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. 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. 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, detection of circulating antibodies to semi-purified or fractionated antigens, and detection of parasite circulating antigens in different host body fluids. 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. However, results of antibody assays, in general, do not correlate well with worm burden, as measured by the egg output, or 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-exchange chromatography. Centrifugation, urea extraction, molecular sieving, and ionic-pore excreted in urine were detected by filtration with Nucleo-ton, MA). Egg counts and other data from microscopic detec-ions and Perry Laboratories, Gaithersburg, MD). The plate was mated Kato slide method. Schistosoma haematobium eggs excreted in urine were detected by filtration with Nucleo-pore® polycarbonate membranes (Corning Separations, Acton, MA). Egg counts and other data from microscopic observations were recorded on patient data sheets. Patients with active excretion of eggs received a full physical exam-ination for evidence of schistosome-associated disease. Blood samples were drawn, and the patients were immediately treated with 60 mg of praziquantel (Biltricide®; Bayer AG, Leverkusen, Germany) per kg of body weight. Serum samples 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 centrifugation, uraection, molecular sieving, and ionic-exchange chromatography.

FAST-ELISA. The FAST-ELISA was performed to detect antibodies to MAMA antigens. The Falcon Assay Screening 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 briefly with distilled water. Three microliters of serum were diluted with 97 µl of PBS/Tween in triplicate into individual wells of a 96-well FAST plate using the Digi±ex 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-rotoral shaker (Bellco, Vineland, NJ). They were then washed and placed in a 96-well plate containing 100 µl of diluted peroxidase-labeled, immunoaffinity-purified goat anti-human antibodies 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′-tetramethylbenzidine 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-rotoral 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-photographically blotted onto a nitrocellulose sheet. After transfer, 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 incubated 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 others 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 washing, 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 samples. 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 according 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 biotinylated second antibody. Samples were tested at the same dilutions used for CAA assay. Streptavidin–alkaline phosphatase was used as the enzyme label. Following the enzyme-conjugate incubation, the plates were washed and incubated with p-nitrophenyl phosphate. Thereafter, 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-
TCA), which contains approximately 3% (w/w) CAA and CCA. The lower detection limits for circulating antigen were 0.3 ng/ml of AWA-TCA for the CAA and CCA detection assays and 0.7 ng/ml for the CCA ELISA. Samples with concentrations above the lower detection limit were considered positive.

**RESULTS**

**Parasitologic data.** Four hundred thirty-four residents of Behbeet village (n = 4,438) were invited to enroll in this 10% survey study. Of these participants, 370 donated blood, urine, and stool, giving a total compliance of 85.2%. One hundred seventy-two were male and 198 were females, with a median age of 23.6 years (range = 5–75). Parasitologic examination demonstrated the presence of live *S. haematobium* eggs in the urine of 67 individuals (18.1%) and *S. mansoni* eggs in stool of two individuals (0.54%). Most of the *S. haematobium*-infected participants (59) showed an egg output of less than 50 eggs/ml (epml). Egg output ranged from 1 to 153 epml; 71.6% of those excreting eggs were males and 28.4% were females. Figure 1 depicts the parasitologic data in relation to the age of the infected villagers. The intensity of infection, as measured by egg output, decreased in group ≥ 20 years of age. In the 10–14-year-old age group, there was a statistically significant difference (P = 0.05) in the intensity of infection between males and females. The geometric mean egg output in this age group was 17.9 epml for males and 7.1 epml for females. A similar trend was seen in the 5–9-year-old age group. Other helminths identified included *Hymenolepis nana*, 6; *Enterobius*, 4; *Ancylostoma duodenale*, 2; *Ascaris*, 1; *Taenia saginata*, 1; and *Strongyloides*, 1.

**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 that 83.6% of the 67 villagers passing eggs in their urine were seropositive (reactivity > 10 U/µl). 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 GP23 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 (19.64%) 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 antibody cross-reactivity 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
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 multi-diagnostic approach combining antibody and circulating an-
tigen detection with clinical data to accurately estimate the prevalence of the disease.

The systematic purification of microsomal antigens from adult worms\(^2\) has allowed the development of rapid, highly specific, and sensitive assays such as the FAST-ELISA\(^18\) and EITB\(^21\) 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*.\(^18\) The EITB is capable of differentiating infections caused by the three species of the genus *Schistosoma* of major importance in humans.\(^22\) 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*.\(^24\) Thus, the FAST-ELISA and EITB were used as screening and confirmatory assays, respectively, for prior or present schistosomiasis.\(^23\)

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 Behbeh; 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\(^23\) 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.\(^26,27\) 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.\(^26\) The sensitivity of the four individual circulating antigen assays reported by van Lieshout and others\(^28\) in 1992 was much higher than the data reported in the present study and matched those of Van Lieshout and others\(^29\) 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.\(^30\) 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.\(^29\)

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.\(^30\) The fact that in our study, with long-standing 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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