Field Evaluation of a PCR Test for *Schistosoma japonicum* Egg Detection in Low-Prevalence Regions of China

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Abstract. Sensitive *Schistosoma japonicum* detection methods are needed to progress from schistosomiasis control to elimination. The sensitivity of the Kato-Katz thick smear and miracidium hatching tests decrease with infection intensity and serological tests cannot always identify current infections. We evaluated a fecal polymerase chain reaction (PCR) assay to detect *S. japonicum* infection in 106 humans and 8 bovines in China. PCR was highly sensitive, detecting *S. japonicum* DNA at 0.5 eggs/g of stool. Comparing PCR examination of a single stool sample to the miracidium hatching test using three consecutive stool samples, more humans were hatching test positive (20%) than PCR positive (15%). However, two individuals were PCR positive in a village where no infections were detected by coprological methods. The sensitivity of PCR makes it a promising tool for schistosomiasis diagnostics and screening, although egg shedding variability and stool sample size present challenges for any detection method in low-transmission areas.

INTRODUCTION

The changing epidemiology of schistosomiasis in countries such as China has fueled the need for more sensitive diagnostic and surveillance methods.[1–4] Infection with the causative species in China, *Schistosoma japonicum*, is typically diagnosed using one of two coprological methods, the Kato-Katz thick smear procedure or the miracidium hatching test, or by immunoassays performed on serum. Both the Kato-Katz and hatching tests are inexpensive, require limited technical training, and are effective in situations of high prevalence and high-intensity infections, but their value is reduced in low-infection intensity settings as the sensitivity of the tests decrease with decreasing schistosome infection intensity.[5–7] The performance of immunoassays such as indirect hemagglutination assay, enzyme-linked immunosorbent assay, and dipstick dye immunoassay has recently been assessed.[8–10] These studies have shown that immunoassays have high sensitivities (> 90%) and ease of use when compared with coprological methods, but issues of specificity and the inability of antibody detection methods to distinguish between current and past infections are still of concern. Moreover, the sensitivity of serological testing, like coprological testing, declines as infection intensity decreases.[11] An expert committee convened by the World Health Organization (WHO) to discuss schistosomiasis elimination concluded "standard parasitological testing…is particularly insensitive for detecting low-level or light infections…"[4] A highly sensitive test is required to accurately assess prevalence of infection and propel the elimination effort forward.

This work evaluates the use of polymerase chain reaction (PCR) as a diagnostic and screening method for detection of *Schistosoma japonicum* infections in humans and bovines in four villages where schistosomiasis has reemerged. The use of PCR as a screening tool is advantageous because of its sensi-

tivity and ubiquity in modern clinical settings. Earlier studies have described PCR methods for detection of *Schistosoma mansoni* and *Schistosoma haematobium* DNA in animal models and in human samples.[12–16] *Schistosoma japonicum* PCR techniques have also been described.[17–22] However, the use of PCR techniques to screen field-collected samples has been limited.[19,21] Conventional PCR rather than quantitative real-time PCR (qPCR) was tested because of the limited need for a quantitative description of infections in a low-prevalence setting and because conventional PCR requires fewer resources than qPCR, making it a more accessible tool for resource-constrained settings. The PCR assay in this study was used to evaluate human and bovine samples and compared with Kato-Katz and miracidium hatching test results, providing, to our knowledge, the first evaluation of a PCR-based *S. japonicum* diagnostic method using field-collected samples from multiple *S. japonicum* host species.

MATERIALS AND METHODS

Study location and sample collection. This study was conducted in four rural, agricultural villages located in regions of Sichuan, China where schistosomiasis reemergence has been documented[23,24]; we define a village as the smallest organizational unit in a region, also referred to as a production group or natural village. Because of the sensitive nature of conducting infection surveys in regions where schistosomiasis transmission control criteria have officially been met, the names and exact locations of the counties and study villages have been withheld.

In November and December 2010, all residents in the study villages, 6–68 years of age, were invited to submit three stool samples on three consecutive days. Bovines in the village were identified by interviewing each head of household. Bovine samples were obtained by confining the animal to a pen or tying the animal until a sample was produced on three consecutive days. Human and bovine samples were collected and transferred to a county laboratory daily and examined using the miracidium hatching test and the Kato-Katz thick smear procedure upon arrival. Approximately 15 g of stool

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from one sample per person and bovine were transferred to a capped tube, frozen for transfer to Chengdu, and stored at −80°C at the Institute of Parasitic Diseases, Sichuan Center for Disease Control and Prevention, Chengdu, China (SIPD) for analysis using PCR in the summer of 2011.

