

Differentiating *Schistosoma haematobium* from Related Animal Schistosomes by PCR Amplifying Inter-Repeat Sequences Flanking Newly Selected Repeated Sequences

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Abstract. In schistosomiasis elimination programs, successful discrimination of *Schistosoma haematobium* from the related animal *Schistosoma* parasites will be essential for accurate detection of human parasite transmission. Polymerase chain reaction assays employing primers from two newly selected repeated sequences, named Sh73 and Sh77, did not discriminate *S. haematobium* when amplifying Sh73-77 intra- or inter-repeats. However, amplification between Sh73 and the previously described DraI repeat exhibited discriminative banding patterns for *S. haematobium* and *Schistosoma bovis* (sensitivity 1 pg and 10 pg, respectively). It also enabled banding pattern discrimination of *Schistosoma curassoni* and *Schistosoma intercalatum*, but *Schistosoma mattheei* and *Schistosoma margrebowiei* did not yield amplicons. Similar inter-repeat amplification between Sh77 and DraI yielded amplicons with discriminative banding for *S. haematobium*, and *S. bovis*; however, *S. mattheei* was detected only at low sensitivity (1 ng). The Sh73/DraI assay detected snails infected with *S. haematobium*, *S. bovis*, or both, and should prove useful for screening snails where discrimination of *S. haematobium* from related schistosomes is required.

INTRODUCTION

Schistosoma haematobium, the cause of urogenital schistosomiasis in humans, afflicts about 130 million people in 53 countries in Africa and the Middle East.¹ We have previously described a polymerase chain reaction (PCR) assay based on amplification of the highly repeated DraI sequence of *S. haematobium*² for sensitive detection of *S. haematobium*-infected intermediate snail hosts (*Bulinus* spp.) from early infection onward.³ We subsequently carried out a large-scale survey of snail infection by *S. haematobium* in coastal Kenya, and demonstrated a correspondence between snail infection rates and local human prevalence and average intensity of infection near the surveyed snail habitats.^{2,3} Those findings suggested the potential of molecular monitoring of snail infection rates to assess focal persistence of parasite transmission.

However, the DraI-PCR assay is group- and not species-specific. That is, it also detects animal schistosome parasite species related to *S. haematobium*, most of which circulate between ruminants and the same bulinid snail hosts of *S. haematobium*. Of these, *Schistosoma bovis* is most often sympatric with *S. haematobium* in endemic areas of Africa and the Middle-East.^{4,5} In these areas, a differential identification of snails infected with *S. haematobium* is required to accurately determine snail infection rates with the human schistosome.^{3,6} This required going deeper into the genome structure of *S. haematobium*, now completely sequenced,⁷ for selecting repeated sequences suitable for differential diagnosis.

Differentiation between snails infected with *S. haematobium* and those infected with *Schistosoma bovis* (or with other related schistosomes) has been successfully accomplished by PCR of an inter-repeat sequence located between the Sh110 sequence of *S. haematobium* and the schistosomal splice-leader sequence.⁸ Although this assay is fairly sensitive (10 pg sensitivity) and species-specific, it does not provide amplicon formation for *S. bovis* or *Schistosoma mattheei* DNA, which

leaves a margin of uncertainty whenever positive species identification is required. A multiplex PCR using primers designed from the cytochrome oxidase subunit 1 (COX 1) has been recently developed at the Natural History Museum of London that enables discrimination of adults and larvae of *S. haematobium* and *S. bovis*. Differentiation, when using this PCR, was based on species-specific amplicons band size, with a detection sensitivity of 0.8 ng of genomic DNA.⁹ However, that assay was not tested for direct identification of infected snails. The COX1-based assay was further refined for use in real-time PCR, with higher detection sensitivity (pg range), but requiring a restriction enzyme fragment length analysis for *S. haematobium*-*S. bovis* species differentiation.¹⁰

The purpose of this study was to develop a simpler and more sensitive PCR assay that enables direct discrimination of *S. haematobium* from related animal schistosomes, notably the more widespread *S. bovis* and *S. mattheei*, by differential identification of electrophoretic bands of corresponding amplicons. The World Health Organization (WHO) is now considering applying schistosomiasis monitoring and diagnosis by loop-mediated isothermal amplification (LAMP). This is expected to facilitate large-scale screening of snail infection prevalence, whether prepatent or patent (i.e., shedding cercariae), in endemic areas where bulinid snails might be infected either with *S. haematobium*, veterinary *Schistosoma* species, or both.

