Research paper

# Potential of antibody test using *Schistosoma mansoni* recombinant serpin and RP26 to detect light-intensity infections in endemic areas

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## A R T I C L E   I N F O

## A B S T R A C T

Schistosomiasis remains a worldwide public health problem, especially in sub-Saharan Africa. The World Health Organization targets the goal for its elimination as a public health problem in the 2030 Neglected Tropical Diseases (NTDs) Roadmap. Concerted action and agile responses to challenges will be necessary to achieve the targets. Better diagnostic tests can accelerate progress towards the elimination by monitoring disease trends and evaluating the effectiveness of interventions; however, current examinations such as Kato–Katz technique are of limited power to detect light-intensity infections. The point-of-care circulating cathodic antigen (POC-CCA) test shows a higher sensitivity compared to the reference standard, Kato-Katz technique, but it still lacks sufficient sensitivity with low infection intensity. In this study, we examined antibody reactions against recombinant protein antigens; *Schistosoma mansoni* serine protease-inhibitor (SmSerpin) and RP26, by enzyme-linked immunosorbent assay (ELISA) in plasma samples with light-intensity infection. The sensitivity using the cock-tail antigen of recombinant SmSerpin and RP26 showed 83.7%. The sensitivity using *S. mansoni* soluble egg antigen (SmSEA) was 90.8%, but it showed poor specificity (29.7%), while the cocktail antigen presented improved specificity (61.4%). We conclude that antibody detection to the SmSerpin and RP26 protein antigens is effective to detect *S. mansoni* light-intensity infections. Our study indicates the potential of detecting antibody against recombinant protein antigens to monitor the transmission of schistosomiasis in low endemicity contexts.

## 1. Introduction

Schistosomiasis is one of the neglected tropical diseases (NTDs) prevalent in sub-Saharan Africa. In the 2021–2030 NTDs Roadmap, the World Health Organization (WHO) targets its elimination as a public health problem as the goal [1]. To achieve the progress towards this goal, interventions such as mass drug administration (MDA) need to be further complemented by multipronged strategies incorporating aspects
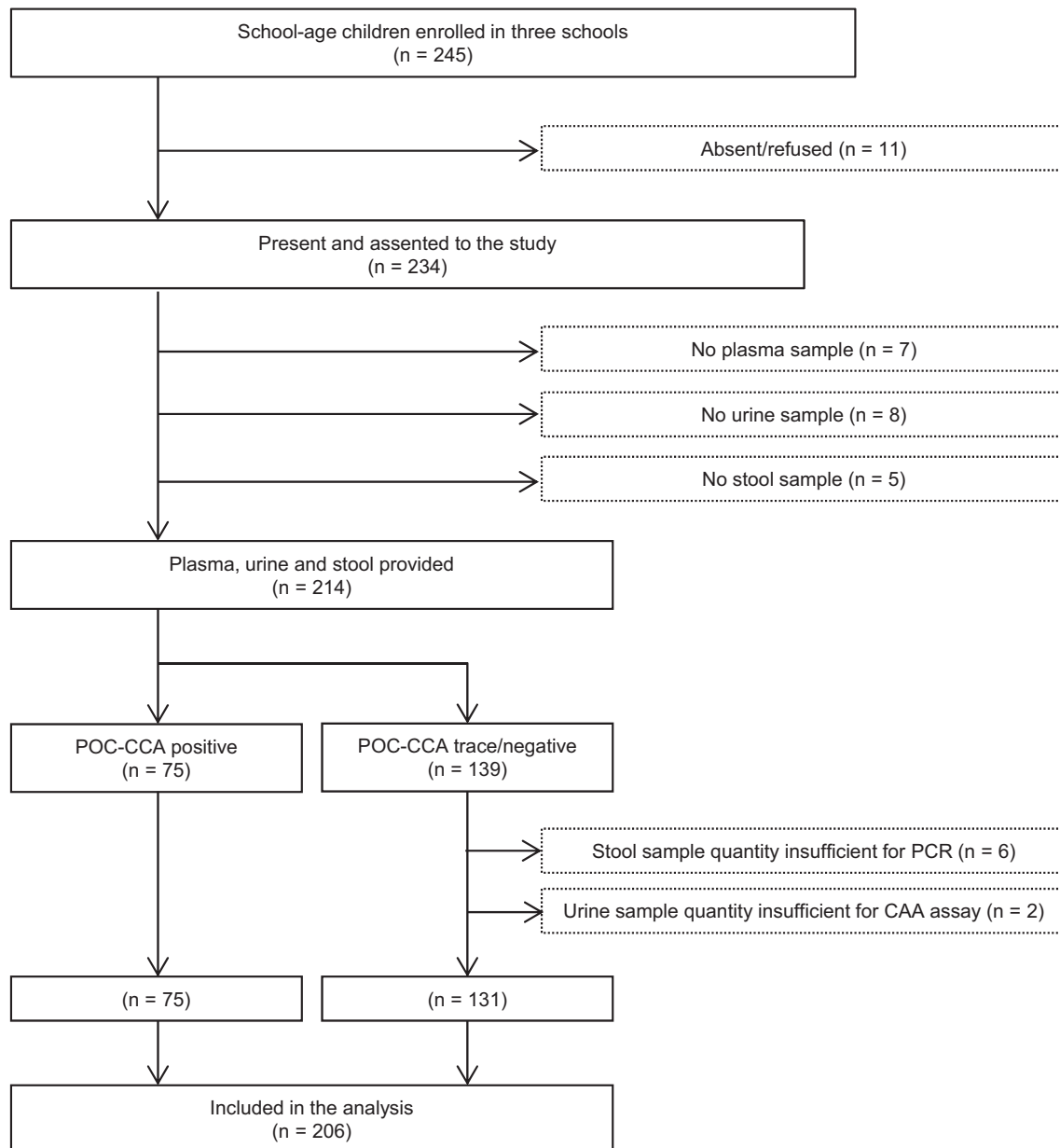
**Fig. 1.** Flowchart of the study enrollment and analyses inclusion criteria.
At the baseline, 245 school-age children were enrolled of which 234 were present and assented to the study. Participants with one or more missing samples (plasma, urine, and/or stool) were excluded from analyses but treatment was provided if applicable. For participants providing all three sample types, 75 individuals testing POC-CCA positive were included in analyses. Of the 139 individuals testing trace/negative for POC-CCA, eight individuals were excluded from the analysis due to insufficient stool or urine volume for subsequent stool-based PCR or urine-based CAA assay. Finally, 206 pupils were included in the analysis. POC-CCA, point-of-care circulating cathodic antigen; CAA, circulating anodic antigen.

such as WASH, infrastructure, education, and targeted treatment based on precise transmission mapping. In schistosomiasis endemic regions, children are initially infected while they are very young [2], and school-age children are a high-risk group both in terms of infection prevalence and intensity [3]. Therefore, school-aged children are the focus of national deworming programs as recommended by WHO [4]. These school-based MDA interventions are gradually reducing schistosomiasis prevalence and infection intensity in the target group [5]. However, multifaceted interventions are needed to progress further towards elimination, and diagnostics play a crucial role in monitoring transmission and evaluating the effectiveness of interventions [1].

