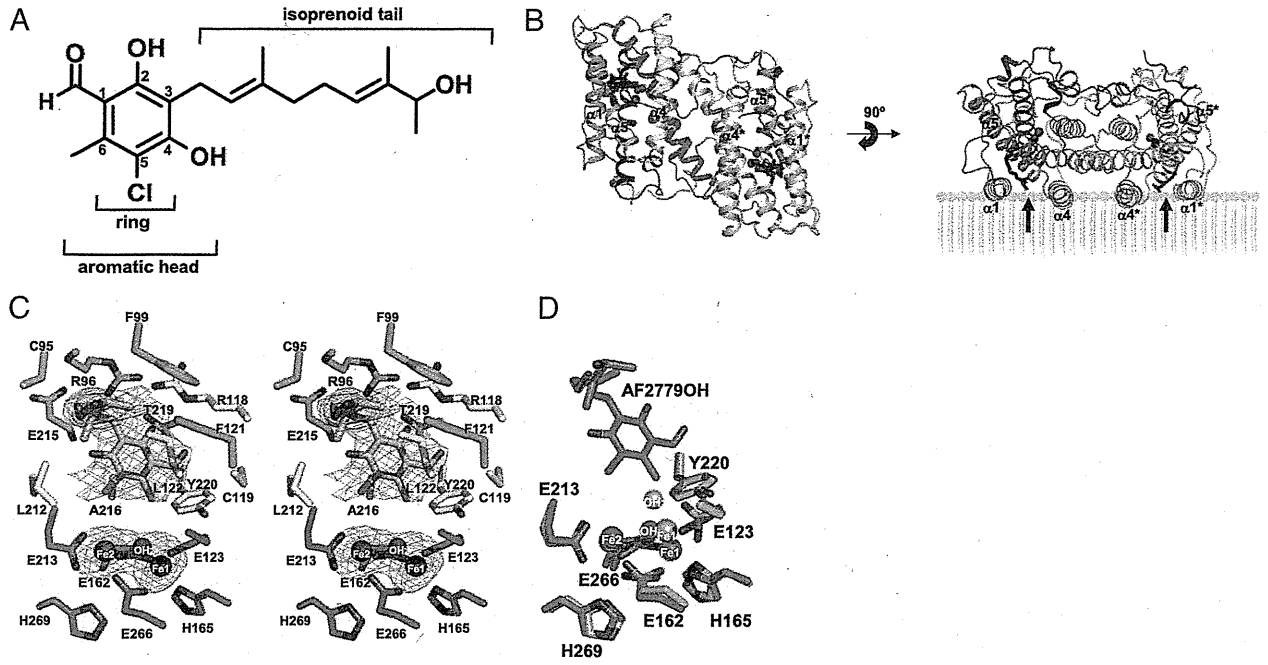

Fig. 3. Structure of the TAO–AF2779OH complex. (*A*) The chemical structure of AF2779OH. (*B*) Overall structure of the TAO–AF2779OH complex. AF2779OH is shown as a red stick. Chains A and B are shown as rainbow (colored blue to red from N to C terminus) and gray, respectively. The AF2779OH-binding cavity is shown by an arrow. (*C*) Stereo view of the AF2779OH binding region of chain A. The residues that interact with AF2779OH (pink stick) -ring and -tail are shown as yellow and cyan sticks, respectively. N, O, and Cl atoms are colored in blue, red, and green, respectively. Sigma-A weighted electron density map calculated from the refined model of the TAO–AF2779OH complex with the diiron centers and AF2779OH molecules omitted from the phase calculation is also shown. Contour levels are 1.0 σ (blue) and 3.0 σ (orange). (*D*) Superimposed diiron active sites of AF2779OH-free (light pink) and -bound (green) forms of TAO. The binding of AF2779OH causes the formation of a coordinate bond between H165 and Fe1.

coordinate with Fe1, unlike H269, which is still separated by 4.3 ± 0.2 Å from Fe2 in both cases (Fig. 3*D* and *SI Appendix*, Fig. S12 and Table S2).

Mutational Analysis of Functionally Relevant Residues. *SI Appendix*, Table S4 summarizes the catalytic activities of the mutated recombinant proteins that were measured in isolated membrane fractions from each *Escherichia coli* culture. It is apparent from *SI Appendix*, Table S4 that all mutated residues that interact either with the diiron (E213A) or the inhibitor (R118A, R118Q, L122A, L122N, E215A, A216L, A216N, T219V, and Y220F; Fig. 4) resulted in almost complete loss of ubiquinol oxidizing activity. Furthermore, the Y246A mutant, which participates in the hydrogen bond network (Fig. 2), also resulted in significant inhibition of catalytic activity. We believe that these residues are important for the correct conformation of the diiron center and interaction with AF2779OH and are consistent with the crystal structure.

Ubiquinol Binding Model. In addition to the inhibitor-binding cavity observed in Figs. 3*B* and 5 *A* and *C*, which is comparable to that observed in other monotopic proteins such as prostaglandin H_2 synthase (37), CAVER protein-analysis software (38) predicts that there is another possible hydrophobic cavity near the membrane surface (Fig. 5 *A* and *D*). This second cavity connects the diiron active site with the membrane exterior and interacts with the inhibitor-binding cavity at the active site. It is formed by residues from helices α1 (R96 and D100), α2 (R118, L122, E123, and A126), α3 (E162 and H165), α5 (L212, E213, E215, A216, and T219), and α6 (E266), which, similar to that observed in the inhibitor-binding cavity, are also highly conserved (*SI Appendix*, Fig. S5). It is apparent from Fig. 5 *A* and *D* that a part of the aromatic head group of AF2779OH enters this second cavity. Based on the structure of the TAO–AF2779OH complex, a ubiquinol-binding model was built by superposing a ubiquinol molecule onto the bound AF2779OH. The model (Fig. 5*B*) indicates that the distance between ubiquinol C4–OH and Fe2 is 4.3 Å and C1–OH is connected to the outside of TAO through a hydrogen bond network, C1–OH···R118···D100 (Fig. 5*B*). On the basis of the structures reported in this study we propose that each hydrophobic cavity binds one ubiquinol close to the active site with their quinol rings located at the bottom of each cavity in a manner similar to AF2779OH. Although the exact route of electron transfer for the four-electron reduction of oxygen to water in any alternative oxidase is unresolved at the present time, we suggest the process involves both ubiquinols and Tyr220

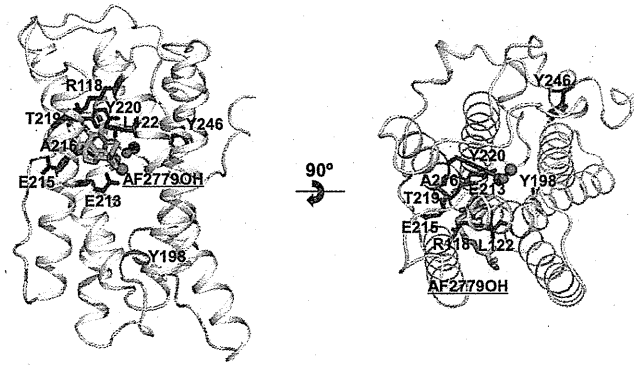

Fig. 4. Location of the recombinant TAO mutations within the protein. Diiron and hydroxo atoms are shown as magenta spheres. AF2779OH is shown as a cyan stick. Red sticks show the mutated residues that almost completely abolished activity (specific activity <10%), whereas yellow sticks show the mutated residues that retained some residual activity (specific activity ≥10%).

(39). During the sequential electron reduction process we suggest that following the activation of oxygen, free radicals are generated on a tightly bound ubiquinol and Tyr220. The ubisemiquinol is then reoxidized by the tyrosine radical generated during the catalytic cycle and the reduction process is completed following full oxidation of a loosely bound ubiquinol (39).

Conclusions

The TAO structures reported in this study are a high-resolution view of a membrane bound diiron-carboxylate protein. Although the crystal structures support earlier modeling studies (13, 14, 22) that suggested that the alternative oxidases are monotopic proteins in which the diiron active site is coordinated by carboxylate and histidine residues, they did reveal that in the oxidized state only carboxylate residues act as the coordinating ligands. Such a primary ligation sphere, although unusual for diiron proteins in the oxidized state, is, however, consistent with our earlier reduced minus oxidized IR difference spectra (26). This study clearly demonstrated that upon reduction of purified TAO there was a net protonation of at least one carboxylate residue in addition to alterations in the signals associated with histidine residues consistent with the notion that the oxidation–reduction cycle of the alternative oxidases involves major conformational perturbations and carboxylate shifts. The structure has also revealed that the redox-active Y220, which is totally conserved across all AOXs (1), is within 4 Å of the active site. Such a close-range electron transfer position, comparable to that observed in the R2 subunit of ribonucleotide reductase (31), is further support for the suggestion that radicals play a key role in the AOX catalytic cycle (39).