MATERIALS AND METHODS

Snails and parasites: *Laboratory snails.* Uninfected laboratory-bred snails (*Bulinus nasutus*) from snail culture in the Division of Vector Borne and Neglected Tropical Diseases (DVBNTD) laboratories in Msambweni, Kenya³ were used as negative controls in experiments involving PCR assays of infected field snails. Snails (*Bulinus truncatus*) obtained from the National Institute of Allergy and Infectious Diseases (NIAID) Schistosomiasis Resource Center, Bethesda, MD, were used in DNA spiking experiments testing PCR sensitivity in detecting genomic parasite DNA.

Field snails were collected and preserved in ethanol by DVBNTD team members based at the Msambweni laboratories

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on the southern coast of Kenya. Snail collection was carried out at Katchetu pond (3.94060°S; 39.54026°E; elevation 146 m), near the Mombasa-Nairobi highway in Katchetu village, Mazeras. This pond contains significant numbers of *Bulinus globosus* infected with schistosomes, as previously determined by DraI PCR (Hamburger and Abbasi, unpublished data). Goats, cattle, and humans frequent this pond.

Adult worms of *S. haematobium*, *S. bovis*, *S. mattheei*, *S. margrebowiei*, *Schistosoma curassoni*, and *Schistosoma intercalatum* preserved in ethanol were provided by Dr. David Johnston of the Natural History Museum, London.⁸ Preparation of DNA from the adult worms was carried out as previously described.⁸ For this purpose 2–3 pairs of male and female adult worms were kept in 300 µL lysis buffer (0.1M EDTA, pH 8.0, 0.1M Tris-HCl pH 7.5, 0.2M NaCl, 1% sodium dodecyl sulfate, 0.2% 2-mercaptoethanol, and 100 µg Proteinase K) at 65°C for 2 hours. The DNA was further purified by phenol/chloroform extraction followed by ethanol DNA precipitation.

Identification of specific repeated genomic sequences. Repeated sequences were identified as previously described.⁸ Briefly, this involved preparing genomic libraries from *S. haematobium* and from five related animal schistosomes (*S. bovis*, *S. mattheei*, *S. margrebowiei*, *S. curassoni*, and *S. intercalatum*), initially selecting clones strongly hybridizing with genomic DNA of the homologous schistosome species and among them, selecting clones that did not hybridize with the DraI repeated sequence of *S. haematobium*. These clones underwent a third cycle of selection involving cross-hybridization with DNA from the other *S. haematobium*-related species and selecting those having hybridization signals only with *S. haematobium* DNA. A fourth level of selection took place after sequence analysis, with final selection of those exhibiting each of the following features: 1) they were not similar to the DraI repeat; 2) they were not similar to known sequences from other schistosomes or to schistosomal sequences presented in the GenBank; and 3) when aligned with other sequences of the same species, the sequences were found to be of high copy number. The nucleotide sequence analysis was carried out using the Dye Terminator Cycle DNA Sequencing Kit (Perkin Elmer, Wallington, UK), according to the instruction of the manufacturer.

Primer design. Primers were designed by using the PRIME software version 9.0, of the Wisconsin Package (Genetic Computer Group [GCG], Madison, WI).

Extraction and purification of DNA from whole snails. The DNA extraction from individual snail tissue was initiated by removing excess ethanol fixative by blotting on tissue paper. The snail bodies were removed from the shell using a fine needle, then macerated with wooden sticks in a 1.5 mL Eppendorf tube after adding 250 µL of snail lysis solution for a snail up to 5 mm, and 500 µL for larger snails. The lysis buffer consisted of 100 mM Tris base, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol, 2% hexadecyltrimethylammonium bromide and Proteinase K. Snails tissue were digested at 60°C for 2 hours followed by phenol extraction and ethanol precipitation. The extracted DNA was suspended in TE buffer and stored at –70°C until used.

