Up-Converting Phosphor Technology-Based Lateral Flow Assay for Detection of Schistosoma Circulating Anodic Antigen in Serum

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Schistosoma sp. circular anodic antigen (CAA) serum concentrations reflect actual worm burden in a patient and are a valuable tool for population screening and epidemiological research. However, for the diagnosis of individual imported schistosomiasis cases, the current enzyme-linked immunosorbent assay (ELISA) lacks sensitivity and robustness. Therefore, a lateral flow (LF) assay was developed to test CAA in serum for individual diagnosis of imported active schistosome infections. Application of fluorescent submicron-sized up-converting phosphor technology (UPT) reporter particles increased analytical sensitivity compared to that of the standard ELISA method. Evaluation of the UPT-LF test with a selection of 40 characterized epidemiologic samples indicated a good correlation between signal intensity and infection intensity. Subsequently, the UPT-LF assay was applied to 166 serum samples of Dutch residents (immigrants and travelers) suspected of schistosomiasis, a case in which group routine antibody detection frequently fails straightforward diagnosis. The UPT-LF assay identified 36 CAA-positive samples, compared to 15 detected by CAA-ELISA. In conclusion, the UPT-LF assay is a low-complexity test with higher sensitivity than the CAA-ELISA, well suited for laboratory diagnosis of individual active Schistosoma infections.

Schistosomiasis (reviewed in references 10 and 18) is considered one of the major helminth diseases in the world. Despite the availability of an effective drug and the implementation of successful control programs, the number of infected cases has not decreased during the last decades and is still estimated to be around 200 million people (9, 26). The main burden of disease occurs in sub-Saharan Africa, where individuals are continuously exposed to new infections while in contact with cercaria-contaminated fresh water. In countries where the disease is not endemic, schistosomiasis is considered the major imported helminth infection, found in immigrant as well as tourist travelers (1–3, 25). Although many infected cases are asymptomatic initially, long-term and heavy infections are associated with severe morbidity. Even light infections may cause serious disease, such as Katayama fever, or neurological and genital complications (10, 18).

Diagnosis of the infection is classically based on the detection of parasite eggs in urine or in feces. However, this method has several disadvantages. The number of excreted eggs is often low and shows a high day-to-day fluctuation. Therefore, stool or urine examination needs to be repeated several times. Alternatively, detection of antibodies is a highly sensitive and specific method to diagnose schistosomiasis. High antibody responses are generally seen with travelers originating from areas where schistosomiasis is not endemic. However, in immigrant travelers with a life-long history of exposure, antibody responses are mostly moderate to low. Some may even become serologically negative, while still excreting viable eggs. In addition, antibody levels are not associated with the actual worm burden and remain unaffected by treatment of the infection. Consequently, serology mostly gives straightforward answers for patients tested within months after their first exposure, but data are difficult to interpret for those who have a history of previous infection (23). A sensitive, serum- or urine-based test demonstrating active Schistosoma infection would be valuable in these cases.

Assays for the detection of Schistosoma circulating antigens (adult worm gut-associated antigens) seem very promising, as serum levels of circulating anodic antigen (CAA) are related to actual worm burden and rapidly decrease following drug treatment (reviewed by van Lieshout et al. [23]). The current monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) determines serum CAA levels for all human Schistosoma species with virtually 100% specificity (7); its value in (sero)epidemiological studies dealing with populations with moderate- to high-intensity infections has been demonstrated previously (17). However, the CAA-ELISA still lacks sensitivity when testing light infections, e.g., in the group of international travelers (24). Moreover, due to the relatively high complexity of the test, it lacks robustness if performed only occasionally for single case identification. This may hamper implementation of the CAA-ELISA within clinical routine diagnostic settings.

