APPLICATION OF IMMUNODIAGNOSTIC ASSAYS: DETECTION OF ANTIBODIES AND CIRCULATING ANTIGENS IN HUMAN SCHISTOSOMIASIS AND CORRELATION WITH CLINICAL FINDINGS

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Abstract. In an initial cross-sectional survey, serum, urine, and stool samples were collected from 370 participants representing about 10% of the population (n = 4,438) in Behbeet village, 50 km south of Cairo, Egypt, an area well known to be endemic solely for Schistosoma haematobium. Diagnosis was approached in two parallel ways. The first approach, which simulated actual conditions in many endemic areas in Egypt, was based on physical examination and urine and stool microscopic analysis. The second approach was based on two advanced immunodiagnostic assay systems. One system detected antibodies to species-specific microsomal antigens, the other detected circulating schistosomal antigens. Microsomal antigens from S. haematobium and S. mansoni were used to detect antibodies in the Falcon assay screening test (FAST®)-ELISA and the enzyme-linked immunoelectrotransfer blot (EITB). Circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) were quantified in serum and urine samples in a sandwich ELISA using monoclonal antibodies. Parasitologically, the prevalence of S. haematobium was 7.01% in females and 25.82% in males, giving an overall prevalence of 15.8%. The combination of urine CCA and serum CAA for detecting circulating antigens and the combination of the S. haematobium adult worm microsomal antigens (HAMA) FAST-ELISA and the HAMA EITB for detecting antibodies significantly improved the sensitivity of detecting S. haematobium circulating antigens and antibodies. Also, including a medical examination as an integral part of field studies and correlating immunodiagnostic results with other clinical and investigational data allowed us to calculate an accurate estimation of S. haematobium prevalence in this area of low endemicity.

Schistosomiasis is one of the most widespread parasitic infections. The disease is endemic in 75 countries, with some 600 million people at risk of infection.1 In Egypt, two species, Schistosoma haematobium and S. mansoni cause urinary and intestinal schistosomiasis, respectively. Despite mass treatment campaigns, schistosomiasis remains endemic and a significant prevalence of the disease still exists. Disease control plans depend mainly on the diagnosis and treatment of infected individuals. Up until now, diagnosis has been dependent on the detection of schistosome eggs in either stool or urine.2,3 However, because of low and sporadic egg production, the risk of undiagnosed individuals is tremendous. Undiagnosed individuals remain infected and transmission of the disease continues. In the last few years, many diagnostic techniques have been developed, including ultrasonography,4,5 detection of circulating antibodies to semi-purified or fractionated antigens,6-10 and detection of parasite circulating antigens in different host body fluids.11-14 Detection of specific antibodies to S. mansoni and S. haematobium adult worm microsomal antigens (MAMA and HAMA, respectively) was found to be 100% specific for both species when used in the FAST-ELISA and immunoblot assays for diagnosis of schistosomiasis.10,11 However, results of antibody assays, in general, do not correlate well with worm burden, as measured by the egg output, nor do they discriminate between previous exposure and current infection. Quantitation of schistosome circulating antigens such as circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) might give more information. The use of reagents to detect circulating antibodies and antigens in field studies has been limited and such assays are usually conducted in highly endemic foci. In this study, we report on the application of immunologic diagnostic methods in a 10% survey of an area in Egypt with low endemicity for S. haematobium to test the sensitivity and specificity of these assays for circulating antigen and antibody when used in combination in a large-scale screening survey. Clinical and other investigational data will be correlated with immunodiagnostic results to enhance patient management in endemic areas in Egypt.

MATERIALS AND METHODS

Population. A 10% systematic sample of houses (every tenth house) in Behbeet village, 50 km south of Cairo, was identified and numbered, and an accurate map was drawn. All occupants more than five years of age were interviewed and invited to participate in the study. Stool, urine, and blood samples were collected from 370 individuals in containers with computer-generated labels. Demographic data from each individual were recorded on data sheets. The investigations were performed in accordance with the Ministry of Health and Human Services guidelines for Clinical Research and Treatment under a protocol approved by the Schistosomiasis Research Project, VACSERA, Egypt, and the Centers for Disease Control and Prevention (CDC-IRB approval # 1381-CDC-IRB).

