Human Antibody Response to Thioredoxin Peroxidase-1 and Tandem Repeat Proteins as Immunodiagnostic Antigen Candidates for *Schistosoma japonicum* Infection

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Abstract. Schistosomiasis continues to be a public health problem in many tropical and subtropical countries. Improving the diagnostic tools for surveillance and monitoring in areas that have reached elimination level will help hasten the possible elimination of this disease. This study therefore aims to develop enzyme-linked immunosorbent assay through the use of recombinant proteins such as thioredoxin peroxidase-1 (SjTPx-1) and four tandem repeat proteins (Sj1TR, Sj2TR, Sj4TR, and Sj7TR). Cutoff values were calculated using 38 serum samples from healthy Japanese volunteers. Sera from 35 schistosomiasis-confirmed patients, four cured from the disease by chemotherapy, and 15 endemic negative controls were used to assess these antigens. SjTPx-1 and Sj7TR both had 85.71% sensitivity. Furthermore, these antigens were also tested against human sera positive for other parasitic infections and showed no or very minimal cross-reaction. These results suggest the potential defined antigens for development of an accurate diagnostic test for schistosomiasis.

INTRODUCTION

Since the identification of the *Schistosoma* parasite in Egypt in 1851,¹ schistosomiasis has continued to be a public health problem in 76-endemic countries.² More than 207 million people are infected worldwide and about 700 million people are at risk of having the disease.² This parasitic disease is far from being eradicated despite national control programs implemented in endemic countries. Improving the diagnostic techniques should be given importance, because schistosomiasis diagnosis is needed for epidemiological studies and evaluating the efficacy of control programs.

Definitive diagnosis of *Schistosoma japonicum* infection in endemic areas still relies on the Kato-Katz technique and/or the sedimentation concentration technique, which are based on the detection of schistosome eggs in the stool of the infected individuals.³ This technique is simple and has a very high specificity, making it the “gold standard” method for schistosomiasis diagnosis. However, this parasitological technique is labor-intensive, requires skilled personnel, has low sensitivity in low prevalence endemic areas,^{4,5} and according to one study, seven repeated Kato-Katz examinations coupled with miracidial hatching is required to have its maximal sensitivity.⁶ Because of these issues in sensitivity, crude egg antigen-based immunodiagnostic techniques such as the circum-oval precipitin test (COPT) and enzyme-linked immunosorbent assay (ELISA) are usually used instead. However, it will be difficult to produce crude egg antigen for large-scale diagnostic purposes. And so far, only a few defined antigens have been identified for serological diagnosis of schistosomiasis.^{7,8} Therefore, there is a need to develop a recombinant diagnostic antigen that is easier to produce.

The recently completed genome of *S. japonicum*,⁹ database available online, made it possible to analyze more antigens to be used possibly in the diagnosis and vaccine development against the parasite. For the diagnostic purposes, pro-

ducing the recombinant antigens is easier than using the crude egg antigen. This can be an important factor in the success of performing mass diagnosis and epidemiological surveys.

Peroxiredoxin (Prx) is considered as the major detoxifying agent against hydrogen peroxide in helminths,¹⁰ which limits the damage done by this reactive oxygen species.¹¹ Based on their amino acid sequences, the Prx have been classified into 1-Cys or 2-Cys Prx.¹² The 2-Cys Prx is also known as thioredoxin peroxidase (TPx) because it uses electrons from the thioredoxin system.¹⁰ In *S. japonicum*, TPx exists in three forms, namely TPx-1, TPx-2, and TPx-3.¹³ TPx-1 is expressed on the tegument of the adult parasite¹⁴ making it more exposed to the host immune system. Among the three types, only TPx-1 was seen in the excretory/secretory products from the adult worm.¹³ In a previous study, it was tested on cattle samples through ELISA and yielded 84.0% sensitivity and 89.0% specificity,¹⁵ whereas the antigen has not been evaluated for human cases. On the other hand, tandem repeat proteins (TRPs) are often targets of humoral responses for protozoan¹⁶ and helminthic parasites.¹⁷ Previous studies showed that computational screening of genomes could identify novel genes encoding TRPs with serological significance from various parasites.^{18–20} However, no such proteins have yet been identified as antigens for serodiagnosis of schistosomiasis. In this study, we evaluated the serological efficiency of TPx-1 and four TRPs for the diagnosis of human schistosomiasis as compared with *S. japonicum* soluble egg antigen (SEA) by ELISA.

