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Multiplex real-time PCR for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* infection in stool samples collected in northern Senegal

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Summary A multiplex real-time PCR assay for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* DNA in faecal samples was developed and evaluated as an alternative diagnostic method to study the epidemiology of schistosomiasis. Primers and probes targeting the cytochrome c oxidase gene were designed for species-specific amplification and were combined with an internal control. Using positive control DNA extracted from adult *Schistosoma* worms and negative control samples ($n = 150$) with DNA from a wide range of intestinal microorganisms, the method proved to be sensitive and 100% specific. For further evaluation, duplicate stool specimens with varying *S. mansoni* egg loads were collected in northern Senegal from pre-selected individuals ($n = 88$). The PCR cycle threshold values, reflecting parasite-specific DNA loads in faeces, showed significant correlation with microscopic egg counts both for *S. mansoni* in stool and *S. haematobium* in urine. The *Schistosoma* detection rate of PCR (84.1%) was similar to that of microscopy performed on duplicate stool samples (79.5%). The simple faecal sample collection procedure and the high throughput potential of the multiplex real-time PCR provide a powerful diagnostic tool for epidemiological studies on schistosomiasis in remote areas, with possibilities for extension to other helminths or protozoa using additional molecular targets.

1. Introduction

Worldwide, an estimated 200 million people are infected with *Schistosoma* spp., causing schistosomiasis or bilharzia

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(Gryseels et al., 2006). On the African continent the two most common species are *Schistosoma mansoni* and *S. haematobium*. Epidemiological studies on schistosomiasis traditionally rely on the detection of parasite eggs in stool (*S. mansoni*) or urine (*S. haematobium*) by microscopy. These methods are relatively inexpensive, easy to perform and provide information on prevalence and intensity of *Schistosoma* infections (Feldmeier and Poggensee, 1993). However, they also have a number of drawbacks. Samples need to be processed within 24—48 h and the processing itself can be rather tedious. Moreover, multiple sampling is needed to obtain an accurate impression of the presence and intensity of infection.

As an alternative for detecting *S. mansoni* infections, antigen-based assays, such as circulating cathodic antigen detection in urine, have proven to be a valuable, field-applicable method. Unfortunately, the test has been shown to be less sensitive for infections with *S. haematobium* (van Dam et al., 2004). *Schistosoma*-specific antibody detection is considered to be highly sensitive, but this method cannot distinguish active from past infection. Blood collection is not easily applicable under field conditions, therefore this method is not recommended for field studies in endemic areas (Gryseels et al., 2006).

PCR-based methods have shown high sensitivity and specificity for the detection of parasitic DNA, yet their use in epidemiological surveys has so far been limited. For a long time, DNA isolation and subsequent DNA amplification was known to be laborious and expensive. However, recent developments in the simplification of DNA isolation procedures and PCR technology, especially real-time PCR, have made DNA amplification a worthy alternative to microscopy-based diagnostic methods (Espy et al., 2006; Klein, 2002; Verweij et al., 2004, 2007a).

To date, conventional PCR methods for the detection of *Schistosoma* DNA in human samples have been published in which the rRNA gene [small subunit (SSU) rRNA], a highly repeated 121 bp sequence of *S. mansoni* or mitochondrial genes (*nad5*, *nad6* and *cox*2) were used as targets (Pontes et al., 2002, 2003; Sandoval et al., 2006). More recently, a real-time PCR using SYBR Green dye for the detection of *S. mansoni* has been published targeting a small fragment of 96 bp on the SSU rRNA gene (Gomes et al., 2006). Although high sensitivity on control DNA was achieved, this real-time PCR only detected *S. mansoni* DNA and did not include an internal control.

The present study describes a real-time PCR for specific detection and quantification of *S. mansoni* DNA in faecal samples as an alternative to microscopy in epidemiological research. Since eggs of *S. haematobium* can also be found in the rectal wall (Azar et al., 1958), a combined real-time PCR was developed to detect additionally *S. haematobium*-specific DNA in faeces using primers and probes targeting the cytochrome c oxidase subunit 1 (*cox*1) gene in the mitochondrial genome. The PCR assay was designed on the *cox*1 gene because the DNA sequences show sufficient divergence between separate *Schistosoma* species whilst a very low level of variation is expected within isolates collected in the same geographical region (Le et al., 2000; Morgan et al., 2005). Additionally, an internal control for the detection of possible inhibition of amplification by faecal contaminants was included in the assay.

