, Promega) and eluted into 60 μL H_2O according to the manufacturer's recommendations. An RNAase treatment step was also used. Then, the quality of parasite gDNA was confirmed by

Table 1 *Schistosoma* samples used in the present study

Species	Geographical origins	Sample codes	Gender of worm
Schistosoma mansoni	Puerto Rico	SM1 (Sman-B)	Unknown
	Puerto Rico	SM20	Unknown
	Egypt (Cairo)	ECSMF1	Female
	Egypt (Cairo)	ECSMM1	Male
Schistosoma haematobium	Cameroon	NCSH2 (Shae-B)	Unknown
	Egypt (Cairo)	ECSHF1	Female
	Egypt (Cairo)	ECSHM1	Male
	Egypt (Cairo)	ECSHF2	Female
Schistosoma japonicum	China (Yunnan)	SJYEIIIF2 (Sjap-B)	Female
	China (Jiangxi)	SJJYM51	Male
	China (Hubei)	SJHWF53	Female
	China (Zhejiang)	SJZJF58	Female
	China (Sichuan)	SJSXM7	Male
	China (Anhui)	SJAGF52	Female
	China (Hunan)	SJHYM59	Male
	Philippines (Leyte)	SJLEYF6	Female
	Philippines (Sorsogon)	SJSORF9	Female
	Philippines (Asuncion)	SJASNM6	Male
	Japan (Yamanashi)	SJYYF11	Female
	Japan (Yamanashi)	SJYYM12	Male
Schistosoma mekongi	Cambodia (Kratie)	SMGF1 (Smek-B)	Female
	Cambodia (Kratie)	SMGF2	Female
	Cambodia (Kratie)	SMGM2	Male
	Cambodia (Kratie)	SMGM1	Male
	Cambodia (Kratie)	SMGM4	Male

amplification of the first internal transcribed spacer of ribosomal DNA (van Herwerden et al. 1998).

Primers, Real-time PCR, and HRM analysis

18S rDNA sequence has been identified as good genetic marker for identification and phylogenetic studies of *Schistosoma* members (Johnston et al. 1993; Attwood et al. 2002; Webster et al. 2006). The 18S rDNA sequence is also quite conserved within a *Schistosoma* species (Yu et al. 2000; Li et al. 2008). Our recent study also demonstrated that 35 *S. japonicum* isolates from mainland China, the Philippines, and Japan have almost identical 18S rDNA sequences, with only one nucleotide variation (Chen et al. 2011). Therefore, a portion of the 18S rDNA of four *Schistosoma* species was chosen as the genetic marker. A pair of primers for real-time PCR coupled with HRM analysis was designed specific for the V4 region (~280 bp) of the nuclear 18S rDNA of four *Schistosoma* species (based on GenBank™ accession numbers AY157226, AY157228, SMU65657, Z11976). Primer names and sequences are as follows: M182U (5′-GGTAACTCCAGCTCCAAAAGCGTAT-3′) and M182D (5′-TTGTTCAAAGTAAAGATGCCGTCCG-3′). The real-time PCR conditions were optimized empirically. Finally, each PCR reaction (20 mL in volume) was performed in 10 μL 2× HRM PCR master mix (QIAGEN), 0.7 μM of each primer, and 1–50 pg genomic DNA in a Rotor-Gene 6000 (HRM)™ (QIAGEN) under the following thermocycling conditions: 5 min at 95 °C to activate HotStarTaq Plus DNA Polymerase, then 40 cycles of three steps: 10 s denaturation at 95 °C, 30 s annealing at 55 °C, and 10 s extension at 72 °C. After amplification, a melt step in the range 78 to 89 °C in 0.2 °C increments, pausing for 2 s per step, was initiated. The increase in Evagreen fluorescence was monitored in real time during the PCR, and the subsequent decrease during the melt phase was measured by acquiring each step to the green channel (470-nm excitation and 510-nm emission) of the Rotor-Gene 6000. Genotypes were then determined by examining normalized and difference melt plots using the Corbett Rotor-Gene 6000 software (Rotor-Gene Q Series Software 2.0.2) with a confidence limit specified as 80 % (a score above this signifying that a sample can be confidently assigned to a particular genotype, and a score below this indicating a sequence variation).

