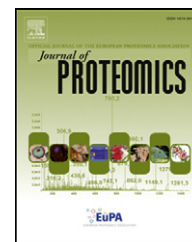


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Identification of proteins inducing short-lived antibody responses from excreted/secretory products of *Schistosoma japonicum* adult worms by immunoproteomic analysis☆



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ABSTRACT

The excretory/secretory antigens of *Schistosoma japonicum* (Sj ESAGs) play important roles in host–parasite immune interactions. In this study, the antibody response patterns to Sj ESAGs in sera of individual rabbits at the healthy stage, 2–6 weeks post-infection and 4–16 weeks after treatment were examined. Antigens inducing short-lived antibody responses were selected by comparing differences in immune recognition of proteins in sera across the different stages by Western blotting and identified by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS). The diagnostic value of these short-lived antibody responses for schistosomiasis was investigated. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified as a major antigen inducing a short-lived antibody response in Sj ESAGs. The antibody response against Sj GAPDH decreased at week 4 and disappeared between weeks 8–12 after effective chemical treatment of rabbits, and this response declined to negative levels in schistosomiasis patients one year after treatment. The intensity of the antibody response against Sj GAPDH was dependent on parasite load in mice. The sensitivity and specificity of IgG antibodies against recombinant Sj GAPDH for schistosomiasis diagnosis were 82.5% and 91.3%. Our findings suggest that Sj GAPDH induces short-lived antibody responses in the host, and detecting IgG against this antigen provides the basis for developing a potential method for diagnosis and evaluating treatment effects for schistosomiasis japonicum.

Biological significance

Schistosomiasis is one of the world's major public health problems. Developing effective diagnostic methods for detecting schistosome-specific antibodies to effectively identify active

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infections is part of a critical strategy for blocking transmission of the parasite and eradicating schistosomiasis. The excretory/secretory antigens of *S. japonicum* (Sj ESAGs) play important roles in host–parasite immune interactions.

In our study, we examine the antibody response patterns to Sj ESAGs within individual rabbits at the healthy, schistosome infection and post-treatment stages by Western blotting. Proteins among the Sj ESAGs inducing short-lived antibody responses were identified by Matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS), and their potential as immune markers for diagnosis and evaluating therapeutic effects in schistosomiasis was evaluated. Our findings suggest that *S. japonicum* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) induces short-lived antibody responses in the host, and detecting IgG against this antigen provides the basis for developing a potential method for diagnosis and evaluating treatment effects for schistosomiasis japonicum.

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1. Introduction

Schistosomiasis continues to be one of the most important tropical parasitic diseases in humans worldwide, with more than 200 million people currently infected among the 779 million people at risk of infection in 76 countries [1,2]. Transmission of schistosomiasis is not only a serious threat to human health, it also tremendously impacts the local social and economic development. Lack of effective diagnostic and epidemiological survey measures for schistosomiasis is still currently a major hindrance to efforts in blocking this parasitic transmission [3,4]. The ova present in stool and the excreted and secretory antigens of *Schistosoma japonicum* adult worms present in serum (also known as circulating antigens) are the objective indicators of an active infection. The gold standard for the diagnosis of schistosome infection is fecal examination by the Kato-Katz technique [5], but the low sensitivity and practicality of this method limit its use for routine veterinary detection as well as human diagnosis [6]. A series of monoclonal antibodies and tests for detecting schistosome-specific circulating antigens have been developed [7–9]. However, large-scale application of praziquantel (PZQ) for treatment of schistosomiasis, especially in China, has decreased the population infection rate and parasite load in endemic areas [10,11]. Thus, the decrease in levels of circulating antigens in serum impedes the application of specific monoclonal antibodies in the clinical diagnosis of schistosomiasis as it results in lower sensitivity and a high rate of false negative detection of circulating antigens.

Antibodies are immune molecules produced by the host to defend against pathogenic infections. During the processes of growth and physiological metabolism, schistosomes continually excrete and/or secrete antigens into the host circulatory system during the course of its parasitic life cycle. These antigens stimulate and/or regulate the function of the host immune system, leading to down-regulation of host T cell responses and abundant antibody production, although the molecular mechanism of immune modulation by schistosome infection remains unclear [12]. However, specific antibodies against schistosome antigens can be utilized as indirect indicators of infection [13]. As the levels of schistosome-specific antibodies are high in the host serum, many methods for detecting them have been developed with the soluble egg antigen (SEA), such as the indirect agglutination assay (IHA),

circum oval precipitin test (COPT), ELISA and dipstick dye immunoassay (DDIA) [14,15], which have been used commonly for screening cases with a history of schistosome infection. As antibodies against SEA persist over a long period of time after treatment, tests which detect these antibodies usually cannot discriminate between current and prior infections in an endemic area and therefore cannot be used to obtain an unequivocal diagnosis of active schistosome infection. Thus, developing effective diagnostic methods for detecting schistosome-specific antibodies to effectively identify active infections in a patient population in endemic areas is part of a critical strategy for blocking transmission of the parasite and eradicating schistosomiasis.

Differences in antigenic structures can induce significantly different antibody responses. Some antibody responses are long-lived and can be maintained up to a lifetime [16,17], while others are short-lived, depending on the persistence of a specific antigen [18–20]. For example, the generation and decline of short-lived antibody responses usually correspond to the appearance and disappearance of the corresponding pathogenic antigen *in vivo*. Alderete et al. [21] and Zang et al. [22] have suggested that measuring short-lived antigen-dependent antibodies can be used in place of direct detection of the pathogen to assess drug treatment effects.

The excreted and secretory antigens (Sj ESAGs) derived from schistosome adult worms parasitizing the cardiovascular system directly encounter the immunological system of the host and stimulate immune responses. The generation of antibody responses to Sj ESAGs has not been well-characterized, and the components of Sj ESAGs inducing short-lived antibody responses and their utility in schistosomiasis diagnosis remain to be investigated. In this study, we observed antibody response patterns to Sj ESAGs within individual rabbits at the healthy, schistosome infection and post-treatment stages by Western blotting. Proteins among the Sj ESAGs inducing short-lived antibody responses were identified by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS), and their potential as immune markers for diagnosis and evaluating therapeutic effects in schistosomiasis was evaluated. This study provides a good foundation for the development of new immune diagnostic methods for schistosome infection.

2. Materials and methods

2.1. Animals, parasite and antigen

2.1.1. Animals

Japanese White Rabbits were obtained from the Jinling Animal Center (Nanjing, China). ICR mice (female, 20 g, 6 weeks old) were purchased from the Experimental Animal Center of Yangzhou University (Yangzhou, China). All animals were raised at the Department of Experimental Animals, Jiangsu Institute of Parasitic Diseases (Wuxi, China). The animal experiments were approved by the Institutional Review Board (IRB00004221) of Jiangsu Institute of Parasitic Diseases (Permit Number: JIPDAERP20110112). All animals used in this study were housed in conventional facilities and were provided food and water *ad libitum*. Animal experiments, including the infection and venous blood sampling of rabbits and mice, were conducted in accordance with recommendations in the Guidelines for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China ([2006]398). Rabbits and mice were sacrificed by carbon dioxide asphyxiation in sealed containers according to the Guidelines. All efforts were made to minimize suffering.

2.1.2. Schistosomes

S. japonicum cercariae (strain isolated in Jiangsu, China) were hatched from infected snails (*Oncomelania hupensis*) provided by the Department of Snail Biology, Jiangsu Institute of Parasitic Diseases. *S. japonicum* adult worms were collected as follows. Ten rabbits were infected with 1500 *S. japonicum* cercariae per rabbit by abdominal skin penetration. At day 42 post-infection, all rabbits were sacrificed, and the adult worms in mesenteric veins were collected by portal venous infusion with anti-coagulant saline containing 0.3% trisodium citrate and 0.7% sodium chloride.

2.1.3. Antigen

Sj ESAGs were prepared following previously published methods [23,24]. Briefly, *S. japonicum* adult worms were collected and washed several times in PBS at room temperature to remove the host blood components on the worm surface. Thereafter, the worms were immersed into cold deionized water containing 100 U/mL protease inhibitor (Sigma, St. Louis, MO, USA) and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and then incubated at 37 °C for 20 min. After the worms were removed, the supernatant containing Sj ESAGs was collected by centrifugation at 20,000 ×g for 30 min at 4 °C to remove non-soluble substances. SEA was also prepared following a previously described method [25]. Protein concentrations of Sj ESAG and SEA were determined using a BCA kit (Pierce Biotechnology, Rockford, IL, USA) before storing the samples at –80 °C.

2.2. Serum collection

2.2.1. Rabbit serum

Rabbits were divided into a treatment group (3 rabbits) and an untreated group (2 rabbits). Infections and serum collection from individual rabbits at the healthy, schistosome infection and post-treatment stages were conducted as follows. Each rabbit was infected with 200 *S. japonicum* cercariae by abdominal skin

penetration. At 6 weeks post-infection, treatment group rabbits were given 160 mg/kg/day artesunate orally for two consecutive days, followed by 150 mg/kg/day praziquantel orally for another two consecutive days. Rabbits of the untreated group did not receive any treatment. Blood samples of all rabbits were obtained before infection (healthy stage) and then collected regularly every two weeks post-infection until 16 weeks post-treatment. The isolated serum samples were stored at –80 °C for subsequent analysis. All rabbits were sacrificed at 16 weeks post-treatment, and adult worms in the mesenteric veins or egg granuloma in the livers were examined to confirm whether the schistosome infection and chemotherapy were successful.

2.2.2. Mice sera

Sera of mice infected with increasing numbers of *S. japonicum* cercariae were prepared as follows. ICR mice were divided into five groups (A to E), with 6 mice in each group. Group A mice served as non-infected healthy controls. Mice in Groups B to E were infected with 5, 10, 15 and 20 cercariae of *S. japonicum*, respectively, by abdominal skin exposure. Blood samples were collected from the caudal vein on day 35 post-infection, and the isolated sera were stored at –80 °C for subsequent analysis. All mice were sacrificed on day 35 post-infection. Adult worms in the mesenteric veins and egg granuloma of livers were surveyed to confirm successful schistosome infection of the mice.

2.2.3. Human sera

Serum samples from 14 schistosomiasis patients, including those taken before treatment and one year after PZQ treatment, were provided by the Jiangxi Institute of Parasitic Diseases. All patients were confirmed by examining eggs in stool samples using the Kato-Katz method [11]. From each of the 14 patients, three stool samples before PZQ treatment and three at one year after PZQ treatment were collected, and the egg content in each sample was determined in three repeated measures by the Kato-Katz method (Supplementary Table 1). Fecal samples from all patients were positive for schistosome eggs before PZQ treatment, and those obtained one year after PZQ treatment were negative (Supplementary Table 1).