In addition to providing a structural insight into the active site of this enigmatic protein our structures have also revealed the nature of the inhibitor binding site. The binding site of our AF derivative was within 4 Å of not only the diiron center but also Y220 and resulted in some dramatic conformational changes such that H165 moved within ligating distance of Fe (1). CAVER protein analysis software (38) suggested that the inhibitor-binding cavity connects at the diiron center with an additional cavity, which could also serve as a ubiquinone binding site.

In conclusion, we believe that the structures presented in this report will contribute to a more complete understanding of the function and inhibition of all AOXs. It will not only be beneficial for the control of trypanosomiasis and other human diseases, such as cryptosporidiosis and candidiasis, but also for the control of plant diseases caused by phytopathogenic fungi (1, 40).

Materials and Methods

Crystallization. The oxidized form of alternative oxidase from *Trypanosoma brucei brucei* was expressed, purified, and crystallized essentially according to the method described previously (23, 24) using 28–34% (wt/vol) PEG 400, 100 mM imidazole buffer (pH 7.4), and 500 mM potassium formate as the reservoir solution. Detailed information is presented in *SI Appendix, SI Materials and Methods.*

Data Collection and Phasing. For phasing by the single-wavelength anomalous dispersion (SAD) method, anomalous scattering effects caused by Fe were measured to 3.2 Å resolution. The dataset was processed and scaled with HKL2000 (41). The program SOLVE (42) was used to locate and refine four "diiron sites" (figure of merit = 0.195). The RESOLVE (43) program was used for solvent flattening (figure of merit = 0.645). The resulting electron density map was clear enough to trace the TAO molecules. Initial models were built

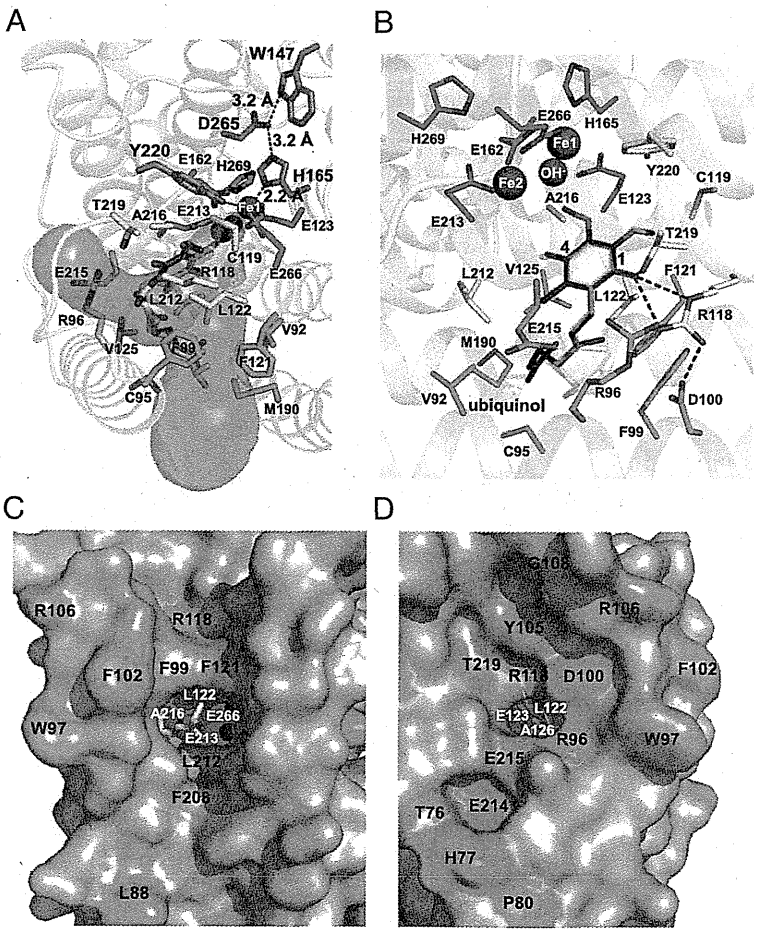

Fig. 5. Putative ubiquinol binding cavities in TAO. (*A*) Two hydrophobic cavities predicted by CAVER protein-analysis software (38). The bound AF2779OH (isoprenoid tail) occupies the green cavity. Putative residues involved in electron transfer are shown as orange sticks. (*B*) Ubiquinol binding model predicted by the superposition of a ubiquinol molecule (purple stick) onto the bound AF2779OH (translucent white stick) of the TAO–AF2779OH complex. Magenta spheres are diiron (Fe–OH^-–Fe), the green stick represents residues coordinating to diiron, and yellow and cyan sticks are residues interacting with the aromatic head and isoprenoid tail, respectively. Hydrogen bonds are depicted as dotted lines. Surface views of the (*C*) green and (*D*) orange cavities shown in *A*. Cyan and pink colors stand for conservation of AOX residues in all eight and over four organisms in *SI Appendix*, Fig S5, respectively.

using RESOLVE (43) and BUCANER (43). Detailed analysis of diffraction data showed that the crystal used for the data collection of Fe-SAD was pseudohemihedral twinning. Amplitude-based twin-refinement using REFMAC5 (45) decreased R_{work}/R_{free} drastically from 0.307/0.363 to 0.250/0.310. X-ray diffraction data of ligand-free TAO and AF derivatives complex crystals were collected to 2.85, 2.6, and 2.3 Å resolution, respectively. All datasets were processed and scaled with HKL2000 (42). Detail information is presented in *SI Appendix, SI Materials and Methods*.

Refinement. The initial model of inhibitor-free TAO was determined by molecular replacement (MR) using the model obtained by SAD (3.2 Å resolution) as a search model. The program Phaser (46) in CCP4i was used for MR. The models of ligand-free TAO and TAO-AF2779OH complex were rebuilt with reference to the well-refined model of the TAO–CCB complex at 2.3 Å resolution. Manual rebuilding and crystallographic refinement of all structures were performed using COOT (47) and REFMAC5 (45). All structures were refined by amplitude-based twin-refinement in REFMAC5 (45) to final R_{work}/R_{free} values of 0.192/0.247 (twin fraction of 0.476), 0.214/0.256 (twin fraction of 0.552), and 0.185/0.227 (twin fraction of 0.527) for ligand-free TAO, TAO–AF2779OH, and TAO–CCB, respectively. The omit electron density maps of ligand-free TAO, TAO–AF2779OH, and TAO–CCB around helix 5 are shown in *SI Appendix*, Fig. S14. On average, about 30 residues of N and C termini of TAO were missing as a result of flexibility. Data collection and structural refinement statistics are summarized in *SI Appendix*, Table S1. Figures showing protein structures were prepared with the graphics program PyMol (www.pymol.org). Detailed information is presented in *SI Appendix, SI Materials and Methods*.

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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, producing 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 parasite[14] 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 protozoan[16] 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 study[26]; 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 GeneDB[27] (accession no. Sjp_0095720.1). The expected length of the PCR product was 555 bp. Twenty microliters of reaction mixture contained 2 μL of buffer, 0.6 μL of 1.5 mM $MgCl_2$, 1.6 μL of 2.5 mM dNTP, 0.4 μL of each 20 pmol/μL primer, 0.2 μL of 5 U/μL *Taq* DNA polymerase (Takara, Otsu, Japan), and 1 μL 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* DH5α 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 μg/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 described[28] 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 μg/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 AHNAK	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.

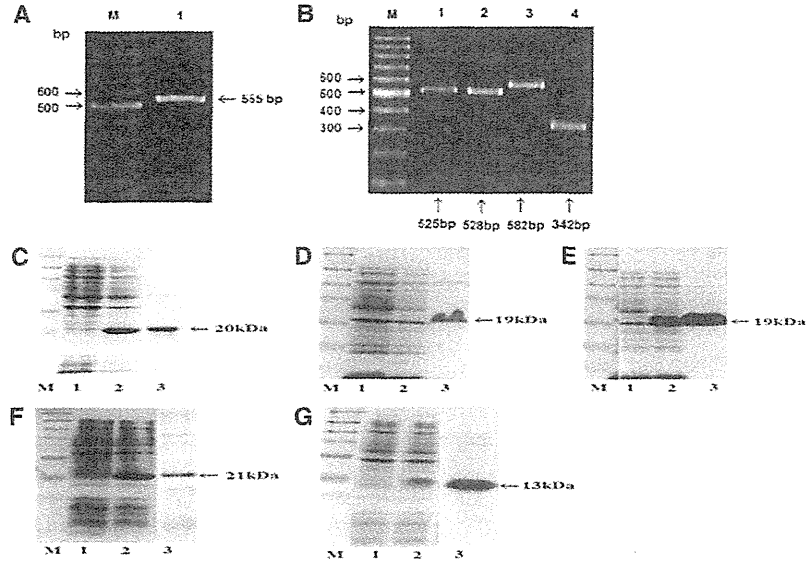

FIGURE 1. Gel electrophoresis and polyacrylamide gel electrophoresis (SDS-PAGE) of the recombinant antigens. (**A** and **B**) The recombinant plasmids were identified by polymerase chain reaction (PCR) and enzyme digestion. (**A**) Lane 1: TPx-1 from the *Schistosoma japonicum* adult worm cDNA library. (**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