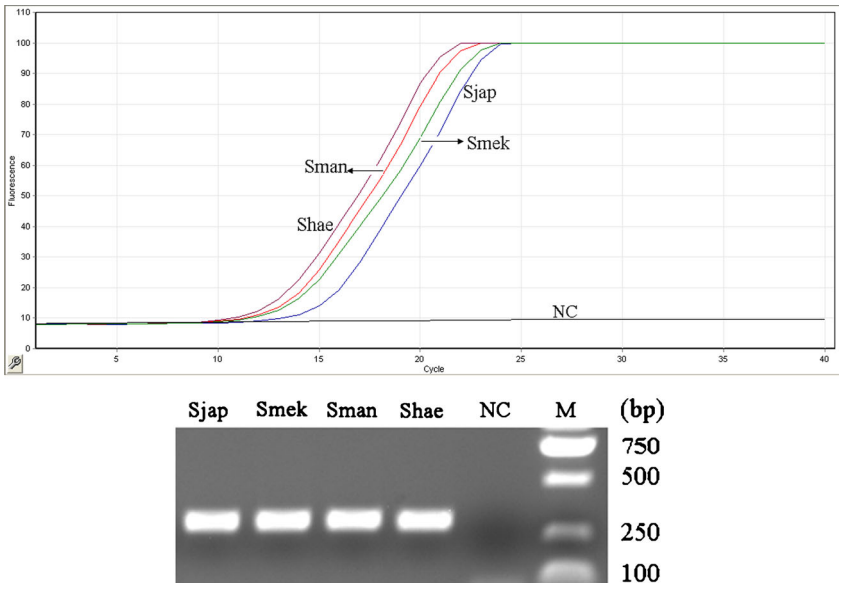

Fig. 1 Real-time PCR amplification curve (*upper panel*) and agarose gel electrophoresis (*lower panel*) of a portion of the 18S rDNA from four *Schistosoma* species. *Sjap S. japonicum*, *Smek S. mekongi*, *Sman S. mansoni*, *Shae S. haematobium*, *NC* negative control; *M* DL2000 marker

Cycle threshold (Ct) values and amplification efficiency were assessed to identify outliers (samples of poor quality) that were then filtered out of subsequent HRM analysis. Ct values would be ideally similar for samples and reference. Therefore, amplicons from randomly chosen *S. japonicum*, *S. mansoni*, *S. haematobium*, and *S. mekongi* individuals were purified and built into recombinant plasmids to establish standard references (namely Sjap-B, Sman-B, Shae-B, Smek-B, respectively, Table 1). Each plasmid stock was diluted so as to give a reaction Ct value between 10 and 20 when used as a standard. To examine their consistency, all standard references of each *Schistosoma* species were tested in triplicate and repeated on several different days using similar reagents and DNA concentrations. After determining the optimized reaction system and conditions, all samples under test were diluted (ddH_2O dilution) or concentrated (DNAmate, TaKaRa, D605A) to match the Ct value of the standards. Any DNA with a Ct≥30 was removed, because it indicated too little starting template or sample degradation. Reactions with amplification efficiency different from the standards or with efficiency less than 1.4 were omitted from evaluation as outliers (Poláková et al. 2008).

