Rapid Detection of a *Schistosoma mansoni* Circulating Antigen Excreted in Urine of Infected Individuals by Using a Monoclonal Antibody

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Schistosomiasis, the second major parasitic disease in the world after malaria, affects about 250 million people worldwide. The current method for the diagnosis of schistosomiasis in areas of endemicity is the microscopic detection of eggs in stool and urine samples, but this assay does not give reliable results, and several measurements on different days are necessary for the precise diagnosis of schistosomiasis (14). Rectal biopsy is required to obtain better results, but it is invasive and its performance requires experienced physicians rather than technicians, and so it is not suitable for use in mass screening (1). Several schistosome serodiagnostic assays designed for the detection of specific anti-schistosome antibodies have been developed over the years. However, it seems difficult to believe how that a test based on antibody measurement may overcome the drawbacks intrinsic to such types of assays, namely, discrimination between active infections, old infections, and re-infections (12, 19). Standardization of reagents, expression of results, and correct interpretation of data are also difficult to achieve (22).

Recently, detection of circulating schistosome antigens secreted by live schistosomes in body fluids with specific monoclonal antibodies (MAbs) has been shown to be a promising approach to the detection of active infection and to the assessment of treatment efficacy and the effectiveness of future vaccines (8, 9, 13, 15, 21). The overall high degrees of sensitivity of antigen detection assays have been confirmed by comparing the results obtained by those assays with those obtained by quantitative parasitological techniques. A sensitivity of 80 to 90% was shown for patients excreting at least 100 eggs per gram (epg) of stool, and a sensitivity of 100% was shown for patients excreting more than 400 epg. The specificities of antigen detection assays, which all rely on the use of MABs, are almost 100% (9–11, 16).

Many of the assays based on antigen detection display both high specificities and high sensitivities (25, 28). However, they require special and highly expensive equipment, and the procedures require long periods of time for their completion such that they cannot be easily adapted for field use. The dot enzyme-linked immunosorbent assay (ELISA) type of immunodiagnostic test is becoming widely used in simple qualitative research applications (23) and has already been reported for use in the detection of schistosomiasis (3). A number of modifications have been described in efforts to produce a more field-applicable assay format.

In the present study we evaluated the sensitivity and specificity of circulating antigen detection in urine by a newly developed fast dot-ELISA assay (FDA) and compared them with those of standard traditional techniques for the rapid and simple diagnosis of human schistosomiasis in the field.

**MATERIALS AND METHODS**

*Study subjects.* A total of 700 Egyptian individuals were included in the present study. They were 542 males and 158 females (age range, 3 to 72 years). A total of 450 individuals were symptomatic, and the remaining 250 individuals were nonsymptomatic. Stool, urine, and blood were collected from all individuals. Rectal biopsies were done for only 394 individuals (309 males and 85 females) among all individuals showing no *Schistosoma mansoni* eggs in their feces.

*Clinical examinations.* Full clinical examinations were done for all individuals and included a medical history. The general examination included assessment of
vital signs, congestion, paratoid gland enlargement, clubbing of fingers, edema of lower limbs, and signs of liver-cell failure. Local examination included abdominal examination (assessment of sizes of the liver and spleen, manifestation of portal hypertension, abdominal masses, and ascites) and chest-heart examination for manifestations of pulmonary hypertension.

**Parasitological examinations.** Simple stool sedimentation by centrifugation for the detection of *S. mansoni* and other parasitic infections was done for 2 or 3 consecutive days for each individual. The Kato thick smear technique was used to count the numbers of *S. mansoni* eggs in the stool specimens as described by Martin and Beaver (20). The individuals positive by the Kato technique were classified as having a mild infection (<100 epg of stool), a moderate infection (101 to 400 epg), or a heavy infection (>400 epg). A rectal biopsy was done by one of the authors, and samples were taken from at least three different sites and were examined by the transparence technique.

**Schistosoma antigenic extract preparation.** *S. mansoni* and *Schistosoma hae- nutobium* antigenic extracts (soluble worm antigen preparation [SWAP], cercarial antigen preparation [CAP], and soluble egg antigen [SEA]) were prepared as described by Da Silva and Ferri (4). The protein content was measured by the method of Lowry et al. (18), and then the specimens were aliquoted and stored at −70°C until use.

