

Assessment of the diagnostic efficacy of enolase as an indication of active infection of *Schistosoma japonicum*

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Abstract Schistosomiasis is a common zoonoses affecting humans. The atypical clinical symptoms, low morbidity, and low degree of infection impede diagnosis and assessment of epidemics. Detecting circulating antigens from adult worms in patients' body fluids should be diagnostically superior to examining eggs in feces. Herein, the excretory-secretory proteins of adult worms were analyzed by using 2-D protein electrophoresis and mass spectrometry. The *Schistosoma japonicum* enolase (*Sj* enolase) was identified as the most abundant excretory-secretory antigen. Purified recombinant *Sj* enolase was prepared, and specific monoclonal and polyclonal antibodies were raised against it. A sandwich enzyme-linked immunoassay (sandwich ELISA) was established that used the monoclonal antibody as a capture antibody and the polyclonal antibody as a detection antibody. The linear

detection range was 0.7–1000 ng/ml (minimum 700 pg/ml). *Sj* enolase could be detected in the sera of infected rabbits and disappeared rapidly postpraziquantel treatment. The sensitivity and specificity of this sandwich ELISA to detect field serum samples of schistosomiasis were 84.61 and 95.83 %, respectively. The cross-reaction rates for clonorchiasis and paragonimiasis were 3.33 and 5 %, respectively. This ELISA assay was used to test 45 matching sera of schistosomiasis patients before treatment and at 3, 6, 9, and 12 months posttreatment. Among the sera, 88.89 % were positive before treatment. At 3, 6, 9, and 12 months postpraziquantel treatment, 93.33, 97.78, 100, and 100 % tested negative, respectively. Therefore, *Sj* enolase can be used to indicate active *Schistosoma* infection, and detecting serum *Sj* enolase is important for diagnosis and evaluating treatment effect.

Hong Gao and Di Xiao contributed equally to this work and are considered co-first authors.

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Introduction

Schistosomiasis is a type of zoonosis that is distributed widely in tropical and subtropical regions around the world. Currently, about 249 million people worldwide are suffering from schistosomiasis and 800 million people may be under the threat of infection (WHO 2014). The pathological damage caused by schistosomiasis becomes a serious public health problem in epidemic areas (Dunne and Pearce 1999; Cheever et al. 2000). There are many reservoir hosts, intermediate hosts, and natural epidemic foci of schistosomiasis in the natural environment; therefore, the composition of the infection source (especially the *Schistosoma japonicum*) is very complex, which makes elimination, prevention, and control difficult. A key approach to controlling schistosomiasis

prevalence is to identify and eliminate the infection sources to prevent the spread of this disease. Therefore, establishing an effective detection method to identify patients with active infection and to define the prevalence of schistosomiasis will be important in formulating appropriate strategies to control schistosomiasis. The existing diagnostic methods of schistosomiasis include pathogenic, immunological, and molecular biological methods. Both the Kato-Katz method, based on the microscopic examination of eggs, and the miracidium hatching method are still the gold diagnostic standards (Colley and Secor 2014; Bergquist et al. 2009) for intestinal schistosomiasis (such as *Schistosoma mansoni* and *S. japonicum*). However, the results of these methods are influenced by multiple factors, such as the degree of infection, the time of egg excretion, and the egg distribution in the stool. The sensitivity of the Kato-Katz method is low, leading to missed diagnoses. Repeated fecal examinations can increase the sensitivity, but reduces the compliance of patients, which limits the effectiveness of this method (especially in low endemic areas). The other disadvantages of the current pathogenic detection methods are manipulation complications and increased time; therefore, they are not suitable for large-scale epidemiological surveys. Testing the specific DNA of *Schistosoma* in the blood and/or excretions of patients by polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) is effective to confirm active infection in patients (Ibironke et al. 2012; Wichmann et al. 2013). However, neither method is suitable for large-scale field application because they need complicated preprocessing of samples, expensive equipment and reagents, as well as support from specialized technicians in a strict laboratory environment. They are also time-consuming and expensive. Immunological diagnostic methods include the circulating antibody test and circulating antigen test. The circulating antibody test is a common approach for the large-scale survey of schistosomiasis. There are also methods to detect short-lived antibody response (Wang et al. 2013) and other universal methods of detecting antibody, including indirect hemagglutination (IHA), circumoval precipitin test (COPT), enzyme-linked immunosorbent assay (ELISA), and immune chromatography; however, these methods cannot distinguish active infection from historic infection, and they also have high cross-reaction rate with other parasites; therefore, their results cannot be used as diagnostic evidence of active infection by *Schistosoma* (Gomes et al. 2014).

The circulating antigens, derived from the worm body, existing in the host body fluid (Deelder et al. 1976, 1980), have been studied as objective evidence of active infection and can be used to evaluate the effect of chemotherapy and the potential impact of prevention strategies. Over the last few decades, studies on detection methods of circulating antigens have focused on testing circulating anodic antigens (CAA) and circulating cathodic antigen (CCA) and have developed a series of detection methods (Deelder et al. 1989a, b; Van

Dam et al. 2004; De Jonge et al. 1990; Van Lieshout et al. 1995a, b); however, the complicated constituents of the CAA and CCA cause differences in specificity and sensitivity and make it difficult to be standardized in methodology. Thus, it would be practical to identify a highly abundant circulating antigen in patients' sera as a detection target and establish standardized methods of detecting the circulating antigen with high sensitivity and specificity to diagnose and prevent schistosomiasis in epidemic areas with low degrees of infection.

In this study, we analyzed the components of excreted and secreted products from *S. japonicum* adult worms using 2-D electrophoresis and matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) to identify highly abundant excreted-secreted protein molecules. The gene of the most abundant protein, *Sj* enolase, was cloned for recombinant expression. Monoclonal antibodies and polyclonal antibodies against *Sj* enolase were prepared and a double antibody sandwich ELISA to detect the circulating antigen *Sj* enolase was established. The performance of this method for the diagnosis and evaluation of the chemotherapeutic effect was assessed.

Material and methods

Reagents

Restriction enzymes (*Sal*I, *Bam*HI), T4 DNA ligase, dNTPs, Wizard® SV Gel and PCR Clean-Up System, Wizard® Plus Minipreps DNA Purification System, and trypsin were purchased from Promega (Madison, CA, USA). Phusion™ high-fidelity DNA polymerase came from the New England Biolabs, Ltd (Beijing, People's Republic of China). High affinity Ni-charged resin came from GenScript Inc. (Nanjing, People's Republic of China). Goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate, goat anti-rabbit IgG-HRP conjugate, and goat anti-human IgG-HRP conjugate came from Bethyl Laboratory Inc. (Houston, Texas Area, USA). Whatman™ nitrocellulose (NC) membrane came from GE Healthcare (Dassel, Germany). The monocomponent 3,3',5,5'-tetramethylbenzidine (TMB) substrate was produced by XinBoSheng Company (ShenZhen, People's Republic of China). BeyoECL Plus was obtained from Beyotime Company (Nantong, People's Republic of China). The Timesaver mRNA purification system, 3-[(3-cholamidopropyl)-dimethylammonium]-propane-sulfonate (CHAPS), protease inhibitor mix, IPG buffer, 24-cm linear IPG strips pH 3–10, and Tris were obtained from GE Healthcare (Piscataway, NJ, USA). The SuperScript™ Choice System came from Invitrogen (Carlsbad, CA, USA). Other chemical reagents were purchased from Sigma-Aldrich (St. Louis, USA).