Currently, the prevalence of human schistosomiasis is mainly monitored by direct parasitological examination. For intestinal schistosomiasis caused by *Schistosoma mansoni,* standard methods are the

traditional determination of eggs in stool by Kato-Katz and the more recent application of parasite antigen detection in urine using the point-of-care circulating cathodic antigen (POC-CCA) rapid diagnostic test [6,7]. Detection of eggs in stool is highly specific, but this method lacks sensitivity especially in settings with low infection intensity and prevalence such as post-treatment situations [8,9]. In comparison, the urine-based POC-CCA test has comparable to higher sensitivity than Kato-Katz-based egg counts depending on infection intensity and amount of stool examined [8,9]. As the POC-CCA test detects the schistosome worm gut-associated proteoglycan-like antigen CCA [10], which is rapidly cleared after successful treatments [11], it is a good tool for monitoring active infection. However, the POC-CCA test is not sensitive enough for detection of very light infections and it is currently recommended for use in moderate to high endemic settings [12]. In settings

with low infection intensity, single use of this test may therefore lead to underestimation of prevalence. Detection of another schistosome antigen, the circulating anodic antigen (CAA), by the up-converting phosphor-lateral flow (UCP-LF) laboratory assay, is highly sensitive and specific, but not commercially available [12]. CAA is a gut-associated proteoglycan-like antigen regurgitated by worms that can be detected in serum as well as urine [13]. *S. mansoni* DNA detection by PCR is very sensitive and specific for identification of egg-positive individuals and very relevant for morbidity risk assessments, however, it is still costly, not yet point-of-care based, and does not reflect pre-patent infections [14,15].

In very low endemic, near and post-elimination contexts, highly sensitive diagnostic tools are needed. Detection of schistosome-specific antibodies is a potential solution for the development of such a sensitive monitoring tool. Antibodies are expected to persist even after parasite clearance and therefore antibody-based measures cannot fully discriminate active from past infections or exposures [16]. This means that residents in endemic areas often present with high antibody levels regardless of presence of active infection or not. Therefore antibody-based tests usually present with low specificity in traditional sense, however, when applied in low transmission and elimination monitoring context the high sensitivity renders it a promising tool [17–19]. Recently, detection of antibodies specific for the recombinant *S. mansoni* serine-protease inhibitor (SmSerpin) and recombinant Sm22.3 (rSm22.3, LGG, or recombinant protein 26; RP26) were suggested as a useful tool to monitor active infection [20]. The diagnostic performances of such single antigens can be limited, but increased sensitivity can be achieved by combining specific antigens in a cocktail approach [21–23].

In this study, we aimed to examine the performance of detection of SmSerpin and RP26 specific antibodies in low-intensity *S. mansoni* infections. We collected plasma samples in Mbita, which is an *S. mansoni* endemic area in Kenya. This area was chosen based on data from previous studies conducted in Mbita, and low infection intensity was expected in areas such as Gembe East [24,25]. We demonstrate that detection of antibodies specific for a combination of the SmSerpin and RP26 antigens may be a useful tool to monitor transmission in low endemic and elimination settings.

## 2. Materials and methods

### 2.1. Ethics statement

This study was reviewed and approved by the scientific and ethics review unit of Kenya Medical Research Institute (KEMRI) (SSC No. 2084) and the ethical review board of the Institute of Tropical Medicine, Nagasaki University (NUITM) (No. 140829127–7). Before sample collection in primary schools in Mbita, we had meetings and explained the purpose and methods of the study to parents/guardians of the pupils, school administrators, and teachers. After the meetings, written consent forms were obtained from parents/guardians while children assented. Blood, urine, and stool samples were collected from the consented children. Children confirmed to be infected with schistosomes either by Kato-Katz or POC-CCA tests (trace included) were treated with 40 mg/kg praziquantel (Prazitel, Cosmos Ltd., Nairobi, Kenya). At the same time, those who were found to be infected with soil-transmitted helminths (STH) by Kato-Katz technique were treated with 400 mg albendazole (ABZ Tablet, Indoco Remedies Ltd., Mumbai, India). The medications were given according to WHO [26] and national guidelines. Pupils were offered a light meal (biscuits and juice) to minimize adverse effects. The local medical officers ensured every pupil swallowed the tablets and observed them to manage any adverse effects. Additionally, negative control blood samples were collected from Japanese healthy volunteers. This was also approved by the ethical review board described above. To evaluate potential cross-reactivity, serum/plasma samples positive for other helminthiases were also tested and approved as follows.

*S. haematobium* positive samples collected in Kwale, Kenya in June 2019 (this study was also approved by SSC No. 2084 in KEMRI and No. 140829127–7 in NUITM and consent obtained as described above). *S. japonicum* and other helminthic infection positive samples stored at the Division of Parasitology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki. These samples were from *S. mansoni* non-endemic area and originally sent to the department for serological diagnoses of parasitic diseases, and utilization of the residual samples was approved by the Research Ethics Review Board of the Faculty of Medicine, University of Miyazaki, under the title of 'Development of comprehensive test and diagnosis system for parasitic diseases – a retrospective study with clinical information and residual specimens' (No. O-0359).

### 2.2. Study area and participants

A cross-sectional study was conducted in Mbita, on the shore of Lake Victoria in Homa Bay County, Western Kenya. The communities along the shores and adjacent islands of Lake Victoria are known to be endemic for *Schistosoma mansoni* [24,27,28]. In Kenya, all schoolchildren in endemic regions have been offered yearly praziquantel and albendazole treatments for schistosomiasis and STHs since 2012 through the national school-based deworming program (NSBDP) [5]. Sample collection was done in two primary schools located in Gembe East and one school in Gembe West. The last school-based MDA in the three schools before our sample collection was conducted in March 2018, and we carried out our sampling from 11 to 13th February 2019. These three schools were expected to have low prevalence (< 30%) and light infection intensity (egg negative or 1–99 EPGs) based on Kato-Katz results from previous studies [24,28]. We initially recruited 245 pupils of which 206 were included in the analysis based on inclusion and exclusion criteria as described in Fig. 1. We could access the date of birth information for 169 pupils out of 206, and the participants were between 5 and 17 years of age with a median of 11. The age distribution was as follows; from 5 to 7-year-old, 10 individuals; from 8 to 10-year-old, 69 individuals; from 11 to 13-year-old, 83 individuals; from 14 to 17-year-old, 7 individuals. The male-to-female ratio among included participant was 1.04.

