JOURNAL OF PROTEOMICS 98 (2014) 289–299

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An integrated immunoproteomics and bioinformatics approach for the analysis of *Schistosoma japonicum* tegument proteins

*Jun-Hu Chen**, *Ting Zhang*, *Chuan Ju*, *Bin Xu*, *Yan Lu*, *Xiao-Jin Mo*, *Shen-Bo Chen*, *Yan-Ting Fan*, *Wei Hu**, *Xiao-Nong Zhou**

National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Key Laboratory of Parasite and Vector Biology of the Chinese Ministry of Health, WHO Collaborating Center for Malaria, Schistosomiasis and Filariasis, Shanghai 200025, People's Republic of China

ARTICLE INFO

Article history:
Received 26 March 2013
Accepted 8 January 2014
Available online 19 January 2014

Keywords:
Schistosoma japonicum
Tegument
Antigen
Immunoproteomics
Bioinformatics

ABSTRACT

Schistosomiasis remains one of the major neglected tropical diseases (NTDs) causing morbidity of humans residing in the tropical countries. Much effort has been devoted to the development of vaccines, since it is recognized that vaccines can be served as an important supplementary component alongside chemotherapy for the future control and elimination of schistosomiasis. To accelerate digging new potential target antigens, it is essential to extensively and intensively search immunogenic proteins in a high-throughput manner using proteomics-microarray techniques. In the present study, an integrated immunoproteomics and bioinformatics approach was used to profile the tegument of the human blood fluke *Schistosoma japonicum*. Results showed that the full-length tegument proteins were high-throughput cloned and expressed and screened with sera from *S. japonicum*-infected patients and normal subjects using protein arrays. Here, thirty highly immunoreactive tegument proteins and 10 antigens with an AUC value greater than 0.90 were identified at first time. In particularly, STIP1, the highest immunoreactive tegument protein has been shown good antigenicity and immunogenicity, and thus makes it to be a potential target for designing anti-parasite drug or vaccine.

Biological significance
The schistosome tegument plays a crucial role in host–parasite interactions and there are several tegument proteins that proved to be potential vaccine candidates. However, vaccines are not yet available, thus it is important to identify new target antigens from schistosome tegument proteome. Herein, we demonstrate that the *S. japonicum* tegument proteins were analyzed by an integrated immunoproteomics and bioinformatics approach. We found that thirty highly immunoreactive tegument proteins and 10 antigens with an AUC value greater than 0.90 were identified for the first time. In particularly, we found 17 of tegument immunoproteomes having putative interaction networks with other proteins of *S. japonicum*. The results will provide clues of potential target molecules for vaccine development and biomarkers for diagnostics of schistosomiasis.

* *Corresponding authors at:* National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, 207 Rui Jin Er Road, Shanghai 200025, People's Republic of China. Tel.: +86 21 34241232.
E-mail addresses: junhuchen@hotmail.com (J.-H. Chen), huw@fudan.edu.cn (W. Hu), ipdzhouxn@sh163.net (X.-N. Zhou).

http://dx.doi.org/10.1016/j.jprot.2014.01.010
1874-3919/

1. Introduction

Schistosomiasis remains one of the major neglected tropical diseases (NTDs) causing morbidity of humans residing in tropical countries, with an estimated 779 million people at risk and 207 million infected people worldwide [1]. Schistosomiasis is usually associated with some severe clinical symptoms and the full impact of schistosomiasis in the world is no less than that caused by other tropical diseases [2–4]. Nevertheless, schistosomiasis is still a NTD, as the transmission of the disease is only marginally affected by chemotherapy, and rapid re-infection easily sustains its prevalence [5]. Indeed, schistosomiasis japonica had re-emerged in several areas where transmission control and elimination had been declared in P. R. China [6]. In spite of the existence of highly effective praziquantel (PZQ) for treatment, there are certain limitations in the disease transmission control by chemotherapy alone, e.g. mass treatment does not prevent re-infections, and the potential risk of parasite resistance has been greatly concerned when repeated mass chemotherapy was delivered in a long term. Therefore, vaccination strategies represent an important supplementary component alongside chemotherapy for sustainable control of schistosomiasis [7].

Understanding the immune response to schistosome infection, both in animal models and in humans, may accelerate the development of a vaccine. For example, the radiation-attenuated (RA) schistosome antigen remains the most effective reagent inducing high levels of protective immunity against schistosome in various animal models (e.g. rodents, pigs and non-human primates) [8–10]. Recently, remarkable efforts were made in finding recombinant antigens with protective efficacy. Immunization with the recombinant proteins of *Schistosoma mansoni*, tetraspanin 2 (SmTSP-2), stomatin-like protein 2 (SmStoLP-2) and SmRho in mice could induce protective immunity with significant reduction of adult worm and liver egg burdens [11–14]. Similar pattern was also observed with *S. japonicum* recombinant proteins including SjTP22.4, insulin receptor 2 and UDP-glucose 4-epimerase protein [15–17]. However, all these antigens reported previously could not produce highly effective immunity for clinical use until now. Apparently, to identify new target antigens should be pursued using the information derived from schistosome genome and proteome studies [18].

PZQ damages the adult schistosome tegument exposing the surface antigens to the host immune system, inducing specific host cellular and humoral immune responses to kill the worms [19,20], resulted in some resistance to re-infection and prevention from the development of severe disease forms of *S. mansoni* and *S. haematobium* [19]. It is the most obvious evidence that the schistosome tegument plays a crucial role in the interaction of parasite and host immune system, therefore, the tegumental proteins are recognized as prime antigens in searching for vaccine candidates [21]. However, the tegument proteins have not yet been extensively and intensively searched. To this end, the proteomic studies are able to identify hundreds of tegumental proteins, which might speed up the development of vaccines against schistosomiasis [22].

In this study, an integrated immunoproteomics and bioinformatics approach was applied to profile tegument proteins of *S. japonicum*, which provide a high-throughput manner for screening hundreds of proteins at one time for immunogenic antigens of interest leading to the clues of potential vaccine target molecules and biomarkers for diagnostics.

2. Materials and methods

2.1. Serum sample collection

Serum samples were obtained from patients in the annual schistosomiasis surveillance in Anhui province, where *S. japonicum* is endemic. The study was approved by the Ethics Committee of the National Institute of Parasitic Diseases (NIPD), China CDC. The study protocol, potential risks, and benefits were explained to the villagers. After their consent to perform the study, the field workers visited the enrolled families, where detailed information was provided to all participants, and questionnaires were applied. All participants in a given household provided written informed consent.

