

# NOVEL REAL-TIME PCR FOR DETECTION OF *SCHISTOSOMA JAPONICUM* IN STOOL

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**Abstract.** Chemotherapy has been used on a large scale in countries where the blood fluke *Schistosoma japonicum* is endemic. This has led to a lower intensity of infections and consequently lower diagnostic values of commonly used diagnostic tests like serology and Kato-Katz stool smear. We designed a novel real-time PCR method for detection of *S. japonicum* in stool samples. Further, we evaluated different versions of an inexpensive, non-commercial extraction method, ROSE, as well as the commercial QIAamp DNA Stool Mini Kit. PCR primer sequences were designed targeting the mitochondrial NADH dehydrogenase I gene. Bovine serum albumin was added to the DNA extracts and SYBR Green was used for detection. The PCR method was evaluated with non-infected stool samples spiked with *S. japonicum* eggs. It demonstrated high sensitivity, even in samples containing a single egg. The two extraction methods were equally effective. The PCR was specific for *S. japonicum* when tested against other *Schistosoma* species, *Trichuris trichiura*, hookworm and *Taenia* sp. We conclude that this novel real-time PCR, in combination with either ROSE or QIAamp DNA Stool Mini Kit extraction, is a sensitive and specific tool for diagnosing *S. japonicum* in human stool samples.

## INTRODUCTION

*Schistosoma japonicum* infections are endemic in China, the Philippines and within a small focus in Indonesia. An estimated 1.2-1.4 million people are infected, of whom 2/3 live in China (Chitsulo *et al*, 2000; Chen and Wang, 2005). Endemic countries have implemented extensive schistosomiasis control programs with large-scale chemotherapy being the main approach since praziquantel became widely available in the mid-1980s. This has resulted in a tremendous reduction in prevalence, thus converting large endemic areas into low transmission zones (Leonardo *et al*, 2002; Chen and Wang, 2005).

Some areas are now aiming for interruption of transmission or complete elimination of the pathogen. This new situation creates special challenges for control programs which were addressed by a recent WHO informal consultation (WHO, 2001). It recommended that algorithms for case detection and surveillance incorporated more sensitive high throughput and preferably inexpensive technologies.

Even when large-scale chemotherapy programs have been successfully applied, there is a need to diagnose human cases in order to eliminate the parasite reservoir and to assess further on-going transmission. A frequently used classification of infection intensity in intestinal schistosomiasis is: i) light  $\leq 100$  eggs per gram (epg) stool, ii) moderate 101-400 epg, and iii) heavy  $> 400$  epg (WHO, 1993). It can be difficult to reach a correct diagnosis when the prevalence and intensities of the infections are low. The two diagnostic strategies commonly used, the Kato-Katz stool smear and antibody detection in serum, both demonstrate serious shortcomings under such epidemiological conditions. The Kato-Katz stool smear is a simple, cheap and quantitative method. However, due to the small

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The results were in part presented at DSP-DSTMH Spring Symposium, Copenhagen, Denmark, March 2005 and XVIth International Congress for Tropical Medicine and Malaria, Marseille, France, September 2005.

amount of stool examined (less than 50 mg), the sensitivity is low in light and moderate infections (Sleigh *et al*, 1982). Yu *et al* (1998) demonstrated that the low sensitivity can be a problem when Kato-Katz stool smear is used for the diagnosis of *S. japonicum* infections in China. When serology is used, the target population is typically tested every one to three years. A common criticism is that antibody tests remain positive long after successful treatment is completed and thus act as false positive infections (Rabello *et al*, 1997; Whitty *et al*, 2000).

The polymerase chain reaction (PCR) is a diagnostic tool with potentially high sensitivity and specificity (Bell and Ranford-Cartwright, 2002). The aim of the present study was to develop a SYBR<sup>®</sup> Green based real-time PCR for detection of *S. japonicum* in stool samples. The assay was evaluated in conjunction with a commercial extraction kit as well as a non-commercial extraction method.

## MATERIALS AND METHODS

### Sample preparation

Stool samples were spiked with eggs from an *S. japonicum* strain originating from Anhui Province, Peoples Republic of China (Sørensen *et al*, 1999). The strain was maintained in *Oncomelania hupensis* snails and in mice at DBL-Institute for Health Research and Development (DBL), Charlottenlund, Denmark. Eggs were recovered from mice livers and counted using glass pipettes and a stereo microscope, and subsequently transferred to either 2 ml Micro tubes (Sarstedt, Nümbrecht, Germany) for the non-commercial ROSE extraction procedure, or 14 ml Falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA) for extraction with the commercial QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). One gram of human stool ( $\pm$  0.1 g) was added to each tube before the samples were spun down and stored at -70°C until use.

