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Research Paper

A Parallel Comparison of Antigen Candidates for Development of an Optimized Serological Diagnosis of Schistosomiasis Japonica in the Philippines

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ABSTRACT

Schistosoma japonicum is stubbornly persistent in China and the Philippines. Fast and accurate diagnostic tools are required to monitor effective control measures against schistosomiasis japonica. Promising antigen candidates for the serological diagnosis of schistosomiasis japonica have generally been identified from the Chinese strain of *S. japonicum*. However, the Chinese (SjC) and Philippine (SjP) strains of *S. japonicum* express a number of clear phenotypic differences, including aspects of host immune responses. This feature thereby emphasized the requirement to determine whether antigens identified as having diagnostic value for SjC infection are also suitable for the diagnosis of SjP infection. In the current study, 10 antigens were selected for comparison of diagnostic performance of the SjP infection using ELISA. On testing of sera from 180 subjects in the Philippines, SjSAP4 exhibited the best diagnostic performance with 94.03% sensitivity and 98.33% specificity using an optimized serum dilution. In another large scale testing with 412 serum samples, a combination (SjSAP4 + Sj23-LHD (large hydrophilic domain)) provided the best diagnostic outcome with 87.04% sensitivity and 96.67% specificity. This combination could be used in future for serological diagnosis of schistosomiasis in the Philippines, thereby representing an important component for monitoring integrated control measures.

1. Introduction

Schistosomiasis japonica is a disease of poverty and remains as a public health issue in China, the Philippines and Indonesia. In China, the epidemiology of schistosomiasis is changing due to extensive integrated control efforts (Collins et al., 2012; Li et al., 2014b). The estimated number of infected people dropped from ~840,000 in 2004 to 185,000 in 2013 (Xu et al., 2016). A number of endemic areas are nearing schistosomiasis transmission interruption (Li et al., 2014b; Xu et al., 2016). In contrast, as of 2010, an estimated 580,000 individuals were reported infected in the Philippines (Rollinson et al., 2013), with very high prevalences recently reported in a number of endemic provinces (Olveda et al., 2016). For China, the need for improved diagnostic tools is urgently required for effective surveillance and determination of elimination; for the Philippines, it is imperative to develop affordable and accurate field diagnostic tools for schistosomiasis control.

There are four major types of methods available for the diagnosis of schistosomiasis: parasitological detection (e.g. the Kato-Katz (KK) method), antibody-detection (AbD), antigen-detection (AgD) and circulating nucleic acids (DNA and RNA) detection (CNAD) (Cai et al., 2016a; Weerakoon et al., 2015). The KK method shows low sensitivity, while AbD based on crude extracted antigens, such as soluble egg antigen (SEA), exhibits cross-reactivity with other helminth infections, which is particularly relevant in many schistosomiasis-endemic areas. There has been considerable focus on the detection of the presence of schistosome ova, or SEA-specific antibodies but these approaches are limited for early diagnosis. More sensitive methods based on PCR technology, including qPCR (Gordon et al., 2015), droplet digital PCR (Weerakoon et al., 2016), and LAMP (Wang et al., 2011a; Xu et al., 2015) have been developed as diagnostic procedures for schistosomiasis. However, the cost of these CNAD tools may limit their practical application for large-scale surveillance of schistosomiasis. Of the available methods, ELISA-based AgD detection has several advantages, such as being affordable, easy-to-operate, there are no requirements for advanced equipment, there is less chance for cross-contamination as reported with PCR methods, such as nested-PCR (Li et al., 2014a) and

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LAMP (Karthik et al., 2014), and provides a balance of sensitivity and specificity once an appropriate antigen is identified and employed.

In this post-genomics era, an increasing number of high-throughput immunological studies have been carried out on schistosomes. These reports have identified a panel of tegumental and excretory-secretory antigens as potential diagnostic targets with high levels of sensitivity and specificity (Chen et al., 2014; Lu et al., 2012; McWilliam et al., 2014; Sangfuang et al., 2016; Xu et al., 2014). For example, Xu et al. identified a saposin-like protein, SjSP-13, as a potential diagnostic candidate based on a genome-wide screening of secretory proteins (Xu et al., 2014), whereas Liu et al. identified other saposin members showing better diagnostic performance than SjSP-13 (Liu et al., 2016). Since different groups employ different human cohorts with variable infection intensity and use different experimental assay conditions, a comparative study was required to compare the clinical diagnostic performance of different candidate *S. japonicum* antigens.

The majority of studies undertaken on the serological diagnosis of schistosomiasis japonica have been carried out using the Chinese strain of *S. japonicum*. However, the Chinese (SjC) and Philippine (SjP) strains of *S. japonicum* show clearly different phenotypes in terms of virulence, fecundity, pathology, drug sensitivity and immunology (Hope et al., 1996; Moertel et al., 2006; Weerakoon et al., 2017). Here, we carried out a comparative study with ten antigens to validate the most promising candidate for diagnosis of schistosomiasis in the Philippines. An optimized formula for serological diagnosis of SjP infection was further suggested based on this parallel comparative study.

2. Materials and Methods

2.1. Ethical Statement

All animal work was conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition) and with the approval of the QIMR Berghofer Medical Research Institute Animal Ethics Committee (Ethics Approval: Project P288). Serum samples from the study participants in the Philippines were collected with informed written consent, and ethical approval was provided by the Institutional Review Board of the Research Institute for Tropical Medicine, Department of Health, Manila, the Philippines (Institutional Review Board Numbers 2012-13-0 and 2015-12) and the Human Research Ethics Committee, QIMR Berghofer Medical Research Institute, Brisbane, Australia (Ethics Approval: Project P524). All serum samples from healthy humans were obtained with informed written consent, and the protocol was approved by the ethics committee of the Institute of Pathogen Biology, Chinese Academy of Medical Sciences (Beijing, China).

2.2. Mice and Parasites

Eight-week-old female BALB/c mice were percutaneously infected with 14 *S. japonicum* cercariae (Chinese mainland strain, Anhui population) or 25 *S. japonicum* cercariae (Philippine strain, Sorsogon population). Blood samples were taken from animals at 4, 6, 7, 9 and 11 weeks post infection (p.i.). Blood samples from five naive mice were used as controls. The liver tissues were collected at 11 weeks post infection. Eggs per gram of liver were calculated as a measure of hepatic egg burden and general infection level, as described (Cai et al., 2015). Six Swiss mice were infected with approximately 25 *S. japonicum* cercariae (Philippine strain). After 6 weeks, these mice were orally administered 150, 200, 250, 300 and 350 mg/kg praziquantel prepared in 2.5% (v/v) Cremophor EL (Sigma, USA) for 5 consecutive days (Chuah et al., 2016). Serum samples were collected before infection and at 2, 4 and 6 weeks post-infection as well as at 1, 2 3, 4, 5, 6 and 7 months after chemotherapy.

2.3. Gene Expression Analysis

A next-generation oligonucleotide microarray was used to determine the expression patterns of the obtained selected target proteins in four developmental stages of *S. japonicum* (eggs, cercariae, hepatic schistosomula and adult worms). The design and construction of the microarray, as well as the hybridization procedures and feature extraction, have been reported previously (Cai et al., 2016b; Cai et al., 2017). Raw data and normalized gene level data from the array have been deposited in the public database Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers for the platform GPL18617, and series GSE57143. The expression pattern of the genes encoding target antigen candidates in the four developmental stages was extracted from the above dataset. A heatmap was generated based on the relative signal intensities of forward probes against the egg stage using HemI 1.0 software.

