DETECTION AND QUANTIFICATION OF SOLUBLE EGG ANTIGEN IN URINE OF SCHISTOSOMA HAEMATOBIUM–INFECTED CHILDREN FROM KENYA

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Abstract. While research on alternative diagnostic and morbidity markers for infection with Schistosoma haematobium has been going on for a long time, egg counts continue to be used as the gold standard, and infection intensity is thought to reflect the severity of the disease. However, this relationship is not always clear and fluctuation in egg output makes it difficult to classify prevalence correctly. The use of circulating adult worm antigen detection as an alternative diagnostic technique has been applied with varying success. However, this is a measure of worm burden and does not reflect the tissue egg load(s). In the present study we have used an assay that detects soluble egg antigen (SEA) in urine of S. haematobium–infected children, and we have evaluated the applicability of the assay as a diagnostic and morbidity indicator. To evaluate this assay, we have studied a group of 470 children from two schools (Tsunguni and Kibaokiche) in the Coast province of Kenya; 84.8% and 77% were egg-positive while the percentage positive as determined by the SEA-ELISA were 78.8% and 76.2% in Tsunguni and Kibaokiche, respectively. In both schools, SEA levels in urine of S. haematobium–infected children significantly correlated with egg counts (Pearson’s r = 0.73, P < 0.0001) and with hematuria (Spearman’s r = 0.65, P < 0.0001). In addition, urinary tract pathology as determined by ultrasound significantly correlated with the SEA levels in urine (Spearman’s r = 0.3, P < 0.001). The SEA-ELISA compared well with microhematuria within egg count classes and with egg counts within hematuria classes.

Detection of Schistosoma haematobium ova in urine of infected individuals remains the leading method for the direct diagnosis of the disease, and the intensity of infection expressed as number of eggs per 10 ml of urine is thought to reflect the worm burden. However, a homogeneous distribution of S. haematobium ova in urine is difficult to achieve, while day-to-day and circadian variation in egg excretion may lead to incorrect estimates in prevalence and intensity of infection. Additionally, the relationship between egg counts and pathology is not always clear. The presence and intensity of microhematuria, proteinuria, and leukocyturia are semi-quantitative measures of S. haematobium infection, and these parameters were found to correlate with infection intensity. However, variations dependent on geographic area, endemicity, cultural practices, age, sex, and even time of the day exist, limiting the application of hematuria as a diagnostic tool. Ultrasound scanning of the urinary tract remains the gold standard for pathology. This technique has been applied in the field and correlates well with standard direct and indirect measurements of S. haematobium morbidity. However, ultrasound equipment is expensive and requires trained physicians to handle the equipment and interpret the results, limiting its use under field conditions. These shortcomings have led to the search for alternative quantitative diagnostic method(s) for infection. In this respect, the detection of circulating antigens in urine and serum of infected individuals takes a prominent place. Two main adult-worm derived antigens, the circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) have been extensively studied. Studies on these antigens have been concentrated on infection with S. mansoni, with comparatively fewer studies for S. haematobium infections. Both CAA and CCA are a measure of the worm load and not necessarily a reflection of the tissue egg load. Recently, however, the detection of soluble egg antigen (SEA) in S. haematobium infections using a specific monoclonal antibody–based dipstick assay has been developed, and has showed promising results.

The present study is part of a project aimed at developing assays that could be used for diagnosis and morbidity assessment in S. haematobium infections. Within this project, an ELISA that sensitively detects eosinophil cationic protein in urine of infected individuals has been developed and was found to relate well with other parameters of infection. Here, we describe further development and application of an ELISA recently developed for the quantitative determination of SEA in urine of school children from two areas in Kenya. The relationship of the antigen levels determined has been compared with egg counts, microhematuria, and ultrasound-detectable pathology.