Heterologous infection sera. Positive reference sera and sera from other heterologous diseases were obtained from CDC (Atlanta, GA). Heterologous infection sera were collected from schistosome-free geographic locations. They included the following infection sera: hepatitis from Alaska and Nevada (United States, n = 18), trichinosis from Louisiana (United States, n = 20), cysticercosis from Peru and
antibodies to MAMA antigens. The Falcon Assay Screen-
centrifugation, urea extraction, molecular sieving, and ionic-
concentrated in urine were detected by filtration with Nucleo-
ton, MA). Egg counts and other data from microscopic
amination for evidence of schistosomiasis-associated disease.
Blood samples were drawn, and the patients were immedi-
ately treated with 60 mg of praziquantel (Biltricide®; Bayer
AG, Leverkusen, Germany) per kg of body weight. Serum
amples were frozen at −85°C; urine samples were stored at
−35°C with 0.1% sodium azide added as a preservative.
**Antigen preparation.** The preparation of MAMA and
HAMA was performed according to the method of Tsang
and others. Briefly, microsomal antigens from *S. mansoni*
or *S. haematobium* adult worms were purified by differential
centration, urea extraction, molecular sieving, and ionic-
exchange chromatography.

**FAST-ELISA.** The FAST-ELISA was performed to detect
antibodies to MAMA antigens. The Falcon Assay Screen-
ing Test (FAST®) polystyrene beads (Becton Dickinson, San
Jose, CA), which are molded onto sticks and attached to 96-
well lids, were sensitized with either MAMA or HAMA (2
µg/ml in 0.05 M Tris-HCl, 0.3 M KCl, 2.0 mM EDTA, pH
8.0) for 2 hr at room temperature with gentle agitation on a
rotatory shaker. The sticks were washed by spraying for 20
sec with 0.3% Tween 20 in phosphate-buffered saline (PBS),
ph 7.2 (PBS/Tween) from a plastic sprayer, then rinsed brief-
ly with distilled water. Three microliters of serum were di-
luted with 97 µl of PBS/Tween in triplicate into individual
wells of a 96-well FAST plate using the DigiFlex automatic
diluter (ICN Biomedicals, Irvine, CA). Standard curves,
from 0 to 100 U/µl for the FAST-MAMA or from 0 to 80
U/µl for the FAST-HAMA, of antibody reactivity were also
included. The beads were incubated with serum for 5 min at
room temperature while mixing on a mini-orbital shaker
(Belco, Vineland, NJ). They were then washed and placed in
a 96-well plate containing 100 µl of diluted peroxidase-
labeled, immunooaffinity-purified goat anti-human antibod-
ies and incubated for 5 min at room temperature while
mixing. The beads were then washed and placed in a 96-
well plate containing 150 µl of 3, 3′, 5, 5′-tetramethylbenz-
idine and H₂O₂ mixed at a ratio of 1:1 (v/v) (Kirkegaard
and Perry Laboratories, Gaithersburg, MD). The plate was
shaken for 2 min at room temperature on the mini-orbital
shaker. The absorbance at 650 nm was measured using a UV
max ELISA plate reader (Molecular Devices Corp., Palo
Alto, CA). The ELISA reader-controlling software (Softmax;
Molecular Devices Corp.) readily processes the digital data
of raw absorbance value into a standard curve from which
FAST ELISA antibody units of activity of unknown samples
can be derived directly from a four-parameter line equation.
Sera with activity greater than 10 U/µl (in the FAST-
MAMA) or 1.2 U/µl (in the FAST-HAMA) were considered
positive.