Sera from patients with clonorchiasis and paragonimiasis, confirmed by egg examination of stool or sputum under a microscope, were provided by the Department of Health Medical Center, Jiangsu Institute of Parasitic Diseases. Sera from patients with schistosomiasis, confirmed by the Kato-Katz analysis of stool samples, were provided by the Hubei Institute of Schistosomiasis Control. Negative control sera came from a healthy population in a non-endemic area for schistosomiasis (Wuxi City, Jiangsu Province, China) and tested negative for antibodies against SEA by ELISA.

2.3. Analysis of antibody response patterns to Sj ESAG components by 1-D Western blotting

Western blot strips were prepared by separation of Sj ESAG proteins (100 mg) on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (made using a 1.0 mm thick preparative comb with 1 reference well, Bio-Rad, Hercules, CA, USA) under constant voltage (120 V) for 90 min. Thereafter, proteins in the gel were transferred onto a nitrocellulose (NC) membrane at constant voltage (20 V) for 30 min using a Trans-Blot Semi-

Dry Electrophoretic Transfer Cell. The membrane was then blocked with 5% skimmed milk powder in PBS-T at room temperature at 4 °C overnight. The blocked membrane was then cut longitudinally into strips of 3-mm width each. The strips were incubated separately with rabbit sera (diluted 1:200) collected at the healthy stage, at 2, 4 and 6 weeks post-infection, and at 2, 4, 6, 8, 10 and 12 weeks post-treatment for 1 h at room temperature. After three washes with Tris-buffered saline-Tween 20 (TBS-T) (10 mM Tris, 150 mM NaCl, pH 7.6, 0.05% Tween-20) (10 min each time), the strips were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:10,000) for 1 h at room temperature. After washing the strips three times in TBS-T again, the color reaction was developed by incubating the strips with 0.6 g/L 3, 3'-diaminobenzidine (DAB) substrate and 0.2% H₂O₂ for 5 min at room temperature and then terminated by washing with distilled water. The antibody response patterns to Sj ESAG components were analyzed by comparing the difference in recognition of each protein band by sera from individual rabbits at different stages.

2.4. Analysis of antibody response patterns to deglycosylated Sj ESAGs

To explore whether short-lived antibody responses were induced by glycoproteins, Sj ESAGs were deglycosylated following methods described by Alarcon et al. [26]. Briefly, Sj ESAG proteins were electro-transferred to a NC membrane, which was blocked as above and then incubated in a sodium metaperiodate solution in 50 mM buffer acetate, pH 4.5, for 60 min at room temperature in the dark. After three washes with acetate buffer (three min each time), the NC membrane was incubated in 50 mM sodium borohydride in PBS for 30 min at room temperature. After three washes (5 min each wash) with PBS-T solution containing 0.05% Tween 20, the NC membrane was cut longitudinally into 3-mm wide strips. The deglycosylation of proteins was confirmed by incubating the above treated NC membranes in a biotin-conjugated lens culinaris agglutinin (LCA) solution (diluted 1:5000) for 1 h, followed by reaction with HRP-conjugated streptavidin (diluted 1:5000, Sigma) for 1 h after 3 washes with TBS-T for 5 min each time. The color was developed with DAB substrate as above.

The strips with deglycosylated Sj ESAGs were reacted with each serum sample, and the antibody response patterns were developed as in Section 2.3 above. The protein bands inducing short-lived antibody responses by non-glycosylated epitopes were identified by comparing differences in the antibody response patterns between the non-deglycosylated and deglycosylated Sj ESAG components.

2.5. Identification of Sj ESAG proteins inducing short-lived antibody responses by 2-D Western blotting and MALDI-TOF/TOF-MS

2.5.1. 2-D Western blotting

The Sj ESAG proteins were cleaned and quantified with the 2-D clean-up kit (GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ, USA) and the 2-D quant kit (GE Healthcare), respectively, following the manufacturer's instructions. Sj ESAGs were then dissolved in lysis buffer containing 2 M thiourea

(Sigma), 6 M urea (GE Healthcare), 1% 1, 4-dithiothreitol (DTT) and 4% CHAPS (GE Healthcare) and stored at –80 °C.

First-dimensional isoelectric focusing (IEF) and second dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to the manufacturer's instructions (GE Healthcare). Equal amounts of Sj ESAG proteins (800 µg in 450 µl rehydration buffer with 8 M urea, 2% CHAPS, 60 mM DTT and 0.5% IPG buffer) were loaded separately onto six immobilized pH gradient DryStrips™ each 24 cm in length [pH 3–10 non-linear (NL), GE Healthcare]. The proteins were initially separated using the Ettan™ IPGphor II™ Isoelectric Focusing system (GE Healthcare) and then focused to their isoelectric points under the following conditions: 30 V for 5 h, 60 V for 6 h, 100 V for 1 h, 300 V for 1 h, 600 V for 1 h, 1000 V for 1 h and 8000 V for 10 h. The total accumulated voltage was 78,126 VhT. After IEF, the proteins on the strips were reduced and bound to SDS by equilibrating each strip in 10 ml of SDS equilibration buffer (50 mM Tris-HCl, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.007% w/v bromochlorophenol) containing 100 mg DTT (1, 4-dithiothreitol) (added immediately before use) for 15 min. A second equilibration step was performed in SDS equilibration buffer containing 480 mg iodoacetamide (added immediately before use) instead of DTT. After equilibration, the immobilized pH gradient strips were loaded onto 12.5% (w/v) homogeneous acrylamide gels (1 mm thick × 24 cm wide × 19 cm long) sealed with 0.5% (w/v) agarose. Proteins were separated using the Ettan™ DALT six electrophoresis units (GE Healthcare) by running the gels at 15 W (2.5 W/gel) for 30 min at 20 °C and then at 108 W (18 W/gel) until the bromochlorophenol blue moved to approximately 1 cm from the lower margin of the gel. One 2-D gel was stained with Coomassie Blue R-350 for 30 min, destained in dehydration solution (alcohol: glacial acetic acid: deionized water = 4:1:5) and then photographed with a digital scanner. Proteins on the other five gels were transferred to polyvinylidene difluoride (PVDF) membranes (25 V, 400 mA, for 60 min) with a Hoefer TE 77 semi-dry transfer unit (GE Healthcare). The membranes were then blocked with 5% skimmed milk in TBS at 4 °C overnight. One membrane was incubated with healthy rabbit serum (before infection, diluted 1:500), while another membrane was incubated with serum (diluted 1:500) of infected rabbits at week 6 post-infection. The third, fourth and fifth membranes were incubated with serum (diluted 1:500) of rabbits at weeks 4, 8 and 12 post-treatment, respectively, for 1.5 h at room temperature. The PVDF membranes were washed three times with TBS-T (containing 0.05% Tween-20) for 10 min each time and then reacted with the goat anti-rabbit IgG-HRP conjugated secondary antibody at 1:10,000 dilution. The membranes were incubated for 1.5 h at room temperature and then washed three times with TBS-T again as above. The protein spots on these five PVDF membranes were visualized with DAB solution (0.6% DAB, 0.1 M Tris-HCl, 0.2% H₂O₂) at room temperature for 5 min and terminated with deionized water. The proteins inducing short-lived antibody responses were analyzed by comparing the antibody recognition profiles of the same protein spots on the different PVDF membranes. Those protein spots which could be recognized by the sera of infected rabbits, but not by those of healthy rabbits and infected rabbits for several weeks post-treatment, were defined as proteins inducing short-lived antibody responses and selected for further analysis by MALDI-TOF/TOF-MS.

2.5.2. MALDI-TOF/TOF-MS

All selected protein spots were excised from the stained acrylamide gels using the One Touch 2D Gel SpotPicker (BioRad, 1.5 mm) and digested as described previously [27]. Briefly, the gel pieces were first washed with 100 μ l 25 mM ammonium bicarbonate (pH 8.0) for 20 min and then washed with 50 μ l 30% acetonitrile containing 0.1 M ammonium bicarbonate until the color of the gel block disappeared. The samples were dried by vacuum freeze-drying and then incubated in digestion buffer (25 mM ammonium bicarbonate and 20 μ l/ml of trypsin) for 16 h at 37 °C, followed by another 60 min incubation after adding 40 μ l of 50% acrylonitrile/5% trifluoroacetic acid. The final sample was absorbed by pre-freezing at –80 °C for 2 h and then vacuum freeze-dried. The material after vacuum freeze-drying was dissolved in 2 μ l of a solution containing deionized water, acetonitrile and trifluoroacetic acid (49.75:49.75:0.5), and 0.5 μ l of this sample solution was used to mix with 0.5 μ l of matrix (saturated matrix solution in 50% acetonitrile and 2.5% trifluoroacetic acid) and then dried at room temperature. All proteins were analyzed with an ABI 4700 MALDI-TOF Mass Spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a 200-Hz Nd:YAG laser operating at a wavelength of 355 nm. The spectra were acquired by using the 4000 Series Explorer™ software version 3.0 (GE Healthcare) in positive-ion reflector mode with a mass tolerance of 20 ppm. The spectrum of every sample was acquired in the mass range between 800 and 4000 Da by using 2000 laser shots and was processed and analyzed by the Global Protein Server Workstation (GPS Applied Biosystems, Foster City, CA, USA), which uses internal Mascot v2.1 software for searching the peptide mass fingerprints. The searches were performed by using the NCBI non-redundant protein database (<ftp://ftp.ncbi.nih.gov/blast/db/FAST/nr.gz>, updated in 2011) with the following criteria: NCBI human database; trypsin digestion; M oxidation and iodoacetamide alkylation as the variable modifications; missed digestion site of 1; and the MS mass error of 0.1 Da. Identifications with a GPS confidence interval greater than 95% were accepted. The inversion database was used to remove false positives.