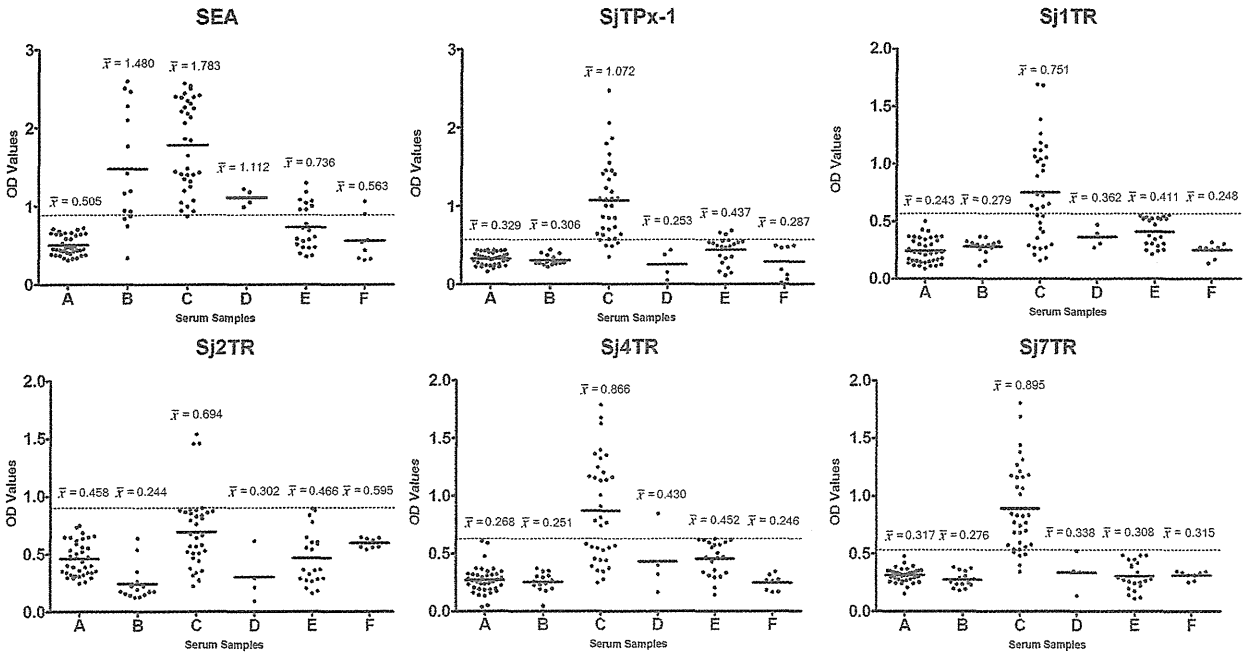

FIGURE 2. Enzyme-linked immunosorbent assay (ELISA) results of soluble egg antigen (SEA) and the recombinant antigens. (**A**) Negative serum samples from Japanese volunteers. (**B**) Negative endemic controls from Filipino volunteers. (**C**) *Schistosoma japonicum* stool-positive serum samples. (**D**) Post-treatment negative serum samples. (**E**) Sera positive for other heminthic 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 chemotherapy[40] 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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PLOS | NEGLECTED TROPICAL DISEASES

Utilization of ELISA Using Thioredoxin Peroxidase-1 and Tandem Repeat Proteins for Diagnosis of *Schistosoma japonicum* Infection among Water Buffaloes

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Abstract

Background: The presence of animal reservoirs in *Schistosoma japonicum* infection has been a major obstacle in the control of schistosomiasis. Previous studies have proven that the inclusion of control measures on animal reservoir hosts for schistosomiasis contributed to the decrease of human cases. Animal surveillance should therefore be included to strengthen and improve the capabilities of current serological tests.

Methodology/Principal Findings: Thioredoxin peroxidase-1 (SjTPx-1) and four tandem repeat proteins (Sj1TR, Sj2TR, Sj4TR, Sj7TR) were initially evaluated against human sera. The previous test showed high sensitivity and specificity for antibody detection against SjTPx-1 and Sj7TR. In this study, the immunodiagnostic potential of these recombinant proteins was evaluated using enzyme-linked immunoassay on 50 water buffalo serum samples collected in Cagayan, the Philippines as compared with the soluble egg antigen (SEA). For specificity, 3 goat serum samples positive with *Fasciola hepatica* were used and among the antigens used, only SEA showed cross-reaction. Stool PCR targeting the *S. japonicum* 82 bp mitochondrial NAD 1 gene was done to confirm the true positives and served as the standard test. Twenty three samples were positive for stool PCR. SjTPx-1 and Sj1TR gave the highest sensitivity among the recombinant proteins tested for water buffalo samples with 82.61% and 78.26% respectively which were higher than that of SEA (69.57%).

Conclusions/Significance: These results prove that SjTPx-1 works both for humans and water buffaloes making it a good candidate antigen for zoonotic diagnosis. Sj1TR showed good results for water buffaloes and therefore can also be used as a possible candidate for detecting animal schistosome infection.

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Introduction

Intensified disease surveillance has become an essential public health instrument in providing necessary information for monitoring the disease and evaluating control measures. Schistosomiasis is considered as a neglected disease caused by *Schistosoma japonicum* in China and Southeast Asia, *S. haematobium* in the Middle East and Africa and *S. mansoni* in Africa. Among them, only *S. japonicum* is known to infect both humans and more than 40 other mammals [1] which complicate the control of the disease. Inclusion of zoonotic surveillance in national control programs in endemic countries might be a necessary tool for the control and elimination of schistosomiasis japonica. Researches have shown how intervention involving animal reservoirs can reduce *S. japonicum* infection in humans [2,3]. Simultaneous treatment of water buffaloes and human has proven to be effective as seen in a five-year praziquantel-based intervention study done around the Poyang Lake in Jiangxi Province, China [2]. However, animal surveillance for schistosomiasis has not yet been fully developed.

In China, a nationwide schistosomiasis survey in 1995 established the high prevalence of *S. japonicum* in water buffalo (9.6%) and cattle (7.2%) [4], showing how important these animals are as reservoir hosts. In Indonesia, domestic animals such as water buffaloes and wild animals were found to be infected with schistosomes (10%) [5]. In the Philippines, a variety of animal reservoir hosts such as rats, cats, dogs, pigs, cattle and water buffaloes were found to be potential hosts for schistosomiasis using different parasitological and immunological assays [6–8]. Among

Author Summary

Schistosomiasis remains to be a public health problem in 76 endemic countries in spite of control efforts that have been done. Among the major causative agents of schistosomiasis, only *Schistosoma japonicum* is known to be zoonotic. However, the role of animal reservoir hosts has not been given much importance which might be the main hindrance in the possible elimination of the disease. In addition, animal surveillance is not part of the current schistosomiasis control program in most of the endemic countries. This study reports the use of recombinant proteins in ELISA for detecting the infection in water buffaloes. These antigens were previously used against humans and showed that SjTPx-1 and Sj7TR can be a good diagnostic antigen. Using the stool PCR as the standard test, SjTPx-1 and Sj1TR were shown to work on the water buffaloes better than the conventional SEA. These antigens can both be useful in the development of intensified animal surveillance for schistosomiasis.

these hosts, water buffaloes had the lowest prevalence of infection [8] and showed no significant role in the *S. japonicum* transmission to humans according to the mathematical modeling done on these prevalence data [9]. A recent study however in one endemic area in Leyte showed prevalence in water buffaloes as high as 51.5% using the highly validated real-time polymerase chain reaction [10]. This may prove that water buffaloes have a major contribution to the transmission of schistosomiasis in the Philippines.

Animal schistosome infection has been usually diagnosed through direct parasitological techniques including Kato-Katz technique and miracidial hatching. The quantitative Kato-Katz fecal smear is simple, practical and useful in quantifying eggs [11,12] and is considered by the World Health Organization as the gold standard method for diagnosing schistosomiasis [13]. However, this method is labor-intensive, requires skilled personnel, has low sensitivity in low prevalence endemic areas [14,15] and seven repeated Kato-Katz examinations coupled with miracidial hatching are needed to reach its maximal sensitivity [16]. On the other hand, molecular detections such as polymerase chain reaction (PCR) are highly sensitive and specific, but they are costly and require expensive equipment. Furthermore, current serological tests utilizing crude antigens like soluble egg antigen-enzyme-linked immunosorbent assay (SEA-ELISA) and circumoval precipitin test (COPT) cause cross-reactions leading to misdiagnosis. Hence there is a need for the development of an easier, more sensitive and specific test for schistosomiasis.