MATERIALS AND METHODS

Study population. The study was carried out in Kaloleni division of Kilifi District in the Coast province of Kenya, an area endemic for S. haematobium. So far, there has been no reported case of S. mansoni in this area. Children from two schools, Tsunguni with a prevalence of 84.8% and Kibaokiche with a prevalence of 77.0%, were selected for the study. Of the 470 children included in the study 53.4% were males and 46.6% were females and their ages ranged from six to 17 years (median = 10 years). For logistic reasons, children in the upper classes (grades seven and eight) were not included in the study. Before commencing the study, permission was obtained from the pupils’ parents, the education office, and the local administration. For ethical reasons all children found positive both at the start and at the end of the study were treated with praziquantel at a dose of 40 mg/kg of body weight. The study protocol was approved by the Ministry of Health’s Ethical Review Committee ( Ministry of Health, Kenya) and the Danish Central Medical Ethics Committee.

Collection and parasitologic examination of urine. Urine for microscopy was collected for a minimum of three consecutive days between 10:00 AM and 2:00 PM. From each
urine sample, a duplicate 10-ml sample (or occasionally one depending on the amount of urine) was filtered using 15-mm polycarbonate filters (Nuclepore®; Costar Europe, Ltd., Badhoevedorp, The Netherlands), which were then placed on a labeled slide. The slides were examined within 6 hr and the number of eggs was counted. The infection status was determined by taking the mean of the two filter egg counts. Egg count adjustments were also performed where the quantity of urine filtered was less than 10 ml. Due to clogging of filters in cases with high egg counts, it was not possible to count the number of eggs accurately; thus, these were expressed as ≥1,000 eggs per 10 ml of urine and treated separately during data analysis. Microhematuria was evaluated semi-quantitatively using reagent strips (Hemastix®; Ames, Bie & Berntsen, Copenhagen, Denmark) and the results ranked as negative, trace, +, ++, or +++ according to the manufacturer’s instructions. Once during the three days other than the day that ultrasound examination was performed, a 10-ml urine sample for the SEA assay was collected in a Nunc (Roskilde, Denmark) polysorb tube and placed in a cool box with ice. The samples were frozen within six hours and stored at −20°C until use. Ninety urine samples from Dutch individuals who had never been to a schistosomiasis-endemic area were included as negative controls.

Clinical examination. The children were examined clinically using an ultrasound machine (SSD-500, 3.5 MHz; Aloka Co., Ltd., Tokyo, Japan) once; the children were asked to drink lemonade before the actual examination took place. Bladder wall thickening, mass or polyp formation, and dilation of the ureter were considered as pathology. The results were recorded according to the Cairo classification on a standard form. For this study, overall pathology was scored as either positive or negative if any of the organs described above showed pathology.

Antigen preparation. Schistosoma haematobium (Egyptian strain) SEA prepared as previously described was used for ELISA standard curves.

Monoclonal antibodies. Anti-egg mouse monoclonal antibody 290-2E6-A (IgM) previously described was used. This antibody was purified from ascitic fluid by ammonium sulfate precipitation followed by hydroxyapatite ion exchange chromatography. It was used both as coating antibody and as detecting antibody after conjugation with biotin dianinocaproyl hydrazide according to the procedure described previously. These were then used in a homologous sandwich ELISA.

Pretreatment of urine samples. Three urine pretreatment methods were evaluated to test the most applicable for the SEA-ELISA. Two methods are routinely used in our laboratory to pretreat urine samples for detection of CAA and CCA: the trichloroacetic acid (TCA) pretreatment and the alkaline pretreatment. For the TCA pretreatment, equal volumes of a 4% TCA solution and urine sample were mixed, vortexed, and incubated for 20 min at room temperature after which the mixture was neutralized with 0.244 M carbonate buffer, pH 9.6. Alkaline treatment involved heating of the urine sample with an equal volume of 0.244 M carbonate buffer, pH 9.6, for 30 min at 70°C. As a third method, which was eventually adopted for this study, samples were heated for 30 min at 70°C without additives. For comparison, untreated samples were also included. The treated samples were used immediately or stored at −20°C until use.