Stool samples from patients containing eggs from different helminths, submitted to the Microbiology Department, University Hospital of North Norway for parasitological examination, were collected. The helminth eggs were identified to species or genus level by microscopy and

the samples frozen at -70°C until extraction with the ROSE-B method.

The following strains of adult *Schistosoma* worms were used after being stored in 70% ethanol: *S. japonicum*-Anhui, the same strain as described above; *S. japonicum*-Zejiang, isolated 1937 from Kashing, Zejiang Province, China; *S. haematobium*, collected in Lake Malawi 2001; *S. bovis*, collected in Iringa, Tanzania 1995; and *S. mansoni* from Brazil.

### DNA extraction

ROSE extraction was performed as described by Pontes *et al* (2003), with minor modifications. The method was evaluated in two alternative versions, without (ROSE-A) and with ethanol precipitation (ROSE-B). One ml of water was added to each of the one gram stool samples. The samples were then vortexed, centrifuged at 500g for two minutes, and the resulting supernatants were discarded. This step was repeated once except that the samples were shaken for 10 minutes by hand or by mechanical shaker. The final pellets were thoroughly mixed with 0.7 ml of ROSE buffer containing 10 mM Tris (pH 8.0), 300 mM EDTA (pH 8.0), 1% w/v sodium dodecyl sulfate (SDS) and 1% w/v polyvinylpyrrolidone (PVPP). The samples were heated to 95°C for 20 minutes and subsequently centrifuged at (18,000g) for ten minutes. Then 100  $\mu$ l and 500  $\mu$ l aliquots of the supernatant were distributed into two new tubes. The 100  $\mu$ l eluate was, unless otherwise stated, diluted 1:170 with distilled water prior to amplification (ROSE-A). The 500  $\mu$ l eluate was subjected to standard ethanol precipitation (Sambrook and Russell, 2001). In brief, 50  $\mu$ l of cold sodium acetate (3 M, pH 5.3) were added and the solution mixed. One ml of ice-cold 96% ethanol was added, the solution mixed again and left on ice for 15 minutes before centrifugation at 18,000g for 10 minutes. After carefully removing the supernatant, 500  $\mu$ l of ice-cold 70% ethanol were added, the sample recentrifuged at 18,000g for two minutes and the supernatant once more carefully removed. The pellet was dried at 37°C for 15 minutes. Resuspension in 100  $\mu$ l of TE-buffer (Ambion, Austin, Texas, USA) was facilitated by heating the mixture to 60°C for 5 minutes. The dissolved pellet was diluted

1:100 in distilled water unless otherwise stated (ROSE-B).

ROSEX buffer was made by adding 5% w/v Chelex 100 (Sigma-Aldrich, St Louis, MO, USA) to ROSE buffer (Zimmermann *et al*, 1998) but was otherwise identical to the corresponding ROSE-A and -B extraction protocols.

Extraction by the QIAamp DNA Stool Mini Kit was done according to the instructions from the manufacturer with one gram stool samples. The protocols "DNA Isolation from Larger Amounts of Stool" and "Isolation of DNA from Stool for Pathogen Detection" were used. A lysis temperature of 70°C was used unless otherwise stated. Bovine serum albumin (BSA) (Sigma-Aldrich) was added to all DNA extracts from stool samples to a final concentration of 0.1 µg/ml unless otherwise stated.

DNA from adult *Schistosoma* worms was extracted by an automated GenoM-48 robot (GenoVision, Philadelphia, USA), using the MagAttract DNA Mini M48 Kit (Qiagen) and the standard tissue protocol. In order to calculate the amplification efficiency of the PCR reaction, DNA from miracidia (*S. japonicum*-Anhui) in physiological saline was extracted using the same procedure as for adult worms. Serial 1:10 dilutions were made from the DNA extract and PCR results were plotted as a standard curve.