### 2.3. Sample collection in Mbita, Kenya

Experienced field surveyors and local medical staffs were responsible for sample collection at each primary school. Prior to the sample collection, the field surveyors brought stool containers to the schools, and school health teachers guided pupils on stool sample collection. On sample collection day, the pupils were given unique identification (ID) numbers for anonymization and underwent blood, urine, and stool collection. Blood collection was performed by local medical staff using sodium heparin tubes. Furthermore, fresh early morning stool samples and spot urine samples were collected separately in the containers labelled with an anonymized ID of each individual. All samples were transferred to the laboratory at the NUITM-KEMRI research station in Mbita. The blood samples were centrifuged, and 2 mL plasma was aliquoted. The plasma samples were first stored at −30 °C and subsequently transported to the Department of Parasitology, NUITM, Japan for antibody ELISA. The urine samples were tested for schistosome CCA using the POC-CCA test. Later, the samples tested negative or trace for CCA were transferred to the Department of Parasitology, Leiden University Medical Centre, the Netherlands for confirmation of schistosome infection status by CAA detection. Stool samples were collected on two consecutive days and the number of eggs determined by microscopy as described below. The remaining stool samples from the POC-CCA negative or trace individuals were transferred to the Parasitology Department, NUITM, Japan for stool-based PCR.

## 2.4. Parasitological examinations

### 2.4.1. Stool examination by the Kato-Katz technique

*S. mansoni* and STH infection burden was evaluated by the Kato-Katz technique using 41.7 mg stool templates [6]. Two thick smear slides were prepared from single stool samples on two consecutive days and were examined by microscopy by two experienced laboratory technicians independently. The slides were examined within one hour for hookworm eggs and later for *S. mansoni*, *Trichuris trichiura*, and *Ascaris lumbricoides* (no *Strongyloides* applicable diagnostic methods were used). The results were recorded as egg counts per slide by the technicians and were converted into eggs per gram of stool (EPGs) by multiplying a factor of 24. The geometric mean was determined based on EPGs. *S. mansoni* infection intensity was categorized as light (1–99 EPGs), moderate (100–399 EPGs), or heavy ($\geq$ 400 EPGs) [4].

### 2.4.2. Point-of-care circulating cathodic antigen (POC-CCA) test

On sample collection day, urine samples were tested for schistosome CCA using the commercially available POC-CCA test (Batch number 180314027 and 181116119, Rapid Medical Diagnostics, Pretoria, South Africa) based on the manufacturer's instructions [29]. Briefly, 2 drops of urine were transferred to the well of the test cassette, and the results were read after 20 min by two independent observers. The test results were recorded as follows; positive (the test line appeared), trace (the test line was very light but visible), or negative (no test line appeared) based on the instructions. The positive results were further categorized into three; strong positive (3+, the test line was more intense than the control line), medium positive (2+, the test line was equally intense with the control line) and light (1+, the test line appeared but was less intense than the test line). In case of discrepancy between observer result, a third observer was consulted.

### 2.4.3. Stool-based PCR

Total DNA was isolated from 200 mg stool aliquots using QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. For identification of *S. mansoni* infection positive or negative, the *Schistosoma*-specific internal-transcribed-spacer-2 (*ITS2*) region was amplified by PCR using a primer set consisting of forward Ssp48F (5′-GGT CTA GAT GAC TTG ATY GAG ATG CT-3′) and reverse Ssp124R (5′- TCC CGA GCG YGT ATA ATG TCA TTA-3′) as described in Obeng *et. al* (2008) [30]. Real-time PCR was performed in a final volume of 20 μL containing 0.25 μM of forward primer, 0.25 μM of reverse primer, 10 μL of SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 7.6 μL of distilled water, and 20 ng of DNA template. DNA extracted from *S. mansoni* eggs was used as a positive control, and sterile double distilled water was used as a negative control. The reaction condition was as follows; initial incubation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Amplifications and amplicon detection were performed using QuantStudio™ 7 Flex Real-Time PCR System (Singapore, Singapore). The results were analyzed on QuantStudio™ Real-Time PCR software v1.3. A PCR result was considered as positive when the cycle threshold (Ct) was within 38 cycles (the cut-off Ct value: 38).

### 2.4.4. Circulating anodic antigen (CAA) detection in urine

Urine samples from participants with a POC-CCA score of negative or trace were aliquoted and transported to LUMC, Netherlands for subsequent testing with the more sensitive UCP-LF CAA laboratory-based assay. Urine CAA concentrations were determined based on 500 μL trichloroacetic acid (TCA) precipitated urine aliquots using the UCP-LF CAA assay (uCAA500) as previously described [12,31]. The cut-off threshold of the assay was 0.6 pg/mL. CAA concentrations above 0.6 pg/mL were regarded as positive, below 0.3 pg/mL as negative, and between 0.3 and 0.6 pg/mL as "indecisive".

## 2.5. Antigen preparations

*S. mansoni* soluble egg antigen (SmSEA) and recombinant antigens, SmSerpin (accession no. CCD60071) and RP26 (accession no. AAB81008) were produced to be used as coating antigens in ELISA (described below). SmSEA was prepared as follows. Purified *S. mansoni* eggs were suspended in 1× PBS and homogenized on ice. The homogenate was frozen at −80 °C and thawed on ice. The freezing and thawing step was repeated five times. The material was centrifuged (15,000 *g*, 4 °C) and SmSEA supernatant was dialyzed against 1× PBS and syringe sterilized (0.22 μm). Recombinant antigens were prepared as previously described [32]. In short, expression plasmids of SmSerpin and RP26 were transformed into *Escherichia coli* BL21 (DE3) for protein expression by IPTG induction. The expressed proteins were purified using His-tag.

