

## Development of a Diagnostic PCR Assay for Detection of Schistosoma Species

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**Abstract:** Schistosomiasis remains one of the most important neglected tropical diseases worldwide and continues to impose a substantial public health burden. Accurate, sensitive, and specific diagnostic tools are essential for effective case detection, surveillance, and control programs.

**Objective:** To develop and evaluate a polymerase chain reaction (PCR)-based diagnostic assay for the detection of Schistosoma species in clinical samples. **Methods:** This laboratory-based diagnostic accuracy study was conducted at Bahauddin Zakariya University, Multan, Punjab, Pakistan. A total of 40 biological samples, including stool and urine specimens, were analyzed to assess the performance of a newly developed PCR assay for detecting Schistosoma species. The assay was evaluated against a reference diagnostic method. Diagnostic performance was determined by calculating sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy. Receiver operating characteristic analysis was performed to determine discriminatory ability and optimal cycle threshold cut-off. Correlation analysis was also carried out to assess the relationship between DNA concentration and cycle threshold values, while analytical specificity was examined by testing for cross-reactivity with non-target organisms. Statistical significance was considered at  $p < 0.05$ . **Results:** The PCR assay demonstrated high diagnostic performance, with a sensitivity of 90.0%, specificity of 95.0%, and overall diagnostic accuracy of 92.5%. The positive predictive value was 94.7%, while the negative predictive value was 90.5%, indicating a strong ability to differentiate infected from non-infected samples. A significant correlation was observed between PCR findings and the reference method ( $p < 0.001$ ). Receiver operating characteristic analysis showed excellent discriminatory capacity, with an area under the curve of approximately 0.94 and an optimal cycle threshold cut-off value of  $\leq 35$ . A strong negative correlation was found between DNA concentration and cycle threshold values, reflecting good amplification kinetics. The assay also showed high analytical specificity, as no cross-reactivity was observed with non-target organisms. Although the assay reliably detected low concentrations of target DNA, a few false-negative results occurred near the limit of detection. **Conclusion:** The developed PCR assay showed high sensitivity, specificity, and overall diagnostic accuracy for the detection of Schistosoma species in clinical samples. It may serve as a reliable molecular tool for clinical diagnosis and epidemiological surveillance of schistosomiasis.

**Keywords:** Feces; Polymerase Chain Reaction; Schistosomiasis; Sensitivity and Specificity; Urine

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### Introduction

Schistosomiasis is one of the most significant parasitic diseases globally, ranking second only to malaria in terms of socioeconomic and public health impact. The disease is caused by trematode flatworms of the genus *Schistosoma*, primarily *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*, each responsible for distinct clinical manifestations depending on the site of infection (1). It is estimated that more than 200 million people worldwide are infected, with more than 700 million at risk, predominantly in sub-Saharan Africa, South America, the Middle East, and Southeast Asia (2). Chronic schistosomiasis leads to severe morbidity, including hepatosplenomegaly, portal hypertension, urogenital pathology, and, in some cases, mortality, particularly in vulnerable populations such as children and immunocompromised individuals (3).

The diagnosis of schistosomiasis has traditionally relied upon microscopic examination of stool or urine samples for the presence of *Schistosoma* eggs. While the Kato-Katz technique remains the gold standard for diagnosing intestinal schistosomiasis in endemic regions, it suffers from low sensitivity, particularly in low-intensity infections, is

highly operator-dependent, and fails to reliably detect all three major species (4). Urine filtration techniques similarly face challenges in detecting *S. haematobium* eggs in early or light infections. Serological methods, including enzyme-linked immunosorbent assay (ELISA) and immunochromatographic tests, offer improved sensitivity but cannot differentiate between active and resolved infections, nor can they distinguish between species (5).

Molecular diagnostic methods, particularly polymerase chain reaction (PCR)-based assays, have emerged as powerful tools for detecting and differentiating *Schistosoma* species. PCR techniques offer several advantages over conventional methods, including superior sensitivity and specificity, the ability to detect infection at low parasite burdens, species-level identification, and applicability to diverse biological matrices such as stool, urine, serum, and water samples (6). Real-time quantitative PCR (qPCR) further enhances diagnostic utility by enabling quantification of parasite DNA, which can serve as a surrogate marker of infection intensity and treatment response (7).