#### Real-time PCR

The PCR was designed to target the mitochondrial NADH dehydrogenase I gene. Sequences from nine *S. japonicum* strains were used, all published in GeneBank (Sørensen *et al*, 1998, 1999). Three of the sequences originated from laboratory strains whereas six were from wild type isolates, and together they represented seven different geographical areas in China and the Philippines. After alignment and BLAST search, two primer sets were selected using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA, USA). After preliminary tests, the primer set SjND1FW (forward: 5' TGR TTT AGA TGA TTT GGG TGT GC3') and SjND1RV (reverse: 5' AAC CCC CAC AGT CAC TAG CAT AA3') was selected for further studies. SjND1FW was designed as a degenerated primer, with half of the primers containing A and half G in position 3, since one strain (*S.*

*japonicum*-Sichuan) had a C→T replacement at the primer binding site. The 82 base-pair (bp) target sequence was otherwise identical in all nine strains. PCR conditions were optimized according to Applied Biosystems guidelines (Primer Express software version 2.0). Several thermal cycler protocols with various annealing temperatures and elongation times were examined. The simplest and most flexible protocol giving the best yield was 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, closing with a dissociation stage (60-95°C over 20 minutes). The reaction mixture contained 1 pmol of each primer, 5 µl of extracted DNA, 10.2 µl of 2x PCR Mastermix for SYBR® Green I Assays (Eurogentec, Seraing, Belgium) and distilled water to a total volume of 25 µl. No-template controls (NTC) contained water instead of extracted DNA. The assays were performed on an ABI PRISM 7900 HT (Applied Biosystems).

#### Detection and quantification

Detection of results in real-time format was done by the thermal cycler software Sequence Detection Systems Software v2.2.1 (Applied Biosystems). It provides an amplification plot as a log plot of SYBR® Green fluorescence against cycle number. In the amplification plot, the fluorescence threshold was set at 0.1 and the manual baseline set at default, 3-15 cycles. The intersection of the increasing fluorescence signal and the fluorescence threshold gave the cycle threshold (Ct) value. The dissociation curve or melting point analysis was performed subsequent to amplification in order to monitor the sequence-dependent melting temperature of the DNA product. Melting point analysis confirmed the melting temperature to be approximately 72°C. A PCR reaction was considered positive only if the fluorescence signal was above the fluorescence threshold and the melting point was in the correct range. PCR reactions were carried out in triplicate from every stool sample. A sample was regarded as positive when at least one reaction was positive. PCR amplicons were also visualized on 2.5% agarose gel as an alternative to the real-time format.

In order to determine whether the assay could be used quantitatively, a standard curve

was prepared by extracting two one-gram stool samples containing 1,000 eggs and no eggs, respectively. Serial 1:10 dilutions were made by diluting extract from the egg-containing sample with extract from the sample without eggs. PCR reactions were run in triplicate for each dilution, and the geometric mean Ct value of each triplet was used in a standard curve. A set of stool samples containing various numbers of eggs (1 egg, 5 egg, 10 egg, 50 egg and 100 egg) were extracted in the same way as the standard curve using ROSE-B extraction method. The geometric mean of the Ct values for each triplet was used to find the predicted number of eggs according to the standard curve.

**Statistical analysis**

Comparisons of ROSE-B extraction with and without BSA by chi-square, as well as the confidence intervals (CI) in the standard curve, were calculated by the SPSS software, version 12.0.1 for Windows (SPSS Inc, Chicago, IL, USA).

**RESULTS**

**Evaluation of extraction methods**

Fig 1 shows the results of 15 stool samples extracted in parallel by ROSE-A and ROSE-B, and with each alternative also tested with and without the addition of BSA. Each sample was run in triplicate. The figure demonstrates that the ROSE-B extraction procedure (with ethanol precipitation) was superior to ROSE-A (without ethanol). The difference between ROSE-B with or without BSA was less clear. One sample was positive in all three reactions with BSA and negative without, whereas the overall difference between 37 and 40 positive reactions was not significant (chi-square = 0.809, p = 0.37).

