

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

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Received 9 April 2008, Revised 9 May 2008,
Accepted 13 May 2008

In the detection of parasitic infection, the traditional methods based on microscopy often have low sensitivity and/or specificity compared with the newer, molecular tests. An assay based on real-time PCR and a reagent strip test for detecting circulating cathodic antigen (CCA) have both now been compared with urine filtration and microscopy, in the detection of *Schistosoma haematobium* infections. Urine samples, obtained from 74 'cases' in areas of Ghana with endemic *S. haematobium* and 79 'controls' from non-endemic areas, were each checked using the three methods. With the results of the filtration and microscopy taken as the 'gold standard', real-time PCR was found to be 100% specific and 89% sensitive whereas the CCA strips were 91% specific and 41% sensitive. With the samples found to contain ≥ 50 eggs/10 ml (indicating relatively intense infections), the sensitivities of the PCR and CCA were higher, at 100% and 62%, respectively. As expected, egg counts were negatively correlated with the number of amplification cycles needed, in the PCR, to give a signal that exceeded the background ($r = -0.38$; $P < 0.01$).

Although the real-time PCR and CCA strip tests are very different, both show promise in the detection of *S. haematobium* infections. The PCR has optimal specificity and high sensitivity but the specificity of the CCA strips and the sensitivity of both tools could still be improved. A more thorough re-evaluation of the sensitivity and specificity of microscopy and these newer diagnostic methods, with an estimation of the cost-effectiveness of each technique, is recommended.

Human schistosomiasis, a disease that affects over 200 million people world-wide, is of particular public-health importance in Africa, where both intestinal and urinary forms of the disease — caused by *Schistosoma mansoni* and *S. haematobium*, respectively — occur (Gryseels *et al.*, 2006). In Ghana, human schistosomiasis is common but focally distributed in several locations, with reported

prevalences reaching 60% in some areas (Bosompem *et al.*, 2004). Most epidemiological assessments of the burden of schistosomiasis have relied on the microscopical examination of formol-ether concentrates or Kato-Katz smears of faecal samples, for *S. mansoni* (Brinkmann *et al.*, 1988; Lengeler *et al.*, 2002), and of membrane filters through which urine samples have been passed, for *S. haematobium* (Peters *et al.*, 1976). In the many poor countries with endemic schistosomiasis, microscopy provides a relatively easy and cheap tool for detecting and estimating the concentration of schistosome

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eggs in faecal and urine samples (Feldmeier and Poggensee, 1993). Despite their feasibility, however, the microscopy-based methods require repeated sampling and careful examination to give high sensitivity, particularly when the infections are light, such as when treatment efficacy is being evaluated (Gryseels and de Vlas, 1996). Unfortunately, in many epidemiological studies, repeated sampling and the careful, time-consuming examination of smears or filters are impractical (because of logistics and because many subjects are reluctant to provide multiple samples).

Alternative diagnostic methods have included the assessment of macro- and micro-haematuria (visible and invisible blood in urine, respectively), the detection of antibodies against various stages of the parasite, circulating anodic antigens (CAA) and circulating cathodic antigens (CCA), and PCR-based methods designed to detect parasite DNA. When French *et al.* (2007) evaluated the assessment of micro-haematuria by use of commercial reagent strips (Hemastix[®]; Bayer Diagnostics, Basingstoke, U.K.), with the results of microscopy taken as the 'gold standard', they recorded sensitivities of $\geq 77\%$ and specificities of $> 90\%$ during a 3-year control programme. Their reasonable cost and ease and speed of use make such reagent strips useful for monitoring the efficacy of control programmes, even though the results are often confounded by haematuria of non-schistosome origin. The use of immunological techniques to detect schistosome-specific antibodies can give high sensitivity (Rossi *et al.*, 1991; Van Lieshout *et al.*, 2000) but cannot distinguish between past and present infections. In addition, the cost of, and difficulty in, collecting the necessary blood samples make such techniques a complicated option for field studies (Gryseels *et al.*, 2006). To overcome these problems with antibody detection, ELISA-based techniques for detecting schistosome CAA and CCA in serum or urine were developed (Deelder *et al.*, 1994; Polman *et al.*, 2000; Van Lieshout *et al.*, 2000).