Plasmid, bacterial strains, and cells

Escherichia coli DH5 α , *E. coli* BL21, and plasmid pET28a(+) were maintained in our laboratory. Murine myeloma cells SP2/0 were bought from cell banks at the Biochemical and Cell Biological College, Chinese Academy of Sciences, Shanghai, People's Republic of China.

Animals

BALB/c mice (20 g, 8 weeks old, female) and Institute for Cancer Research (ICR) mice (20 g, 6 weeks old, female) were purchased from the Experimental Animal Center of Yangzhou University (Yangzhou, People's Republic of China). Japanese White rabbits were obtained from the Jinling Animal Center (Nanjing, People's Republic of China).

All animals were raised at the Department of Experimental Animals, Jiangsu Institute of Parasitic Diseases (Wuxi, China) and provided with food and water ad libitum. The animal experiments were approved by the Institutional Review Board (IRB00004221) of Jiangsu Institute of Parasitic Diseases (Permission Number: JIPDAERP20110112). Animals, namely rabbits and mice, were infected and venous blood was obtained 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 to minimize suffering.

Parasite and antigens

Cercariae of *S. japonicum* (Jiangsu 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* soluble egg antigen (*Sj* SEA), adult worm antigen (*Sj* AWA), soluble *Clonorchis sinensis* worm antigen (SCA), and *E. coli* BL21 protein were prepared following previously published methods (Chappell and Dresden 1986; Boros and Warren 1970). The excreted and secreted antigens of *S. japonicum* adult worm (*Sj* ESA) were prepared according to published methods (Kirinoki et al. 1998). Briefly, the infected snails were incubated in illumination conditions and hatched in dechlorination of water at 25 °C for 2–3 h. Each rabbit was infected with 1000–1500 *S. japonicum* cercariae by abdominal skin penetration. On the 42nd day after infection, the adult worms in the mesenteric veins were collected by portal venous infusion and washed with saline 2–3 times to remove the attached host proteins. About 2000 adult worms were immersed in 20 ml phosphate buffer (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 2 mM, pH 7.2–7.4) for 15 min at 37 °C. The upper solution without worms was

termed the *Sj* ESA. The *Sj* ESA was centrifuged at 16,000 \times g for 30 min. The supernatant was placed in 1.5 ml sterile Eppendorf tubes. All the antigens were quantified using a BCA Protein Assay Kit and stored at –80 °C.

Sera

Sera of animals infected by S. japonicum

Rabbits were divided into the treatment group (four rabbits) and the untreated group (two rabbits). Each rabbit was infected with 500 *S. japonicum* cercariae by abdominal skin penetration. Rabbits of the treatment group were given 160 mg/kg/day artesunate orally for two consecutive days from the 39th day to the 40th day postinfection, followed by 150 mg/kg/day praziquantel orally for another two consecutive days from the 41st day to the 42nd day after infection. Rabbits in the untreated group were not treated with any medicine. Blood samples were obtained before infection (healthy stage) and then collected regularly every 2 weeks until the 18th week postinfection. The isolated serum samples were stored at –80 °C for subsequent analysis. The adult worms in the mesenteric veins of rabbits were collected by portal vein perfusion to confirm whether the schistosome infection and chemotherapy were successful at the end of the experiment.

Human sera

Serum samples from 143 schistosomiasis patients were collected from the epidemic areas of schistosomiasis by the Jiangling Institute of Schistosomiasis Control, Hubei Province, People's Republic of China. Among these patients, blood samples were collected from 45 of them before treatment and at 3, 6, 9, and 12 months after praziquantel (PZQ) treatment, respectively. Infections in the patients were confirmed by examining eggs in stool samples using the Kato-Katz method and the miracidium hatching method. From each of the 45 patients, three stool samples before PZQ treatment and at 3, 6, 9, and 12 months after PZQ treatment were collected, and the eggs in each stool sample were counted in three repeated measures by the Kato-Katz method and the miracidium hatching method. Fecal samples from all patients were positive for schistosome eggs and/or miracidium hatching before PZQ treatment, and those obtained at 3, 6, 9, and 12 months after PZQ treatment became negative. All study procedures were approved by the Ethics Committee of Jiangsu Institute of Parasitic Diseases and Jiangling Institute of Schistosomiasis Controlling. All participants were informed of the purpose of this study and gave informed consent. Thirty serum samples of clonorchiasis infection were obtained from the epidemic region in the Guangxi Zhuang Autonomous Region, China. All fecal samples of the patients were positive for *C. sinensis* eggs. Twenty serum samples of

paragonimiasis were obtained from the Department of Clinic Medical Center, Jiangsu Institute of Parasitic Diseases. The IgG antibody against *Paragonimus westermani* was positive in all sera. Sera from 96 healthy donors were collected from a nonendemic area, in which the IgG antibody against SEA of *S. japonicum* was negative. All the sera samples were kept at -30°C .

Methods

Identification of highly abundant circulating antigen molecules in *Sj* ESA

We used 2-D electrophoresis to analyze the composition of excreted and secreted protein of *S. japonicum* adult worms. The *Sj* ESA proteins were cleaned and quantified using the 2-D clean-up kit (GE Healthcare, Piscataway, NJ, USA) and the 2-D Quant kit (GE Healthcare), respectively, following the manufacturer's instructions. *Sj* ESA proteins were 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). *Sj* ESA proteins (800 μg in 450 μl rehydration buffer with 8 M urea, 2 % CHAPS, 60 mM DTT, and 0.5 % IPG buffer) were loaded onto an immobilized pH gradient (DryStrips™ 24 cm in length [pH 3–10 nonlinear (NL)]). The proteins were initially separated using the Ettan™ IPGphor IITM 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 and 126 V. After IEF, the proteins on the strips were reduced and bound to SDS by equilibrating 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 0 (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 \times 24 cm wide \times 19 cm long) sealed with 0.5 % (w/v) agarose. Proteins were separated using a Ettan™ Dalt unit (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. All selected protein spots were excised from the stained acrylamide gels using the One Touch 2D Gel SpotPicker (Bio-Rad, 1.5 mm) and digested. Briefly, the gel pieces were first washed with 100 μl 25 mM ammonium bicarbonate (pH 8.0) for 20 min and then washed with 50 μl 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 a digestion buffer (25 mM ammonium bicarbonate and 20 $\mu\text{g}/\text{ml}$ of trypsin) for 16 h at 37°C , followed by another 60 min incubation after adding 40 μl of 50 % acrylonitrile/5 % trifluoroacetic acid. The final sample was absorbed by prefreezing at -80°C for 2 h and then vacuum freeze-dried. The material was dissolved in 2 μl of a solution containing deionized water, acetonitrile, and trifluoroacetic acid (49.75:49.75:0.5), and 0.5 μl of this sample solution was used to mix with 0.5 μl of a matrix solution (saturated matrix solution in 50 % acetonitrile and 2.5 % trifluoroacetic acid) and then dried at room temperature. All proteins were analyzed using 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 using 2000 laser shots and was processed and analyzed by the Global Protein Server Workstation (GPS Applied Biosystems), which uses internal Mascot v2.1 software to search the peptide mass fingerprints. The searches were performed by using the NCBI nonredundant protein database (<ftp://ftp.ncbi.nih.gov/blast/db/FAST/nr.gz>, updated in 2011) with the following criteria: NCBI human database, trypsin digestion, 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 (Zhang et al. 2009).