Collection of serum samples was conducted with the approved protocol. All personal identifiers and medical information were delinked from the serum samples and concealed. Stool specimens were examined with the Kato–Katz thick smear procedure as described [23]. Normal human serum samples used as control in the study were collected in Shanghai, a non-endemic region.

2.2. PCR amplification and In-Fusion cloning

The genes encoding full-length tegument proteins were selected from the previous database of *S. japonicum* proteome (http://function.chgc.sh.cn/sj-proteome/index.htm) [22]. A total of 200 unique genes encoding tegument proteins of *S. japonicum* were used for PCR amplification and In-Fusion cloning. Among them, 54 proteins have specific protein domain analyzed by SMART (Simple Modular Architecture Research Tool) software (http://smart.embl-heidelberg.de/) [24], 19 proteins have signal peptide (http://www.cbs.dtu.dk/services/SignalP/) and 16 proteins have transmembrane domain [24,25].

Gene-specific primers were designed with Invitrogen OligoPerfect Designer (http://tools.invitrogen.com/content.cfm?pageid=9716) [26,27]. The nucleotide sequences with the signal peptide were excluded from the gene expression constructs [25]. Each gene-specific sense and antisense primers were converted into In-Fusion PCR primers for extending at the 5′-terminus with the sequence 5′-GGG CGG ATA TCT CGA G-3′ and 5′-GCG GTA CCC GGG ATC CTT A-3′, respectively. Plasmid DNA and ds cDNA of *S. japonicum* were used for PCR amplifications of target genes according to the previous reports [26,27].

The pEU-His vector (derived from pEU, CellFree Sciences, Matsuyama, Japan) was used for In-Fusion cloning. The vector was first linearized by double digestion with restriction enzymes *Xho* I and *Bam*H I (Takara, Japan) and cloned with PCR products in a high-throughput manner as described previously [26,27]. A target colony was selected for plasmid

preparation using the Midi Plus™ Ultrapure plasmid extraction system (Viogene, Taipei, Taiwan), according to the manufacturers' instructions. Highly purified DNA was eluted in 50 μl 0.1 × TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and the concentration was tested by a NanoVue Plus Spectrophotometer (GE Healthcare).

2.3. High-throughput expression of *S. japonicum* proteins and western-blot analysis

S. japonicum proteins were expressed by a wheat-germ cell free (WGCF) expression system, using the bilayer translation reaction method which was described previously [28]. The total fraction of 42 proteins was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The separated proteins were transferred to 0.45 μm PVDF membranes (Millipore, Billerica, MA). After blocking with 5% skim milk in TBS/T, Penta·His antibody (QIAGEN) and secondary HRP-conjugated goat anti-mouse IgG (Pierce) were used to detect His-tagged recombinant proteins. The immunoblots were incubated with Diaminobenzidine (DAB) and the results were documented by a ScanJet 5300C Scanner (Hewlett-Packard).

2.4. Protein expression analyzed by protein array

OPEpoxy glass slides (75 × 25 mm) were used for protein arrays (CapitalBio, Beijing, China). Teflon tapes with 200 holes (25 × 8, diameter 1.5 mm) each were attached to the modified glass slides to prepare well-type amine arrays [26]. 1 μl of each crude *S. japonicum* protein solution was spotted to each well of the arrays and incubated for 2 h at 37 °C. In addition, the wells spotted with purified His-tagged *S. japonicum* saposin (unpublished data) and wheat germ lysate without any plasmid vector were set as the positive and negative controls, respectively. The array was first blocked with 5% bovine serum albumin (BSA) in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at 37 °C and incubated with Penta·His antibody (1 μl, 10 ng/μl) in PBS-T for 1 h at 37 °C. The reactions were visualized with Alexa Fluor 546 goat anti-mouse IgG (10 ng/μl, Invitrogen) in PBS-T for 1 h at 37 °C and scanned in a fluorescence scanner (LuxScan 10 K, CapitalBio, Beijing, China) [27]. Fluorescence intensities of array spots were quantified by the fixed circle method using ScanArray Express software version 4.0 (PerkinElmer). The positive cut-off value was calculated as the mean fluorescence intensity value of the negative controls plus two standard deviations (SD).

2.5. Serum screening using protein arrays

Sera samples of pooled 10 *S. japonicum* patients and pooled 10 normal subjects were used for primary screening on well-type amine arrays. Briefly, 1 μl of each protein solution was spotted in duplicate to each well of the microarrays and incubated for 2 h at 37 °C. The chips were then probed with the pooled serum samples (1:200) which were pre-absorbed against wheat germ lysate (1:10) to block the anti-wheat germ antibodies. The bound antibodies were visualized with Alexa Fluor 546 goat anti-human IgG (10 ng/μl, Invitrogen) in PBS-T and quantified as described above. The higher response of a probed protein against the patient serum sample was considered to be positive, when the relative ratio of signal intensity (SI) was >2.0 compared with the response to the normal sera. The Benjamini–Hochberg method was used to correct the false discovery rate using MULTTEST procedure in version 8.0 of SAS/STAT software [29].

After primary screening, 30 proteins with high immunoreactivity were screened again with 10 individual positive serum samples and 10 individual normal serum samples which were used to constitute pooled samples in the primary screening. The second screening was run on one array for further confirmation.

2.6. Cloning, production, and purification of recombinant STIP1 and PPase

The genes encoding top 2 immunogenic proteins (STIP1, SJCHGC06661 and PPase, SJCHGC07024) were cloned into pET-28a vector using In-Fusion PCR primers between two restriction sites: *Bam*H I and *Xho* I. The vectors were then transformed into *Escherichia coli* strain BL21 (DE3) for expression of the recombinant STIP1 and PPase proteins. Bacteria were cultures in LB broth at 37 °C to an A_{600} nm of 1.4. Expression was induced with 0.7 mm isopropyl 1-thio-β-D-galactopyranoside (IPTG), and bacteria were grown for a further 9 h at 37 °C. The suspension was harvested by centrifugation at 5,000 rpm (4 °C) for 8 min and the pellet was resuspended in Ni column buffer A (300 mM NaCl, 50 mM phosphate buffer pH 7.4, 10 mM imidazole, 10% glycerol) containing 5 mM PMSF and lysed by sonication. The lysate was clarified by centrifugation 13,000 rpm (4 °C) for 30 min, and the supernatant was filtered by 0.22 μm filter membrane and purified by an affinity chromatography column of nickel-nitrilotriacetic acid (Ni-NTA) resin, using an ÄKTA purifier 10 (GE Healthcare). The eluted protein was

Table 1 – Efficiency of PCR amplification and cloning of parasite genes.