2.4. Cloning, Expression and Purification of Recombinant Proteins

Primers were designed to amplify a specific region of the target proteins (Supplementary Table 1). The DNA fragments were amplified by PCR from cDNA isolated from adult worms of *S. japonicum* (Philippine strain). After digestion with restriction enzymes, DNA fragments were cloned into the pET-28a vector. Recombinant plasmids were confirmed by sequencing and transformed into *E. coli* BL21 (DE3). Expression of the recombinant proteins was induced by 0.5 mM IPTG. Recombinant proteins were purified under native or denaturing conditions using Ni-NTA agarose (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified recombinant proteins were analyzed by 12% (w/v) SDS-PAGE.

2.5. Western Blotting

Purified recombinant protein samples were loaded onto 12% (w/v) SDS-PAGE and transferred to 0.2-μm PVDF membranes at 100 V for 10 min and then at 100 mA for 1 h using a wet western blotting system. After blocking with 5% (v/v) non-fat milk in PBST for 90 min, the membrane was incubated with a mouse anti-His monoclonal antibody (Sigma-Aldrich Co, MO, USA) at 4 °C overnight. After washing, the membrane was incubated with an HRP-conjugated goat anti-mouse IgG (H + L) antibody (ThermoFisher Scientific, MA, USA) for 1 h at room temperature. SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, MA, USA) was used to detect signals.

2.6. Human Serum Samples

Serum samples from schistosomiasis patients were obtained from Northern Samar, the Philippines. For the first serum cohort ($n = 180$), 67 and 113 patients were confirmed as positively and negatively infected, respectively, in 2012 by examining eggs in stool samples using the Kato-Katz method. The second serum batch ($n = 412$) were obtained from the same endemic area in 2015, two years after a program of mass drug (i.e., 40 mg/kg praziquantel) administration. Of these, 108 and 304 patients were confirmed as positive and negative infection, respectively, using the Kato-Katz method (Olveda et al., 2017). Serum samples of healthy individuals were obtained from Heilongjiang Province, a non-endemic area for schistosomiasis in China.

2.7. Evaluation of Diagnostic Candidates for Schistosomiasis Japonica by ELISA

Recombinant proteins were quantified by the bicinchoninic acid assay (BCA assay) (ThermoFisher Scientific, MA, USA). For determining IgG levels induced in SjC or SjP-infected BALB/c mice, all recombinant proteins were diluted to a final concentration of 1 μg/mL with coating buffer overnight at 4 °C with 100 μL added per well. All wells were

blocked by blocking buffer (1% BAS in PBST) at 37 °C for 1 h. All tested serum samples were diluted at $1:10^2$ with blocking buffer (100 μL/well) and incubated at 37 °C for 1 h. A biotin-SP-conjugated goat anti-mouse IgG (Fc specific)-biotin antibody (Jackson ImmunoResearch, PA, USA) was used as secondary antibody (1:10,000, 100 μL/well) and samples were incubated for 1 h at 37 °C. Streptavidin-HRP (BD Pharmingen, CA, USA) (1:10,000) was then applied to each well (100 μL/well). PBST washes were applied 5 times after each step, 2 min between each step. Reactions were developed using TMB substrate (100 μL/well) for 5 min and stopped using 2 M sodium hydroxide (50 μL/well). Optical density (OD) values were read at 450 nm using a microplate reader, and all tests were run in duplicate on each test plate. A positive antibody response was defined as an OD value higher than 2.1 times the mean of OD values of the serum samples from control mice. To determine IgG titers against the ten antigens in SjP-infected BALB/c mice at 7 weeks p.i., the same procedure was applied except that a series of serum dilutions ($1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$) were used.

For small scale assays, determining the level of human IgG generated against these antigens, all procedures were similar to those described above except that all recombinant proteins were diluted to a final concentration of 1 μg/mL, serum samples were used at two dilutions ($1:10^2$ and $1:10^3$) and a mouse monoclonal anti-human IgG (Fc specific)-biotin antibody (Sigma-Aldrich Co, MO, USA) was employed as secondary antibody (1:20,000, 100 μL/well). In large scale assays with the first human cohort, four dilutions of serum samples (1:100, 1:250, 1:500 and 1:1000) were tested for the detection of IgG antibodies against Sj23-LHD, SjSAP4, SjSAP5, SjSAP4 plus Sj23-LHD, and SjSAP5 plus Sj23-LHD. For the latter two combinations, 50 ng of each antigen were mixed per well. With the second human cohort, only one optimized serum dilution (1:250) was used to detect IgG antibodies against these antigens or antigen combinations. A positive antibody response was defined as an OD value higher than 2.1 times the mean of OD values of the control serum samples from healthy individuals.

2.8. Statistical Analysis

All results are reported as means ± SEM (standard error of the mean). For analysis of the IgG levels generated against the ten antigens during the SjC- or SjP-infection course, one-way ANOVA followed by Holm-Sidak multiple comparison was used. For analysis of IgG levels at each time point between the SjC- and SjP-infection in the BALB/c mice, two-way ANOVA followed by Holm-Sidak multiple comparisons were used to compare statistical differences. For analysis of the worm burden between the SjC and SjP-infections in the BALB/c mice, the Man-Whitney test was used. *P*-values of <0.05 were considered statistically significant. We defined sensitivity and specificity according to the following formulae: sensitivity = number of true positives / (number of true positive + number of false negatives) and specificity = number of true negatives / (number of false positives + number of true negatives).

3. Results

3.1. General Information for the 10 Selected Proteins

Five secretory (SjSP-13, SjSAP4, SjSAP5, SjSP-163 and SjSP-189) and five tegumental-associated (Sj23-LHD, SjSTIP1 (stress-induced phosphoprotein 1), SjPPase (pyrophosphatase), SjPGM (phosphoglycerate mutase) and SjRAD23 (radiation sensitive 23)) proteins were selected for comparison as antigen candidates based on previous studies of SjC (Table 1). The DNA sequences encoding these antigens were cloned from a cDNA library of SjP and protein sequences deduced. Comparative sequence analysis indicated SjSP-189 shared 90% with the SjC homologue, whereas the other nine antigens shared 98–100% identity between the two strains (Table 1).

Table 1
Ten *S. japonicum* antigen candidates selected for comparison.

Antigen	Accession No.	Sequence identity[a]	References
SjSP-13	AY222880	98%[b]	(Liu et al., 2016; Xu et al., 2014)
SjSAP4	FN315320	99%[b]	(Liu et al., 2016)
SjSAP5	AY222887	99%[b]	(Liu et al., 2016)
SjSP-163	AY814943	98%[b]	(Xu et al., 2014)
SjSP-189	AY815838	90%[b]	(Xu et al., 2014)
Sj23-LHD	M63706	100%	(Jiang et al., 2010; Jin et al., 2010; Wang et al., 2011b)
SjSTIP1	AY816102	99%	(Chen et al., 2014)
SjPPase	AY814211	99%	(Chen et al., 2014)
SjPGM	FN315287	100%	(Zhang et al., 2015)
SjRAD23	FN314619	99%	(Zhang et al., 2015)

[a] Between the SjP and SjC orthologs.
[b] Signal peptide sequence excluded.