## 2.6. Enzyme-linked immunosorbent assay (ELISA)

Wells of 96-microwell plates (Thermo Scientific, Roskilde, Denmark) were coated overnight at 4 °C with 50 ng per well of each antigen (SmSEA, SmSerpin, RP26, and antigen cocktail of SmSerpin and RP26) in 50 μL of 0.1 M carbonate-bicarbonate buffer (pH 9.6). For the antigen cocktail, 25 ng of each SmSerpin and RP26 were mixed in 50 μL coating buffer. The wells were washed three times with PBS-T (0.05% Tween 20 in PBS, pH 7.6) and blocked with 150 μL of 1% casein in 1× PBS for 2 h at room temperature. Then, 50 μL of standard reference sample dilution series and participant plasma samples in 1:1000 dilution were applied in duplicates, and the plates were incubated for 1 h at 37 °C. After washing with PBS-T, 50 μL of 1:10,000 diluted HRP-conjugated goat anti-human IgG (ab6858, Abcam plc, Cambridge, UK) was added and incubated for 1 h at 37 °C. The plates were washed again, and 50 μL of 1-Step™ Ultra TMB-ELISA (Thermo Fisher Scientific, Rockford, USA) was added for colour development for 20 min in the dark. Finally, 50 μL of 1 M sulfuric acid was added to stop the reaction. Absorbance was measured at 450 nm light using Multiskan FC microplate reader (Thermo Fisher Scientific, Vantaa, Finland). A pooled mixture of 20 *S. mansoni*-infection positive plasma was used as the standard reference serial dilution series on all plates. This standard reference plasma was 3-fold serially diluted in nine steps starting from a 1:1000 dilution. As the specific IgG antibody concentration in the standard reference plasma mix was unknown an arbitrary concentration unit (AU) was defined where 1:1000 equaled 10 AU (dilution factor * 10ˆ4). Standard curves based on 5-parameter logistic regression fits were generated using an online tool (www.elisaanalysis.com, Elisakit.com Pty Ltd., 2012) to translate measured absorption (OD450) to the arbitrary antigen-specific IgG antibody concentration in participant samples. Samples presenting with OD values higher than the top reference standard value (1:1000; 10 units) were further diluted and re-analyzed. The intra-assay variation between duplicates was calculated for all samples, and samples with % coefficient variation over 10% were re-analyzed. The cut-off levels for samples being positive were defined as the geometric mean + 3 standard deviations of the specific IgG antibody levels measured in non-endemic controls. The cut-off levels of SmSEA, SmSerpin, RP26, and SmSerpin-RP26 mixture were 0.03, 0.56, 0.36, and 0.67 AUs, respectively.

## 2.7. Samples for cross-reactivity evaluations

To evaluate cross-reactivity of SmSEA, SmSerpin, RP26, and the SmSerpin/RP26 mix, we tested samples with other helminthic infections. Test samples were 131 samples; schistosomiasis haematobia (*n* = 20), schistosomiasis japonica (*n* = 12), paragonimiasis (n = 20), fascioliasis (n = 20), clonorchiasis (n = 10), sparganosis (*n* = 19), gnathostomiasis (n = 10), and toxocariasis (n = 20). Schistosomiasis haematobia samples were from Kwale, *S. haematobium* endemic area in Kenya. The samples with schistosomiasis japonica, paragonimiasis, fascioliasis, clonorchiasis, sparganosis, gnathostomiasis and toxocariasis were from *S. mansoni* non-endemic area. We prioritized selecting cases

**Table 1**

*S. mansoni* infection determined by POC-CCA and Kato-Katz technique among 199 individuals and confirmation of infection status in the 124 individuals with POC-CCA negative/trace scores by stool-based PCR and urine-based UCP-LF CAA assay.

| POC-CCA | n (%) | Kato-Katz | | PCR | | CAA | |
|---|---|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative | Positive | Negative |
| Positive | 75 (37.7%) | 9 | 66 | N/A | | N/A | |
| Trace | 26 (13.1%) | 1 | 25 | 1 | 25 | 6 | 20 |
| Negative | 98 (49.2%) | 0 | 98 | 3 | 95 | 16 | 82 |
| n/total (%) | 199 | 10/199 (5.0%) | 189/199 (95.0%) | 4/124 (3.2%) | 120/124 (96.8%) | 22/124 (17.7%) | 102/124 (82.3%) |

Samples testing indecisive for urine CAA were excluded (n = 7).
N/A, not available.
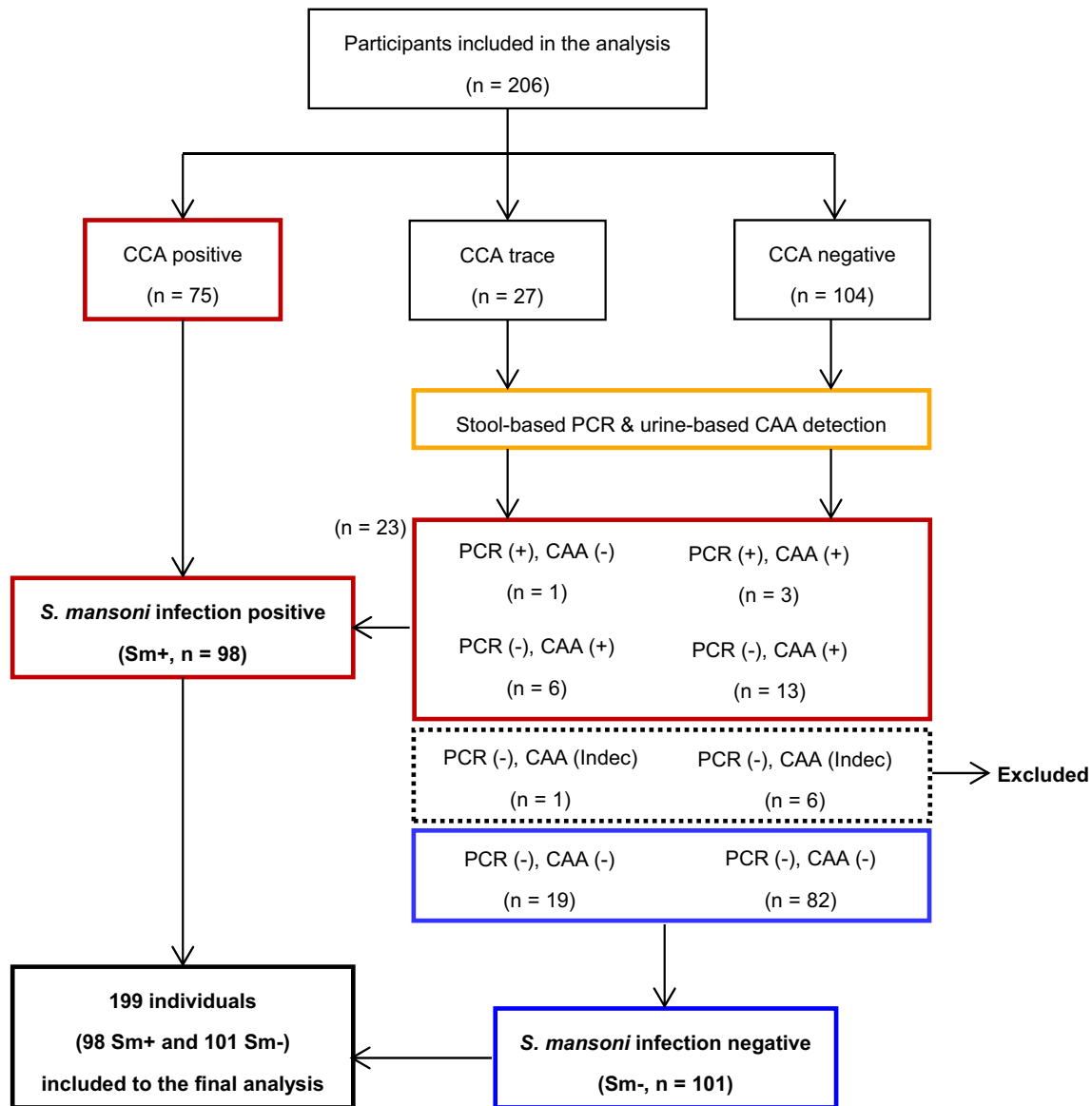