	PCR (%)	Cloning methods	
		In-Fusion cloning (%)	Homologous recombination (%)
S. japonicum	97.0% (194/200)	99.0% (192/194)	–
P. vivax[a]	90.8% (99/109)	92.9% (92/99) *	–
P. falciparum[b]	79.2% (152/192)	90.8% (138/152) *	–
Schistosoma[c]	88.1% (244/277)	–	100.0% (244/244)
P. falciparum[d]	96.0% (922/960)	–	97.0% (715/737)

[a, b, c, d] The results of PCR amplification and cloning of parasite genes were published in the previous reports [26,27,33,34].
* $p < 0.05$.

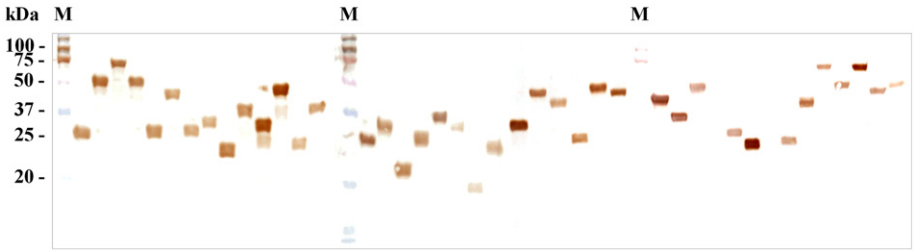


Fig. 1 – Western blot analysis of the expression level of *Schistosoma japonicum* proteins. Forty-two of *S. japonicum* proteins were detected by anti-His$_6$-tag antibody.

dialyzed overnight against 50 mM Tris–HCl pH 7.4, 100 mM NaCl, 10% glycerol. The purified proteins were separated by SDS-PAGE under reducing conditions and transferred to 0.45 μm PVDF membranes (Millipore, Billerica, MA). After blocking with 5% skim milk in TBS/T, Penta·His antibody (QIAGEN) and secondary HRP-conjugated goat anti-mouse IgG (Pierce) were

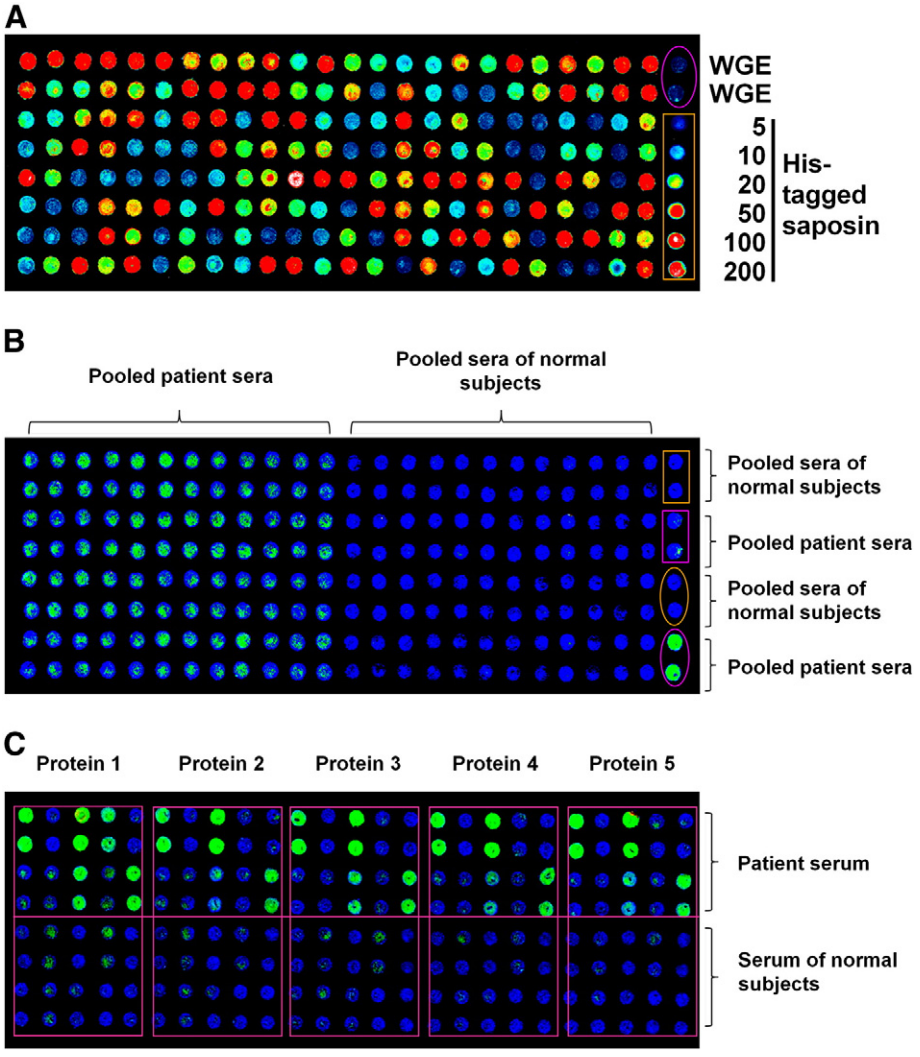


Fig. 2 – Antibody profiling of *Schistosoma japonicum* tegument proteins by protein arrays. A), Protein arrays probed with anti-His$_6$-tag antibody and detected by Alexa Fluor 546 goat anti-mouse IgG. Control reactions of wheat germ lysate (WGE) that lacked vector template (circle) and reactions of purified His-tagged *S. japonicum* saposin (rectangular box). B), Crude *S. japonicum* tegument proteins react with pooled serum from naturally exposed *S. japonicum* patients (left panel) and pooled serum from normal subjects (right panel). Wheat germ lysate that lacked vector template (hexagons) and purified saposin (circles) probed with patient and normal serum served as inner negative and positive controls, respectively. C), Crude *S. japonicum* tegument proteins with high immunoreactivity were screened again with sera from these 10 cases of naturally exposed *S. japonicum* patients (top panel) and 10 normal subjects (bottom panel) together on one array.

used to detect His-tagged recombinant proteins. The immunoblots were incubated with DAB.