3.2. Transcriptional Profiling of the ten Antigens in four Developmental Stages

The expression levels of the 10 *S. japonicum* antigen candidates in eggs, cercariae, hepatic schistosomula and adult worms were further analyzed based on data extracted from a next-generation schistosome DNA microarray dataset. SjSP-13 and SjSAP4 were mainly expressed in cercariae, hepatic schistosomula and adults; SjSAP5 and SjSP-189 were most highly expressed in hepatic schistosomula and adults; while one secretory protein, SjSP-163, and five tegumental-associated antigens were consistently expressed across all four stages (Fig. 1a,b).

3.3. Production of Recombinant Proteins

The gene sequences of the ten proteins were confirmed as correct by DNA sequencing. The ten recombinant proteins were successfully expressed in *E. coli*. Four and six recombinant proteins were purified under native and denaturing conditions, respectively (Supplementary Fig. 1). The ten purified antigens were further probed by Western blotting with an anti-his-tag antibody.

3.4. Kinetics of Specific IgG Antibodies in the Sera of S. japonicum-infected Mice

Serum samples from six infected mice were collected prior to infection (0 week) and at 4, 6, 7, 9 and 11 weeks p.i. with SjC or SjP cercariae. The time point when specific IgG became elevated during the course of infection against the ten antigens varied among the different candidates. In SjC-infected mice, significant increases in IgG antibody levels against Sj23-LHD and SjSAP4 were detected from 4 weeks p.i. (1-Way ANOVA, $P < 0.0001$ and $P < 0.001$, respectively) and subsequently, and a significant rise in IgG antibody level against SjSAP5 occurred from 6 weeks p.i. onwards (1-Way ANOVA, $P < 0.0001$); with the remaining seven antigens, a significant increase in specific IgG was observed from 7 weeks p.i. (Fig. 2a). During the course of SjP infection, the IgG antibody level against Sj23-LHD rose significantly from 4 weeks p.i. onwards (1-Way ANOVA, $P < 0.0001$). A significant increase in SjSAP4- and SjSAP5-specific IgG antibody levels was observed at 6 weeks p.i. (1-Way ANOVA $P < 0.01$, and $P < 0.05$, respectively) (Fig. 2b). The IgG levels against SjSP-13 and SjRAD23 increased significantly from 7 weeks p.i. (1-Way ANOVA $P < 0.05$) and 11 weeks p.i. (1-Way ANOVA $P < 0.01$), respectively. With the remaining antigens, a significant increase in specific IgG levels was found at 9 weeks p.i. onwards (Fig. 2b). We carried out comparative analysis of IgG responses during the course of the SjC and SjP infections. These were no differences detected in IgG responses against either Sj23-LHD or SjSAP5 in mice infected with SjC or SjP for any of the time points selected. SjSAP4 elicited a significantly higher level of IgG in the SjC-infected mice from 4 to

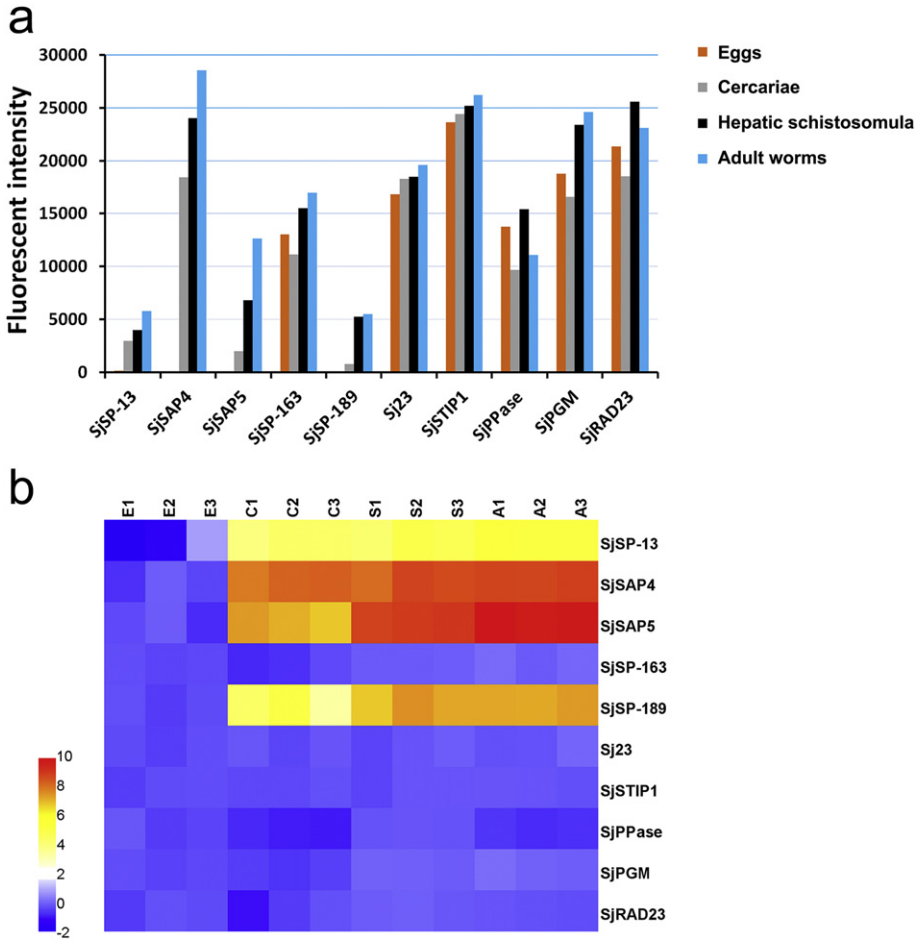

Fig. 1. Expression profiles of the 10 *S. japonicum* antigens in the four developmental stages. (a) The transcriptional profiles of the antigen candidates. The data were extracted from an available next-generation schistosome microarray dataset (Cai et al., 2017). The column represents the mean of normalized fluorescent intensity value. (b) Heatmap showing the relative transcriptional levels of the 10 *S. japonicum* antigen candidates in: E, eggs; C, cercariae; S, hepatic schistosomula; A, adult worms.

7 weeks p.i. Significantly higher IgG levels were induced against SjSP-189 and SjRAD23 in SjC-infected mice at 7 and 9 weeks p.i., while with the other antigens, significantly higher IgG levels were induced in these mice from 7 weeks p.i. onwards (Fig. 3). There was no difference in the worm burden between the SjC- and SjP-infections (Supplementary Fig. 2).

3.5. Determination of IgG Titers against the 10 Antigens in the Sera of SjP-infected BALB/c Mice

We then determined the IgG titer against the 10 antigens in the sera of SjP-infected BALB/c mice at 7 weeks p.i. We found that the highest IgG titer (between $1:10^4$ and $1:10^6$) was raised against Sj23-LHD. High IgG titers (between $1:10^3$ and $1:10^4$) were also induced by SjSAP4 and SjSAP5. With SjSP-189, the IgG titer was between $1:10^2$ and $1:10^3$. Consistent with the results obtained for the IgG kinetics during SjP infection of BALB/c mice, the remaining antigens elicited low IgG titers. Accordingly, Sj23-LHD appeared to be the most sensitive antigen candidate for the diagnosis of schistosomiasis japonica (Supplementary Table 2).

