A PCR-based Assay for the Detection of *Schistosoma japonicum* from Human Samples

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RESEARCH ARTICLE

Abstract

Background and Objective: *Schistosoma japonicum* is the causative agent of schistosomiasis in the Philippines. Current diagnostics suffer from low sensitivity and accuracy, hence an accurate and reliable diagnosis of schistosomiasis is essential for its prevention and control. In this study, a PCR-based assay for the detection of *Schistosoma japonicum* for patient stool and serum samples was developed.
Methodology: Three candidate primer sets targeting mitochondrial genes *COX3*, *NAD4*, and *NAD5* were assessed. *COX3* primer pair was used for the rest of the study for sensitivity, specificity, and performance testing. Lastly, the assay using *COX3* primer pair was compared to Kato-Katz and circumoval precipitin test (COPT).
Results: *COX3* and *NAD5* primers showed suitability for the assay as sequencing analyses gave high similarities of 96-98% for *S. japonicum*, while *NAD4* showed no similarity to any organisms. The PCR-assay was shown to have a detection limit of 4 ng/ul DNA and was specific only to *S. japonicum*. The assay detected seven out of ten *S. japonicum*-spiked stool samples and ten out of ten *S. japonicum*-spiked serum samples. Comparative performance testing with Kato-Katz and COPT showed high specificity of 100% for both samples, but low sensitivity for formalin-fixed stool samples and stored serum samples.
Conclusion: This study developed a sensitive and specific PCR-based assay to detect *S. japonicum* from human samples. Results suggest that this PCR assay could be useful for the detection of *S. japonicum* in fresh clinical samples and can be further improved as a reference to improve other diagnostic assays for schistosomiasis.

Keywords: *Schistosoma japonicum, schistosomiasis, PCR, COX3*

Introduction

Schistosomiasis is an acute and chronic parasitic disease caused by blood flukes or trematode worms of the genus *Schistosoma* [1]. It is one of the most prevalent diseases in the world with an estimated 200 million cases and a mortality rate of 41,000-280,000 per year [2,3]. Schistosomiasis is endemic to tropical countries including the Philippines but in non-endemic areas, the disease represents an increasing problem due to immigrants and tourists contracting the disease [4,5,6]. In the Philippines, *Schistosoma japonicum* is the only species of schistosome and is the sole causative agent of schistosomiasis in the country [7]. This parasite is considered to be the hardest to control because of its zoonotic nature, enabling infection of 40 species of mammalian hosts including humans, cattle, rats, dogs, and cats [8,9,10,11,12].

Various methods of diagnosis have been established for human schistosomiasis [6]. Traditional parasitological methods which involve detection of eggs using microscope in stool samples, and immunological detection which includes serological tests and detection of specific antigens are still currently being employed [3]. Stool examination remains to be the gold standard for detection because it is relatively inexpensive and simple, however, it requires highly trained and experienced technicians and lacks sensitivity when the prevalence and intensity of infection are low [11,13,14,15,16]. Immunological methods on the other hand, also suffer from low sensitivity, as well as distinguishing reliably between current and recent schistosome infections [3,17]. Recently, molecular techniques have been gaining popularity in the field of diagnosis. PCR-based assays for the

detection of Schistosoma species are presently being developed to circumvent the pitfalls of existing diagnostic methods [15,18,19]. These molecular-based assays are known to provide greater sensitivity and specificity provided that reliable molecular genetic markers are used [6,20].

In this study, a PCR-based assay was developed to detect *Schistosoma japonicum* in patient stool and serum samples. Few studies have assessed the application of PCR to diagnose schistosomiasis from human samples in the Philippines using conventional PCR, real-time PCR, and droplet digital PCR. These methods, especially the latter two, are expensive and require advanced and specialized equipment [21,22,23,24]. Moreover, three primer pairs were used targeting three separate mitochondrial genes, namely: cytochrome c oxidase subunit 3 (*COX3*) and NADH hydrogenase subunits 4 and 5 (*NAD4* and *NAD5*) aside from the usual mitochondrial targets such as *NAD1* and *COX1* genes [9,21, 22, 23, 24].