The recombinant STIP1 and PPase proteins were detected in the pooled serum from 10 cases of *S. japonicum*-exposed individuals and unexposed subjects (1:200), respectively. Bound antibodies were detected by incubation in secondary HRP-conjugated goat anti-human IgG (Sigma) and the immunoblots were incubated with DAB. The results were documented by a ScanJet 5300C Scanner.

2.7. Inducing polyclonal antibodies against recombinant STIP1 and PPase

Eight to ten week-old C57 mice were subcutaneously inoculated with 20 μg of recombinant STIP1 and PPase emulsified in Freund's complete adjuvant (FCA) on day 0. Two boosters were given on days 14 and 28, using the same proteins emulsified in Freund's incomplete adjuvant (FIA), PBS was set as the negative control. Animal care was performed in accordance with institutional guidelines. Animal sera were collected at days 28. A previously described ELISA test was used for determining inoculated protein immunogenicity. Briefly, each of the inoculated proteins was coated in 96-well ELISA plates and incubated at 1:200 dilution of each serum and a HRP-conjugated goat anti-mouse IgG (Pierce) was used as the secondary antibody.

2.8. Data analysis

Differences in proportions were compared using a χ^2 test. The area under the receiver operating characteristic (ROC) curve (AUC) and a two-tailed unpaired Student *t*-tests were used to evaluate the immunoreactivity of the recognized antigens by GraphPad Prism software, version 5.0 (GraphPad, San Diego, CA). Statistical differences of $p < 0.05$ were considered significant. The heatmap of antibody responses was drawn using the TIGR multi-array experiment viewer (MeV) software [30]. Data of conserved cysteine residues was analyzed by WebLogo (http://weblogo.berkeley.edu/logo.cgi) [31]. Interaction networks of tegument proteins were analyzed by STRING tools (http://string-db.org/) [32].

3. Results

3.1. PCR amplification of genes and In-Fusion cloning

The design flow of In-Fusion cloning primers for the PCR amplification has shown in [26]. Out of 200 *S. japonicum* genes selected for PCR amplification, 194 genes were amplified successfully (Table 1). Four colonies per transformation were selected at random for screening by colony PCR to evaluate the efficiency of In-Fusion cloning. The screening results showed that of the 194 genes screened, 192 were successfully cloned (99.0%), which is significantly high than the efficiency of the cloning of *Plasmodium* genes ($p < 0.05$; Table 1). However, the results were consistent with the reports by other studies for high-throughput construction of expression vectors of *Plasmodium falciparum* and *Schistosoma* using the homologous recombination in *E. coli* [33,34].

3.2. High-throughput expression of *S. japonicum* proteins

To confirm the expression level of the *S. japonicum* proteins expressed by WGCF, approximately one fifth (i.e. 42/192) of the recombinant *N*-terminal His_6-tag fusion proteins were detected by western blot with Penta·His antibody specific to the His_6-tag epitope of the His_6-tagged fusion proteins. Forty of the 42 open reading frames (ORFs) (95.2%) yielded protein products (Fig. 1). Though the genes from *S. japonicum* have higher A/T contents (64% in average) than that of *P.vivax* (58% in average), the tegument proteins of *S. japonicum* are easily expressed by WGCF system [35]. Moreover, this result was similar to data reported by our previous studies for high-throughput expression of *Plasmodium* proteins by WGCF [26,27].

3.3. Antibody profiling by protein arrays

We evaluated the efficiency of *S. japonicum* protein expression with the Penta·His antibody by comparing protein arrays (Fig. 2A). About 98.4% (189/192) of the *S. japonicum* proteins were positive for the His_6-tags on the array indicating successful expression. Two arrays coated with the target proteins were probed with the pooled positive and pooled normal serum samples, respectively (Fig. 2B, left panel and right panel). The positive serum showed significantly higher

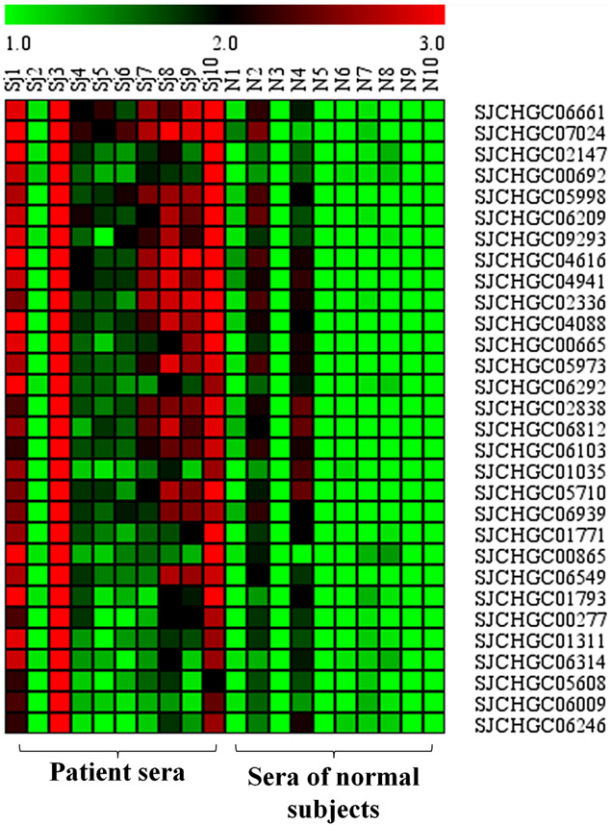


Fig. 3 – Tegument immunogenic proteins of *Schistosoma japonicum*. The immunoreactivities of the proteins probed with serum samples from *S. japonicum* patients (Sj1–Sj10) and normal subjects (N1–N10) are depicted as a heatmap with antigen IDs listed on the right.

reactivity to the WGCF expressed proteins, while the normal serum with low reactivity. After primary screening, 30 proteins with high immunoreactivity were re-screened, but using the individual serum samples from *S. japonicum* patients and normal subjects on the same array (Fig. 2C). The patterns of antibody response to the antigens were obviously different between the wells probed with positive and normal serum samples.