2.6. Gene cloning and protein expression of *S. japonicum* glyceraldehyde-3-phosphate dehydrogenase (Sj GAPDH)

The results of MALDI-TOF/TOF-MS showed that Sj GAPDH was a major component of Sj ESAG which induced a specific short-lived antibody response. To further study the immunogenicity of Sj GAPDH, we expressed this protein in *Escherichia coli*. Briefly, the total RNA of adult worms was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and first strand cDNA was synthesized with the SuperScript™ Choice System (Invitrogen). The following primers for amplifying the open reading frame of the DNA fragment encoding the Sj GAPDH gene were designed according to the published sequence (AB034728.1): forward primer 5'-atgctagcatg tcgaaggctaaaggttggtatcaat-3' and reverse primer 5'-aagagctcttaag aatggtcgactctatgc-3'. An *Nhe* I site was introduced into the 5' end of the upstream primer, and a *Sac* I site was introduced into the 5' end of the downstream primer. The PCR-amplified DNA fragment encoding Sj GAPDH from the *S. japonicum* adult worm cDNA was cloned into a TA cloning vector (pGEM-T Easy, Promega,

Madison, WI, USA) to construct the recombinant plasmid pGEMT-SjGAPDH. The inserted Sj GAPDH DNA fragment was confirmed by sequencing (Invitrogen, Shanghai, China) and then subcloned into the expression plasmid pET28a(+) at the *Nhe* I and *Sac* I restriction sites to form the recombinant plasmid pET28a-SjGAPDH. This recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells, and positive transformants were selected on a Luria-Bertani (LB) media plate containing kanamycin (50 μ g/mL) and induced by isopropyl β -D-1-thiogalactopyranoside (IPTG, 1 mM) to express the Sj GAPDH protein. The expressed products were analyzed by SDS-PAGE, and the recombinant Sj GAPDH protein was purified with nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Valencia, CA, USA) following the manufacturer's instruction.

2.7. Analysis of antibody responses to recombinant Sj GAPDH protein

Antibody responses to recombinant Sj GAPDH protein in sera of individual rabbits at the healthy, schistosome infection and post-treatment stages were observed by indirect ELISA with recombinant Sj GAPDH (rSj GAPDH-ELISA) and Western blotting. For the ELISA, the flat-bottom 96-well ELISA plate (Greiner Bio-One) was coated with 1 μ g (100 μ l) per well of the recombinant Sj GAPDH protein in coating buffer (0.05 M sodium carbonate solution, pH 9.6) and kept at 4 °C overnight. To start the assay, plates were blocked with 350 μ l/well of blocking buffer (5% w/v skim milk, 0.05% Tween-20, Tris 10 mM, NaCl 150 mM, pH 8.0) for 1 h at room temperature. After the plates were washed three times with PBS-T, 100 μ l of serum diluted 1:100 in PBS containing 1% BSA was added to each well and incubated at 37 °C for 1 h. After the plate was washed three times as above, 100 μ l of the HRP-labeled goat anti-mouse IgG secondary antibody diluted 1:10,000 was added into each well, and the plate was incubated at 37 °C for 1 h. After the plate was washed three times as above, the color reaction was developed by adding 50 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) substrate to each well and incubating at room temperature for 5 min in the dark. The color development was stopped by adding 50 μ l/well of 2 M sulfuric acid solution (H₂SO₄), and the optical density (OD) in each well was measured at 450 nm using an ELISA plate reader. The OD₄₅₀ values of sample wells above 2.1 times that of negative control wells were judged as positive [28]. Meanwhile, antibody responses to recombinant Sj GAPDH protein in sera were also examined by Western blotting. The recombinant Sj GAPDH protein was run on an SDS-PAGE gel, transferred to NC membranes and reacted with the sera of individual rabbits at various stages pre- and post-infection as described above in Section 2.3.

Changes in specific antibody levels in sera of the 14 individual schistosomiasis patients from before treatment to one year post-treatment were detected by rSj GAPDH-ELISA following the procedure described above, and SEA antibody levels were also detected by a traditional ELISA (SEA-ELISA) as a control in the same samples.

The relationship between the intensity of antibody response against recombinant Sj GAPDH protein and degree of schistosome infection in mice was analyzed by detecting the serum IgG antibody level of animals infected with different amounts of *S. japonicum* cercariae by rSj GAPDH-ELISA as in the above procedure.

2.8. Evaluating the diagnostic value of recombinant *Sj* GAPDH protein

Sera of 40 schistosomiasis, 30 clonorchiasis and 20 paragonimiasis patients as well as 40 healthy individuals were examined by r*Sj* GAPDH-ELISA and by SEA-ELISA as a control. The sensitivity, specificity and efficacy of detecting specific antibodies against *Sj* GAPDH were calculated as described by Galen [29] to evaluate the diagnostic value of recombinant *Sj* GAPDH protein for schistosomiasis.

2.9. Statistical analysis

Antibody response levels were compared and analyzed with GraphPad Prism software 5.0 and the Statistical Package for Social Sciences (SPSS) software, version 13.0 (Chicago, IL, USA). All data were expressed as means $\pm$ standard deviation.

3. Results

3.1. Partial components of *Sj* ESAGs induce short-lived antibody responses

Sj ESAG proteins were separated by 1-D SDS-page according to the molecular weight and transferred onto NC membranes. The NC strips were reacted with sera of individual rabbits at stages before and after infection, as well as after treatment, which showed varying antibody response patterns to the different components of *Sj* ESAG. Most of the *Sj* ESAG proteins could be recognized by sera of infected rabbits but not by those of non-infected rabbits. Among them, the antibody response against a 95 kDa protein band with sera of treatment group rabbits was maintained through the infection and post-treatment stages. Antibody responses against the 27/30 kDa protein bands gradually became stronger with the extension of time. However, the intensity of antibody responses against the 16, 20, 23, 32, 36/37, 45, 48, 67 and 78 kDa bands with rabbit sera from 4 weeks post-treatment gradually became weak. In particular, the intensity of the antibody response against the 36/37 kDa bands was strong with rabbit sera at 4 weeks post-infection but decreased quickly and disappeared or became very weak at 8 weeks post-treatment (Fig. 1). Conversely, the antibody responses against 16, 20, 23, 32, 36/37, 48, 52, 67, 78 and 95 kDa protein bands with sera of untreated infected rabbits remained relatively strong throughout the infection period; however, antibody responses against the 32 and 36/37 kDa protein bands slightly decreased at 16 weeks after schistosome infection, and those to the 27/30 kDa bands gradually became stronger (Fig. 2). There was a significant difference in the antibody responses against the 16 and 20 kDa protein bands among the individual rabbits. These two protein bands could only be recognized by sera of rabbits 1 and 2 in the treatment group and rabbit A in the untreated group, but they did not react with sera of rabbit 3 in the treatment group and rabbit B in the untreated group. Overall, the intensities of antibody responses against the 16, 45, 48 and 95 kDa protein bands were weaker than those of other protein bands. It is worth noting that the 14, 22, 40 and 56 kDa protein bands could not be recognized by rabbit sera at any stage. The

antibody response against the 52 kDa protein band was not related to any stage of infection, as this protein could be recognized by sera of individual rabbits both before (healthy stage) and after (schistosome infection and post-treatment stages) infection (Figs. 1 and 2).

Sj ESAGs were deglycosylated, which was confirmed by the LCA affinity blot assay, and used to distinguish the specific antibody responses to glycoproteins. The amount of deglycosylated protein components of *Sj* ESAGs combined with LCA was far less than that of the corresponding non-deglycosylated proteins (Fig. 3), indicating that the deglycosylation procedure was successful. The deglycosylated *Sj* ESAGs on the NC membrane strips were reacted with sera of individual rabbits from the healthy, schistosome infection and post-treatment stages, and the antibody response patterns were similar to those with non-deglycosylated *Sj* ESAGs (Fig. 4). On the whole, intensities of antibody responses to all deglycosylated protein bands, except for the 36/37 kDa bands, were weaker than those of non-deglycosylated *Sj* ESAGs. The antibody response in rabbit sera against the deglycosylated 36/37 kDa protein bands became stronger over the period of 4–6 weeks post-infection, but it disappeared rapidly during post-treatment, compared with the corresponding antibody responses to non-deglycosylated *Sj* ESAGs.

3.2. Genes of *Sj* ESAG components inducing short-lived antibody responses

To identify genes of *Sj* ESAG components inducing short-lived antibody responses, *Sj* ESAGs were separated by 2-DE (24 cm IPG, PI 3–10), and approximately 700 protein spots were visualized on an SDS-PAGE gel after Coomassie Blue R350 staining with molecular weights of 10–130 kDa and PI values in the range of 3–10. All *Sj* ESAG protein spots were transferred onto PVDF membranes and reacted with sera of individual rabbits from the healthy stage, schistosome infection stage at week 6 post-infection and post-treatment stage at weeks 4, 8 and 12. The protein spots inducing short-lived antibody responses, which did not react with the sera of healthy rabbits (Fig. 5B) but could be recognized strongly with sera of schistosome-infected rabbits at week 6 post-infection (Fig. 5C), were mainly located between 30–37 kDa with pI 6–9 and 25–36 kDa with pI 4–5 on the 2-DE gels. The intensity of these spots reacted with sera of treated rabbits declined at week 4 and disappeared or became very weak at weeks 8–12 post-treatment (Fig. 5D–F). The 36/37 kDa protein spots appeared as a horizontal line on the 2-D SDS-PAGE gels as their molecular masses were similar, but their pI values were different (Fig. 5A). Forty-two strongly stained protein spots inducing short-lived antibody responses were determined with an ABI 4700 mass analyzer, and 21 the protein names among them were identified successfully (Table 1). Among them, protein spots 2–9 and 11 were identified as *S. japonicum* GAPDH (36.5 kDa); protein spots 12, 14 and 15 were identified as *S. japonicum* fructose-1, 6-bisphosphate aldolase (FBA, 39.5 kDa) (mass fingerprints are shown in Supplementary Figure 1); protein spots 34 and 35 were identified as SJCHGC05757; protein spots 26, 27 and 30 were identified as SJCHGC01391; and protein spots 18–21 were identified as SJCHGC02266 (Fig. 6). Other protein spots were not identified.

