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Nilanjan Lodh

Marquette University, nilanjan.lodh@marquette.edu

James C. L. Mwansa

Johns Hopkins University

Mabel M. Mutengo

Johns Hopkins University

Clive J. Shiff

Johns Hopkins University

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Diagnosis of *Schistosoma mansoni* without the Stool: Comparison of Three Diagnostic Tests to Detect *Schistosoma mansoni* Infection from Filtered Urine in Zambia

Nilanjan Lodh, James C. L. Mwansa, Mable M. Mutengo, and Clive J. Shiff*

Department of Molecular Microbiology and Immunology, School of Public Health, Johns Hopkins University, Baltimore, Maryland; University Teaching Hospital, Department of Pathology and Microbiology, Lusaka, Zambia

Abstract. Diagnosis for intestinal *Schistosoma mansoni* lacks sensitivity and is arduous to conduct. The standard diagnostic tests, Kato-Katz (KK) and circulating cathodic antigen (CCA) both lack sensitivity and with KK, require obtaining, transporting, and examining fresh stool. We compared diagnostic efficacy of KK, CCA, and polymerase chain reaction (PCR) to detect *S. mansoni* infection (species-specific DNA) from 89 filtered urine samples collected in Zambia. The PCR was the strongest indicator of positive cases with sensitivity and specificity of 100% in comparison to CCA (67% and 60%) and KK (50% and 100%). High positive and negative predictive values (100%) were also indicative of robustness of PCR. The same pattern was observed when stratified for sex and age group-specific analysis. Diagnosis of *S. mansoni* from filtered urine samples by PCR is an effective means to detect low intensity infection and would enhance the effectiveness of surveillance and control programs of schistosomiasis.

INTRODUCTION

Schistosomiasis is one of the most widespread parasitic infectious diseases that affect humankind. Globally an estimated 200 million people are currently infected and an estimated 800 million people are at risk in 74 countries with an annual death of about 20,000 patients.¹ The disease is widely distributed in Africa, Western Asia, South America, and South West Asia affecting a high proportion of populations, especially children in developing countries causing major socioeconomic and public health consequences. Its intestinal form, *Schistosoma mansoni* is responsible for severe liver and intestinal damage, physical growth retardation, and cognition and memory problems.² Mass drug administration (MDA) is now being applied to control large-scale schistosomiasis in Africa and elsewhere.³ The impact of MDA has been measured by change in disease prevalence and infection intensity.⁴ Because the infection is easily spread, to implement a better control and surveillance strategy before and after MDA, it must rely upon proper diagnosis of asymptomatic cases especially for areas with low disease prevalence.

Currently most epidemiological assessment to detect infected individuals with *S. mansoni* depends on direct examination of Kato-Katz (KK) smears of fecal samples or else the use of commercially available urine reagent strip tests for the detection of circulating cathodic antigen (CCA) produced by viable worms. The KK parasitological technique, which depends on diagnosing parasite eggs in the human excreta, is the method of choice⁵ to detect parasites in infected individuals. However, there are several shortcomings of this method. In chronic and low intensity infections these eggs appear sporadically and are difficult to detect, therefore it lacks sensitivity.^{6–9} Moreover, this technique is limited caused by day-to-day fluctuation of egg output,¹⁰ especially in a post-treatment situation.¹¹ As an alternative, a point-of-care CCA urine test has been developed for the diagnosis of

*S. mansoni*¹² and the CCA test has also been evaluated in low endemic settings to assess its diagnostic performances.¹³

The CCA test depends on the detection of major antigen regurgitated by adult worms.¹⁰ It has been easy to use in field condition and needs minimal practical training; its use as a field diagnosis, requiring improved sensitivity has been extolled by Bergquist¹⁴ and recently demonstrated by Colley and others.¹⁵ However, there are detractors. Positivity of CCA is strongly correlated with *S. mansoni* infection intensities¹⁰ and sensitivity of CCA decreases in low infection settings; light to moderate infections are frequently seen as negative by reagent strip test.^{11,16} Moreover, CCA is also prone to produce false positive results¹⁷ and inaccurately estimates disease prevalence¹³ to light *S. mansoni* infections. As MDA initiatives expand in many countries, the subsequent decline in schistosome infection will require a more sensitive diagnostic test for *S. mansoni*.