Methodology

Clinical Sample Collection and DNA Extraction

Formalin-fixed stool and serum samples were obtained from the College of Public Health, University of the Philippines Manila. The protocol for handling the human samples was reviewed and approved by the UP Manila Research Ethics Board (UPMREB-2013-NIH-P2-052). Stool samples were already previously diagnosed with schistosomiasis via Kato-Katz method for stool samples and via COPT for serum samples. Ten stool samples were used for DNA extraction using QiaAmp Mini Stool DNA Kit™ (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Since Kato-Katz-negative stool samples were absent, distilled water was used instead. For the serum samples, ten COPT-positive and five COPT-negative samples were used for DNA extraction using DNeasy Blood & Tissue Kit™ (QIAGEN, Hilden, Germany) following manufacturer's protocol. DNA from *Schistosoma japonicum*-positive snails, previously collected in Leyte, was extracted using QiaAmp Mini Stool DNA Kit™ (QIAGEN, Hilden, Germany) and served as the positive control. All DNA extracts were then stored at -20°C prior to PCR.

Primer Selection, PCR Amplification and Sequencing

Primers specific to *COX3*, *NAD4*, and *NAD5* genes of *Schistosoma japonicum* were retrieved from Zhao *et al*. as shown in Table 1 [9].

Table 1. *Primers for S. japonicum retrieved from Zhao et al. [9].*

Target Gene	Forward and Reverse Primers (all 5' → 3')	Expected Size (bp)
NAD5	GKTTATCTGGKTTTCCWTTTA TGGGTGAGATCTCYTACTTG GAAAGAAAG	680
NAD4	GTGGTTAGTTGGTTCTAAGG ATAACAAGTATACCCCATTTA CGAG	747
COX3	GAGTTTATTTTCATTAGTAAAA TTACTAACAAATACACAATGG TAAA	643

PCR reactions were done using MyCycler™ Thermal Cycler (Bio-Rad, California, USA) thermocycler and the cycling was performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 30 s at 94°C, annealing at 30 s at 50°C for both *COX3* and *NAD5* primers while 45°C for NAD4 primers, extension at 1 min at 72°C; followed by a final extension of 5 min at 72°C. Amplicons were visualized via agarose gel electrophoresis (AGE) and GelDoc™ (Bio-Rad, California, USA). Further confirmation was done by sending PCR products for sequencing to Macrogen Korea. Sequenced PCR products were compared to all target genes namely, *COX3*, *NAD4*, and *NAD5* of *S. japonicum* using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Assessment of Sensitivity and Specificity

Only the *COX3* primer set was used for the rest of the study, while the *NAD5* primer set will be used for future studies. To evaluate the specificity of the PCR assay, common bacteria, namely: *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus epidermidis*, and one trematode worm *Paragonimus westermani* (same class as *S. japonicum*) were used. No genomic DNA was used as negative control. The sensitivity was assessed by performing PCR on one *S. japonicum* positive control with decreasing DNA concentrations and the detection limit was determined as the smallest DNA template amount at which a PCR product was still visible.

Performance of the PCR Assay via Spiking Studies

Ten *Schistosoma japonicum*-positive snails, previously collected in Leyte, were placed in a microcentrifuge tube, crushed and homogenized in sterile double distilled water. Aliquots of the resulting homogenate were used for serum and stool spiking. Approximately five grams of *S. japonicum*-

negative formalin-free stool sample was homogenized in 1X PBS. Aliquots were transferred to ten 1-mL tubes and the first tube was spiked with the same volume of snail homogenate, then a four two-fold dilution was prepared. Two replicates per dilution were prepared for a total of ten spiked samples. The serum sample was directly spiked with the snail homogenate following the same spiking protocol for the stool sample. After the samples were spiked, DNA extraction was done using QiaAmp Mini Stool DNA Kit™ (QIAGEN) and DNeasy Blood & Tissue Kit™ (QIAGEN) for stool and serum samples, respectively. PCR amplification was carried out using the cycling conditions for *COX3* primer set and the amplicons were likewise analyzed and viewed via AGE and GelDoc™ (Bio-Rad).

Comparative Performance Testing

Preliminary performance was compared to Kato-Katz and COPT methods using previously diagnosed formalin-fixed stool samples and serum samples. For the comparative performance testing, the accuracy and efficiency measurements were based on two parameters: specificity and sensitivity as compared to Kato-Katz and COPT results. The calculations were done as follows:

Specificity = D/(B+D) x 100%	Sensitivity = A/(A+C) x 100%

Table 2. *Corresponding proportions of A, B, C, and D values to be used for comparative performance testing.*

PCR Assay	Kato-Katz or COPT	
	Positive	Negative
Positive	A	B
Negative	C	D
Total Samples	A+C	B+D

Legend: A = no. of samples positive for both PCR and negative for Kato-Katz or COPT. B = no. of samples positive for PCR and negative for Kato-Katz or COPT. C = no. of samples negative for PCR and positive for Kato-Katz or COPT. D = no. of samples negative for both PCR and Kato-Katz or COPT.