MATERIALS AND METHODS

Parasite. The Yamanashi strain of *S. japonicum* was maintained using the *Oncomelania nosophora* snails and imprinting control region mice to complete its life cycle.²¹ Each mouse was infected with 40 to 60 cercariae by exposing its tail to a cercarial water suspension. After 7 to 8 weeks, the mice were killed to obtain the adult parasite. The adults were collected from the mesenteric veins of the intestine, which were cleaned briefly with normal saline solution. The animal experiments in this study were carried out in compliance with

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the Guide for Animal Experimentation at Dokkyo Medical University Japan.

Human sera. Non-endemic control sera were collected from 38 healthy Japanese volunteers from Tochigi prefecture in May 2003.²² These subjects were without any risk of contracting *S. japonicum* infection and had no history of traveling to schistosomiasis-endemic areas. Fifteen endemic control sera and four post-treatment samples (1 year after chemotherapy) were collected from Gonzaga, Cagayan, the Philippines. These individuals were confirmed negative through stool examination. The schistosomiasis-positive serum samples were collected from 35 human patients from Leyte, the Philippines.²³ They were diagnosed by the detection of the parasite eggs in their stool. Sera from patients with other parasites, including *Trichuris trichiura* ($N = 1$), *Plasmodium falciparum* ($N = 4$), *Plasmodium vivax* ($N = 1$), and *Entamoeba histolytica* ($N = 4$) were collected from a schistosomiasis-free area in the Philippines. They were diagnosed through either microscopic examination or detection of antibodies by immunofluorescent assay. *Paragonimus westermani*-positive samples ($N = 11$) were taken from Japanese patients and *Opisthorchis viverrini*-positive sera ($N = 10$) were collected from Thailand diagnosed through either clinical manifestations or antibody detection. Blood samples were taken from these subjects after informed consent in their local language were obtained by a medical staff member from each patient or their guardians. This study was done according to the ethical guidelines for epidemiological studies provided by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan. The University of the Philippines, Manila also issued favorable technical and ethical reviews of this study.

Computational search and synthesis of tandem repeats (TR) genes. DNA sequence data from *S. japonicum* (*Sjaponicum*_Genes.v3) were obtained from GeneDB.²⁴ Tandem Repeats Finder, a program to locate and display TR in DNA sequences,²⁵ was used to identify TR genes as previously described.¹⁸ In this study, the genes were regarded as TR genes if the scores obtained from the Tandem Repeats Finder analysis were 500 or higher. The biochemical properties of each of the top 20 *S. japonicum* TR genes/proteins were analyzed for 1) a protein's molecular mass, isoelectric point, presence of a signal sequence, trans-membrane domain(s), or a GPI-anchor signal; 2) known antigenicity and/or functions by Blast searches against the National Center for Biotechnology Information (NCBI) database using both DNA and deduced amino acid sequences; 3) evidence of protein expression by comparison with a previous study²⁶; 4) transcriptomic expression levels by comparison with the NCBI EST database. On the basis of these analyses, four TR genes were selected for production of recombinant proteins. Nucleotides coding a partial TR domain of the chosen TRPs were synthesized by GenScript USA Inc. (Piscataway, NJ).

Cloning and sequencing of TPx-1. Total RNA was extracted from *S. japonicum* adult worms using TRIzol (Invitrogen, Madison, WI). First strand synthesis of complementary DNA (cDNA) was done using the Ready-To-Go T-Primed First Strand Kit (Amersham Biosciences, UK) and oligo (dT) primer. From the cDNA of the adult worm, SjTPx-1 was amplified by polymerase chain reaction (PCR) using the primer set 5'-TTA GGA TCC ATG GTA CTG ATT CCA AAT-3' and 5'-TTA AAG CTT TAA TCA GTG ATT CAC TTT-3' (*Bam*HI