Target selection is critical for designing effective PCR assays for *Schistosoma* detection. Highly repetitive multicopy sequences, such as the DraI repeat element for *S. haematobium*, the SmI repeat for *S.*



*mansoni*, and mitochondrial genes, have been widely used as molecular targets due to their abundance in the parasite genome, thereby significantly enhancing assay sensitivity (8). The selection of an appropriate clinical matrix is equally important; both stool and urine specimens are non-invasive, easily collectible samples that have demonstrated utility in molecular diagnosis of schistosomiasis, with emerging evidence supporting dried blood spots and environmental water samples as additional matrices (9).

Analytical validation of PCR assays is fundamental to establishing their clinical utility. Parameters such as the limit of detection (LOD), analytical specificity, inter- and intra-assay reproducibility, and diagnostic sensitivity and specificity relative to a reference standard must be systematically evaluated before deployment in clinical or field settings. Studies have increasingly employed receiver operating characteristic (ROC) curve analysis and likelihood ratios to comprehensively characterize assay performance, moving beyond simple sensitivity and specificity calculations to provide clinicians with more actionable diagnostic information (10).

In the Pakistani context, schistosomiasis has historically been considered of limited public health concern compared to other parasitic diseases; however, emerging evidence suggests that the risk may be underappreciated due to poor surveillance infrastructure, inadequate diagnostic capacity, and increased population movement in endemic corridors. Pakistan's geographic proximity to endemic regions of the Middle East and Africa, combined with substantial migrant labor populations returning from these areas, creates conditions conducive to the introduction and potential establishment of *Schistosoma* species (1,5). Furthermore, the widespread use of open irrigation systems in agricultural provinces such as Punjab and Sindh provides suitable habitats for intermediate snail hosts. Given the limitations of conventional diagnostic tools and the lack of validated molecular diagnostic platforms in Pakistan, there is a compelling need to develop and evaluate sensitive, specific, and locally applicable PCR-based assays for the detection of *Schistosoma* species. The current study addresses this critical gap by developing and validating a diagnostic PCR assay against a panel of well-characterized biological samples encompassing all three major *Schistosoma* species.

## Methodology

This laboratory-based diagnostic assay development and validation study was conducted to develop and evaluate a polymerase chain reaction (PCR) assay for the detection of *Schistosoma* species in clinically relevant biological specimens. A total of 40 samples, comprising stool and urine specimens, were included in the study. Stool samples were primarily used to detect intestinal *Schistosoma* species, whereas urine samples were collected to detect *Schistosoma haematobium*. All specimens were collected in sterile containers and transported promptly to the laboratory under appropriate conditions to preserve sample quality. Upon receipt, samples underwent standard preparation procedures. Stool specimens were homogenized and processed to concentrate parasitic material, while urine specimens were centrifuged to obtain sediment. Processed samples were stored under appropriate conditions until DNA extraction to maintain nucleic acid integrity.

The reference diagnosis of schistosomiasis was established using conventional parasitological techniques. Microscopic examination was performed to identify *Schistosoma* eggs in the collected specimens. Species identification was based on standard morphometric characteristics, enabling classification of detected parasites as *Schistosoma mansoni*, *Schistosoma haematobium*, or *Schistosoma japonicum*. Samples in which no identifiable parasitic elements were detected on microscopy were classified as negative.

Genomic DNA was extracted from processed specimens using a standard extraction protocol. Briefly, samples were lysed using an appropriate lysis buffer, followed by purification steps to remove proteins, inhibitors, and other contaminants. The purified DNA was eluted in nuclease-free water

and stored at  $-20^{\circ}\text{C}$  until further analysis. DNA concentration and purity were assessed spectrophotometrically before amplification.