The performance of the multiplex real-time PCR assay was evaluated using a range of DNA controls as well as stool samples collected in a remote area of northern Senegal, where both *S. mansoni* and *S. haematobium* are endemic.

2. Materials and methods

2.1. Controls

Positive control DNA was extracted from adult *S. mansoni* and *S. haematobium* worms and eggs derived from infected hamsters as well as from human faecal samples confirmed positive for *S. mansoni* by microscopy.

The specificity of the multiplex PCR was tested using: (i) DNA isolated from individual adult worms of *Trichuris trichiura*, *Ascaris lumbricoides*, *Necator americanus*, *Opisthorchis felineus* and *Fasciola hepatica* and DNA of *Strongyloides stercoralis* first-stage larvae and *Ancylostoma duodenalis* third-stage larvae. Furthermore, *Entamoeba histolytica*, *Entamoeba dispar*, *Dientamoeba fragilis*, *Giardia lamblia*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* DNA was tested in the PCR (Verweij et al., 2007a); (ii) DNA obtained from 15 different bacterial/yeast cultures of *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Enterobacter aerogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, *Shigella flexneri*, *Proteus mirabilis*, *Candida albicans*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella boydii* and *Campylobacter upsaliensis*; and (iii) 80 DNA extracts from stool specimens that were positive by microscopy and confirmed by species-specific PCR for *E. histolytica* ($n = 20$), *E. dispar* ($n = 20$), *G. lamblia* ($n = 20$) and *C. parvum* ($n = 20$). The PCR assay was also tested on 40 unpreserved stool samples from individual patients with a negative result in microscopy using formalin—ether sediments and modified acid-fast staining as well as a negative *Giardia* antigen test. Two subsequent stool samples of these 40 patients also tested negative by microscopy and *Giardia* antigen test.

2.2. Field samples

For evaluation of the multiplex PCR, 176 stool samples from 88 subjects (age range 2—83 years, median 20 years; 58% female) were collected over a 2-week period in August 2006 in a village in northern Senegal. The village is endemic for both *S. mansoni* and *S. haematobium* (de Clercq et al., 1999) and no mass treatment programme with schistosomicides had taken place in the village in recent years. Participants were selected on delivery of at least two stool and two urine samples and representing a wide range of egg excretions based on microscopic analysis of the first stool sample. Within 24 h after production, faecal suspensions ($\cong 0.25$ g/ml) were prepared in 70% ethanol. No major abnormalities, such as bloody appearance, were observed in stool consistency. Suspensions were stored and transported at room temperature until further processing was performed at the Laboratory for Parasitology, Leiden University Medical Centre, The Netherlands, within a couple of weeks after collection.

Table 1 Oligonucleotide primers and detection probes for real-time PCR for the simultaneous detection of *Schistosoma mansoni* and *S. haematobium* as well as phocin herpes virus 1 (PhHV-1) as an internal control

Target organism/oligonucleotide name	Oligonucleotide sequence	GenBank accession no. or literature reference of target sequence
Schistosoma mansoni		
Smcyt748F	5′-CCCTGCCAAATGAAGAGAAAAC-3′	NC_002545
Smcyt847R	5′-TGGGTGTGGAATTGGTTGAAC-3′	
Smcyt785T	FAM-5′-CCAAAACCAGACCCCTCTCAAATTG-3′-BHC1	
Schistosoma haematobium		
Sh307F	5′-CCTCCATTATCCATATCTGAGAATTCA-3′	AY157209
Sh447R	5′-AGAAGTCTTAAAATCGACACGACTAATAATC-3′	
Shaem377MGB	NED-5′-ACCAACTAGTCTAGATACAC-3′-MGBNFQ	
PhHV-1		
PhHV-267s	5′-GGGCGAATCACAGATTGAATC-3′	Niesters, 2002
PhHV-337as	5′-GCGGTTCCAAACGTACCAA-3′	
PhHV-305tq	Cy5-5′-TTTTTATGTGTCCGCCACCATCTGGATC-3′-BHQ2	

2.3. Microscopy

For *S. mansoni*, egg excretion was determined by duplicate 25 mg Kato examinations on each stool sample (Katz et al., 1972). Intensities of infection were expressed as eggs per gram of faeces (EPG). For *S. haematobium*, eggs were quantified using the urine filtration method (Peters et al., 1976). Results were expressed as eggs per 10 ml of urine (EP10 ml).