Miracidium hatching test. All human and bovine stool samples were examined using the miracidium hatching test.[25] Approximately 30 g of stool were filtered through 80 head mesh to remove large particles, and then strained with 260 head nylon mesh to concentrate schistosome eggs. Tap water was used to wash the sample until rinse water ran clear. The sediment was then transferred to a flask and water was added to reach the narrow neck of the container for improved visualization. Because the miracidium hatching is sensitive to chlorine and pH, we resuspended sediment using water with pH between 6.5 and 7.5 and free chlorine < 1 ppm, and confirmed using a colorimeter (Colorimeter C301, NovaTech, Houston, TX). Samples were left undisturbed in a room with ambient temperatures between 28 and 30°C. Human samples were examined for the presence of miracidia 2, 5, and 8 hours after preparation. Because we have observed that *S. japonicum* eggs may hatch more rapidly in bovine stool because of the higher water volume, bovine samples were examined 1, 3, and 5 hours after sample preparation. Samples were examined for the presence of miracidia for at least 2 minutes at each observation point. A sample was classified as hatching test positive if any miracidia were observed.

Kato-Katz thick smear procedure. Using the Kato-Katz thick smear procedure, three slides were prepared using 41.7 mg of homogenized stool for each slide from the first stool sample submitted by each person.[26] Each slide was also examined for the presence of *S. japonicum* and the soil-transmitted helminthes (STHs), e.g., *Ascaris lumbricoides*, *Trichuris trichiura*, and *Ancylostoma* spp., to check for cross-reactivity. Because hookworm eggs degrade rapidly,[27] slides were examined 2 hours after preparation, using a light microscope, for the presence of hookworm eggs. Slides were then stored for 24 hours out of direct sunlight before examination for *S. japonicum*, *A. lumbricoides*, and *T. trichiura*. Infection intensity, expressed in eggs per gram of stool (EPG), was calculated as the total number of *S. japonicum* eggs divided by the total sample weight. A sample was classified as Kato-Katz positive for schistosomiasis if at least one *S. japonicum* egg was detected.

DNA extraction. DNA was extracted from one stool sample per person and bovine using the "isolation of DNA from larger volumes of stool" protocol from the QIAamp DNA Mini Stool Kit (QIAGEN, Valencia, CA), with slight modifications. A stool lysis buffer composed of 10 mM Tris pH 8.0, 300 mM EDTA pH 8.0, and 1% w/v sodium dodecyl sulfate was used for the first lysis step and the first incubation was conducted at 95°C. Extracted DNA was eluted with 50 μL of QIAGEN AE buffer. The DNA was extracted from 1 g of stool from each person and bovine.

PCR. PCR was performed on all extractions using previously designed primers[22]; the primers targeted the highly repetitive retrotransposon SjR2 of *S. japonicum* and resulted in a 230 bp product. The sequences of the primers were: 5′-TCTAATGCTATTGGTTTGAGT-3′ (forward) and 5′-TTC CTTATTTTCACAAGGTGA-3′ (reverse). The reaction mixture was composed of 0.2 μL of each 25 μM primer, 0.2 μL 10 mM dNTP, 0.04 μL 5 Units/μL GoTaq DNA polymerase (Promega, Madison, WI), 2 μL 5× buffer with 7.5 mM MgCl₂,

0.4 μL bovine serum albumin, 1.5 μL template DNA, and distilled water to a final volume of 10 μL. The initial denature step was run at 95°C for 5 minutes, followed by 35 cycles of 94, 53, and 72°C for 1 minute each. The final extension was run at 72° for 7 minutes. Amplified products were run on a 2% agarose gel with GoldView (SBS Genetech Co., Ltd., Beijing) for visualization.

Validating protocol and assessing sensitivity. *Schistosoma japonicum* worms from an infected rabbit were provided by SIPD and had been stored at 4°C in 96% EtOH. Each worm was washed three times in distilled water and then suspended in 10 μL of lysis solution (0.02M NaOH, pH 12) and incubated at 95°C for at least 30 minutes. The resulting lysate was cooled to 4°C and 10 μL of neutralizing solution (40 mM Tris-HCl, pH 5) was added. The expected 230 bp PCR product was seen when worm lysate was used as the DNA template. The DNA extracted from the stool sample of an individual from an *S. japonicum* non-endemic population was used to test the primer specificity.

