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Molecular Diagnosis of *Schistosoma* Infections in Urine Samples of School Children in Ghana

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Abstract. Recent studies using an internal transcribed spacer (ITS)-based real-time polymerase chain reaction (PCR) for the detection of *Schistosoma* DNA in urine samples has shown high sensitivity and specificity when performed on controls and known microscopy-positive samples. In this study, using 730 urine samples collected from children in five primary schools from different communities in the Greater Accra region of Ghana, specific detection of *Schistosoma* DNA showed excellent sensitivity of 100% and 85.2% in urines with > 50 eggs/10 mL urine and ≤ 50 eggs/10 mL of urine, respectively. Additionally, *Schistosoma*-specific DNA was amplified in 102 of 673 samples in which *Schistosoma* eggs could not be detected with microscopy. Taking microscopy and/or PCR-positive samples as true positives, the negative predictive value calculated was 94.6–100% for each school sampled as compared with 54.3–95.7% using microscopy. This ITS-based real-time PCR proves to be a powerful tool in epidemiological surveys of schistosomiasis providing more precise and sensitive results than microscopy.

INTRODUCTION

Schistosomiasis is one of the most important human helminth infections in terms of morbidity and mortality.[1] The disease is endemic in many developing countries affecting largely children, farmers, and women who depend primarily on water bodies, which may harbor intermediate host snails. Millions of people are infected worldwide, with schistosomes of various species leading to the loss of 1.53 million disability-adjusted life years, with the majority of morbidity (85%) and mortality occurring in sub-Saharan Africa.[2]

Diagnosis plays a vital role in the identification of schistosomiasis infections generating results that influence decisions on individual and community treatment, estimations of prognosis and assessment of morbidity, evaluation of chemotherapy, and other control measures.[3] However, there are limitations with some of the diagnostic techniques. Although tedious and time-consuming, most epidemiological assessments of the burden of schistosomiasis have relied on microscopy, providing a relatively easy and cheap tool for detecting and estimating the intensity of schistosome eggs in fecal and urine samples in many schistosomiasis-endemic countries.[4] However, inadequate sensitivity as a result of great fluctuations of egg output in urine or stool are well known, and the difficulties in meeting the multiple sampling requirements for classical parasitological diagnosis often lead to suboptimal results.[5]

Antibody-based assays are sensitive but cannot distinguish history of exposure from active infection; their cross-reaction with other helminths has been shown, and these assays are not easily applicable under field conditions.[2] Antigen-based assays, such as circulating cathodic antigen detection in urine, have proven to be a valuable, field applicable method for detecting *Schistosoma mansoni* infections, unfortunately, the test has shown to be less sensitive for infections with *Schistosoma haematobium*.[6,7]

Polymerase chain reaction (PCR)-based methods have shown high sensitivity and specificity for the detection of parasitic DNA and have been used for the detection of a broad range of parasites.[5,8,9] Until now their use in human epidemiological surveys has been limited with more studies exploring the potential of schistosome-specific PCR focusing more on the snail vector rather than its definitive host.[10,11] Recent developments in the simplification of DNA isolation procedures and PCR technology, especially real-time PCR, have made DNA amplification a worthwhile alternative for microscopy-based diagnostic methods.[8,12–14]

In this study, the prevalence and intensity of urinary schistosomiasis in Ghanian school children was assessed using real-time PCR.

METHODS

Study area and sample collection. The study was conducted in five primary schools (children 5–19 years of age, median age 11 years) from different communities in Greater Accra region of Ghana. The schools selected included Anyamam Presby Primary (AN), Goi Presby Primary (GP), Pantang D/A Primary (PA), Nii Okai Basic (NB), and DeYoungsters (DY) representing schools with a schistosomiasis prevalence based on microscopy ranging from ≈0% to 18%. Three of these schools (i.e., AN, GP, and PA) are located in rural areas where as the other two can be found in urban areas of Accra. There had been no mass treatment of schistosomiasis in these areas in the 3 years before sample collection. Urine samples (N = 730) were collected in 50 mL containers between 10.00 and 14.00 when schistosome eggs excretion is known to be highest[15] and left at ambient temperature between the collection sites and the Noguchi Memorial Institute for Medical Research (NMIMR) laboratory. A 2 mL aliquot of each sample was kept frozen at –80°C for PCR. The study was performed within a larger epidemiological study on allergy and parasitic infections ("GLOFAL GHANA") approved by the Institutional Review Board of the NMIMR, Accra, Ghana. A written informed consent was received from parents or guardian of each child. Mass drug administration took place in the same year after the samples were collected.