Although these tests had limited application in individual patient diagnosis, they led to the development of a one-step reagent-strip test for the detection of schistosome CCA in urine, in which colloidal carbon acted as the visible label (Van Dam *et al.*, 2004). Further development and up-scaling resulted in a colloidal-gold-based, field-applicable, commercial strip test that has been evaluated against *S. mansoni* infections in Uganda and *S. haematobium* infections in Zanzibar (Stothard *et al.*, 2006). In this evaluation, the test worked well with *S. mansoni* but not with *S. haematobium*; the prevalence of *S. mansoni* infection estimated using the CCA strip was found to be highly correlated with that based on the microscopical examination of Kato-Katz smears, but the strips did not give any positive results with the *S. haematobium* infections (Stothard *et al.*, 2006). The test was therefore taken back to the laboratory so that the labelling and coating procedures could be modified to allow detection of at least medium-high *S. haematobium* infections. The current version of this test is again based on a colloidal carbon label, with the quality-control procedures and the test's stability optimised so that reproducible research batches, of 5000 to 10,000 strips each, can be manufactured (unpubl. obs.). Although not completely formatted for end-stage use, the test is easily applicable in the field and stable at normal room temperatures, without the need for cold-chain logistics (unpubl. obs.).

Methods based on PCR, which typically have high sensitivity and specificity, have been successfully used to reveal the presence of DNA from a broad range of parasites, including hookworms (Verweij *et al.*, 2007a), microsporidia (Verweij *et al.*, 2007b) and *Schistosoma* in stool samples (Ten Hove *et al.*, 2008). Most of the earlier limitations in the use of PCR to detect parasitic infections, including difficulties in DNA isolation and problems with contamination, have been overcome in recent years. The isolation of parasite DNA has been improved and simplified (Subrungruang *et al.*, 2004), real-time

PCR, with fluorescent probes, have been developed (Klein, 2002), internal controls have been improved (Verweij *et al.*, 2007b), and the possibility of combining two or more assays has been explored (Verweij *et al.*, 2007b). Although both conventional (Sandoval *et al.*, 2006) and real-time PCR (Gomes *et al.*, 2006) have already been used to detect schistosome DNA in human samples, such molecular methods have been largely targeted against *S. mansoni* rather than *S. haematobium*. In addition, in the investigations that have focused on *S. haematobium*, PCR-based methods have primarily been used to detect the parasite's DNA in its snail vector rather than in its definitive human hosts (Hamburger *et al.*, 2004; Abbasi *et al.*, 2007).

The main aim of the present study was to compare the performances of real-time PCR and the newest version of the carbon-labelled CCA strip tests in detecting *S. haematobium* infection in subjects from endemic and non-endemic areas of Ghana. The results of traditional microscopy, with just one urine sample/subject, were used as the 'gold standard'. The potential for using non-microscopic diagnostic methods in epidemiological studies of urinary schistosomiasis, with just one urine sample/subject, was thus explored.

SUBJECTS AND METHODS

Subject Recruitment and Field Samples

For the present study, one urine sample and one stool sample were collected from each of 153 schoolchildren aged 6–16 years: 79 'controls' (randomly selected from the children attending De Youngster's school, which is in a non-endemic part of Accra) and 74 'cases' (a random selection of the schoolchildren, from the *Schistosoma*-endemic areas of the Dangme West and Accra districts, who were found, by microscopy, to be excreting *S. haematobium* eggs in their urine). These children were a subset of those enrolled in a larger epidemiological