Sandwich ELISA. After further optimization of the ELISA previously developed, the following ELISA was adopted. Microtiteration plates (Maxisorp®; Nunc) were coated by overnight incubation at 4°C with 100 µl/well of a solution containing 5 µg/ml of the purified antibody in 0.1 M sodium carbonate buffer, pH 9.6. Plates were washed four times in 2 mM phosphate-buffered saline (PBS) and blocked by incubation for 1 hr at 37°C with a 0.1% bovine serum albumin (BSA) solution in carbonate buffer. The plates were washed as before and used immediately. Pretreated urine samples and the SEA standard were diluted in assay buffer (0.035 M PBS, 0.1% BSA, 0.3% Tween 20) and 80 µl/well was added. Urine samples were tested column-wise in a four-step, two-fold, serial dilution format. The standard SEA was tested in a duplicate, two-fold dilution series starting at 1,000 ng/ml. The plates were then incubated for 1 hr at 37°C, washed, and incubated with 100 µl/well of biotinylated antibody diluted 1:400 in assay buffer plus 2% fetal calf serum for 1 hr at 37°C. The plates were then washed and streptavidin–alkaline phosphatase (1:8,000 in assay buffer) was added and incubated for 25 min at 37°C. The amount of coupled conjugate was determined by incubation with 0.1% p-nitrophenylphosphate in 0.1 M diethanolamine buffer containing 0.5 mM MgCl₂, pH 9.6. The substrate incubation was done overnight at 4°C and the absorbance was measured at 405 nm. The cut-off point was calculated as the mean absorbance of the buffer row plus two standard deviations.

Data analysis. Since neither the egg counts nor the SEA concentration data were normally distributed, a logarithm transformation log(x + 1) was performed. The Student’s t-test was applied accordingly to test for differences and the Pearson’s and Spearman’s correlation coefficients (as applicable) were computed to check the associations between the SEA concentrations and other parameters.

RESULTS

A comparison of the three pretreatment methods showed that heating the samples without additives for 30 min at 70°C gave the best performance; this procedure was then adopted for the whole study. With the ELISA, SEA concentrations in urine samples of the infected children were found to range from 0 to 37,349 ng/ml (median = 343.0, 95% confidence interval = 1,670.4, 2,618.0). All 90 urine samples of the uninfected negative controls had absorbance values less than the mean background. Table 1 summarizes the results of the parasitologic examination, hematuria, and the SEA-ELISA. In Tsunguni, 84.8% of the children were egg positive; in Kibaokiche this value was 77.0%. The results of microhematuria mirrored these findings with 83.3% of the children in Tsunguni being positive compared with 75.4% in Kibaokiche. With the SEA-ELISA, 78.8% and 76.2% of the children in Tsunguni and Kibaokiche, respectively, were positive. Although the SEA positivity was similar in the two schools, the geometric mean concentrations differed significantly, with a mean value of 232.4 ng of SEA/ml for the children in Tsunguni and a mean of 125.8 ng of SEA/ml for the children in Kibaokiche. We observed a significant difference between the percentage positive determined by egg
Soluble Egg Antigen in Urine of *S. haematobium*-Infected Children

**Table 1**

<table>
<thead>
<tr>
<th>Egg counts</th>
<th>Hematuria</th>
<th>SEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>% positive</td>
<td>gmean</td>
</tr>
<tr>
<td>Tsunguni</td>
<td>198</td>
<td>84.8</td>
</tr>
<tr>
<td>Kibaokiche</td>
<td>252</td>
<td>77.0</td>
</tr>
<tr>
<td><em>P = 0.04</em></td>
<td>NS</td>
<td><em>P = 0.04</em></td>
</tr>
</tbody>
</table>

**Sex**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Egg counts</th>
<th>% positive</th>
<th>gmean</th>
<th>Hematuria</th>
<th>% positive</th>
<th>gmean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>244</td>
<td>85.2</td>
<td>28.2</td>
<td>85.7</td>
<td>81.6</td>
<td>269 (183, 393)</td>
</tr>
<tr>
<td>Females</td>
<td>206</td>
<td>74.8</td>
<td>15.5</td>
<td>70.9</td>
<td>72.3</td>
<td>103 (67, 160)</td>
</tr>
<tr>
<td><em>P = 0.005</em></td>
<td><em>P = 0.005</em></td>
<td><em>P &lt; 0.001</em></td>
<td><em>P = 0.007</em></td>
<td><em>P &lt; 0.001</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Age (years)**