**BRL4 MAb.** An anti-Schistosoma MAb (BRL4 MAb) has been generated by the establishment of mouse hybridomas (2). In brief, inbred female BALB/c mice were infected with 50 *S. mansoni* cercariae. Five months later, a fusion was done with spleen cells from the infected animals and P3-X63-Ag8-U1 mouse myeloma cells. Hybridomas were screened for antibodies against SWAP by indirect ELISA. One of the highly reactive cell lines (BRL4) was injected intraperitoneally into mice for ascitic fluid production. The isotype of BRL4 MAb was determined with different anti-mouse immunoglobulin subclasses (Binding Site, Birmingham, United Kingdom).

**Immunoblotting.** Polyaclaylamide gel electrophoresis was carried out in thick, 10% vertical slab gels (Bio-Rad) under reducing conditions by the method of Laemmli (17). After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gel was electroblotted onto nitrocellulose (NC) filter at 60 V for 2 h as described previously (24). After blocking with 5% nonfat milk, the NC filter was incubated overnight with the BRL4 MAb diluted 1:50. Goat anti-mouse immunoglobulin G (IgG) alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was used at a dilution of 1:200 at room temperature. 5-Bromo-4-chloro-3-indolyl phosphate–Nitro Blue Tetrazolium (BCIP-NBT) premixed substrate solution (Sigma) was used to visualize the reaction. The reaction was stopped with distilled water. Then the filter was dried and kept in the dark.

**Indirect ELISA.** A polystyrene flat-bottom microtiter plate was coated with 2.5 µg of SWAP per ml in carbonate-bicarbonate buffer (pH 9.6). After blocking, 50 µl (per well) of 1:200-diluted human serum samples in 0.05% (vol/vol) phosphate-buffered saline (PBS)–Tween 20 (PBS-T20) was added and the plate was incubated at 37°C for 2 h. After washing, 50 µl (per well) of anti-human IgG alkaline phosphatase conjugate (whole molecule; Sigma) diluted 1:500 in 0.2% (wt/vol) non-fat milk in PBS-T20 was added and the plate was incubated at 37°C for 1 h. One milligram of para-nitrophenyl phosphate (Sigma) per ml was used as a substrate, and the absorbance was read at 405 nm with an EL311 microplate autoreader (Bio-Tek Instruments).

**FDA.** The FDA was carried out with a Hybi-Dot Manifold (Bethesda Research Laboratories, Richmond, Calif.) to detect the schistosome circulating antigen in urine. The NC filter (Sigma) was washed in a bath of distilled water and soaked in a bath of PBS (pH 7.2), each for 1 min. Then, the NC filter was overlaid on a filter paper pressed PBS and held in the manifold. Fifty microliters of a urine sample was added to each well, and after suction, the NC filter was air dried and then washed in 0.3% PBS-T20 for 1 min on a shaker. Blocking of nonspecific binding sites on the filter was done with 2% (wt/vol) nonfat dry milk in PBS-T20 for 15 min on a shaker. After removal of the blocking solution, 1 µl of the BRL4 MAb was added in a dilution of 1:500 in PBS-T20 for 5 min with continuous shaking. After washing, anti-mouse IgG alkaline phosphatase conjugate (Sigma) was added at a dilution of 1:500 in blocking buffer for 5 min with continuous shaking. The filter was washed with PBS for 3 min. An insoluble purple product was developed after the addition of the BCIP-NBT premixed substrate solution (Sigma) for about 3 min. The reaction was stopped with distilled water, and the filter was dried and kept in the dark. Positive controls for the assay were the affinity-purified antigen either from SWAP or from the urine of infected individuals and neat urine samples from infected individuals. Negative controls were urine samples from noninfected individuals. FDA allows a semiquantitative reading of the resulting colored spot in case of antigen detection (i.e., a positive test result). The purple color that was produced varied in its intensity from weak (1 or 2+) to strong (3+ or 4+). Positive controls with these different color intensities were used. A colorless spot was produced in the case of a negative test result. The resulting color for the tested sample was then compared and related to the color of one of the positive and negative controls with the naked eye.

**RESULTS**

A total of 700 individuals were subjected to stool analysis and/or rectal biopsy examination. A total of 433 individuals were parasitologically diagnosed as having *S. mansoni* infection (152 patients by stool analysis and 281 patients by rectal biopsy examination). All the infected individuals were symptomatic. A total of 267 individuals were parasitologically diagnosed as being noninfected (113 of them had no *S. mansoni* eggs in their rectal biopsy specimens). Counting of the eggs was done by the Kato technique for 100 of 152 infected individuals with eggs in their feces. Fifty-four patients had mild infections (10 of them had 20 epg of feces), 37 individuals had moderate infections, and 9 individuals had heavy infections.