Recently, schistosome-specific DNA has been successfully detected in *Schistosoma haematobium* from filtered urine¹⁸ and *S. mansoni* from feces, serum,^{19,20} and urine.²¹ The amplification reaction is capable of detecting as little as 1 fg of a highly repetitive *S. mansoni* DNA sequence, showed no cross-reactivity with other related helminthes,²² and always showed high sensitivity and specificity. This diagnostic technique depends on the polymerase chain reaction (PCR) and so far has not been extensively used in human schistosomiasis detection²²; it is now important to demonstrate the usage of PCR as a surveillance tool to monitor schistosomiasis prevalence in high and low endemic areas.

In this study, we used a simple tool to collect and transport urine to laboratories¹⁸ and amplified highly repetitive DNA fragments of *S. mansoni* that spans more than 10% of the parasite genome.¹⁹ The diagnostic performance of this test was also compared with traditional KK and CCA tests obtained through fecal and urine samples of the subjects from endemic areas of Zambia. This is the first study that compared all three known and well-studied diagnostic tests for *S. mansoni*.

MATERIALS AND METHODS

Study population. The study was conducted among 100 participants living in an area known to be endemic for

*Address correspondence to Clive J. Shiff, Department of Molecular Microbiology and Immunology, School of Public Health, Johns Hopkins University, 615 North Wolfe Street, E5142, Baltimore, MD 21205. E-mail: cshiff@jhsph.edu

S. mansoni. Participants were adults 18–50 years of age and resident in the study area for not less than a year. This age group was chosen because of the perception of decreasing sensitivity of egg detection and parasite load with advancing age.

Study design. The study was a component of the cross-sectional study conducted to establish the prevalence of *S. mansoni*-related morbidity and role of immunogenetics in hepatosplenic schistosomiasis in four rural communities of Kaoma district in Western province of Zambia. Participants were randomly selected for the study after receiving consent from participants. Single stool samples were processed and examined for the presence of *S. mansoni* parasites. Other than detection of *S. mansoni* CCA, urine samples were also examined for possible *S. haematobium* infections.

The study was reviewed and approved by the University of Zambia biomedical research ethics committee. Participant samples were identified with unique study identification numbers and results were only known to the study clinician and individual participants. Those found infected with *S. mansoni* were treated by praziquantel by using a dose of 40 mg/kg body weight.

KK and CCA test. Stool samples collected from each of the recruited participants was processed and examined in the field. Duplicate stool smears were prepared from single stool samples using the KK technique as previously described.^{23,24} Briefly, smears were prepared using a 41.7 mg template, stained with malachite green, and examined microscopically for the presence or absence of *S. mansoni* eggs.

The presence of *S. mansoni* infections was also tested with the CCA rapid test (Rapid Medical Diagnostics, Pretoria, South Africa). The test was conducted according to the manufacturer's recommendation. Briefly, one drop of urine was added into the cassette well and a drop of the reagent buffer was added. The cassette was left undisturbed for 20 min before the results were recorded. Two results are expected with this test; either positive or negative. For a positive result, both the control band and the test band appear red, whereas in a negative test only the control line appears red. The person performing this test was blinded from results of the KK and vice versa.

Urine collection. Urine samples were collected between 10 am and 12 pm for optimum egg passage. All the participants provided urine samples in plastic bottles and ~50 mL urine was passed through a 12.5 cm Whatman No. 3 (Whatman International, Maidstone, UK) filter paper, folded in a cone marked with subject identification. This grade of paper was selected because it is coarse, maintains a cone shape when folded, and it retained both schistosome eggs and DNA fragments from urine during filtration.¹⁸ The cone was set in a plastic funnel to pass the urine. To avoid contamination, plastic cups and funnels were used only once. After filtration, the paper disc was opened and allowed to dry under a fly-proof bed net before being packed with desiccant in individual plastic sleeves until further use.

DNA extraction from urine. Inner quadrants of the folded marked cones were used to extract the DNA. A consistent number of 12 punches (~1 mm in diameter) of the paper made by a regular paper puncher were subject for DNA extraction. Each time the paper puncher was cleaned with 10% bleach solution and dried to avoid contamination. All the paper discs from each sample were placed into a 1.5 mL eppendorf tube containing 600 μ L nuclease-free water. The

tubes were incubated at 95°C for 10 min and then placed on a rotator at room temperature overnight (16–18 hrs). The water containing DNA was transferred to a Qiagen QIAmp 2 mL column tube. The DNA was precipitated and concentrated using the QIAmpDNA Blood Mini Kit (Qiagen, MD) according to the manufacturer's protocol. The DNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and stored at –20°C for future use.