Gene cloning, protein expression, and characterization of *Sj* enolase

The results of MALDI-TOF/TOF-MS showed that *Sj* enolase was the most abundant protein of *Sj* ESA. To study the diagnostic value of *Sj* enolase as a circulating antigen further, we expressed this protein in *E. coli*. Briefly, the messenger RNA (mRNA) of adult worms was extracted with a Timesaver mRNA purification system (GE Health), following the manufacturer's instructions, and the first-strand complementary DNA (cDNA) was synthesized using the SuperScript™ Choice System (Invitrogen). The following primers for amplifying the open reading frame of the DNA fragment encoding the *Sj* enolase gene were designed according to the published

sequence (gi46201125): forward primer: 5'-ttggatccatgaaaatggcaattatagcgat-3' and reverse primer: 5'-ttgtcgactcaaatgaggatggcggaagt-3'. A *Bam*HI site was introduced into the 5' end of the upstream primer, and a *Sal*I site was introduced into the 5' end of the downstream primer. The DNA fragment encoding *Sj* enolase from the *S. japonicum* adult worm cDNA was amplified by the Phusion high-fidelity enzyme and cloned into a TA cloning vector (pGEM-T Easy, Promega) to construct the recombinant plasmid pGEM-T-*Sj* enolase. The inserted *Sj* enolase DNA fragment was confirmed by restriction enzyme analysis and DNA sequencing (Invitrogen, Shanghai, China) and then subcloned into the expression plasmid pET28a(+) at the *Bam*HI and *Sal*I restriction sites to form the recombinant plasmid pET28a-*Sj* enolase. 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 µg/ml) and induced by isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) at different temperatures to express the *Sj* enolase protein. The expressed products were analyzed by SDS-PAGE, and the recombinant *Sj* enolase protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) resin following the manufacturer's instructions.

To determine the immunogenicity of r*Sj* enolase, five ICR mice (6 weeks old, female) were immunized with 50 µg of purified r*Sj* enolase in complete Freund's adjuvant for the first injection and boosted with the same dose of the purified r*Sj* enolase in incomplete Freund's adjuvant for three further times. The immunization interval was 2 weeks, and the level of serum antibody against r*Sj* enolase was measured by a universal ELISA with an r*Sj* enolase-coated plate. The AWA and r*Sj* enolase were analyzed by SDS-PAGE, transferred onto the NC membranes with a Bio-Rad semidry transfer unit, and then reacted with the immunized mice serum against r*Sj* enolase and schistosomiasis patients' sera, respectively, to observe the immunogenicity and immunoreactivity of recombinant *Sj* enolase.

The r*Sj* enolase transferred on NC membranes was also reacted with the sera of clonorchiasis, paragonimiasis, and healthy human sera, respectively, to observe the specificity of immunoreaction of r*Sj* enolase.

Preparation of antibodies against r*Sj* enolase

Polyclonal antibodies

Rabbits were immunized with complete adjuvant with the r*Sj* enolase, and each rabbit was injected intradermally with 2 mg protein in neck skin and boosted with incomplete adjuvant with r*Sj* enolase at the same concentration three times, with intervals of 2 weeks. The sera of immunized rabbits were collected by carotid

artery bleeding after 2 weeks after the final immunization. The titer of serum antibody against the r*Sj* enolase was determined by a conventional ELISA method. The polyclonal antibody of the immunized rabbit serum was purified by affinity chromatography using Protein G Sepharose resin.

Monoclonal antibodies

Immunization Mice (BALB/c, female, 8 weeks old) were immunized with complete Freund's antigen with r*Sj* enolase (50 µg) intradermally in the first immunization and then boosted three times with the same amount of r*Sj* enolase with incomplete adjuvant antigen at intervals of 2 weeks; the final boost injected the same dose of r*Sj* enolase in PBS into the mice abdomens, 1 week after the fourth immunization.

Fusion and screening The single spleen cells were prepared from immunized mice spleens and were fused with SP2/0 cells at logarithmic phase in the proportion of 6:1 using 50 % PEG-4000 (Sigma). The fusion cells were seeded in 96-well plates with HAT selective medium/RPMI 1640 medium (containing 15 % fetal bovine serum, 1000 U/ml penicillin, 100 mg/ml streptomycin, hypoxanthine, aminopterin, thymidine). The medium was changed every week at half the amount with HAT (Gibco), and the hybridoma cells in each well were monitored by microscopic examination. The wells in which the hybridoma secreted the specific antibody against *Sj* enolase were selected by ELISA, and the hybridoma cells were subcloned by the limited dilution method several times until the specific antibody against *Sj* enolase of all wells became positive. Those cells that could secrete the monoclonal antibody steadily over a long term were stored in liquid nitrogen.

Each BALB/c mouse (8 weeks old, female) was inoculated with $0.5\text{--}1.0 \times 10^6$ hybridoma cells, which secreted the specific antibody, in their abdomens to grow the hybridomas. The ascetic fluid was being collected several weeks later. The monoclonal antibody in ascetic fluid was purified by affinity chromatography using Protein G Sepharose resin, following the manufacturer's instructions.

The affinity constant of the antibody against *Sj* enolase was tested by noncompetitive ELISA (Beatty et al. 1987). The microtiter plates were coated with two appropriate concentrations of r*Sj* enolase separately, then reacted to the polyclonal antibody and monoclonal antibody at different dilution ratios, respectively. An antibody reaction curve was drawn with antibody dilution as the abscissa and A_{450} values as the ordinate. The affinity constant (K_{aff} value) was calculated with half

of the maximum A_{450} value at the corresponding antibody concentration as follows:

$$K_{\text{aff}} = \frac{(n-1)}{2(n[\text{Ab}']_t - [\text{Ab}]_t)}$$

In the equation above, t represents the temperature, $[\text{Ag}]_t$ represents the concentration of the high-concentration antigen, $[\text{Ag}']_t$ represents the concentration of the low-concentration antigen, $[\text{Ab}]_t$ represents the antibody concentration corresponding to the high-concentration antigen, $[\text{Ab}']_t$ represents the antibody concentration corresponding to low-concentration antigen, and n represents the value of $[\text{Ag}]_t / [\text{Ag}']_t$.

The specificity of the monoclonal antibody against rSj enolase was analyzed by western blotting to check whether the monoclonal antibody recognized the SEA, ESA, AWA, SCA, and *E. coli* BL21 proteins, which were separated by SDS-PAGE and transferred onto an NC membrane. HRP-labeled sheep anti-mouse IgG was used as the secondary antibody. Reactive proteins on the NC membrane were stained by a chemiluminescence reagent (BeyoECL Plus).