Serum samples from 433 individuals with schistosomiasis and 267 noninfected individuals were tested by indirect ELISA for detection of human anti-schistosome IgG antibodies. ELISA detected schistosomiasis with a sensitivity of 90% and had false-positive results for 312 of 267 noninfected individuals, with a specificity of 56% (Table 1).

Fresh urine samples from *S. mansoni*-infected patients and noninfected individuals were subjected to FDA. The assay detected the circulating antigen in 401 of 433 infected patients with a sensitivity of 93%. However, FDA could detect the circulating antigen in patients with mild infections (20 epg of feces) as well as in patients with heavy infections (more than 400 epg of feces). The assay gave false-positive results for 29 of the noninfected individuals, and this revealed an 89% specificity (Table 1). All 433 *S. mansoni*-infected patients were classified into four groups according to the clinical examination: 241 had simple intestinal bilharziasis, 100 had hepatosplenomegaly, 34 had shrunken liver and splenomegaly, and 58 had ascites. FDA had a sensitivity range of 90 to 94% (Table 2).

The cross-reactivity with other parasitic infections was studied on the basis of microscopic examination of stools from 267 noninfected individuals. FDA showed a specificity of 100% for stools from individuals infected with *Hymenolepis nana* (10

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**TABLE 1. Advantages of FDA as an alternate assay for the detection of human schistosomiasis based on antigen detection compared with stool analysis and the anti-schistosomal antibody detection test**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA</td>
<td>93 [401(401+32)]</td>
<td>89 [238(238+29)]</td>
<td>93 [401(401+29)]</td>
<td>88 [238(238+32)]</td>
<td>91 [401+238]</td>
</tr>
<tr>
<td>Stool analysis</td>
<td>35 [152(152+28)]</td>
<td>100 [267(267+0)]</td>
<td>100 [152(152+0)]</td>
<td>49 [267(267+28)]</td>
<td>60 [152(267)]</td>
</tr>
<tr>
<td>Antibody test</td>
<td>90 [390(390+43)]</td>
<td>56 [135(135+32)]</td>
<td>73 [390(390+132)]</td>
<td>80 [135(135+43)]</td>
<td>75 [390(135)]</td>
</tr>
</tbody>
</table>

* a Sensitivity, number true positive/(number true positive + number false negative).
* b Specificity, number true negative/(number true negative + number false positive).
* c Positive predictive value, number true positive/(number true positive + number false positive).
* d Negative predictive value, number true negative/(number true negative + number false negative).
* e Efficiency, (number true positive + number true negative)/total number.

* FDA for detection of Schistosoma circulating antigen excreted in urine.

Detection of anti-schistosome IgG antibodies in serum samples against SWAP by indirect ELISA technique.
for the diagnosis of schistosomiasis in the field be developed. Available reagent strip assays for the demonstration of blood in the urine of S. haematobium-infected individuals are rapid and simple and have been shown to correlate with the parasitological diagnosis, but they are not specific and remain of no use for the detection of S. mansoni or Schistosoma japonicum infections. Recently, a rapid reagent strip assay based on the detection of schistosoma circulating cathodic antigen in urine was developed (26). The assay can be completed in only 75 min and showed more than 95% sensitivity and specificity, and this assay was also applied for the assessment of cure of schistosomiasis patients (27).

In the present study, the FDA developed on the basis of an IgG2a MAb for the detection of Schistosoma circulating antigen excreted in urine is a simple, rapid, sensitive, and specific enzyme immunoassay. The assay could therefore be used in the field as part of a mass screening program. The urine sample was used neat, i.e., without any treatment, the assay needs no sophisticated equipment, and 96 urine samples could be run in about 30 min. In addition, all assay steps were done at room temperature.

We evaluated the sensitivity and specificity of our assay for the detection of S. mansoni in comparison with those of stool analysis and testing for antibody by ELISA. We found that FDA had a higher sensitivity (93%) than microscopic examination of eggs in stool (35%) and a higher specificity (89%) than anti-schistosomal antibody detection in serum by ELISA (56%). Moreover, FDA had sensitivities ranging from 90 to 94% for the different clinical stages of schistosomiasis. Also, the assay could detect the schistosome antigen by the Kato technique in urine samples from patients with light infections of 20 epg of feces. The circulating antigens were detected in individuals with low egg counts (9, 10).