3.6. Initial Evaluation of the Sensitivity of the 10 Antigens for the Diagnosis of Human SjP Infection

For initial screening of the 10 antigens, we used nine serum samples from SjP-infected patients and three healthy individuals as controls. Six antigens exhibited seropositivity being detected at a serum dilution of 1:100, among which SjSAP4 and SjSAP5 exhibited the highest sensitivity, followed by SjSP-13. Unexpectedly, Sj23-LHD-ELISA showed a moderate sensitivity of 55.5%, and four other tegumental proteins, SjSTIP1, SjPPase, SjPGM and SjRAD23, were seronegative with these serum samples in ELISA (Table 2).

3.7. Comparison of Sj23-LHD-, SjSAP4-, SjSAP5-, SjSAP4 + Sj23-LHD- and SjSAP5 + Sj23-LHD-ELISA for Diagnosis of SjP Infection in the First Patient Cohort

Three antigens (Sj23-LHD, SjSAP4 and SjSAP5) were thus selected for diagnosing clinical serum samples on a larger scale. Sj23-LHD was included due to its earlier diagnostic performance, whereas SjSAP4 and SjSAP5 were selected as a result of their high sensitivity in the diagnosis of SjP infection. Also, SjSAP4 + Sj23-LHD and SjSAP5 + Sj23-LHD, were employed to determine whether these two combinations could achieve a better diagnostics performance than the single antigens employed alone. To obtain optimized assay conditions, a series of serum dilutions (1:100, 1:250; 1:500 and 1:1000) was used, since a highly sensitive biotin-streptavidin system was employed in the ELISA (Fig. 4a,b,c,d). After testing the serum samples from the first cohort, we found that 1:250 was the best dilution to achieve a balance of sensitivity and specificity of the assays (Table 3). With this dilution, the SjSAP4-ELISA showed the best performance with a sensitivity of 94.03% for KK-positives and of 72.57% for KK-negatives, and a specificity of 98.33%. This was followed by the SjSAP4 + Sj23-LHD-ELISA, exhibiting 91.04% sensitivity for KK-positives and 67.26% sensitivity for KK-negatives, and a specificity of 96.67%. The Sj23-LHD-ELISA

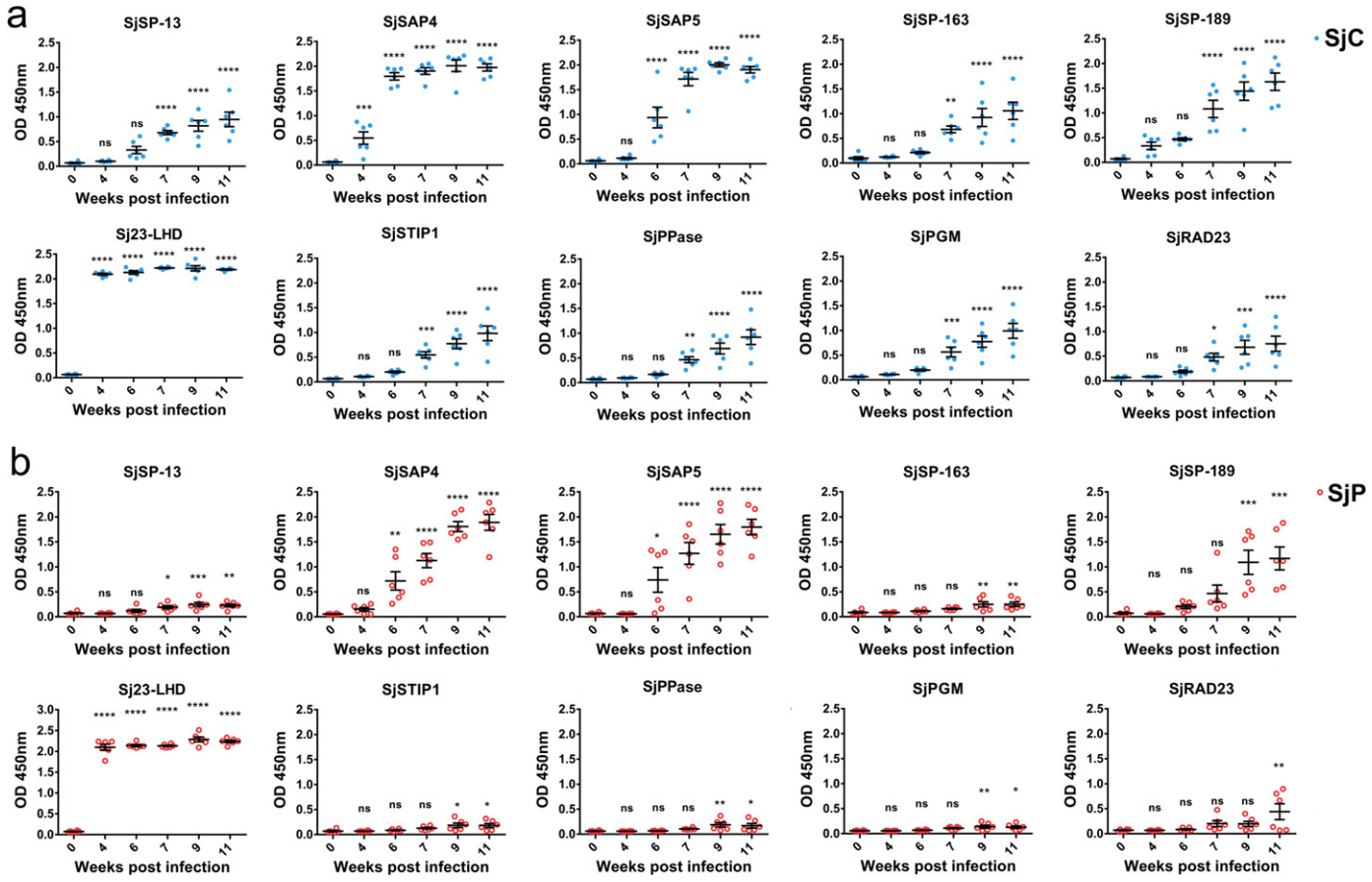

Fig. 2. IgG responses against the 10 antigens during the course of *S. japonicum* infection in BALB/c mice. (a) SjC and (b) SjP infection. Statistical significance between infected and naive mice was determined using 1-Way ANOVA. (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$, ns = no significant difference). Serum dilution: 1:100.

showed quite moderate sensitivity (53.73% for KK-positives and 21.24% for KK-negatives), yet a high specificity (95.00%) (Fig. 4b, Table 3).

