

Rapid Diagnosis of Schistosomiasis by Antigen Detection in Urine with a Reagent Strip

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For the rapid and simple diagnosis of schistosomiasis, a reagent strip assay for detection of circulating cathodic antigen (CCA) in urine was developed. The test was based on a previously developed sandwich enzyme-linked immunosorbent assay (ELISA) using a combination of two anti-CCA monoclonal antibodies. For the reagent strip assay, as the capture matrix, monoclonal antibody was coated onto a nitrocellulose membrane and mounted on polyvinyl chloride strips. Urine samples were then tested in an assay consisting of a combined incubation step of the urine sample and biotinylated detecting antibody followed by incubation in streptavidin peroxidase and a subsequent staining. The specificity and the sensitivity of the assay, as determined with urine samples of 61 uninfected controls and 67 *Schistosoma mansoni*-infected individuals, were 96.7 and 95.5%, respectively. The results of the reagent strip assay compared very well with microscopical parasitological diagnosis by the standard Kato-Katz method for the same individuals. The reagent strip test has a lower detection limit of 1 ng of CCA per ml and can be completed in 75 min. By the inclusion of two reference bands on the strips, standardized reading could be achieved. This reagent strip assay is a promising tool for qualitative diagnosis of *S. mansoni* infections in control programs.

Schistosomiasis is a major parasitic disease, afflicting about 200 million individuals in the tropics. The major features of the chronic phase of the disease are diarrhea, dysentery, abdominal pain, lack of appetite, loss of weight, hepatomegaly, splenomegaly, and ascites in intestinal schistosomiasis (*Schistosoma mansoni* and *S. japonicum*) or distal hematuria in an established *S. haematobium* infection. *S. mansoni* worms live in the branches of the vena mesenterica inferior in the wall of the colon, and some of the eggs are excreted with the feces, while other eggs remain in the body and are the main cause of pathology. The intensity of schistosomal infection is currently measured by quantitative egg counts in feces. This parasitological method, however, is often complicated by the great fluctuation of the egg output and/or low concentration of eggs.

Measurement of circulating anodic antigen (CAA) and of circulating cathodic antigen (CCA), both genus-specific glycoconjugates associated with the gut of the adult worm, by the enzyme-linked immunosorbent assay (ELISA), has been shown to be a good alternative to egg counts for the diagnosis of *S. mansoni*, *S. haematobium*, and *S. japonicum* infections (2, 4, 6, 16).

The technique is, however, relatively cumbersome and requires an appropriately equipped laboratory. For use under field conditions, an assay for detection of schistosomiasis should be rapid, specific, sensitive, and easy to perform.

A number of modifications of the ELISA have been described in efforts to produce a more field-applicable assay format. The dot immunobinding assay, a modification of the ELISA using a nitrocellulose membrane as a test matrix, is becoming widely used in simple qualitative research applications (9, 14). Dot ELISA-type immunodiagnostic tests, detect-

ing either antibodies or antigen, have been used successfully for immunodiagnosis of a variety of parasitic infections (8) and have already been reported for schistosomiasis (1). Recently, a dot dye immunoassay was described by Snowden and Hommel (13), and this assay format has been used successfully for the diagnosis of schistosomiasis in detecting immunoglobulin G antibodies against soluble egg antigen (11). However, antibody levels in general show no correlation with the worm burden, and the presence of specific antibodies does not indicate an active infection since antibodies are demonstrable in serum even a long time after successful chemotherapy. Detection of circulating antigens is preferable since it is a more direct measure of the worm burden, giving an indication of the activity and intensity of infection. Moreover, since several circulating antigens are present in urine, a noninvasive method of sampling can be applied.

Both CAA and CCA are detectable in serum and urine, but as previous studies showed, in urine, CCA levels are considerably higher than those of CAA (3, 7). After successful chemotherapy, CCA levels in serum and urine decrease rapidly (after 1 week) (15). The present study was undertaken to develop an assay that is more field applicable than the ELISA for testing of urine samples. A reagent strip assay (dipstick) was adapted from the previously developed sandwich ELISA for CCA by using monoclonal antibodies (4). Assay conditions were optimized, and the assay was evaluated for the diagnosis of *S. mansoni* infection.