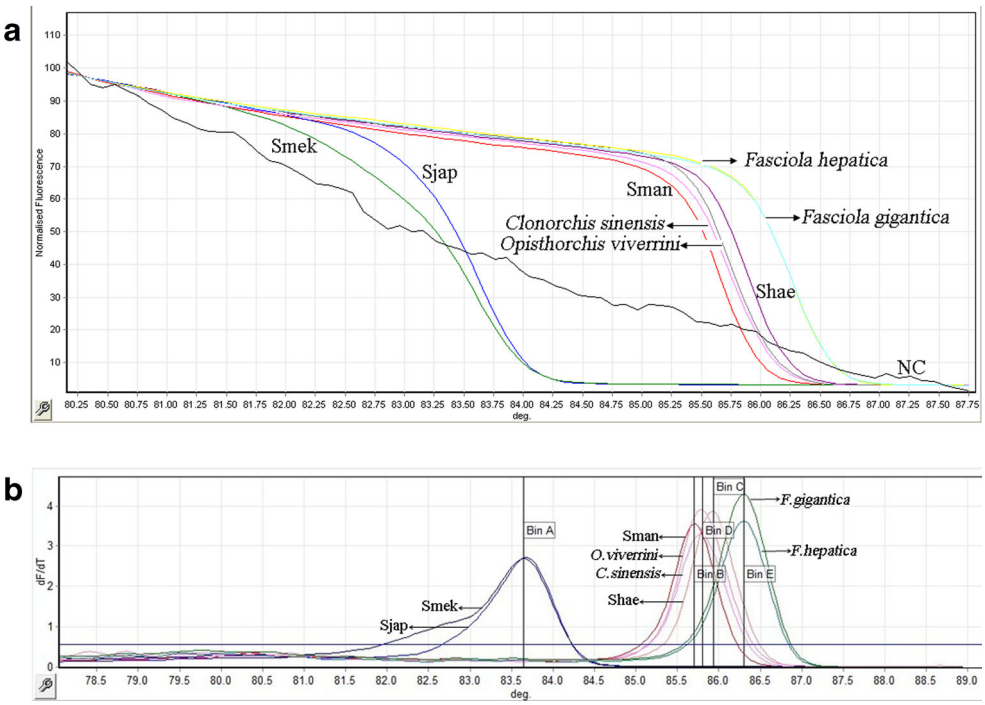

Fig. 2 Specificity of HRM analysis of normalized and temperature-shifted melting curves of *Schistosoma* spp. and other common zoonotic trematodes. *Sjap S. japonicum*, *Smek S. mekongi*, *Sman S. mansoni*, *Shae S. haematobium*, *NC* negative control. Melting peaks of trematodes, Bin A includes *S. japonicum* and *S. mekongi*, Tm, 83.65 °C; Bin B represents *S. mansoni*, Tm, 85.65 °C; Bin C represents *S. haematobium*, Tm, 85.85 °C; Bin D includes *C. sinensis* and *O. viverrini*, Tm, 85.78; and Bin E includes *Fasciola hepatica* and *Fasciola gigantica*, Tm, 86.30 °C

Table 2 Melting points for the peaks and HRM curve profiles of 18S rDNA PCR products from species of *Schistosoma*

Species/standards	Sample codes	Melting peak (°C)	HRM genotype	HRM confidence (%)
Standards	Sman-B	85.65 BinB	Sman	92.35
	Shae-B	85.85 BinC	Shae	92.24
	Sjap-B	83.65 BinA	Sjap	99.60
	Smek-B	83.65 BinA	Smek	91.65
S. mansoni	SM20	85.65 BinB	Sman	85.87
	ECSMF1	85.65 BinB	Sman	85.87
	ECSMM1	85.65 BinB	Sman	85.85
S. haematobium	ECSHF1	85.85 BinC	Shae	86.10
	ECSHM1	85.85 BinC	Shae	86.10
	ECSHF2	85.85 BinC	Shae	86.08
S. mekongi	SMGF2	83.65 BinA	Smek	83.75
	SMGM2	83.65 BinA	Smek	83.80
	SMGM1	83.65 BinA	Smek	83.80
	SMGM4	83.65 BinA	Smek	83.77
S. japonicum	SJJYM51	83.65 BinA	Sjap	95.35
	SJHWF53	83.65 BinA	Sjap	98.78
	SJZJF58	83.65 BinA	Sjap	83.46
	SJSXM7	83.65 BinA	Sjap	87.28
	SJAGF52	83.65 BinA	Sjap	89.89
	SJHYM59	83.65 BinA	Sjap	92.60
	SJLEYF6	83.65 BinA	Sjap	92.36
	SJSORF9	83.65 BinA	Sjap	90.22
	SJASNM6	83.65 BinA	Sjap	94.20
	SJYYF11	83.65 BinA	Sjap	95.00
	SJYYM12	83.65 BinA	Sjap	97.28
Clonorchis sinensis	Cs2	85.78 BinD	Variation	–
Opisthorchis viverrini	Ov1	85.78 BinD	Variation	–
Fasciola hepatica	Fh6	86.30 BinE	Variation	–
Fasciola gigantica	Fg7	86.30 BinE	Variation	–
	NC		Variation	–