2.4. DNA isolation

For isolation of DNA, 250 μl of ethanol—faeces suspension was centrifuged. The pellet was washed twice with PBS and finally suspended in 200 μl of PBS containing 2% polyvinylpolypyrrolidone (Sigma, Steinheim, Germany). The suspension was heated for 10 min at 100 °C and following sodium dodecyl sulphate—proteinase K treatment (2 h at 55 °C) DNA was isolated using QIAamp Tissue Kit spin columns (QIAgen, Hilden, Germany) (Verweij et al., 2001). Within the isolation lysis buffer, 10^3 plaque-forming units/ml of phocin herpes virus 1 (PhHV-1) was added to serve as an internal control (Niesters, 2002).

2.5. PCR amplification and detection

Schistosoma mansoni- and *S. haematobium*-specific PCR primers and detection probes were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) on the *cox*1 sequence (GenBank accession numbers **NC_002545** and **AY157209**, respectively). For detection of *S. mansoni*-specific DNA, primers Smcyt748F and Smcyt847R were selected to amplify a fragment of 99 bp, which is detected by the double-labelled probe Smcyt785T (Biolegio, Nijmegen, The Netherlands). For detection of *S. haematobium*-specific DNA, primers Sh307F and Sh447R were selected to amplify a fragment of 143 bp, which is detected by the Minor Groove Binding (MGB) TaqMan probe Shaem377MGB (Applied Biosystems, Warrington, UK). For an internal control, the PhHV-1-specific primers and probe set consisted of forward primer PhHV-267s, reverse primer PhHV-337as and the specific double-labelled probe PhHV-305tq (Biolegio) (Niesters, 2002). All primers and detection probes are described in Table 1.

For amplification of DNA extracted from individual worms, eggs or stool specimens, 5 μl of eluted DNA was used as a template in a final volume of 25 μl with PCR buffer (HotStarTaq Master Mix; QIAgen), 2.5 μg of bovine serum albumin (Roche Diagnostics Nederland B.V., Almere, The Netherlands), 5 mM $MgCl_2$, 2.0 pmol of each *S. mansoni* primer, 5.0 pmol of each *S. haematobium* primer, 3.75 pmol of each PhHV-1-specific primer, and 2.5 pmol of *S. mansoni*, *S. haematobium* and PhHV-1 detection probes. Amplification consisted of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification, detection and data analysis were performed with the Applied Biosystems 7500 Real Time PCR System and Sequence Detection Software version 1.2.2.

Serial 10-time dilutions of *S. mansoni* and *S. haematobium* control DNA were used to optimise the conditions for DNA amplification. Furthermore, each dilution series was tested for cross-reaction between the two species and to assess the ability to detect mixed infections.

2.6. Data analysis

Results of microscopy and real-time PCR analysis were stored in a Microsoft Access database (Microsoft, Redmond, WA, USA). For statistical analysis, data were entered in SPSS 11.0.1 (SPSS Inc., Chicago, IL, USA).

Intensity of infection as determined by the cycle threshold (Ct) value of the real-time PCR (reflecting faecal parasite-specific DNA loads) or microscopy (i.e. EPG or EP10ml) were described for the first and second stool and urine samples separately. Data were depicted as number, range and median value of positive subjects. Analysis agreement between the subject's outcome by real-time PCR analysis and microscopy was expressed using the κ statistic, considering a subject as positive for a diagnostic test when at least one of the

Table 2 Number (%) of positive cases and detected values (range and median of positive cases) of 88 subjects showing *Schistosoma mansoni* eggs in stool by Kato[a] or *S. haematobium* eggs by urine filtration technique[b] compared with the number and detected values of subjects showing *S. mansoni* or *S. haematobium* DNA in stool (Ct values) by real-time PCR