MATERIALS AND METHODS

Urine samples. For initial laboratory evaluation of the reagent strip assay, a total of 128 urine samples were tested, and 67 of these samples had been derived from *S. mansoni*-infected individuals from Tshamaka, Zaire. The endemic situation of *S. mansoni* in this region has been described by Polderman et al. (10). The urine samples were stored frozen at

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-20°C until use and mixed prior to testing. Schistosome egg excretion per gram of feces had been determined by the Kato-Katz method (5) and ranged from 13 to 15,440 (median, 460 eggs per g of feces). The other samples derived from 61 healthy Dutch individuals with no history of visiting schistosomiasis-endemic areas.

For all urine samples, CCA concentrations had been determined previously by the sandwich ELISA (4), in which assay samples were pretreated by heating in alkaline buffer (7). This ELISA had a lower detection limit of 120 pg of CCA per ml and showed a specificity of 98%, as determined with 102 urine samples from healthy Dutch individuals who had no history of visiting an area where schistosomiasis is endemic.

Reagent strip. Protein A-purified mouse immunoglobulin G3 anti-CCA monoclonal antibody (line 54-5C10-A, 150 µg/ml) and two dilutions (1:250 and 1:600) of rabbit anti-mouse immunoglobulin (RAM-Ig; Dakopatts, Glostrup, Denmark) in 0.035 M phosphate-buffered saline (pH 7.8; PBS), were adsorbed (19.6 µl/10-2mm band) onto a nitrocellulose membrane (pore size, 0.45 µm; Schleicher & Schuell, Dassel, Germany), giving strips with three 2-mm-wide coated bands. After the nitrocellulose membrane was rinsed four times with PBS, the membrane was mounted with double-sided tape to transparent 0.15-mm-thick polyvinyl chloride for easy handling, subsequently postcoated with 2% (wt/vol) bovine serum albumin (BSA) and 3% (wt/vol) Dextran T10 (Pharmacia, Uppsala, Sweden), and air dried.

A strip of the coated membrane (15-mm wide) was then adhered with doubled-sided tape to 0.40-mm-thick polyvinyl chloride, after which reagent strips (3 by 80 mm) were cut. These reagent strips could be used immediately or stored dry at ambient temperature, thereby remaining stable for at least 6 months.

Reagent strip test procedure. For assaying, reagent strips were incubated for 1 h at room temperature on a shaker in 500 µl of undiluted urine sample to which, in a final dilution of 1:200, biotinylated anti-CCA mouse immunoglobulin M monoclonal antibody (line 8-3C10-A) (4) was added. Reagent strips were rinsed briefly (approximately 3 s) with tap water and incubated for a further 5 min on a shaker (at room temperature) in a 500-µl solution of 1:1,000 streptavidin-conjugated peroxidase (Dakopatts) in 0.035 M PBS (containing 0.02% [vol/vol] Tween 20, 0.5% [wt/vol] polyethylene glycol 1000, and 0.1% [wt/vol] BSA). Reagent strips were rinsed as before, and bands were detected after immersion of the strips for 30 s in a metal-enhanced diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) substrate solution. The reagent strips were then washed again with water and allowed to dry, after which positive and negative reactions were determined. The substrate was made by preparing a mixture of the substances 0.05% (wt/vol) diaminobenzidine, 0.05% (wt/vol) NiSO₄·6H₂O, 0.05% (wt/vol) imidazole, 0.15% (wt/vol) NaBO₂·H₂O₂·3H₂O, and 2.2% (wt/vol) K₂HPO₄. Alternatively, a dry mixture of these substances was prepared, and immediately before use, demineralized water was added. When stored dry at ambient temperature, the mixture remained stable for at least 6 months.

In each assay, double dilutions of the trichloroacetic acid-soluble fraction of the adult worm antigen (AWA-TCA) ranging from 30 to 480 ng of AWA-TCA per ml, as positive controls and negative controls were tested together with the samples. In addition, AWA-TCA contains approximately 3% CCA (14a). Direct binding of the biotinylated detecting antibody by RAM-Ig allowed control of proper test conditions and provided semiquantitative reference values for the test band. The staining of the 1:600 RAM-Ig band corresponded with

TABLE 1. CCA detection by the ELISA and reagent strip assay of urine samples of uninfected Dutch individuals and of *S. mansoni*-infected individuals from Tshamaka, Zaire

Assay	No. of infected individuals (n = 67) positive	No. of uninfected individuals (n = 61) negative	% Sensitivity	% Specificity
Reagent strip	64	59	95.5	96.7
ELISA	59	61	88.1	100

about 30 ng of AWA-TCA per ml, the lower detection level, which was taken as the cutoff value; the 1:250 RAM-Ig band corresponded with approximately 240 ng of AWA-TCA per ml. Results were considered positive when three bands were present and the intensity of the test band was equal to or higher than the 1:600 RAM-Ig signal.