The addition of Chelex 100 in the ROSEX protocol showed no advantage compared to ROSE extraction, neither with extraction alternatives A or B nor with or without BSA (data not shown). The addition of water to DNA extracts (1:170 and 1:100, respectively) is commonly used to dilute inhibitors of the PCR reaction. Dilution proved essential for all amplifications regardless of the extraction protocol employed

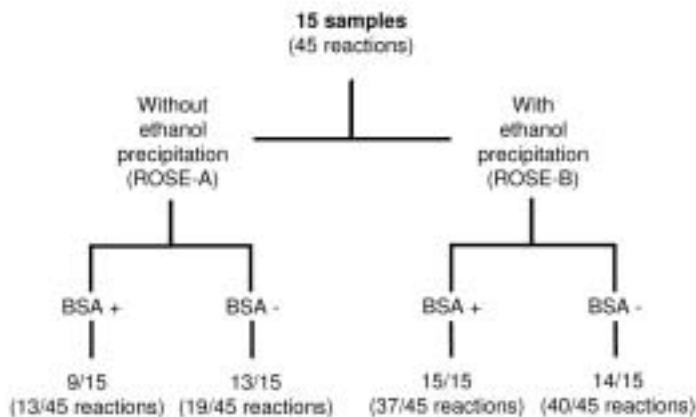


Fig 1—Number of positive samples / total number of samples in alternative ROSE extraction protocols. Each one-gram stool sample was spiked with various number of eggs per gram (10 egg n = 4, 5 egg n = 7, 1 egg n = 4), and PCR reactions were performed in triplicate for each sample.

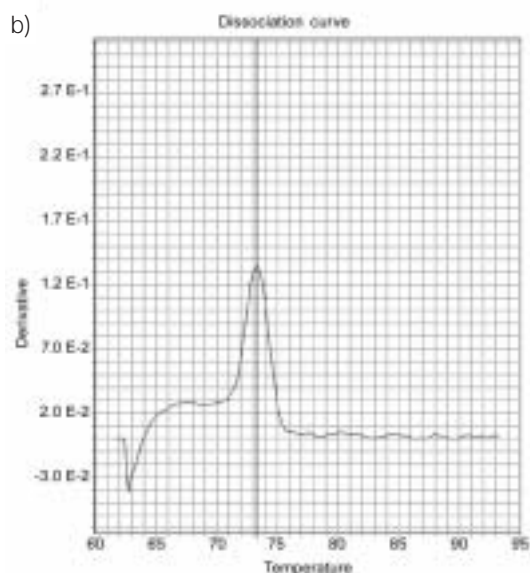
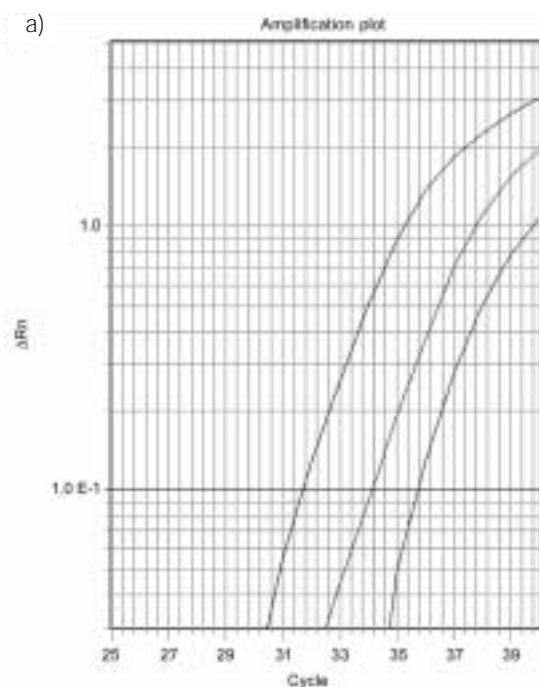
Table 1

The PCR results of one-gram stool samples, spiked with various number of *Schistosoma japonicum* eggs, after ROSE-B extraction (n = 30) or QIAamp DNA Stool Mini Kit extraction (n = 25).

Number of eggs per one gram of sample	Number of positive samples/ total number of samples	
	ROSE extraction	QIAamp extraction
>1,000	1/1	
500		3/3
100	4/4	4/4
50	2/2	2/2
10	7/7	2/2
5	7/7	6/8
1	8/9	6/6
Total	29/30	23/25

(ROSE-A, ROSE-B or ROSEX, with or without BSA) (data not shown).

We found that increasing the lysis temperature from 70°C to 90°C, as suggested in the QIAamp DNA Stool Mini Kit handbook, offered no advantage when it was used on samples containing 5 egg (n = 3) and 50 egg (n = 2).