Microscopy. For each subject, the urine sample was examined for ova of *S. haematobium* by the urine-filtration

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method, which allowed the intensity of infection to be expressed as the numbers of *S. haematobium* eggs/10 mL urine with counts of 1–49 and > 50 eggs/10 mL indicative of low and high intensity of infections, respectively.

DNA extraction. The DNA was isolated from all urine samples using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) protocol after a 200 μL subsample of each urine sample was heated for 10 min at 100°C before treatment with sodium dodecyl sulphate and proteinase K for 2 h at 55°C. As an internal control, phocin herpes virus 1 (PhHV-1) was added to the lysis buffer (at 1,000 plaque-forming units/mL).

Real-time PCR. *Schistosoma* genus-specific primers amplifying a 77-bp fragment of the internal transcribed spacer-2 (ITS2) sub unit described by Obeng and others,[15] consisting of Ssp48F (5′-GGT CTA GAT GAC TTG ATY GAG ATG CT-3′) and Ssp124R (5′-TCC CGA GCG YGT ATA ATG TCA TTA-3′) detected by the probe, Ssp78T [FAM-5′-TGG GTT GTG CTC GAG TCG TGGC-3′-Black Hole Quencher (Biolegio, Nijmegen, The Netherlands)], were selected for amplification and detection of *Schistosoma* DNA.

Amplification of each DNA sample was performed in a 25 μL reaction mixture containing PCR buffer (HotstarTaq mastermix; QIAGEN), 5 mM $MgCl_2$, 12.5 pmol of each *Schistosoma* genus-specific primer, 15 pmol of each PhHV-1-specific primer, 2.5 pmol each of the *Schistosoma* genus-specific and PhHV-1-specific double-labeled probes, and 5 μL of the DNA sample. The thermocycler used was set to give 15 minutes at 95°C, followed by 50 cycles, each of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. Amplification, amplicon detection and the related data analysis were performed with the CFX96 real-time PCR detection system (Bio-Rad, Veenendaal, The Netherlands) using the CFX Manager version 1.6.514 (Bio-Rad). The PCR output from this system consists of a cycle-threshold (Ct) value, representing the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence, indicating the parasite-specific DNA load in the urine sample tested.

The DNA isolation and setup of the PCR reactions were performed using a custom-made Hamilton robot platform.

Data analysis. Statistical analysis was done using the version 16.0 of the SPSS software package (SPSS, Chicago, IL). Real-time PCR results were stratified into high (Ct < 30), moderate (30 ≤ Ct ≥ 35), low (Ct > 35) DNA load and negative (no amplification detected in 50 cycles). The positive and negative predictive values of microscopy and real-time PCR were calculated with true positives as microscopy and/or PCR positive and microscopy and PCR negatives assumed to be true negatives.

RESULTS

Microscopy. Microscopy revealed an overall prevalence of 7.8% (57 of 730) for *S. haematobium* eggs in the total samples used in this study. Prevalence and intensity within schools is represented graphically in Figure 1 showing GP with 18.2% infection followed by NB with 15.3% infection.