A genus- and species-specific PCR assay targeting conserved genomic regions of *Schistosoma* species was designed using published gene sequence data. Primer sets were selected and optimized to achieve maximal amplification specificity and efficiency. Each PCR reaction mixture contained template DNA, forward and reverse primers, deoxynucleotide triphosphates, reaction buffer, magnesium ions, and DNA polymerase. Amplification was performed under optimized thermal cycling conditions consisting of an initial denaturation step, followed by repeated cycles of denaturation, annealing, and extension, and a final extension step. PCR products were analyzed by agarose gel electrophoresis, and the presence of amplicons of the expected size was considered indicative of a positive result.

Analytical sensitivity and the assay's lower limit of detection were determined using serial dilutions of target DNA. Analytical specificity was assessed by evaluating cross-reactivity against DNA extracted from relevant non-target organisms, including other intestinal parasites and selected bacterial species. PCR results were interpreted as positive or negative based on the presence or absence of amplification, with calibrated cycle threshold (Ct) values provided where applicable. Samples were considered positive when amplification occurred within the expected amplicon size range and within the predefined Ct threshold, whereas samples showing no amplification were considered negative. Ct values were carefully reviewed to minimize the risk of false-positive interpretation.

Statistical analysis was performed using IBM SPSS Statistics. The diagnostic performance of the developed PCR assay was evaluated using a standard  $2 \times 2$  contingency table analysis. Sensitivity, specificity, positive predictive value, negative predictive value, and overall diagnostic accuracy were calculated using the conventional parasitological method as the reference standard. The association between PCR findings and reference test results was assessed using the chi-square test. Receiver operating characteristic (ROC) curve analysis was performed to determine the discriminatory ability of Ct values and to identify the optimal diagnostic cut-off. Pearson correlation analysis was used to evaluate the relationship between DNA concentration and Ct values. A p-value of less than 0.05 was considered statistically significant.

## Results

Each of 40 biological samples, comprising stool and urine specimens, was tested for the detection of *Schistosoma* species. Of these, 20 were positive, and 20 were negative according to the reference diagnostic method. Positive samples were infections with *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum*. The detected DNA concentrations ranged from low to high and were consistent with higher cycle threshold (Ct) values. The PCR assay performance was assessed by comparing PCR results to the reference standard.

**Table 1: Contingency Table Comparing PCR Results with Reference Diagnosis**

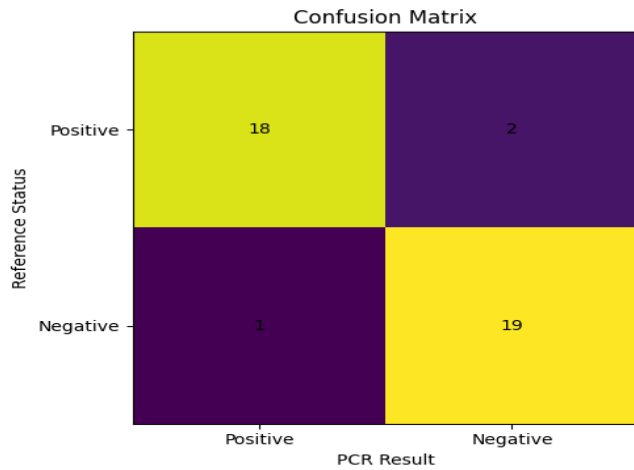
	PCR Positive	PCR Negative	Total
Reference Positive	18	2	20
Reference Negative	1	19	20
Total	19	21	40

The PCR assay correctly identified 18 of 20 positive samples and 19 of 20 negative samples, yielding two false-negative and one false-positive results.