In a previous study, thioredoxin peroxidase-1 (SjTPx-1, GeneDB accession no. Sjp_0095720.1) and four tandem repeat proteins (TRP) namely Sj1TR, Sj2TR, Sj4TR and Sj7TR (GeneDB accession nos. Sjp_0099630, Sjp_0086200, Sjp_0059850, Sjp_0110390 respectively) were evaluated against human sera [17]. SjTPx-1 and Sj7TR both showed high sensitivity and specificity making them promising diagnostic antigens for human schistosomiasis. Using ELISA, these recombinant proteins were tested on water buffaloes and the results were compared with stool PCR assay and the conventional SEA-ELISA and COPT. This study therefore examined the immunodiagnostic potential of the recombinant antigens in water buffaloes which might lead to the development of a more reliable and accurate diagnostic test for animal schistosomiasis. Strengthening the diagnostic test is crucial in both the human and animal schistosome infection surveillance in areas where elimination is in sight and might be vital in the prevention of emergence and re-emergence of schistosomiasis japonica leading to the possible control of this neglected parasitic disease.

Materials and Methods

Samples

Serum and stool samples were taken from 50 water buffaloes in Gonzaga, Cagayan, the Philippines. Stool samples collected by intrarectal means from water buffaloes were placed in code-labeled cups and stored with 10% neutralized formalin until processing. None of the stools were found positive for *S. japonicum* eggs using the formalin-ether concentration technique (FECT). Non-endemic negative control sera were taken from 18 water buffaloes in Nueva Ecija and Batangas in the Philippines. All the owners of the water buffaloes were informed about the study and gave consent to use their water buffaloes in this study. Sera positive for *Fasciola hepatica* were collected from experimentally infected goats ($N = 3$). They were diagnosed through the detection of the parasite in the stool. This study was done according to ethical guidelines for the use of animal samples permitted by Animal Care and Use Committee, Dokkyo Medical University (Permit No. 0029) in accordance with the Guidelines for the Care and Use of Laboratory Animals, Dokkyo Medical University, The Law Concerning Kind Treatment and Management of Animals (Law No. 221) and Japanese Government Notification on Feeding and Safe-keeping of Laboratory Animals (No. 6), as well as by Obihiro University of Agriculture and Veterinary Medicine (Permit No. 23–153).

Stool DNA Extraction

FECT was done prior to DNA extraction to maximize the quantity of schistosome eggs in the collected stool if positive and to remove fecal debris. Although formaldehyde is known to degrade DNA, DNA extraction was not deterred since neutral-buffered formaldehyde was used [18,19] and the PCR target is less than 400 bp [20]. DNA extraction from stool samples was done using QIAamp DNA Stool Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol and stored at −20°C until use. DNA was also extracted from cattle stool in non-endemic area (Obihiro, Hokkaido, Japan) to serve as the negative control.

Stool PCR

PCR was done on the stool samples collected from 50 water buffaloes targeting the 82 bp mitochondrial NADH dehydrogenase I gene (SjND1) [21]. The primer set SjND1 forward 5′-TGR TTT AGA TGA TTT GGG TGT GC3′ and reverse 5′ AAC CCC CAC AGT CAC AGT CAC TAG CAT AA3′ was used according to a previous research [22]. Twenty microliters of reaction mixture contained 2 µl of buffer, 0.6 µl of 1.5 mM $MgCl_2$, 1.6 µl of 2.5 mM dNTP, 0.4 µl of each 20 pmol/µl primer, 0.2 µl of 5 U/µl *Taq* DNA polymerase (Takara, Otsu, Japan) and 1 µl of template. The conditions for PCR were as follows: 95°C for 10 mins, followed by 40 cycles of 95°C for 15 secs, 60°C for 1 min, 72°C for 1 min, 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 2.5% agarose gel and visualized by ethidium bromide staining. PCR reactions were done in triplicates for every stool sample and a sample is regarded as positive when at least one reaction was positive.

Recombinant Antigen Preparation

Recombinant molecules of SjTPx-1 and the four TRPs from *S. japonicum* used in this study were prepared as previously described [17]. In brief, SjTPx-1 was cloned using PCR from *S. japonicum*

Yamanashi strain adult worm cDNA while the nucleotides coding a partial tandem repeat domain of the 4 TRPs were synthesized by GenScript USA Inc. (Piscataway, NJ). The genes were then digested with their respective restriction enzymes, inserted into the pET28 vector (EMD Biosciences, San Diego, CA) and transfected into *Escherichia coli* BL21 grown in SOB medium (Sigma-Aldrich, St. Louis, MO). The recombinant proteins were recovered using the Ni-NTA agarose (Qiagen Inc., Valencia, CA), dialyzed and eluted with 20 mM Tris, pH 8.0. The integrity and purity of the proteins were evaluated by 15% polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and subsequent Coomassie Brilliant Blue staining. The concentration of each expressed protein was measured using the BCA Protein Assay (Thermo Scientific, Rockford, IL).

Serological Tests

(i) COPT. COPT was performed as previously described [23]. One drop of serum sample was mixed on a slide with approximately 5 μg of lyophilized schistosome eggs. The eggs were collected from a rabbit infected with *S. japonicum* for 12 weeks. A cover slip was then placed over a nail polish ring surrounding the egg suspension. The slides were then sealed with paraffin and incubated at 37°C for 48–72 h. Bleb and segment formation for positive samples were detected under the microscope.

(ii) ELISA. The conventional ELISA was done according to a previously described method [24] with slight modifications. Horseradish peroxidase (HRP)-conjugated Protein G (Rockland Inc., Gilbertsville, PA) served as the secondary antibody in this study and 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD) was used as the substrate for HRP. Ninety-six wells microplates (Nunc Maxisorp, Thermo Fisher, Rockland, IL) were sensitized separately with SEA (1 μg/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-1%BSA), the serum samples were placed on the antigen-coated wells. The test sera (0.1 ml) were diluted 200-fold in T-PBS-1%BSA while the secondary antibody (0.1 ml) was diluted in 10,000-fold. Optical density (OD) at 450 nm was determined using a microplate reader (MTP-500, Corona Electric, Tokyo, Japan). Each ELISA reaction was performed with positive (8-weeks post-infected rabbit serum) and negative controls (diluting buffer). All the tests were done in triplicates and data represent mean values. The cut-off value was calculated as the mean absorbance value of the 18 negative controls plus 3 standard deviations. A sample was considered positive when the mean absorbance value of each sample was higher than the cut-off value.

Statistical Analysis

The validity of the ELISA assays using the recombinant proteins was estimated by the sensitivity, specificity and predictive values using the stool PCR as the reference standard. Kappa value was used to estimate the agreement between the antigens [25]. To test for the statistical significance of the difference between the mean OD values of the PCR positive and PCR negative samples on the ELISA using the crude and recombinant antigens, two-tailed p-value was calculated using unpaired *t* test with 95% confidence interval.

Results

Stool PCR

Stool PCR was performed to serve as the standard test by determining the positives for *S. japonicum* infection. Stool DNA from a non-endemic cattle served as the negative control and *S. japonicum* DNA template served as the positive control. Of the 50 water buffalo samples, 23 were positive. As seen on Figure 1, a band having approximately 82 bp was found in the positive samples while none on the negative samples. The band was also seen in the positive control but not in the negative control.

Serological Tests

COPT was done initially on the 50 water buffalo samples for the purpose of comparing it with the ELISA using the recombinant proteins. Seventeen samples turned out to be positive as shown by bleb or segment formation after 48 h incubation. All of the samples positive for COPT were also PCR positive.

The ELISA was performed using sera from 50 water buffaloes from an endemic area in the Philippines. Cut-off values were calculated using 18 water buffalo serum samples from non-endemic areas in the Philippines. Twenty samples were positive for both SjTPx-1 and Sj1TR, 18 for SEA and 14 samples for Sj2TR, Sj4TR and Sj7TR. As shown on Table 1, 16 out of the 18 SEA positive, 19 out of the 20 SjTPx-1 positive and 18 out of the 20 Sj1TR positive samples were also PCR positive. There were 2 PCR negative samples detected only by SEA and Sj1TR, and of which, 1 was also detected by SjTPx-1. Furthermore, there were 4 samples detected only by PCR and negative for all the recombinant proteins and SEA. The mean OD values for PCR negative were lower than that of the PCR positive samples (Figure 2) for the crude and the recombinant

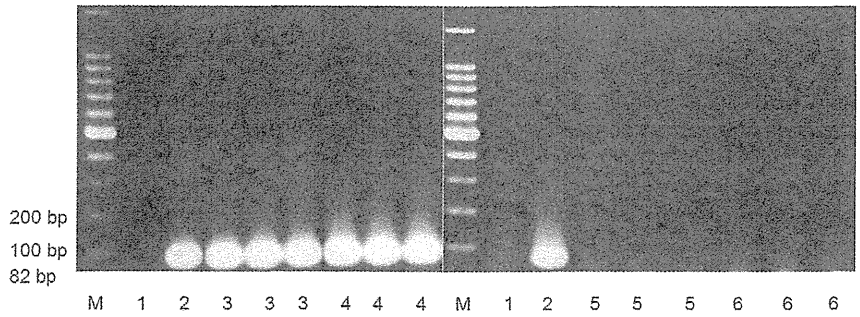

Figure 1. Gel electrophoresis of the stool PCR for water buffaloes targeting the *Schistosoma japonicum* NAD 1 gene. *M*, marker. *Lane 1*, negative control (stool DNA from non-endemic cattle). *Lane 2*, positive control (*S. japonicum* adult DNA template). *Lane 3–6*, water buffalo stool samples done in triplicates. Positive control, lanes 3 and 4 show positive results with bands at 82 bp while none is seen on negative control, lanes 5 and 6.
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Table 1. Results tally of the samples for *Schistosoma japonicum* using serological tests and stool PCR.

