A POLYMERASE CHAIN REACTION ASSAY FOR DETECTING SNAILS INFECTED WITH BILHARZIA PARASITES (SCHISTOSOMA MANSONI) FROM VERY EARLY PREPATENCY

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Abstract. In the present study, we adapted a polymerase chain reaction (PCR) assay, previously shown by us to be very sensitive for detecting cercariae in water, for the sensitive detection of Schistosoma mansoni DNA in infected snails from early prepatency. Polymerase chain reaction primers were designed based on the 121-basepair highly repeated sequence we previously identified in the genome of S. mansoni. The DNA was prepared from the snails by a simple alkaline extraction procedure, and the PCR assay enabled a clear differentiation between infected and normal snails. Infected snails were detected as early as one day after penetration of a single miracidium. The high sensitivity of the test enabled identification of a single infected snail even when its DNA was pooled with material from up to 99 uninfected snails, thus demonstrating the possibility of mass diagnosis in pools of snails. The assay has the potential for large-scale determination of prepatent infection prevalence in snails, thus offering new possibilities for the evaluation of schistosomiasis transmission and for schistosomiasis control, as discussed.

Schistosomiasis, is caused in humans by three main species of blood flukes (Schistosoma mansoni, S. haematobium, and S. japonicum) and is transmitted by freshwater snails. It continues to plague many developing countries in the tropics with an estimated 200 million infected people worldwide.

Freshwater snails, intermediate hosts of schistosomes, become infected by larvae (miracidia) released from schistosome eggs that reach the water with excreta. After several weeks of asexual multiplication within the snail’s tissues, larvae infective to humans by active penetration through the skin (cercariae), are released into the water. Snail infection and contact patterns of humans with water infected with cercariae are important factors in the transmission of schistosomiasis, and risk of infection is normally estimated by detecting infected intermediate hosts in sites where humans come in contact with water. Infection rates in field populations of snails are routinely determined by searching for cercariae shed from snails with patent infection. In contrast, prepatent infections, which can constitute a significant proportion of infected snails populations, are not determined routinely because of a lack of a suitable method. Microscopic examination of crushed snails in search of developing cercariae and intramolluscan-multiplying larvae (the sporocysts) is sometimes used for this purpose but is highly inaccurate at early prepatency, and unsuitable for routine large-scale screening of snail populations. Another approach is to examine nonsheddng field snails maintained for several weeks in the laboratory for protracted shedding. However, this method is time-consuming, it cannot be done routinely on a large scale, and it can be highly inaccurate if snails mortality is high. The omission of data on prepatent infections leaves out important information for the evaluation and mathematical modeling of transmission dynamics of schistosomes.

In an attempt to facilitate determination of prepatent infections in snails, we have previously developed monoclonal antibodies and a simple ELISA test procedure for detecting antigens of S. mansoni in the hemolymph of Biomphalaria from the second week of infection onwards. In another study, we have demonstrated that prepatent infection of S. mansoni can be detected one week after infection by dot hybridization with a total DNA probe. Detection of prepatent infections of snails has also been accomplished very recently by polymerase chain reaction (PCR) amplification of the mitochondrial DNA minisatellite region of S. mansoni, and by nested PCR amplification of 18S sequences of rDNA. A reliable and efficient detection of prepatent infections in snails from very early stages after the invasion of a miracidium, especially if manageable for routine large-scale tests, should enable an extended outlook on the epidemiology of schistosome infection of snails, and facilitate mathematical modeling of transmission. Early detection of prepatent infections of snails may also enable early detection of the effect of community-based control measures on transmission if control should affect the degree of water contamination by schistosome eggs as do a variety of other factors.

In the present study, we adapted a PCR assay and a simple procedure for DNA extraction for identifying S. mansoni in infected snails very early after infection with a single miracidium. The PCR primers were designed based on a highly abundant repeated sequence constituting about 10% of the genome of S. mansoni. Similar primers were previously used in a sensitive PCR assay at conditions adapted for the sensitive detection of cercariae in water.

MATERIALS AND METHODS

Parasites and laboratory maintenance. The Egyptian strain of S. mansoni was originally obtained from the Welcome Laboratories (London, United Kingdom), and was maintained at the Hebrew University in outbred albino mice and in snails (Biomphalaria glabrata). Mice were infected by subcutaneous injection of about 350 cercariae, and nine weeks later eggs in homogenates of their livers were used for harvesting miracidia. For maintenance of the life cycle, snails (8–10 mm in size) were infected by 10 miracidia/snail. For experimental purposes, snails of similar size were infected by exposure to one miracidium.
Preparation of DNA from worms and from snails. Adult worms were collected from the livers of infected mice by perfusion\(^\text{17}\) for preparation of high molecular weight DNA as previously described.\(^\text{15}\) For preparing DNA from snails, a simple DNA preparation protocol was adapted according to a method previously described.\(^\text{6}\) Each snail (shell and all) was triturated vigorously with two wooden applicators in a plastic tube with 300 \(\mu\)l of 1 M NaOH plus 1\% Triton X-100. The mixtures were then left at room temperature for three days (or alternatively heated to 65\(^\circ\)C for 1 hr), then neutralized with HCl, boiled for 5 min, cooled quickly on ice, and centrifuged at 10,000 rpm for 15 min to remove debris. The supernatants were transferred to another tube, and precipitation with alcohol was carried out directly (without the addition of salts) by adding 2.5 volumes of cold ethanol, cooling to \(-70\)^\circ\)C for 30 min, and centrifugation. The pellet was washed three times with 70\% cold ethanol and dissolved in 100 \(\mu\)l of TE buffer (10 mM Tris, 1mM EDTA, pH 8.0). The extraction products were kept at \(-20\)^\circ\)C until used.