study on allergy and parasitic infections ('GLOFAL GHANA') that had been approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, Accra, Ghana. Although several other schools were invited to participate in the larger study, data were only collected from each school where at least 150 subjects consented to participate. Once the parents of an eligible child gave their informed consent (by signing or thumb printing a consent form), that child was enrolled in the study. The background prevalences of schistosome infection in the participating schools varied between 0% and 3.0% for *S. mansoni* and between 0% and 20.5% for *S. haematobium*. The median egg outputs of the infected children in the schools were 80 eggs/g for *S. mansoni* (with a range of 40–720 eggs/g) and 11 eggs/10 ml urine for *S. haematobium* (with a range of 1–779 eggs/10 ml). In the 3 years prior to sample collection, there had been no community-wide treatment for schistosomiasis in the study areas.

The urine samples were checked for evidence of *S. haematobium* infection (see below) not only by microscopy (in Ghana) but also, after storage at -80°C , by real-time PCR (in The Netherlands) and by CCA strip tests (also in The Netherlands). The stool samples were checked for *S. mansoni* eggs by microscopy (see below). Urine samples were left at the ambient temperature between the collection sites and the research laboratory in Ghana whereas the stool samples were transported on ice.

Microscopy

For each subject, the urine sample was checked for *S. haematobium* eggs by the urine-filtration method (Peters *et al.*, 1976) and one stool sample was checked for *S. mansoni* eggs by the microscopical examination of a 25-mg Kato–Katz smear (Katz *et al.*, 1972). The use of these methods allowed the intensities of infection to be expressed as the

numbers of *S. haematobium* eggs/10 ml urine — with counts of <50 and \geq 50 eggs/10 ml considered indicative of low- and high-intensity infections, respectively — or the numbers of *S. mansoni* eggs/g faeces.

DNA Isolation and Real-time PCR

A 200- μ l 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. The DNA was then isolated using a spin column from the QIAamp DNA mini kit (QIAGEN, Hilden, Germany). As an internal control, phocin herpes virus 1 (PhHV-1) was added to the lysis buffer (at 1000 plaque-forming units/ml), and primers specific for this virus and a corresponding, PhHV-1-specific, Cy5 double-labelled detection probe (Biolegio, Nijmegen, The Netherlands) were included in each reaction mixture.

The *Schistosoma*-specific primers and probe used for the real-time PCR were chosen using the Primer Express software package (Applied Biosystems, Foster City, CA), on the basis of the internal-transcribed-spacer-2 (ITS2) sequences for *S. haematobium* (GenBank accession DQ677661), *S. mansoni* (AF503487) and *S. intercalatum* (U22166). With a positive sample, the *Schistosoma*-specific primers, Ssp48F (5'-GGT CTA GAT GAC TTG ATY GAG ATG CT-3') and Ssp124R (5'-TCC CGA GCG YGT ATA ATG TCA TTA-3'), amplified a 77-bp fragment. The double-labelled Ssp78T [FAM-5'-TGG GTT GTG CTC GAG TCG TGGC-3'-Black Hole Quencher (Biolegio)] was used as a probe, to detect any *Schistosoma*-specific amplification. The PCR, which was optimised using a 10-fold serial dilution of *S. haematobium* DNA, had been found to offer 100% specificity when tested against 150 DNA controls derived from a wide range of intestinal microorganisms (Ten Hove *et al.*, 2008).

The amplification of each DNA sample was performed in a 25- μ l reaction mixture

containing PCR buffer (HotstarTaq mastermix; QIAGEN), 5 mM MgCl₂, 12.5 pmol of each *Schistosoma*-specific primer, 15 pmol of each PhHV-1-specific primer, 2.5 pmol each of the *Schistosoma*-specific and PhHV-1-specific double-labelled probes, and 5 μ l of the DNA sample. The thermocycler used was set to give 15 min at 95°C, followed by 50 cycles, each of 15 s at 95°C and 60 seconds at 60°C. Amplification, amplicon detection and the related data analysis were performed with the AB7500 real-time detection system (Applied Biosystems). The PCR output from this system consisted of a cycle-threshold (Ct) value, representing the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence, and indicating the parasite-specific DNA load in the urine sample tested.