	COPT		SEA		SjTPx-1		Sj1TR		Sj2TR		Sj4TR		Sj7TR	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Negative by PCR	0	27	2	25	1	26	2	25	1	26	0	27	0	27
Positive by PCR	17	6	16	7	19	4	18	5	13	10	14	9	14	9

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antigens. The p-values obtained to show the significance of the difference between the mean OD values of PCR positive and PCR negative samples were all less than 0.05 and were considered statistically significant (data not shown).

To test for specificity, 3 serum samples from goats experimentally infected with *Fasciola hepatica* were also used. Only SEA showed cross-reaction with 2 samples having high OD values (data not shown).

Based on the statistical analysis, SjTPx-1 and Sj1TR showed high agreement with the stool PCR done on the samples based on the kappa values as seen in Table 2. The specificity and the positive predictive values of these 2 recombinant proteins were higher than those of SEA.

Discussion

The lack of importance given to the role of animal hosts in the transmission of *S. japonicum* has turned into a loophole in the control efforts for schistosomiasis. Efficient and highly sensitive diagnostic tools for animal surveillance should be employed as a strong support in ensuring control of the parasitic infection among the reservoir hosts. This study aims to determine the possible use of the recombinant antigens in the diagnosis of schistosomiasis among the water buffaloes. Results of the study are expected to contribute to clearer insights in the role of this animal in the transmission of the disease.

SjTPx-1, which has a sensitivity of 85.71% for humans [17] and 84.0% for cattle [26] in previous studies, showed a comparable 82.61% sensitivity among the water buffaloes. However, it should be noted that the infection standard between these studies are different, with stool PCR confirmed samples used in this study and microscopy confirmed samples in the previous studies. But despite this difference, SjTPx-1 showed good immunodiagnostic potential in all these studies and therefore might be an effective diagnostic

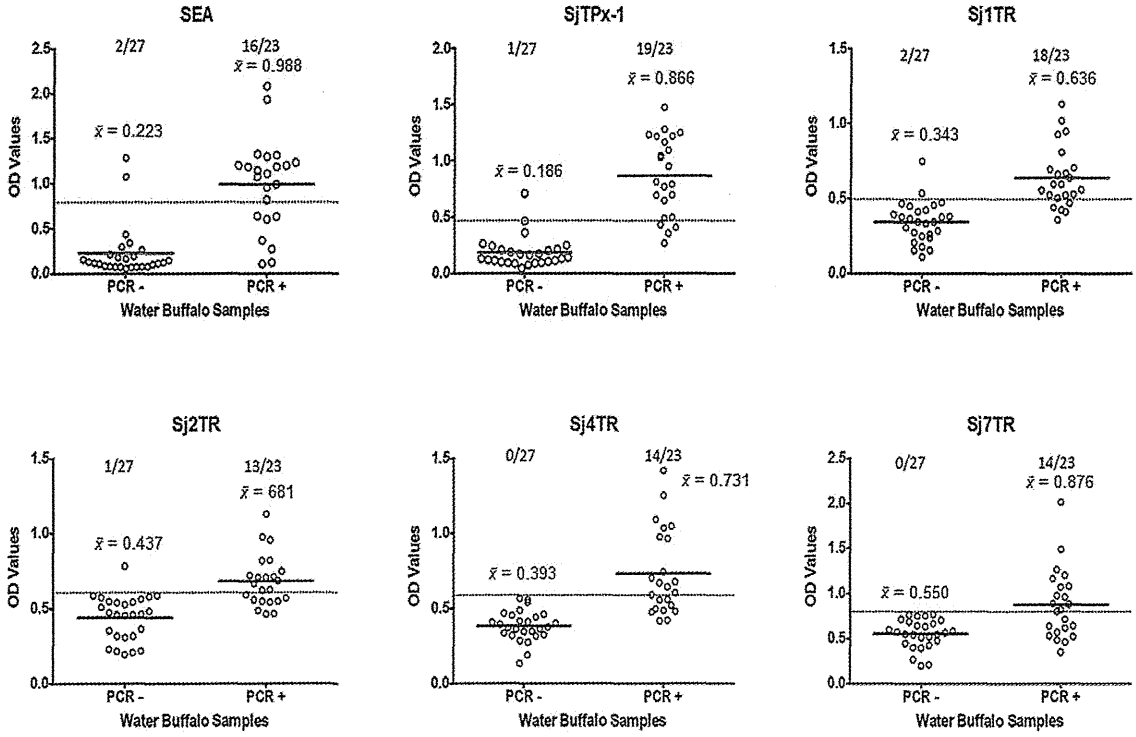

Figure 2. Difference in OD values among stool PCR negative and positive in ELISA using SEA and the recombinant proteins. The graph shows that SjTPx-1 and Sj1TR have the highest number of positives among the PCR positive samples. PCR negative samples that are positive for ELISA using the recombinant proteins show OD values minimally higher than the cut-off values. Mean OD values for each set were also shown.
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Table 2. Statistical analysis of the ELISA results of SEA and the recombinant proteins.

Antigen[a,b]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa[c]
SEA	69.57	86.67	80	78.79	0.571
SjTPx-1	82.61	96.67	95	87.88	0.805
Sj1TR	78.26	93.33	94.74	84.85	0.765
Sj2TR	56.52	96.67	92.85	74.36	0.557
Sj4TR	60.87	100	100	76.92	0.638
Sj7TR	60.87	100	100	76.92	0.638

[a]Stool PCR served as the reference standard.
[b]ELISA = enzyme-linked immunosorbent assay; SEA = soluble egg antigen; PPV = positive predictive values; NPV = negative predictive values.
[c]Kappa value of SjTPx-1 showed high agreement with the stool PCR based on the previously reported criteria set [25].
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antigen candidate for both humans and animals. Furthermore, Sj1TR performed better in water buffaloes (78.26%) than in humans (68.57%) while Sj7TR did not show good antigenicity in water buffaloes (60.87%) as it did in humans (85.71%). These differences in antigenicity can be explained by the differences in immune responses among various host species.

On the other hand, results showed that SEA has lower sensitivity than SjTPx-1 and Sj1TR, and causes cross-reaction with *F. hepatica* positive samples. Both the conventional SEA-ELISA and COPT therefore are not adequate enough to properly diagnose cases of schistosomiasis. Given this and the difficulty in scaling up production of SEA for mass screening, the use of recombinant proteins has proven to be a good alternative for schistosomiasis diagnosis.

In this study, we used stool PCR as the standard test instead of stool microscopy. Coprological methods such as Kato-Katz technique have been the commonly accepted gold standard in schistosomiasis diagnosis. However, it will be difficult to detect schistosome eggs in the stool of large animals due to the size of their excreta which might affect the sensitivity of the test. The adequacy of the stool PCR in diagnosing true positives has been already validated by a previous study done among water buffaloes in the Philippines [10]. Their results showed a marked difference in the number of positive water buffaloes with 51.5% prevalence for stool PCR as against to the 3.7% prevalence using the coprological tests including DBL and Kato-Katz technique. Stool PCR has been tested also in other helminthic [27] and protozoan [28] infection and was found to possess higher sensitivity and specificity as compared to stool microscopy.

Furthermore, samples which were PCR negative and positive for the recombinant protein-based ELISA should be investigated further. It was shown in the initial assessment of the stool PCR that its sensitivity can be affected by the degree of infection [22]. For example, in an infection higher than 10 schistosome eggs per gram (epg) of stool, the sensitivity can go as high as 95 to 100%. The sensitivity goes down to 78 to 85% when the infection is less than 10 epg. It is therefore very important to adjust the diagnostic capabilities of the recombinant proteins to detect cases even in very low infections which are undetectable even with molecular techniques such as PCR. Furthermore, the extent of time that the antibodies against these recombinant proteins will be present in the blood circulation should also be analyzed. It was widely known that one of the limitations of antibody-based serological tests is that it cannot distinguish past and present infection. In addition, the infection in water buffaloes is self-limiting [29] which further complicates the possible diagnosis of active infection. In the previous paper using these recombinant proteins [17] however, serum samples from human individuals collected one year after treatment with praziquantel tested negative for the recombinant antigens. This somehow suggests that the recombinant antigens might be used to detect current infection in humans. However, it was not yet studied in animals and it will be very useful if these recombinant antigens can also be used to diagnose present animal infection as well.