Amplification by PCR. Amplification by PCR was carried out in a total volume of 50 \(\mu\)l containing 10 mM Tris-HCl pH 9.2, 7.5 mM KCl, 3.5 mM MgCl\(_2\), 200 mM of each dNTPs, 20 pM of the two amplification primers, 2.5 units of \(Taq\) polymerase (Red Hot \(Taq\), Advanced Biotechnologies, Epsom, United Kingdom), 2 \(\mu\)l of DNA sample, and \(H_2O\). The nested primers designed, based on the 121-base pair (bp) highly repeated sequence of \(S.\ mansoni,\)\(^\text{15}\) were 5'-GAAATCGTTGTTATCTCCG-3' and 5'-GTTGACCTG-CCTAAAAATAC-3'. The amplification was carried out in a DNA thermal cycler (MiniCycler,\(^\text{18}\) MJ Research, Watertown, MA). The reaction mixtures were initially heated at 94\(^\circ\)C for 5 min and were then subjected to 35 cycles at 95\(^\circ\)C for 1 min, 60\(^\circ\)C for 1 min, and 72\(^\circ\)C for 30 sec. Finally, an elongation step at 72\(^\circ\)C for 10 min was included.

Detection of infected snail DNA pooled with DNA from uninfected snails. A 1-\(\mu\)l aliquot of DNA from a snail infected for three days by one miracidium was mixed with 10, 20, 30, 40, 50 and 100 equal aliquots of DNA from uninfected snails. Two microliters of the DNA pools were then subjected to PCR amplification results. Subsequently, pooled DNA from infected snails; lane 14, infected snail; lane 15, negative control (no target DNA).

results in multiple bands with DNA from uninfected snails, but differentiation from infected snails was still possible by their different banding pattern (Figure 1). In contrast, DNA from uninfected snails at the given high-stringency conditions was not amplified, thus enabling clear differentiation between infected and normal snails.

Identification of infected snails at very early prepatency. We initially found that prepatently infected snails at one week postexposure to one miracidium can be easily detected by the PCR. We subsequently attempted detection of infected snails at earlier prepatency, namely at three days and one day postexposure. All experiments were done with 20 snails infected by a single miracidium for demonstrating detection at the extreme sensitivity probably required in the field. Preliminary experiments with different numbers of miracidia suspended in buffer showed that DNA extraction and DNA amplification by the PCR will result in a sensitive and consistent identification of even a single miracidium.

For control purposes, DNA from 20 uninfected snails was individually collected, assayed by the PCR, and showed negative amplification results. Subsequently, pooled DNA from uninfected snails was used as a negative control in each amplification run in the thermocycler.

When DNA extracted from individual infected snails was examined different number of bands of amplification products were obtained even one day after miracidial penetration (Figure 2). Since the number of amplification ladder bands seems to be related to the schistosomal DNA concentration,\(^\text{16}\) the results suggests that different amount of schistosomal DNA were successfully extracted by the extraction procedure.

Among 10 snails exposed to one miracidium, eight (80\%) were PCR positive one week later (Table 1). Among 20 snails tested three days postexposure, 16 (80\%) were found to be PCR positive (Table 1), while among the 20 snails tested one day postexposure, 16 snails (80\%) were also PCR positive (Figure 2 and Table 1). A group of 21 snails each exposed to a single miracidium was left for nine weeks, and a repeated shedding test carried out from the sixth week after
infection indicated that 17 snails (81%) were infected. These results, as summarized in Table 1, demonstrate that the PCR sensitivity is 100%. Similar results were obtained in two amplification experiments. Statistical analysis was not done if the results were clear. The results indicate that detection of prepatent infections from very early prepatency, and with maximal sensitivity, is possible.

**Identification of an infected snail by PCR in DNA pools.** Identification of an infected snail when its DNA is pooled with DNA aliquots from several uninfected snails should increase the feasibility of using a PCR assay for mass screening of prepatent infections. This is expected to be possible only when the detection sensitivity is very high. In our experiments, the detection limit was reached when DNA from one infected snail was diluted with 40 equal aliquots of DNA from uninfected snails (Figure 3A), but when double amount of primers were used in the PCR, the test could detect a single, infected snail in pooled material from 100 snails (Figure 3B). These results indicate the feasibility of rapid mass screening.