PCR. The PCR reactions were carried out in a volume of 25 µL using ready-mix PCR tubes, which contain Taq polymerase, buffer, and dNTPs (Syntezza, Jerusalem, Israel). Each reaction was performed by mixing 1 µL of the direct

TABLE 1

Sequence information of the designed primers based on the newly selected Sh73 and Sh77 repetitive sequences, for comparison, the DraI reverse primer previously used in *Schistosoma haematobium* DraI PCR amplification

Sequence	Primer name	Primer sequence
Sh73 repeat	73d	CCTTGGTCACTGATTTC
	73r	CTCCACCTTATGCCGGTC
Sh77 repeat	77d	CCTGGGTGTGCACGTCTTGT
	77r	CTAAATGCCTAGCAACGGGGC
DraI repeat	DraI reverse primer	TCACAACGATACGACCAAC

and reverse primers (20 pmoles each), and 18 µL of double distilled water (DNA/RNA free). A total quantity of the 20 µL was added to each ready-mix tube. This was followed by adding 5 µL of the test DNA (purified parasite genomic DNA or snail-extracted DNA). Primer sequences are listed in Table 1, and the thermocycler profile as previously described by Hamburger and others² for *S. haematobium* DNA detection. Briefly, the thermal profile involved 5 minutes at 95°C followed by 35 cycles each of 1 minute at 95°C, 1 minute at 58°C followed by 1 minute at 72°C, and a final elongation step at 72°C for 10 minutes. Gel electrophoresis using 1.5% agarose then followed.

RESULTS

Newly selected repetitive genomic sequences of *S. haematobium*. The DNA sequence analysis, which included multiple sequence alignments and the basic local alignment search tool (BLAST) comparison searches in GenBank, were applied to the repetitive sequences that emerged from our four-step selection process (see Materials and Methods) for clones from genomic libraries of the different *Schistosoma* spp. having terminal spined ova. Using this approach, it was possible to select two promising repetitive DNA sequences of 73 and 77 bp, subsequently named Sh73 and Sh77, respectively (Table 2). These fragments were not similar to any previously identified *Schistosoma* sequences in GenBank, but should be included within the now completely sequenced genome of *S. haematobium*.⁷ Based on these sequences, new primers (Table 1) were designed and developed for use in sensitivity and specificity testing in PCR assays.

PCR assays employing Sh73 and Sh77 primers. Primers designed from these newly selected repeated sequences were next used in PCR assays to amplify corresponding sequences of genomic DNA in *S. haematobium*, *S. bovis*, and *S. mattheei*.

Amplification was accomplished with primers of both sequences, yielding a banding pattern typical of tandemly

TABLE 2

DNA sequence analysis of the two newly selected repeated *Schistosoma haematobium* segments (Sh77 and Sh73)*

Repeat name	DNA sequence
(Sh77)	5'- CCTGGGTGTG CACGTCTTGT TTGTGCAC TCCAAACCTCAA GGGTCACCGT GGTG GAGCCCCGTGTGCTAGG CATTAG-3'
(Sh73)	5'- CCTTGGTCACTGATTTCATTTTCA GTTTGCC CACCTGATGCT GGCTGCCCA CCTC GACCGGCATAAGGTGG AGC-3'

*The bold sequences represent the segments used to construct direct and reverse primers used in this project's polymerase chain reaction (PCR) amplification.

arranged repeated sequences (results not shown). Although reactions employing these primers reached a sensitivity limit of 0.1 pg for amplifying *S. haematobium* genomic DNA, they also amplified low concentrations of DNA from *S. bovis* and *S. mattheei*. For the separate sets of Sh73 and Sh77 primers, the similar amplification patterns from the three *Schistosoma* species (results not shown) did not provide any useful discriminative test.

We also used mixed Sh73 and Sh77 primers in amplification experiments attempting to amplify inter-repeats between them. Amplification was accomplished in these experiments, but again, without a discriminative banding pattern (results not shown).

PCR assays employing combinations of primers for identifying inter-repeat sequences. Subsequently, trials for inter-repeat amplification between the new Sh73 and Sh77 repeat sequences and the previously identified DraI repetitive sequence² were undertaken, employing different primer combinations from the Sh73 and Sh77 sequences and from the DraI sequence. The primer combination of DraI reverse primer and Sh73 direct primer (73d) yielded a *S. haematobium* amplicon with a banding pattern that included a band at 200 bp, and another one of 350 bp (Figure 1A). A ladder of larger bands is also shown Figure 2. When using these primers for amplifying *S. bovis* DNA, the banding pattern obtained included a 150 bp band and another one of 300 bp. This banding pattern of *S. bovis* could be clearly differentiated from that of *S. haematobium*. However, PCR of *S. mattheei* DNA by the Sh73/DraI primers did not yield an amplicon

(Figure 1A). The sensitivity of *S. haematobium* detection was 1 pg, whereas *S. bovis* detection was 10 pg (Figure 2).