Establishment and optimization of a double antibody sandwich ELISA for detecting circulating antigen *Sj* enolase

The sandwich ELISA for detecting circulating antigen *Sj* enolase (referred to as *Sj* enolase sandwich ELISA) was established and optimized as follows. The purified monoclonal antibody or polyclonal antibody was used as the capture antibody for *Sj* enolase, and purified rabbit polyclonal antibody or monoclonal antibody was used as the detection antibody, respectively. HRP-labeled goat anti-rabbit IgG or HRP-labeled goat anti-mouse IgG was used as the second detection antibody, and the substrate TMB was used for color development. The capture antibody was diluted with carbonate buffer (50 mM HCO_3^- , pH 9.6), and the detection antibody was diluted with phosphate buffer (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM, pH 7.2–7.4). The capture antibody was coated on ELISA plates at different concentrations (10.0, 5.0, 2.5, 1.25, 0.625, 0 $\mu\text{g}/\text{ml}$) and kept at 4 °C overnight. Each well was blocked with PBSM (PBS containing 5 % skimmed milk powder) for 1 h, at 37 °C, washed by PBST (PBS containing 0.5 % Tween-20) three times, and reacted with rSj enolase solution (dissolved in PBS) at 10 $\mu\text{g}/\text{ml}$ for 1 h at 37 °C. Then the plate was washed three times and reacted with the detection antibody at different concentrations (5.0, 2.5, 1.25, 0.625, 0 $\mu\text{g}/\text{ml}$) for 1 h at 37 °C. The plates were washed three times with PBST, and then reacted HRP-labeled second antibody was diluted 1:10,000 at 37 °C for 1 h. Finally, the plates were stained with

the TMB substrate for 5 min at room temperature. The color development was stopped by adding 50 $\mu\text{l}/\text{well}$ of 2 M sulfuric acid solution. The optical density (OD) of each well was measured at 450 nm by an ELISA plate reader. The optimal antibody matching and its working concentration of the double antibody sandwich ELISA were determined by comparing the ratio of the OD_{450} value of the positive well to a negative well (P/N). On the above basis, the optimized coating time and working concentration of the capture antibody, the optimized time of the capture antibody reacting with the antigen and antigen reacting with the detection antibody, and the optimized reaction time and working concentration of the HRP-labeled secondary antibody were determined. The rSj enolase was diluted in gradient and tested by the optimized double antibody sandwich ELISA. A standard curve was drawn with the natural logarithm of concentrations as the independent variables and with the OD_{450} value as the dependent variable. The experiment was repeated five times to determine the minimum detection limit and the linear detection range of the method.

Detection of serum samples

The dynamics of *Sj* enolase in the serum of the same rabbit at different time points before the infection, after infection, and after praziquantel treatment were observed. Briefly, 1 μl of serum at different time points was dotted on an NC membrane piece (4 mm×4 mm), respectively. The NC pieces were kept at 37 °C in an incubator for 2 h, blocked with 5 % PBSM for 1 h, washed three times with PBST, and reacted with the monoclonal antibody against *Sj* enolase at 37 °C for 1 h. The pieces were washed as above and then reacted with the HRP-labeled sheep anti-rabbit IgG diluted by 1:10,000 at 37 °C for 1 h. The NC pieces were washed three times, each wash lasting 10 min. Finally, the dots were stained using the DAB substrate. The change in the level of *Sj* enolase in the serum of the same infected rabbits was determined by observing the color change among the serum spots at different time points.

The serum samples of schistosomiasis (including 45 matching sera from the same patients before treatment and at different time points after treatment), paragonimiasis, clonorchiasis, and healthy donors were detected in parallel by the *Sj* enolase sandwich ELISA and SEA ELISA (which detects the specific antibody IgG against SEA by universal ELISA). The cut-off threshold was determined by the area under the receiver-operating characteristic curve (ROC) curve, and the sensitivity (SE), specificity (SP), positive predictive value (PPV), negative predictive value (NPV), and Youden index of this method were also calculated.

Statistical analysis

Antibody level analysis and the linear curve were drawn by GraphPad Prism software 5.0. The ROC curve was analyzed by the Statistical Package for Social Sciences (SPSS) software (Yu 2003; Hanley and McNeil 1982; Zweig and Campbell 1993), version 17.0 (Chicago, IL, USA). The description of the data was performed by tables, arithmetic mean, standard deviation, and rates. McNemar's *t* test was applied for group comparison of sensitivity and specificity. A significance level of 0.05 was used.

Results

The composition of the high-abundant protein in *Sj* ESA

The *Sj* ESA was analyzed by 2-D electrophoresis. The spots on the gel were mainly distributed in the range of isoelectric point from 4 to 8. MALDI-TOF/TOF-MS analysis identified 456 protein spots, of which 76 kinds of proteins came from *S. japonicum* (Electronic supplementary material 1). The highly abundant protein molecules included 2-glyceric acid phosphate dehydratase (enolase), 70-kDa heat shock protein (HSP70), fructose diphosphate aldolase (FDA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glyceric acid phosphate kinase, glutathione transferase, thioredoxin peroxidase, protein 14-3-3, and adjustment subunit type II of PKA. Among these proteins, the content of *Sj* enolase was the highest, representing the most abundant circulating antigen (Fig. 1).

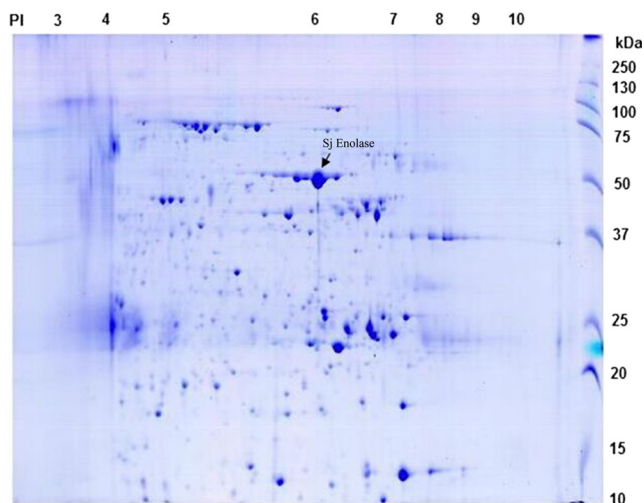


Fig. 1 Profile of *Sj* ESA analyzed by 2-D electrophoresis. The majority of spots on the gel were distributed in the range of isoelectric point from 4 to 8, and a total of 456 protein spots were identified, of which 76 kinds of proteins came from *Schistosoma japonicum*. The arrow shows the largest spot corresponding to *Sj* enolase

Expression and characteristics of *Sj* enolase

The cDNA fragment encoding *Sj* enolase was amplified from the mRNA of adult worms by RT-PCR and confirmed by the restriction enzyme analysis and DNA sequencing after TA cloning. The nucleotide sequence of the cDNA fragment was identical to the published gene sequence (gi46201125) of *Sj* enolase. The cDNA fragment encoding the *Sj* enolase was subcloned into expression plasmid pET28a(+) to construct the recombinant expression plasmid pET28a-*Sj* enolase (Supplementary Fig. 1). The recombinant expression plasmid was transformed into component cells of *E. coli* BL21. Bacteria containing pET28a-*Sj* enolase expressed a 50-kDa protein when cultured at 18 °C, the molecular mass of which was slightly larger than the predicted value because of the 6-His tag on the N-terminus of the recombinant *Sj* enolase. A highly pure preparation of the recombinant *Sj* enolase protein was obtained successfully by Ni-NTA affinity chromatography (Fig. 2).

ICR mice were immunized with purified r*Sj* enolase, and the titer of serum IgG against r*Sj* enolase exceeded 1:100,000, which indicated that r*Sj* enolase is strongly immunogenic. Western blotting demonstrated that r*Sj* enolase could be recognized only by the serum of schistosomiasis, which suggested that the immunogenicity and antibody reactivity of r*Sj* enolase are similar to those of the natural *Sj* enolase in worms (Fig. 3).