3.4. The immunogenic proteins of *S. japonicum* tegument

The profiles of 30 highly immunoreactive tegument proteins (15.0% of total target proteins) are shown in Fig. 3, and the SI (signal intensity) of the reactivity of each antigen by individual serum samples is shown in a colorized matrix. All of the 30 tegument proteins identified are previously uncharacterized *S. japonicum* antigens. Fifteen of these proteins were recognized by more than 5 individual patient sera. Moreover, one protein (SJCHGC07024) was recognized by 9 individual patient sera (90%). Phosphoglycerate kinase (SJCHGC09293) was recognized by 7 individual patient sera (70%), which was a common antigenic protein in human parasitic worms. This protein has already been found in the patients of *S. mansoni* and *Clonorchis sinensis* [36,37]. A 78 kDa glucose-regulated protein (GRP78, SJCHGC06292) was recognized by 4 individual patient sera (40%), which has identified to be a homologue protein of *Leishmania donovani* with strong immunogenicity [38].

The AUC value 1.0 denotes a perfect differentiation of reactivity generated by patient sera and normal control, respectively. An AUC of 0.5 for an antigen suggests that the distribution of intensity values of patient sera and normal control cannot be distinguished. Above 0.5, the higher the AUC value of an antigen, the better differentiation is likely made between the reactivities presented by patient sera and normal control [33]. When ranked by AUC values, all of the 30 antigens have an AUC value greater than 0.70, among them 10 antigens (33.3%) > 0.90 ($1.36e{-}4 < p > 5.5e{-}3$), 17 antigens (56.7%) > 0.80 ($9.41e{-}4 < p > 2.58e{-}2$) and 3 antigens (10%) > 0.7 ($3.01e{-}2 < p > 7.11e{-}2$) (Table 2). One protein (STIP1, SJCHGC06661) with TPR/STI1 domain has a highest AUC value (0.95, $p = 1.36e{-}4$) and could be recognized by 8 patients' sera.

From a total of 192 screened *S. japonicum* proteins in the array for antibody response, 8 tegument proteins contain specific protein domain namely TPR domain/STI1 domain, ACTIN domain, A1pp domain, PDZ domain, RRM domain, S1 domain, PHB domain and SapB domain, respectively (Table 2). When gene family and domain variations were compared between schistosomes and metazoans, it has shown that ~1,000 protein domains have been abandoned by *S. japonicum*, including some involved in basic metabolic pathways and

Table 2 – Features of *S. japonicum* tegument immunogenic proteins.

Protein ID	AUC	*p* value	Recognition [a]	MW (kDa)	pI	SP [b]	TMD [c]	Protein domain [c]
SJCHGC06661	0.95	1.36E−04	80%	36.2	6.09	No	0	TPR/STI1 domain
SJCHGC07024	0.93	2.63E−04	90%	32.7	5.93	No	0	No
SJCHGC02147	0.93	5.50E−03	40%	53.6	5.76	Yes	0	No
SJCHGC00692	0.93	2.10E−03	30%	41.7	5.30	No	0	ACTIN domain
SJCHGC05998	0.91	3.72E−04	70%	13.6	4.68	Yes	0	No
SJCHGC06209	0.91	1.63E−03	70%	20.9	7.70	No	0	A1pp domain
SJCHGC09293	0.90	1.40E−03	70%	44.3	7.06	No	0	No
SJCHGC04616	0.90	6.94E−04	70%	21.0	9.30	No	0	PDZ domain
SJCHGC04941	0.90	6.38E−04	70%	22.3	5.40	No	0	No
SJCHGC02336	0.90	1.23E−03	60%	38.7	7.31	No	0	No
SJCHGC04088	0.89	9.41E−04	60%	13.4	9.73	No	0	No
SJCHGC00665	0.89	2.79E−03	50%	38.9	5.88	No	0	No
SJCHGC05973	0.88	1.47E−03	60%	19.2	7.86	No	0	No
SJCHGC06292	0.88	1.50E−02	40%	71.5	5.03	Yes	0	No
SJCHGC02838	0.87	2.23E−03	60%	58.6	8.87	Yes	0	No
SJCHGC06812	0.87	2.55E−03	60%	16.2	5.46	No	0	No
SJCHGC06103	0.86	2.16E−03	60%	33.9	7.17	No	0	No
SJCHGC01035	0.86	2.58E−02	30%	23.2	11.58	No	0	RRM domain
SJCHGC05710	0.86	2.64E−03	50%	37.0	4.68	No	0	S1 domain
SJCHGC06939	0.86	1.82E−03	50%	20.6	8.84	No	0	No
SJCHGC01771	0.85	5.40E−03	40%	28.3	7.35	No	0	No
SJCHGC00865	0.85	1.12E−02	30%	46.1	5.77	No	0	PHB domain
SJCHGC06549	0.83	2.66E−03	50%	14.5	4.37	No	0	No
SJCHGC01793	0.83	1.35E−02	40%	44.2	9.24	No	0	No
SJCHGC00277	0.82	1.91E−02	50%	16.0	5.92	No	1	SapB domain
SJCHGC01311	0.81	1.42E−02	30%	55.7	6.51	Yes	0	No
SJCHGC06314	0.81	2.54E−02	30%	22.4	8.28	No	0	No
SJCHGC05608	0.79	3.01E−02	30%	32.9	8.19	No	0	No
SJCHGC06009	0.77	3.47E−02	30%	26.7	5.50	No	0	No
SJCHGC06246	0.72	7.11E−02	30%	28.7	11.35	No	0	No

[a] Total number of positive antibody reaction with individual 10 patient serum samples.
[b] SP, signal peptide was analyzed by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/).
[c] TMD, transmembrane domain and protein domain were analyzed by SMART (http://smart.embl-heidelberg.de/).

defense, implying that loss of these domains could be, at least partly, a consequence of the adoption of a parasitic way of life [35].

Proteins containing SapB domain are popularly seen in the schistosome genome and proteome. By searching through the complete *S. japonicum* genome sequence data, 14 genes identified in this study were found to be conformable to the characteristics of a gene family containing 21 SapB domains (Fig. 4A), the saposin-like protein (SAPLIP) family, which composed of distantly related polypeptides that have six conserved cysteine residues forming three disulfide bridges (Fig. 4B). SAPLIPs have been reported from the gastrodermis of related flukes, and at least one is hemolytic and a promising vaccine antigen [39].