PCR test. The PCR was administered on all 89 samples of both sexes and different age groups. The PCR was carried out by using forward (5' GATCTGAATCCGACCAACCG 3') and reverse (5' ATATTAACGCCACGCTCTC 3') primers²⁰ to amplify the 110 bp region from a highly repeated 121 bp fragment of *S. mansoni* described by Hamburger and others.¹⁹ The repetitive fragment constitutes at least 12% (600,000 copies/cell) of the *S. mansoni* genome. All DNA samples had a concentration of 1–3 ng/ μ L. For amplification, 1 μ L of DNA served as PCR template. The total PCR reaction volume was 10 μ L and consisted of 5 μ L of PCR Master Mix, 2X (Promega, Madison, WI), 0.5 μ L (10 μ M) of each of the amplification primers, 0.5 μ L of 25 mM MgCl₂, and 2 μ L of water to complete the final volume. The amplification profile was an initial denaturation of 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, annealing temperature of 63°C for 45 sec, and elongation at 72°C for 30 sec, followed by a final extension step at 72°C for 5 min. Electrophoresis of 4 μ L amplified PCR products was carried out in a 2% agarose gel stained with ethidium bromide (10 mg/ μ L) with the help of 50 bp ladder (New England BioLabs, Inc., Ipswich, MA) to estimate band sizes.

Statistical analyses. Only samples with data from three diagnostic tests were considered for analysis. Results were recorded for all tests and converted to numerical values (1 = positive and 0 = negative) for analysis. The positive and negative assessment of each sample was based on the following predictions.

- If KK+, then sure presence of infection
- If PCR+, then assume presence of infection
- True Positive (TP): If PCR+, CCA+, and KK+
- True Negative (TN): If PCR–, CCA–, and KK–
- Positive: If PCR+ and KK–
- False Negative (FN): If KK+ and PCR or CCA–
- If CCA– and PCR or KK+
- False Positive (FP): If CCA+ and PCR or KK–

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive and negative likelihood ratio was calculated using MedCalc 12.4.0 (MedCalc Software, Belgium). Disease prevalence was calculated based

TABLE 1
Comparative evaluation of the Kato-Katz (KK), circulating cathodic antigen (CCA) and polymerase chain reaction (PCR) for diagnosis of *Schistosoma mansoni*-infected people from Zambia

Diagnostic test	True positive	False positive	True negative	False negative	Total
KK	45	0	10	34	89
CCA	53	4	6	26	89
PCR	79	0	10	0	89

TABLE 2

Estimation of prevalence, sensitivity, specificity, predictive values, and likelihood ratios of three diagnostic tests for identifying *Schistosoma mansoni* infection

Diagnostic test	Disease prevalence*	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (PPV)	Negative predictive value (NPV)	Positive likelihood ratio†	Negative likelihood ratio‡
KK	51%	57% (45–68%)	100% (69–100%)	100%	23%	0	0.43
CCA	60%	67% (56–77%)	60% (26–88%)	93%	19%	1.68	0.55
PCR	89%	100% (95–100%)	100% (69–100%)	100%	100%	0	0

*Disease prevalence = number of positives by each test out of total number of samples that had been analyzed.

†Positive likelihood ratio = ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease.

‡Negative likelihood ratio = ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease. CI = confidence interval; KK = Kato-Katz; CCA = circulating cathodic antigen; PCR = polymerase chain reaction.

on number of positive cases by each diagnostic test. Agreement statistics, which included Kappa value and Bowker's Symmetry, were determined (JMP v9, SAS Institute Inc., Cary, NC) to establish the agreement between two diagnostic tests. Sex-specific and age group (7–19 yrs, 20–39 yrs, and 40–85 yrs)-specific evaluation (JMP v9, SAS Institute Inc.) of positivity and negativity of each diagnostic test were also done to establish the robustness of the PCR test.

RESULTS

The comparison of three diagnostic methods to detect *S. mansoni* infection in the rural Zambian population is based on performance of each test when a schistosome egg is present in feces and most importantly when an egg is not present or there is a low level of infection (asymptomatic infection). The PCR is the best indicator of disease prevalence among three tests irrespective of age, sex, and infection level of a population. Of the 89 samples that were tested, 79 were True positive (TP) by PCR, whereas 45 and 53 tested positive by KK and CCA. The PCR did not provide any False positive (FP) or False negative results (FN; Table 1). The number FN was highest with KK (34) and 26 by CCA. In comparison KK positive is diagnostic, no FP, whereas CCA did show 4 FP.