**Enzyme-linked immunoelectrotransfer blot (EITB).**
Electrophoresis and electrotransfer were done according to
Tsang and others. The MAMA or HAMA were resolved by
sodium dodecyl sulfate gradient (5–22%)–polyacrylamide
gel electrophoresis. The resolved bands were then electro-
phoretically blotted onto a nitrocellulose sheet. After trans-
fer, the nitrocellulose was cut into 2-mm wide strips and the
strips were exposed to individual sera diluted 1:50 with PBS/
Tween/5% nonfat milk for 1 hr at room temperature. Strips
were washed four times with PBS/Tween. After the last
wash, goat anti-human IgG-peroxidase was added and in-
cubated for 1 hr at room temperature. Strips were washed
four times with PBS/Tween and once with PBS. Antigen
bands with bound antibodies were visualized with 3,3′-dia-
minobenzidine/H₂O₂ substrate. Sera recognizing MAMA
GP30 and/or HAMA GP23 were recorded.

**Circulating antigen detection by ELISA.** Before ELISA
testing, both serum and urine samples were pretreated for 30
min at 70°C with alkaline buffer (carbonate-bicarbonate
buffer, pH 9.6) at 1:4 and 1:2 dilutions, respectively. The
ELISA techniques for quantitative determination of CAA
and CCA were performed as described by Deelder and oth-
ers and De Jonge and others. In the CAA assay, the anti-
CAA monoclonal antibody (MAb) 120–1B10-A served both
as the capture antibody and as the alkaline phosphatase–
conjugated second antibody. Maxi-sorb microtitration
plates (Nunc, Roskilde, Denmark) were coated with CAA
mouse MAb (ascitic fluid diluted 1:1,000) and, after wash-
ing, blocked with 0.033% bovine serum albumin. Plates
were washed and stored dry at −70°C until use. Samples
were tested in four two-fold dilution series, starting with an
initial dilution of 1:4 for sera and 1:2 dilution for urine sam-
ple. After the plates were washed, alkaline phosphatase–
conjugated protein A-Sepharose–purified mouse MAb 120–
1B10-A was added to the wells. The CCA levels in serum and urine were determined ac-
cording to the procedures of De Jonge and others, using
anti-CAA IgG3 MAb 54–5C10-A as a capture antibody and
IgM MAb 8–3C10 as the alkaline phosphatase-labeled conju-
gate. The change in color was monitored at 405 nm using the UV
max ELISA plate reader (Molecular Devices Corp.). The ELISA reader-controlling software (Softmax)
readily processes the digital data of raw absorbance value
into a standard curve from which CAA or CCA concentra-
tion of unknown samples can be derived directly from a four-parameter line equation, using a standard dilution curve
of trichloroacetic acid–treated adult worm antigen (AWA-
Immunodiagnostic data: assay sensitivity. Sera were tested for the presence of schistosome-specific antibodies by the FAST-ELISA and EITB. Screening with the FAST-ELISA using MAMA showed 83.6% of the 67 villagers passing eggs in their urine were seropositive (reactivity > 10 U/mL). This may be due in part to the reactivity of both *S. mansoni* and *S. haematobium*-infected patients with MAMA in the FAST-ELISA. The FAST-ELISA screening with the homologous antigen (HAMA) showed an enhanced sensitivity of 98.5% (Table 1). Sera from the 67 egg-excreting patients were tested for reactivity with the species-specific GP30 band of HAMA* in the EITB assay. Sixty-three were blot-positive. The overall sensitivity of the EITB assay was therefore 94.0%. By screening with the HAMA FAST-ELISA and the EITB, all egg-passing individuals were detected and the sensitivity of antibody detection reached 100% (Table 1). Interestingly, the MAMA EITB detected a substantial percentage (19.6%) of the participants possessing antibodies to the species-specific band GP30, thus indicating a current or previous infection with *S. mansoni*.