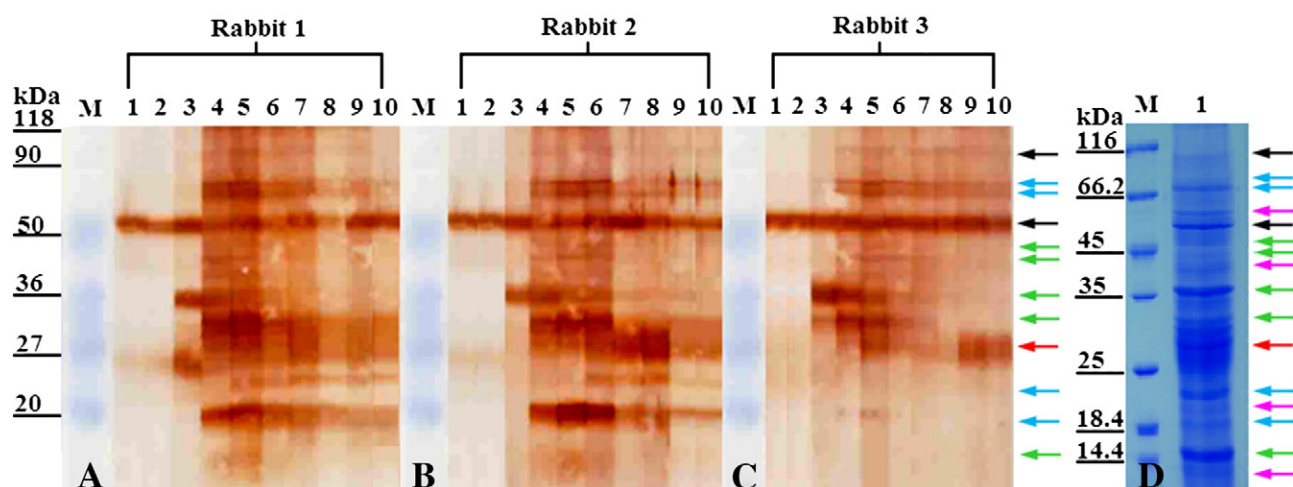


Fig. 1 – Antibody response patterns of Sj ESAGs reacting with sera of individual rabbits at the healthy stage, *S. japonicum* infection stage and post-treatment stage. (A, B, C) Antibody response patterns to Sj ESAGs with sera of rabbit 1, rabbit 2 and rabbit 3, respectively. Lane 1, Sj ESAGs reacted with rabbit serum at the healthy stage (before infection); lanes 2–4, Sj ESAGs reacted with rabbit sera at weeks 2, 4 and 6 post-infection, respectively; lanes 5–10, Sj ESAGs reacted with rabbit sera at weeks 2, 4, 6, 8, 12 and 16 post-treatment, respectively; lane M: prestained standard protein molecular weight marker. Color-coded arrows indicate recognized protein bands: black arrows, unchanged antibody response; blue arrows, antibody response gradually became weaker; green arrows, antibody response diminished quickly; red arrows, antibody response was enhanced gradually. (D) SDS-PAGE analysis of Sj ESAGs. Lane 1, Sj ESAGs; lane M, prestained standard protein molecular weight marker. The arrows represent protein bands and are color-coded as above. In addition, the pink arrows indicate protein bands which did not induce an antibody response.

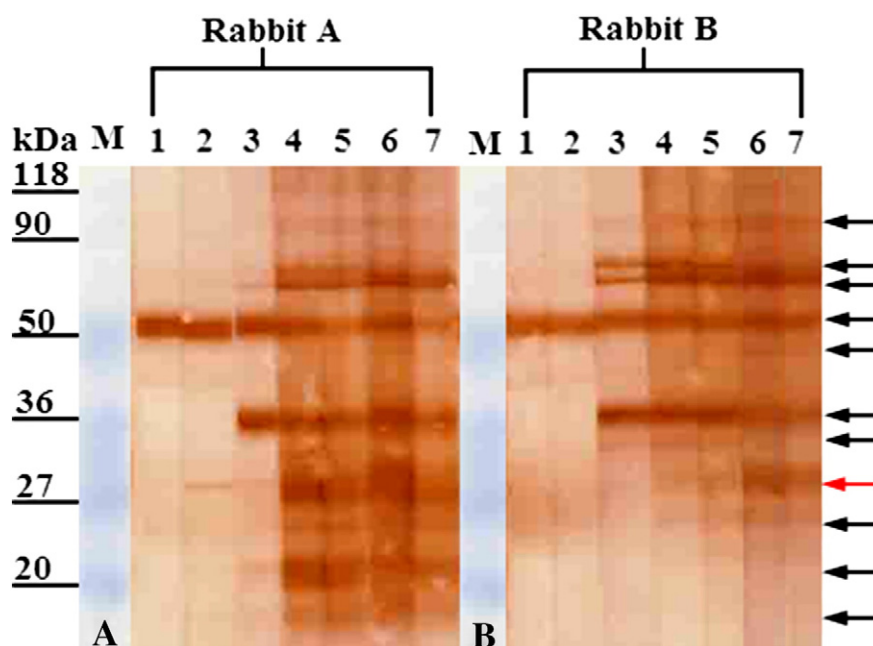


Fig. 2 – Antibody response patterns to Sj ESAGs with sera of untreated *S. japonicum*-infected rabbits. (A, B) Antibody response patterns to Sj ESAGs with sera of rabbit A and rabbit B of the untreated infection group, respectively. Lane 1, Sj ESAGs reacted with rabbit serum at the healthy stage (before infection); lanes 2–7, Sj ESAGs reacted with sera of untreated rabbits at weeks 2, 4, 6, 8, 12 and 16 post-infection, respectively; lane M, prestained standard protein molecular weight marker. Color-coded arrows indicate recognized protein bands: black arrows, unchanged antibody response; red arrows, antibody response was enhanced gradually.

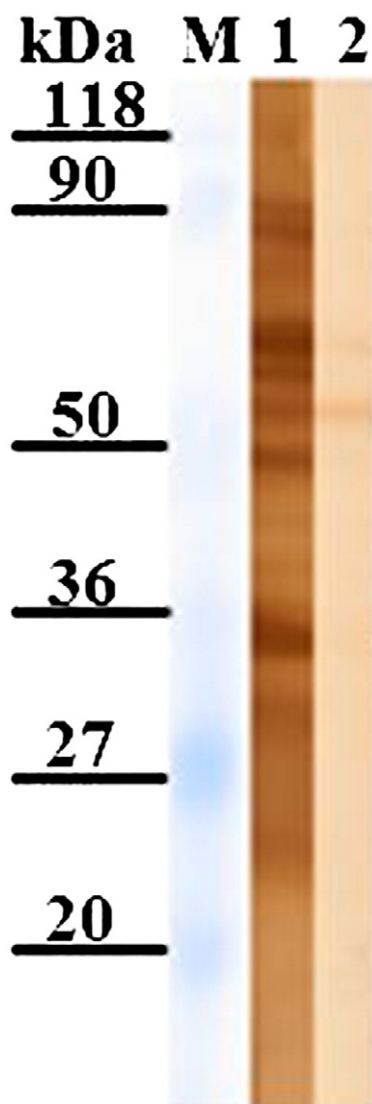


Fig. 3 – Deglycosylation of Sj ESAGs. Lane 1, non-deglycosylated Sj ESAGs reacted with biotin-labeled LCA; lane 2, deglycosylated Sj ESAGs reacted with biotin-labeled LCA; lane M, prestained standard protein molecular weight marker.

3.3. Antibody responses to recombinant GAPDH protein

To verify immunological features of Sj GAPDH which could induce a short-lived antibody response, we cloned its coding gene fragment and constructed the recombinant expression plasmid pET28a-Sj GAPDH (Supplementary Figure 2A). Bacteria transformed with the pET28a-Sj GAPDH plasmid expressed a 39 kDa protein, the molecular mass of which is a bit larger than the predicted value due to the 6-His tag on the N-terminus of the recombinant Sj GAPDH. A highly pure preparation of the recombinant Sj GAPDH protein was obtained successfully via Ni-NTA affinity chromatography (Supplementary Figure 2B, C).

Both ELISA and Western blotting methods were used to detect the presence of IgG against recombinant Sj GAPDH in the sera of individual rabbits at different stages of schistosome infection and treatment. The ELISA results showed that the

serum IgG antibody level against Sj GAPDH could be detected as early as week 4 post-infection, and then the titer rose rapidly and peaked at week 6 post-infection. At week 4 post-treatment, the serum IgG titer rapidly decreased and dropped nearly to the negative cut-off value at 8–12 weeks post-treatment (Fig. 7A). Meanwhile, in the untreated group, the IgG antibody titer against recombinant Sj GAPDH was maintained at a high level throughout the infection process, with only a slight reduction at the late period of infection (Fig. 7B). Consistent with the ELISA results, Western blotting analysis also indicated that the recombinant Sj GAPDH protein could be recognized specifically by sera of schistosome-infected rabbits, and the intensity of the Western blot bands with the sera of treated rabbits decreased quickly to a negative level between weeks 8–12 post-treatment (Fig. 7C); meanwhile, the Sj GAPDH-specific antibody levels of untreated rabbits remained relatively high, compared to the early stage of infection, and did not decline obviously over time (Fig. 7D).

3.4. Correlation between degree of schistosome infection and intensity of Sj GAPDH-specific antibody response

ICR mice of groups A to E were infected with different numbers of cercariae, and adult worms in the mesenteric veins were calculated on day 35 post-infection. The average adult worm burdens of groups A to E were 0.0 ± 0.0 , 3.8 ± 2.4 , 6.7 ± 3.8 , 10 ± 5.9 and 16 ± 7.0 , respectively (Fig. 8). Egg granulomas of groups A to E in the mouse livers are shown in Supplementary Figure 3. These results confirmed that the schistosome infection of mice was successful. The anti-rSj GAPDH IgG level detected by ELISA positively correlated with the schistosome worm burden in mice (Fig. 8); that is, the Sj GAPDH-specific antibody levels rose along with the degree of schistosome infection, and significant differences were observed in those antibody titers among groups A to E ($P < 0.05$).

3.5. Changes in anti-rSj GAPDH serum IgG antibody within individual schistosomiasis patients before and after treatment

Sera from 14 schistosomiasis patients before treatment and one year post-treatment were examined by rSj GAPDH-ELISA and SEA-ELISA at the same time. While serum samples from all 14 patients before treatment were positive by rSj GAPDH ELISA and SEA-ELISA, the anti Sj GAPDH antibody IgG levels in the 14 patients at one year post-treatment were significantly reduced to below the negative threshold (Fig. 9A). However, sera of the 14 patients obtained one year post-treatment remained positive by SEA-ELISA with a high titer of anti-SEA IgG antibody (Fig. 9B).