3.8. Comparison of Sj23-LHD-, SjSAP4-, SjSAP5-, SjSAP4 + Sj23-LHD- and SjSAP5 + Sj23-LHD-ELISA for Diagnosis of SjP Infection in the Second Patient Cohort

To validate the results, we analyzed serum samples from a larger size cohort with lower intensity *S. japonicum* infections. A comparison of the infection intensities based on EPG between the first and the second cohort is presented in Table 4. With the second cohort, we observed reduced sensitivity, regardless of which antigen or antigen combination was used. In contrast to the results obtained with sera from the first cohort, the SjSAP4 + Sj23-LHD-ELISA exhibited the best diagnostic performance with a sensitivity of 87.04% for KK-positives and 58.55% for KK-negatives, and a specificity of 96.67%, followed by the SjSAP4-ELISA, which showed a sensitivity of 84.26% for KK-positives and of 56.25% for NN-negatives, and a specificity of 96.67% (Fig. 5, Table 5). For KK-negatives, a relatively high positivity rate was observed with the SjSAP4-ELISA (56.25%), the SjSAP5-ELISA (51.64%), the SjSAP4 + Sj23-

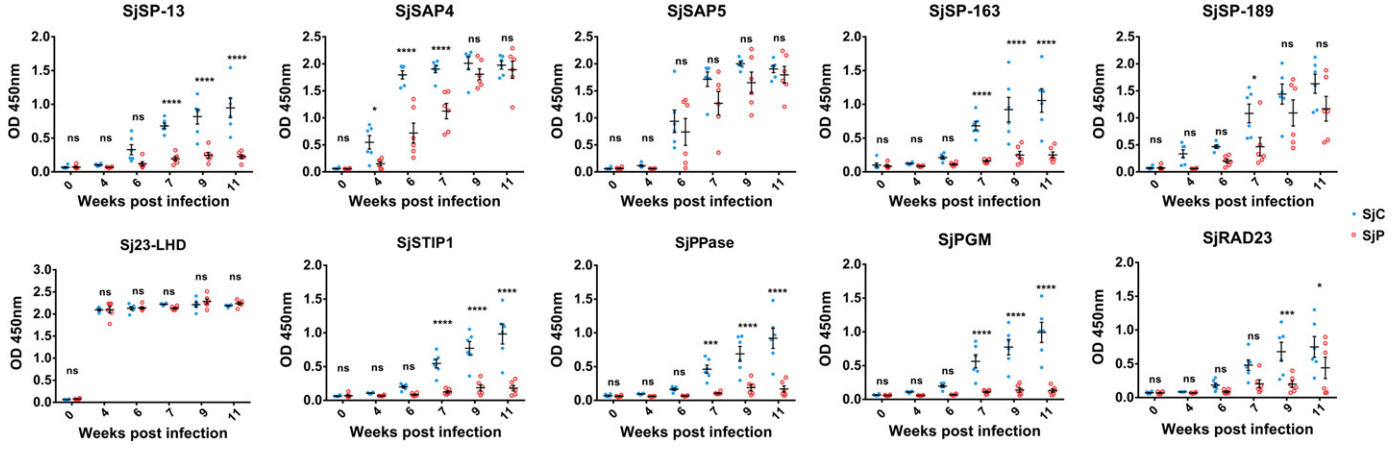

Fig. 3. Comparison of IgG responses against the 10 antigens in BALB/c mice infected with SjC or SjP. Statistical significance was determined using 2-Way ANOVA. (* = $P < 0.05$, *** = $P < 0.001$, **** = $P < 0.0001$, ns = no significant difference).

Table 2
Reactivity of the six selected proteins with 9 serum samples from SjP infected patients with varying infection intensities.

No.	EPG	Antigens (R value)											
		SjSP-13		SjSAP4		SjSAP5		SjSP-163		SjSP-189		Sj23-LHD	
1	43.3	3.92	2.75	20.79	3.36	11.67	5.71	1.66	0.99	1.62	1.03	1.51	0.91
2	10.0	4.73	2.33	31.81	16.47	9.62	5.38	2.09	1.10	7.82	1.65	5.82	1.23
3	6.7	1.72	1.06	31.51	11.01	4.93	3.70	1.27	1.01	15.65	3.68	9.70	1.47
4	2750.0	2.75	1.39	31.51	19.14	9.96	5.42	1.12	1.07	9.31	1.79	20.20	2.64
5	323.3	1.69	1.07	27.32	6.00	11.27	3.97	1.72	1.21	1.85	1.06	1.31	0.92
6	23.3	2.09	1.23	27.28	6.55	5.00	1.98	0.81	0.93	0.93	1.01	0.94	0.97
7	3.3	4.63	1.81	27.49	6.62	12.46	5.52	2.49	1.02	2.24	1.05	1.43	0.93
8	186.7	5.47	3.37	31.98	24.67	13.39	9.48	3.84	1.12	3.33	1.15	4.86	1.04
9	650.0	5.20	3.40	28.13	14.70	12.41	5.34	3.44	1.05	3.54	1.33	11.58	1.78
Sensitivity		66.7%	44.4%	100%	100%	100%	88.9%	33.3%	0%	66.7%	11.1%	55.5%	11.1%
Serum dilution		$1:10^2$	$1:10^3$	$1:10^2$	$1:10^3$	$1:10^2$	$1:10^3$	$1:10^2$	$1:10^3$	$1:10^2$	$1:10^3$	$1:10^2$	$1:10^3$

R value = $OD450_{patient}$/Mean of $OD450_{healthy}$.
Serum samples with R value ≥ 2.1 were considered as seropositive.
EPG: eggs per gram of faeces.