Sensitivity of the PCR assay was assessed by spiking stool samples from the non-endemic individual with *S. japonicum* eggs. Eggs from rabbit liver were provided by SIPD and had been stored at −20°C in 96% EtOH. Each spiked sample was prepared in triplicate with 1, 2, 3, 4, 5, 10, and 20 egg(s) in 1 g of stool, respectively. The three 0.5 EPG samples were prepared by spiking 2 g of stool with 1 egg. The full 2 g of spiked stool was used for DNA extraction. All DNA was extracted using the previously described DNA extraction protocol.

Ethical approval and treatment. All participants provided written informed consent before participating in this study. The research protocol was approved by the Sichuan Institutional Review Board and the University of California, Berkeley, Committee for the Protection of Human Subjects. Each person who tested positive for *S. japonicum* was provided treatment with praziquantel by the county Anti-Schistosomiasis Control Station. All bovines testing positive were referred to the county veterinary station for treatment with praziquantel.

RESULTS

The PCR assay was shown to be highly sensitive in detecting *S. japonicum* DNA in spiked stool samples. *Schistosoma japonicum* DNA was detected at 0.5 EPG as well as 1–5, 10, and 20 EPG.

A total of 142 people from the four study villages submitted stool samples. Individuals who submitted stool samples were, on average, older (mean age 49) and more likely to be female (50% female) than those who did not participate in the infection survey (mean age 39, 43% female), as many young adults, particularly males, leave the study villages to work or attend school in urban areas. We tested 106 people using all three parasitological testing methods (because of inadequate stool sample size, 26 individuals lacked results from all three tests). The prevalence of infection for these 106 individuals was 10.4%, 19.8%, and 15.1% according to the Kato-Katz thick smear, miracidium hatching test, and PCR assay, respectively. The miracidium hatching test was positive for 13.2% of individuals based on their first stool sample, 9.4% based on the second stool sample, and 13.9% based on the final stool sample. Mean infection intensity was 3.0 EPG (range 0–88). Of the 11 Kato-Katz positive individuals, seven

TABLE 1

Detection of human *Schistosoma japonicum* infection by three different testing methods in four villages located in regions where schistosomiasis has reemerged in China

Village	Tested	Number infected by		
		Hatching test	Kato-Katz	PCR
A	40	8	4	7
B	35	13	7	7
C	20	0	0	2
D	11	0	0	0
Total (%)	106	21 (19.8)	11 (10.4)	16 (15.1)

Each participant was requested to submit three stool samples, one per day from three consecutive days. Each sample was examined using the miracidium hatching test. One sample per person was examined using the Kato-Katz thick smear procedure (preparing three slides per person) and polymerase chain reaction (PCR).

had only 1–3 eggs detected (8–24 EPG). At the village level, *S. japonicum* infections were detected by Kato-Katz and the hatching test in two of the four villages, and by PCR in three of the four villages (Table 1).

Table 2 shows the concordance between the three tests. Two samples were PCR positive only, four samples were hatching test positive only, and zero samples were Kato-Katz positive only. When we examined only the first miracidium hatching test, four individuals were classified as hatching test positive, but PCR negative. When all stool samples were analyzed using the miracidium hatching test, eight individuals were classified as hatching test positive but PCR negative.

We tested eight bovines using PCR and the miracidium hatching test. Six bovines were positive by both tests. One bovine was PCR negative and hatching test positive, and one bovine was PCR negative and hatching test negative. All eight bovines were from the same village.

Concomitant STH infections were seen, with a prevalence of 16% for hookworm infection, 6.6% for *A. lumbricoides*, and 3.8% for *T. Trichuris* infection among the 106 participants. No additional amplified bands were observed in the samples from individuals with STH infections.

DISCUSSION

PCR-based detection methods for *S. japonicum* have been evaluated using experimental animal models[17,18,20,22]; how-

TABLE 2

Comparison of three different diagnostic tests for *Schistosoma japonicum* infection in human stool, the Kato-Katz thick smear, the miracidium hatching test, and a PCR assay, conducted in regions where schistosomiasis has reemerged in China

	PCR+	PCR−
Miracidia hatching test (1 sample) and Kato-Katz		
Kato-Katz+ and Hatch+	5	3
Kato-Katz+ and Hatch−	2	1
Kato-Katz− and Hatch+	5	1
Kato-Katz− and Hatch−	4	85
Miracidia hatching test (all samples) and Kato-Katz		
Kato-Katz+ and Hatch+	6	4
Kato-Katz+ and Hatch−	1	0
Kato-Katz− and Hatch+	7	4
Kato-Katz− and Hatch−	2	82