BinA-E was calculated automatically and the corresponding curve information refers to Fig. 2

The identity of each amplicon was further confirmed by sequencing (Sangong Biotech, China). The acquired sequences were aligned with those of published *Schistosoma* species from GenBankTM, and phylogenetic analyses (MEGA5.2) (Tamura et al. 2011) were conducted subsequently to verify the accuracy of the genotype results obtained by the established HRM analysis.

Assessment of specificity and sensitivity of the detection assay

To evaluate the specificity of the designed HRM assay, some of the common zoonotic trematodes which are genetically closely related to *Schistosoma* and used in previous studies (Zhao et al. 2010, 2012b), namely, *Fasciola hepatica*, *F. gigantica*, *Clonorchis sinensis*, and *Opisthorchis viverrini*, were chosen as "controls." For the sensitivity analysis, the concentration of DNA in each *Schistosoma* sample was determined using a spectrophotometer, and then tested in tenfold serial dilutions. The detection limit was determined as the smallest genomic DNA amount at which Ct value <30.

Validation of the HRM assay for identification and differentiation of isolates belongs to the four *Schistosoma* species

After optimization, 21 representative specimens representing four *Schistosoma* species from 15 geographical localities in seven endemic countries were randomly chosen to assess the capacity of the real-time PCR coupled with HRM assay (Table 1).

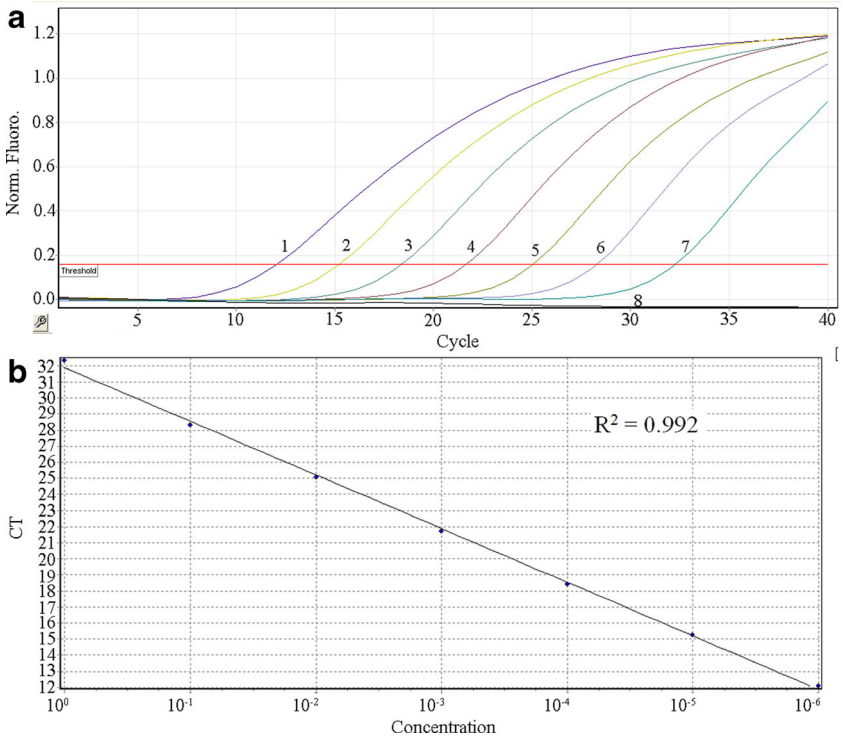

Fig. 3 Assessment of sensitivity of the HRM assay for detection of *Schistosoma* spp. **a** *Lanes 1–7* represent serial dilutions of genomic DNA of *S. japonicum*, 1–7: dilutions 1 to 10^{-6} ng, respectively. **b** A linear correlation is shown between the Ct and the tenfold diluted DNA of *Schistosoma* spp. *NC* negative control