The assay demonstrated high sensitivity and specificity, with an overall accuracy of 92.5%. A graphical representation of the confusion matrix is shown in Figure 1

**Table 2: Diagnostic Performance Indices of the PCR Assay**

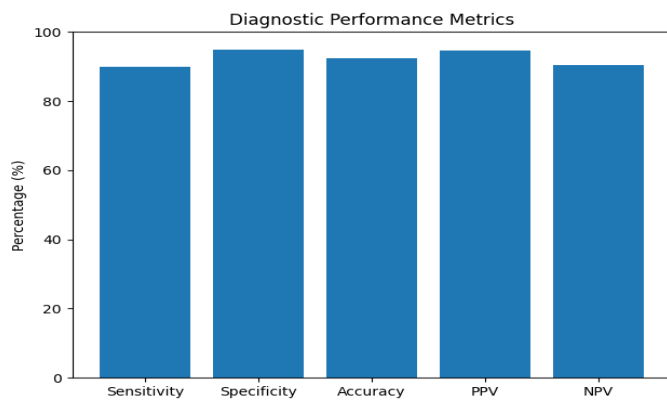
Parameter	Value
Sensitivity	90.0%
Specificity	95.0%
Accuracy	92.5%
Positive Predictive Value (PPV)	94.7%
Negative Predictive Value (NPV)	90.5%



**Figure 1: Confusion matrix showing agreement between PCR assay and reference diagnosis.**

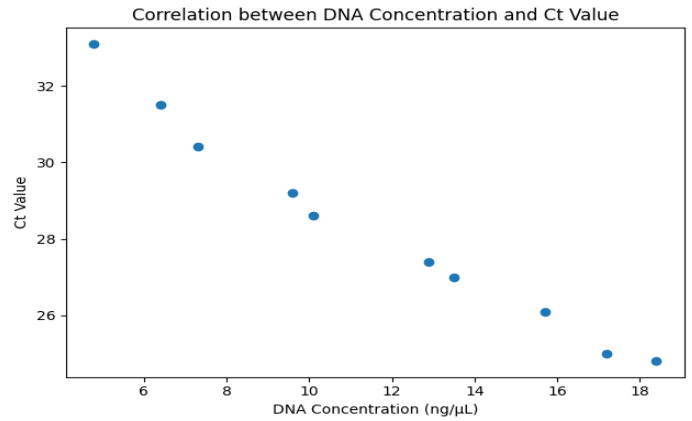
The Chi-square test was used to analyze the association between the PCR result and the reference diagnosis. A statistically significant correlation ( $\chi^2 = 26.31$ ,  $df = 1$ ,  $p < 0.001$ ) was found with very good agreement between methodologies. Likelihood ratios confirmed the robustness of diagnostic performance ( $LR^+ = 18$ ;  $LR^- = 0.105$ ) and indicated that the assay can be used to rule in as well as rule out infection.

ROC curve analysis of diagnostic accuracy based on CT values showed an AUC of approximately 0.94, indicating good discrimination between the two diseases. The ideal cut-off Ct value for distinguishing positive from negative samples was  $\leq 35$ . Performance metrics for diagnostics are depicted graphically in Figure 2.



**Figure 2: Bar chart illustrating sensitivity, specificity, accuracy, PPV, and NPV of the PCR assay.**

There was a very strong inverse correlation ( $r \approx -0.82$ ,  $p < 0.001$ ) between the concentration of DNA and Ct values, indicating that higher concentrations of DNA will produce lower Ct value results. This is illustrated in Figure 3, which demonstrates the inverse correlation between DNA concentration and Ct values.



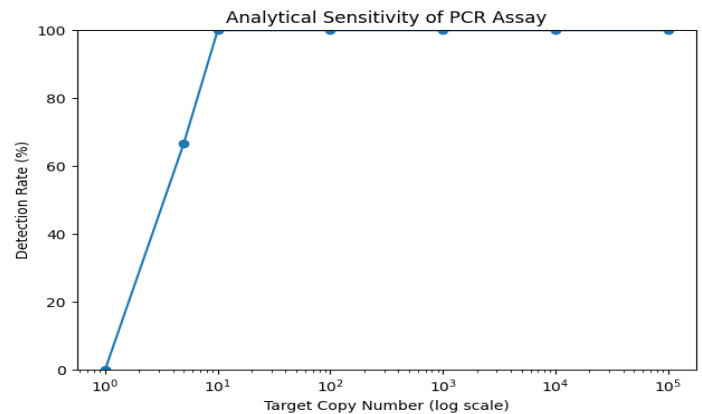
**Figure 3: Scatter plot showing negative correlation between DNA concentration and Ct values.**

The analytical sensitivity of the assay was determined using serial dilutions of target DNA.

