PCR detection of *Schistosoma japonicum* cercariae: a potential tool for the field

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Abstract Despite decades of prevention and control efforts, the water-borne parasitic disease schistosomiasis remains endemic in 74 of the world's developing nations. Five species of the *Schistosoma* trematode are pathogenic to humans; *S. japonicum* is the infective agent of schistosomiasis in China and the Philippines. The purpose of this research is to develop a sensitive and specific technique for the detection of *S. japonicum*'s larval life-stage (known as cercariae) from water samples. In the field, this will potentially provide a direct measure of the cercarial hazard of irrigation ditch water in villages within China's Sichuan Province. Endemic for schistosomiasis, these agricultural communities are currently being monitored and assessed for local variations in risk by a study at UC Berkeley's School of Public Health. To detect free cercariae from water samples, a qualitative polymerase chain reaction (PCR) assay has been developed targeting regions of the retrotransposon *SjR2* for amplification. Samples of cercariae obtained from a laboratory population of snail intermediate hosts were assayed following DNA extraction to test the technique. The results demonstrate that the PCR assay is capable of detecting the presence of as little as one cercaria in a water sample, and is not inhibited by additional organic matter contaminants such as those found in local creek water. Further modifications for the field setting will allow this methodology to be tested at the study sites in question. This research was conducted with the assistance of Jennifer Kyle, a graduate student in Dr. Eva Harris' laboratory in the School of Public Health, Division of Infectious Diseases.

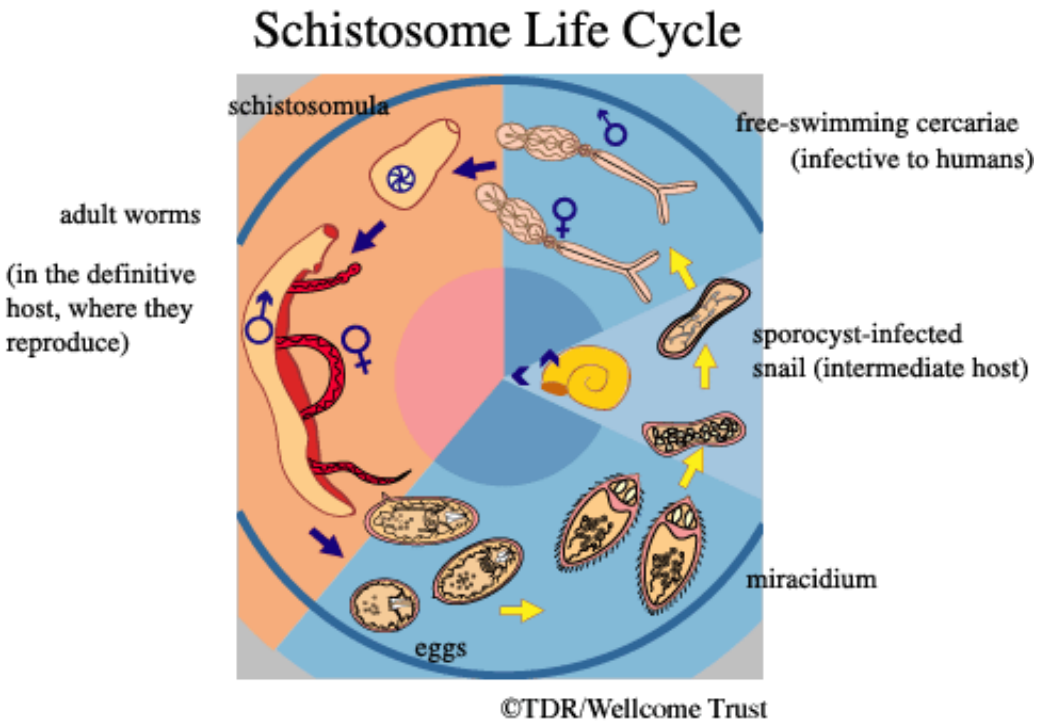
Introduction

According to the World Health Organization, schistosomiasis is second only to malaria in socio-economic and public health importance in tropical and sub-tropical regions of the world (WHO 1996). An estimated 200 million people in 74 countries are currently infected with the water-borne parasitic disease, with an additional 600 million at risk (WHO 2002). The majority of known cases of schistosomiasis occur in sub-Saharan Africa and are most often due to intestinal and urinary infection with *S. mansoni* and *S. haematobium*, respectively. However, the disease is also endemic to parts of Latin America and the Caribbean, as well South-East Asia and the Western Pacific, where *Schistosoma japonicum* causes intestinal schistosomiasis.