Real-time PCR. Amplification of the internal control was detected at the expected Ct value of ≈30 in all samples and *Schistosoma*-specific DNA was detected in 152 of 730 samples. Prevalence and intensity of infection as reflected by low, moderate, and high DNA load in the schools are shown in Figure 1. The GP showed the highest prevalence of 55.6%, with the majority of subjects showing a high to moderate DNA load with Ct-values < 35. The AN followed next with 29.7% positives with 21.3% containing high to moderate DNA load. In DY, which was revealed as negative by microscopy,

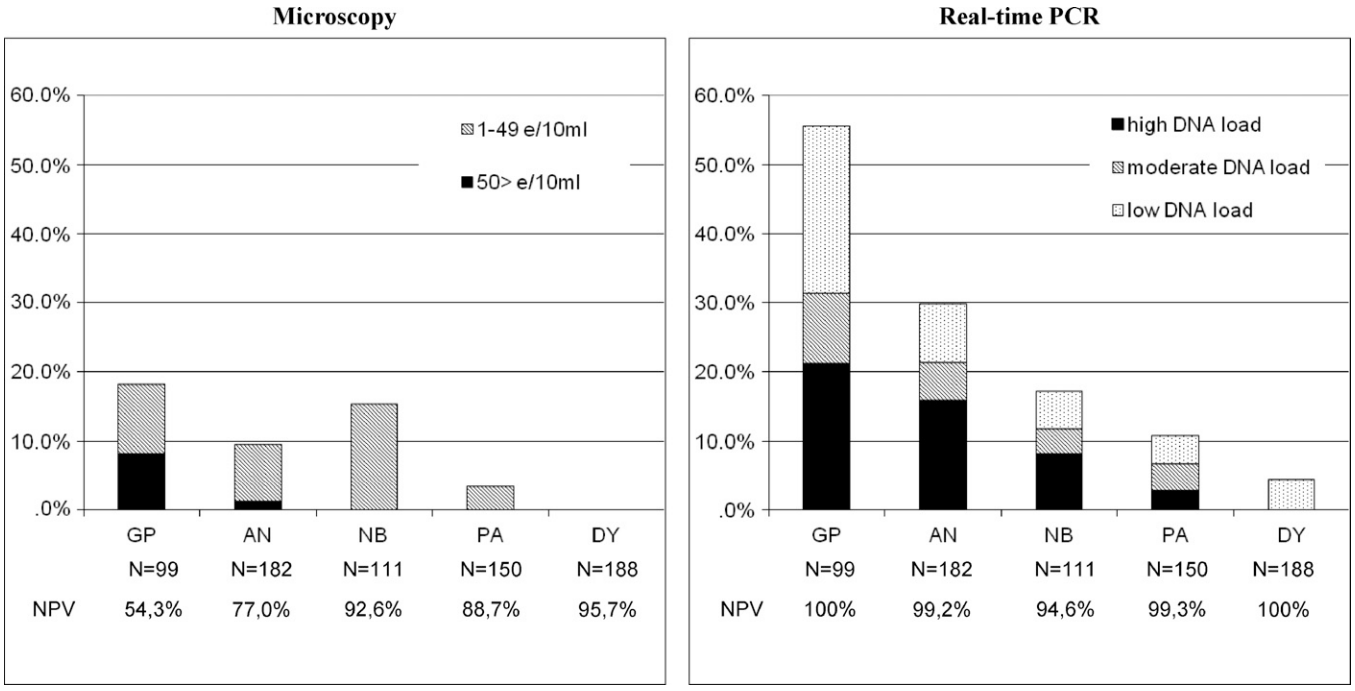

FIGURE 1. Prevalence and intensity of infection as measured with detection of *Schistosoma* eggs using microscopy and *Schistosoma* DNA using real-time PCR in urine samples from five schools in Ghana (N = 730) with the negative predicted values as calculated with a positive result of microscopy and/or real-time PCR as true positive.

TABLE 1

Results of microscopy with the numbers of *S. haematobium* eggs/10 mL urine divided into egg counts of 1–49 and > 50 eggs/10 mL indicative of low and high intensity of infections respectively and real-time PCR results divided into high (Ct < 30), moderate (30 ≤ Ct ≥ 35), and low (Ct > 35) DNA loads and negative (no amplification detected in 50 cycles)*