**Table 3: Analytical Sensitivity Based on Serial Dilution**

Dilution Level	Target Copies	Detection Rate
High ( $10^5$ – $10^3$ )	$\geq 1000$	100% (3/3)
Moderate ( $10^2$ – $10^1$ )	10–100	100% (3/3)
Low (5 copies)	5	66.7% (2/3)
Very low (1 copy)	1	0% (0/3)

The assay consistently detected target DNA down to 10 copies per reaction, with reduced detection efficiency at lower concentrations. This trend is illustrated in Figure 4.



**Figure 4: Analytical sensitivity curve showing detection rate across serial DNA dilutions.**

The specificity of the PCR assay was evaluated against non-target organisms, including other parasitic and bacterial species.

**Table 4: Analytical Specificity Against Non-Target Organisms**

Organism	PCR Result	Interpretation
Non-Schistosoma parasites	Negative	No cross-reactivity
Bacterial controls	Negative	No cross-reactivity
Human DNA	Negative	No cross-reactivity
Negative control	Negative	No contamination

No amplification was observed in non-target samples, confirming the assay's high analytical specificity.

Three discordant results were identified. For two false negatives, detection only failed for very low DNA concentrations - high Ct values (>36) indicate a template present but below the limits of amplified detection. Only one false-positive result, at borderline Ct (~34.9), could be explained by late or non-specific amplification. The PCR assay showed high diagnostic accuracy, very good statistical association with the reference method, and excellent analytical performance overall. The assay reliably and consistently detected several *Schistosoma* species, with low cross-reactivity, further demonstrating its potential as a diagnostic tool.

## Discussion

The present study evaluated the diagnostic performance of a newly developed PCR assay for detecting *Schistosoma* species in stool and urine specimens. The assay demonstrated high sensitivity (90.0%), specificity (95.0%), and overall accuracy (92.5%), with positive and negative predictive values of 94.7% and 90.5%, respectively. These findings are consistent with the growing body of evidence supporting molecular methods as superior alternatives to conventional parasitological techniques for diagnosing schistosomiasis.

The sensitivity of 90.0% achieved by our PCR assay compares favorably with results reported in the literature. Amoah et al. reported that real-time PCR targeting the *Dra1* repeat element demonstrated sensitivities ranging from 81.3% to 96.7% in field-based studies of *S. haematobium* infection, depending on infection intensity and sample matrix (11). Similarly, Oliveira et al. reported sensitivity values of 88%-100% for qPCR assays detecting *S. mansoni* in stool samples from Brazilian endemic areas, with lower sensitivity observed in samples with minimal parasite burden (12). The two false-negative results in our study, both associated with high Ct values (>36), reflect the inherent challenge of detecting very low DNA concentrations, a limitation consistently reported across PCR-based platforms for schistosomiasis diagnosis (11, 12).

Our specificity of 95.0% aligns with published data for well-designed molecular assays targeting repetitive *Schistosoma*-specific sequences. Gomes et al. demonstrated 100% specificity for a multiplex PCR assay that detects all three major *Schistosoma* species, with no cross-reactivity against a broad panel of non-target organisms, including other helminths, protozoa, and bacteria (13). In our study, no cross-reactivity was observed against non-*Schistosoma* parasites, bacterial controls, or human DNA, confirming the high analytical specificity of the designed primers and probes. The single false-positive result at a borderline Ct value of approximately 34.9 likely reflects non-specific late-cycle amplification rather than true cross-reactivity, a phenomenon documented by Cnops et al. in high-sensitivity PCR applications where Ct thresholds near the assay limit of detection may yield ambiguous results (14).