Diagnostic parameters were calculated and showed 100% sensitivity (95% CI: 95–100) and specificity (95% CI: 69–100) for PCR (Table 2). The predictive values of sensitivity for PCR were significantly higher than KK (57%, 95% CI: 45–68; specificity, 100%, 95% CI: 69–100) and CCA (67%, 95% CI: 56–77; 60%, 95% CI: 26–88). The PCR was also more sensitive determining the disease prevalence (89%) based on positive cases than KK (51%) and CCA (60%). The PPV values for all three tests were close, ranging from 93% to 100%, but the NPV were variable, as it was 100% for PCR and significantly lower for KK (23%) and CCA (19%). In addition, the positive and negative likelihood ratio was also reflective of PPV and NPV values for three diagnostic tests.

The agreement statistics also replicate our findings regarding sensitivity and specificity of three tests. The PCR showed a significantly low degree of agreement with KK (kappa: 0.23; 95% CI: 0.10–0.34; $P < 0.05$) and CCA (kappa: 0.14; 95% CI: 0.04–0.31; $P < 0.05$), whereas KK had a relatively high degree of agreement with CCA (kappa: 0.50; 95% CI: 0.33–0.68; $P < 0.05$; JMP v9, SAS Institute Inc.; Table 3). Kappa values show the agreement between two tests compared at a time (-1 = negative association, 0 = random, 1 = complete agreement). The symmetry for lack of agreement (Bowker Symmetry; JMP v9, SAS Institute Inc.) between comparisons of two tests at a time was all significant ($P < 0.05$). This test checks for symmetry in two-way tables and the test decision is based on X^2 approximation of the distribution of the test statistic.²⁵ The Bowker Symmetry is significantly different from random ($P < 0.05$) for all comparisons, indicating that test positives by three diagnostic tests are highly unlikely to be the same.

We extended our comparative analysis for three diagnostic tests for both males and females. Out of 89 people, 51 were female and 38 were male. For PCR the rate of positive infection identification was 86% for females and 92% for males (Table 4). In comparison it was 67% (female) and 61% (male) for CCA and even lower for KK (51% female, 50% male). Most importantly, more than half of the individuals tested negative by KK and CCA for both males and females came out positive by PCR, which is an indication of high sensitivity.

For age group analysis, PCR was a stronger predictor for infected individuals in comparison with KK and CCA. We categorized all 89 individuals into three age groups (see Materials and Methods) and assessed positive and negative individuals by each test for each age group. The PCR detected almost or more than 90% (82.1–93.7%) positive individuals for all groups, whereas detection by KK and CCA was widely distributed (KK: 32.1–75%; CCA: 46.4–73.3%; Table 5). Interestingly, PCR remained consistent with detection of infected individuals clearing all phases of life; on other hand,

TABLE 3

Agreement statistics were calculated for three diagnostic tests

Diagnostic test	Degree of agreement - Kappa value	95% CI	<i>P</i> value	Bowker's Symmetry test	<i>P</i> value
KK vs. CCA	0.50	0.33–0.68	0.0001*	6.55	0.0105*
KK vs. PCR	0.23	0.10–0.34	0.0003*	34	0.0001*
CCA vs. PCR	0.14	0.04–0.31	0.0463*	16.13	0.0001*

* Significant.

KK = Kato-Katz; CCA = circulating cathodic antigen; PCR = polymerase chain reaction.

TABLE 4
Positive and negative identification of *Schistosoma mansoni* infection by three diagnostic tests for both female and male*

Diagnostic test	Female		Total	Male		Total
	Positive	Negative		Positive	Negative	
KK	26 51%	25 49%	51	19 50%	19 50%	38
CCA	34 67%	17 33%	51	23 61%	15 39%	38
PCR	44 86%	7 14%	51	35 92%	3 8%	38

*KK = Kato-Katz; CCA = circulating cathodic antigen; PCR = polymerase chain reaction.

both KK and CCA had sensitivity as a person aged (KK: 32.1%; CCA: 46.4%).