	PCR Ct Values					
	Negative	Ct > 35	30 > Ct < 35	Ct < 30	Total	Median Ct-value in PCR (range)
Microscopy egg class						
Negative	571 (84.6%)	56 (8.3%)	21 (3.1%)	25 (3.7%)	673 (100%)	35.3 (20.5–39.4)
1–49 eggs/10 mL	7 (14.8%)	3 (6.3%)	9 (19.1%)	28 (59.6%)	47 (100%)	25.8 (17.8–39.1)
≥ 50 eggs/10 mL	0 (0%)	0 (0%)	0 (0%)	10 (100%)	10 (100%)	23.1 (16.1–28.1)
Total	578 (79.2%)	59 (8.1%)	30 (4.1%)	63 (8.6%)	730 (100%)	31.9 (16.1–39.4)

*PCR = polymerase chain reaction; Ct = cycle-threshold.

Schistosoma-specific DNA amplification was detected in 8 of 188 samples (4.3%) all showing a low DNA load.

Comparing microscopy detection and PCR amplification. The results of microscopy and PCR are summarized in Table 1. Cycle threshold values were significantly higher for urine samples in which *S. haematobium* eggs were not found by microscopy ($N = 102$, median 35.3, range 20.5–39.4) than for those from urines in which *S. haematobium* eggs were seen by microscopy ($N = 50$, median Ct 24.7, range 16.1–39.1) (Mann–Whitney test; $P < 0.0001$).

DISCUSSION

In a previous study, using well-defined DNA and urine samples as controls, and 74 urine samples known to contain *S. haematobium* eggs, the *Schistosoma* real-time PCR achieved 100% specificity and sensitivities of 100% and 87% in urines with > 50 eggs/10 mL urine and ≤ 50 eggs/10 mL of urine, respectively.[15] In this study using 730 urine samples collected from children in five primary schools from different communities in Greater Accra region of Ghana, the real-time PCR for the specific detection of *Schistosoma* DNA again showed excellent sensitivities of 100% and 85.2%, in urines with > 50 eggs/10 mL urine and ≤ 50 eggs/10 mL of urine, respectively (Table 1). Additionally, *Schistosoma* DNA was detected with PCR using 200 μL of urine in samples in which eggs were not detected by microscopy using 10 mL of urine. Although it is not clear why *Schistosoma* DNA could not be amplified in seven of the microscopy positive samples (1–12 eggs/10 mL) apparently *Schistosoma* DNA, sometimes with high DNA loads, can be present even in the absence of eggs in the urine sample. A higher sensitivity of the PCR in samples with low egg counts might be achievable by using a larger volume of the urine sample by applying filter-based DNA isolation methods[16,17] In the schools included in this study, the higher sensitivity of the real-time PCR results in much higher negative predictive values (> 94.6%) as compared with microscopy (54.3–95.7%).

The apparent poor sensitivity of the microscopy in this study might have resulted in an overestimate of the PCR sensitivity. Analysis using a (Bayesian) latent class model for analysis without a "true gold standard," performing PCR and microscopy on multiple samples from the same individuals, and standardization of converting DNA-loads into egg counts is subject for further study. Further evaluation is needed in particular in low-prevalence communities. It would also be interesting to assess the application of the *Schistosoma* genus PCR testing urine samples in areas where only *S. mansoni* is endemic.

Facilities for real-time PCR are available in an increasing number of research centers in low- to middle-income countries. Sample collection and transportation to a central laboratory for PCR results into simplification of the complex organization of labor-intensive field studies performing microscopy on the spot, especially when thinking about integrated control in which urine samples can be collected together with stool samples for molecular screening for intestinal schistosomiasis and soil-transmitted helminths.

In conclusion, the results of this study indicate that real-time PCR can serve as a powerful alternative in determining the prevalence and intensity of *Schistosoma* infections using urine samples as template. This can lead to a more accurate assessment of the prevalence and intensity of the disease and evaluating the effectiveness of treatment programs in endemic areas.

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REFERENCES

1. McManus DP, Loukas A, 2008. Current status of vaccine for schistosomiasis. *Clin Microbiol Rev 21:* 225–242.
2. Gryseels B, Polman K, Clerinx J, Kestens L, 2006. Human schistosomiasis. *Lancet 368:* 1106–1118.