When using the DraI reverse primers and the Sh77 direct primer (77d), the banding patterns of the three schistosome species enabled species differentiation but with less sensitive detection (Figure 1B). The banding pattern of *S. haematobium* amplicon consisted of two bands (150 and 300 bp) plus a smear, with detection sensitivity of 1 ng. The banding pattern of *S. bovis* amplicon included one clear band (200 bp) plus a smear with a vague second band within. The banding pattern of *S. mattheei* amplicon in this case exhibited a single clear band of 150 bp, similar to the small first band of *S. haematobium*. The larger band of *S. haematobium* (300 bp) did not appear in the *S. mattheei* amplicon, and therefore the banding patterns of these species are discriminative. The sensitivity of detection in this case was 1 ng for *S. haematobium* and *S. bovis*, and perhaps also for *S. mattheei*, where a very weak band was shown with 1 ng DNA (Figure 1B).

Because the detection sensitivity for amplifying the inter-repeat when using Sh77-derived primer was too low for our purposes, and because the primers required yielded a heavy primer-dimer response, we did not further pursue this line of investigation

Detection of *S. haematobium* and *S. bovis* in snail tissues.

DNA spiking experiments in laboratory-bred snails. Extracts of laboratory bred *B. nasutus* were spiked with different concentrations of genomic DNA from *S. haematobium*, *S. bovis*, or a mixture thereof. The PCR was subsequently performed using a combination of Sh73-direct and DraI-reverse primers. The

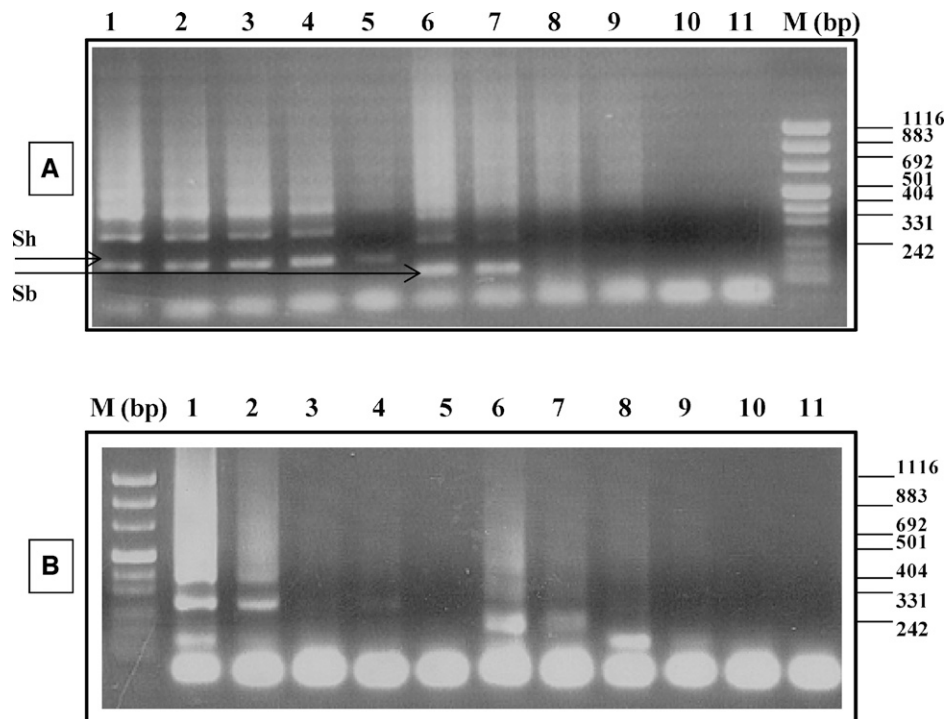


FIGURE 1. Successful species discrimination using inter-repeat polymerase chain reaction (PCR) amplification with reverse primer from *Schistosoma haematobium* DraI repeat, and direct primer from newly selected Sh73 repeat (4297-73d, panel A), or direct primer from Sh77 repeat (4297-77d, panel B). The amplification assay at different concentrations of *S. haematobium* genomic DNA is shown (lane 1: 10 ng; lane 2: 1 ng; lane 3: 0.1 ng; lane 4: 0.01 ng; lane 5: 1 pg), *Schistosoma bovis* DNA (lane 6: 10 ng; lane 7: 1 ng), and *Schistosoma mattheei* DNA (lane 8: 10 ng; lane 9: 1 ng). Lanes 10–11: negative controls, M: DNA size marker. Arrows point to specific bands of *S. haematobium* (Sh) and *Schistosoma bovis* (Sb).