Results

Comparison of the Three Candidate Primers

Three candidate primers were used in this study, all of which were retrieved from Zhao *et al.*, targeting three mitochondrial genes namely *COX3*, *NAD4*, and *NAD5* genes [9]. The primers were used to amplify *S. japonicum* DNA via PCR for validation and amplicons were sequence-verified. Retrieved DNA sequences for both *COX3* (617-621 bp) and *NAD5* (668-669 bp) primer pairs had lengths comparable to the expected sizes of 643 and 680, respectively. For the *NAD4* primer, on the other hand, the PCR amplicon of 429 bp was lower than the expected size of 747 bp, showing a truncated product. The same sequences were ran using the BLAST software and have shown that *COX3* and *NAD5* primer sets had % identities of 96-98% to *S. japonicum* mitochondrion, while *NAD4* showed no similarity to any *Schistosoma* species.

Assessment of Sensitivity and Specificity of the PCR-based Assay

Using the *COX3* primer set, the sensitivity of the PCR assay was assessed using a decreasing dilution of *S. japonicum* DNA. The *NAD5* primer set, on the other hand, will be used for future studies. The limit of detection is the

Table 3. *Sequencing and BLAST results of amplified products of the three candidate primer sets.*

Primer Set		Sequencing Results		
		Length	BLAST Result	% Identities
COX3	F	621	*Schistosoma japonicum mitochondrion*	96
	R	617	*Schistosoma japonicum mitochondrion*	98
NAD4	F	429	-	-
	R	429	-	-
NAD5	F	668	*Schistosoma japonicum mitochondrion*	96
	R	669	*Schistosoma japonicum mitochondrion*	98

smallest amount of detectable DNA and was determined to be 4 ng/ul of DNA. The electropherogram is shown in Figure 1.

The specificity of the assay was tested against other microorganisms specifically, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, and *Staphylococcus epidermidis,* and one trematode worm *Paragonimus westermani*. No amplicons were amplified for the tested organisms except *S. japonicum* DNA making the assay specific only to *S. japonicum* as shown in Figure 2.

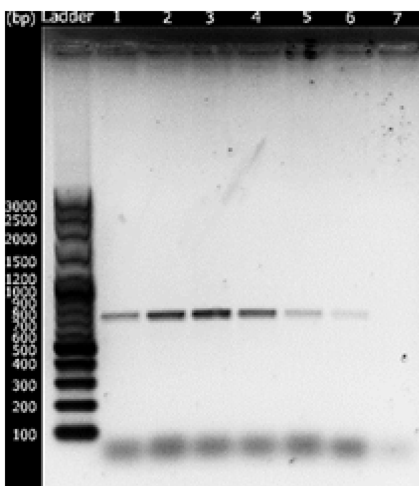

Figure 1. *Electropherogram for the sensitivity test using COX3 primer set. Ladder = VC 100bp Plus DNA ladder (Vivantis). Lane 1 = Positive control, Lanes 2-6 represent decreasing template concentration of 32, 24, 16, 8, 4, 2 ng/ul, respectively.*

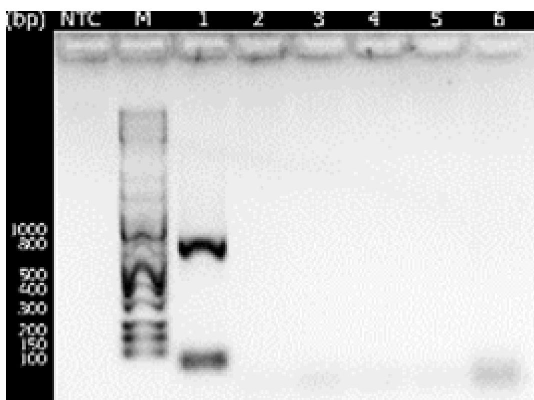

Figure 2. *Electropherogram for specificity test using COX3 primer set. M = KAPA Universal Ladder (Kapa Biosystems). NTC = no template control, Lane 1 = S. japonicum, Lane 2 = P. westermani, Lane 3 = E. coli, Lane 4 = S. epidermidis, Lane 5 = S. aureus, Lane 6 = E. aerogenes.*

Performance of the PCR Assay via Spiking Studies

Spiking was done to determine the performance of the assay when used in *S. japonicum*-free formalin-free stool samples. The same spiking was also done for a serum sample. In the ten spiked stool samples, seven out of ten bands were detected as shown in Figure 3. For the spiked serum samples, all ten spiked samples showed the appropriate bands as shown in Figure 4.