<table>
<thead>
<tr>
<th>Age</th>
<th>Egg counts</th>
<th>% positive</th>
<th>gmean</th>
<th>Hematuria</th>
<th>% positive</th>
<th>gmean</th>
</tr>
</thead>
<tbody>
<tr>
<td>6–9</td>
<td>170</td>
<td>71.2</td>
<td>13.2</td>
<td>71.2</td>
<td>70.0</td>
<td>81 (51, 131)</td>
</tr>
<tr>
<td>10–13</td>
<td>225</td>
<td>84.0</td>
<td>26.9</td>
<td>82.7</td>
<td>82.3</td>
<td>286 (191, 427)</td>
</tr>
<tr>
<td>14–17</td>
<td>55</td>
<td>94.5</td>
<td>34.7</td>
<td>87.3</td>
<td>80.0</td>
<td>232 (102, 533)</td>
</tr>
<tr>
<td><em>P = 0.0002</em></td>
<td><em>P = 0.004</em></td>
<td><em>P = 0.006</em></td>
<td><em>P = 0.02</em></td>
<td><em>P = 0.003</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*NS = not significant.

† Geometric mean (log x + 1) egg counts/10 ml of urine and SEA concentrations in ng/ml of urine. Values in parentheses are 95% confidence intervals.

Counts compared with the SEA-ELISA for Tsunguni (*P = 0.013*) but not for Kibaokiche (*P = 0.53*). The SEA, hematuria, and egg count prevalences for the different age groups were comparable in Kibaokiche but not in Tsunguni (Table 1). In both schools, males, females and the different age groups differed significantly in the prevalence and the intensity of infection determined either by the SEA-ELISA or the egg counts. Within the different egg output classes (Figure 1), the sensitivity of the SEA-ELISA was comparable with that of the hematuria dipstick assay, with both detecting approximately 100% of the children having 50 or more eggs/10 ml of urine, while 74% of the lightly infected children (1–49 eggs/10 ml of urine) were positive in the ELISA. A comparable performance was also observed between egg counts and the ELISA within the different classes of hematuria. There was no difference in the results between these two schools. A significant correlation was observed between egg counts and levels of SEA in urine (Pearson’s *r* = 0.73, *P < 0.0001). Hematuria also had a significant correlation with levels of SEA in urine (Spearman’s *r* = 0.65, *P < 0.0001).

Pathology ranged from bladder wall thickening, polyp formation and masses due to egg deposition in the bladder wall. Furthermore, hydronephrosis and ureter dilatations were observed. Bladder pathology accounted for most of the pathology observed. In Tsunguni, 65.3% of the children compared with 58.4% in Kibaokiche had detectable urinary tract pathology, while more males (*P < 0.05*) had pathology than females. The frequency of children with pathology increased with increasing levels of SEA concentration. In addition, significant correlations were observed between the egg counts and SEA on one hand and overall pathology and bladder pathology on the other (Table 2). However, it was observed that of the children who had no ultrasonography-detectable pathology, 66% were positive as determined by hematuria dipstick and SEA-ELISA, and 71.9% were egg positive.

We observed that within the group of zero egg counts (Figure 1) 32% and 31% of the children were positive for SEA or hematuria, while 49% showed pathology.

To evaluate the effect of chemotherapy on antigen levels, a small sample of children (*n = 34*) from the group under study was selected and urine samples were tested with the SEA-ELISA. Before treatment, 93.5% of the children were egg positive with a mean egg count of 96.5 eggs per 10 ml of urine.
of urine. Eight weeks after treatment the prevalence was 35.5% and the mean egg count decreased to 3.8 eggs per 10 ml. With the SEA-ELISA, before treatment 83.5% of the children were SEA positive with a mean antigen concentration of 1,373 ng of SEA/ml. After treatment, only 5.9% of the children had detectable SEA in their urine (mean = 1.3 ng of SEA/ml).