Carbon-labelled CCA Strips

Urine samples were analysed using CCA strips, as previously described (Van Dam *et al.*, 2004). Briefly, 75 μ l CCA buffer were added to a tube with air-dried colloidal carbon (SS4; Degussa, Frankfurt, Germany) conjugated to the 54-4C2-A monoclonal antibody. The contents of the tube were then thoroughly mixed with 25 μ l urine before being probed with a CCA test strip — a strip of nitrocellulose (Prima 125; Whatman, Dassel, Germany) coated with the 54-5C10-A monoclonal antibody. Lateral flow was then allowed to proceed until the strip was dry. The other reagents and materials employed were identical to those described by Van Dam *et al.* (2004). Each test line was interpreted against the results obtained using five reference samples produced by adding a trichloroacetic-acid-soluble fraction of adult *S. haematobium* antigens (containing 3% CCA), at 0, 10, 100, 1000 or 10,000 ng/ml, to a pool of negative-control urine samples. The line intensities obtained with these five samples were given scores of 0, 0, 1, 2 and 3, respectively, and a urine sample was only

considered CCA-negative if its test line matched a reference line scored 0.

Data Analysis

The data collected were stored in Access (Microsoft) and Excel (Microsoft) databases and later transferred to version 14.0 of the SPSS software package (SPSS, Chicago, IL), for statistical analysis. A Spearman's rank-correlation test was used to evaluate the level of correlation between the counts of *S. haematobium* eggs/10 ml urine and the corresponding Ct values from the PCR. The sensitivity and specificity of the reagent-strip test and the real-time PCR were estimated, with the results of the microscopy taken as the 'gold standard' (i.e. the cases were assumed to be true-positives and the control subjects were assumed to be true-negatives). McNemar's test was used to see if any diagnostic test was statistically more sensitive than any other (in identifying a case as positive). A *P*-value of <0.05 was considered indicative of a statistically significant difference.

RESULTS

In terms of age distributions, the 79 controls were similar to the 74 cases, with mean (S.D.) ages of 10.4 (2.1) and 10.8 (2.1) years, respectively. Most (57%) of the controls and 47% of the cases were female.

The median value (and range) for the egg outputs in the urine of the cases was 11 (one to 493) eggs/10 ml urine. Only two of the subjects, both cases, were found positive for *S. mansoni*, with egg outputs of 640 and 720 eggs/g faeces.

The results of the CCA-strip tests and PCR, for both controls and cases, are summarized in the Table. In the PCR, none of the urine samples from the controls showed any amplification of *Schistosoma*-specific DNA, although all showed amplification of the viral DNA from the internal controls (with a Ct of about 33). Urine samples from seven of the controls were found CCA-positive, the intensity of the test strip in each positive control being scored 1.

Of the cases with low-intensity *S. haematobium* infections (i.e. those excreting ≤ 50 eggs/10 ml urine), 87% were found PCR-positive for *Schistosoma* and only 36% were found CCA-positive (two of the 22 CCA-positives scoring 2 and the rest scoring 1). The performances of the PCR and strip tests appeared better among the cases with high-intensity *S. haematobium* infections, however, with 100% of such cases found PCR-positive and 62% found CCA-positive (all the CCA-positives scoring 1).