In this report we describe the development of a rapid test strip designed to detect CAA in single serum samples by utilizing the same genus-specific monoclonal antibodies as the current standard CAA-ELISA. Sensitivity of the test is increased by up-converting phosphor technology (UPT) using instrument-assisted assay analysis (5). The important advantages of UPT reporters compared to other (conventionally...
backing and cut into 4-mm-width LFS strips as described earlier (4). Strips were absorbent pads (filter paper 470; Schleicher & Schuell) mounted on plastic sample application pads (glass 33; Schleicher & Schuell, Keene, NH), and a paper Scientific, Inc., Muttenz, Switzerland). The striped nitrocellulose, a glass fiber (Sigma). Antibodies were diluted to 0.45 mg/ml in TM buffer (10 mM Tris, pH 7.4) provided with a flow control line composed of a rabbit anti-mouse antibody by using a load of 175 ng antibody per 4 mm. The membrane was furthermore composed of mouse monoclonal anti-CAA antibody 147 (LUMC, Parasitology) was coupled to 400 nm UPT reporter particles (OraSure Technologies, Inc., Bethlehem, PA) as described earlier (4), utilizing a conjugation load of 25 μg antibody per mg reporter particles. The resulting UPTMCA reporter conjugate is stable for at least 6 months in a refrigerator at 4°C. Directly before use, the stock solution of the conjugate was homogenized, and a desired amount was sonicated (1 min, water bath sonicator, 100 W) and diluted in assay buffer (see below) to 1 ng/μl. The UPT assay buffer used in these assays is a buffer developed for direct testing of serum and plasma samples (6): 100 mM HEPES, pH 7.2, 270 mM NaCl, 0.5% (wt/vol) Tween 20, 1% (wt/vol) bovine serum albumin.

UPT-LF CAA assay. A schematic illustration of the assay is depicted in Fig. 1. In detail, the UPT-LF CAA assay consists of four steps: (i) mixing of 100 μl assay buffer containing 1 ng/μl UPTMCA reporter conjugate with 10 μl CAA test sample in a microtiter plate well or 1.5-ml tube; (ii) incubation in an orbital shaker (1,200 rpm) at 37°C for 60 min; (iii) addition of the mixture to the sample pad of a CAA-specific UPT-LF strip placed in a well of a microtiter plate (sample pad down), allowing chromatography to continue until strips are dry (between 30 min and 1 h); and (iv) scanning of the strips by using a modified Packard Fluorocount meter (4). Test line signals were normalized to control line signals of each individual strip; the results are expressed as ratio signals (UPT value). The cutoff threshold for the UPT-LF CAA assay was determined with 30 Dutch blood bank donors without a known history of schistosomiasis.

CAA-ELISA. CAA concentrations were determined in serum as described previously (16). Serial dilutions of the trichloroacetic acid (TCA)-soluble fraction of Schistosoma adult worm antigen (AWA-TCA) were assayed simultaneously on each ELISA plate to calculate CAA concentrations. AWA-TCA contains approximately 5% (wt/wt) CAA. The lower limit of detection (LOD) of the CAA-ELISA is 10 pg CAA/ml.

MATERIALS AND METHODS

CAA-specific UPT-LF strips. A laminated nitrocellulose membrane (HiFlow Plus HF09004; Millipore Corp., Bedford, MA) was provided with a test line composed of mouse monoclonal anti-CAA antibody 147 (LUMC, Parasitology) by using a load of 175 ng antibody per 4 mm. The membrane was furthermore provided with a flow control line composed of a rabbit anti-mouse antibody (Sigma). Antibodies were diluted to 0.45 mg/ml in TM buffer (10 mM Tris, pH 8, 1% [vol/vol] methanol) and applied using a Linomat IV stripper (Camag Scientific, Inc., Muttenz, Switzerland). The striped nitrocellulose, a glass fiber sample application pad (glass 33; Schleicher & Schuell, Keene, NH), and a paper absorbent pad (filter paper 470; Schleicher & Schuell) were mounted on plastic backing and cut into 4-mm-width LFS strips as described earlier (4). Strips were stored dry in containers with silica and are stable for up to 1 year. A schematic of the LF strip is presented in Fig. 1.