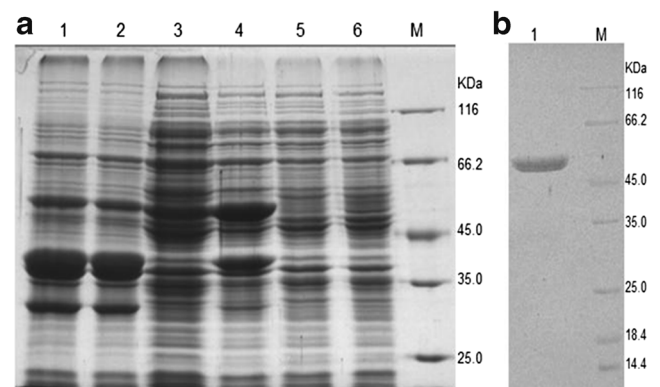


Fig. 2 Analysis of expression products of recombinant *Sj* enolase by SDS-PAGE. **a** Lane M: standard protein molecular weight markers. Lane 1: the lysate of *E. coli* BL21 transformants containing recombinant plasmid pET28a-*Sj* enolase induced by 0.5 mM IPTG at 18 °C. Lane 2: the lysate of *E. coli* BL21 transformants containing recombinant plasmid pET28a-*Sj* enolase induced by 0.1 mM IPTG at 18 °C. Lane 3: the lysate sediment of *E. coli* BL21 transformants containing recombinant plasmid pET28a-*Sj* enolase induced by 0.1 mM IPTG at 18 °C. Lane 4: the lysate supernatant of *E. coli* BL21 transformants containing recombinant plasmid pET28a-*Sj* enolase induced by 0.1 mM IPTG in 18 °C. Lane 5: the lysate of *E. coli* BL21 transformants containing plasmid pET28a. Lane 6: the lysate of *E. coli* BL21. **b** Lane M: standard protein molecular weight marker. Lane 1: purified recombinant r*Sj* enolase protein on SDS-PAGE gel stained with Coomassie Blue

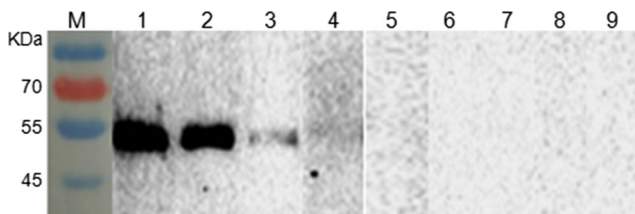


Fig. 3 Specificity analysis of r*Sj* enolase by western blotting. *Lane M*: standard protein molecular weight markers. *Lanes 1–3*: r*Sj* enolase recognized by sera from rabbits, mice, and patients infected with *Schistosoma japonica*, respectively. *Lanes 4–6*: r*Sj* enolase recognized by sera from patients infected with *Sparganum mansoni*, *Clonorchis sinensis*, and *Paragonimus westermani*, respectively. *Lanes 7–9*: r*Sj* enolase recognized by sera from healthy rabbits, mice, and humans, respectively

Characteristics of monoclonal antibodies and the polyclonal antibody against *Sj* enolase

The BALB/c mice were immunized with purified r*Sj* enolase. When the titer of mouse serum antibody IgG against r*Sj* enolase exceeded 1:100,000, the single spleen cell of immunized mouse was fused with SP2/0 cells to select the hybridoma, which could secrete the specific monoclonal antibody against r*Sj* enolase. Finally, five monoclonal hybridoma strains, named as 13G8, 13A3, 11F6, 4G3, and 1G9, were obtained, all of which could secrete a specific monoclonal antibody against r*Sj* enolase steadily and effectively. The subclass of the monoclonal antibody IgG secreted by the hybridoma of 13G8, 13A3, 11F6, 4G3, and 1G9 was ascribed to be IgG1, IgG1, IgG2a, IgG1, and IgG2b, respectively. The titer of cultured supernatant of these five hybridomas could reach to 1:200,000, 1:120,000, 1:100,000, 1:150,000 and 1:100,000, respectively. Both titers of ascites from mice inoculated with hybridoma 13G8 and 4G3 were over 1:204,800. Meanwhile, polyclonal antibodies against *Sj* enolase were prepared by immunizing rabbits with r*Sj* enolase, the titer of which was over 1:200,000. The specific monoclonal antibody IgG and polyclonal antibodies were purified from the mice ascites and immunized rabbit blood, respectively, by affinity chromatography with Protein G Sepharose (Supplementary Fig. 2).

The r*Sj* enolase was coated on ELISA plates at 10.0, 5.0, 2.5, 1.25, 0.625, and 0 µg/ml, respectively, and reacted with different dilutions of the monoclonal antibody or polyclonal antibodies to determine the affinity constants. The affinity constants of monoclonal antibodies 13G8 and 4G3 and the polyclonal antibodies were 8.2×10^7 , 4.87×10^7 , and 2.7×10^8 mol/l, respectively (Supplementary Fig. 3). Ultimately, monoclonal antibody 13G8 (McAb 13G8), with the highest affinity, was selected for further research. The results of western blotting showed that McAb 13G8 could recognize both the r*Sj* enolase and the natural *Sj* enolase of SEA and AWA, but did not cross react with the protein molecules of *E. coli* BL21 and SCA (Fig. 4), which indicated that the monoclonal antibody 13G8 had high specificity.

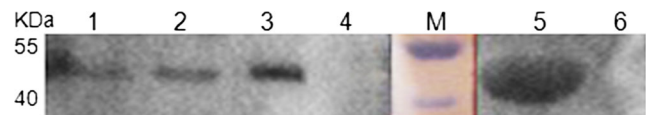


Fig. 4 Specificity analysis of McAb 13G8 by western blotting. *Lane M*: standard protein molecular weight markers. *Lane 1*: SEA recognized by purified McAb 13G8. *Lane 2*: AWA recognized by purified McAb 13G8. *Lane 3*: AWA recognized by purified McAb 13G8. *Lane 4*: *C. sinensis* protein (SCA) recognized by purified McAb 13G8. *Lane 5*: the purified r*Sj* enolase recognized by purified McAb 13G8. *Lane 6*: the lysate of *E. coli* BL21 recognized by purified McAb 13G8

The optimized test conditions and detection limit of the double antibody sandwich ELISA

A double antibody sandwich ELISA to detect *Sj* enolase was established. The reaction volume is 100 µl, and the optimized antibody match was determined by checkerboard phalanx titration. The specific monoclonal antibody was used as the capture antibody, and the polyclonal antibodies were used as the detection antibody. The optimized coated amount of the capture antibody was 6.25 µg/ml, and the optimized concentration of the detection antibody was 1.25 µg/ml. The favorable test conditions of the sandwich ELISA for detecting the circulating antigen *Sj* enolase were as follows: the wells of ELISA plate were coated with the capture antibody at 4 °C overnight and blocked with PBS containing 5 % skimmed milk powder at 37 °C for 1 h. The plate was washed three times with PBST (containing 0.05 % Tween-20), and then the circulating antigen solution containing *Sj* enolase or serum samples were added into the wells. The plate was incubated at 37 °C for 2 h and then reacted with detection antibody at 37 °C for 1 h after washing. The plate was washed three times again and reacted with HRP-labeled goat anti-rabbit IgG diluted by 1:10,000 at 37 °C for 1 h. The plate was washed with PBST three times again, and 50 µl of the substrate TMB solution was added into each well of the plate. The plate was kept at room temperature for 5 min to develop the color, which was stopped by adding 50 µl of 2 M sulfuric acid. The cut-off value was 0.192, which was 2.1 times of the OD₄₅₀ value of the negative wells (without adding r*Sj* enolase). The detection limit of this method was 700 pg/ml and the linear range covered from 0.7 to 1000 ng/ml (Fig. 5).