As this study proved the serological applicability of SjTPx-1 and Sj1TR in water buffaloes, this might be also used in the development of rapid immunochromatographic tests that can detect animal schistosome infection in the field. Although the possible reservoir animal hosts in endemic areas can also undergo mass drug administration as previously done in China, serological tests utilizing these recombinant proteins will be useful in epidemiological studies and surveillance of animal infection in areas that have reached elimination level. It was reported that inappropriate surveillance system was one of the factors attributed to the re-emergence of schistosomiasis in one province in China [30]. Infection rate among the cattle in that province was reported to have reached as high as 22.3%. Mammalian reservoir hosts might serve as the sentinel population in schistosomiasis transmission as they have the potential to be the key source of *S. japonicum* infection in re-emerging regions [31]. The World Health Organization noted that case detection will be a problem when elimination of the disease is at hand [32]. Environmental monitoring was said to be important in knowing the scale of the transmission mechanism in such low transmission environment [33]. Strengthening therefore the diagnostic capabilities of serological tests might be one of the vital keys in the possible prevention of such re-emergence of the disease.

On the other hand, the emergence of schistosomiasis in new endemic foci is also a threat to the possible elimination of the disease. The site used in this study, Cagayan Valley, was not known to be endemic of schistosomiasis until 2002 [34]. Based on the results of this study, schistosome infection among water buffaloes has a positivity rate ranging from 24% to 46% using ELISA and stool PCR respectively. Animal infection might play a big role in the transmission of the disease in that area. A more specific and sensitive animal surveillance is therefore also needed to prevent spreading of the disease in other areas. Furthermore, the recombinant antigens used in this study should also be tested against other animals like dogs, pigs and rats as previous studies showed that they are also important reservoir hosts for *S. japonicum* [9,35]. This will also provide a more realistic epidemiological picture of the disease which is very important in the control program.

In the future studies, the use of these recombinant antigens should be also validated in areas with different levels of endemicity both for humans and animals. This stage is relevant as the challenge now is to optimize the diagnostic use of these recombinant antigens to the different stages of active control. Appropriate diagnostic tools were strongly needed to evaluate effectiveness of community interventions, verify local disease elimination and detect resurgence of the disease at the earliest time possible [36].

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Author Contributions

Conceived and designed the experiments: JMA YG SK. Performed the experiments: JMA MK MA EAV. Analyzed the data: JMA YG SK. Contributed reagents/materials/analysis tools: NI MK YC PTR LRL SK. Wrote the paper: JMA YG LRL SK.

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Short communication

Localization and expression profiling of a 31 kDa antigenic repetitive protein Sjp_0110390 in *Schistosoma japonicum* life stages

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ABSTRACT

Sj7TR is a 13 kDa repetitive region of a 31 kDa protein in *Schistosoma japonicum* known as Sjp_0110390 that showed high sensitivity and specificity in antibody detection against schistosomiasis patients. However, the current database for *S. japonicum* genes characterized it only as an expressed protein. A more thorough understanding of this antigenic protein is therefore necessary to possibly give more information about the nature of this protein and its role in the parasite. In this study, immunolocalization and expression profiling were done for Sjp_0110390 on the different stages of the parasite. Immunofluorescent assay showed that Sjp_0110390 was expressed in the young stages of the parasites including the schistosomula, eggs, aquatic and intra-molluscan stages. This was supported by the reverse-transcriptase PCR which confirmed the stage-specific expression of Sjp_0110390 and Western blot test which detected the protein in the extracted eggs proteins, but not in the adults. Furthermore, it was also highly expressed in infected *Oncomelania hupensis nosophora* snails suggesting that Sjp_0110390 might have a role in the development of the parasite inside the intermediate host. This result also suggests that Sj7TR might be used not only for human diagnosis but to detect snail infection as well.

Schistosoma japonicum is the major causative agent of schistosomiasis in South East Asia and China. Current available serological techniques for the diagnosis of schistosomiasis still depend on the use of crude egg antigen. However, the use of crude antigen has proven to produce false positive results, which can affect the disease surveillance in areas that already reached elimination level. Also, large-scale production of crude antigen is difficult which requires maintaining the parasite's whole life cycle inside the laboratory. The use of recombinant antigens therefore might be used to address these problems in schistosomiasis diagnosis. One recombinant antigen already tested for diagnostic purposes is a 13 kDa repetitive region of a 31 kDa protein Sjp_0110390 (GeneDB ID: Sjp_0110390). In a previous paper, the tandem repeat was labeled as Sj7TR and was tested against human sera [1], being a good candidate as a diagnostic antigen. However, Sjp_0110390 was not yet characterized and its expression in the life stages of *S. japonicum* has not yet been known. Available EST sequences (GenBank ID: CV42804.1, CV746246.1, CV750916.1, CV736328.1) corresponding to this gene were reported at the ones whose developmental stages were unknown [2].

Diagnostic antigens like Sj7TR need to be characterized to know their possible function in each stage of the parasite. This may help understand the principles behind their serological value in the mammalian hosts as well as determine what other possibilities this antigen might be capable of. Histidine-rich protein II of *Plasmodium falciparum* for example was found to be secreted through the surface membrane of the infected erythrocytes [3] making it a good antigen for the diagnostic tests developed years later [4,5]. Lactate dehydrogenases in *Taenia solium* were not specific antigens for immunodiagnosis but because of their characterization were suggested to be candidate targets for anti-*Taenia* drugs and vaccine development [6]. In this study, immunolocalization and expression

Abbreviations: ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tag; IFAT, indirect immunofluorescent antibody test; NSS, normal saline solution; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PVDF, polyvinyliden difluoride; RT, room temperature; SDS, sodium dodecyl sulfate; T-PBS, phosphate buffered saline with Tween-20.

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profiling were done on Sjp_0110390 in both the intra-mammalian and intra-molluscan life stages to give some insights on this schistosome protein.

The cDNA sequence of Sjp_0110390 comprised a 822 bp ORF encoding 273 amino acid residues with the predicted molecular mass of 31.04 kDa and theoretical isoelectric point of pH 3.84 [7]. Based on an online tandem repeats finder (http://tandem.bu.edu/trf/trf.html), the sequence has 4.9 copies of the 165 bp-long repeat unit [8]. Sequence analysis reveals that it is highly hydrophilic, does not possess any transmembrane domain [9] and has no signal peptide [10]. The protein was also shown to not contain nuclear localization signal [11] and predicted to be cytoplasmic [12].

Yamanashi strain of *S. japonicum*, maintained using standard laboratory procedures in female ICR mice and their snail hosts, *Oncomelania hupensis nosophora*, was used for this study. Animal experiments done in this study were conducted according to ethical guidelines for the use of animal samples permitted by Animal Care and Use Committee, Dokkyo Medical University (Permit No. 0029) in accordance with the Guidelines for the Care and Use of Laboratory Animals, Dokkyo Medical University, The Law Concerning Kind Treatment and Management of Animals (Law No. 221) and Japanese Government Notification on Feeding and Safe-keeping of Laboratory Animals (No. 6), as well as by Obihiro University of Agriculture and Veterinary Medicine (Permit No. 23-153). Infected mice were anesthesized using ether before they were sacrificed. Perfusion method with normal saline solution was done to collect the intra-mammalian stages of the parasites. Schistosomules were taken from the perfused fluid of a 2 weeks post-infected mouse while the juvenile and fully developed adults were collected from the portal and mesenteric veins of 2 weeks and 10 weeks post-infected mice respectively. *S. japonicum* eggs were isolated from infected mouse intestines by a digestion method using actinase E (No. 122, Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) and collagenase (C6885, Sigma–Aldrich, St. Louis, MO). Miracidia were collected after the eggs were hatched in fresh water. *O. h. nosophora* snails were infected with 5 miracidia and 6 months later were sacrificed to collect both the sporocysts and cercarial stages. The sporocysts were isolated by crushing the snails and separating them from the snail tissues whereas the cercariae were shed from the crushed snails. Another batch of *O. h. nosophora* snails were infected with 100 miracidia and were sacrificed 10 days later for histological sectioning. Tissues from non-infected snails were also taken as samples.