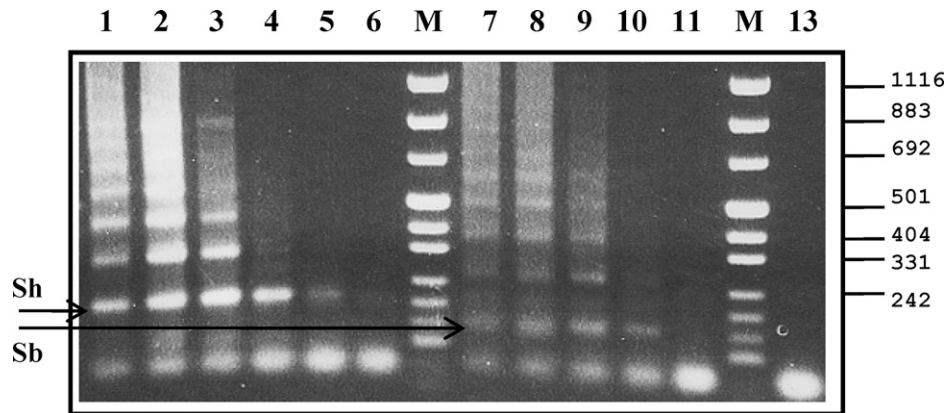


FIGURE 2. Comparison of polymerase chain reaction (PCR) amplicon banding patterns and sensitivity for *Schistosoma haematobium* and *Schistosoma bovis* genomic DNA using the new inter-repeat approach (primers 4297-73d). The amplification reactions shown here used different concentrations of *S. haematobium* genomic DNA (lane 1: 10 ng; lane 2: 1 ng; lane 3: 0.1 ng; lane 4: 0.01 ng; lane 5: 1 pg; 6: 0.1 pg), or *S. bovis* genomic DNA (lane 7: 10 ng; lane 8: 1 ng; lane 9: 0.1 ng; lane 10: 0.01 ng; lane 11: 1 pg). Lane 13: No DNA (negative control) and M: DNA size marker. Arrows point to specific bands of *S. haematobium* (Sh) and *S. bovis* (Sb).

primers were found to retain their sensitivity in amplifying DNA from each of the two species, and even showed a third amplicon banding pattern for a mixed DNA from these two *Schistosoma* species (Figure 3).

Field collected snails. Examination of field snails from Katchetu pond (where snail infections by either *S. haematobium* or *S. bovis* was likely) by PCR employing the 73d and the DraI reverse primers indicated the presence of both *Schistosoma* species, including single infections and mixed infections within individual snails (Figure 4, Lanes 2, 3, and 6).

PCR assay with DNA from different schistosome species belonging to the *S. haematobium* group (species with terminal spined ova). Although this study focuses on differentiating *S. haematobium* from *S. bovis* (the most prevalent and widespread schistosome of ruminants) we also examined its ability to differentiate *S. haematobium* from other non-*bovis* species belonging to the same group. The PCR amplification using the 73d/DraI reverse primer combination showed very different banding patterns for DNA from these other schistosomal species. Figure 5 summarizes these results and shows that if larger concentrations of DNA are used (10 ng), different banding patterns can be obtained with amplicons from *S. curassoni*, *S. intercalatum*, *S. bovis*, and *S. haematobium*.

By contrast, *S. margrebowiei* and *S. mattheei* DNA did not yield detectable amplicons.

DISCUSSION

Schistosoma haematobium-related animal parasites are present in many locations of *S. haematobium* transmission and can infect the same intermediate-host snail species as *S. haematobium*, thus interfering with accurate determination of infection rates of snails infected with the human schistosome.