Examination[c]	No. (%) of positive cases	Range	Median
1st Kato	64 (73)	20—5580	200
2nd Kato	53 (60)	20—9280	180
Both Katos	70 (80)	15—7080	128
1st urine	55 (63)	1—2180	14
2nd urine	48 (55)	1—596	23
Both urines	63 (72)	1—1093	17
1st *S. mansoni* PCR	61 (69)	21.1—38.5	27.1
2nd *S. mansoni* PCR	53 (60)	21.7—38.4	25.8
Both *S. mansoni* PCRs	64 (73)	21.9—39.5	27.6
1st *S. haematobium* PCR	32 (36)	28.9—45.9	37.2
2nd *S. haematobium* PCR	39 (44)	29.1—44.1	40.7
Both *S. haematobium* PCRs	48 (55)	29.0—47.5	41.2

[a] Eggs per gram of faeces.
[b] Eggs per 10 ml of urine.
[c] Stool and urine examination of first and second sample, and both samples, where a case is considered positive if in at least one of the two samples eggs or DNA were detected. For further explanation see the Materials and Methods section.

duplicate stool or urine samples shows a positive outcome.

In addition, the mean number of egg counts of the subject's duplicate stool or urine samples was calculated as well as the mean Ct value of the two stool samples. For this purpose, negative values were redefined with a value representing an infection just below the detection limit of the diagnostic test used, i.e. one-half the detection limit in the case of microscopy and 3 Ct values above the highest detected Ct value for real-time PCR, i.e. a Ct of 42 and 49 for the *S. mansoni* and *S. haematobium* PCRs, respectively.

Spearman's non-parametric correlation coefficient (ρ) was used to calculate concordance in intensity of infection as determined by microscopy and real-time PCR, respectively. Statistical significance was considered at $P < 0.05$.

3. Results

The real-time PCR was optimised first as a monoplex with 10-time dilution series of *S. mansoni* and *S. haematobium* DNA. Thereafter, monoplex real-time PCRs were compared with the multiplex PCR with *S. mansoni* and *S. haematobium* assays combined with PhHV-1 internal control. The Ct values obtained from testing the dilution series of *S. mansoni* and *S. haematobium* in the individual assays and the multiplex assays showed no systematic deviation in amplification curves, and the same analytical sensitivity was achieved. One hundred femtograms of both *S. mansoni* and *S. haematobium* DNA could be detected, also in the presence of 1 ng of DNA of the other *Schistosoma* species or internal control.

The specificity of the multiplex real-time PCR was evaluated using 150 DNA controls derived from a wide range of intestinal microorganisms as described in Section 2.1. Amplification of *Schistosoma* DNA was not detected in any of these samples; only amplification of the internal control was detected at the expected Ct of approximately 33.

For a more elaborate evaluation of the multiplex real-time PCR, a selection of duplicate stool and urine samples from 88 subjects were used. Microscopic and PCR results are summarised in Table 2. The numbers of subjects showing *S. mansoni* eggs in stool and *S. haematobium* eggs in urine were 70 (80%) and 63 (72%), respectively. In 49 subjects (56%) both *Schistosoma* species were detected, and 4 subjects (5%) showed no *Schistosoma* egg excretion. Amplification and detection of the PhHV-1 internal control was within the range of expected Ct values in all faecal samples (median 32.7). Specific amplification was detected for *S. mansoni* in 64 subjects (73%) and for *S. haematobium* in 48 subjects (55%). Specific amplification of both *S. mansoni* and *S. haematobium* was detected in 38 subjects (43%), and 14 subjects (16%) showed no DNA amplification for either of the two *Schistosoma* species. Statistical analysis showed a κ value of 0.44 between the detection of *S. mansoni* infection by microscopy and real-time PCR ($n = 88$; $P < 0.001$).