#### Efficiency, specificity and sensitivity of real-time PCR

The slope ( $s$ ) of the standard curve, made from miracidial eluates, was  $-3.59$ , which by the equation,  $\text{Efficiency} = 10^{-s} - 1$  (Rutledge *et al*, 2003), gave a slope-derived estimate of amplification efficiency of 90%. Patient stool samples containing *S. mansoni* ( $n = 3$ ), hookworm ( $n =$

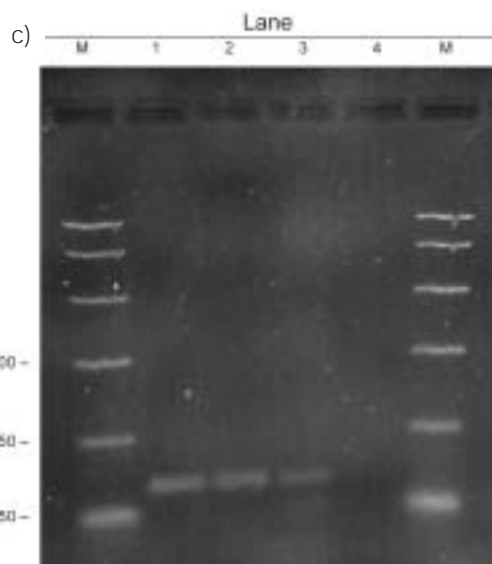


Fig 2—Results after PCR for stool samples containing 100 eggs [left curve(a) and lane 1(c)], 10 eggs [middle curve(a) and lane 2(c)] and 1 egg [right curve(a), curve(b) and lane 3(c)]. Fig 2a is the real-time amplification plot. Fig 2b is the dissociation curve or melting point analysis. Figure 2c shows the PCR amplicons on agarose gel. Lane M= PCR marker, fragments with 50, 150 and 300 base-pair size are indicated. Lane 4= no-template control.

2), *Trichuris trichiura* ( $n = 1$ ) and *Taenia* sp ( $n = 1$ ), tested negative. When extracts from adult *Schistosoma* worms were tested, both the *S. japonicum*-Anhui and the *S. japonicum*-Zhejiang strains were positive, whereas *S. mansoni*, *S. haematobium* and *S. bovis* were negative.

The sensitivity of the real-time PCR in stool samples containing various concentrations of *S. japonicum* eggs is shown in Table 1. The results with both ROSE-B and QIAamp DNA Stool Mini Kit extraction are shown. PCR reactions were carried out in triplicate for each sample. In samples containing 10 egg or more, 40/42 (95%) of the PCR reactions were positive with ROSE-B extraction and 33/33 (100%) with QIAamp. In positive samples containing less than 10 egg, 35/45 (78%) of the reactions were positive with ROSE-B and 33/39 (85%) with QIAamp extraction. Fig 2a-c shows an example of an amplifi-

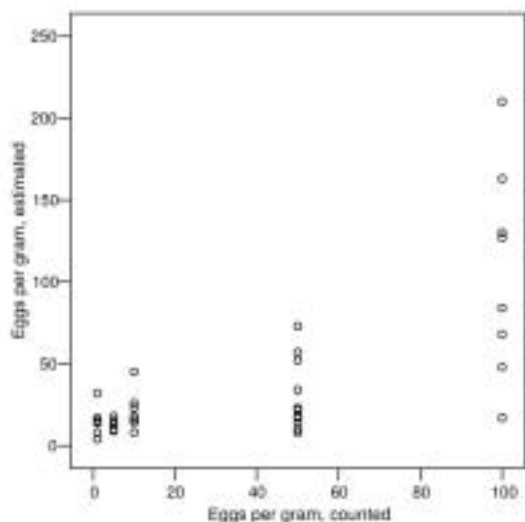


Fig 3—Plot of 40 stool samples with actual egg counts against the numbers of eggs estimated by using a standard curve.

cation plot, a dissociation curve and PCR amplicons on an agarose gel. The bands on the gel are compatible with the predicted 82 bp product.

#### Quantification

Fig 3 shows the correlation between actual egg counts and standard curve estimated number of eggs in the same samples. The mean estimated egg for all samples with 1-50 counted eggs together is 21 (95% CI: 15-27). The mean estimated egg for samples with 100 counted eggs is 106 (95% CI: 53-159).

## DISCUSSION

In this study we present a novel SYBR<sup>®</sup> Green based real-time PCR for detection of *S. japonicum* in stool samples. We chose PCR primers based on *S. japonicum* sequences from seven different geographical areas. The PCR was specific for *S. japonicum* when it was tested on different *Schistosoma* species and negative for three commonly occurring intestinal helminths. We used one-gram stool samples, spiked with various numbers of eggs, to test the sensitivity of the PCR and found it to be high, even in samples spiked with a single egg.