Results and discussion

Alignment of sequences from *Schistosoma* spp. showed that the ribosomal 18S gene is highly conserved within a species but exhibits some consistent interspecific differences (Johnston et al. 1993). One pair of primers (M182U and M182D), based on conserved portions of the sequences of the V4 region of the 18S rDNA, generated an amplicon from all tested *Schistosoma* species that could be used for detection and specific identification of these. The primers did not amplify host DNA. Samples representing four *Schistosoma* species were randomly chosen to establish the standard reaction. All samples amplified a product of about 280 bp with a good Ct value around 10 to 20. Unique HRM profiles were produced for

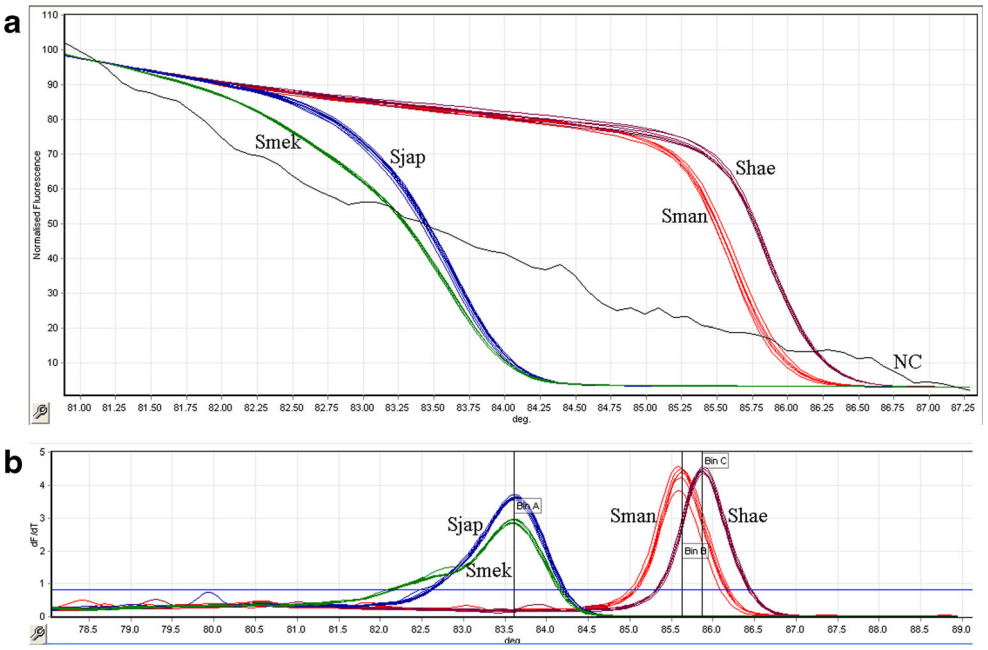

Fig. 4 Real-time PCR and HRM analyses for all *Schistosoma* spp. samples. **a** Normalized melting curves for *Schistosoma* spp. isolates. **b** Melting peaks of *Schistosoma* spp. Bin A includes *S. japonicum* and *S. mekongi*, Tm, 83.65 °C; Bin B includes *S. mansoni*, Tm, 85.65 °C; and Bin C includes *S. haematobium*, Tm, 85.85 °C. *Sjap S. japonicum*, *Smek S. mekongi*, *Sman S. mansoni*, *Shae S. haematobium*, *NC* negative control

each of the four *Schistosoma* species (Fig. 1). Nucleotide sequence analyses and phylogenetic analysis of the amplicons confirmed the identity of each species (not shown).