Table 3 shows the PCR results per *S. mansoni* egg count class. Discrepancies between the outcome of microscopy and PCR only occurred in subjects with very low egg counts or when no eggs were observed. *Schistosoma mansoni*-specific amplification was detected in all subjects with high, moderate and low *S. mansoni* egg counts. Additionally, *S. mansoni* PCR-positive samples showed a decreasing median Ct value with increasing egg count category, and an overall significant correlation was found between egg counts (EPG) and Ct values ($\rho = -0.77$; $n = 88$; $P < 0.001$).

A κ value of 0.31 was shown between the outcomes of microscopy performed on urine and *S. haematobium* DNA detection in stool ($n = 88$; $P < 0.005$). *Schistosoma haematobium*-specific amplification in faecal DNA samples was detected in 17 (89%) of 19 subjects in whom more than 50 *S. haematobium* EP10ml were detected (Table 3). The discrepancy between real-time PCR and microscopy increased when fewer than 50 *S. haematobium* EP10ml urine were detected. Nevertheless, a decreasing median Ct value of *S. haematobium* PCR-positive samples is observed

Table 3 Comparison of *Schistosoma mansoni*- and *S. haematobium*-specific real-time PCR performed on DNA isolated from stool samples with Kato results for the detection of *S. mansoni* and with urine filtration results for the detection of *S. haematobium* from 88 subjects, categorised by number of eggs

Egg count category[a]	*N*	No. (%) PCR +ve	Ct value[b]	
			Range	Median
Schistosoma mansoni				
No eggs	18	6 (33)	24.4—39.5	35.4
Very low (15—100 EPG)	32	20 (63)	25.0—38.0	33.1
Low (101—400 EPG)	16	16 (100)	24.5—38.3	27.1
Moderate (401—1000 EPG)	14	14 (100)	23.3—37.3	25.6
High (>1000 EPG)	8	8 (100)	21.9—27.2	23.4
Total	88	64 (73)	21.9—39.5	27.6
S. haematobium				
No eggs	25	7 (28)	43.3—47.5	45
Low (1—50 EP10 ml)	44	24 (55)	31.8—46.1	41.5
High (>50 EP10 ml)	19	17 (89)	29.0—46.6	37.8
Total	88	48 (55)	29.0—47.5	41.2

[a] EPG: egg count per gram faeces; EP10 ml: egg count per 10 ml urine.
[b] The range and median Ct value are calculated for positive PCR samples per egg count category.

with increasing egg count categories, and Ct values as detected in the *S. haematobium*-specific real-time PCR showed significant correlation ($\rho = -0.59$; $n = 88$; $P < 0.001$) with the quantitative interpretation of eggs counted in urine.

Figure 1 shows the cumulative number of schistosomiasis positives by microscopy and PCR, respectively. After analysing the first stool series, 64 (72.7%) of 88 subjects were found to be positive by microscopy whereas 69 (78.4%) were found to be positive by PCR. Six more subjects (70/88; 79.5%) became positive after analysing the second stool series by microscopy and five more (74/88; 84.1%) by PCR.

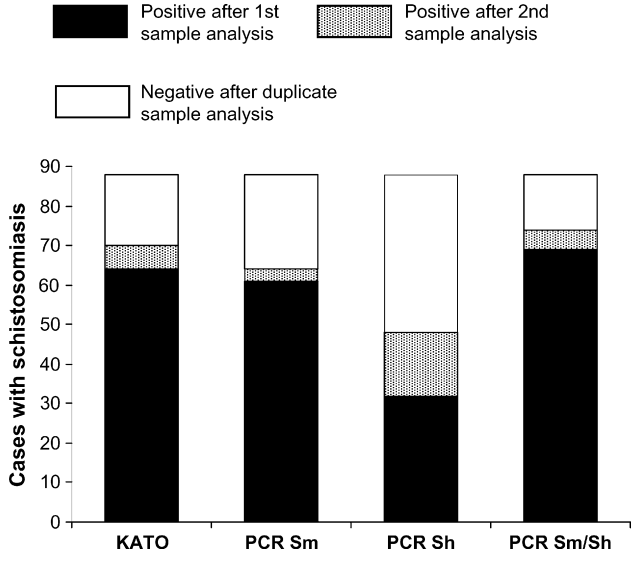

Figure 1 Cumulative number of subjects ($n = 88$) found to be positive for schistosomiasis after first and second analysis of stool samples by Kato or real-time PCR. Sm, *Schistosoma mansoni*; Sh, *S. haematobium*.