Each participant was requested to submit three stool samples, one per day from three consecutive days. Each sample was examined using the miracidium hatching test. One sample per person was examined using the Kato-Katz thick smear procedure (preparing three slides per person) and polymerase chain reaction (PCR). The table presents the results of the miracidium hatch test performed on the first stool sample ("1 sample") and the combined results of the miracidium hatching tests from all samples from an individual ("all samples").

ever, PCR-testing of clinical samples from humans and field-collected samples from other mammalian hosts has been limited.[19,21] Here, we have demonstrated a highly stable and sensitive PCR method able to detect schistosomiasis infection in a region where schistosomiasis had reemerged and infection intensity was low (mean infection intensity: 3.0 EPG). The ability to detect *S. japonicum* infection in low-prevalence areas has important implications for the clinical management of schistosomiasis and public health surveillance.

Schistosomiasis morbidity is generally proportional to infection intensity, as the quantity of eggs can increase fibrosis and the risk of anemia.[28,29] However, low-intensity schistosomiasis infection when combined with low-intensity STH infection may synergistically increase the risk of anemia,[30] underscoring the importance of sensitive diagnostics in areas where multiple helminth species are present, as in the case in our study region. In addition, the ability to detect low-intensity infections will be a critical tool in the determination of a morbidity profile of such infections, allowing us to more fully characterize their clinical effects.

A highly sensitive and stable assay for the detection of *S. japonicum* infections in humans and bovines is needed to attain long-term interruption of schistosomiasis. Our study demonstrates a viable detection method for *S. japonicum* infections in two host species. The bovine results are preliminary and suggestive, rather than definitive, because of the limited number of samples that were used. Future studies testing this method on a larger sample size of both humans and bovines will be worthwhile.

Schistosoma japonicum transmission is persistent even in regions with very low parasite concentrations, and models suggest modest parasite inputs can lead to the initiation of transmission in formerly parasite-free environments.[31] Thus, identification and treatment of all infections, including those of animal hosts, is crucial to elimination efforts. The detection of infection by PCR in the village where infection prevalence was 0% according to Kato-Katz and hatching test demonstrates the value of PCR-based surveillance. Because control programs occur at the village level, more accurate village-level prevalence measures will better direct control efforts and provide insight to possible reasons for reemergence. The use of pooled samples may provide a method for cost-effective monitoring. The PCR method described here is also being piloted to detect *S. japonicum* DNA in snails and stool pits, which are candidates for environmental sampling.

Notably, the PCR results did not correspond exactly to the results of the two standard assays for *S. japonicum* egg detection. Two people were PCR positive for *S. japonicum* but negative according to the Kato-Katz and hatching test. We strongly believe these positive PCR results are true positives because of the absence of contamination in any of the negative control samples, which were analyzed with each set of field samples, underscoring the potential of the PCR method to offer a more sensitive diagnostic tool. In addition, eight samples were PCR negative for *S. japonicum* but hatching test positive. Based on the sensitivity of the PCR method in detecting *S. japonicum* DNA in the spiked stool samples, we do not believe that the fewer number of PCR positive results was caused by false negatives, but rather, to the absence of *S. japonicum* eggs in these samples. The hatching test results were based on the examination of a larger stool volume (30 g of stool per sample versus 1 g for the PCR assay) and the

examination of stool from 3 days versus 1 day. When the results of the first miracidium hatching test, based on a single stool sample, were compared with PCR, the number of samples that were PCR negative but hatching test positive decreased to four, suggesting some individuals may have been infected with *S. japonicum*, however eggs may not be clinically detectable in every stool sample. Variability in egg shedding, caused by day-to-day variability in stool output, clustering of eggs within stool, and inter-individual variance,[32–34] as well stool sample size could explain why overall, more samples were found to be hatching test positive than PCR positive.