Recombinant Sj7TR protein (rSj7TR) was prepared as previously described [1,13]. Polyclonal antibody was produced using male ICR mice immunized intraperitoneally each with 100 μl of the recombinant rSj7TR coupled with complete Freund's adjuvant (Sigma–Aldrich, St. Louis, MO) for the first immunization and incomplete Freund's adjuvant (Sigma–Aldrich, St. Louis, MO) for the subsequent booster immunization. The rSj7TR was administered at 2 weeks intervals and blood samples were collected before and after the immunization. The presence of specific antibodies was detected via ELISA. Anti-rSj7TR IgG was purified using MAbTrap Kit (GE Healthcare, Waukesha, WI). Non-specific mouse IgG was also purified in the same manner from a non-immunized ICR mouse serum to serve as negative control.

Immunolocalization was done to know the distribution of Sjp_0110390 in different stages of *S. japonicum*. Two types of protocols for indirect immunofluorescent antibody test (IFAT) were used in this study namely: (1) for the intra-mammalian stages of *S. japonicum*, Qdot nanocrystals 655 (Quantum Dot Corp., Hayward, CA) conjugated anti-rSj7TR was used producing red fluorescence; and (2) for the aquatic/intra-molluscan stages, non-conjugated anti-rSj7TR and a secondary antibody Alexa Fluor 488 conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) producing green fluorescence was utilized. The negative controls used were Qdot 655 conjugated non-specific mouse IgG for the first protocol and just the secondary antibody for the latter. Conjugations of anti-rSj7TR and non-specific mouse IgG with QDot 655 were done according to the product instructions and confirmed by incubating and binding them with Protein G agarose (Boehringer Mannheim, Germany) producing fluorescence. Stages from the mice were treated with RPMI 1640 (Sigma–Aldrich, St. Louis, MO) and 10% fetal bovine serum (Filtron Pty Ltd., Victoria, Australia) overnight at 37 °C to remove the host antigens. For the IFAT samples, either the whole parasite (miracidium, sporocyst, cercaria, schistosomula, juvenile adult, eggs), frozen section (adult, eggs, infected snails, normal snails) or paraffin section (juvenile adult, adult, infected snail) were used. For staining whole parasite bodies, the samples were fixed in a mixture of 50% methanol and 50% acetone for 20 min at −20 °C. After washing with phosphate buffered saline (PBS) containing 0.05% Tween 20 (T-PBS), blocking was done with 1% bovine serum albumin in T-PBS for 30 min at room temperature (RT). The samples then were treated with anti-rSj7TR for 1 h at RT and washed three times with T-PBS. For the protocol using the non-conjugated anti-rSj7TR, additional incubation with the secondary antibody was done for 1 h at RT and then washed again three times with T-PBS. Anti-rSj7TR was diluted 1:100 with PBS for both protocols while 1:200 for the secondary antibody. For the snail samples and adult schistosomes, nuclear staining was done by incubating it with 2 μg/ml Hoechst 33342 (Molecular Probe, Eugene, OR) for 15 min at RT. IFAT smears were observed using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan).

Fig. 1A shows the localization of Sjp_0110390 in the intra-mammalian stages of *S. japonicum*. Sjp_0110390 was seen localized on the ventral (VS) and oral sucker (OS) of the schistosomulum (SS) whereas it was not found in the juvenile adults (JA) or in the well-developed adults (DA). However, cut sections of the schistosome egg revealed the presence of Sjp_0110390 inside (EG). Sjp_0110390 has been proven to work against human sera for antibody detection against *S. japonicum* [1]. This mechanism might be explained by the expression of the protein on the schistosome eggs which are exposed to the host immune system once they are expelled by the gravid female and carried through the bloodstream.

In Fig. 1B, expression of Sjp_0110390 in the aquatic and intra-mammalian stages of the parasite were shown. Sjp_0110390 was seen concentrated in the apical gland/secretory duct (AG/SD) of the miracidium (MC). This might have contributed to the invasion processes of the parasite into the intermediate host snail. It will be interesting to know whether Sjp_0110390 has a function in the invasion of *S. japonicum* into the snail or in the development of the parasite inside the intermediate hosts.

Section of infected snails showed that Sjp_0110390 was highly concentrated on the parasite (Sj) inside the snail tissues (IOH) as compared to the normal snail section (NOH). Snail infection surveillance is one of the dim areas in schistosomiasis control program. Currently, snail infection was mainly determined by cercarial shedding or snail crushing method to reveal the presence of the cercaria in the intermediate hosts [14]. Recent technologies proved that polymerase chain reaction and loop-mediated isothermal amplification assay can be used to detect snail *S. japonicum* infection [15]. The strong expression of Sjp_0110390 in the infected snail may prove that this protein can be used as a target antigen for diagnosing snail infection. This protein can be a candidate antigen in the development of immunochromatographic test that can rapidly detect snail infection directly on endemic sites. Snail surveillance is a neglected integral part of schistosomiasis control program and improving this will therefore hasten the possible elimination of schistosomiasis. Accurate information on snail infection will definitely help in designing cost-effective control interventions other than mass drug administration.

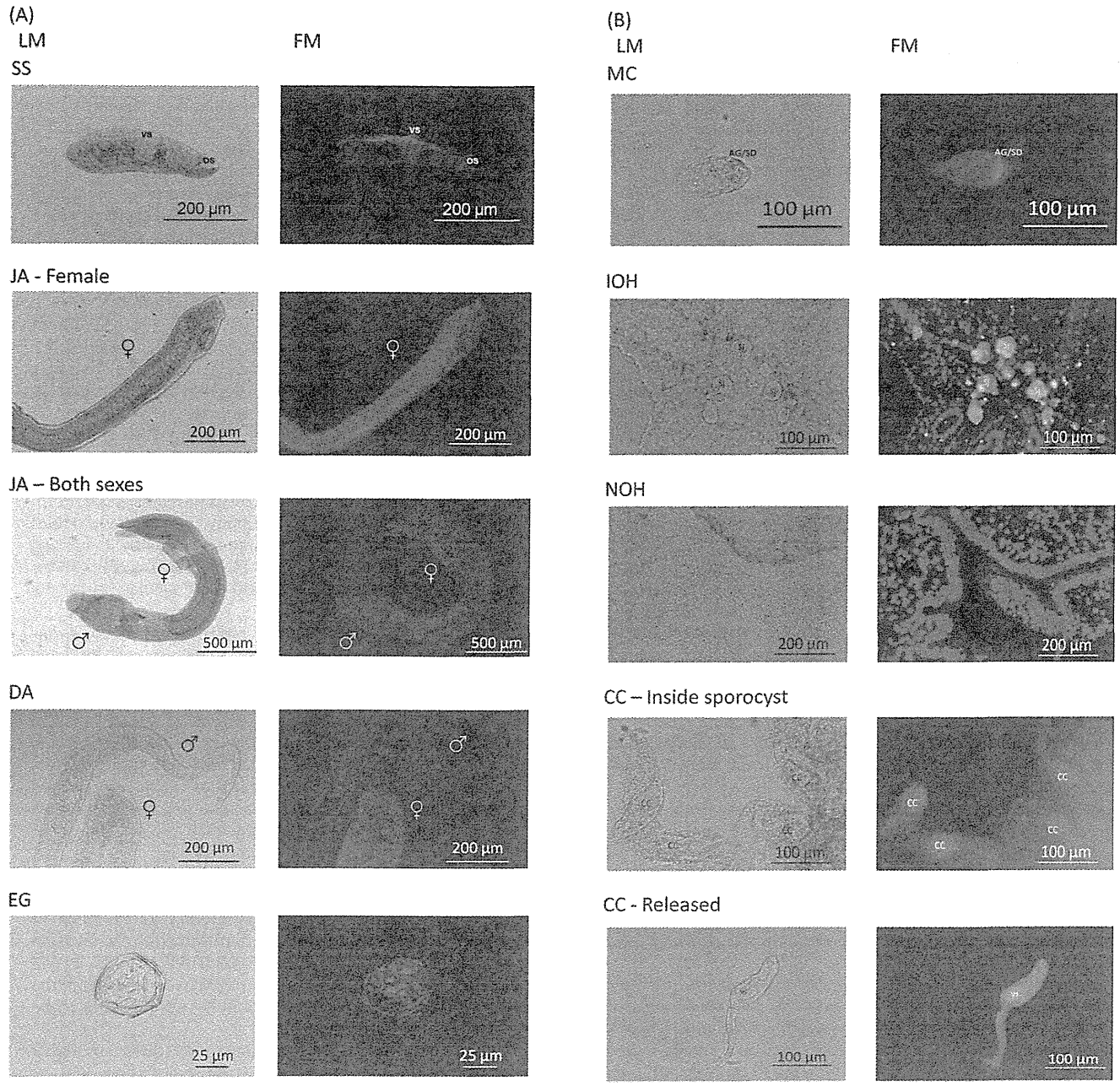

Fig. 1. Immunolocalization of Sjp_0110390 in different stages of *Schistosoma japonicum* (A) intra-mammalian stages of *S. japonicum*. Schistosomulum (SS), juvenile adults (JA), well-developed adults (DA) and egg (EG) were shown in light (LM) and fluorescence microscopy (FM). The samples were stained with QDot-conjugated anti-Sj7TR (red fluorescence) and Hoechst stain (blue fluorescence). (B) Aquatic/intra-molluscan stages of *S. japonicum*. Miracidium (MC), the infected *Oncomelania hupensis nosophora* tissues (IOH), the normal snail tissue (NOH) and cercariae (CC) were shown in light and fluorescence microscopy. The samples were stained with QDot-conjugated anti-rSj7TR (red fluorescence) or anti-rSj7TR/Alexa Fluor 488 (green) and Hoechst stain (blue). AG/SD, apical gland/secretory duct. OS, oral sucker. VS, ventral sucker. Sj, *S. japonicum*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The cercaria inside the sporocyst sac and the released cercariae (CC) revealed the localization of Sjp_0110390 on the ventral sucker (VS) of the parasite. Furthermore, the parasite or the snail tissues used as negative controls did not react either with the Qdot 655 conjugated non-specific mouse IgG or with the secondary antibody alone (data not shown).