and *Hind*III sites were underlined) based on the sequence obtained from GeneDB²⁷ (accession no. Sjp_0095720.1). The expected length of the PCR product was 555 bp. Twenty microliters of reaction mixture contained 2 μ L of buffer, 0.6 μ L of 1.5 mM $MgCl_2$, 1.6 μ L of 2.5 mM dNTP, 0.4 μ L of each 20 pmol/ μ L primer, 0.2 μ L of 5 U/ μ L *Taq* DNA polymerase (Takara, Otsu, Japan), and 1 μ L of template. The conditions for PCR were as follows: 94°C for 5 min, followed by 35 cycles of 30 sec in 94°C, 45 sec in 60°C, and 45 sec in 72°C, and a final extension of 72°C for 10 min. The PCR was performed using Veriti 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, CA). The PCR products were separated by electrophoresis in 1.5% agarose gel and visualized by ethidium bromide staining. The amplified DNA sequence was cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). *Escherichia coli* DH5 α were transformed with the plasmid. Selected clones were sequenced to verify the identity of the cloned sequences using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Recombinant protein preparation. The SjTPx-1 gene was digested with the restriction enzymes *Bam*HI and *Hind*III, whereas the four synthesized TRP genes with *Nde*I and *Eco*RI (the enzyme sites added at 5' and 3', respectively). The resulting digested genes were inserted into the pET28 vector (EMD Biosciences, San Diego, CA). *Escherichia coli* BL 21 was transfected with pET28 plasmids containing the individual genes and were grown in LB medium (Sigma-Aldrich, St. Louis, MO) supplemented with 50 μ g/mL of kanamycin for cloning. The expression of the recombinant proteins in SOB medium (BD, Sparks, MD) was induced with 0.5 mM isopropyl-thio- β -D-galactoside (IPTG) and maintained for 3 h. The recombinant proteins were recovered using the Ni-NTA agarose (Qiagen Inc., Valencia, CA) according to the manufacturer's instruction. SjTPx-1, Sj1TR, Sj2TR, and Sj7TR were purified as soluble proteins, whereas Sj4TR as an insoluble protein. The proteins were eluted and dialyzed with 20 mM Tris, pH 8.0. The integrity and purity of the proteins were evaluated with 15% polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and subsequent Coomassie Brilliant Blue staining (MP Biomedicals, Solon, OH). The concentration of each expressed protein was measured using the BCA Protein Assay (Thermo Scientific, Rockford, IL).

ELISA. A conventional ELISA was done as previously described²⁸ with slight modifications. In this study, horseradish peroxidase (HRP)-conjugated anti-human immunoglobulin G (IgG) goat serum (Rockland Inc., Gilbertsville, PA) was used for the secondary antibody and 3,3',5,5'-tetramethylbenzidine (KPL, Gaithersburg, MD) was used as the substrate for HRP. The wells of the microplates (Nunc Maxisorp, Thermo Fisher, Rockland, IL) were sensitized separately with SEA (1 μ g/well) or each of the recombinant proteins (200 ng/well). Proteins were diluted with carbonate/bicarbonate buffer at pH 9.6. After blocking with 1% bovine serum albumin (BSA) in phosphate buffered saline with 0.05% Tween 20 (T-PBS) (T-PBS-0.1%BSA), the antigen-coated well was filled with the serum. The test sera (0.1 mL) were diluted 1:400 with T-PBS-0.1%BSA and while the secondary antibody (0.1 mL) was diluted in 1:10,000. Optical density (OD) was measured at 450 nm using a microplate reader (MTP-500, Corona Electric, Tokyo, Japan). All the tests were done in triplicates.