The target antigen of our BRL4 MAb was identified at 74 kDa in the urine of S. mansoni-infected individuals and in three developmental stage antigenic extracts of S. mansoni. This antigen has been characterized as a protein in nature, with 56.9% hydrophilic amino acids and 43.1% hydrophobic amino acids (2). Only the 30-kDa degradation product was identified in the urine samples, and this may be due to the unsuitable environment of urine. In addition, the BRL4 MAb identified a 74-kDa antigen in the three developmental stage antigenic extracts of S. haematobium. Preliminary data from a diagnostic

### DISCUSSION

Several immunodiagnostic assays based on MAbs for the detection of schistosome antigens in the serum and urine of schistosomiasis patients have been described. Deelder et al. (5–7) and De Jonge et al. (9, 10) used a mouse MAb against the gut-associated proteoglycans, the circulating anodic antigen, the circulating cathodic antigen, or the M antigen in several enzyme immunoassays. These assays cannot be easily applied in the field, where the sample must be pretreated with trichloroacetic acid followed by dialysis, and the assay also requires a long time for its completion and special and highly expensive equipment.

So, it is very important that a rapid, simple, and reliable test for the diagnosis of schistosomiasis in the field be developed. Available reagent strip assays for the demonstration of blood in the urine of S. haematobium-infected individuals are rapid and simple and have been shown to correlate with the parasitological diagnosis, but they are not specific and remain of no use for the detection of S. mansoni or Schistosoma japonicum infections. Recently, a rapid reagent strip assay based on the detection of schistosoma circulating cathodic antigen in urine was developed (26). The assay can be completed in only 75 min and showed more than 95% sensitivity and specificity, and this assay was also applied for the assessment of cure of schistosomiasis patients (27).

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### TABLE 2. Detection of Schistosoma circulating antigen by FDA in the four clinical stages of human schistosomiasis

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>No. of specimens</th>
<th>No. of specimens positive by FDA</th>
<th>% Sensitivity</th>
</tr>
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<tbody>
<tr>
<td>Simple intestinal bilharziasis</td>
<td>241</td>
<td>225</td>
<td>92</td>
</tr>
<tr>
<td>Hepatosplenomegaly</td>
<td>100</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>Shrunken liver and splenomegaly</td>
<td>34</td>
<td>32</td>
<td>94</td>
</tr>
<tr>
<td>Ascaris</td>
<td>58</td>
<td>52</td>
<td>90</td>
</tr>
<tr>
<td>Total</td>
<td>433</td>
<td>401</td>
<td>93</td>
</tr>
</tbody>
</table>

FIG. 1. Random screening of schistosomiasis patients by FDA. The assay allows a semiquantitative reading; i.e., faint spots (1+ or 2+ color intensity) are considered weak positive and dark spots (3+ or 4+ color intensity) are considered strong positive. Wells A1 to C1 represent positive controls, and wells D1 to H1 represent negative controls.

FIG. 2. Identification of the Schistosoma antigen recognized by the BRL4 MAb by Western blotting. Lane A, urine from a noninfected individual; lanes B and C, urine from S. mansoni-infected patients; lanes D to F, antigenic extracts of S. mansoni (lane D, CAP; lane E, SWAP; lane F, SEA); lanes G to I, antigenic extracts of S. haematobium (lane G, CAP; lane H, SWAP; lane I, SEA). Molecular size standards (not shown) were triose phosphate isomerase, 34.1 kDa; lactadhexygenase, 40.8 kDa; fumarase, 57.8 kDa; pyruvate kinase, 72.7 kDa; fructose-6-phosphate kinase, 91.8 kDa; β-galactosidase, 117.0 kDa; and α-2-macroglobulin, 191.0 kDa.
study performed for the detection of *S. haematobium* infection showed that FDA with BRL4 MAb detected *S. haematobium* eggs in more than 80% of individuals with eggs in their urine samples.

On the basis of these results, FDA has a number of advantages that make it a preferable technique over the other diagnostic assays. It is a simple, rapid, noninvasive, specific, and sensitive assay for the detection of schistosome antigens among humans with all clinical stages of schistosomiasis. This will enhance the application of this assay in the field and for mass screening and control programs of *S. mansoni* and *S. haematobium* schistosomiasis.

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