Transmission to humans occurs when people come into contact with bodies of water containing the cercarial (larval) stage of *S. japonicum* (see figure below). The free-swimming cercarial life stage directly penetrates human skin through a process of mechanical movements and lytic secretions (He, *et al* 1990). In the human host, the larval forms develop into adult worms that mate, migrate to the host's mesenteric veins, and produce eggs. The highly antigenic properties of the eggs elicit a myriad of immune responses from the host, such as fibrosis of the liver and spleen, and in severe cases, fatal damage to the lungs and brain. Multiple studies suggest that in certain areas of China schistosomiasis contributes significantly to the population's morbidity (Li *et al*. 1993, and Wiest *et al*. 1992). In China, as well as other regions of East Asia, *S. japonicum* infection is associated with hepatocellular and colorectal carcinoma (Ishii *et al*. 1994), childhood malnutrition and growth abnormalities (McGarvey *et al*. 1993) and gynecological complications such as proximal tubal obstruction (Letterie *et al*. 1992), rupture of the appendix during pregnancy, and fetal anoxia and subsequent death (Moore and Smith 1989).

S. japonicum eggs migrate from the mesenteric veins to the lumen of the intestine and are transported from their mammalian host back to the environment via excreted fecal matter. Once in the water, the eggs hatch and release a free-swimming miracidium that seeks out an appropriate species of snail as an intermediate host. This stage of the life cycle can only be completed if the necessary snail is within close range of the miracidium. In the case of the *S. japonicum* strain found in China, the required snail

species is *Oncomelania hupensis.* Of concern to control efforts, one study has implicated the completion of the Three Gorges Dam on the Yangtze River in the expansion of the habitat of *O. hupensis* (Hotez *et al*. 1997). After a period of maturation in the snails, sporocysts containing hundreds of cercariae develop which are subsequently released from the snail into the water, completing the cycle.



Since the 1950s, efforts have been made in the People's Republic of China to disrupt this life cycle in the interest of preventing schistosomiasis transmission to humans (Minggang and Zheng, 1999). Of particular challenge to disease control is the fact that other animals besides humans can be the definitive host for *S.japonicum*. In farming communities that have close associations with these animals, risk of transmission is increased by practices such as using cattle dung mixed with human excrement as a fertilizer termed "nightsoil" (Spear *et al.* 2002). With the financial support of a World Bank Loan to the government of China (1992-1998), morbidity control has been attempted through health education, chemotherapy for humans and cattle, and the reduction of snail populations, including the use of molluscicides. Despite drastic improvements in some regions, the disease remains endemic other areas. Lake and

mountainous regions have proven especially challenging, demonstrating that intervention tactics are not without their limitations. For example, chemical molluscicides have successfully controlled some snail populations, but not others (Spear *et al*. 2002). Praziquantel can be an effective treatment under appropriate social and economic conditions, but already research has indicated an increase in praziquantel resistant strains (Black 2002). In short, no single intervention has proven sufficient to control schistosomiasis transmission across China's diverse environment.

Over the past decade, a study lead by Dr. Robert Spear of the School of Public Health at the UC Berkeley has developed a mathematical model to describe schistosomiasis transmission in the mountainous Sichuan Province of China - a "problem area for the control programme" due to extensive snail habitats and socio-economic underdevelopment (Minggang and Zheng, 1999). The model employs several parameters and calibrates them to local conditions using field data with the objective of developing a tool that will better inform the combination of interventions to be applied in a particular setting, in addition to predicting their effectiveness (Spear *et al*. 2002). Parameters include established biologic values (e.g. the number of cercariae produced per sporocyst, per day), measurable data (including variables such as estimates of the snail population and human exposure to hazardous water) and environmental inputs such as rainfall and water temperature. The input and measurable parameters rely on data from the field, and the integrity of the model as a whole is influenced by the quality of these measurements.