Reverse transcriptase (RT-PCR) was done to confirm the expression of the gene on all the stages of the parasite. Total RNA from the schistosome eggs, schistosomula, adult parasites and *S. japonicum* infected *O. h. nosophora* containing the intra-molluscan stages of the parasite were extracted using TRIzol (Invitrogen, Carlsbad, CA) and treated with DNase I (Takara, Otsu, Japan) to degrade any DNA contamination. Non-infected *O. h. nosophora* was also used as a negative control for the snail intermediate host. RT-PCR was done using PrimeScript™ High Fidelity RT-PCR kit (Takara, Otsu, Japan) following the manufacturer's protocol with primer pair 5′-ATG CCT GCT GAAC-3′ and 5′-TAA ACT AGG TAG GAT ATT ACT TAGT-3′. Gel electrophoresis of the PCR products showed that a band was seen at approximately 700 bp from the infected snail, schistosome egg and schistosomula confirming the presence of Sjp_0110390 on

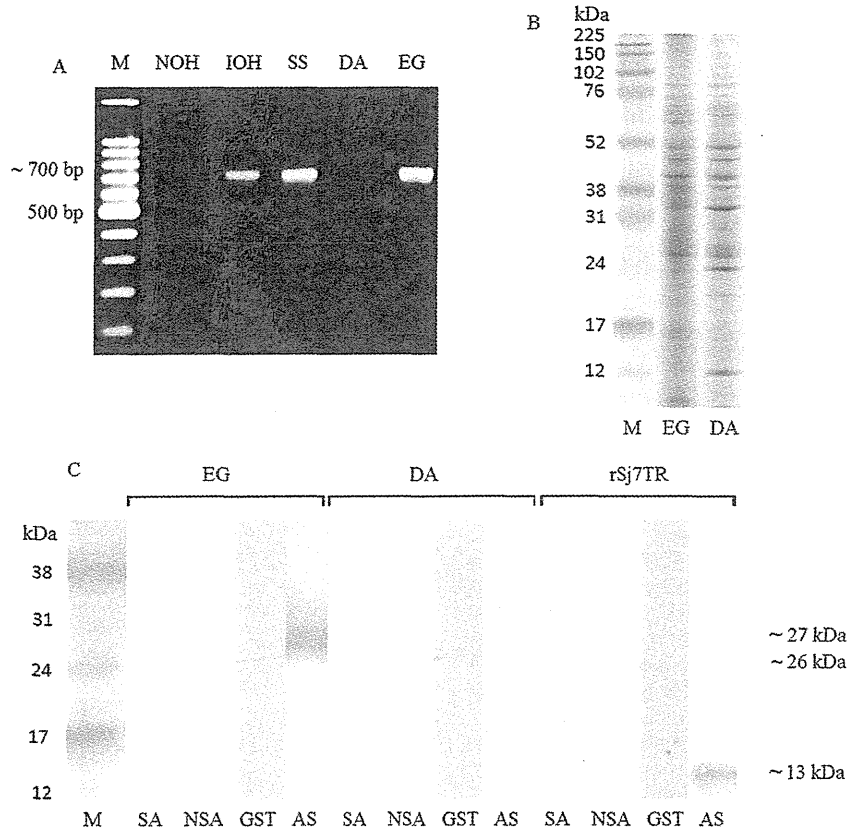

Fig. 2. Gel electrophoresis of reverse transcriptase-PCR products, polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of Sjp_0110390 in different stages of *Schistosoma japonicum*. Gel electrophoresis (A) shows the presence of cDNA band at ~700 bp in the *S. japonicum*-infected *Oncomelania hupensis quadrasi* snail (lane IOH), schistosomula (lane SS) and schistosome eggs (lane EG) but none in the non-infected snail (lane NOH) and adult parasites (lane DA). SDS-PAGE (B) showed the fractionated proteins of the schistosome egg extract (lanes EG) and the adult parasite extract (lanes DA), M, marker. (C) These extracts and the recombinant Sj7TR (lanes rSj7TR) were transferred onto a PVDF membrane and reacted individually with anti-rSj7TR antisera (AS), anti-GST polyclonal antibody (GST), non-specific mouse IgG (NSA) and anti-mouse IgG (SA).

these stages while none on the adult parasite and the non-infected snail (Fig. 2A). Sequencing was then done to verify the identity of the cloned sequences using the ABI Prism 100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA).

Western blotting using anti-rSj7TR antisera was performed to examine the expression levels of Sjp_0110390. Sodium dodecyl sulfate (SDS) extraction was done for both *S. japonicum* eggs and adults. The eggs and adults were homogenized in SDS buffer with a Potter-Elvehjem homogenizer. The homogenates were incubated at 100 °C for 5 min and centrifuged at 10,000 × *g* for 20 min at 4 °C. The SDS protein extract of both the eggs and adult were collected and fractionated on 15% SDS-polyacrylamide gel. The extracts were electrically transferred onto polyvinyliden difluoride (PVDF) membranes (Immobilon, Millipore, Billerica, MA). Blocking was done with 2% skim milk in T-PBS for 1 h, and then the membranes were incubated with anti-rSj7TR diluted 1:50 with the blocking buffer for 1 h. After washing with T-PBS, the membranes were incubated with the secondary antibody HRP-conjugated anti-mouse IgG (Southern Biotechnology Associates, Birmingham, Al) at 1:200 dilution for 1 h. Specific binding of anti-rSj7TR was detected using 3,3-diaminobenzidine (Merck, Darmstadt, Germany) as the substrate. PVDF membranes containing the adult and egg extract proteins incubated with the non-specific mouse IgG and the secondary antibody separately served as negative controls and with the mouse-derived polyclonal antibody against *S. japonicum* glutathione S-transferase (GST) as a positive control.

Immunoblotting result showed the presence of a band in the infected snail, schistosomula and egg protein extract (Fig. 2C) whose molecular sizes is found to be approximately 27 kDa. This result also coincides with the RT-PCR result showing a band at approximately 700 bp. According to the database, the expected size of the protein is 31 kDa [16] but based on the Western blot, the protein was expressed in nature lower than the expected molecular size. This was further confirmed by the sequencing done on the Sjp_0110390 cDNA showing that instead of having five repeats, it just possessed four repeats which made the protein smaller than expected (Supplementary Fig. 1). Tandem repeat analysis of this sequence revealed 4.2 copies of the 165 bp-long repeat unit [8] lower than that in the previous analysis of the sequence from the database. These differences might be due to strain differences of the parasites used (Yamanashi strain) against the one used in the whole genome sequence available online (Anhui strain). On the other hand, the recombinant Sj7TR showed a band with the molecular size of 13 kDa as expected. Furthermore, no bands were seen on the negative controls used while an approximately 26 kDa band was seen for the anti-GST positive control as expected on both the adult and egg lysates. The Western blot result is consistent with that of the IFAT and RT-PCR showing that Sjp_0110390 was not expressed in the adult stage of the parasite.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2012.12.002.

In conclusion, Sjp_0110390 was expressed mainly at the young stages of the parasite including the eggs, aquatic and intramolluscan stages and the schistosomula. However, it was not expressed on the adult forms of the parasite. Interestingly, the

significant antibody titer against Sj7TR found among *S. japonicum* infected individuals in the previous study [1] may be caused by the parasite's eggs or by continuous exposure to the cercaria but not from the adults. The titer value therefore might somehow be related to the number of eggs dislodged in the host's tissues eliciting immune response. It is known that the main inflammatory response seen on infected humans was caused by the schistosome egg deposition resulting to a strong egg antigen-specific cell-mediated granulomatous reaction [17].

It was also shown that the antigen was localized in the developing suckers of the parasite suggesting that it might be important in the feeding mechanism of the young schistosomes. However, the role and function of Sjp_0110390 in the young stages should be further studied through RNA interference techniques [18–21] to fully characterize the protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2012.12.002.

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