The performance of the circulating antigen detection assay for the different egg output groups is summarized in Table 2. Generally, the percentage of positively detected cases in CAA and CCA detection assays increased with increasing egg excretion, especially those excreting > 50 epml. The highest sensitivity was achieved with the urine-CCA assay, which was capable of identifying 52 (78%) of 67 active cases, followed by the serum CAA assay with 37 cases (55%). The maximum sensitivity reached was 87%, where 58 of 67 active cases tested positive for at least one of the two assays. The parasite burden positively correlated with the urine CCA—detected level (Spearman’s ρ = 0.4, P < 0.01; n = 52). Meanwhile, the detection of urine CCA and serum CAA in persons excreting eggs correlated with detection of antibodies by HAMA-EITB (Spearman’s ρ = 0.81, P < 0.001; n = 58).

Immunodiagnostic data: assay specificity. Heterologous parasitic infection sera (n = 240) from persons residing in schistosome-free geographic locations were tested for antibodies cross-reactive with HAMA and for antigens recognized in the CAA and CCA assays. The heterologous sera had a reactivity less than 1.2 U/µl in the HAMA FAST-ELISA and failed to react with GP23 in the HAMA EITB (Figure 2). The ELISA showed that serum CAA and urine CCA were not detected in the tested sera except in 20% (4 of 18) of *Wuchereria bancrofti*-infected individuals and 20% (3 of 14) of *Onchocerca volvulus*-infected individuals in the CAA and CCA assays, respectively. Thus, at the cutoff levels used in the present study, the specificities of the serum CAA and urine CCA assays were 97.8% and 95.5%, respectively. In a population coinfected with *W. bancrofti* or *O. volvulus*, the specificities of the assays would decrease to about 80%. For the antibody assays, the HAMA FAST-ELISA, and the HAMA EITB, the specificities were 100%.

Clinical data. Analysis of the clinical data in relation to...
**TABLE 2**

Sensitivity of immunodiagnostic assays for detection of *Schistosoma haematobium* infections*

<table>
<thead>
<tr>
<th></th>
<th>epml = 0</th>
<th>epml &lt; 20</th>
<th>epml = 20–50</th>
<th>epml &gt; 50</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of individuals</td>
<td>303</td>
<td>45</td>
<td>14</td>
<td>8</td>
<td>67</td>
</tr>
<tr>
<td>Mean epml</td>
<td>0</td>
<td>7.0</td>
<td>26.9</td>
<td>91.4</td>
<td>21.3</td>
</tr>
<tr>
<td>Range epml</td>
<td>0</td>
<td>1–18</td>
<td>20–38</td>
<td>55–153</td>
<td>1–153</td>
</tr>
<tr>
<td>Serum CAA</td>
<td>18 (6%)</td>
<td>23 (51%)</td>
<td>8 (57%)</td>
<td>6 (75%)</td>
<td>37 (55%)</td>
</tr>
<tr>
<td>Serum CCA</td>
<td>29 (10%)</td>
<td>3 (7%)</td>
<td>2 (14%)</td>
<td>2 (25%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>Serum CCA and</td>
<td>90 (29%)</td>
<td>32 (71%)</td>
<td>12 (86%)</td>
<td>8 (100%)</td>
<td>52 (78%)</td>
</tr>
<tr>
<td>urine CCA</td>
<td>172 (57%)</td>
<td>37 (82%)</td>
<td>13 (93%)</td>
<td>8 (100%)</td>
<td>58 (87%)</td>
</tr>
<tr>
<td>FAST MAMA</td>
<td>156 (51%)</td>
<td>38 (84%)</td>
<td>13 (93%)</td>
<td>7 (87%)</td>
<td>56 (83%)</td>
</tr>
<tr>
<td>FAST HAMA</td>
<td>252 (83%)</td>
<td>42 (93%)</td>
<td>14 (100%)</td>
<td>8 (100%)</td>
<td>66 (98%)</td>
</tr>
<tr>
<td>EITB MAMA</td>
<td>75 (25%)</td>
<td>28 (62%)</td>
<td>7 (50%)</td>
<td>4 (50%)</td>
<td>39 (58%)</td>
</tr>
<tr>
<td>EITB HAMA</td>
<td>150 (50%)</td>
<td>41 (91%)</td>
<td>14 (100%)</td>
<td>8 (100%)</td>
<td>63 (94%)</td>
</tr>
<tr>
<td>FAST HAMA and</td>
<td>258 (85%)</td>
<td>45 (100%)</td>
<td>14 (100%)</td>
<td>8 (100%)</td>
<td>67 (100%)</td>
</tr>
</tbody>
</table>