3. Feldmeier H, Poggensee G, 1993. Diagnostic techniques in schistosomiasis control: a review. *Acta Tropica 52:* 205–220.
4. Lengeler C, Utzinger J, Tanner M, 2002. Questionnaires for rapid screening of schistosorniasis in sub-Saharan Africa. *Bull World Health Organ 80:* 235–242.
5. ten Hove RJ, Verweij JJ, Vereecken K, Polman K, Dieye L, van Lieshout L, 2008. Multiplex real-time PCR for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* infection in stool samples collected in northern Senegal. *Trans R Soc Trop Med Hyg 102:* 179–185.
6. van Dam GJ, Wichers JH, Ferreira TM, Ghati D, van Amerongen A, Deelder AM, 2004. Diagnosis of schistosomiasis by reagent strip test for detection of circulating cathodic antigen. *J Clin Microbiol 42:* 5458–5461.
7. Stothard JR, Kabatereine N, Tukahebwa EM, Kazibwe F, Rollinson D, Mathieson W, Webster JP, Fenwick A, 2006. Use of circulating cathodic antigen (CCA) dipsticks for detection of intestinal and urinary schistosomiasis. *Acta Trop 97:* 219–228.
8. Verweij JJ, Brienen EA, Ziem J, Yelifari L, Polderman AM, van Lieshout L, 2007a. Simultaneous detection and quantification of *Ancylostoma duodenale*, *Necator americanus*, and *Oesophagostomum bifurcum* in fecal samples using multiplex real-time PCR. *Am J Trop Med Hyg 77:* 685–690.
9. Verweij JJ, Ten Hove R, Brienen EA, van Lieshout L, 2007b. Multiplex detection of *Enterocytozoon bieneusi* and *Encephalitozoon* spp. in fecal samples using real-time PCR. *Diagn Microbiol Infect Dis 57:* 163–167.
10. Barber KE, Mkoji GM, Loker ES, 2000. PCR-RFLP analysis of The ITS2 region to identify *Schistosoma haematobium* and *S. bovis* from Kenya. *Am J Trop Med Hyg 62:* 434–440.
11. Abbasi I, King CH, Sturrock RF, Kariuki C, Muchiri E, Hamburger J, 2007. Differentiation of *Schistosoma haematobium* from related schistosomes by PCR amplifying an inter-repeat sequence. *Am J Trop Med Hyg 76:* 950–955.
12. Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JDC, Wengenack NL, Rosenblatt JE, Cockerill FR, Smith TF, 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical Microbiology 19:* 165–256.
13. Klein D, 2002. Quantification using real-time PCR technology: applications and limitations. *Trends Mol Med 8:* 257–260.
14. Verweij JJ, Blange RA, Templeton K, Schinkel J, Brienen EA, van Rooyen MA, van Lieshout L, Polderman AM, 2004. Simultaneous detection of *Entamoeba histolytica, Giardia lamblia* and *Cryptosporidium parvum* in fecal samples using multiplex real-time PCR. *J Clin Microbiol 3:* 1220–1223.
15. Obeng BB, Aryeetey YA, de Dood CJ, Amoah AS, Larbi IA, Deelder AM, Yazdanbakhsh M, Hartgers FC, Boakye DA, Verweij JJ, van Dam GJ, van Lieshout L, 2008. Application of a circulating-cathodic-antigen (CCA) strip test and real-time PCR, in comparison with microscopy, for the detection of *Schistosoma haematobium* in urine samples from Ghana. *Ann Trop Med Parasitol 102:* 625–633.
16. Ibironke OA, Phillips AE, Garba A, Lamine SM, Shiff C, 2011. Diagnosis of *Schistosoma haematobium* by detection of specific DNA fragments from filtered urine samples. *Am J Trop Med Hyg 84:* 998–1001.
17. Ibironke O, Koukounari A, Asaolu S, Moustaki I, Shiff C, 2012. Validation of a new test for *Schistosoma haematobium* based on detection of Dra1 DNA fragments in urine: evaluation through latent class analysis. *PLoS Negl Trop Dis 6:* 1464.