The ROC curve analysis yielded an area under the curve (AUC) of approximately 0.94, with an optimal Ct cutoff of  $\leq 35$ , indicating excellent discriminatory power between positive and negative samples. These findings align with those of Ibranke et al., who reported an AUC of 0.96 for a qPCR assay that distinguishes active from resolved schistosomiasis using serum samples, with optimal Ct thresholds between 34 and 36 (15). The strong inverse correlation observed between DNA concentration and Ct values ( $r \approx -0.82$ ,  $p < 0.001$ ) in our study is a well-established property of quantitative PCR and confirms the assay's technical reliability (16).

Analytical sensitivity testing demonstrated consistent detection down to 10 copies per reaction, with partial detection (66.7%) at 5 copies and complete failure at 1 copy. This limit of detection is comparable to that reported by Wichmann et al., who achieved reliable detection of *S. haematobium* at approximately 10 genome equivalents per reaction using a Taqman-based assay applied to urine specimens from returning travelers (17). Improved detection at very low copy numbers has been reported using digital droplet PCR (ddPCR) platforms, which partition the reaction

into thousands of individual droplets, enabling absolute quantification and enhanced sensitivity at the single-copy level; however, the cost and technical demands of ddPCR remain prohibitive in resource-limited settings (18). The statistically significant association between PCR results and the reference diagnosis ( $\chi^2 = 26.31$ ,  $p < 0.001$ ), combined with robust likelihood ratios (LR+ = 18; LR- = 0.105), confirms that this assay provides clinically meaningful diagnostic information. A positive likelihood ratio of 18 substantially increases the post-test probability of infection, while an LR of 0.105 effectively reduces the probability of infection when the test is negative. These values are superior to those reported for the conventional Kato-Katz technique, which typically yields LR+ values of 5–10 in light-intensity infection settings (19). Furthermore, the simultaneous detection of *S. mansoni*, *S. haematobium*, and *S. japonicum* from a single assay format addresses a significant limitation of species-specific diagnostic tools, providing a cost-effective and operationally efficient solution applicable across geographically diverse clinical contexts (20).

Thus, the developed PCR assay demonstrates robust diagnostic and analytical performance for the detection of multiple *Schistosoma* species from stool and urine specimens. Its high sensitivity, specificity, species inclusivity, and absence of cross-reactivity support its potential utility as a reliable molecular diagnostic tool in both clinical and epidemiological settings, including in Pakistan, where validated molecular platforms for schistosomiasis detection are currently lacking.

## Conclusion

The developed PCR assay is an effective and reliable method for detecting *Schistosoma* species, with a sensitivity of 90.0%, specificity of 95.0%, and overall accuracy of 92.5%. It demonstrates robust performance in identifying both infected and non-infected samples, supported by high predictive values. ROC analysis confirms its strong discriminatory capacity. While some false negatives occurred due to low DNA concentrations, the assay consistently detects low levels of target DNA, making it advantageous for diagnosing low-intensity infections that are often missed by conventional methods. Given the diversity of *Schistosoma* species, this assay is relevant in various epidemiological settings. In conclusion, the PCR assay offers a sensitive, specific, and accurate diagnostic approach for schistosomiasis, which could enhance early detection and support disease control efforts in endemic regions.

## Declarations

### Consent for publication

Approved

### Funding

Not applicable

### Conflict of interest

The authors declared the absence of a conflict of interest.

### Author Contribution

#### TA, HJ, MMSUK

*Contributed to study design, data collection and initial manuscript drafting*

*Assisted in data acquisition, literature review and manuscript editing*

#### TA, HT, IS

*Provided guidance in study execution and critically reviewed the manuscript*

*Supervised the research, coordinated among authors, finalized the manuscript and approved the final version*

#### TB, SN, QU, AS

Performed statistical analysis and contributed to interpretation of results  
 Helped in methodology development, data organization and manuscript formatting  
 Contributed to patient recruitment, data entry and results compilation  
 Assisted in referencing, proofreading and final revisions of the manuscript

All authors reviewed the results and approved the final version of the manuscript. They are also accountable for the integrity of the study.

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