3.5. Interaction networks of *S. japonicum* tegument immunogenic proteins

Interaction networks of *S. japonicum* tegument immunogenic proteins were analyzed and 17 highly immunoreactive proteins (56.7%, 17/30) were found to be interactive with at least one protein of *S. japonicum*, in which 12 proteins have interactions with more than 10 other proteins. Interaction networks of the highest immunogenic tegument protein (STIP1, SJCHGC06661) with other proteins of *S. japonicum* were shown in Fig. 5A. The interaction behavior of other 3 tegument proteins including heat shock protein HSP 90-alpha 2 (HSP90, SJCHGC00820), DnaJ homolog subfamily A member 1 (HSP40, SJCHGC01085) and heat shock 70 kDa protein 9 precursor (HSP70, SJCHGC06312) was noted though these tegument proteins have low immunoreactivity (Fig. 5B). Generations of compounds with high affinity and specificity for different cochaperone proteins can be used as chemical probes to dissect the complex Hsp90–cochaperone interactions, with the potential to be developed into novel antiparasitic drugs [40].

3.6. Antigenicity and immunogenicity of the recombinant STIP1 and PPase

SDS-PAGE analysis showed that the bands of purified recombinant STIP1 and PPase proteins appeared at about 36 kDa and 32 kDa, respectively, which were similar to the predicted size (Fig. 6A&B). Furthermore, western blot analysis showed the specific bands of STIP1 and PPase detected by anti-His antibody (Fig. 6A&B). The recombinant STIP1 and PPase proteins were also detected in the pooled serum from 10 cases of *S. japonicum*-exposed individuals rather than unexposed subjects (Fig. 6A&B), which indicated the naturally acquired humoral immune responses of recombinant STIP1 and PPase against *S. japonicum* infections.

Antibody titers of the recombinant STIP1 and PPase proteins were determined in immunized C57 mice. Stronger antibody titers were generally observed in the recombinant

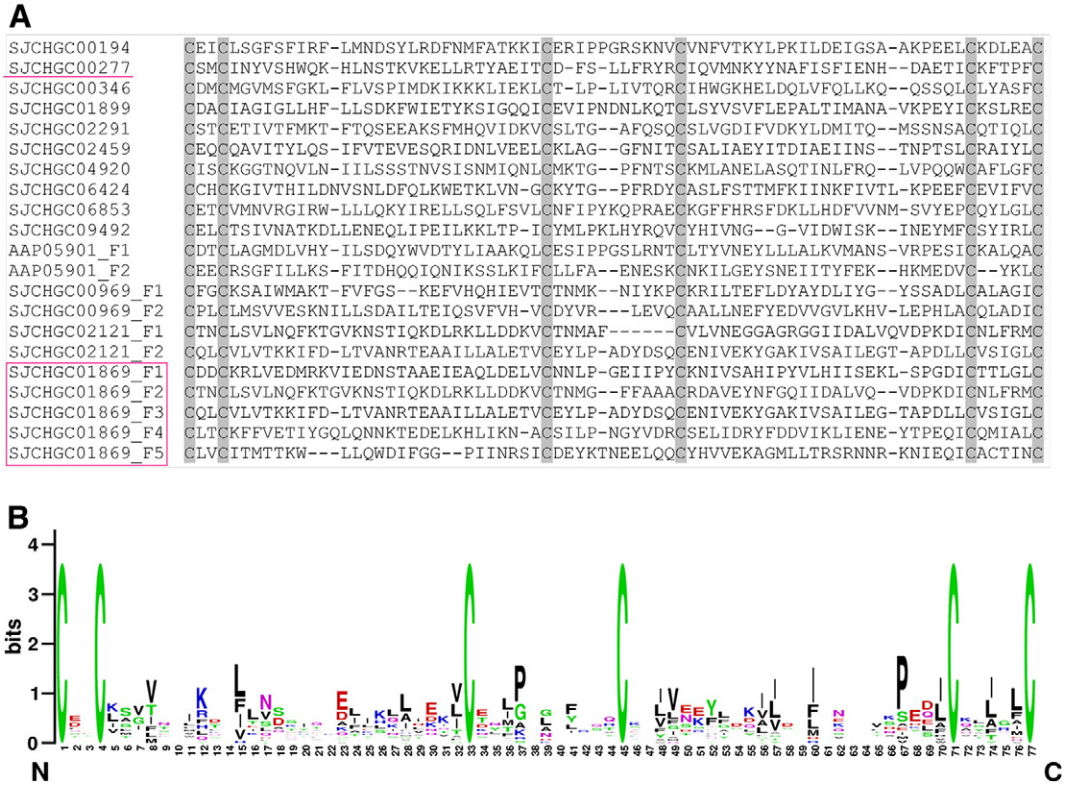
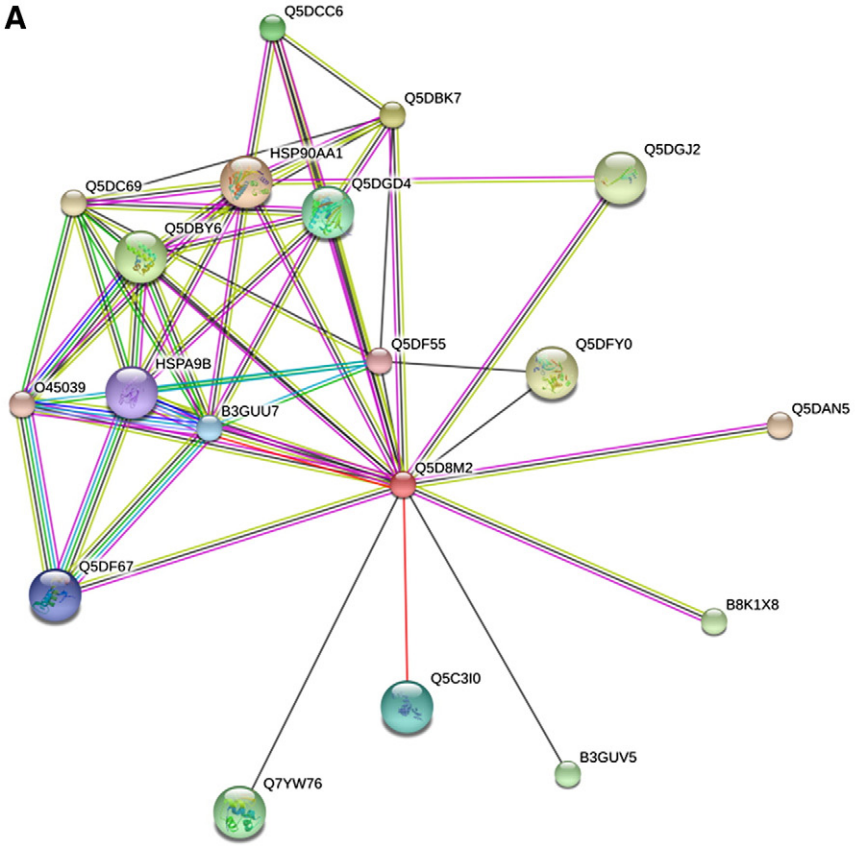