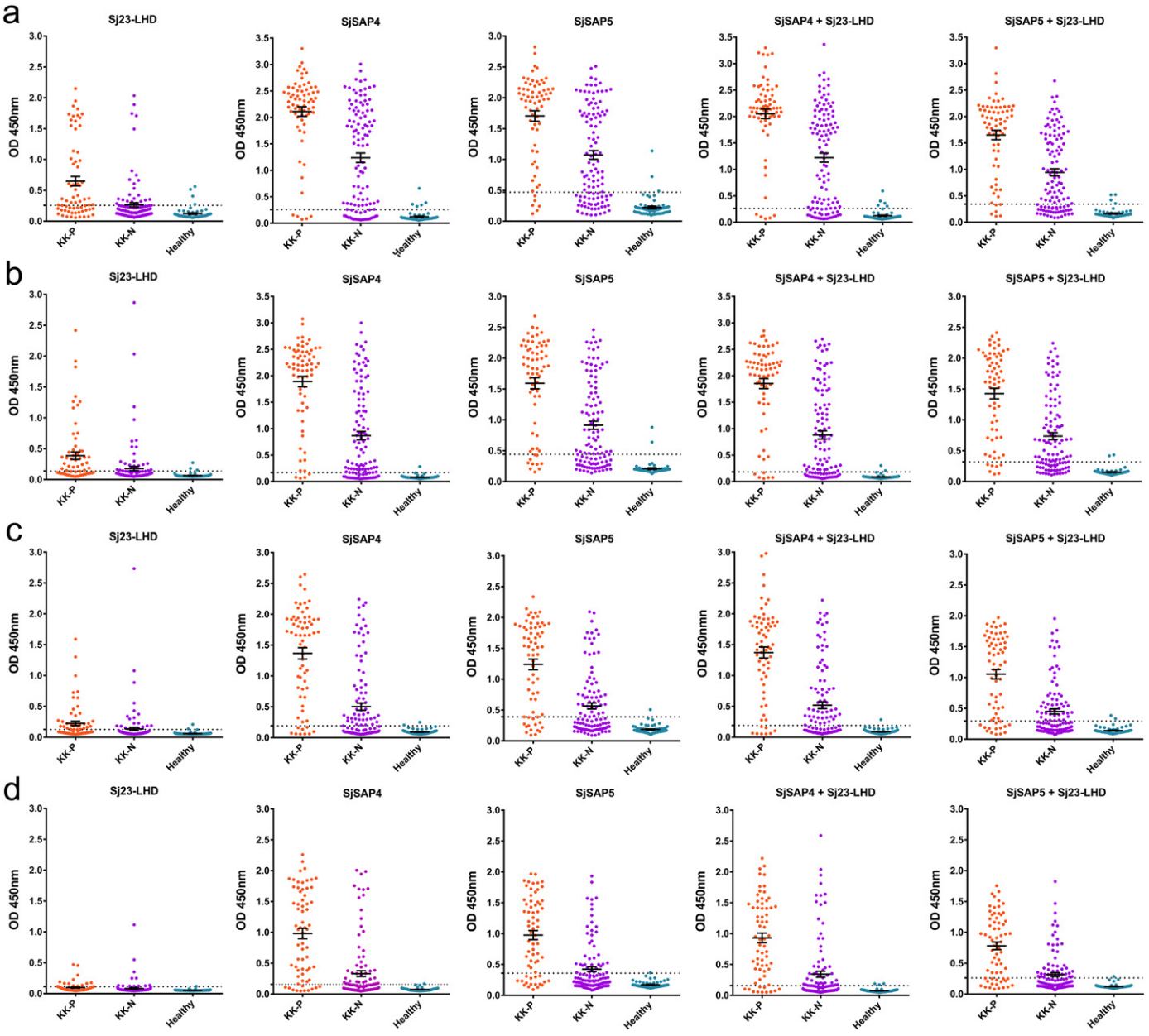

Fig. 4. Scatter plots showing the responses of Sj23-LHD-, SjSAP4-, SjSAP5-, SjSAP4 + Sj23-LHD- and SjSAP5 + Sj23-LHD-ELISA for the diagnosis of schistosomiasis japonica. Different serum dilution (a) 1:100, (b) 1:250; (c) 1:500; (d) 1:1000 were used for optimization. 'KK-P': Kato-Katz positive; 'KK-N': Kato-Katz negative.

Table 3
Sensitivity and specificity of Sj23-LHD-, SjSAP4-, SjSAP5-, SjSAP4 + Sj23-LHD- and SjSAP5 + Sj23-LHD-ELISA tested against 180 serum samples from the first SjP patient cohort.

Serum dilution	Antigens	Sensitivity (KK-positive $n = 67$)	Sensitivity (KK-negative $n = 113$)	Specificity
1:100	Sj23-LHD	61.19%	22.12%	93.33%
	SjSAP4	92.54%	75.22%	91.67%
	SjSAP5	91.04%	66.37%	95.00%
	SjSAP4 + Sj23-LHD	92.54%	76.99%	91.67%
	SjSAP5 + Sj23-LHD	91.04%	68.14%	93.33%
1:250	Sj23-LHD	53.73%	21.24%	95.00%
	SjSAP4	94.03%	72.57%	98.33%
	SjSAP5	85.07%	61.95%	96.67%
	SjSAP4 + Sj23-LHD	91.04%	67.26%	96.67%
	SjSAP5 + Sj23-LHD	89.55%	65.49%	96.67%
1:500	Sj23-LHD	46.27%	16.81%	96.67%
	SjSAP4	86.57%	50.44%	96.67%
	SjSAP5	76.12%	45.13%	98.33%
	SjSAP4 + Sj23-LHD	88.06%	53.10%	98.33%
	SjSAP5 + Sj23-LHD	79.10%	46.90%	96.67%
1:1000	Sj23-LHD	19.40%	10.62%	98.33%
	SjSAP4	83.58%	38.05%	98.33%
	SjSAP5	74.63%	33.63%	98.33%
	SjSAP4 + Sj23-LHD	82.09%	39.82%	95.00%
	SjSAP5 + Sj23-LHD	73.13%	37.17%	98.33%

LHD-ELISA (58.55%) and the SjSAP5 + Sj23-LHD-ELISA (49.01%) (Fig. 5, Table 5). Stool and serum samples, collected from the same cohort, were subjected to a sensitive droplet digital PCR (ddPCR) assay, which detects *Sjnad1*, a cell-free DNA marker for diagnosis of SjP infection (Weerakoon et al., 2017). A positive ddPCR rate of 66.12% and 57.57% were obtained for KK-negatives when testing the stool and serum samples, respectively (Supplementary Table 3).

3.9. Antibody Responses against Sj23-LHD, SjSAP4 and SjSAP5 before and after Drug Treatment in a Mouse Model

To compare the duration of Sj23-LHD-, SjSAP4- and SjSAP5-specific IgG antibodies post chemotherapy, six Swiss mice were infected with cercariae of the SjP strain, and after 6 weeks, all mice were treated with praziquantel for 5 consecutive days. The levels of IgG antibodies to the three antigens were determined at 2, 4, and 6 weeks p.i. and 1–7 months after completion of the drug treatment in the mice. In five of the six mice, the Sj23-LHD-specific IgG antibody started to drop at 2 months post drug treatment and decreased to relatively low levels at 7 months post-chemotherapy. In only one out of six mice, did the levels of SjSAP4- and SjSAP5-specific IgG antibodies decrease at 7 months post-chemotherapy (Fig. 6).

4. Discussion

While remarkable success has been achieved in combating schistosomiasis japonica in China, it is still a devastating disease in the Philippines with high prevalences and morbidity reported in several provinces (Olveda et al., 2016; Rollinson et al., 2013; Xu et al., 2016).

Table 4
Comparison of *S. japonicum* infection intensity in the two patient cohorts.

EPG	1st cohort (KK positive n = 67) n (percentage)	2nd cohort (KK positive $n = 108$) n (percentage)
>400	5 (7.46%)	0 (0.00%)
100–400	13 (19.40%)	4 (3.70%)
40–99	5 (7.46%)	8 (7.41%)
10–39	21 (31.34%)	18 (16.67%)
<10	23 (34.33%)	78 (72.22%)

EPG: eggs per gram of faeces.

A major obstacle preventing its effective management in the Philippines may, in part, be due to the scarcity of affordable and accurate diagnostic tools and ELISA-based serological diagnosis remains an attractive option to achieve this goal. Currently, antigens for diagnosis of *S. japonicum* infection predominantly use antigens identified in the SjC strain. A validation was thus required before applying SjC antigen candidates for the diagnosis of SjP infections which we report here.