3.6. Diagnostic value of recombinant Sj GAPDH

Batches of sera from patients with schistosomiasis or other parasitic diseases and from healthy donors were detected by Sj GAPDH-ELISA and SEA-ELISA as a control. The positive rates of antibodies against Sj GAPDH and SEA in sera of 40 schistosomiasis patients were 82.5% and 100%, respectively. The rates of cross-reactivity of sera from 30 patients infected with *Clonorchis sinensis* to Sj GAPDH and SEA were 6.7% and 20%, respectively, and those of sera from 20 patients infected with *Paragonimus westermani* to Sj GAPDH and SEA were 25% and 65%, respectively. The false positive rates of Sj GAPDH

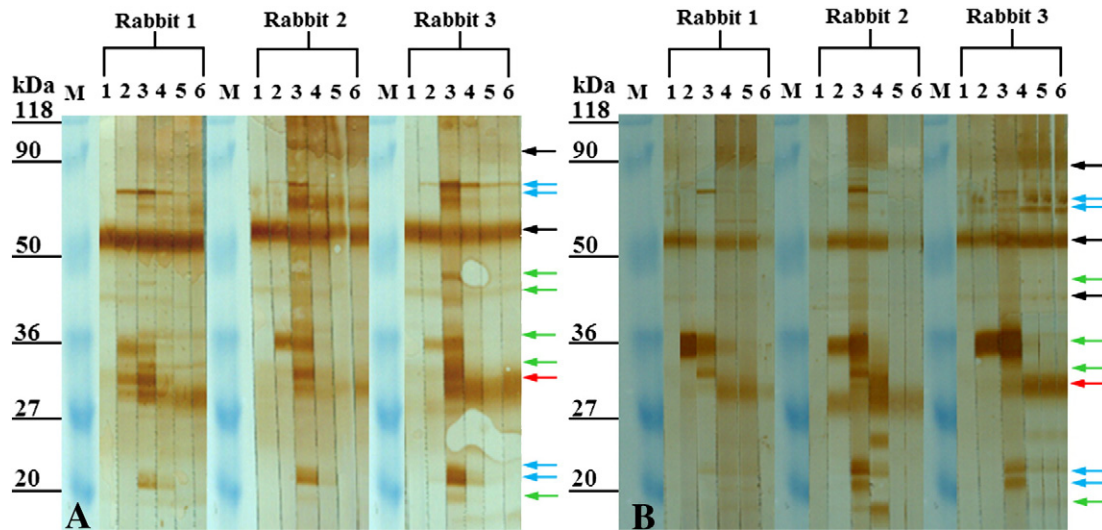


Fig. 4 – Rabbit serum antibody response patterns to non-deglycosylated and deglycosylated *Sj* ESAs. (A) Antibody response patterns to non-deglycosylated *Sj* ESAs. (B) Antibody response patterns to deglycosylated *Sj* ESAs. Lane 1, non-deglycosylated and deglycosylated *Sj* ESAs reacted with rabbit serum at the healthy stage; lanes 2–3, non-deglycosylated and deglycosylated *Sj* ESAs reacted with rabbit sera at weeks 4 and 6 post-infection, respectively; lane 4–6, non-deglycosylated and deglycosylated *Sj* ESAs reacted with rabbit sera at weeks 8, 12 and 16 post-treatment, respectively; lane M, prestained standard protein molecular weight marker. Color-coded arrows indicate recognized protein bands: black arrows, unchanged antibody response; blue arrows, antibody response gradually became weaker; green arrows, antibody response diminished quickly; red arrows, antibody response enhanced gradually.

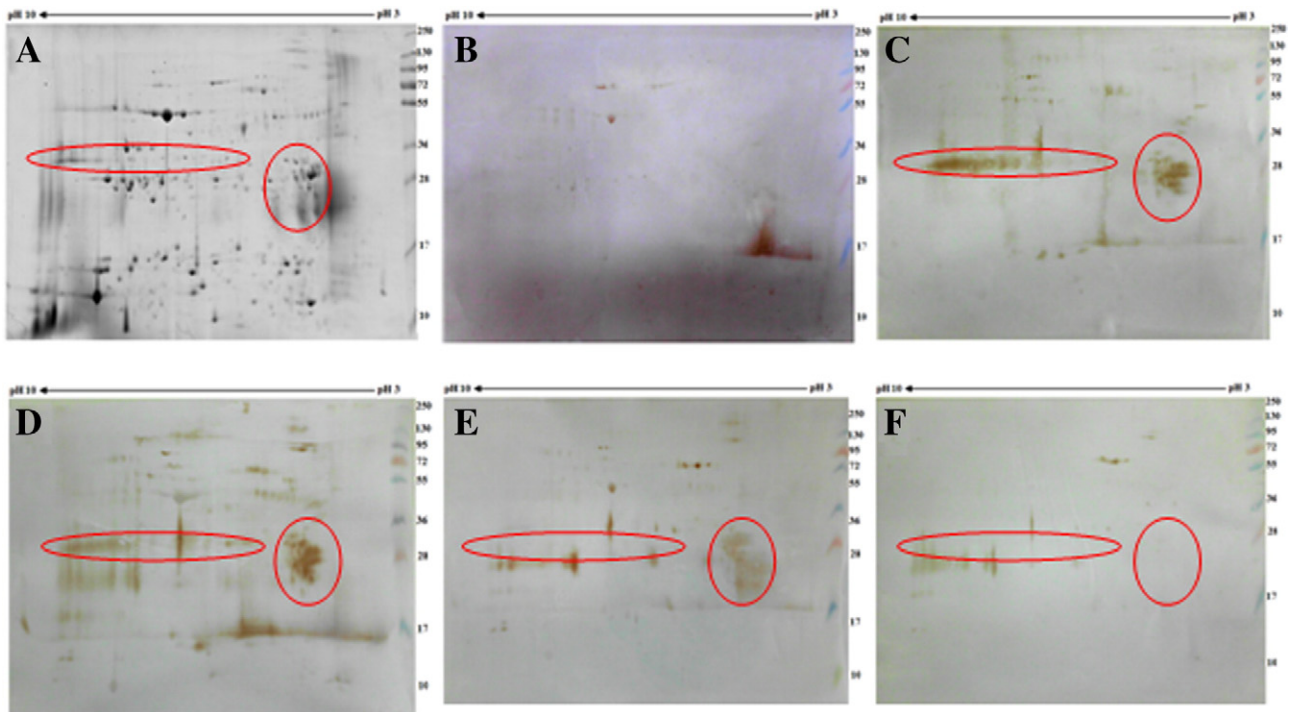


Fig. 5 – Antibody response patterns in sera of individual rabbits at the healthy stage, *S. japonicum* infection stage and post-treatment stage reacted with *Sj* ESAs by 2-D Western blotting analysis. (A) Image of 2-DE gel of *Sj* ESAs stained with Coomassie Brilliant Blue. (B) *Sj* ESAG protein spots reacted with sera of healthy rabbit. (C) *Sj* ESAG protein spots reacted with sera of rabbits at week 6 post-infection. (D) *Sj* ESAG protein spots reacted with sera of rabbits at week 4 post-treatment. (E) *Sj* ESAG protein spots reacted with sera of rabbits at week 8 post-treatment. (F) *Sj* ESAG protein spots reacted with sera of rabbits at week 12 post-treatment.

Table 1 – MALDI-TOF/TOF-MS identification of proteins inducing short-lived antibody responses.

Dots	Protein	Accession no.	MW	pI	Score	Score C.I.%
2–9,11	glyceraldehyde-3-phosphate dehy-drogenase (<i>S. japonicum</i>)	gi 257209379	36578.8	8.74	117	100
12,14,15	fructose-1,6-bisphosphate aldolase (<i>S. japonicum</i>)	gi 95113705	39540.4	6.33	199	100
34,35	SJCHGC05757 protein (<i>S. japonicum</i>)	gi 76155211	28955.6	4.93	70	48.3
26,27,30	SJCHGC01391 protein (<i>S. japonicum</i>)	gi 56757962	48565.7	5.65	199	100
18–21	SJCHGC02266 protein (<i>S. japonicum</i>)	gi 56752649	42720.9	5.0	298	100

and SEA to 40 healthy donor sera were both 0%. Upon comparison of the ELISA results with those of microscope examination of eggs in stool samples, the sensitivity and specificity of Sj GAPDH-ELISA for schistosomiasis diagnosis were 82.5% and 91.3%, respectively (Table 2). The positive predictive value (PPV) and the negative predictive value (NPV) were 82.5% and 91.3% (Table 3), respectively. For the SEA-ELISA, the sensitivity and specificity were 100% and 76.3%, while its PPV and NPV were 67.8% and 100%, respectively (Tables 2 and 3).

4. Discussion

During the process of schistosome infection, the host immune response gradually shifts from the T helper type 1 (Th1) in the acute phase to the T helper type 2 (Th2) profile in the chronic phase [30]. The excreted and secretory proteins of adult schistosome worms and eggs play an important role in the immune regulation and immune evasion by the parasite [31–34], but they also induce robust antibody responses. Antibodies specific for schistosome antigens present in the serum indicate that the host is suffering a current infection or has had a history of schistosomiasis; therefore, these antibody responses can be used for screening schistosome infection in common methods including ELISA, IHA, COPT and dipstick test. Generally, SEA has been used as the major antigen for developing antibody detection methods for schistosomiasis. Antigens released from schistosome eggs that become trapped in the host liver, bowel and/or bladder tissues for an extended period of time after treatment can induce antibody responses, some of which may be maintained permanently and do not

necessarily depend on antigen reactivation or the presence of the parasite [35]. Moreover, SEA and the soluble adult worm antigens (AWA) are composed of complicated mixtures of components, some of which have strong cross-reactivity with other parasites [36]. For these reasons, antibodies against SEA or AWA cannot be used as an unequivocal diagnostic indicator of an active schistosome infection.

Sj ESAs, derived from the living adult schistosome worm, are the main antigens that stimulate the host immune system to produce specific antibody responses. Theoretically, antibody response patterns differ depending on the specific characteristics of an antigen. Antigens may induce non-specific or schistosome-specific antibody responses. Some antibody responses are persistent [17], while other antigen-dependent responses are short-lived and decline rapidly when the antigen disappears *in vivo* [18]. Because serum antibody responses can be more easily screened than worm eggs in fecal samples, identification of specific excreted and secretory antigen components of *S. japonicum* adult worms that can induce these short-lived antibody responses would be helpful for establishing a highly sensitive and specific method of diagnosis and evaluating curative effects for schistosomiasis.