The dynamics of *Sj* enolase in the serum of the infected rabbit

The results of Dot-ELISA showed no *Sj* enolase in the sera of healthy rabbits before *Schistosoma* infection, but the quantity of *Sj* enolase in the serum of the infected rabbits increased gradually and remained at a high level until the end of the this experiment in the untreated group (18 weeks postinfection). In the treated group, the quantity of *Sj* enolase in the infected rabbits increased continually in the first 2 weeks after

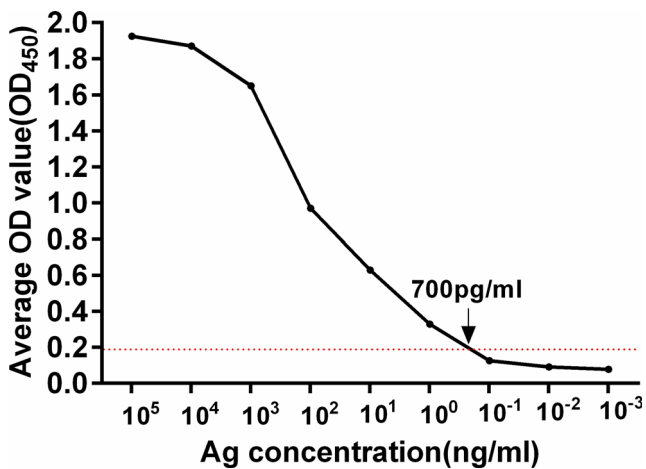


Fig. 5 Relationship of *Sj* enolase concentration and OD₄₅₀ value of the *Sj* enolase sandwich ELISA. The capture antibody was present at 6.25 µg/ml, and the detection antibody was at 1.25 µg/ml. The amount of r*Sj* enolase was diluted sequentially. The cut-off value was 0.192, which was the average OD₄₅₀ value of the negative wells (without adding r*Sj* enolase) plus three standard deviation. The arrow indicates the lowest concentration of r*Sj* enolase that could be detected by the *Sj* enolase sandwich ELISA in each sample. The detection limit of this method was 700 pg/ml and the linear range covered 0.7 to 1000 ng/ml

praziquantel treatment and then decreased rapidly, reaching an undetectable level at 12 weeks after praziquantel treatment (18 weeks postinfection) (Fig. 6). These results suggested that the level of circulating antigen *Sj* enolase in the serum of rabbits correlated with the number of *S. japonicum* worms present in vivo.

The diagnostic performance of *Sj* enolase sandwich ELISA

The sera of schistosomiasis, other parasitic diseases, and healthy subjects were detected by the *Sj* enolase sandwich ELISA, with a cut-off threshold of 0.134, which was calculated according to the area under the ROC curve (Supplementary Fig. 4). Among 143 sera of schistosomiasis (all were positive by feces examination), 121 sera were positive in the *Sj* enolase sandwich ELISA (positive rate 84.61 %). Ninety-two of 96 healthy subjects were negative (specificity 95.83 %), and the Youden index was 0.8. The positive predictive value (PPV)

and negative predictive value (NPV) were 95.31 and 86.17 %, respectively (Table 1).

Among the 30 sera of clonorchiasis, only one serum was positive, giving a cross-reaction rate of 3.33 %. Among the 20 sera of paragonimiasis, only one serum was positive, giving a cross-reaction rate of 5 %. Meanwhile, these sera were also detected by SEA ELISA, which displayed cross-reaction rates with clonorchiasis and paragonimiasis of 16.67 and 65.00 %, respectively (Table 2).

The correlation between the degree of *Schistosoma* infection and the quantity of *Sj* enolase in the serum of schistosomiasis

Sera from 89 schistosomiasis patients, whose feces egg examination was positive, were tested by the *Sj* enolase sandwich ELISA. These patients were categorized (WHO 1993) as follows: three cases of severe infection (egg amount of per gram feces (EPG) > 400), 11 cases of medium infection (EPG 101–400), and 75 cases of light infection (EPG < 100), according to the eggs per gram in feces accounted by the Kato-Katz method. The results showed a positive correlation ($r=0.768$, $p=0.000$) between the quantity of *Sj* enolase in schistosomiasis serum and the EPG: the quantity of *Sj* enolase in the serum of patients increased along with the rising degree of *Schistosoma* infection (Fig. 7). The results indicated that the sensitivity of *Sj* enolase sandwich ELISA was higher than SEA ELISA for detecting both light and nonlight infection; the difference was not statistically significant ($p>0.05$) (Table 3).

The value of evaluating the chemotherapeutic effect of *Sj* enolase sandwich ELISA

Matching sera from 45 schistosomiasis patients, which included the sera before treatment and at 3, 6, 9, and 12 months after praziquantel treatment, were detected by the *Sj* enolase sandwich ELISA. The positive rate was 88.89 % before praziquantel, and the negative rates at 3, 6, 9, and 12 months postpraziquantel treatment were 93.33, 97.78, 100, and 100 %, respectively. By contrast, the same 45 matching sera were tested by SEA ELISA to detect the IgG against SEA: the positive rate was 95.56 % before treatment and the negative

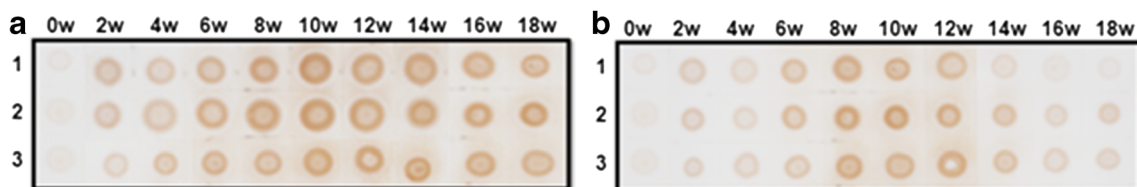


Fig. 6 The dynamics of *Sj* enolase in sera of infected rabbits detected by Dot-ELISA with McAb 13G8. **a** The level of *Sj* enolase in rabbit sera of the untreated group detected by Dot-ELISA at 0, 2, 4, 6, 8, 10, 12, 14, 16,

and 18 weeks after infection. **b** The level of *Sj* enolase in rabbit sera of the praziquantel-treated group at 0, 2, 4, and 6 weeks after infection and at 2, 4, 6, 8, 10, and 12 weeks after praziquantel treatment

Table 1 Comparison of the detecting efficacy between *Sj* enolase sandwich ELISA and SEA ELISA

| | Truly positive | False negative | Truly negative | False positive | Sensitivity (%)* | Specificity (%)** | PPV (%) | NPV (%) |
|----------------------------------|----------------|----------------|----------------|----------------|------------------|-------------------|---------|---------|
| SEA ELISA | 129 | 14 | 78 | 18 | 90.21 | 81.25 | 82.79 | 89.25 |
| <i>Sj</i> enolase sandwich ELISA | 121 | 22 | 92 | 4 | 84.61 | 95.83 | 95.31 | 86.17 |

PPV positive predictive value, NPV negative predictive value

* $p > 0.05$, ** $p < 0.01$ when compared by McNemar's test

rates at 3, 6, 9, and 12 months posttreatment were only 28.89, 28.89, 37.78, and 42.22 %, respectively (Fig. 8, Supplementary material 2). These results indicated that the circulating antigen *Sj* enolase disappeared along with the elimination of worms in patients given an effective treatment. This result suggested that it is of great value to detect the circulating antigen *Sj* enolase to evaluate the chemotherapeutic effect on schistosomiasis.