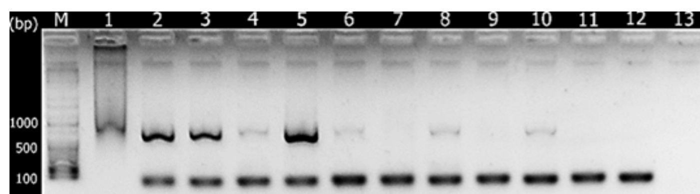

Figure 3. *Electropherogram of the PCR assay for S. japonicum-spiked formalin-free stool sample. M = KAPA Universal ladder (Kapa Biosystems). Lane 1 = positive control, Lanes 2-11 = spiked samples, Lane 12 = unspiked sample, Lane 13 = NTC.*

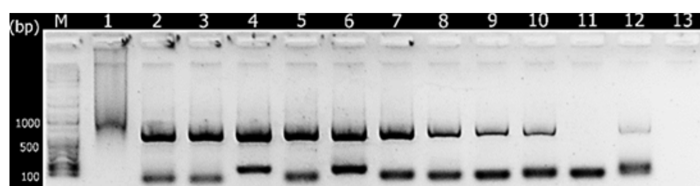

Figure 4. *Electropherogram of the PCR assay for S. japonicum-spiked serum sample. M = KAPA Universal ladder (Kapa Biosystems). Lane 1 = positive control, Lanes 2-10 and 12 = spiked samples, Lane 11 = unspiked sample, Lane 13 = NTC.*

Comparative Performance Testing

The performance of the PCR-based assay was compared to both Kato-Katz and COPT methods measuring specificity and sensitivity. The comparative testing results of the assays are tabulated in Table 4.

Table 4. *Summary of the comparative testing of the PCT assay, Kato-Katz, and COPT methods.*

PCR	Kato-Katz		COPT	
	Positive	Negative	Positive	Negative
Positive	2	0	0	0
Negative	8	10	10	5
Total	10	10	10	5

The computed performance of the PCR assay compared to Kato-Katz and COPT is shown in Table 5. Notably, the specificity of the assay was 100% compared to both methods, while the sensitivity values were 20% for Kato-Katz method and 0% for COPT.

Table 5. *Computed performance of the PCR assay compared to Kato-Katz and COPT methods.*

Parameter	vs Kato-Katz	vs COPT
Specificity	100%	100%
Sensitivity	20%	0%

Discussion

Validation of the Candidate Primer Pairs

The *COX3* and *NAD5* primer pairs were able to produce the correct amplicon sizes and the amplified sequences were positive for *Schistosoma japonicum* (96-98% identities) as confirmed via BLAST. The high % identities mean that the percent similarity between the query sequences and the subject sequence is high. Moreover, the results confirm the results of Zhao *et al*. that the *COX3* and *NAD5* genes are capable of detecting *Schistosoma japonicum* [9]. Meanwhile, the *NAD4* primer set did not show the correct amplicon size and the sequencing results showed no similarity to any organism using BLAST. The results for *NAD4* mean that this primer set was not able to detect *S. japonicum* DNA. Other studies have targeted other mitochondrial genes including *COX1* and *NAD1*, which suggest that these genetic regions are also useful markers for detecting *Schistosoma japonicum* [21,22,23,24]. The % identities for both *COX3* and *NAD5* primer sets are the same which means both of these primers can be used to identify *S. japonicum* from human samples. For the rest of the study, the *COX3* primer set was randomly chosen and used to minimize reagent use and costs. On the other hand, the *NAD5* primer set will be used for future studies.

Assessment of Sensitivity and Specificity of the Assay

The sensitivity of the assay using *COX3* primer pair was determined to be the smallest amount detectable DNA and was shown to be 4 ng/ul of *S. japonicum* DNA. Other PCR studies for *S. japonicum* detection have reported limits of detection of 0.8 pg DNA using standard PCR and 0.08 fg DNA using loop-mediated isothermal amplification (LAMP), which is lower compared to the present assay [25,26]. This implies that the developed PCR assay has lower sensitivity compared to the assays of Xia *et al*. and Xu *et al*. [25,26]. However, it should be taken into consideration that *S. japonicum*-infected snails were used for positive controls, hence, the limit of detection for the developed assay will be even lower than the calculated 4 ng/ul. The PCR assay was also shown to be specific only to *S. japonicum*, since amplicons were absent from the tested organisms except *S. japonicum*. These results show that the PCR assay using *COX3* primer pair is sensitive and specific only to *S. japonicum*.