**Fig. 2.** Definition of composite reference to determine schistosome infection status (Sm+/−). Of the 206 included participants, 75 individuals with POC-CCA positive scores were regarded as *S. mansoni* infection positive (Sm+). 131 samples tested POC-CCA trace/negative and these samples were further examined by stool-based PCR and urine-based UCP-LF CAA assay. Of these, 23 individuals showed positive results for *S. mansoni* infection by PCR and/or CAA assay and were regarded as positive for *S. mansoni* infection. CAA indecisive results (n = 7) were excluded from the final analysis. A composite reference positive was defined as samples with POC-CCA, CAA, and/or PCR positive results (Sm+, 98 individuals in total, shown in red boxes). 101 individuals tested negative for both PCR and CAA were regarded as negative for *S. mansoni* infection (composite reference negative definition; POC-CCA trace/negative, CAA negative and PCR negative; Sm-, shown in blue boxes). CCA, circulating cathodic antigen; CAA, circulating anodic antigen; Indec, indecisive. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Sensitivities and specificities of EPG by Kato-Katz, POC-CCA test and antibody ELISA tests.

| Composite reference n (%) | Kato-Katz | | POC-CCA | | | Ab SmSEA | | Ab SmSerpin | | Ab RP26 | | Ab SmSerpin-RP26 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Positive | Negative | Positive | Trace | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| Sm+ 98 (49.2) | 10 | 88 | 75 | 7 | 16 | 89 | 9 | 64 | 34 | 73 | 25 | 82 | 16 |
| Sm- 101 (50.8) | 0 | 101 | 0 | 19 | 82 | 71 | 30 | 38 | 63 | 43 | 58 | 39 | 62 |
| Sn; Sp (%) (95% CI) | Sn 10.2% (5.0–18.0) | Sp 100% (96.4–100.0) | Sn Tr- 76.5% (66.9–84.5) | Sn Tr+ 83.7% (74.8–90.4) / Sp Tr- % 100%* | Sp Tr+% 81.2% (72.2–88.3) | Sn 90.8% (83.3–95.7) | Sp 29.7% (21.0–39.6) | Sn 65.3% (55.0–74.6) | Sp 62.4% (52.2–71.8) | Sn 74.5% (66.7–82.8) | Sp 57.4% (47.2–67.2) | Sn 83.7% (74.8–90.4) | Sp 61.4% (61.2–70.9) |

*S. mansoni* infection status (Sm+ or Sm-) was determined by a composite reference consisting of four diagnostic tests; POC-CCA, Kato-Katz, PCR, and UCP-LF CAA detection. Abbreviations in table: Ab; antibody, Sn, sensitivity; Sp, specificity; Ab SmSEA; detection of SmSEA specific IgG antibodies; Ab SmSerpin, detection of SmSerpin specific IgG antibodies; Ab RP26, detection of RP26 specific IgG antibodies; Ab SmSerpin-RP26, detection of a combination of SmSerpin and RP26 specific IgG antibodies; Tr-, traces regarded as negatives; Tr+, traces regarded as positives.
95% confidence intervals are shown in brackets for Sn and Sp for each method used.
* Note that Tr- specificity for POC-CCA is per definition 100% as no alternative diagnostic measurement was used to obtain number of false positives. Additionally, specificity for POC-CCA Tr + is based on an assumption of 100% specificity for POC-CCA positive (score 1, 2, 3).

positive for worms/eggs, but this data was not available for a substantial number of schistosomiasis japonica, paragonimiasis, fascioliasis, clonorchiasis, gnathostomiasis, and toxocariasis. Therefore, these cases were chosen based on clinical symptoms and strong antibody reaction against the relevant parasites' antigens in ELISA. As for the cases of *S. haematobium* infection, diagnoses were made by CAA detection and strong antibody levels against *S. haematobium* SEA in plasma ELISA.

### 2.8. Data analyses

To calculate sensitivity and specificity of each diagnostic test, we used a composite reference based on the combined results of the four diagnostic tests; stool examination by Kato-Katz technique, POC-CCA test, stool-based real-time PCR, and urine-based CAA detection. Per definition, a participant was considered positive for *S. mansoni* infection if testing positive in at least one of the four tests. As for the POC-CCA trace scores, if both the real-time PCR and the urine CAA detection results were negative, the individual was considered to be negative. All statistical analyses and visualizations were performed on GraphPad Prism 7.04. To compare the antibody levels between the *S. mansoni* infected (Sm+) and uninfected (Sm-) groups, Mann-Whitney's U tests were used. Statistical significance was set at $p < 0.05$.

### 3. Results

#### 3.1. Prevalence of S. mansoni infection among the participants

To determine the prevalence of *S. mansoni* infection among the study participants, stool examination by Kato-Katz technique and POC-CCA urine tests were applied on samples from 206 individuals. In addition, those tested POC-CCA trace or negative ($n = 131$) were further examined by stool-based PCR and urine-based UCP-LF CAA assay. As CAA indecisive results cannot be marked as truly positive or negative, seven individuals testing indecisive for urine-based CAA were then excluded. Therefore, 199 individuals were included in the final analysis. The results of the four diagnostic tests are summarized in Table 1 and Fig. 2.

Of the 199 participants, 75 (37.7%) were POC-CCA positive, 26 (13.1%) were trace, and 98 (49.2%) were negative. In contrast, only 10 individuals (5.0%) tested positive for eggs in stool by microscopy based on two Kato-Katz slides from two samples on consecutive days. All of those 10 individuals were categorized as light infection intensity (varied from 24 to 83 EPGs), and the geometric mean EPGs was 36. In terms of STH infections, only one individual tested positive for *Trichuris trichiura* with a mean egg count of 72. Among the pupils with POC-CCA trace or negative scores, CAA detection in urine and/or PCR of DNA in stool added 23 individuals positive for *S. mansoni* infection. According to the defined composite diagnostic reference (Sm+: POC-CCA, CAA, and/or PCR positives; Sm-: POC-CCA negative/trace, CAA and PCR negatives), 98 pupils (49.2%) were positive (Sm+, Table 2). From these results of composite reference, *S. mansoni* infection intensity among our study participants was low, although almost half of them were positive for infection.

Based on the infection status definition by the composite reference, the sensitivity and specificity of the Kato-Katz technique compared to the composite reference was 10.2% (95% CI 5.0–18.0%) and 100% (96.4–100.0%), respectively. The sensitivity of the POC-CCA test was 76.5% (66.9–84.5%, trace as negatives) whereas the specificity per definition was 100% as POC-CCA positive samples were not measured by CAA or PCR. For trace measures as positives, POC-CCA is 83.7% (74.8–90.4%) sensitive and 81.2% (72.2–88.3%) specific.