DISCUSSION

Diagnostic tools appropriate for undertaking interventions to control helminth infections are the key to their success.²⁶ The accurate identification of infected populations at highest risk and with low infection is necessary to implement an effective control program for an endemic area, which will improve the assessment of drug efficacy and patient management. Much will be lost in local elimination programs if a significant number of low-level infections are not detected. The potential for expansive transmission is real when even small numbers of miracidia are released in water.²⁷ This is the first study to evaluate the performances of three diagnostic tests to detect *S. mansoni*-infected individuals. For this study, we used the novel urine filtration technique (which was also used for *S. haematobium*) to isolate and amplify small fragments (121 bp) of a highly repetitive DNA sequence of *S. mansoni* genome. This enables detection of the parasite in urine because, first, high representation of the DNA fragment obviates high detection sensitivity, and second, such sequences evolve more rapidly than the rest of the genome.¹⁹

With traditional parasitological techniques, the possibility of false negatives and multiple sample requirements often lead to suboptimal results.²⁸ The KK examines 50 mg of a stool specimen that is likely to be several 100 g in mass. Hence, low-level infections are missed. In our study 34 of 89 samples (38%) were FN (Table 1), which is a clear indication of loss of sensitivity of KK (57%, Table 2). An antigen capture test, CCA test of *S. mansoni*, now in use as a point-of-care rapid diagnostic, has only been validated against the KK test with similar sensitivity problems.²⁹ In fact, our study also indicates loss of sensitivity (67%, Table 2) for CCA, which is evident by the number of FN (26 of 89, 29%, Table 1). It has also been reported by several authors that even though it is easy to use, CCA shows reduced sensitivity in detecting urine CCA in low endemic areas of *S.*

mansoni.^{11,13,16,29} Moreover, urine CCA produces similar disease prevalence of multiple KK thick smears¹⁰ and even multiple CCA testing does not improve the overall disease prevalence in comparison with a single CCA test,¹⁵ this task is made more difficult to rationalize when, as shown here, neither CCA nor KK can detect all cases of parasitemia. The high level of specificity of infection in the current study is also related to the detection of *S. mansoni*-infected individuals, where KK and PCR perform exceptionally well (100%) and on the other hand CCA performs moderately (60%, Table 2).

This study has shown several significant advances in the diagnosis of *S. mansoni*, and these are relevant for consideration both in the laboratory and in the field, particularly where programs are under way to reduce or eliminate transmission. First, the demonstration that parasite-specific DNA from urine can be detected in filter paper as shown by Ibranke and others¹⁸ obviates the need for taking stool samples. This is a major change in logistics and makes handling of specimens so much easier and safer. Second, there is a substantive increase in sensitivity. Our evidence shows that KK missed 34 of 79 positive cases in a prevalence study in rural Zambia, and CCA missed 26 of these cases (Table 1). In endemic situations where treatment interventions are planned, this omission is significant because even if all positive cases are cured, there is still a population of viable parasites to continue the parasite cycle, and the multiplicative stage for the parasite is in the snails. Essentially, this and other studies, e.g.,¹⁵ have cast doubt on the efficacy of the KK test for use as a standard diagnostic unless there is a need for some estimate of egg load, and that should be planned with circumspection.

This study used PCR as the appropriate means to amplify parasite DNA. However, use of thermocycler machines to amplify DNA is not suited to field situations where electric power is unstable or intermittent. There is a real need to make these techniques that increase sensitivity available under such conditions so that they can be used much closer to the real life situation. To this end, the loop-mediated isothermal amplification method has been developed for other

TABLE 5
Effectiveness of three diagnostic tests across different age groups for diagnosis of *Schistosoma mansoni* infection*

Diagnostic test	Age group A (7–19 yrs)		Age group B (20–39 yrs)		Age group C (40–85 yrs)	
	Positive	Negative	Positive	Negative	Positive	Negative
KK	12 (75%)	4	24 (53.3%)	21	9 (32.1%)	19
CCA	11 (68.7%)	5	33 (73.3%)	12	13 (46.4%)	15
PCR	15 (93.7%)	1	41 (91.1%)	4	23 (82.1%)	5

*KK = Kato-Katz; CCA = circulating cathodic antigen; PCR = polymerase chain reaction.

situations including detection of schistosome material in infected snails.³⁰ This should be a priority for an improved detection method for human infection of schistosomes.

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Authors' addresses: Nilanjan Lodh and Clive J. Shiff, Department of Molecular Microbiology and Immunology, School of Public Health, Johns Hopkins University, Baltimore, MD, E-mails: nlohd@jhsph.edu and cshiff@jhsph.edu. James C. L. Mwansa and Mable M. Mutengo, University Teaching Hospital, Department of Pathology and Microbiology, Lusaka, Zambia, E-mails: mwansaj@gmail.com and mmutengo@yahoo.com.

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