**DISCUSSION**

The PCR assay described in the present study was based on a similar assay previously shown to be very sensitive for detecting cercariae in water. The high detection sensitivity of this assay was demonstrated by the detection of 10 fg of *S. mansoni* DNA, and of a single cercaria. This assay was also shown to be specific for detecting schistosomes rather than other trematodes, but further specificity analyses are required by cross-amplification assays with DNA from a variety of animal schistosomes. In the present study, the PCR assay identified infected snails at one day (Figure 2 and Table 1), three days, and one week after they were exposed to a single miracidium, with an actual sensitivity of 100% (Table 1). In comparison, detection of prepatent infections by presence of schistosomal antigens in snail hemolymph was possible only from the second week after exposure to miracidia and onward, and schistosomal DNA was detected by dot hybridization only one week after exposure to 10 miracidia. Recently, a PCR assay based on amplification of a mitochondrial DNA minisatellite region enabled detection of infected snails from one week after snail exposure to 10 miracidia, and another assay based on a nested PCR of 18S rDNA sequences also enabled the detection of infected snails one day after exposure to a single miracidium, but this required two successive PCRs, its sensitivity was somewhat lower, and testing of pools of samples was not attempted. The early and sensitive detection of snails infected with a single miracidium, and the possibility of testing pools of DNA samples (Figure 3), as described in the present study and discussed below, is expected to be useful for large-scale screening of snail populations in nature. Furthermore, the clear positive versus negative differentiation between infected and uninfected snails should be superior to differentiation by banding pattern of the PCR products as shown by others. A clear distinction between positive and negative results should also enable application of a simple ELISA-type

**Table 1**

<table>
<thead>
<tr>
<th>Detection method*</th>
<th>No. tested</th>
<th>No. positive</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR amplification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 1 day PE</td>
<td>20</td>
<td>16</td>
<td>80</td>
</tr>
<tr>
<td>At 3 days PE</td>
<td>20</td>
<td>16</td>
<td>80</td>
</tr>
<tr>
<td>At 1 week PE</td>
<td>20</td>
<td>16</td>
<td>80</td>
</tr>
<tr>
<td>Cercarial shedding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6–8 weeks PE</td>
<td>21</td>
<td>17</td>
<td>81</td>
</tr>
</tbody>
</table>

*PE = postexposure.
assay for determining positivity or negativity of the amplification products.

The NaOH procedure used in the present study was previously used for extracting DNA from cercariae,16 snails,9 and sputum of people infected with the lymphatic filaria Wuchereria bancrofti.19 The simple DNA extraction procedure should greatly facilitate establishment of the PCR approach for monitoring infected snails during early prepatency. Furthermore, collection of snails in EDTA was shown (Hamburger J and others, unpublished data) as suitable for long-term preservation of the DNA at ambient temperature, as has been previously shown with blood19 and sputum.18 Since collection of snails is simple and can be done by village workers,20 one can envisage a logistically convenient separation of snail collection and storage from the high technology required for their testing by PCR in central laboratories.

The PCR in pools of snail material (Figure 3) should greatly facilitate large-scale surveys for determining the prevalence of S. mansoni in snail populations as previously done for determining the prevalence of Onchocerca volvulus in pools of blackflies.21 In the present study, DNA aliquots were pooled. Up-scaling of the DNA preparation to pools of snails is likely to succeed as we have found in preliminary tests with pools of 10 snails, but further up-scaling is still required. Mass screening of pools of snails for schistosomal infection may enable coverage of large areas requiring snail surveys for establishing schistosomiasis transmission. This can be considered particularly important for periodical monitoring where the prevalence of snail infection is very low and only a few infected snails can be found among thousands of uninfected ones (particularly during the maintenance phase after effective control, or in new water development schemes where the danger of the spread of schistosomiasis exists). The pool approach for mass screening by PCR was also proposed for lymphatic filariasis by testing blood, mosquitoes,22 and sputum pools (Hamburger J and others, unpublished data). The establishment of this approach in developing countries for mass-monitoring purposes in patients and in vectors requires further development for a variety of conditions and proof of cost effectiveness.

Making data on the prevalence of prepatent snail infection regularly and easily available is expected to enable a more complete evaluation of snails’ infection prevalence, a facilitated application of mathematical models, and a better evaluation of water contamination by schistosome eggs. In the latter regard it should be mentioned that the force of infection from humans to snails, which is the measure of water contamination by egg-bearing human excreta, is currently evaluated by age (size) prevalence of patent infection.12,13 Since prepatent infections in snails are the earlier outcome of water contamination by eggs, their prevalence in relation to the age (size) of the snails may enable a more rapid and exact evaluation of changes in water contamination by schistosome eggs. Furthermore, resurgent infection in a community previously treated may also be rapidly detected by an increase in the prevalence (or a corresponding shift in age-prevalence distribution) of prepatently infected snails. If this should be the case, then early identification of resurgent infection may be considered by the proposed approach, rather than by repeated community surveys, for timing re-treatment and for targeting it at the most vulnerable groups who frequent the sites monitored.21 Snail surveys for timing retreatment was previously proposed,20 because repeated community surveys often result in a progressive decrease in community cooperation.24

The usefulness of the PCR assay developed in the present study for the proposed purposes remains to be demonstrated by suitable field studies. The availability of the required technology makes such field studies highly feasible.

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REFERENCES