Information about host snail distribution and the presence of *S. japonicum* in spatially distinct snail populations are two key variables necessary to the development of a mathematical model for schistosomiasis transmission. Global positioning system (GPS) technology is currently used to map the density of the *Oncomelania* snail population measured via field surveys. Because not all snails are infected, cercarial bioassays using mice are also employed to measure the spatial variation in cercarial concentration in water (Spear *et al*. 2002). While the low spatial resolution of cercarial data is a serious limitation of the mouse bioassay method, the technique is robust when applied to turbid waters found in the field, and avoids the problems associated with the stickiness of *S. japonicum* cercariae, which typically makes them difficult to sample with traditional water quality apparatus (Spear *et al*. 2003).

Despite these advantages, however, the use of mouse bioassays is an expensive and time-consuming endeavor. The intent of this project is to develop a method in the laboratory for the detection of *S. japonicum* cercariae from water samples. This method can then be adapted for and applied to field samples with the objective of improving the accuracy of the estimate of cercarial hazard of surface waters in the study described above and, possibly, to eliminate the need for mouse bioassays. Polymerase chain reaction (PCR) is a promising technique for accomplishing this objective.

A rapid method of amplifying a specific genetic sequence, PCR exhibits high sensitivity and specificity, limited only by the current genetic information available for the target species. PCR assays have been successfully developed and described for *S. mansoni* and *S. haematobium* (Hamburger *et al*. 2001) and for bird schistosomes (Hertel *et al*. 2001) and used with pure lab samples as well as water samples taken from the field. Furthermore, PCR does not require high concentrations of the target organism in the sample, as is the case with some immunological detection methods, such as enzyme-linked immunosorbent assay (ELISA). PCR assays conducted on *S. mansoni* have successfully demonstrated a detection level of as few as one cercaria per sample (Hamburger *et al*. 1998). PCR is also less susceptible to cross-contamination in the field than immunological techniques, although there is a risk of cross-hybridization to species with very similar genomes, depending on the genetic sequence selected for amplification.

In the present study, a highly sensitive PCR assay was developed for the successful identification of *S. japonicum* cercariae in laboratory samples. The primers were designed based on the sequence of a retrotransposon that is present in multiple copies in the *S. japonicum* genome.

Methods

Thirteen infected *Oncomelania hupensis* (subspecies *hupensis*) snails and seventeen infected *Oncomelania hupensis (*subspecies *chiui*) snails were obtained from the laboratory of Dr. Fred Lewis at the National Institutes of Health for the purposes of this study. All were exposed to *S. japonicum* miracidia in December of 2003 and the majority were determined to be sporocyst-positive prior to their shipment. The snail population was maintained in Petri dishes with a mixture of autoclaved mud, deionized water, and an

algal food source for approximately two weeks following their arrival. Over the course of these weeks, the infected snails actively shed cercariae when submerged in water and exposed to a light source. Appropriate measures were taken in accordance with the guidelines of the UC Berkeley Biosafety committee to ensure the safety of everyone in the lab.

To perform the PCR assays, samples of individual cercariae were harvested from the snails and their DNA was extracted with the Qiagen DNEasy® commercial animal tissue DNA extraction kit. Samples collected included uninfected snail tissue, infected snail tissue, and stream and deionized water containing both high and low densities of individual free-swimming cercariae. To obtain high-density (more than 100 cercariae) samples, snails were crushed and the cercariae released artificially. The shells of these snails were carefully cracked between the top and bottom of a Petri dish and separated from the snail tissue with dissecting needles. The tissue was then placed in a well slide under a dissecting microscope, whereupon 2-3 drops of deionized water were added, facilitating the release of the cercariae. A micropipette was used to transfer the cercariae into microcentrifuge tubes in preparation for DNA extraction.