* epml = eggs/ml of urine; CAA = circulating anodic antigen; CCA = circulating cathodic antigen. For definitions of other abbreviations, see Table 1.

parasitologic results revealed that the presence of the disease is significantly correlated with some clinical manifestations and symptoms such as hematuria (26.9%), leukocyturia (60.0%) (as indicated by the urine strips), and urinary tract complaints currently or during the last six months before physical examination (35.8%) (Table 3). These parameters and others listed in Table 4 were also significantly correlated with the EITB-HAMA results. Meanwhile, urine analysis of 68% of the persons excreting eggs showed significant leukocyturia (n = 46; P < 0.001); 86% (n = 48) of the leukocyturia-positive donors tested positive for circulating antigen (urine CCA) (P < 0.01).

**Estimation of prevalence.** In parasitologically negative subjects (n = 303), the percentage of seropositive individuals with reactivity in the FAST-HAMA more than 1.2 U/ml and recognizing the *S. haematobium*-species specific GP23 band in the EITB assay was 40.5% (n = 123). Meanwhile, 57% (n = 172) of the parasitologically negative villagers tested positive for serum CAA or urine CCA. Clinically, 59 of the 303 parasitologically negative persons were recorded as having almost the same schistosomiasis-indicative symptoms observed in the infected participants group, that is, hematuria, leukocytosis, and current urinary tract complaints. The percentage of persons who excreted no eggs, but had circulating antigens and antibodies and reported clinical findings, was 19.5%. Therefore, integrating the clinical and immunodiagnostic data with the conventional parasitologic data gave an estimated overall prevalence in this area of 37.6% instead of 18.1%.

**DISCUSSION**

Diagnosis of schistosomiasis has mainly depended upon finding ova in patients’ excreta. Serologic testing has been used to enhance our ability to detect the disease. However, none of the studies reported in the literature applied a multidiagnostic approach combining antibody and circulating antigen (U-CCA), FAST-ELISA with *Schistosoma haematobium* adult worm microsomal antigens (HAMA), and enzyme-linked immunoelectrotransfer blot (EITB) with HAMA. To determine specificity, the immunodiagnostic assays were performed on a panel of eight heterologous infection sera: hepatitis (Hep, n = 18), trichinosis (Tric, n = 20), cysticercosis (Cyst, n = 41), filariasis (Fil, n = 18), amebiasis (Ameb, n = 52), onchocerciasis (Oncho, n = 14), echinococcosis (Echin, n = 19), and taeniasis (Taen, n = 18). To determine sensitivity, the immunodiagnostic assays were performed with *S. mansoni* Puerto Rico (SMPR, n = 23) and *S. haematobium* infection reference sera (SHRS, n = 18). The four immunodiagnostic assays were evaluated based on the percent of sera in each group positive by the assay criteria.
tigen detection with clinical data to accurately estimate the prevalence of the disease.

The systematic purification of microsomal antigens from adult worms' has allowed the development of rapid, highly specific, and sensitive assays such as the FAST-ELISA and EITB for detecting antibodies in schistosomiasis. When MAMA was used in the FAST-ELISA, the sensitivity and specificity were 98% and 99%, respectively, in detecting sera infected with *S. mansoni*. The EITB is capable of differentiating infections caused by the three species of the genus *Schistosoma* of major importance in humans. Antibody reactivity with GP30 of MAMA identifies a *S. mansoni* infection, GP18, GP23, or GP29 of *S. japonicum* adult worm microsomal antigens (JAMA) identifies *S. japonicum*, and GP23 of HAMA identifies *S. haematobium*. Thus, the FAST-ELISA and EITB were used as screening and confirmatory assays, respectively, for prior or present schistosomiasis.