Statistics. Comparison between the dipstick, the ELISA, and the Kato-Katz methods was assessed by using the statistical package SPSS/PC+, version 4.0, for an IBM personal computer. Because the results were measured on a nominal scale (positive and negative), the McNemar test was done to investigate whether the dipstick method performed equally as well as the two other methods.

RESULTS

The optimal assay conditions were determined in a standard protocol with consecutive incubation steps by using double dilutions of AWA-TCA in negative urine samples ranging from 30 to 480 ng of AWA-TCA per ml and negative control urine. Incubation at higher temperature did not improve the reaction intensity, and shaking during incubation proved to be necessary since results were worse without shaking.

In efforts to produce a more field-applicable assay, combinations of different steps were examined. A single incubation procedure did not yield good results nor did the combination of biotinylated detecting monoclonal antibody and enzyme conjugate. However, a combination of the antigen and biotinylated monoclonal antibody incubation step showed even better results than when these steps were performed separately.

The protocol of 1 h of incubation for the combined antigen-biotinylated second antibody step, 5 min of incubation with the enzyme, and 30 s staining, all performed at room temperature, was applied in further experiments. Rinsing the dipsticks between the incubation steps with water showed to be sufficient. Under these conditions, approximately 30 ng of AWA-TCA per ml, which corresponds with 1 ng of CCA per ml, was still detectable.

Throughout the study, staining of the reference bands corresponded well with staining of 30 and 240 ng of AWA-TCA and thus allowed standardized interpretation of the results.

Table 1 summarizes the results of the reagent strip assay and the ELISA. The strips were scored independently by two observers, giving exactly the same results. Although for this group the specificity of the reagent strip assay was 96.7% (versus 100% for the ELISA), the observed sensitivity of 95.5% (versus 88.1% for the ELISA) was surprisingly good. The McNemar test showed no significant difference in results obtained with the reagent strip assay in comparison with those determined by the Kato-Katz method ($P = 0.25$; $n = 67$) or the ELISA ($P = 0.07$, $n = 67$).

DISCUSSION

There is an urgent need for simple and reliable tests which can be used in endemic areas for the diagnosis of schistosomiasis and for the follow-up of chemotherapy, preferably based on urine samples. It has been demonstrated that detection of CCA in urine can be used as a sensitive method to monitor the efficacy of chemotherapy (15).

Although reagent strip assays for the demonstration of blood in the urine of *S. haematobium*-infected individuals are available and have shown to correlate well with parasitological diagnosis (12), such assays are of no use for *S. mansoni* or *S. japonicum* infections. The present assay, based on the detection of a major adult schistosome antigen which is specific for the genus *Schistosoma*, appears to fulfill the requirements for a simple and rapid parasite-specific assay. The present assay needs no expensive equipment and can be performed in 75 min, which is twice as fast as the ELISA, and in contrast to the ELISA, urine samples do not need a time-consuming pretreatment.

To avoid problems with run-to-run variability or within-run variability, standardized interpretation of the reagent strips was considerably improved by the inclusion of two reference bands. These reference bands served two purposes, namely, the presence of a cutoff value and a positive control. We found that this approach allowed repeated testing without significant differences in the observed positivity rate.

Although the lower detection level of the reagent strip assay is not as good as that of the ELISA, it is still 1 ng of CCA per ml. However, in comparison with the ELISA, the reagent strip assay resulted in a higher sensitivity with a good specificity. This higher sensitivity might be explained in part by the slightly lower specificity observed with the reagent strip assay. Furthermore, undiluted urine samples and a larger sample volume are tested with this assay, which might increase the sensitivity. No significant difference was found in the results of the reagent strip assay and diagnosis by the Kato-Katz method or the ELISA.

The reagent strip assay is not a quantitative reaction, although additional reference bands on the strips would allow a semiquantitative reading. Although quantitative assessment can be important, since the level of antigen is likely to reflect the intensity of infection (3) in many instances, e.g., control programs, a simple yes or no answer would be more than sufficient. This study shows that the developed dipstick is a promising tool for the sensitive and rapid diagnosis of infection with *S. mansoni*. Further studies will be concerned with the application of this assay to infections with other schistosome species and with the evaluation of its applicability to the follow-up of chemotherapy.

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