Statistical analysis. The validity of the antigens was estimated by the sensitivity, specificity, and predictive values using the

stool analysis as the reference standard. The agreement between the antigens was estimated by the kappa value.²⁹

RESULTS

TR gene analysis. A total of 12,657 gene sequences were analyzed by Tandem Repeats Finder, of which 134 genes were found to have TR regions based on the arbitrary cutoff score of 500. Only eight of them had a score higher than 2,000, which is very few as compared with previous studies on other parasites such as *Leishmania infantum*,³⁰ *Trypanosoma brucei*,³⁰ and *P. falciparum*.³¹ The top 20 TR genes with the highest scores are shown in Table 1, and four TR genes were selected for production of recombinant proteins based on conservation in other organisms and expression evidence. For example, ubiquitin (Sjp_0031660 and Sjp_0066050) and splicing factor 3G subunit 4 (Sjp_0031090) were excluded from further study because of the high conservation. Although Sjp_0059850 (Sj4TR) showed some similarity to proteins from other organisms, those included higher animals but not pathogens causing diseases to mammalian hosts. Furthermore, genes without expression evidence based on previous proteomic and transcriptomic studies were avoided for further study because they were more likely to be just putative genes.

Cloning and expression of proteins. The PCR amplified SjTPx-1 gene was 555 bp (Figure 1A) similar to the size reported in the database (GeneDB). The gene showed 98% identity with *S. japonicum* TPx-1 gene (Sjp_0095720.1) in both the nucleotides and amino acid sequences. The gene was conserved in other parasites, with 82% identity to *Schistosoma mansoni* TPx-1 and 61–68% identity to non-schistosome parasites. Understandably, it showed a high percent identity with *S. mansoni*, but the parasitic diseases, which should be considered for possible cross-reaction, are those that can also be seen in the schistosomiasis-endemic areas. In Southeast Asia, schistosomiasis shares geographical endemicity with *Plasmodium* spp. (63% identity), *O. viverrini* (63% identity) and *E. histolytica* (66% identity).

The SDS/PAGE showed that the recombinant proteins in expected size (SjTPx-1, 20 kD; Sj1TR, 19 kD; Sj2TR, 19 kD; Sj4TR, 21 kD, and Sj7TR, 13 kD) were expressed and purified as a single band (Figure 1C–G).

ELISA. The ELISA was performed using sera from 38 Japanese controls, 15 Filipino endemic negative controls, 4 post-treated negative samples, and 35 stool-confirmed schistosomiasis japonica patients. To check their cross-reactivity, sera from 11 *P. westermani*, 10 *O. viverrini*, 1 *T. trichiura*, 3 *P. falciparum*, 1 *P. vivax*, and 4 *E. histolytica* positive patients were included for the ELISA. The cutoff OD value was calculated from the values of the 38 Japanese controls as mean + 3 SD. Thirty-four of the 35 schistosomiasis-confirmed sera were positive for SEA (Figure 2). Eleven of the 15 endemic negative controls were positive for SEA, whereas none for the recombinant antigens. For the post-treatment samples, all were positive for SEA and only one for Sj4TR. SjTPx-1 and Sj7TR both had 30 samples positive of the 35, having 85.71% sensitivity. Sj1TR had 24 positive samples (68.57%) and Sj4TR with 20 positive samples (57.14%). Only three samples were positive for Sj2TR making it not a good candidate for human schistosomiasis diagnosis. For the *P. westermani*, *Plasmodium* spp., and *E. histolytica*-positive samples, results showed no cross-reaction with the recombinant antigens. In contrast, 3 of the 11 *P. westermani*, 5 of the 10 *O. viverrini*, and 2 of the 4 *E. histolytica*-positive samples show high OD values for SEA. Among the recombinant proteins, only 3 of the *O. viverrini*-positive samples showed a very minimal reaction with SjTPx-1.

To test for reproducibility, five independent assays for each recombinant protein using one non-endemic control and one stool-positive control were done on three different plates at the same time. Intra-assay coefficient of variation was below 4% for all the tests (data not shown).

Based on the statistical analysis, SjTPx-1 and Sj7TR showed high agreement with the stool analysis done on the samples based on the kappa values (Table 2). The specificity and the positive predictive values of the four recombinant antigens (SjTPx-1, Sj1TR, Sj4TR, and Sj7TR) were higher than those of SEA.