A year-long snail survey by PCR employing primers designed from the DraI highly repeated sequence (which also amplifies DNA from *Schistosoma* spp. related to *S. haematobium*) was previously carried out in the Msambweni District, on the south coast of Kenya, where animal schistosomes are rare.³ The results indicated that DNA testing could serve as a useful marker of water contamination as part of control surveillance programs aiming to detect residual transmission.⁶ As a result, evaluation of the effect of elimination activities on snail infection rates in Zanzibar, where animal schistosomes of the *S. haematobium* group are absent, will be based on DraI-based PCR testing (Rollinson D, personal communication). However, in many other areas of sub-Saharan

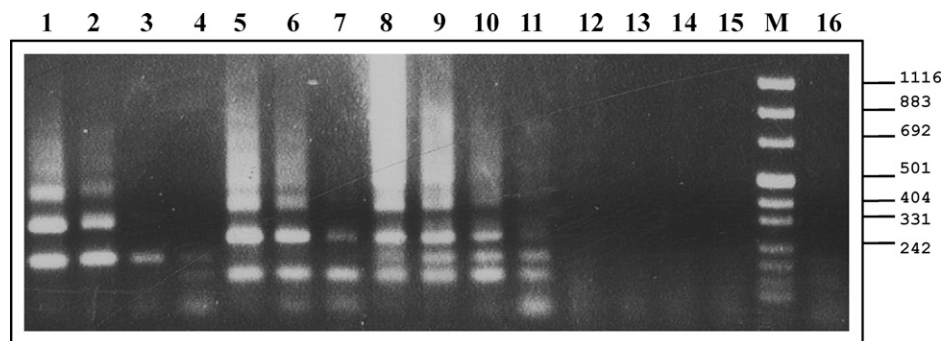


FIGURE 3. Inter-repeat polymerase chain reaction (PCR) amplification using DraI reverse primer and 73 direct primers at different concentrations of *Schistosoma haematobium* genomic DNA (lane 1: 0.1 ng; pg). Lanes 8–11, represent PCR amplification of “mixed target DNA” composed of 1 pg *S. haematobium* genomic DNA with different added concentrations of *Schistosoma bovis* genomic DNA (lane 8: 1 ng; lane 9: 0.1 ng; lane 10: 10 pg; lane 11: 1 pg). Lanes 12–15: show negative PCR amplification results for extracts from different uninfected *Bulinus* snails. Lane 16: No DNA (negative control), and M: DNA size marker.

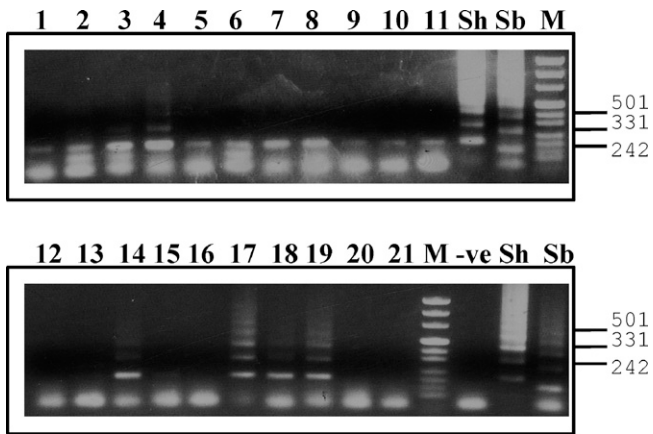


FIGURE 4. Inter-repeat polymerase chain reaction (PCR) amplification using *Dra*I reverse primer and 73 direct primer targeting different *Bulinus* snails (1–21) that were collected from a region co-endemic for *Schistosoma haematobium* and *Schistosoma bovis*. M: DNA size marker.

Africa and the Middle East, *S. haematobium* and its related schistosome species share habitat, and can infect the same host snail species.^{4,11,12} Of these species, *S. bovis* is the most widespread and prevalent,^{4,5} and thus has the widest potential to interfere in testing for *S. haematobium* infection.

Given the abundance and wide distribution of *S. bovis*, our present study focused on differentiating *S. haematobium* from *S. bovis* (Figures 1–4), although differentiation from other schistosomes was also addressed (Figures 1 and 5). Thus, *S. mattheei* could be differentiated from *S. haematobium* by lack of amplicon when amplification used the Sh73-direct and the *Dra*I-reverse primers (Figure 1A), or from both *S. haematobium* and *S. bovis* by different electrophoretic pat-