In this study, we used serum samples from mice and patients to compare the diagnostic performance of 10 antigen candidates selected based on studies undertaken on SjC. However, murine models of schistosomiasis cannot completely reflect authentic immunoreactivities in patients. Firstly, the levels of IgG antibodies induced by the 10 antigens were only observed for 11 weeks p.i. since the BALB/c mouse infections were terminated for ethical reasons. Secondly, in mice, the presence of only one worm pair represents a high infection dose if body weight is taken into consideration. In murine models of schistosomiasis, the dynamic IgG levels against different antigens vary during the course of infection. Sj23, being a tegumental antigen, is exposed to the host directly after invasion and induces a rapid immune response as was observed here and in previous investigations (Jiang et al., 2010; Krautz-Peterson et al., 2017), and has been suggested as a potential diagnostic target for early detection (Wang et al., 2011b). We further showed that, following drug treatment, that the level of Sj23-LHD-specific IgG antibodies dropped more rapidly than those generated against SjSAP4 and SjSAP5. Theoretically, the diagnostic performance of Sj23-LHD should have been superior to SjSAP4 and SjSAP5 based on the results obtained in mice. However, this was not the case when clinical serum samples were tested. The low sensitivity of the Sj23-LHD-ELISA, using serum samples from KK-positive subjects, indicates that Sj23-LHD-specific IgG levels may decline in patients during the late course of an infection, since the infection intensity is lower than in mice. In contrast, the two saposin family members, SjSAP4 and SjSAP5, being intestinal tract secretory proteins (Liu et al., 2016), may be released slowly into the host circulation thereby inducing longer lasting antibody responses.

Differing results were obtained when testing serum samples from the two human Philippines cohorts. Firstly, diminished sensitivity was observed when the second cohort was tested, regardless of which antigen or antigen combination was used. This is likely due to the comparatively lower *S. japonicum* infection intensity levels of the second cohort compared with the first cohort based on the egg burden of those individuals positive by the Kato-Katz procedure. In contrast to the first cohort, where about 30% KK-positive individuals had an EPG < 10, this number was >70% in the second cohort. Secondly, with the first cohort, the SjSAP4-ELISA exhibited the best diagnostic performance, followed by the SjSAP4 + Sj23-LHD-ELISA, whereas this diagnostic outcome was reversed with the second cohort. The reason for including Sj23-LHD as a diagnostic marker was to contribute to the detection of individuals with early infections of *S. japonicum*. We did find individuals in the second cohort showing positive serological reactivity against Sj23-LHD, but negative against SjSAP4 and SjSAP5. Mass praziquantel drug administration had been applied to the second cohort 2 years prior to blood collection, but re-infection may have occurred subsequently in these individuals. This reflects the current epidemiological picture in *S. japonicum*-endemic areas of the Philippines (Inobaya et al., 2015), which strengthens the case for including Sj23-LHD to increase the sensitivity of available diagnostic assays and the earlier detection of schistosomiasis which no doubt will contribute to its monitoring and control.

For KK-negative individuals, relatively high positivity rates were observed in the ELISAs, which necessitated using an alternative diagnostic tool to validate these serological results. Detection of cell-free DNA (cfDNA) has proven a reliable and sensitive diagnostic tool for human parasitic infections (Weerakoon and McManus, 2016). Accordingly, we thus employed a novel ddPCR developed by our group (Weerakoon et al., 2017) to probe serum samples from the second cohort, and found similar positivity rates when the serum samples were tested by this method (Supplementary Table 3), which substantiates

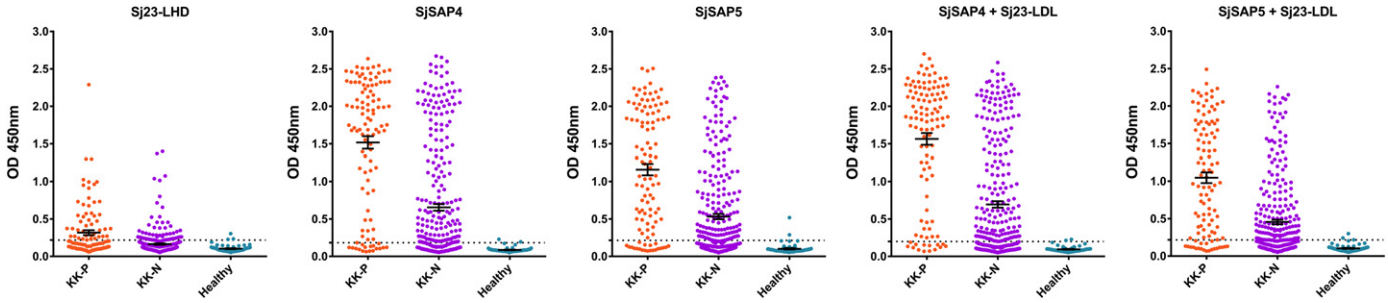

Fig. 5. Scatter plots showing the responses of the Sj23-LHD-, SjSAP4-, SjSAP5-, Sj23-LHD + SjSAP4- and Sj23-LHD + SjSAP5-ELISAs for the diagnosis of schistosomiasis japonica with serum samples from the second patient cohort. A serum dilution of 1:250 was used in the assays. 'KK-P': Kato-Katz positive; 'KK-N': Kato-Katz negative.

the serology data we report. It is noteworthy that conflicting results regarding the duration of detectable schistosome cfDNA in mammalian hosts after drug treatment have been reported. It has been shown by one group that DNA (a 230-bp fragment of the highly repetitive retrotransposon *SjR2* of *S. japonicum*) was undetectable by conventional PCR in the serum of SjC-infected rabbits 10 weeks after a single PZQ treatment (Xia et al., 2009). In contrast, another group reported that the time to total elimination of *Schistosoma*-derived cell-free DNA fragments from plasma following treatment was projected to exceed one year (Wichmann et al., 2009). These conflicting studies are cautionary, suggesting that the time point when the detection of parasite-derived cfDNA becomes negative after chemotherapy depends on multiple factors, such as infection intensity, the concentration of the target cfDNA, the sample volume for DNA extraction, and the sensitivity of the PCR method used. We propose that the KK-negatives may have harboured a low intensity infection (likely due to unsatisfactory treatment/drug compliance issues (Olveda et al., 2016)), in the early stage of a re-infection or antibodies against the target antigens remained from a previous infection.

For several of the selected antigens we found inconsistent diagnostic performance for detecting SjC or SjP infection both with mouse and human serum samples. We found that in mice there was no significant difference in worm burden between the SjC and SjP infections, but the IgG responses induced by SjSP-13, SjSP-163, SjSTIP1, SjPPase and SjPGM were significantly different from 7 weeks p.i. onwards in the two types of infection (Fig. 3). Given these antigens are expressed in the gastrodermis or tegument, the process of vesicle-mediated transport may play an important role in the localization of these proteins. Previously, it has been shown that some of the components associated with vesicle-mediated transport have lower copy number in SjP compared with SjC worms (Gobert et al., 2013), which may impact on the exposure of these antigens to the host. Further, the rSjSTIP1- and rSjPPase-ELISAs showed a sensitivity of 80% and 90%, respectively, on testing a small number of patient sera with SjC-infection (Chen et al., 2014). rSjPGM- and rSjRAD23-ELISA achieved a sensitivity of 91.35% and 88.46%, respectively, when sera from SjC-infected buffaloes were tested (Zhang et al., 2015). However, these antigens had no diagnostic value for detecting SjP infection. These differing results may have been due to the variable *S. japonicum* infection intensities among the different samples. But it is more likely to reflect differences in the host response against the two *S. japonicum* strains. In addition, the rSj23-LHD-ELISA exhibited 89.9% sensitivity when probing sera of SjC-infected bovines (Jin et al., 2010), but here, a sensitivity of only 42.59–53.73% was achieved when sera from SjP patients were tested. The reactivity of SjSP-13 with sera from humans infected with SjC parasites previously showed circa 90% sensitivity (Liu et al., 2016; Xu et al., 2014), whereas a sensitivity of only 66.7% was achieved when tested with 9 sera from SjP-infected individuals. These findings emphasize the importance of validating SjC antigen homologues for the diagnosis of SjP infections.