#### 3.2. S. mansoni antigen specific IgG levels in Mbita samples

Next, the total IgG levels against SmSEA, SmSerpin, RP26, and the mixture of SmSerpin and RP26 in 199 Mbita plasma samples were analyzed by ELISA. Then, the sensitivities and specificities of IgG

## a. SmSEA



## b. SmSerpin



## c. RP26
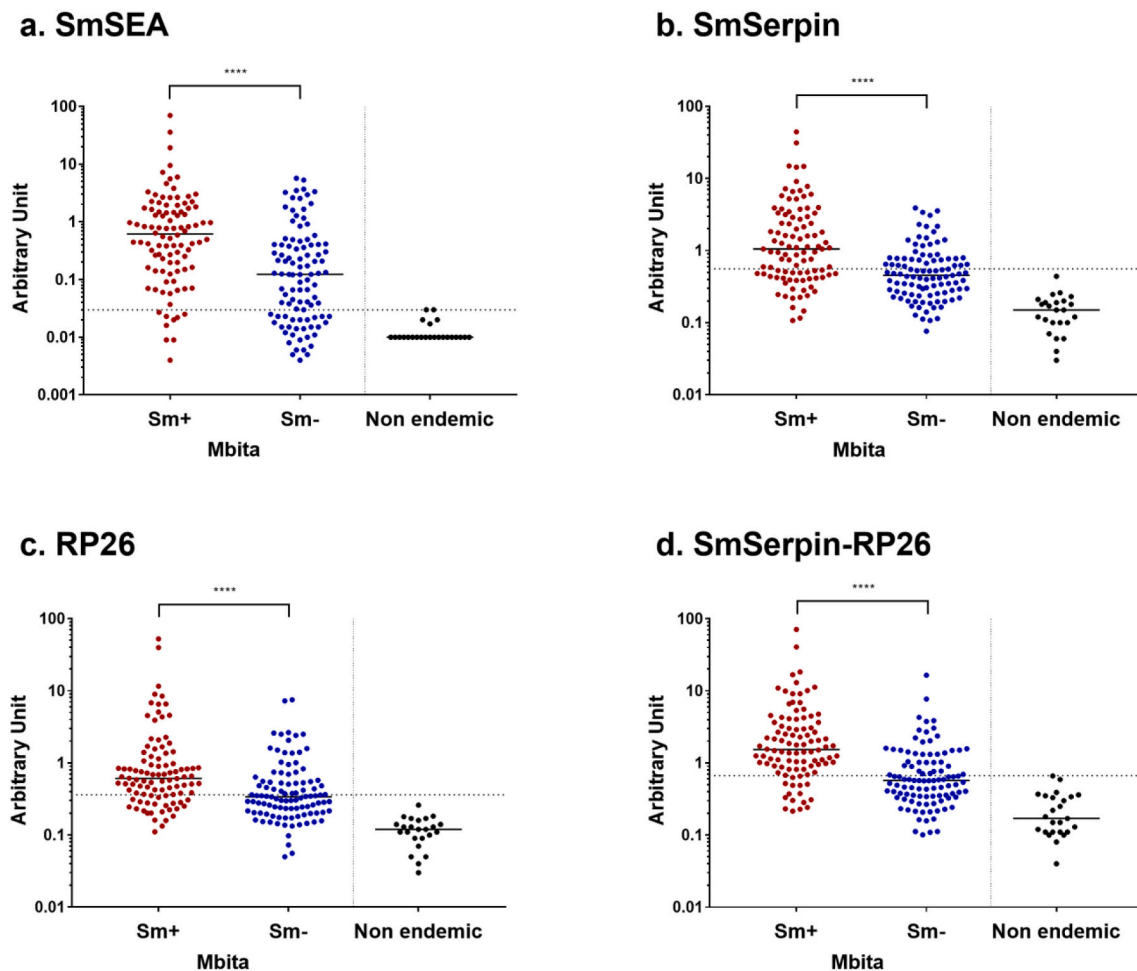


## d. SmSerpin-RP26



**Fig. 3.** IgG antibody levels against SmSEA, SmSerpin, RP26, and SmSerpin-RP26 in Mbita and non-endemic control samples. The IgG antibody levels against (a) SmSEA, (b) SmSerpin, (c) RP26, and (d) the mixture of SmSerpin and RP26 in the *S. mansoni* positive group (Sm+, $n = 98$, shown in red dots) and the negative group (Sm-, $n = 101$, shown in blue dots) as defined by composite diagnostic reference. The cut-off value for each assay is shown as a dotted line. The cut-off is based on antigen-specific IgG levels measured in 25 non-endemic controls, which are included on the graphs as reference (Non-endemic, black). The antibody levels in the Sm + and Sm- groups were compared using Mann-Whitney's *U* tests. Statistical significance was set at $p < 0.05$, and is shown using asterisks: **** = $p < 0.0001$. Horizontal bars indicate the medians. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detection against the four sets of antigens were calculated based on the composite reference data. The results are summarized in Table 2.

For all antigens, the infection positive group (Sm+) showed a higher level of IgG response compared to the negative (Sm-) group (Fig. 3). As expected, anti-SmSEA IgG was detectable in most of the endemic area samples, yielding the highest sensitivity of 90.8% (83.3–95.7%) and the poorest specificity of 29.7% (21.0–39.6%) based on the composite reference infection status (Sm+, Sm-). The recombinant antigens, SmSerpin, and RP26 showed lower sensitivities compared to SmSEA. The sensitivity of anti-RP26 IgG detection (74.5%) was comparable to that of POC-CCA, while the sensitivity of anti-SmSerpin IgG detection was lower (65.3%). The specificity of antibody detection against recombinant antigens was poorer than POC-CCA as is to be expected (62.4% for SmSerpin and 57.4% for RP26). When mixed together, the cocktail antigen of SmSerpin-RP26 showed improved sensitivity (83.7%, 74.8–90.4%) compared to antibody assays using single recombinant antigen and POC-CCA (76.5%). The specificity (61.4%, 61.2–70.9%) was lower than POC-CCA, but was far better than that of SmSEA assay. When Receiver-Operating Characteristic (ROC) analysis was applied to the Sm + and Sm- samples from Mbita, the cocktail antigen of SmSerpin and RP26 presented the greatest value of the area under curve (AUC) among the four sets of antigens, suggesting the best diagnostic performance. The ROC curves and the AUC values are shown in Supplemental

Fig. 1.