TABLE 1
Top 20 tandem repeats (TR) genes of *Schistosoma japonicum*

	Gene ID	Product	Score	Identity*	Proteome†	EST	
1	Sjp_0099630	Expressed protein	5085	No	A	yes	Sj1TR
2	Sjp_0086200	Cytoplasmic dynein 1 light intermediate chain 1	3558	No	no	yes	Sj2TR
3	Sjp_0047310	SRP40, C-terminal, domain-containing	2522	No	na	no	
4	Sjp_0002410	Expressed protein	2454	No	na	no	
5	Sjp_0070530	Expressed protein	2357	No	na	no	
6	Sjp_0002010	Melanoma inhibitory activity protein 3 precursor	2189	No	na	no	
7	Sjp_0031660	Ubiquitin C	2154	100%	CSAEM	yes	
8	Sjp_0023440	Mediator of DNA damage checkpoint protein 1	2024	No	na	no	
9	Sjp_0088690	Neuroblast differentiation-associated protein AHNAC	1978	No	na	no	
10	Sjp_0066050	Ubiquitin B	1763	100%	na	yes	
11	Sjp_0012130	Thrombospondin type 3 repeat	1758	42%	na	yes	
12	Sjp_0059850	1-phosphatidylinositol-4-phosphate 5-kinase	1743	53%	C	yes	Sj4TR
13	Sjp_0020150	Kringle-like fold, domain-containing	1715	no	na	no	
14	Sjp_0008730	Polypeptide N-acetylgalactosaminyltransferase	1570	54%	CSM	yes	
15	Sjp_0031090	Splicing factor 3B subunit 4	1562	93%	no	yes	
16	Sjp_0087830	Conserved hypothetical protein	1536	no	no	yes	
17	Sjp_0089830	HMG-I and HMG-Y, DNA-binding	1463	no	na	no	
18	Sjp_0030930	Erythrocyte band 7 integral membrane protein	1457	50%	na	no	
19	Sjp_0110390	Expressed protein	1391	no	no	yes	Sj7TR
20	Sjp_0069600	Protein kinase PKN/PRK1, effector, domain-containing	1389	no	CM	yes	

*The highest percent identity of the repeat motif to proteins from organisms other than *Schistosoma* species. "no" indicates that the motif was not found in the other organisms.

†Protein expression evidence was based on Liu and others.²⁶ C = cercariae; S = hepatic schistosomula; A = adults; E = eggs; M = miracidia; No = not detectable; na = data not available.

FIGURE 1. Gel electrophoresis and polyacrylamide gel electrophoresis (SDS-PAGE) of the recombinant antigens. (A and B) The recombinant plasmids were identified by polymerase chain reaction (PCR) and enzyme digestion. (A) Lane 1: TPX-1 from the *Schistosoma japonicum* adult worm cDNA library. (B) Synthesized TRP genes. Lane 1: Sj1TR; Lane 2: Sj2TR; Lane 3: Sj4TR; Lane 4: Sj7TR. M = Marker. (C–G) Expression and purification of the recombinant proteins. M = Marker. Lane 1: *Escherichia coli* culture before adding isopropyl-thio- β -D-galactoside (IPTG) and Lane 2: after adding IPTG. (C) SjTPx-1. (D) Sj1TR. (E) Sj2TR. (F) Sj4TR. (G) Sj7TR.

DISCUSSION

To date, there are only a few defined antigens evaluated for diagnostic purposes in schistosomiasis, most of which were only tested against animal sera.^{15,32–34} Previous results showed

high sensitivity and specificity of the candidate antigens but further study is needed to know their true immunodiagnostic potential on human schistosomiasis. Therefore, other antigens should still be tested and examined for comparative evaluation with those already used. In this study, we assessed the

FIGURE 2. Enzyme-linked immunosorbent assay (ELISA) results of soluble egg antigen (SEA) and the recombinant antigens. (A) Negative serum samples from Japanese volunteers. (B) Negative endemic controls from Filipino volunteers. (C) *Schistosoma japonicum* stool-positive serum samples. (D) Post-treatment negative serum samples. (E) Sera positive for other heminthetic infection (*Paragonimus westermani*, $N = 11$; *Opisthorchis viverrini*, $N = 10$; *Trichuris trichiura*, $N = 1$). (F) Sera positive for protozoan infection (*Plasmodium falciparum*, $N = 3$; *Plasmodium vivax*, $N = 1$; *Entamoeba histolytica*, $N = 4$). Mean optical density (OD) values ($\bar{x}$) are given on each category of samples.