Table 5
Sensitivity and specificity of the Sj23-LHD-, SjSAP4-, SjSAP5-, Sj23-LHD + SjSAP4- and Sj23-LHD + SjSAP5-ELISA for diagnosis of 412 serum samples from the second patient cohort.

Serum dilution	Antigens	Sensitivity (KK-positive n = 108)	Sensitivity (KK-negative *n* = 304)	Specificity
1:250	Sj23-LHD	42.59%	18.09%	95.00%
	SjSAP4	84.26%	56.25%	96.67%
	SjSAP5	80.56%	51.64%	96.67%
	SjSAP4 + Sj23-LHD	87.04%	58.55%	96.67%
	SjSAP5 + Sj23-LHD	80.56%	49.01%	95.00%

Further, the poor diagnostic performance of SjSP-163, SjSP-189, SjSTIP1, SjPPase, SjPGM and SjRAD23 triggered us to re-consider the criteria for candidate antigen selection based on their subcellular localization, relative expression levels and biological functions. SjSP-163, being a putative orthologue of the endoplasmic reticulum-residing protein, nucleotide exchange factor SIL1, acting as a molecular chaperone facilitating protein folding, translocation and degradation (Yan et al., 2011), may have less chance to be exposed to the host immune system. The transcript level of SjSP-189 in the intravascular stage was similar to that of SjSP-13, but lower than those of other antigens (Fig. 1). STIP1 acts as a co-chaperone of Hsp70 and Hsp90 (Cross et al., 2016), and the subcellular localization of schistosome TIP1 was suggested by the results of a previous proteomic study showing that Hsp70 and Hsp90 are cytosolic proteins in *Schistosoma bovis* (Perez-Sanchez et al., 2008). Inorganic PPases are highly conserved enzymes responsible for catalyzing the hydrolysis of inorganic pyrophosphate (PPi) into two molecules of orthophosphate (Pi), providing a thermodynamic driving force for a variety of biosynthetic reactions, such as DNA, RNA, protein, polysaccharide, and lipid synthesis (Guimier et al., 2016). There are two groups of PPases: soluble PPases and membrane-bound PPases, but the latter only occurs in algae, plants, and selected species of protozoan parasites, bacteria and archaea (Nita et al., 2016). PGM is involved in the energy metabolism required for parasite activity and survival, and it has been found that the PGM ortholog in *S. bovis* is localized to the cytosol (Perez-Sanchez et al., 2008). RAD23 is known to be associated with DNA-damage repair (Ng et al., 2003), so it seems most likely that SjRAD23 may be located in the nuclei of the subtegumental cell bodies of *S. japonicum*. Although these latter four antigens (SjSTIP1, SjPPase, SjPGM and SjRAD23) have been suggested to be tegument-associated (Chen et al., 2014; Zhang et al., 2015), they may not be directly exposed to the host vascular system. Tegumental debris shed from the blood flukes may be the source of these antigens resulting in the induction of low levels of antibodies. Consequently, the most important selection criteria for diagnosis are that a candidate antigen should be continuously released into or exposed to the bloodstream of the host by an intact viable parasite and that it has a relatively high level of expression and antigenicity.

An inevitable dilemma in using serology for schistosomiasis diagnosis is its limited ability to distinguish between ongoing and previous schistosome infections. Here we investigated IgG titers generated

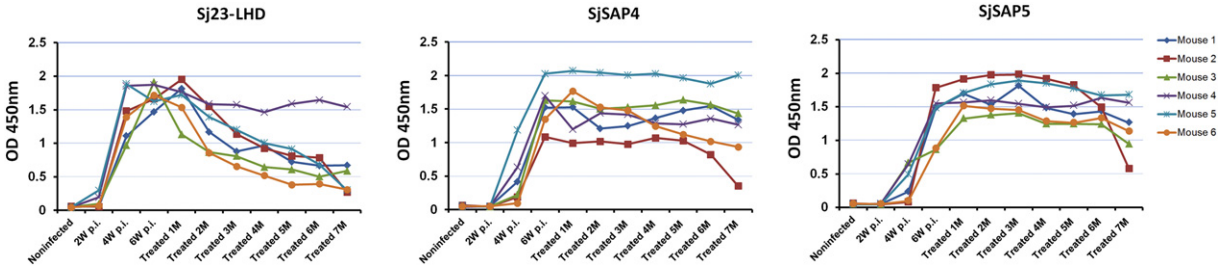

Fig. 6. ELISA detection of Sj23-LHD, SjSAP4- and SjSAP5-specific IgG antibodies in the sera of Swiss mice prior to and post drug treatment (Serum dilution for Sj23-LHD-, SjSAP4- and SjSAP5-ELISA: 1:10,000).

against Sj23-LHD, SjSAP4 and SjSAP5 over several months in PZQ-cured mice previously infected with *S. japonicum*. While IgG responses to Sj23-LHD declined with time after treatment, those to SjSAP4 and SjSAP5 in the main did not (Fig. 6). As mentioned earlier, clinical infections in the Philippine are now mostly low intensity, so IgG titers against SjSAP4 and SjSAP5 in cured patients may not last as long as those observed in mice, but this still needs to be evaluated. In this context, further steps (such as adjusting the ratio of antigens in the combination ELISA) to optimize the assay to improve the ability to distinguish active from past infections are warranted.

In summary, our findings highlight the fact that during the course of a SjC and SjP infection, different IgG levels may be induced by antigen orthologs. Three antigens, Sj23-LHD, SjSAP4 and SjSAP5, elicited high IgG titers in both SjC- and SjP-infected BALB/c mice. SjSAP4 exhibited the best diagnostic performance when probing sera from the first SjP patient cohort with relatively high infection intensity. With the second cohort, where the majority of infections were of low intensity and early stage infection may have occurred, the combination of SjSAP4 + Sj23-LHD yielded the best diagnostic performance. This combination could be used in future for serological diagnosis of schistosomiasis japonica in the Philippines, thereby representing an important component for monitoring and sustaining integrated control measures.

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Conflicts of Interest

All authors: No reported conflicts.

Author Contribution

P.C. and D.P.M. conceptualized the study design and directed the project; P.C. and Y.M. performed all the experiments; P.C., S.L., X.P. and Q.C. contributed to the DNA chip data; K.G.W., D.U.O., X.P., R.M.O., Q.C. and A.G.R. contributed to the collection of clinical samples; P.C., K.G.W., Y.M. and D.P.M. analyzed, reviewed and interpreted the data; P.C. drafted and D.P.M. revised the manuscript. All authors approve of the final version of the manuscript.

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Appendix A. Supplementary data

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