To illustrate, previous studies have shown that the number of eggs produced by one pair of worms, over time, follows the negative binomial distribution such that

$$E \sim NB(\bar{E}, r), \tag{1}$$

where $\bar{E}$ is the mean egg output in EPG, and r is the aggregation parameter, a measure of the variability in egg output by a given worm pair. Assuming that egg output of each worm pair is independent (a reasonable assumption at low infection density), for an individual with N pairs of worms, the probability that no eggs are present in a one-gram stool sample is given by

$$\Pr(E_{obs} = 0 | P_w = N) = \left(1 + \frac{\bar{E}}{r}\right)^{-Nr}. \tag{2}$$

Following the estimations of Hubbard and others,[34] we set $\bar{E} = 1.44$ EPG and consider three scenarios corresponding to low, medium, and high levels of aggregation (Figure 1). In individuals infected with a single worm pair, the probability a one-gram stool sample contains zero eggs is 45–62%, depending on the aggregation parameter used. Although this probability decreases exponentially as the number of worm pairs increases, there is still a 10% chance of no egg production corresponding to $N = 5$ and $r = 0.25$. Therefore, one of the greatest challenges in correctly identifying infected individuals in low-prevalence regions may be simply obtaining a sample that contains *S. japonicum* DNA.

Collecting samples from multiple days can increase the probability that eggs are present in at least one sample. Equation 2 can also be used to calculate the probability that no eggs are present in stool samples obtained from an individual infected with *one* pair of worms on N different days, by substituting the number of worm pairs (P_w) with the number of days of sampling (D_s). If samples are collected on three different days, there is a 9–24% chance that no eggs will be present in any sample. For PCR analysis to be more accurately assessed as a possible diagnostic or screening method in very low infection intensity areas, it may be necessary to explore PCR assays that use DNA extracted from more than one gram of stool, or to collect samples on multiple days.

Though more expensive than the Kato-Katz and hatching test, PCR-based detection is a viable option in China because of the resources currently available at the province level and the willingness of the Chinese government to invest in public health infrastructure. At provincial anti-schistosomiasis control stations, there is both the equipment and technical knowledge necessary to perform PCR assays. At the time of our study, the county level anti-schistosomiasis control stations did not have the capacity to conduct PCR assays, but recent construction of new, standardized laboratories for these control stations and increased budget allowances may soon allow PCR capability. Although PCR requires the capital investment in a thermal cycler, the operating cost and process is relatively inexpensive and simple, especially with the use of master mixes made in batches and a small final mix volume. qPCR, although valuable for quantification, is more resource intensive than qualitative PCR and is not critical in a setting that only requires a binary outcome.

Countries that operate at a more low-resource setting than China may need detection methods that require less equipment than PCR. Loop-mediated isothermal amplification (LAMP) is another DNA amplification method that has been shown to be even more sensitive than PCR in the detection of *S. japonicum* DNA[35] and does not require the use of a thermal cycler or gel visualization. Tools such as LAMP have the potential to be very valuable in detecting low-intensity infections in low-resource settings, but because many of China's laboratory facilities are already able to perform PCR, this
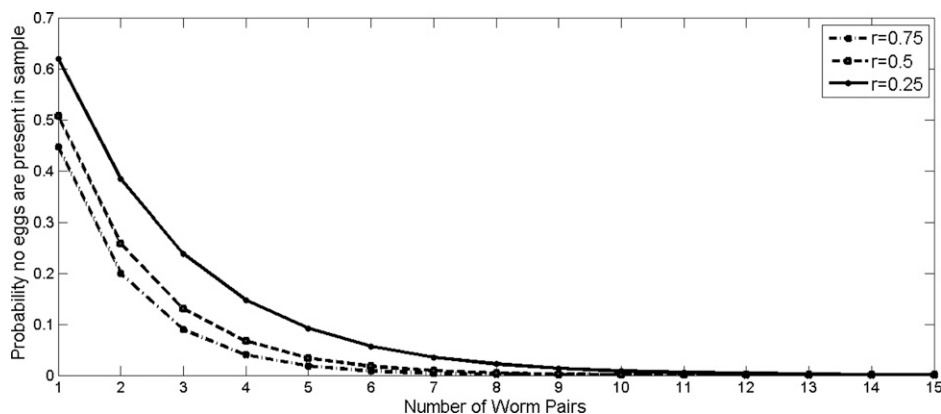

FIGURE 1. Probability that no eggs are present in a one-gram stool sample in an individual infected with N worm pairs, estimated using three estimates of variability in egg output, (r). We assume egg shedding follows a negative binomial distribution, with a mean egg output $\bar{E}$, set to 1.44,[34] and aggregation (a measure of variability in egg output), r. Probabilities were calculated for high, medium, and low estimates of r.

stable and validated assay is currently the most promising option for a highly sensitive diagnostic and screening method.

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