Discussion

The implementation of the long-term and large-scale schistosomiasis prevention and control strategy has kept the schistosomiasis prevalence under control effectively. Currently, a low degree of infection and a low morbidity are the new normal levels of schistosomiasis prevalence in China (Lei et al. 2014). The traditional pathogenic detection methods (such as the Kato-Katz method and the miracidium hatching method) cannot meet the needs of identifying patients with low infection degree and for schistosomiasis epidemiologic surveys. Thus, new methods with high sensitivity and specificity must be developed (Zhu 2005; Wu 2002). The level of serum circulating antigens should be positively correlated with worm burdens in patients and can be detected as objective indicators of active *Schistosoma* infection (Van Lieshout et al. 1995b). Detecting the circulating antigens is considered a vital diagnostic method, which not only can diagnose an active infection but also reflect the severity of infection. Therefore, it can be used to assess the efficiency of chemotherapy and control programs (Zhou et al. 2010). The circulating antigens derived from the *Sj* ESA mainly focused on membrane-associated antigens (MAAs) and gut-associated antigens (GAAs). However, the components of these circulating antigens are complicated, and the amount of the different components in

serum varied significantly. In addition, the metabolic kinetics was not clear. The targets of monoclonal antibodies prepared by spleen cells from immunized mice immunized with *Schistosoma* by traditional methods were not clear (Abdeen et al. 1999), and the results of the circulating antigens detected by these monoclonal antibodies were too ambiguous to believe. With the development of proteomics, it is possible to analyze the protein components and the quantity of each component of the *Sj* ESA. Evidently, a protein present at high abundance in the *Sj* ESA should exist at a high concentration in the serum of schistosomiasis patients. Thus, proteomics could select highly abundant protein molecules in the *Sj* ESA as optimized targets for detection methods, and these methods would improve the specificity and sensitivity of detection of circulating antigens (Liu et al. 1988; Sulbarán et al. 2010; Shen et al. 2000). In this study, we determined that the most abundant protein in the *Sj* ESA was *Sj* enolase.

Enolase has multiple biological functions and is a key enzyme in cell glycolysis. It also acts as a heat shock protein (Sirover 1996) or a proto-oncogene regulatory protein (Ejeskär et al. 2005). Pérez-Sánchez et al. (2008) observed that enolase was located on the surface of *Schistosoma bovis* and was presumed to be involved in gene regulation and the infection process in hosts. Zhao et al. (2007) noted the presence of enolase in protein maps of larvae, male and female *S. japonicum*, which indicated that the enolase may play an important role in the process of growth and differentiation of *Schistosoma*. Cheng et al. (2005) identified a membrane protein present specifically in the female adult worm of *S. japonicum*, which may be enolase. Yang et al. (2010) showed that enolase was expressed in each developmental stage of *S. japonicum* and was widely distributed on the surface and in the body of *Schistosoma*. The above reports suggested that enolase is an important functional protein and is very abundant in the worm body, which might explain why *Sj*

Table 2 Comparison of the cross-reaction rate between *Sj* enolase sandwich ELISA and SEA ELISA

| | Case no. | SEA ELISA | | | Sandwich ELISA of <i>Sj</i> enolase | | |
|----------------------|----------|-----------|----------|-------------------------|-------------------------------------|----------|-------------------------|
| | | Positive | Negative | Cross reaction rate (%) | Positive | Negative | Cross reaction rate (%) |
| <i>C. sinensis</i> | 30 | 5 | 25 | 16.67 | 1 | 29 | 3.33 |
| <i>P. westermani</i> | 20 | 13 | 7 | 65.00 | 1 | 20 | 5 |

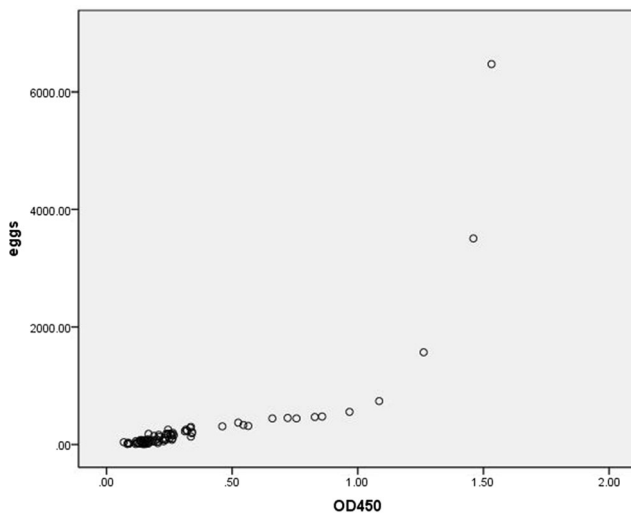


Fig. 7 Relationship between the *Sj* enolase quantity in sera and EPG. The total EPG of three feces egg counts of each patient was drawn as the abscissa, and the average OD₄₅₀ values of five tests were calculated as the ordinate. The relationship was analyzed by Pearson correlation in the Statistical Package for Social Sciences (SPSS) software, version 17.0. The results showed a positive correlation ($r=0.768$, $p=0.000$) between the OD₄₅₀ and the EPG

enolase was so abundant in the *Sj* ESA. Therefore, the gene of *Sj* enolase was cloned and expressed in vitro, and the recombinant *Sj* enolase was used to produce antibodies that could recognize not only r*Sj* enolase but also the natural *Sj* enolase in SEA, ESA, and AWA. This indicated that the r*Sj* enolase was immunologically similar to the natural *Sj* enolase, but with stronger immunogenicity and immunoreactivity. The immunized rabbit sera did not react with the proteins of SCA and *E. coli*, which suggested that the immunogenicity of *Sj* enolase was highly specific. All of the above implied that *Sj* enolase was a potential target molecule for immunological diagnosis.

Five hybridomas producing monoclonal antibodies against r*Sj* enolase were produced, among which hybridoma 13G8 showed the highest titer ($>1:204,800$) and an affinity constant of 8.2×10^7 mol/l. Western blotting demonstrated that McAb 13G8 could recognize not only r*Sj* enolase but also the natural *Sj* enolase of SEA and AWA, but not *E. coli* and SCA proteins. Thus, McAb 13G8 showed high specificity and affinity and

Table 3 Sensitivity comparison of *Sj* enolase sandwich ELISA and SEA ELISA for testing patient sera with different infection degrees

| | Light infection ($n=75$) | | Nonlight infection ($n=14$) | |
|---------------|----------------------------|---------|-------------------------------|---------|
| | Anti-SEA IgG | Enolase | Anti-SEA IgG | Enolase |
| Positive | 68 | 71 | 13 | 14 |
| Negative | 7 | 4 | 1 | 0 |
| Positive rate | 89.71 % | 94.67 % | 92.31 % | 100 |