In the PCR, the median Ct value for the cases with low-intensity *S. haematobium* infections (28.1) was also higher than that for the cases with more intense infections, indicating a negative correlation between Ct

TABLE. Comparison of the results of the carbon-labelled circulating-cathodic-antigen (CCA) strip tests and the *Schistosoma*-specific real-time PCR with those of urine filtration and microscopy

Subjects	No. and (%):			Median Ct value in PCR and (range)
	Investigated	CCA-positive	PCR-positive	
Controls (all found negative by microscopy)	79	7 (8.9)	0 (0)	
CASES WITH:				
≤ 50 eggs/10 ml urine	61	22 (36.1)	53 (86.9)	28.1 (16.5–40.6)
>50 eggs/10 ml urine	13	8 (61.5)	13 (100)	24.0 (19.5–30.0)
At least one egg/10 ml urine	74	30 (40.5)	66 (89.2)	27.4 (16.5–40.6)

Ct, The cycle-threshold value, representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence.

value and the egg count determined by urine filtration. In a Spearman's rank-correlation test, this correlation was subsequently found to be statistically significant ($r = -0.38$; $P < 0.01$).

Overall, 89% of the cases were found PCR-positive and 41% were found CCA-positive. Of the eight (egg-positive) cases found PCR-negative, six had egg counts of < 10 eggs/10 ml and were found CCA-negative. The other two cases found negative in the initial PCR had slightly higher egg counts (12 and 27 eggs/10 ml), were found CCA-positive, and were found PCR-positive (with Ct of 35 and 27) when the real-time PCR was repeated using the same urine samples. Correlation between egg output and CCA-strip score could not be assessed because all but two of the CCA-positive subjects were scored 1.

With the results of the microscopy used as the 'gold standard', the PCR appeared to be significantly more sensitive than the strip test ($P < 0.01$).

DISCUSSION

In routine fieldwork and large epidemiological studies, the difficulties in meeting the multiple sampling requirements for classical parasitological diagnosis often lead to sub-optimal results (because of the false-negative cases). Highly sensitive diagnostic tools that could provide an accurate result using just one or two samples/subject would be very valuable in research and the evaluation of treatment programmes, and also, possibly, in routine diagnosis. Human schistosomiasis, which remains a major public-health problem in Ghana, is often assessed in the field using questionnaires and subsequent microscopy (Moestue *et al.*, 2003). The knowledge and perceptions of many endemic communities mean that even symptom-aware subjects often do not seek treatment (Wagatsuma *et al.*, 2003; Danso-Appiah *et al.*, 2004), and they may not volunteer the information on schistosomiasis-associated conditions that

would help investigators determine the presence or prevalence of the disease.

An ELISA-based monoclonal-antibody dipstick developed for the detection of *S. haematobium* in urine was evaluated by Bosompem *et al.* (2004) and was, until the present study, the only alternative to microscopy for the diagnosis of schistosomiasis that had been evaluated in the field in Ghana. In the present study, two recently developed field tests for the detection of schistosome CCA or DNA in urine samples were compared with the results of filtration/microscopy, under field conditions, using just one sample/subject. The evaluation was conducted with cases selected from endemic rural areas with low-moderate prevalences of *S. haematobium* and with controls who came from a non-endemic area in the urban capital of Ghana, Accra. When tested against urine samples from the cases and controls, the PCR-based assay showed high sensitivity (89%) and maximal specificity. There was also a significant negative correlation between the Ct values from the PCR and the egg counts, indicating the potential value of PCR in determining the intensity as well as the presence of infection. Discrepancies between the results of the PCR and microscopy were limited to the lightly infected cases who were excreting very low numbers of eggs. The sensitivity of the real-time PCR could be increased by testing urine samples twice. As all the PCR results with the internal and positive controls in the initial real-time PCR were as expected if the assay was working correctly, it is unclear why a few egg-positive urine samples were found PCR-negative. The volume of urine from which the parasite DNA was isolated was, however, 50 times less than the volume filtered for the microscopy (200 μ l *v.* 10 ml). With very light infections, the amount of schistosome genetic material present in 200 μ l of the host's urine may not reach levels that are detectable using the PCR-based technique. This may explain why it was only with the cases with high-intensity infections that the PCR was found to be 100% sensitive. The true sensitivity of

the PCR (compared against microscopy) would have been better assessed in a prospective study that included 'endemic normals' (i.e. subjects who, though living in an endemic area, appeared microscopy-negative for schistosome infection).