Most of the literature available including the most recent study of Fornillos *et al*. only show qualitative detection either by the presence or absence of *S. japonicum* DNA and not by how much parasite DNA can be detected [21,22,23,24,27]. At present, the absence of a true gold standard for quantitative correlations to actual worm burden remains a significant challenge [28]. And as such we are unable to make a comparison of the amount of *S. japonicum* DNA that may exist in an infected sample because there is the inherent problem of how many *S. japonicum* eggs contain this amount of DNA. This will be affected by other factors such as the number of eggs produced, amount of eggs released or excreted, amount of eggs detected, etc. [28,29].

Performance of the PCR Assay via Spiking Studies

PCR results of the spiked *S. japonicum*-negative formalin-free stool samples indicate that the spiking experiment was successful as the correct amplicon bands were present for seven out of ten spiked samples while the unspiked stool sample and negative control showed no bands. Moreover, the PCR assay was able to detect the *S. japonicum* DNA even at the lowest dilutions. The varying thickness of the bands may be attributed to the quality of the extracted DNA since stool samples contain large amounts of inhibitors and other contaminants that can affect the PCR [18,30]. All spiked serum samples showed the correct amplicon size and the unspiked serum sample and negative control showed no bands. Since the serum contains fewer contaminants as compared to stool samples, the expected gradient for band thickness was observed in the gel. The results signify that the PCR-based assay can be used to detect *S. japonicum* in stool and serum samples and the PCR assay works even at low concentrations of the parasite DNA.

Preliminary Comparative Performance Testing

The assay was tested to detect *S. japonicum* from previously diagnosed stool and serum samples using Kato-Katz method and COPT, respectively. The specificity of the designed PCR assay was higher than the PCR assays of Pontes *et al*. with 88% and Cai *et al*. with 42.4% and comparable to the PCR developed by Gobert *et al*. with 100% [15,24,31]. This means that anyone who tested positive will have the disease since specificity measures the proportion of people

without the disease who have a negative result [32]. In terms of sensitivity, which measures the proportion of people with a disease who have a positive result, only 20% was obtained for the stool samples and 0% for the serum samples, as compared to the high sensitivities of 85.7%, 94.4%, and 96.7% achieved by Pontes *et al.*, Cai *et al*., and Gobert *et al*., respectively [15,24,31,32]. The low sensitivity and specificity values can be attributed to the low sample size used in the study or the PCR inhibition by contaminants present in stool samples [15,30,31]. Other factors include DNA degradation during field transportation or the long storage time and use of formalin as a fixing agent since formalin crosslinks with DNA which can ultimately hinder PCR reaction [31,33]. Lastly, the specificity and sensitivity results were evaluated under the assumptions that false negatives and false positives are absent using the Kato-Katz and COPT methods.

Other studies have used droplet digital PCR and real-time PCR in detecting *Schistosoma japonicum* in human samples, but the high cost per sample run poses a challenge on the use of these methods as diagnostic tools [22,23,24]. Conventional PCR offers advantages such as lower cost compared to the other two PCR techniques but still having high sensitivity and specificity needed to detect pathogens. Compared to the usual Kato-Katz and serological methods of diagnosis, all PCR techniques can process large number of samples in a shorter amount of time, and more importantly, it is reproducible. In epidemiological studies, tests that offer high sensitivity and specificity are needed which can be supplemented using PCR [15,18].

Conclusion and Recommendations

The study successfully developed a sensitive and specific PCR-based assay for the detection of *Schistosoma japonicum*. Results of the spiking experiment suggest that for the assay in this study to work properly, samples should be prepared appropriately, handled with care, and if possible, submitted to the laboratory immediately for analysis. Lastly, to fully compare the performance of the assay with present diagnostic methods, it is suggested that the sample size be increased and samples to be used be of high integrity. The developed assay can be further improved and used as a reference to develop and improve other diagnostic assays for schistosomiasis.

Better diagnostic tests for schistosomiasis are still required, and in this case, should apply to both the field and the clinic. This PCR assay is reliable, specific, cheap, and can readily be used in extension services for the common folk who are affected by this parasitic infection. This study presented a simpler platform, that can be realistically implemented in the current setting.

Another important thing that can be accomplished is to detect schistosomiasis from the snail host. Identification of snails carrying the parasite can help in monitoring control and elimination programs. The assay in this study used infected snail samples and spiked them in a range of dilutions in serum and stool. This study has been successful in detecting this via PCR and this can be used in the future to assess the situation and prevent further infection.

Improvements in the diagnosis of *Schistosoma japonicum* are essential for future plans involving the development of effective drugs, control and elimination programs, and monitoring infectivity and morbidity.

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