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Sample pretreatment and standards. All serum samples tested by CAA-ELISA or UPT-LF were pretreated with TCA to remove interfering proteins and to dissociate immune complexes (8). After TCA extraction, the samples were subjected to a neutralization step, resulting in a 1:4 dilution compared to the original serum. A standard serial dilution of AWA-TCA was used to determine the analytical sensitivity of the UPT-LF assay.
Characterized samples from areas of schistosomiasis endemicity. For an initial laboratory evaluation of the UPT-LF assay, four sets (10 serum samples each), representing different 
Schistosoma infection levels, were selected. These well-defined samples were collected earlier as part of epidemiological studies on the prevalence and intensity of 
S. mansoni infections. Ten low-reactive serum samples were selected from banked specimens from a study performed at Saracca, Surinam, an area with low 
S. mansoni transmission (22). The CAA concentrations as determined by ELISA ranged from 190 pg/ml to 7.5 ng/ml (median, 1.9 ng/ml). Ten moderate- and ten high-reactive serum samples were selected from banked specimens from a study performed at Mwanza, Tanzania (13), an area with moderate to high transmission. The moderate-intensity-infection set had CAA concentrations ranging from 3.5 to 28.5 ng CAA/ml (median, 11.1 ng/ml), whereas in the high-intensity-infection set the CAA concentrations ranged from 34.5 to 114.0 ng CAA/ml (median, 66.2 ng/ml).

The specificity of the assay was tested with 10 serum samples selected from banked specimens from a study performed in an area of Senegal where schistosomiasis is not endemic (16). No Schistosoma eggs were found in the latter group following extensive stool and urine examination, and all samples tested negative in the CAA-ELISA.

Schistosomiasis-suspect samples from areas of schistosomiasis nonendemicity. A second evaluation study of the UPT-LF assay was performed with 166 serum samples sent to our reference laboratory by general practitioners or other diagnostic laboratories within The Netherlands. All samples originated from individuals living within The Netherlands and were sent with the request of 
Schistosoma antibody testing because of suspected schistosomiasis. This group represents recent travelers or immigrants from a region of schistosomiasis 
endemicity. Routine testing implied screening of serum by two “in-house” antibody assays, an immunofluorescence assay for anti-adult worm antibodies (IFA-AWA) and an ELISA for anti-soluble egg antigen antibodies (ELISA-SEA) (23). IFA-AWA titers of ≥16 and ELISA-SEA titers of ≥32 were considered positive. Based on serology outcome, the group was divided into an antibody-negative group (n = 43) and an antibody-positive group (n = 123). Samples were tested in the CAA-ELISA for comparison with the UPT-LF assay.

RESULTS

Analytical sensitivity of the UPT-LF assay. The analytical sensitivity of the UPT-LF assay was determined by analyzing an AWA-TCA dilution series. The result of a typical experiment with direct comparison of the UPT-LF assay and the CAA-ELISA (n = 6) is shown in Fig. 2A. In this set of experiments, the UPT value obtained with 1 pg/ml CAA was well above the zero control. A more detailed analysis (Fig. 2B) in the lower concentration range demonstrated that the UPT-LF assay was able to detect 0.5 pg/ml CAA (lowest concentration tested). The UPT-LF value obtained at this concentration was 0.083, relevantly higher than the 0.038 value obtained with the zero control.

Cutoff threshold value for serum samples. UPT-LF tests performed with the normal human sera (negative samples obtained from 30 Dutch blood bank donors) resulted in UPT values ranging from 0.027 to 0.064 (median, 0.034), with an average of 0.036 and a standard deviation (SD) of 0.0087. The cutoff threshold above which a sample was designated “CAA positive” was set to ≥0.081 (highest negative plus 2 SD), and the cutoff threshold below which a sample was designated “CAA negative” was set to ≤0.053 (average plus 2 SD). Samples resulting in ratio values between 0.053 and 0.081 were designated “potentially positive.” The positions of the positive and negative cutoff thresholds determined with TCA extracted sera are indicated in Fig. 2B.