**DISCUSSION**

In the present study we have clearly shown that SEA can be detected in urine of *S. haematobium*-infected children and that measurement of SEA can be used as a diagnostic tool or indicator of morbidity. From the correlation coefficients, it appears that the antigen(s) detected are primarily derived from the urine-borne eggs although we cannot disregard the influence of circulating SEA. In addition, significant correlations were found between the SEA concentration and urinary tract pathology as detected by ultrasonography, and this indicates that the SEA-ELISA has the potential of being a morbidity assessment tool.

Bosompem and others have recently described a *S. haematobium*-specific monoclonal antibody–based dipstick assay detecting SEA in urine, but the performance of this assay in comparison with specific egg counts was not evident from their study. In our study, we found significant correlations between the ELISA and egg counts (Pearson’s *r* = 0.73) and hematuria (Spearman’s *r* = 0.65). The sensitivity of the ELISA increased with increasing intensity of infection, with most of the heavily infected children (50 or more eggs/10 ml of urine) being positive while 74% of the children harboring light infections (1–49 eggs/10 ml of urine) were diagnosed as positive with this assay. The sensitivity of the ELISA compared well with hematuria within the egg output classes and with egg counts within the hematuria classes. This is noteworthy because these two diagnostic measures are well-established measures of infection. An interesting observation was that within the group of zero egg counts, 32% and 31% of the children were positive for SEA or hematuria. This observation illustrates that due to variation, egg excretion may be misclassified and emphasizes that one-point egg counts may not be the best indicator of infection status. The differences in sensitivity observed between the SEA-ELISA and the other parameters in the two schools may be due primarily to light infections for the children in Tsunguni that were not detected in the ELISA. After chemotherapy the detected antigen seems to clear faster than eggs, although most of the eggs detected might be dead eggs. In an earlier study, we have noted that the antibody we are using in our assay might be able to differentiate between dead/immature eggs and live/antigen-excreting eggs. This is of importance especially in quantifying the true effect of chemotherapy.

The gold standard for morbidity assessment in *S. haematobium* infections is the visualization of urinary tract pathology by ultrasonography. This requires trained and experienced medical staff and the equipment is expensive, limiting the use of this method for routine field application in most endemic situations. In addition, due to variation in egg excretion, the relationship between intensity of infection and pathology is not always clear. This therefore calls for the development of simpler quantitative field applicable techniques that can be used within the setting of disease control programs. In this first study, significant correlations were observed between the SEA levels in urine and urinary tract pathology, a good indication that the assay might be useful in morbidity assessment. However, more studies are required to understand more about the dynamics of pathology resolution and reappearance after treatment in relation to SEA levels in urine and factors that might influence the above mentioned relationship.

Immunodiagnosis of schistosomiasis by antigen detection is increasingly gaining momentum and application of the CAA and CCA assays in epidemiologic studies has proved successful. However, most of the work has concentrated on *S. mansoni*, with comparatively fewer studies on *S. haematobium*. Detection of SEA in urine might provide complementary information as opposed to adult-worm derived antigens, including more information on anti-fecundity immunity, and the use of this assay in assessment of the effect of anti-fecundity vaccines that are currently under development is envisaged. Recent studies using serum-CAA determinations have suggested an age-related reduction in fecundity in *S. haematobium*, but not *S. mansoni*, and an SEA-ELISA could provide additional information in this regard.

Since this was a noninvasive study, we did not take blood samples. To our knowledge, this was the first study in which the relationship between the detected SEA and other parameters of infection and morbidity in *S. haematobium* infections has been analyzed. The SEA assay has the potential for both diagnosis and morbidity assessment. Further work will aim at the standardization and simplification of the assay to make it more field applicable. Since egg counts and hematuria are known to exhibit a diurnal and day-to-day variation, further investigations will examine whether SEA in urine follows the same pattern as egg counts and hematuria. Furthermore, in follow-up and reinfection studies after chemotherapy, the dynamics of soluble antigen levels, egg counts, and hematuria will be investigated during clearance and reappearance of urinary tract pathology post-treatment.
REFERENCES