4. Discussion

In this study, a multiplex real-time PCR assay for the detection of *S. mansoni* and *S. haematobium* was developed and evaluated using well defined DNA and stool samples as controls. In the pre-selected study population, 100% sensitivity of *S. mansoni* PCR was shown for subjects with on average more than 100 EPG detected in two stool samples, and a significant association was demonstrated between egg excretion and Ct values, representing the amount of parasite DNA in faeces. Discrepancies between microscopy and PCR analysis are observed both ways only in samples with very low egg counts or low parasite DNA concentration. Significant correlation between egg counts and Ct values was still achieved if only *S. mansoni*-specific PCR results of the first collected stool samples were included (data not shown), indicating that molecular analysis of a single stool sample already provides sufficient data. In this study, microscopy-positive samples were overrepresented. Therefore, a cross-sectional study is planned that would include a higher proportion of samples without detected eggs to obtain a more precise estimation of sensitivity.

Molecular-based diagnostic techniques for schistosomiasis have been described in previous reports (Gomes et al., 2006; Pontes et al., 2002, 2003; Sandoval et al., 2006). A valid comparison with these methods is difficult as they are all very diverse in their use of DNA targets, DNA isolation, PCR procedure and methods of sample selection.

Although eggs of *S. haematobium* were not observed in stool samples, the presence of *S. haematobium* DNA was detected successfully. As expected, the sensitivity of *S. haematobium* DNA detection in faecal samples is lower than the sensitivity achieved by microscopic examination of urine samples. A preliminary study using the real-time PCR on DNA isolated from urine samples, collected in a *S. haematobium*-endemic area in Gabon, already shows promising results (unpublished data).

Obviously, because of the relative high costs, this PCR procedure is not intended for routine diagnosis in clinical settings in *Schistosoma*-endemic countries. On the other hand, prices of equipment and consumables are becoming more and more attractive. This trend, in combination with rapid improvements in technical performances, has already resulted in an increasing number of centrally located research centres within low income countries having real-time PCR technology available, for example for HIV viral load determination. In our opinion, the described *Schistosoma* PCR provides a powerful alternative or additional diagnostic tool for these research centres. The use of ethanol-suspended stool samples for storage and transport at room temperature makes collection of samples at remote areas more attractive. This has already been used successfully for hookworm multiplex real-time PCR, where microscopic egg or larva counts were also reflected by the Ct values (Verweij et al., 2007b). This also illustrates that the procedure can easily be extended towards real-time PCR assays for the detection of a whole range of intestinal microorganisms, such as *G. lamblia*, *Cryptosporidium* sp., *E. histolytica* or microsporidia (ten Hove et al., 2007; Verweij et al., 2004, 2007a).

In conclusion, the multiplex real-time PCR for the detection of *S. mansoni* and *S. haematobium* has been shown to be a powerful tool for epidemiological studies and can provide additional value in the evaluation of control programmes for the following reasons: (i) possible storage of stool samples at room temperature without need for direct sample processing; (ii) high throughput potential; (iii) quantitative output; (iv) the combination of multiple molecular targets and internal control in one assay; and (v) the possibility of using the isolated DNA for targeting additional intestinal microorganisms.

Authors' contributions: RJtH and JJV designed the real-time PCR; LvL and KP designed the study protocol; KV and RJtH were responsible for sample collection; KV and LD were responsible for microscopic data collection; RJtH carried out the DNA isolation and real-time PCR analysis; RJtH, LvL and JJV were responsible for data analysis and interpretation of the data; RJtH wrote the paper, with substantial intellectual input by JJV and LvL, and KP made significant comments. All authors read and approved the final manuscript. RJtH and LvL are guarantors of the paper.

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Conflicts of interest: None declared.

Ethical approval: This study is part of a larger investigation on schistosomiasis epidemiology and control in Senegal, for which approval was obtained from the ethical committees of the Institute of Tropical Medicine in Antwerp, Belgium, and the Ministry of Health in Dakar, Senegal.

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