Evaluation of UPT-LF with a set of characterized samples from areas of schistosomiasis endemicity. An evaluation of the UPT-LF assay with a set of 40 characterized epidemiological serum samples indicated excellent correlation with infection status as determined by the CAA-ELISA (Fig. 3). The UPT values of the 10 samples from the Surinam area of low schis-
TABLE 1. UPT-LF and ELISA analysis of 166 clinical serum samples suspected of schistosomiasis

<table>
<thead>
<tr>
<th>UPT result</th>
<th>No. of serology-positive samples by CAA-ELISA</th>
<th>No. of serology-positive samples negative by CAA-ELISA</th>
<th>No. of serology-negative samples by CAA-ELISA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>12</td>
<td>7</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Potentially positive</td>
<td>3</td>
<td>13</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>88</td>
<td>42</td>
<td>130</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>108</td>
<td>43</td>
<td>166</td>
</tr>
</tbody>
</table>

* Samples were divided in two groups according to their antibody responses (123 serology-positive samples and 43 serology-negative samples). A UPT value of ≥0.081 was considered positive, and a UPT value of ≥0.053 was considered negative. IFA-AWA titers of ≥16 and/or ELISA/SEA titers of ≥32 were considered positive. Samples with ≥40 pg CAA/ml were considered positive by CAA-ELISA.

DISCUSSION

CAA excreted by adult Schistosoma parasites is cleared rapidly from human circulation, and the concentration in serum is correlated to the intensity of the infection (23). The presence of CAA in serum as detected by the CAA-ELISA therefore is a valuable tool to diagnose active infection of Schistosoma. However, the current ELISA does not meet the sensitivity level to effectively diagnose imported schistosomiasis cases in countries where schistosomiasis is not endemic; these cases for a large part represent very light infections (24). Also, implementation of the ELISA in routine clinical diagnostics is complex as ELISA may lose robustness when performed only occasionally. Moreover, the ELISA was not developed for single case identification.

Recently a rapid immunochromatography test strip for the detection of Schistosoma circulating cathodic antigen in urine has been introduced. This simple-to-use field test has shown its value in epidemiological surveys, particularly in remote areas of S. mansoni endemicity (19, 20). The applied immunochromatography or LF format is suitable for single case diagnosis. In this paper, we present a UPT-LF assay for detection of CAA that was initially designed for lab-based applications. Serum assays are appropriate and readily available for this setting, whereas CAA detection in general has a higher sensitivity and specificity than circulating cathodic antigen detection (16). The UPT-LF assay, as with the ELISA, is performed on TCA extracted serum samples and utilizes the same mouse monoclonal antibody to build a CAA immunosandwich (7). The ELISA detection format is replaced with an LF-based detection of CAA-bound UPT-reporter particles.

Analytical sensitivity: comparison of UPT-LF with ELISA.

The analytical sensitivity of UPT-LF indicated an LOD of 0.5 pg CAA per ml, more than 10-fold better than the 10 pg/ml for the ELISA. Assuming a CAA molecular mass between 10 and 100 kDa and a 10-μl sample volume, this translates to a detection level between 10^6 and 10^5 target molecules per LF strip (in agreement with LODs as determined in other UPT-LF assays [5, 29]).

The dynamic range of the UPT-LF assay is 4 orders of magnitude, spanning CAA concentrations of 0.5 pg/ml through 500 pg/ml. The 0.5-pg/ml value represents the LOD of the UPT-LF assay whereas 500 pg/ml indicates a UPT plateau value. It is possible to perform quantitative measurements above 500 pg/ml by increasing the number of UPT reporter particles in the assay; this, however, negatively affects the LOD. In the very few cases (in regions where schistosomiasis is not endemic) that UPT-LF diagnosis indicates concentrations above 500 pg/ml and further quantification is required, e.g., for determination of accurate drug treatment, the ELISA is applicable up to concentrations of 30,000 pg/ml. Alternatively, the UPT-LF analysis could be repeated simply with a dilution of the TCA-treated sample.