In this study, the excreted and secretory antigens of *S. japonicum* were separated by SDS-PAGE and electro-transferred to NC membranes to detect IgG response patterns in sera of individual schistosome-infected rabbits, with or without treatment. The Western blotting results showed that the antibody response patterns of Sj ESAs were diversified. The antibody response against the 52 kDa protein band was not related to a particular stage of infection, as it could be recognized by sera of the same rabbits at different stages, including the healthy, schistosome infection and post-treatment stages (Fig. 1). This

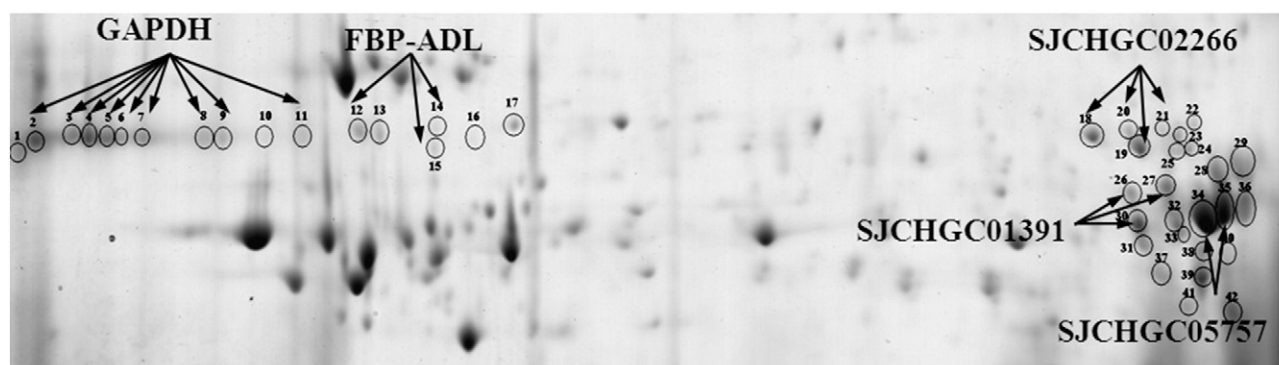


Fig. 6 – MALDI-TOF/TOF-MS of 36/37 kDa protein spots of Sj ESAs. Proteins (pI 6–9, MW 30–36 kDa and pI 4–5, MW 25–36 kDa) inducing short-lived antibody response in a 2-DE gel. Proteins of spots 2, 3, 4, 5, 6, 7, 8, 9 and 11 in red ovals were identified as GAPDH; spots 12, 14 and 15 were identified as FBA; spots 18, 19, 20 and 21 were identified as SJCHGC02266; spots 26, 27 and 30 were identified as SJCHGC01391; both spots 34 and 35 were identified as SJCHGC05757; other protein spots could not be identified.

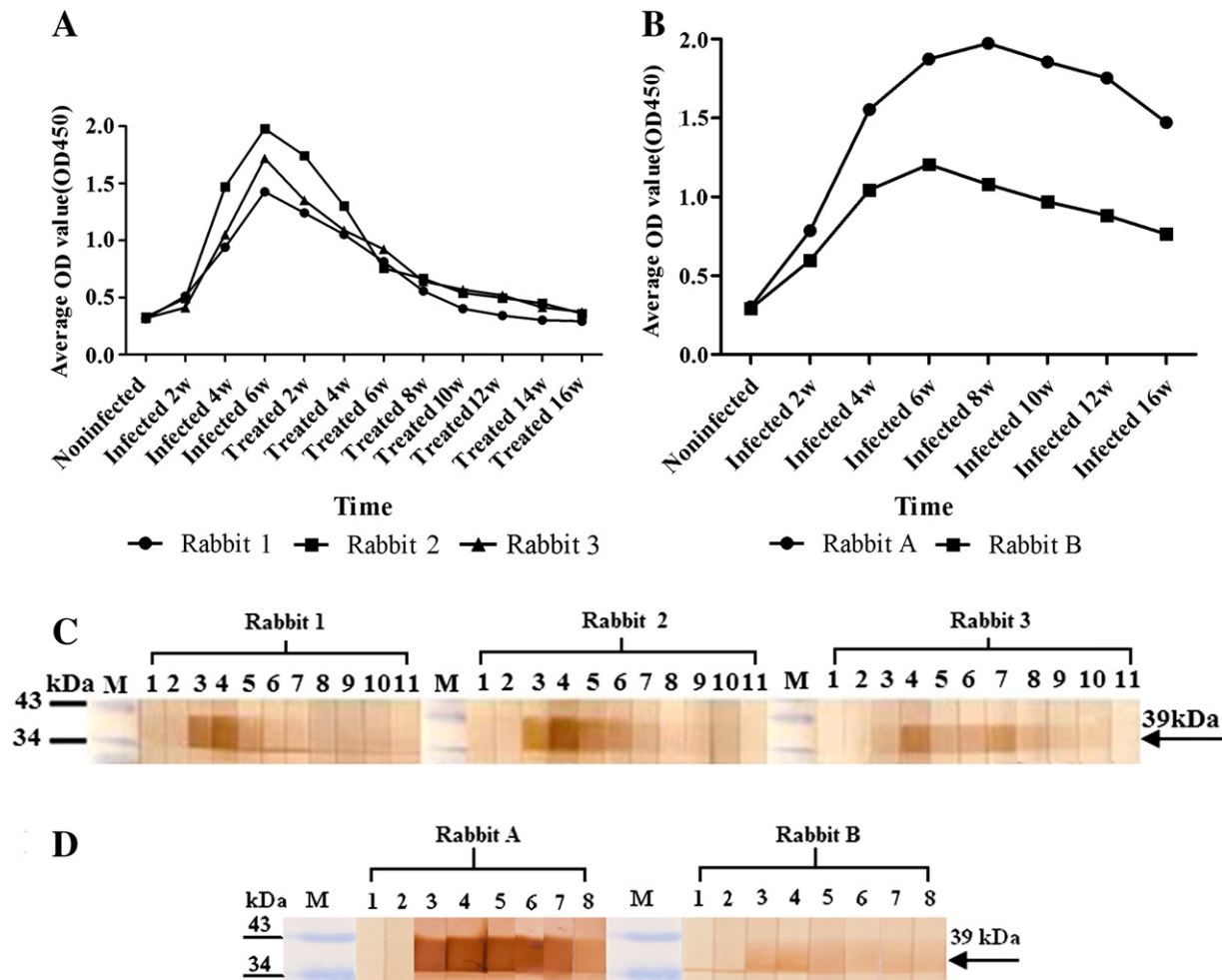


Fig. 7 – Changes in rabbit serum levels of antibodies recognizing recombinant *Sj* GAPDH. (A) Dynamics of IgG antibodies against recombinant *Sj* GAPDH in sera of individual rabbits at the healthy stage, *S. japonicum*-infection stage at weeks 2, 4 and 6, and at weeks 2, 4, 6, 8, 10, 12, 14 and 16 post-treatment detected by ELISA. (B) Dynamics of IgG antibodies against *Sj* GAPDH in sera of untreated *S. japonicum*-infected rabbits, including at the healthy stage and during the *S. japonicum*-infection stage at weeks 2, 4, 6, 8, 10, 12, 14 and 16 by ELISA. (C) Dynamics of IgG antibodies recognizing recombinant *Sj* GAPDH in sera of individual rabbits (1, 2, and 3) at the healthy stage, *S. japonicum*-infection stage at weeks 2, 4 and 6, and at weeks 2, 4, 6, 8, 10, 12 and 16 post-treatment detected by Western blotting. (D) Dynamics of IgG antibodies recognizing *Sj* GAPDH in sera of untreated *S. japonicum*-infected rabbits (rabbits A and B), including at the healthy stage and during *S. japonicum*-infection stage at weeks 2, 4, 6, 8, 10, 12 and 16 by Western blotting.

protein band could also react with the secondary antibody against IgG (data not published), indicating that it is similar to IgG or may be contaminated with IgG protein derived from host blood. Although the weaker intensities of antibody responses against the 45, 48 and 95 kDa protein bands could be related to their low expression levels in *Sj* ESAs, the low antibody response against the abundant 16 kDa protein band in *Sj* ESAs indicated that the immunogenicity of this molecule was lower. Meanwhile, the 14, 22, 40, 48 and 56 kDa protein bands were not recognized by any rabbit sera at the healthy, schistosome infection or post-treatment stage, indicating that these antigens could not induce host immune responses or that the antibody responses induced by these antigens were too weak to be detected by the methods used in this study (Fig. 1). These parasite proteins, with no or low immunogenicity may be highly similar in structure and function to host proteins,

resulting in tolerance by the host immune system and down-regulation of immune responses against these proteins. Clarifying the mechanism for the low immunogenicity of these molecules may be important for efforts in developing a new efficient vaccine for schistosomiasis japonicum. The 27/30 kDa protein bands could not be recognized by sera of healthy rabbits but were recognized specifically by sera from the schistosome infection stage and post-treatment stage of the same rabbit. The maintenance of the antibody titer to the 27/30 kDa bands over time after treatment indicated that this was a long-lived antibody response (Fig. 1). Interestingly, the 36/37 kDa proteins could not be recognized by sera of healthy rabbits, but they could be recognized specifically by sera of schistosome-infected rabbits; however, the intensity of these antibody responses declined gradually at 4 weeks post-treatment and became very weak or absent between 8 and 12 weeks post-treatment (Fig. 1).

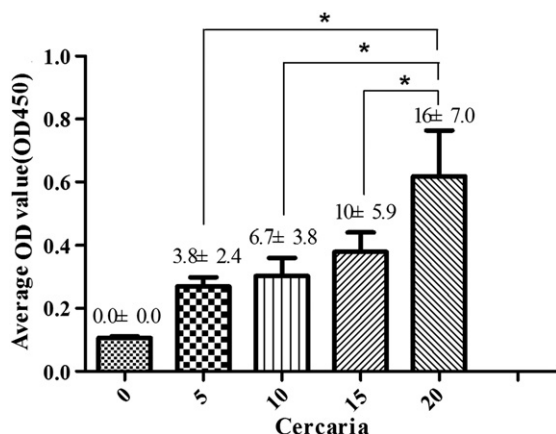


Fig. 8 – Correlation between anti-rSj GAPDH IgG antibody level and degree of *S. japonicum* infection in mice. Anti-rSj GAPDH IgG levels of mouse groups infected with 0, 5, 10, 15, 20 cercariae are shown. Data are means $\pm$ SD of the average number of recovered adult worms from mice of each group (each group have six mice). * $P < 0.05$ between groups.

Meanwhile, the intensity of the antibody response to these 36/37 kDa protein bands in sera from the untreated rabbits at weeks 14–16 post-infection (the equivalent time points to weeks 8–10 post-treatment in the treatment group) remained high (Fig. 2), although there was a slight decrease at the chronic stage of schistosome infection. While the reason for the decline in

antibody response against Sj GAPDH at the chronic stage of infection remains to be investigated, these results indicated that the antibody responses against 36/37 kDa proteins were short-lived and antigen-dependent. With antigen levels reduced or absent post-treatment upon removal of an active schistosome infection, antibody responses to the 36/37 kDa proteins also fell subsequently or became negative. Compared to the patterns of antibody responses to non-deglycosylated Sj ESAGs, those to deglycosylated 36/37 kDa proteins with infected rabbit sera were enhanced, and the antibody response in sera of treated rabbits to deglycosylated 36/37 kDa proteins fell more quickly (Fig. 3). The antibody titers to other antigens declined overall after deglycosylation. These results also suggested that the short-lived antibody responses against the 36/37 kDa proteins of Sj ESAG were induced by a peptide epitope (s) and not by a glycosylated epitope (s).