Cercariae that were allowed to emerge naturally from submerged snails were used for the low-density samples. In this method of harvesting, two to three snails were placed in the bottom of a 50 mL plastic conical tube containing either deionized water or stream water from Strawberry Creek on the UC Berkeley campus. Each tube was placed beneath a 60-watt light bulb to attract the cercariae to the surface. The optimal time to allow the cercariae to emerge and swim to the surface of the water was determined to be 12-15 hours. After this amount of time, an average of 20 free-swimming cercariae could be collected per 80 µL of surface water. After the cercariae died, they sank to the bottom of the tube.

Once water samples containing a specified amount of cercariae were obtained, their DNA was extracted. The Qiagen DNA extraction kit allowed for the purification of cellular DNA with silica-gel membrane technology instead of organic extraction or direct ethanol precipitation. The procedure for DNA extraction involved lysing the cells with proteinase K and using a variety of buffers to facilitate the binding of DNA to the silica-gel membrane. Following cell lysis and incubation at high temperatures in a hot water

bath, contaminants were flushed from the membrane through a series of centrifugation steps, and the DNA was eluted in buffer in order to be made available for use in the PCR assays.

The PCR assay was based on a standard protocol with the variants being the gene sequence selected for amplification and the oligonucleotide primers that correspond to its 5' and 3' ends (Harris 1998). In this case, the target gene sequences were selected from *SRj2*, a 3.9 kilobase (kb) non-long terminal repeat retrotransposon described by Dr. Paul Brindley and others (Laha *et al* 2002). Hybridization analysis indicated that approximately 10,000 copies of the retrotransposon can be found among the *S. japonicum* chromosomes, which is equivalent to approximately 14% of the entire genome. The frequent occurrence of this retrotransposon made a sequence within it an ideal candidate for PCR amplification, especially from samples containing very few cercariae.

One long (1328 basepair (bp)) and one short (916 bp) sequence were chosen as target sequences from within the endonuclease domain of the retrotransposon, a region more likely to be species specific than the highly conserved reverse transcriptase domain (see Table 1). The same 20 nucleotide (nt) upper primer was used for both sequences, so the shorter target sequence is essentially equivalent to the first two-thirds of the longer target sequence. The primers were designed using Oligo Explorer® primer design software and were synthesized by the Qiagen® corporation. Before selecting the primers, the *SjR2* sequence was compared to the *SR2* sequence from *S. mansoni* (which belongs to the same clade of non-long terminal repeat retrotransposons) using Clustal X genome analysis software to ensure that an area of significant homology was not selected as the target sequence.

SjR2 Primer	Primer Sequence	Primer length (nt)	Target sequence length (kb)
Upper Primer	AGCCCAGTTTCTTTTTCAGG	20	
Lower Primer 1	ATGTCAACCGATGTCTTTCC	20	916
Lower Primer 2	TGCCGAGGATCTATCAGTTC	20	1328

Table 1. *SjR2* Primer design

Each PCR assay was performed using 2 microliters (µL) of DNA in a 25µL reaction mixture containing 2.5µL of 10X buffer containing $MgCl_2$ (New England Biolabs), 0.5µL deoxynucleotide triphosphates (dNTPs) (10 mM, Roche), 0.25µL *Taq* polymerase (5

units/mL, New England Biolabs), and 18.5µL distilled DNAase and RNAase free water. Also included were 0.625µL of each primer at 10µM. An automated thermocycler was used for the denaturating, annealing, and amplification steps. The program was set to 94°C for two minutes, and then underwent 35 cycles of 94°C, 55°C, and 72°C for one minute each. For samples containing only one cercariae, the number of cycles was extended to 40 to increase amplification. The final extension step took place at 72°C for two or five minutes. Certain samples were run at an annealing temperature of 57°C for 40 cycles, with twice the primer concentration (1.25µL). The amplification products were separated by gel electrophoresis with 1% agarose gel in tris-acetate-EDTA (TAE), stained with ethidium bromide and visualized with a UV transilluminator. A 100bp DNA ladder (Invitrogen) was used as the size marker.