The specificity of the detection assay was examined by comparison with some other common zoonotic trematodes. Amplicons could be produced from all worm species using these primers, but visual assessment of the conventional and normalized curves could distinguish all species, with the genotypes of other trematodes represented as "variation" after analysis of the melt curve by the software (Fig. 2, Table 2). The assay sensitivity was assessed by using tenfold serial dilutions of predefined *Schistosoma* genomic DNA (recombinant plasmids). Besides, there was a good linear correlation coefficient (R^2=0.992) between the log concentrations of purified DNA (1–10^{-6} ng) and the Ct value (Fig. 3). The lower limit of detection was 10^{-5} ng (Fig. 3), which was four orders of magnitude more sensitive than conventional agarose electrophoresis (Li et al. 2011; Zhao et al. 2012b) and three orders of magnitude higher than polyacrylamide gel detection (Zhao et al. 2010). Our detection assay displayed a high degree of sensitivity and reproducibility.

To assess the capacity of HRM curve analysis for the detection and identification of specimens of different species, 21 individual specimens of *Schistosoma* from different endemic regions were analyzed: *S. mansoni* (n=3), *S. haematobium* (n=3), *S. mekongi* (n=4), and *S. japonicum* (n=11). Visual assessment of the conventional and normalized curves could distinguish all species (Fig. 4, Table 2) with high confidence values. No signal was detected in the negative controls. The melting curves were characterized by peaks of 83.65 °C for *S. japonicum* and *S. mekongi*, 85.65 °C for *S. mansoni*, and 85.85 °C for *S. haematobium* (Fig. 4). Although *S. japonicum* and *S. mekongi* melting peaks were identical to each other, their melting profiles are significantly different. In *S. mekongi*, a minor peak on the left shoulder of the major peak is a distinctive feature (Fig. 4). These are known to be closely related species from Asia (Chen et al. 2011, 2015), so the similarity is not surprising. *S. mansoni* and *S. haematobium* are both from Africa and rather distantly related to the Asian species (Chen et al. 2011; Zhao et al. 2012c).

Detection of characteristic eggs of parasitic worms, including *Schistosoma* species, is the "gold standard" for diagnosis. However, direct detection of eggs is not possible in prepatent infections, and difficult in low-endemicity areas, hence rendering specific diagnosis difficult especially in travelers and expatriates with mixed infections (Meltzer and Schwartz 2013).

In recent years, with the increased numbers of travelers and expatriates worldwide, the potential risk of *Schistosoma* spp. coinfection in endemic areas or discovery of infection in returning travelers and immigrants has attracted broad attention. Development of a rapid and easy screening method for identification and differentiation of species of blood flukes is a problem to be solved. Many PCR and real-time PCR methods (Kato-Hayashi et al. 2010; Cnops et al. 2012; Ten et al. 2008) have been previously reported but showed limitations for rapid interspecies identification. Marx et al. (2000) used a genome-wide melting curve scanning method to distinguish between *S. mansoni*, *S. japonicum*, and *S. haematobium*. Then, Kongklieng et al. (2013) made real-time PCR with high-resolution melting analysis to differentiate *S. japonicum* and *S. mekongi*. Compared with traditional melting curve analysis of Marx et al. (2000), the HRM detection assay established in the present study provides more information than just the calculated melting temperature (Tm), permitting differentiation on the basis of curve shape, even when they define the same Tm values (Jin et al. 2012). In addition, the detection method for two schistosoma species established by Kongklieng et al. (2013) showed limited detecting scale compared with the present study which made rapid detection and identification of four major *Schistosoma* species. Furthermore, the entire process from PCR to HRM curve analysis can be completed within 3 h, which is a great time saving.

In conclusion, the present study developed a real-time PCR coupled with HRM analysis assay for detection and differential identification of *S. mansoni*, *S. haematobium*, *S. japonicum*, and *S. mekongi*. This method has the advantage of being rapid, sensitive, and inexpensive. It has important implications for epidemiological studies and could be applicable to diagnosis of a wide range of microorganism species, especially when these are closely related.

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