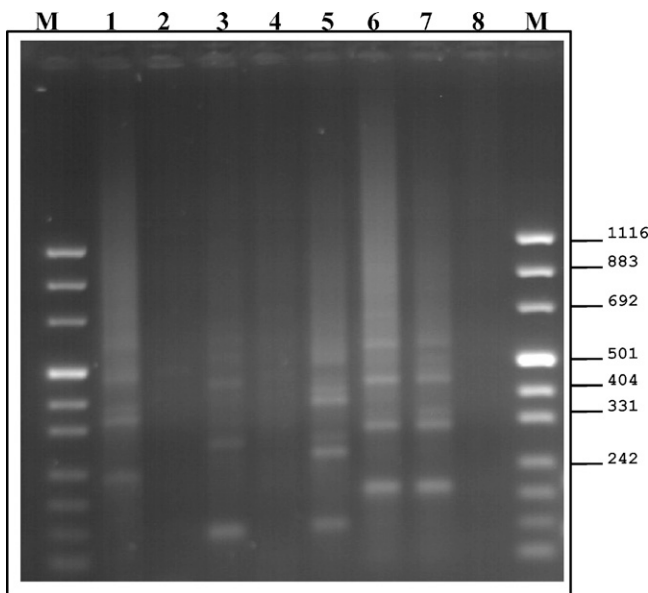


FIGURE 5. Inter-repeat polymerase chain reaction (PCR) amplification using *Dra*I reverse primer and 73 direct primer for different species of terminal spined schistosomes. Lane 1: *Schistosoma curassoni*, 10 ng; lane 2: *Schistosoma margrebowiei* 10 ng; lane 3: *Schistosoma intercalatum* 10 ng; lane 4: *S. mattheei*, 10 ng; lane 5: *Schistosoma bovis*, 10 ng; lane 6: *Schistosoma haematobium* 1 ng; lane 7: *S. haematobium* 0.1ng; lane 8: no DNA. M: DNA size marker.

terns (Figure 1B) when employing the Sh77-direct and *Dra*I-reverse primers. Differentiation of *S. haematobium* from *S. curassoni*, *S. margrebowiei*, and *S. intercalatum* was also possible by differential banding patterns (Figure 5). The possibility of detecting a mixed infection now enables studying various aspects of mixed infections (e.g., possibility of interference or enhancement, which may affect infectivity of the human schistosome).

Detection sensitivity will affect practical aspects of using this test for screening infection rates. In this study, the detection sensitivity for *S. haematobium* was 1 pg as compared with 10 pg detection sensitivity for *S. bovis* when amplification used the 73d and the *Dra*I reverse primers (Figure 1A and 2). This sensitivity enabled detection of mixed genomic DNA and of snail infections with both *S. haematobium* and *S. bovis* (Figures 3 and 4). Because the estimated DNA concentration of a single miracidium is about 1–2 ng/ μ L,⁹ it can be assumed that detection of snails infected with *S. haematobium* will be accomplished from very early prepatency and infection with *S. bovis* infection will be detected a very short time after infection, taking into consideration that less than a tenth of the extracted DNA is used for amplification. With the other related species yielding positive amplification signals at the 1 ng DNA level (Figures 1B and 5), it is expected that PCR signals of *S. haematobium* will be readily detected earlier in prepatency, whereas the signals of the other schistosomes may not be detected at all in the majority of the snails examined. This outcome should actually facilitate discrimination of *S. haematobium* from these other species of veterinary parasites for purposes of surveillance of human-to-snail parasite transmission.

The situation of a positive PCR signal with *S. haematobium* and no signal with the related schistosomes⁸ could also enable direct identification of *S. haematobium* in snails by more user-friendly LAMP assays.^{13,14} However, in cases where all suspected schistosome species yield amplicons (e.g., *S. haematobium* with *S. bovis*, Figures 1–2, and 5), adaptation of the assay to a LAMP format cannot provide direct species differentiation without using specific hybridization probes in an additional step (Abbasi and others, unpublished data). Another matter that can be addressed by species-specific PCR is a better definition of animal schistosome distribution, resolving questions of inter-species competition¹² or reaffirming species identity, as in the case of *S. curassoni*.¹¹ In addition, the present PCR assay differentiating *S. haematobium* from *S. bovis*, yielding positive amplification signals for both, may help clarify the matter of hybridization between the two, which has been tentatively reported as causing an emerging disease in humans.¹⁰

We hope this study will lead to the development of assay kits for discriminating *S. haematobium* from related schistosome species for use in field laboratories performing surveillance of transmission sites. Such assays are expected to be possible after further validation using larger numbers of field snails for large-scale monitoring of post-intervention residual transmission.

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