Results

Initial PCR amplifications were performed using primers for both the short and long target sequences on samples of uninfected snail tissue, infected snail tissue, and varying densities of cercariae in water (Figs. 1a and b). Both the short and long products could clearly be visualized for all samples containing free cercariae, regardless of their density, the manner in which they were collected, or the characteristics of the water they were sampled in. No product appeared in the uninfected snail tissue samples but a non-specific stain of DNA appeared in the infected snail tissue sample. The final lane in Figure 1a (lane 10) is the amplification of the same DNA sample as lane nine, using the short product lower primer instead of the long product lower primer. With the DNA ladder as a guideline, the short PCR product appears as a distinct band at about 916kb and the long PCR product appears at about 1328kb. All lanes containing a product also demonstrated some degree of non-specific binding (the DNA that can be viewed is not confined to a band). This result was addressed in later assays by raising the PCR annealing temperature from 55°C to 57°C.

To test the sensitivity of the assay, two samples containing one cercaria each as well as a sample containing two cercariae were tested with the short primers (Fig. 2). Although faint, product bands can be seen for both samples containing an individual cercaria, and a band can be clearly seen for the sample containing two cercariae. To

increase the product yield for the single cercaria samples, the primer concentration in the PCR mixture was doubled and the number of PCR cycles was increased from 35 to 40. In addition, the annealing temperature was raised to 57°C in a subsequent assay in order to reduce the non-specific binding by the primers (Fig. 3).

Figure 3 reveals clearer product bands for the single cercaria sample as a result of the PCR optimization steps that were taken. In Figure 3, no PCR product is present in the sample containing infected snail tissue, despite the optimization steps that were taken in Figure 3 to provide adequate concentrations of primers and to reduce non-specific binding of primers. In every lane except those containing snail tissue DNA, very short products can be seen at the bottom of the gel, demonstrating the presence of excess primer in the form of primer self-hybridization.

Before the samples were subjected to PCR amplification, their genomic DNA content was assessed with a spectrophotometer (data not shown) and with gel electrophoresis visualization (Fig. 4). The DNA content for the samples containing cercariae only were below the detection threshold of the spectrophotometer. Figure 4 reveals DNA in both samples containing some form of snail tissue, but in neither of the cercariae-only samples. Successful PCR amplification of target sequences using the same samples indicates that the assay is sensitive enough to detect amounts of DNA that cannot be otherwise quantified.

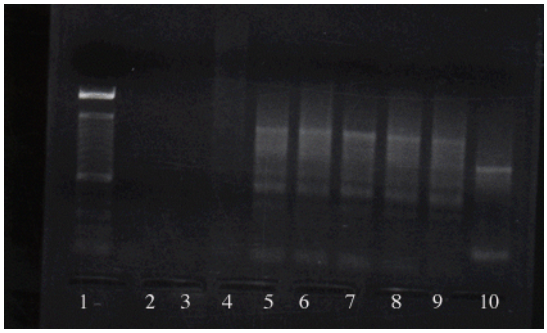


Figure 1a. Amplification of long (1328bp) target sequences with various samples (except last lane). (55°C annealing temp, 1x primer concentration, 35 PCR cycles). Lane 1 is the DNA ladder, 2 is the water control, 3 is uninfected snail tissue, 4 is infected snail tissue, 5 is a sample with four cercariae, 6 is a sample with 25 cercariae, 7 is a sample of many cercariae released into deionized water, 8 is many cercariae released into creek water, 9 is cercariae from a crushed snail, and 10 is the same sample as in lane 9, amplified with the short primers instead of the long primers.

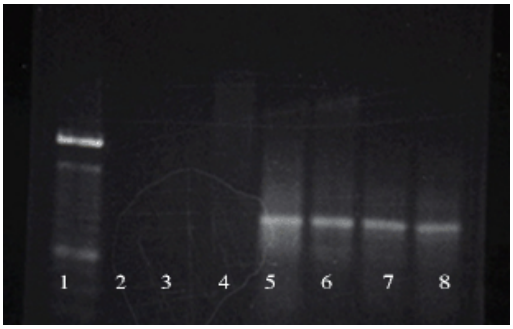


Figure 1b. Amplification of short (916bp) target sequences with various samples. (55°C annealing temp, 1x primer concentration, 35 PCR cycles). Lane 1 is the DNA ladder, 2 is the water control, 3 is uninfected snail tissue, 4 is infected snail tissue, 5 is a sample with four cercariae, 6 is a sample with 25 cercariae, 7 is a sample of many cercariae released into deionized water, 8 is many cercariae released into creek water. There appears to be less non-specific binding than with the long primers.