### 3.3. Cross-reactivity of S. mansoni antigens with other helminth infections

Lastly, the four *S. mansoni* antigen sets were examined for the cross-reactivity with other helminth infections. As shown in Fig. 4 and Table 3, all antigens showed cross-reactivity with samples collected from patients infected with other schistosome species (Sh: *S. haematobium* and Sj: *S. japonicum*) and to a lesser extent with other trematodes. SmSEA had the highest cross-reactivity (58% of 131 samples, and 45% of 99 non-schistosome-infected samples), while the recombinant antigens showed lower cross-reactivities. The SmSerpin antigen displayed a low cross-reactivity to *S. haematobium* positive samples, indicating species-specificity. In contrast, RP26 was cross-reactive with all *S. haematobium* infection samples. This result was consistent with the results from the previous report that RP26 is a useful antigen for detecting both *S. mansoni* and *S. haematobium* infection [20,33]. Both SmSerpin and RP26 reacted with limited number of *S. japonicum* cases. The SmSerpin-RP26 mixture had the lowest number of cross-reactive non-schistosome-infected samples.

## a. SmSEA



## b. SmSerpin



## c. RP26
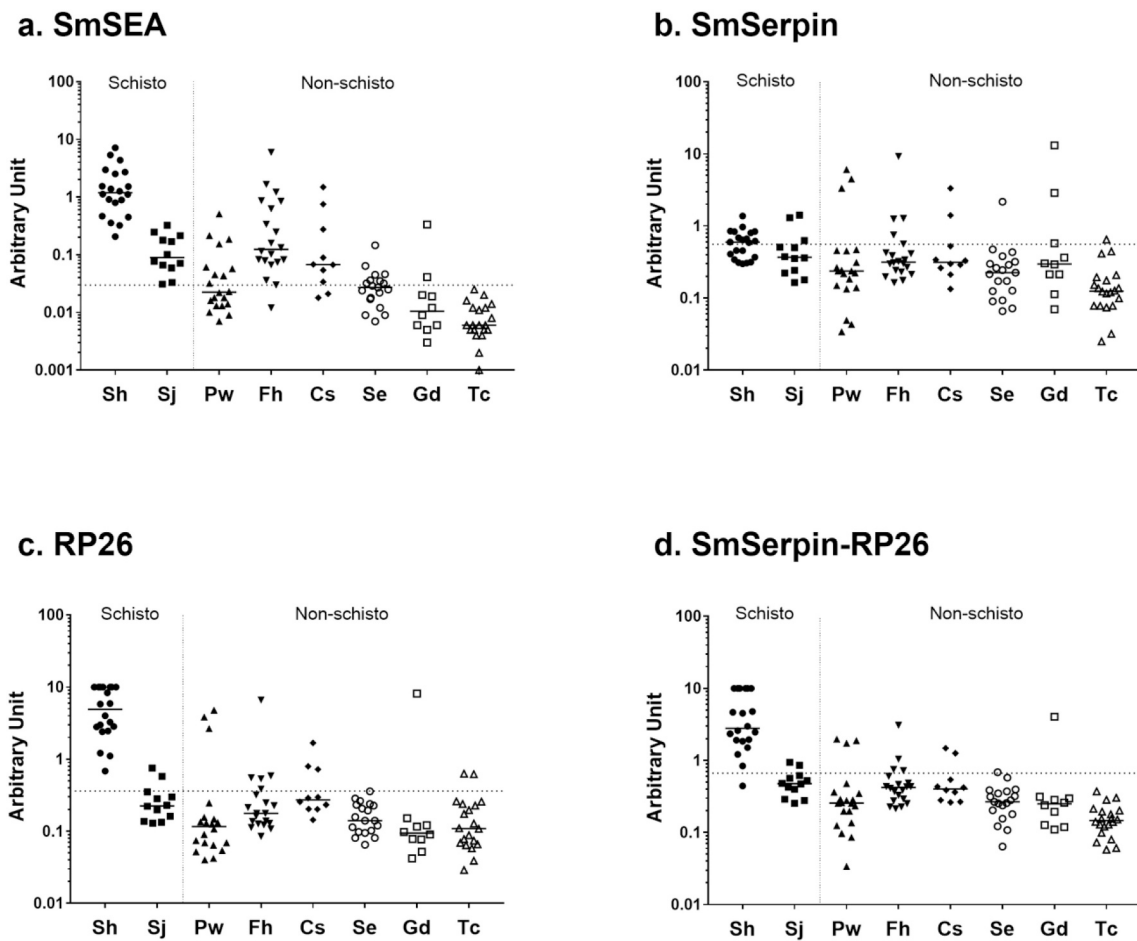


## d. SmSerpin-RP26



**Fig. 4.** Cross-reactivity with other helminthic infections. The IgG antibody levels against (a) SmSEA, (b) SmSerpin, (c) RP26, and (d) the mixture of SmSerpin and RP26 among 131 samples with other helminthic infections than *S. mansoni*. The samples were from patients with schistosomiasis haematobia (Sh; $n = 20$)), schistosomiasis japonica (Sj, $n = 12$), paragonimiasis (Pw; n = 20), fascioliasis (Fh; n = 20), clonorchiasis (Cs; n = 10), sparganosis (Se; $n = 19$), gnathostomiasis (Gd; n = 10), and toxocariasis (Tc; n = 20).

**Table 3**
Cross-reactivity of *S. mansoni* antigens with other helminth infections.

| Antigen | Parasitic infection (number of samples that cross-reacted) | | | | | | | | Non-Schisto (%) | All (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sh ($n = 20$) | Sj ($n = 12$) | Pw ($n = 20$) | Fh ($n = 20$) | Cs ($n = 10$) | Se ($n = 19$) | Gd ($n = 10$) | Tc ($n = 20$) | | |
| SmSEA | 20 | 11 | 9 | 18 | 8 | 8 | 2 | 0 | 45 (45.5) | 76 (58.0) |
| SmSerpin | 11 | 3 | 3 | 4 | 2 | 1 | 2 | 1 | 13 (13.1) | 27 (20.6) |
| RP26 | 20 | 2 | 3 | 5 | 3 | 0 | 1 | 2 | 14 (14.1) | 36 (27.5) |
| SmSerpin-RP26 | 19 | 2 | 3 | 4 | 2 | 0 | 1 | 0 | 10 (10.1) | 31 (23.7) |

The results of IgG antibody binding to SmSEA, SmSerpin, RP26, and the mixture of SmSerpin and RP26 in 131 samples with other helminth infections than *S. mansoni*. The samples were from patients with schistosomiasis haematobia (Sh), schistosomiasis japonica (Sj), paragonimiasis (Pw), fascioliasis (Fh), clonorchiasis (Cs), sparganosis (Se), gnathostomiasis (Gd), and toxocariasis (Tc). The "All" column shows the total number (%) of cross-reactive samples of the 131 test samples. The "non-schisto" column shows the number and percentage of cross-reactive non-schistosoma samples (Pw, Fh, Cs, Se, Gd and Tc).

## 4. Discussion

In this study, we assessed the diagnostic potential of antibody detection assays based on recombinant proteins SmSerpin and RP26 for the detection of low-intensity infections in the *S. mansoni* endemic area, Mbita. These antigens have been shown to be promising candidates for monitoring schistosomiasis in a previous study [20]. In Tanigawa et al. (2015), limited sample size and the insensitive Kato-Katz technique as the reference test were used. Therefore, this study presents an improved design with increased sample size and a sensitive composite reference test to assess the diagnostic performance of these recombinant antigens more accurately.