A cheap, non-commercial extraction method,

ROSE-B, gave results comparable to the commercial extraction kit QIAamp DNA Stool Mini Kit.

PCR as a tool for diagnosing *S. mansoni* has been evaluated by Pontes *et al* (2002). In a clinical trial they found the prevalence of infection to be 30.9% when three stool samples were examined by Kato-Katz, whereas 38.1% were positive in a single sample using PCR (Pontes *et al*, 2003). They used conventional PCR with polyacrylamide gel to detect the product from a tandemly repeated DNA sequence from the schistosomal genome (Hamburger *et al*, 1991). Their PCR product was of varying lengths due to multiples of the repeated DNA sequence, hence making it unsuitable for SYBR<sup>®</sup> Green detection in real-time PCR. We designed a PCR targeting the mitochondrial NADH dehydrogenase I gene. The mitochondrial genome has the advantage of being present in several copies in each cell, thus potentially increasing the sensitivity of the assay.

We have shown that the product from the present PCR also can be detected by agarose gel if real-time PCR is not available. However, there are several advantages of using PCR in a real-time format: i) increased speed and a potential for high throughput; ii) elimination of post PCR handling, thus decreasing the risk of contamination; iii) possible quantification without extra operations.

Creating a quantitative test was not the primary goal of this study and Fig 3 demonstrates a wide range in estimated values for each category of counted eggs. This might be due to different levels of inhibition or varying numbers of target copies in the eggs. The assay seems to be inadequate to quantify egg concentrations accurately on an individual level. When the samples were stratified into groups of 1-50 egg and 100 egg, we found a statistically significant difference between estimated mean values. A semi-quantitative gradation of the concentration of eggs present in stool samples is usually sufficient for clinical purposes and is useful for monitoring the burden of disease on a community level. However, all samples tested here resemble light infections according to the WHO classification. Samples containing a wider range of egg concentrations must be included if this PCR is

to be fully evaluated as a quantitative or semi-quantitative test. Our results suggest that the PCR could be used as a semi-quantitative method.

Cheap, non-commercial extraction methods for stool samples are rarely evaluated. Steiner *et al* (1995) introduced the Rapid One-Step Extraction (ROSE) buffer, and modifications of the method have later been evaluated (Zimmermann *et al*, 1998; Pontes *et al*, 2003). We investigated several variants of the ROSE extraction protocol. In our experience, the best results were achieved when extraction with ROSE buffer was followed by ethanol precipitation (ROSE-B) and a 1:100 dilution of the DNA eluate.

Stool samples are challenging in PCR due to the high content of inhibitory substances. Addition of bovine serum albumin to the PCR mixture has been shown to increase amplification efficiency in such cases (Kreder, 1996; Abu Al-Soud and Padstrom, 2000). However, in our study the advantage of BSA was less obvious, but we have occasionally seen samples being positive with BSA and negative without it, and that addition of BSA repeatedly resulted in more well-defined dissociation curves after extraction with ROSE-B and QIAamp. We therefore recommend adding BSA to the eluate for both extraction methods.

Several aspects need further investigation: i) whether inhibition of the PCR becomes a problem when it is used on a wider spectre of stool samples; ii) further testing of specificity, especially towards intestinal flukes; iii) how the test performs post treatment. The price of the assay as well as the availability of equipment is also an issue, even though we tried to keep the operating costs as low as possibly by evaluating a non-commercial extraction method and by using SYBR<sup>®</sup> Green for detection. On the other hand, PCR equipment and knowledge of how to use it is spreading, also in countries where *S. japonicum* is endemic. In certain situations, when a diagnostic test with high sensitivity is needed, PCR can be a useful tool. This may include epidemiological studies and studies aiming at evaluating other diagnostic tests. Furthermore, it may be used as a confirmative test in antibody posi-

tive subpopulations.

Further studies are warranted in order to evaluate this PCR method, including studies in animal models and community based studies in *S. japonicum* endemic areas.

## ACKNOWLEDGEMENTS

Rasmus Goll and Anne Mette Asfeldt are thanked for their contribution to the manuscript preparation. Susanne Kronborg from DBL – Institute for Health Research and Development is thanked for supplying eggs and worms.

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