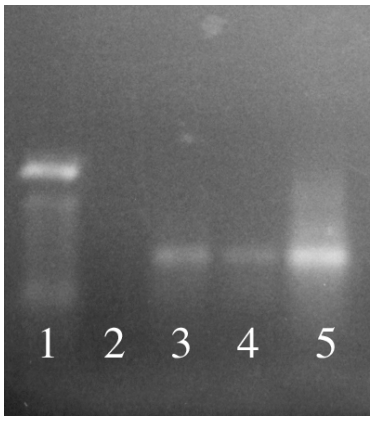


Figure 2. Amplification of the short product from a single cercaria demonstrates the assay's sensitivity. (55°C annealing temperature, 1x primer concentration, 35 PCR cycles). Lane 1 is the DNA ladder; lane 2 is the water control. Lanes 3 and 4 represent separate samples containing one cercaria each. Lane 5 is a sample containing two cercariae.

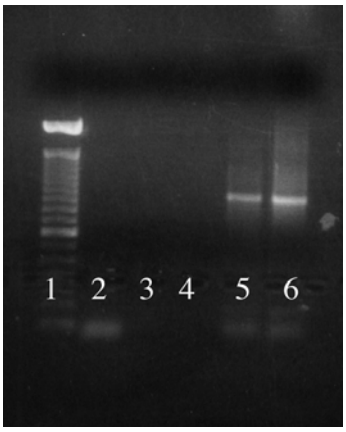


Figure 3. Snail tissue is retested with twice the amount of primer. (57° annealing temperature, 2x primer concentration, 40 PCR cycles). Lane 1 is the DNA ladder; lane 2 is the water control. Short primer self-hybridizing products can be seen at the bottom of the gel in all samples except 3 and 4, the uninfected and infected snail tissue samples, respectively. Lane 5 is a single cercaria sample and lane 6 is a sample containing four cercariae.

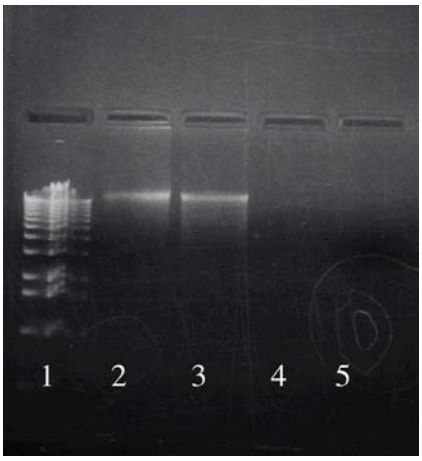


Figure 4. Genomic DNA visualized prior to amplification. Lane 1 is a 1kb DNA ladder. Lanes 2 and 3 contain uninfected and infected snail tissue, respectively. Lane 4 is a sample with four cercariae, and lane 5 is a sample with 25 cercariae.

Discussion

These results demonstrate the successful PCR amplification of two target sequences from the *SjR2* retrotransposon in laboratory samples of *S. japonicum* cercariae, with the potential for applying this method of detection in the field. Samples containing a high density of cercariae were collected in order to make initial assessments of the capability of the assay to detect the schistosomal DNA and to confirm that valid primers were designed. Once this was established, lower density samples were used to test the sensitivity of the assay. Consistent product amplification of single cercaria suggests that the sensitivity level of this assay is as low as one cercaria per water sample. The product yield for the single cercaria samples was improved by increasing the number of PCR amplification cycles from 35 to 40. Although non-specific amplification products could be seen in the samples with an annealing temperature of 55°C, these products were reduced when the annealing temperature was raised to 57°C. The results also demonstrate that the organic matter present in a creek water sample does not inhibit amplification. Snail tissue assays were performed to ensure that the sequence being amplified was not present in the snail genome as well as to test whether cercariae could be detected in infected snail tissue.