Fig. 4 – Saposin-like proteins of *Schistosoma japonicum*. A), The alignment of Saposin-like proteins of *S. japonicum*. Tegument proteins are marked with pink underline and rectangular box. B), The corresponding sequence logo reveals the conserved features. The invariable cysteine residues are displayed in green. Amino acids are colored according to their chemical properties: polar amino acids are green, basic are blue, acidic are red and hydrophobic amino acids are black.

STIP1 and PPase protein immunized mice (mean $OD_{450\ nm}$, 0.51 and 0.51) than PBS immunized mice (mean $OD_{450\ nm}$, 0.05 and 0.02) with significant difference ($p < 0.001$) (Fig. 6C). The formulations have been shown good immunogenicity of the recombinant STIP1 and PPase proteins.

4. Discussion

Most commercially available vaccines rely specifically on the induction of neutralizing antibodies that block the function of



B

Your Input:

Q5D8M2	SJCHGC06661 protein (319 aa) *(Schistosoma japonicum)*	

Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
HSP90AA1	SJCHGC00820 protein (719 aa)				•	•		•		0.890
Q5DBK7	SJCHGC01423 protein (343 aa)				•			•		0.875
Q5DBY6	SJCHGC01085 protein (400 aa)				•	•		•		0.810
Q5DCC6	SJCHGC02843 protein (188 aa)					•				0.803
Q5DGD4	SJCHGC06677 protein (797 aa)					•				0.688
Q5C3I0	SJCHGC08637 protein (129 aa)			•						0.647
B3GUU7	Putative uncharacterized protein (648 aa)							•		0.634
Q5DF67	SJCHGC06021 protein (335 aa)									0.588
HSPA9B	SJCHGC06312 protein (653 aa)							•		0.576
Q5DF55	T-complex protein 1 subunit alpha (551 aa)				•					0.524
O45039	HSP70 (512 aa)							•		0.523
Q5DAN5	SJCHGC01391 protein (431 aa)									0.489
Q5DC69	10 kDa heat shock protein, mitochondrial (Hsp10); Eukaryotic CPN10 homolog which is essential f [...] (102 aa)				•					0.488
Q5DFY0	Glycylpeptide N-tetradecanoyltransferase (EC 2.3.1.97); Adds a myristoyl group to the N-termina [...] (463 aa)				•					0.475
Q5DGJ2	SJCHGC09289 protein (390 aa)							•		0.465
B8K1X8	p50 immunophilin (EC 5.2.1.8) (424 aa)									0.451
B3GUV5	Putative uncharacterized protein (134 aa)				•					0.428
Q7YW76	Endothelial differentiation-related factor 1 (134 aa)				•					0.402

Fig. 5 – Analysis of the interaction networks between highest immunogenic tegument protein (SJCHGC06661) and other proteins of *Schistosoma japonicum*. A), Map of interaction networks between SJCHGC06661 and other proteins of *S. japonicum*. B), Detail description of *S. japonicum* proteins involving the interaction network. Three tegument proteins we expressed, heat shock protein HSP 90-alpha 2 (HSP90, SJCHGC00820), DnaJ homolog subfamily A member 1 (HSP40, SJCHGC01085), and heat shock 70 kDa protein 9 precursor (HSP70, SJCHGC06312) interact with tegument protein (SJCHGC06661) which has six TPR domains and one STI1 domain.

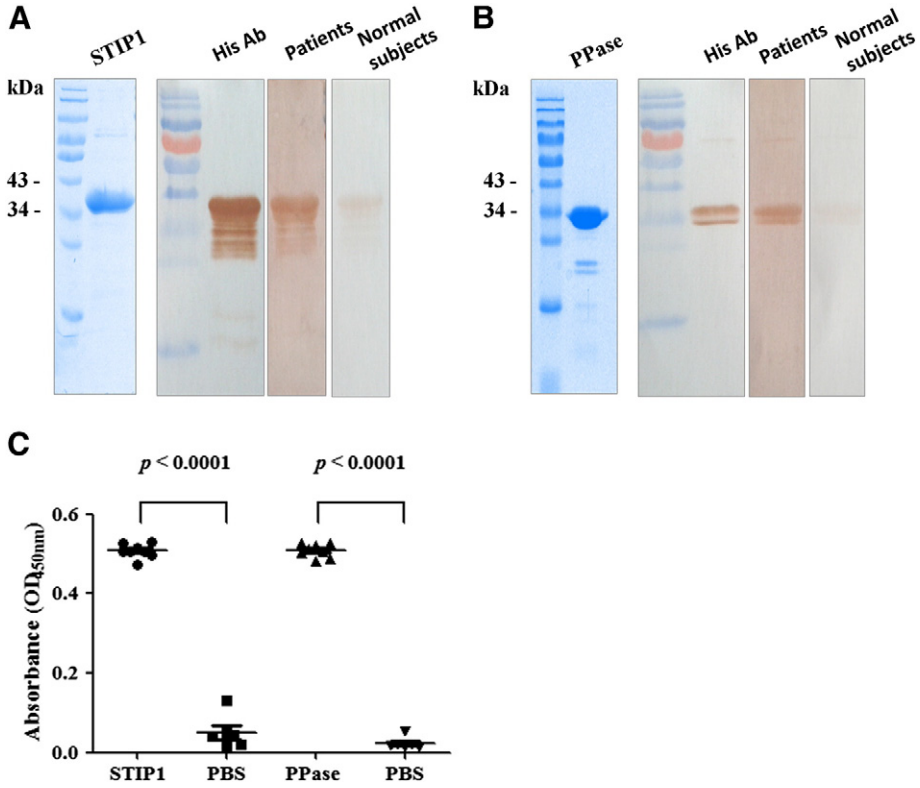


Fig. 6 – Antigenicity and immunogenicity of the recombinant STIP1 and PPase proteins of *Schistosoma japonicum*. A), SDS-PAGE analysis of the expression of recombinant STIP1 (left panel) and western blot analysis of the recombinant STIP1 against anti-His antibody, *S. japonicum*-exposed individuals and unexposed subjects, respectively (right panel). B), SDS-PAGE analysis of the expression of recombinant PPase (left panel) and western blot analysis of the recombinant PPase against anti-His antibody, *S. japonicum*-exposed individuals and unexposed subjects, respectively (right panel). C), Stronger antibody titers were generally observed in the recombinant STIP1 and PPase protein immunized mice than PBS immunized mice ($p < 0.0001$).