n is the number of patients

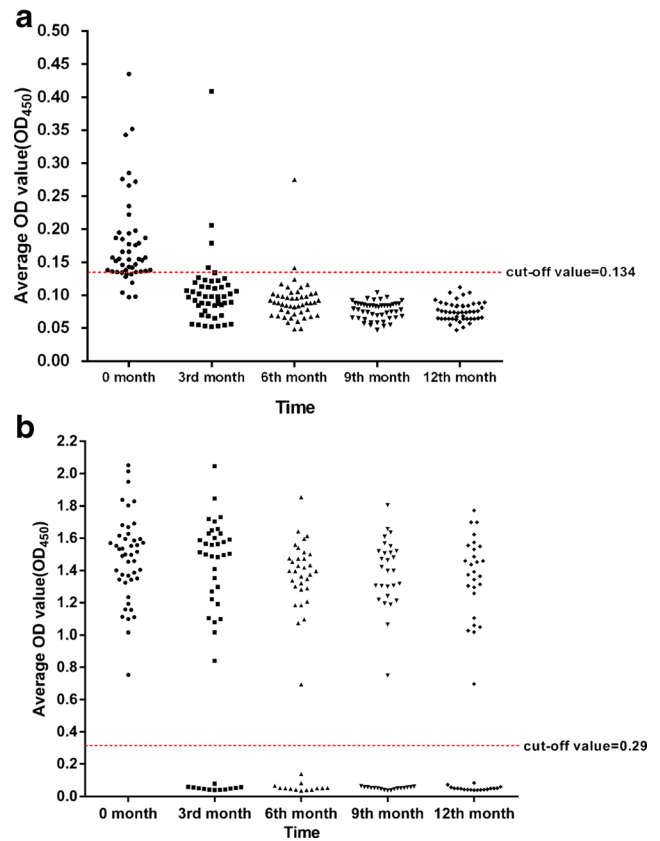


Fig. 8 The value of evaluating the chemotherapeutic effect of *Sj* enolase sandwich ELISA and SEA ELISA in treated patients. Patients' sera were collected before treatment or at 3, 6, 9, and 12 months after praziquantel treatment. **a** The results tested by *Sj* enolase sandwich ELISA. The line represents the cut-off value, which was obtained from a ROC curve. **b** The results tested by SEA ELISA. The line represents the cut-off value, which was 2.1 times of the average A_{450} value of the negative wells (adding healthy sera)

was suitable for developing a new diagnostic method. Using McAb 13G8 to detect the dynamics of *Sj* enolase in rabbit sera at different time points indicated that *Sj* enolase of the untreated group increased gradually and remained high until the end of the experiment (18 weeks postinfection). By contrast, enolase in the sera of the treated group increased in the first 2 weeks after treatment and then decreased rapidly to a negative level, which indicated that *Sj* enolase disappeared as the worms were eliminated from the blood. As Feldmeier et al. (1986) reported, *Sj* enolase may be used as an objective indication of active infection of *S. japonicum* and may be an excellent target molecule for circulating antigen detection. Detecting the presence or absence of the *Sj* enolase molecule in patients and the dynamic change may have great potential value for both diagnosing active schistosomiasis and evaluating the curative effect of chemotherapy.

We established a double antibody sandwich ELISA to detect circulating antigen *Sj* enolase. The optimal antibody matching was determined using McAb 13G8 as the capture antibody and the polyclonal antibodies against *Sj* enolase as the detection

antibody. The linear range of antigen concentration detected by this method was between 0.7 and 1000 ng/ml, and the minimum detection in a 100- μ l reaction was 70 pg. The sensitivity of this method was higher than the published circulating antigen detection method, whose minimum was between 5 and 80 ng/ml (Lei et al. 2009; Nibbeling et al. 1988; Fu and Carter 1990; Hassan et al. 1992). By contrast, the sensitivity of the method using the HRP-labeled monoclonal antibody against *Sj* enolase as the detection antibody was rather lower (data not published), possibly because the activity of the HRP-labeled monoclonal antibody prepared in our laboratory was too low.

Based on the test results of healthy human serum samples, the clinical cut-off threshold of the double antibody sandwich ELISA was 0.134. According to the threshold, the sensitivity and specificity of the double antibody sandwich ELISA for detecting 143 active schistosomiasis sera and 96 healthy sera were 84.61 and 95.83 %, respectively, and the Youden index was 0.8. The quantity of *Sj* enolase in the serum of patients was positively correlated with the EPG, which indicated that the amount of *Sj* enolase in the serum of patients effectively reflected the infection degree. When the sera of other patients infected with other parasites were detected by this double antibody sandwich ELISA, the cross-reaction rate was 3.33 % with clonorchiasis and 5 % with paragonimiasis, which indicated that this double antibody sandwich ELISA has a better specificity than previous methods. Although the sensitivity of the double antibody sandwich ELISA was slightly lower than that of the SEA ELISA, it was not statistically significant ($p>0.05$). The specificity of the double antibody sandwich ELISA was obviously higher than that of SEA ELISA, and the statistical difference was significant ($p<0.05$). Compared with the diagnostic method of detecting antibody IgG, the double antibody sandwich ELISA has the advantage of distinguishing the active schistosomiasis from historic infection, which showed a good clinical diagnostic efficacy.

The dynamic change of *Sj* enolase in the serum of 45 schistosomiasis patients before treatment and at 3, 6, 9, and 12 months posttreatment was monitored by the double antibody sandwich ELISA and by the anti-SEA IgG test in the same patients. The results proved that the anti-SEA IgG could persist for a long time in patients after treatment, but the circulating antigen *Sj* enolase decreased rapidly after praziquantel treatment in a short time; therefore, detecting the dynamic changes of *Sj* enolase in serum could assess the chemotherapeutic effect effectively.

Recently, some detection methods of CCA have been evaluated in endemic areas of schistosomiasis (Van Dam et al. 2015). Some of them showed high sensitivity and were suitable for screening the patients with low infection degree. What the detected target molecules are and how the dynamics of the target molecules work in vivo are not yet clear. Will the target antigen of this method disappear rapidly after praziquantel treatment in vivo? Is it useful to assess the therapeutic effect

of schistosomiasis? Answers to the above questions require further research. In addition, the serum samples used in this method must be preprocessed by trichloroacetic acid precipitation, which is time-consuming and not suitable for massive sample detection in the field. The serum sample detected by the double antibody sandwich ELISA developed in this research does not need to be preprocessed. The sensitivity and specificity of this method were 84.61 and 95.83 %, respectively, in a low degree of *Schistosoma* infection epidemic area (infection rate between 1 and 5 %), which showed the advantages of this method to screen for patients with active infections in a low degree of infection in an epidemic area.

By the end of 2008, the prevalence rate of schistosomiasis in China was less than 5 % and will fall to 1 % in 2015. However, the lack of an effective method for diagnosing active schistosomiasis in the endemic area with a low degree of infection might delay the process of schistosomiasis elimination in China. Some methods of detecting circulating antigens of *S. japonicum* have been developed previously (Lei et al. 2009; Wen et al. 2011); however, their sensitivity and specificity were too low to develop diagnostic kits for application in the field. The double antibody ELISA to detect the circulating antigen *Sj* enolase developed in this study showed a good and comprehensive performance, such as higher sensitivity and specificity, low cross-reaction rate with other parasitic diseases, and evaluation of the chemotherapeutic effect. We expect that this method will make up the shortfall of an effective detection method in schistosomiasis prevention and control programs for a low degree of *Schistosoma* infection in endemic areas in China in the future. If the technique of HRP-labeled monoclonal antibody could be improved, or the chemiluminescence technology and time-resolved fluorescence technology could be adapted in subsequent research, the sensitivity of detecting the circulating antigen *Sj* enolase could be improved effectively, and the performance and application of this method would be further enhanced.

Conclusion

Sj enolase proved to be a powerful indicator of active *Schistosoma* infection, and the double antibody sandwich ELISA developed in this study could diagnose patients with a low degree of *S. japonicum* effectively.

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Competing interests The authors declare that they have no competing interests.

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