The results of 2-D Western blotting showed that the antigens inducing short-lived antibody responses in Sj ESAG fell into two groups of molecules: 36/37 kDa proteins with pI 6–9 and 25–36 kDa proteins with pI 4–5. The 36/37 kDa protein molecules were arranged in a horizontal line on the SDS-PAGE gel, which included 17 protein spots. While their molecular masses were similar, their pI values were different. Among the 42 protein spots analyzed by MALDI-TOF/TOF-MS which could potentially induce short-lived antibody responses, 21 genes were identified successfully: nine protein spots were Sj GAPDH, three protein spots were Sj FBA, four spots were SJCHGC02266, three spots were SJCHGC01391, and two spots were SJCHGC 05757 (Table 1). These results suggested that Sj GAPDH, Sj FBA, SJCHGC02266, SJCHGC01391 and SJCHGC05757

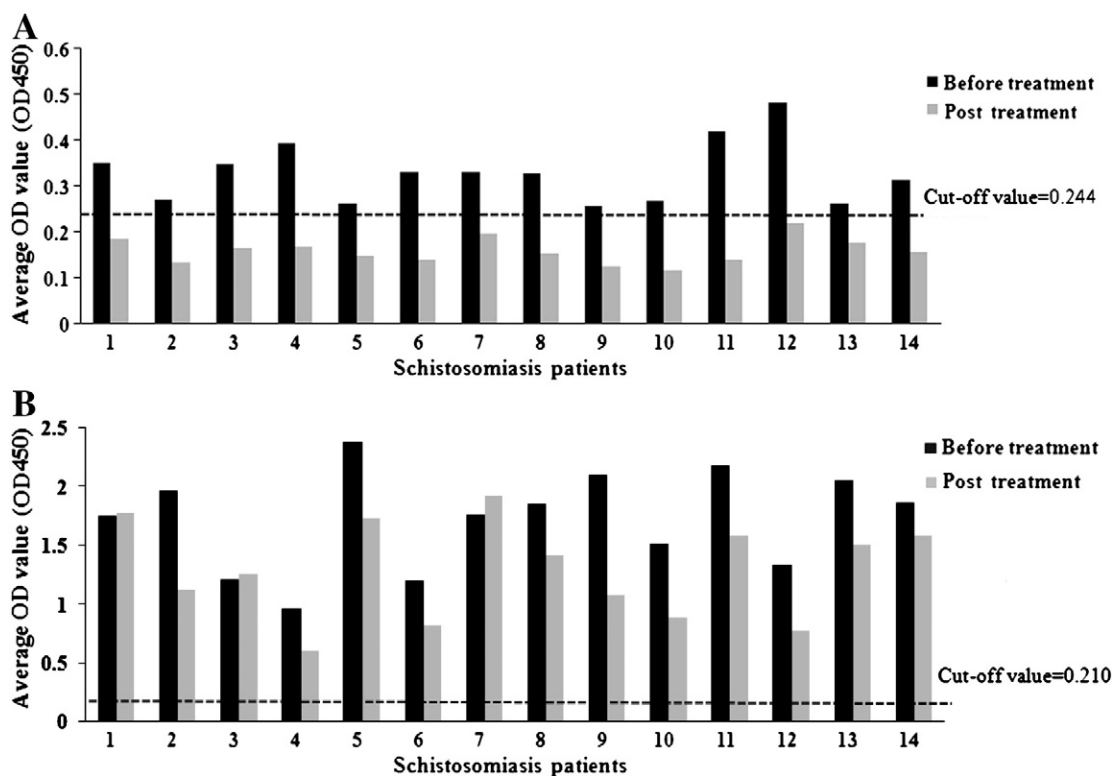


Fig. 9 – Comparison of antibody levels in schistosomiasis patients before and after treatment. (A) Changes of IgG antibody levels against rSj GAPDH in 14 schistosomiasis patients. (B) Changes of IgG antibody levels against SEA in 14 schistosomiasis patients.

Table 2 – Comparison of positive rates of antibodies against Sj GAPDH and SEA detected in patient sera by ELISA.

Samples	SEA-ELISA			Sj GAPDH-ELISA	
	Total no.	No. positive	Positive rate (%)	No. positive	Positive rate (%)
Healthy donor	30	0	0	0	0
<i>S. japonicum</i> -infected patient	40	40	100	33	82.5
<i>C. sinensis</i> -infected patient	30	6	20	2	6.7
<i>P. westermani</i> -infected patient	20	13	65	5	25

are multi-functional proteins with different isoelectric points. The multiple functions of a protein are realized by post-translational modifications, which would produce proteins with different isoelectric points [37,38]. The antibody response patterns on 2-DE Western blots in this study demonstrated that the proteins with different isoelectronic points could induce similar short-lived antibody responses, perhaps because these proteins have the same amino acid sequence which may share same epitopes. Furthermore, our findings confirmed Sj GAPDH and Sj FBA as major components of Sj ESAs which have been identified in previously published work [39,40].

Recombinant FBA derived from the Formosan strain of *S. japonicum* has been confirmed as an effective diagnostic antigen for schistosomiasis [41]. The results of 2-DE immunoblotting showed that the levels of IgG antibody against the 25 to 36 kDa protein molecule group (i.e., SJCHGC05757, SJCHGC0319 and SJCHGC02266) in rabbit serum after treatment declined slowly (Fig. 5). Thus, the potential of detecting the antibody level changes of antibodies to molecules in this group for evaluating the chemotherapeutic effect of schistosomiasis may be lower than that of Sj GAPDH. As a multifunctional protein, GAPDH is not only involved in glycometabolism, but it is also a virulence factor in a number of pathogenic organisms, which enables intracellular parasites to disable cytoprotective mechanisms by redirecting the host phagocytic vesicles [42]. It is of interest to determine whether detecting the change of serum antibodies against Sj GAPDH in the host can provide unequivocal diagnosis of active schistosome infection and to evaluate curative effects of treatments. Therefore, to explore the immunodiagnostic value of Sj GAPDH, we prepared the purified recombinant Sj GAPDH protein in *E. coli*. Analyses by Western blotting and ELISA showed that the recombinant Sj GAPDH could be recognized specifically by sera of schistosome-infected rabbits, but the intensity of the Sj GAPDH antibody response in sera of treated rabbits declined gradually along with the

extension of treatment time (Fig. 7). This antibody response pattern was similar to that of natural Sj GAPDH in *S. japonicum* adult worms. These results confirmed again that the short-lived antibody response against Sj GAPDH was induced by peptide epitope(s) and not by glycosylated epitope(s) of Sj GAPDH.

The level of IgG antibody against recombinant Sj GAPDH rose with the severity of schistosome infection in mice in a typical dose-dependent relationship (Fig. 8). Moreover, the recombinant Sj GAPDH was used to screen the sera of 14 patients with schistosomiasis japonicum. The IgG antibody against recombinant Sj GAPDH in all 14 patient sera before treatment were positive but then became negative after one year of treatment; meanwhile, SEA-specific IgG antibodies in all 14 patients were still detected at one year post-treatment (Fig. 9). These results suggested that Sj GAPDH could also induce short-lived antibody responses in humans, and detecting changes in Sj GAPDH antibodies in patient serum before and after treatment would aid in the evaluation of chemotherapeutic effects against schistosomiasis.

An indirect ELISA for detecting Sj GAPDH antibodies was used to evaluate reactivity of sera from healthy donors and patients with various parasitic infections. The sensitivity of the assay was 82.5% for schistosomiasis, while the cross-reactivity rate was 6.7% for clonorchiasis and 20% for paragonimiasis. These results suggested that antibodies against Sj GAPDH are highly specific and have the potential for discriminating between infections with other flatworms, as the amino acid sequence homology between Sj GAPDH and Cs GAPDH is only 74.5%. Whether the unique amino acids and their post-translational modifications in Sj GAPDH contribute to the specific immunogenicity of this protein remains to be further studied. Although the sensitivity for detecting anti-Sj GAPDH IgG antibodies was slightly lower than that for detecting anti-SEA antibodies, the opposite was true for the specificity of the assay (i.e., specificity for anti-Sj GAPDH IgG antibodies was higher than that for anti-SEA antibodies). While these

Table 3 – Comparison of positive predictive value (PPV) and negative predictive value (NPV) of Sj GAPDH and SEA detected in patient sera by ELISA.

	SEA-ELISA		Sj GAPDH-ELISA	
	Schistosomiasis patients +	Schistosomiasis patients –	Schistosomiasis patients +	Schistosomiasis patients –
Tested positive	40 (A)	19 (B)	A (33)	B (7)
Tested negative	0 (C)	61 (D)	C (7)	D (73)
PPV of SEA = $A/(A + B) \times 100\% = 40/(40 + 19) = 67.8\%$.				
NPV of SEA = $D/(C + D) \times 100\% = 61/(0 + 61) = 100\%$.				
PPV of GAPDH = $A/(A + B) \times 100\% = 33/(33 + 7) = 82.5\%$.				
NPV of GAPDH = $D/(C + D) \times 100\% = 73/(7 + 73) = 91.3\%$.				

specifications may meet the needs of schistosomiasis clinical diagnostic tests, more sensitive detection methods, such as the biotin-avidin signal amplification system and time resolution fluorescence immunoassay, would further improve the sensitivity of detecting antibodies against Sj GAPDH. Since the level of anti-Sj GAPDH IgG antibodies was found to correlate with the severity of schistosome infection, it can possibly replace current methods of detecting worm eggs and circulating schistosome antigens as a defined indicator of schistosomiasis.

As the rate in decline of serum antibody against Sj GAPDH post-treatment is related to host genetics and schistosome worm burden, the time for this antibody level to become negative post-treatment will vary among different individuals. Therefore, the practical value of detecting specific antibodies against Sj GAPDH for the diagnosis and assessment of treatment effect for active schistosome infection should be further verified by testing a large number of cases in an endemic area.

5. Conclusions

This study demonstrated that the GAPDH protein identified among the *S. japonicum* adult worm excretory-secretory antigens could induce an antigen-dependent specific short-lived antibody response in the host. Detecting antibody responses against Sj GAPDH provides a potential basis for developing a new method for diagnosis and evaluation of chemotherapeutic effects for schistosomiasis.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.05.003>.