their target proteins, and this appears to also be the case for helminth vaccines, where neutralizing antibodies block proteins that have biological roles [5]. PZQ treatment damages the adult schistosome tegument and then resulting in some resistance to re-infection of *S. mansoni* and *S. haematobium*, thus it is important to identify new target antigens from schistosome tegument proteome [19].

The ability of protein array technique to detect specific antibody responses to a number of antigens in parallel in a high-throughput way has significant implications for vaccine target discovery [34,41]. Recently, protein arrays were used to characterize antibody reactivity profiles of *P. vivax* and *P. falciparum* infection [26,27]. Protein arrays were also used to characterize antibody reactivity profiles with the serum from *Schistosoma* infected rabbits [34,42]. In the present study, the same platform of protein arrays was used to characterize antibody reactivity profiles of *S. japonicum* infection in human. From 192 tegument proteins screened by protein arrays, 30 (15.6%, 30/192) highly immunoreactive proteins were identified. All the highly immunoreactive tegument proteins were not characterized from previous literatures.

Many surface proteins share similar characteristics containing cysteine-rich domains that are of potential significance in adherence and vaccine targets. They include 6-cys family of *Plasmodium* and the surface antigen (SAG) related sequence (SRS) super-family of *Toxoplasma* [43,44]. Recently, lytic proteins were identified from two liver fluke species: clonorin from *C. sinensis* and FhSAP1 and FhSAP2 from *Fasciola hepatica* [45,46]. Recombinant FhSAP2 showed protective efficacy against challenge infection with *F. hepatica* and provided cross-protection in mice experimentally infected with *S. mansoni* [47]. The results indicate that SAPLIPs have the potential as attractive broad-spectrum protective targets for parasite vaccines. One *S. mansoni* SAPLIP (Sm-SLP-1) is expressed in the gastrodermis of the worm, which is immunogenic in humans and mice, but not protective in its current form. However, there was no experimental evidence of immunogenicity and protective effect for other SAPLIPs of *S. mansoni*. Most recently, the first three-dimensional crystal structures of SAPLIPs from hookworms and ecdysozoan organisms, namely Na-SLP-1 and Ac-SLP-1 were reported and both SAPLIPs share the property of membrane binding activity [48].

In our tegument immunoproteome study, one highly immunoreactive tegument protein (SJCHGC00277) containing a SapB domain belongs to SAPLIP family. It is a diverse family of lipid-interacting proteins, of which their cellular functions were only partially understood [49]. A search of the complete *S. japonicum* genome sequence identified 14 protein members conforming to the characteristics of this family and most of

S. japonicum SAPLIPs only had one SapB domain (data not shown). Another tegument protein (SJCHGC01869), which has five SapB domains, has showed low immunoreactivity. It may be attributed to the low expression level due to the high molecular weight of the protein (922 aa and 104.2 kDa). The gene family coding saposin-like proteins (SAPLIPs) may play important roles in host–pathogen interactions and have high potential as vaccine targets against schistosome infection.

The tetratricopeptide repeat (TPR) domain is a degenerate 34 amino acid sequence that occurs in tandem repeats in the protein. Proteins containing TPRs are involved in a variety of biological processes, such as cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis, protein folding and protein binding [50,51]. A TPR-domain protein *P. falciparum* (PfPP5) is destined to have a definitive role in parasite growth and signaling pathways. This is exemplified by the interaction between PfPP5 and the cognate chaperone HSP90 [52]. The highest immunogenic tegument protein (STIP1, SJCHGC06661) with six TPR domains and one STI1 domain had an AUC of 0.95 and was recognized by 8 cases (80%) of *S. japonicum* patient sera. Analysis of the biological process of STIP1 showed that it may involve in physiological process and response to stress.

Schistosome heat shock proteins (HSPs) were found to exert dual immunoregulatory effects, including immunostimulatory and immunosuppressive roles [53,54]. SJCHGC06661 is a putative stress inducible protein 1 (STIP1) and might have an important role in interaction with HSP complex. In fact, STIP1 was predicted to have a direct functional interaction with three other tegument proteins (HSP90, HSP70 and HSP40) that we expressed, and to have a STIP1 binding site. The recombinant STIP1 (SJCHGC06661) was recognized by *S. japonicum*-exposed individuals and has been shown good immunogenicity in immunized mice, indicating that STIP1 could be one of the key factors for the response to stress, and a potential target for designing anti-parasite drug or vaccine.

5. Conclusion

In present study, the *S. japonicum* tegument proteins were analyzed by an integrated immunoproteomics and bioinformatics approach. Thirty highly immunoreactive proteins were identified for the first time, which will provide clues of potential target molecules for vaccine development, biomarkers for diagnostics, and the function identification of unknown and hypothetical proteins in the *S. japonicum* genome.

Acknowledgments

This work was supported in part by the Foundation of National Science and Technology Major Program (Grant no. 2012ZX10004-220, 2008ZX10004-011, and 2009ZX10004-302), the China Postdoctoral Science Foundation (20100480360), and Diagnostics Innovation of Tropical Diseases in PR China (International S&T Cooperation 2010DFA33970). We would like to thank Prof. Zheng Feng and Dr. Ming-bo Yin for critical reading of the manuscript, and to thank all participants in the study and the staffs for collection of serum sample from *S. japonicum* patients and normal subjects at Anhui Institute of Parasitic Diseases and Shanghai Municipal Center for Disease Control and Prevention, respectively.

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