The initial results of the snail tissue assays were inconclusive. The uninfected snail sample resulted in negative amplification while a non-specific smear of DNA appeared in

the lane containing the infected snail tissue. A positive amplification product was expected from infected snail tissue based on the results of similar testing for *S.mansoni* in *Biomphalaria glabrata* snails (Hamburger *et al.* 1998). In the Hamburger study, the sensitivity of the assay was tested by combining increasing amounts of uninfected snail DNA with samples of infected snail DNA. Their results showed that large amounts of uninfected snail DNA inhibited the detection of cercarial DNA from infected snails, presumably due to the non-specific binding of primers to snail DNA. The negative results were addressed by increasing the primer concentration, which lead to an amplification product. In accordance with the demonstrated success of this approach, we doubled the primer concentration for the samples containing infected snail tissue. Although there remained no amplification product (Fig. 3), an excess of primer (indicated by primer self-annealing bands) appeared in every sample except those containing snail tissue, indicating that the concentration of primer may still be a limiting factor. If this is the case, primer is likely to be binding non-specifically to the snail tissue to the extent that there is not sufficient primer available for the amplification of the cercarial DNA. More samples with increased primer concentrations need to be run in order to test this hypothesis.

The sample variants tested in this study could be tested more thoroughly to improve our confidence in the results. Multiple samples of infected snail tissue should be tested and the results compared to see if the same negative results appear. Additional primer should also be added to the sample until primer bands are also seen at the bottom of the snail tissue lanes. Once these technical issues are sufficiently addressed, further research on the integrity of the parasitic DNA within snail tissue should be considered if infected snails continue to yield negative results. Tests with additional substances such as the tissue of other aquatic organisms should also be performed to determine if they inhibit amplification as well, or if the phenomenon is unique to the snail host. The sensitivity limit of the assay could be addressed further with samples containing cercarial fragments (such as a head or tail) or residual cercarial DNA. This will give an indication of whether a PCR assay might yield positive results in a situation where there are no viable cercariae present.

The specificity of this PCR assay at the species level is yet to be determined. It must be tested against other schistosome species (as well as additional non-schistosome

organisms present at the study sites) to ensure the absence of cross-hybridization effects. Because there is no genetic information available for other schistosomes present in the *S. japonicum* habitat, these tests must take place with samples containing the organisms in question and cannot be approximated using software tools. In the event that cross-hybridization does occur with other species, it may be possible to digest the PCR samples with a restriction enzyme after amplification in order to differentially cut the *S. japonicum* product into a unique distribution discernable from the products of other schistosomes (Barber *et al.* 2000). PCR assays for additional target sequences may also be developed from other described elements of the *S. japonicum* genome, such as the *Gulliver* long terminal repeat retrotransposon (Laha *et al.* 2001). Different target sequences will contain different levels of homology and it is likely that one that produces no cross-hybridization can be found.

The results obtained to this point indicate that, with further tests and slight modifications, the PCR assay developed in this study may be a useful tool for the direct determination of cercarial hazard of surface waters in China's Sichuan province. High levels of sensitivity and specificity are crucial for this purpose due to the low density of cercariae in the water, as well as the presumed existence of other schistosome species at the study sites. Before testing the PCR assay in the field, a suitable water sampling technique must be developed, as well as an appropriate method for DNA extraction from large volumes of water. Once these steps are taken, samples of water can be tested in parallel using the mouse bioassays and the PCR detection assay, as suggested by other cercariometry studies (Prentice *et al.* 1984), in order to validate and compare the PCR assay to established methods. Finally, establishing a quantitative PCR assay that estimates of the number of cercariae in a given sample is vital to improving the risk-predicting capabilities of the mathematical model currently in use. Thus, the assay presented here is a valuable first step in achieving the eventual goals of informing risk assessments in endemic regions and prescribing more appropriate control strategies for the people of China's Sichuan province.

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