Ten Hove *et al.* (2008) described a real-time PCR (with primers, specific for *S. haematobium* and *S. mansoni*, targeted at the parasites' cytochrome c oxidase genes) that successfully amplified *Schistosoma* DNA from stool samples. In the present study, an ITS2-based, *Schistosoma*-genus-specific, real-time PCR was found to give a high level of agreement with the results of urine filtration/microscopy. Together, these results indicate the potential usefulness of real-time PCR as a diagnostic tool for both urinary and intestinal schistosomiasis. It would be interesting to assess the application of the ITS2-based PCR in testing urine samples in areas where only *S. mansoni* is endemic. In general, PCR-based methods are more expensive than microscopy. The development of multiplex systems (Verweij *et al.*, 2007a, b), that allow the detection and identification of several different parasites in a single assay, and of simpler procedures for the isolation of DNA may, however, make the use PCR a viable option for diagnosis, at least in a research setting. By adding ethanol as a 'DNA-friendly' preservative, it should be possible to store urine and stool samples without refrigeration or freezing for a few months without affecting the results of the PCR. Unfortunately, compared with the microscopy-based methods for detecting schistosome infection, the isolation of DNA from such samples and the use of PCR still require a better equipped laboratory and more specialist knowledge and training.

Compared with the PCR, which has to be laboratory-based, the field-applicable carbon-labelled CCA strip that was evaluated in the present study showed lower sensitivity (41%) and specificity (91%). It was not possible to analyse the semi-quantitative scoring of the strips as an indicator on

infection intensity, as only two strips were scored higher than 1. As with the PCR, however, the sensitivity of the strips was better (at 62%) for the samples from the high-intensity infections (i.e. those with >50 eggs/10 ml urine) than for those from the cases with lighter infections. Although a higher sensitivity would be optimal, the strips should still be useful in areas of high endemicity for urinary schistosomiasis.

Previously, an earlier version of the CCA strip (EVL), that used gold as the visible label, was found to be highly sensitive for *S. mansoni* (Stothard *et al.*, 2006; Legesse and Erko, 2007), like the corresponding CCA-ELISA (Polman *et al.*, 1995; De Clercq *et al.*, 1997; Van Lieshout *et al.*, 2000). The potential usefulness of the CCA strips in the diagnosis of urinary schistosomiasis has, however, been a matter of controversy (De Jonge *et al.*, 1989; Kremsner *et al.*, 1994). Stothard *et al.* (2006), for example, could not detect any positive *S. haematobium* cases using the gold-labelled strip, although, in other studies, the same strip did detect some *S. haematobium* infections, albeit with low sensitivity (unpubl. obs.). Ayele *et al.* (2008) have recently reported a sensitivity of 52% and a specificity of 63.3% for the EVL strips when used to detect *S. haematobium*. In the present study, in showing evidence of *S. haematobium* infection in urine samples, the performance of the carbon-labelled CCA strips was found to be similar to that of the CCA-ELISA, with a moderate sensitivity that may restrict the routine use of the strips to areas with generally moderate or high intensities of infection.

In conclusion, the results of the present study indicate that real-time PCR may potentially serve as a gold standard in determining the prevalence and intensity of *S. haematobium* infection. Further evaluation is needed, however, in cross-sectional studies, in particular in low-prevalence communities. The potential for the latest version of the CCA strips to be used as a diagnostic field test in areas with moderate to high intensities of infection has also been

demonstrated. The sensitivity of these strips has to be improved, however, if they are to be used successfully in areas of low endemicity for urinary schistosomiasis.

ACKNOWLEDGEMENTS. The authors wish to thank the community leaders, school head teachers and the parents and subjects involved in this study, for their invaluable contribution. They are also grateful to L. Tamatey in particular and to the staff of the Department of Parasitology of the NMIMR, for their assistance with the field and laboratory work.

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