Almost half of our study participants were positive for infection, while the infection intensity was low. Under such a setting, the POC-CCA test detected a 7.5-fold higher number of infections compared to the Kato-Katz technique (Table 1). This result had a similar trend with the previous study in low-endemic areas after multiple rounds of MDAs [34]. Our observations ascertained that the sensitivity of Kato-Katz technique is insufficient for transmission monitoring in endemic areas with low-intensity infections [14,35]. The POC-CCA test showed 76.5% sensitivity (trace scores regarded as negative, Table 2), which was much functional. Still, part of the light *S. mansoni* infections were under the

detection limit of the POC-CCA test (Table 2). We anticipate that the number of light infections missed by POC-CCA test will increase as the control interventions will be further extended. Under such circumstances, an antibody-based assay for monitoring transmission would be very useful.

We found that the antibody ELISA using *S. mansoni* antigens was a sensitive assay for the detection of infections in the setting with low-intensity infection. SmSEA showed the highest sensitivity of 90.8%, followed by the SmSerpin-RP26 mixture, which showed 83.7% sensitivity (Table 2). These sensitivities were shown to be better than the POC-CCA test. The single recombinant antigens (SmSerpin and RP26) had poorer sensitivity (65.3% and 74.5%, respectively). While the sensitivity of the antibody assay using recombinant antigens was improved by applying a cocktail of the two antigens, the performance of SmSerpin-RP26 still needs to be optimized. Diagnostic performance of an antigen cocktail depends on the antibody response type induced by each antigen [36] and is also assumed to be influenced by antigen competition [37]. Therefore, sensitivity optimization may be achieved by including other antigens or changing number and ratio of antigens to mix [21,37,38]. Although SmSEA showed better sensitivity, the advantage of using recombinant antigens is the achievability of consistent high-quality scalable material and better specificity, which is necessary for mass-surveillance and product development. By using recombinant antigens, products such as ELISA kit or point-of-care kit using finger-prick blood might be achievable in the future. In contrast, crude antigen preparations such as schistosome SEA, which consist of native parasite material, are difficult to standardize and produce in sufficient quantities. Furthermore, SEA production requires upkeeping and maintenance of the schistosome lifecycle, which is very costly.

While sensitivity of a test is extremely important in areas approaching elimination, specificity is also an important parameter to minimize unnecessary re-examination/treatments and to achieve targeted control. Our results show that the specificity of antibody detection of anti-SmSEA was very poor (Table 2), which can be ascribed partly to past infections and partly to cross-reactivity of protein and particularly, also glycan epitopes in this crude antigen mixture [39]. In contrast, the specificities of antibody detections against the recombinant protein antigens were much better. These observations allude that the antibody response to these recombinant antigens reflects active infection. In a previous murine study, anti-SmSerpin and anti-RP26 IgG1 (equivalent to human IgG4) were shown to decline after treatment [40], and it supports the idea that the same phenomenon could occur in humans. Part of the explanation of RP26 specific antibody decreasing post-treatment after clearance of worms could be that this protein is not expressed in eggs [41]. Whereas, schistosome serpin is expressed in all stages [42], and further studies are needed to clarify the dynamics of these antibody responses after treatments in humans. Although low specificity affected by past infection is a limitation to use of antibody tests in endemic areas, its application to young children and/or new outbreak foci will not be restricted by this limitation. In such scenarios, an antibody test based on schistosome-specific recombinant proteins can be both highly sensitive and specific.

Cross-reactivity also affects the specificity of the antibody-based assays. Schistosome crude SEA is a heterogeneous mixture containing numerous antigens [43] and is known to show cross-reactivity with other types of schistosomes and other parasites such as *Fasciola hepatica*, *Paragonimus westermani,* and hookworms [44–47]. In the present study, SmSEA showed extensive cross-reactivity with other parasitic infections (Table 3 and Fig. 4). These cross-reactions were markedly reduced for the recombinant SmSerpin and RP26, although some samples showed high antibody reactions. Serpins have been suggested to be involved in immune regulation and are identified in many parasitic helminths [48]. By BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the amino acid sequence of SmSerpin is similar to serpins of *S. haematobium* (accession no. AAA19730, 76% identical) and *S. japonicum* (accession no. AAA57435, 65% identical). The serpins of *F. gigantica* and *F. hepatica*

(accession no. TPP60406 and THD24816) and *Clonorchis sinensis* (accession no. RJW66304) also have a homologous sequence with SmSerpin. The functions of the protein encoded by cDNA of *S. mansoni* RP26 are unknown, but the sequence has saposin-like domains [41]. Saposin-like domains are also found in proteins of other parasites. As for schistosomes, identical sequences with RP26 are found in saposin containing proteins of *S. haematobium* (accession no. XP_035589485, 77% identical) and *S. japonicum* (accession no. CAX71050, 55% identical). In addition, the alignment of RP26 saposin-like domain has partial identity with clonorin in *Clonorchis sinensis*, Pw10.9 in *P. westermani*, and FhSap-1 in *F. hepatica* [41]. The cross-reactivity with other parasitic infections observed in recombinant protein antigens can possibly be explained by these homologies in the amino acids sequence of SmSerpin and RP26 with other parasites' proteins. In the cocktail-form, the cross-reactivity was reduced, and it might be because of antigen competition by two antigens. In this study, we did not examine our participants in Mbita for other helminth infections, but the improved specificities of the recombinant antigens may also be described by reduced cross-reactivity with other parasitic infections. Unfortunately, the prevalence of STH infections was too low to assess any cross-reactive potential to the recombinant antigens in our study. In Kenya, parasitic infections such as fascioliasis and sparganosis are also transmitted, but the prevalence among humans is unknown [49,50], and further studies are needed to estimate the effects of other parasitic infections on the specificities.

In conclusion, our study demonstrates that the ELISA detecting a mixture of anti-SmSerpin and anti-RP26 IgG has potential for detection of light intensity *S. mansoni* infections in endemic areas. The specificity of the SmSerpin-RP26 mixture was far better than SmSEA, and it is more suitable to be adopted in near-elimination settings. We propose that the antibody tests using the SmSerpin-RP26 cocktail antigen would be useful to monitor transmission in future low endemic, near, and post eliminations setting as the intensified interventions envisioned by the 2030 roadmap push schistosomiasis towards elimination as a public health problem.

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| | AUC (95% CI) |
|---|---|
| SmSEA | 0.716 (0.645-0.788) |
| SmSerpin | 0.720 (0.650-0.791) |
| RP26 | 0.689 (0.615-0.762) |
| SmSerpin-RP26 | 0.772 (0.706-0.837) |