TABLE 2

Statistical analysis of the ELISA results of SEA and the recombinant proteins*

Antigen	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa†
SEA	97.14	71.76	58.62	98.38	0.577
SjTPx-1	85.71	96.47	90.91	94.25	0.836
Sj1TR	68.57	100	100	88.54	0.756
Sj4TR	57.14	98.82	95.24	84.85	0.634
Sj7TR	85.71	100	100	94.44	0.895

* ELISA = enzyme-linked immunosorbent assay; SEA = soluble egg antigen; PPV = positive predictive values; NPV = negative predictive value.

† Kappa values of SjTPx-1 and Sj7TR showed high agreement with the stool analysis based on the previously reported criteria set.²⁹

immunodiagnostic potential of SjTPx-1 and four TR proteins using human samples.

The recombinant SjTPx-1 and Sj7TR both showed an 85.71% positivity rate on the schistosomiasis-confirmed human samples as compared with the 97.14% positivity rate of SEA. Of the 35 stool-positive samples, 32 were positive for either one or both of the SjTPx-1 and Sj7TR; therefore, complementing these two antigens, there will be a possible maximal yield of 91.43% sensitivity. Fusion proteins have been shown effective in the serodiagnosis of other parasites.^{35,36} On the basis of the ELISA results, Sj7TR is a promising candidate antigen for diagnostic purposes, although GeneDB defined it only as an expressed protein. Immunolocalization and expression profiling therefore of this schistosome antigen should be done to understand its role for the parasite.

The sensitivity of SjTPx-1 using human samples was comparable to that of water buffaloes in a previous study.¹⁵ However, the recombinant TRPs should also be tested for their applicability to other animal reservoirs. Sj1TR, which detected lower antibodies in human schistosomiasis patients than Sj7TR, showed higher reactivity to sera from water buffaloes with suspected infection of *S. japonicum* (Angeles and others, unpublished data). Such variation in antigenicity between different hosts has been found in other parasitic diseases.³⁷ Differences in immune responses to *S. japonicum* antigens between humans and other animal hosts may be derived from different survival mechanisms in these hosts because of their diverse immunological backgrounds depending mostly on the host's immunological memory. This includes the immunodominance of the primary response,³⁸ which means to which epitopes did the host responds; and to which of the primary epitopes has the host retained its memory. It will be intriguing to further characterize such differentially recognized antigens in terms of expression levels and functions in such hosts. In addition, strain diversity of these recombinant proteins should also be studied in future researches.

The relationship between the intensity of infection using the number of eggs per gram and the antibody titer measured against the recombinant antigens was also studied (data not shown). The result showed no correlation between the two quantitative measures. This might be explained by the idea that antibody production is not merely caused by the number of eggs released by the schistosome parasite, but also to the host's ability to produce antibody against certain epitopes of the antigen.

The use of *S. japonicum* SEA in immunological tests has been known to cause false-positive results with other parasitic and viral diseases.³⁹ As seen in the results, SEA showed cross-reaction with *P. westermani*, *O. viverrini*, and *E. histolytica*-

positive samples. Only SjTPx-1 showed a very minimal cross-reaction with *O. viverrini*-positive sera, whereas none for the recombinant tandem repeat proteins. This proves that recombinant proteins are more specific than the crude antigen.

Mass chemotherapy⁴⁰ has been the main strategy in the control of schistosomiasis in high prevalence, whereas selective treatment is used in low prevalence areas. Hence, there is a need for a sensitive diagnostic tool for the cases to be identified and given treatment. In this study, endemic negative controls and samples from persons previously treated for schistosomiasis tested positive only with the crude antigen. This is proof that recombinant proteins can be used to identify true positives in schistosomiasis-endemic areas. The use of the recombinant proteins will therefore be critical in surveillance and monitoring in areas where the prevalence level has reached the elimination level. Furthermore, this may also be applied to epidemiological studies and animal reservoir surveillance of the disease, which need highly specific tests. Hence, the real epidemiological picture of schistosomiasis can be shown, which can further help in the possible elimination of the disease.

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