The UPT-LF cutoff threshold value used in this study was determined from samples obtained from 30 Dutch blood bank donors. Although UPT values were presented as ratio values (allowing interassay comparison [4]), an indeterminate group (potentially positive) was included because assays were performed with different batches of LF strips. All LF strips were produced manually in small batches of 40 strips. The average UPT value of 0.036 (median, 0.034) with an SD of 0.0087, determined for the blood bank donors, indicates that a negative cutoff threshold of 0.053 is not unrealistic. When using this 0.053 threshold, only one of the 30 blood bank samples and one of the serology-negative samples scored CAA positive. A precise assay cutoff threshold needs to be determined in future with a higher number of negative controls (from regions of schistosomiasis endemicity as well as nonendemicity) by using strips from large production batches.

Evaluation of the UPT-LF assay. A retrospective analysis of four defined sets of epidemiological samples demonstrated 100% diagnostic agreement between the CAA-ELISA and the
The UPT-LF assay is of potential value in laboratory diagnosis of Schistosoma-positive serum sets, this result was not surprising, as all samples were selected based on CAA-ELISA results and the UPT-LF assay obviously demonstrated a better analytical sensitivity. Although the UPT-LF assay initially was not designed to function as a quantitative assay, excellent correlations were seen in these three serum sets between the UPT ratio and the serum CAA concentration as determined by the CAA-ELISA. Only the differentiation between moderate- and high-intensity-infection sets was less pronounced with the UPT-LF assay than with the CAA-ELISA. This is a consequence of reaching a plateau value in the UPT ratio above 500 pg CAA per ml. In addition, the specificity of the UPT-LF assay was found to be high, as samples collected in a region of Senegal where schistosomiasis is not endemic all tested clearly negative.

The performance of the UPT-LF assay was analyzed in 166 serum samples referred to our diagnostic laboratory because of suspected schistosomiasis. These samples were tested routinely by antibody serology, which is the standard procedure to identify a Schistosoma infection in a setting where schistosomiasis is not endemic (25). The UPT-LF assay identified 27% more CAA-positive samples than the ELISA and 140% more cases when including the potentially positive group. With respect to the results obtained with the negative controls, the majority of the samples from the potentially positive group are likely to be truly positive. Similarly to previous publications indicating antibody detection to be a poor indicator of an active Schistosoma infection (25), UPT values did not correlate with absolute serology values. Although more active cases were identified by the UPT-LF assay than by the ELISA, the majority of antibody-positive individuals had no detectable CAA levels. Based on previous data, there is no indication that lack of detectable CAA serum levels is related to the Schistosoma species involved (23). Unfortunately, data regarding geographical location and time period of possible exposure, microscopic stool or urine examination, and clinical signs of infection were mostly not available. The antibody-positive, CAA-negative group may include several individuals where adult worms are not yet fully developed (very recent infection), cases with only minimal antigen production (low number of parasites), or individuals who may have lost their infection. Additional studies are planned to further explore the clinical diagnostic value of the UPT-LF assay, evaluating anamnestic data and following patients who may have lost their infection. Additional studies are planned to further explore the clinical diagnostic value of the UPT-LF assay.

**Conclusions.** The UPT-LF assay is a straightforward, easy-to-use single-sample test for detection of CAA in TCA extracted serum samples. It shows higher analytical sensitivity and better accuracy than the CAA-ELISA when testing clinical samples. Although precise determination of the UPT-LF cutoff threshold value awaits a study with a wider selection and higher number of negative controls and more controlled LF strip production (large batches), it can be concluded that the UPT-LF assay is of potential value in laboratory diagnosis of Schistosoma infections.

**ACKNOWLEDGMENTS**

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