In this study, detection of schistosome-specific antibodies directed to microsomal antigens in an *S. haematobium*-endemic area was evaluated using both MAMA and HAMA. The sensitivity of the MAMA FAST-ELISA was 83.6%, whereas the HAMA FAST-ELISA screening was able to detect a significantly higher percentage (P < 0.001) of the infected persons (98.5%). When screening was done with the HAMA FAST-ELISA and EITB, all egg-passing individuals were detected and the sensitivity of antibody detection reached 100%. Therefore, in *S. haematobium*-endemic areas, screening with HAMA, rather than MAMA, in the FAST-ELISA would increase the sensitivity of detecting egg-excreting persons. The EITB confirmatory test is not only capable of determining the infecting species, but also complements the FAST-ELISA in detecting all infected persons. Only *S. haematobium* is known to be endemic in Behbeet; consequently, stool samples were usually overlooked and only urine examination was performed. The detection of a substantial percentage (19.64%) of the participants possessing antibodies to *S. mansoni* GP30 certainly emphasizes the importance of immunodiagnosis for proper patient management. Therefore, treatment based solely on the presence of ova is inadequate and allows patients with low egg outputs to remain undiagnosed and continue to transmit the disease. A new assay format that could combine the simplicity of the FAST-ELISA and the capability of the EITB to determine the species would be very useful in field studies.

The different sensitivities achieved with different circulating antigen assays were recorded in other studies with *S. mansoni* infections. The urine CCA assay was capable of identifying 52 of the 67 active cases (78%), followed by the serum CAA assay with 37 cases (55%), while 87% of the active cases were positive for at least one of these two assays. The improvement of sensitivity by parallel testing on more than one antigen has been reported previously in a study of Egyptian schistosomiasis patients. The sensitivity of the four individual circulating antigen assays reported by van Lieshout and others in 1992 was much higher than the data reported in the present study and matched those of Van Lieshout and others in 1994. This is consistent with the previous observation that the sensitivity of the assays is not only dependent on the intensity of infection, but also on the prevalence in the area where the patients come from. However, it is possible that the apparent association of assay sensitivity with prevalence of infection is actually due to parasite strain differences in the production of CAA and CCA.

Recently, circulating antigen detection was also applied in a newly established, heavily *S. mansoni*-infected community in the northern part of Senegal, where fecal egg excretion was found in 91% of the population. Similar to our study, the urine CCA assay was found to have the highest sensitivity, immediately followed by the serum CAA assay, while CAA could only be demonstrated in 55% of the urine samples and in remarkably low concentrations. Results were explained by a possible difference in clearance dynamics of this antigen due to the very recent nature of the infection in the study population. The fact that in our study, with longstanding infections and low egg excretion, levels of CAA in urine were also found to be very low indicates that other mechanisms may play a role in this phenomenon.

Based on the results of this study, we conclude that the combination of urine CCA and serum CAA for detecting circulating antigens and the combination of the HAMA FAST-ELISA and the HAMA EITB for detecting circulating antibodies significantly improves our ability to detect *S. haematobium* circulating antigens and antibodies. With the increased sensitivity of the assays, one can determine the prevalence of infection with higher accuracy. In addition, this study highlights the value of integrating immunodiagnostic tools and clinical findings. The clinical findings of hematuria, leukocyturia, and urinary tract complaints were significantly associated with egg-positive patients. The clinical findings of hematuria, leukocyturia, urinary tract complaints, tender abdomen, and supra-pubic tenderness were significantly associated with antibody-positive patients. Therefore, including these clinical findings as a key to look closely for evidence of infection is important in patient management. Correlating immunodiagnostic results with other clinical and investigational data should allow us to provide recommen-
dations to physicians practicing in rural endemic areas, which in turn would significantly enhance the efficiency of the national anti-schistosomiasis control program in Egypt.

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