Authors' contributions

CXY and JZZ designed and supervised the study and critically revised the manuscript. JW performed the laboratory work, analyzed data and drafted the manuscript. FZ performed the 24-cm 2D-blot. DX identified protein spots by MS and performed database searches. LJS and XRY expressed and purified the recombinant GAPDH fusion protein. WZ participated in the immunoblot assays. HQW, CYQ and XDK contributed to data analysis. SS, YLX and JY prepared sera from infected rabbits and mice. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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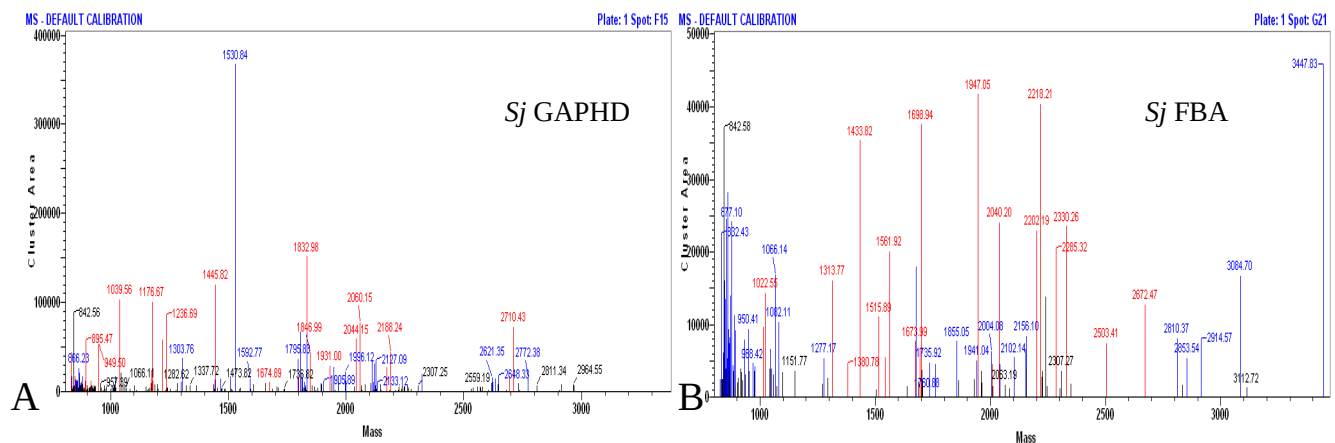
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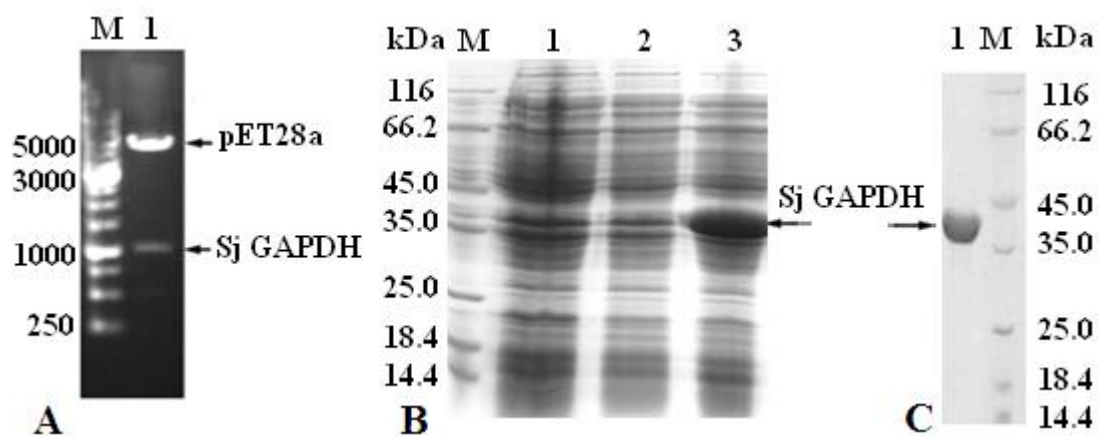
Supplemental Table 1. Worm egg burden by fecal examination of 14 schistosomiasis patients before and after praziquantel treatment

village	Code	Sex	Age BT	Age PT	Epg					
					BT1	BT 2	BT 3	PT 1	PT 2	PT 3
XinHua	1301102	M	48	49	0	2	1	0	0	0
XinHua	1301106	F	43	44	0	1	1	0	0	0
XinHua	1303204	F	34	35	2	1	6	0	0	0
XinHua	1303904	F	33	34	3	3	5	0	0	0
XinHua	1304802	F	44	45	0	0	2	0	0	0
XinHua	1304904	F	45	46	17	11	-1	0	0	-1
XinHua	1305902	F	63	64	0	0	1	0	0	0
XinHua	3600602	F	58	59	1	2	1	0	0	0
XinHua	3603402	F	56	57	0	4	8	0	0	0
XinHua	3605902	F	61	62	0	2	2	0	0	0
XinHua	3607201	M	66	67	0	1	2	0	0	0
XinHua	3608501	M	41	42	0	0	3	0	0	0
XinHua	4503302	F	62	63	0	2	11	0	0	0
XinHua	4508604	F	13	14	0	0	1	0	0	0

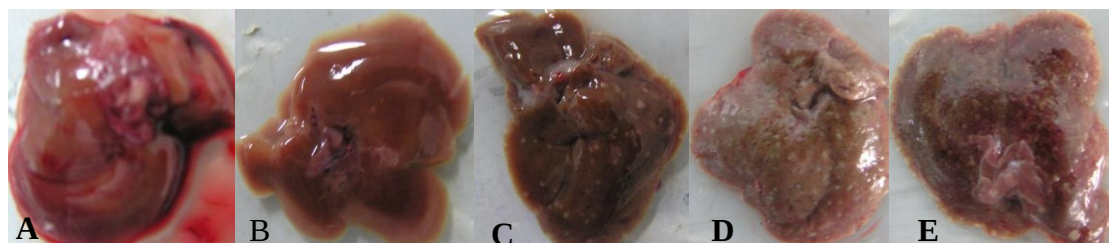
BT: before treatment, PT: post-treatment., Epg: the number of egg per gram stool, -1 indicates that the stool sample was not obtained from the patient



Supplementary Figure 1. MALDI-MS fingerprints of *Sj* GAPDH and *Sj* FBA

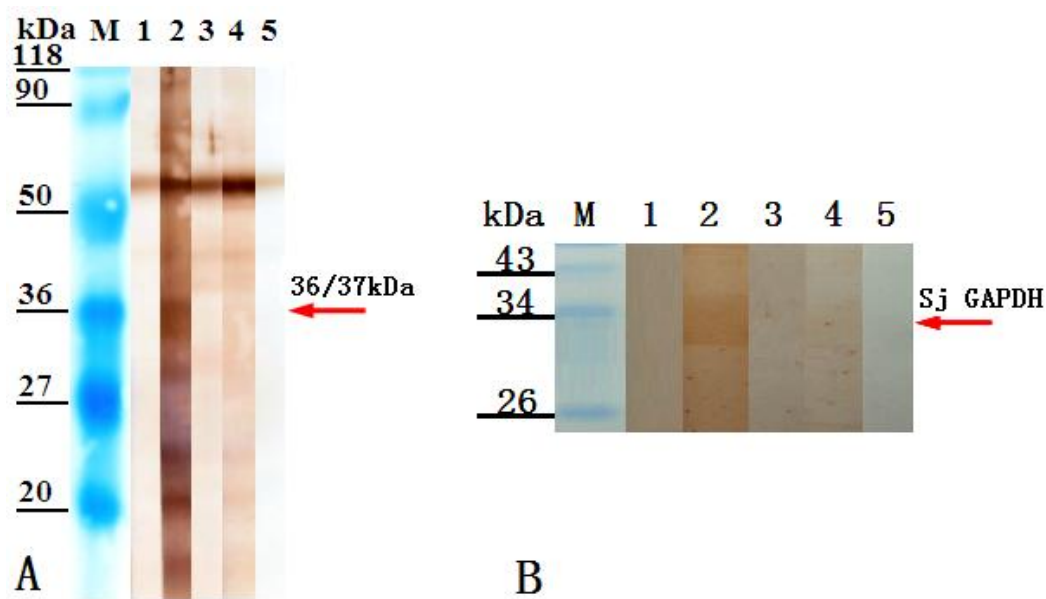


Supplementary Figure 2. Expression and purification of recombinant *Sj* GAPDH protein. (A) Restriction analysis of expression plasmid pET28a-*Sj* GAPDH. Lane M, 1 kb DNA standard molecular weight marker; lane 1, restriction products of pET28a-*Sj* GAPDH digested with *NheI/SacI*. (B) Expression of recombinant *Sj* GAPDH. Lane 1, lysate of *E. coli* BL21; lane 2, lysate of *E. coli* BL21 transformed with plasmid pET28a; lane 3, lysate of *E. coli* BL21 transformed with recombinant plasmid pET28a-*Sj* GAPDH; lane M, standard protein molecular weight marker. (C) Purification of recombinant *Sj* GAPDH protein. Lane M, standard protein molecular weight marker; lanes 1, The purified recombinant *Sj* GAPDH protein.



Supplementary Figure 3. Pathological examination of healthy and *S. japonicum*-infected mouse livers.

(A) Liver of healthy mouse in control group A. (B to E) Livers of mice in experimental groups B to E, which were infected with 5, 10, 15 and 20 cercariae of *S. japonicum*, respectively.



Supplementary Figure 4. The specific antibody response of Sj ESAGs reacting with different parasites serum

(A) Lane 1, ESAG reacted with mixed sera of ten healthy individuals; Lane 2, ESAG reacted with mixed sera of ten schistosomiasis; Lane 3, ESAG reacted with mixed sera of ten clonorchiasis; Lane 4, ESAG reacted with mixed sera of ten paragonimiasis; Lane 5, ESAG reacted with HRP labeled goat anti-human IgG; Lane M, Prestained protein molecular weight standard marker.

(B) Lane 1, Sj GAPDH reacted with mixed sera of ten healthy individuals; Lane 2, Sj GAPDH reacted with mixed sera of ten schistosomiasis; Lane 3, Sj GAPDH reacted with mixed sera of ten clonorchiasis; Lane 4, Sj GAPDH reacted with mixed sera of ten paragonimiasis; Lane 5, Sj GAPDH reacted with HRP labeled goat anti-human IgG; Lane M, Prestained protein molecular weight standard marker.