Studies on the evaluation of potential antigens for antigen and antibody-based serodiagnostic assays for *Schistosoma japonicum* infection

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抗原及び抗体の検出に基づく血清診断法の開発のための日本住血吸虫抗原の評価に関する研究

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Abbreviations

AWA	adult worm antigen
DAS-ELISA	double-antibody sandwich enzyme-linked immunosorbent assay
HAT	hypoxanthine-aminopterin-thymidine
HsPrx-1	*Homo sapiens* peroxiredoxin-1
mAb	monoclonal antibody
MDA	mass drug administration
MmPrx-1	*Mus musculus* peroxiredoxin-1
NTD	neglected tropical disease
pAb	polyclonal antibody
POCT	point of care test
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	soluble egg antigen
SjCatB	*Schistosoma japonicum* cathepsin B
SjTPx-1	*Schistosoma japonicum* thioredoxin peroxidase-1
SmCCA	*Schistosoma mansoni* circulating cathodic antigen
WHO	World health organization

General Introduction

1. Schistosomiasis

Schistosomiasis is a serious debilitating disease found in the tropical and subtropical regions of the world (Ross *et al.,* 2002; Mitra *et al.,* 2017). Thus far, the disease has been reported in 78 countries with current official estimates suggesting that approximately 240 million people affected; of these, approximately 120 million people show symptoms while 20 million people suffer from severe illness (WHO, 2017; Chitsulo *et al.,* 2000). In the tropics, it is considered to be the second most devastating parasitic disease after malaria (CDC, 2012). Several species belonging to the genus *Schistosoma* are known to infect humans, including: *S. haematobium, S. mansoni, S. mekongi, S. intercalatum* and *S. japonicum.* The World Health Organization (WHO) has designated schistosomiasis a neglected tropical disease (NTD) and aims to seek its active control. Three of the previously mentioned species, namely, *S. haematobium, S. mansoni* and *S. intercalatum* are found in sub-Saharan Africa, while *S. mansoni* is the main species found in Brazil, Venezuela and the Caribbean. *S. japonicum* is the main species found in Asia, including China, the Philippines and parts of Indonesia. The closely related species *S. mekongi* is only found in Cambodia and Laos along the Mekong River (Chitsulo *et al.,* 2000). Among all of the species causing human schistosomiasis, *S. japonicum* is the most pathogenic due to the large number of eggs that it lays (approximately 3500 eggs/female/day) (Mahmoud, 2001).

2. *Schistosoma japonicum*

S. japonicum was first discovered in Japan in 1904 by Dr. Fujiro Katsurada who recorded an accurate description of the parasite (Despommier *et al.,* 1995). Initially, the eggs, which are passed out from the feces of an infected host, subsequently hatch into free-swimming miracidia. At this stage, the parasite actively seeks to infect the semi-aquatic intermediate snail hosts: *Oncomelania hupensis hupensis* in China and *O. h. quadrasi* in the Philippines. Inside the snail, the parasite undergoes clonal replication and develops into numerous cercariae, the infective stage of this parasite (Mahmoud, 2001).

The ability of *S. japonicum* to infect several mammalian species, including domestic animals, sets it apart from other *Schistosoma* species that are infective to humans (Mahmoud, 2001). Animals in close association with humans, such as cattle, water buffaloes, dogs, and pigs, are the most susceptible to infection (He *et al.,* 2001). This has made the control of the disease particularly complicated. Infection with *S. japonicum* is characterized by marked hepatic portal fibrosis, which leads to ascites (Mahmoud, 2001). Aberrant eggs may also cross the blood brain barrier causing seizure, as in the case of cerebral schistosomiasis. However, the precise disease mechanism is not fully understood (Imai *et al.*, 2011). The huge number of eggs laid by a single female contributes to the higher potential of this disease to spread in the environment and cause pathologies associated with the localization of deposited eggs in vital organs such as the liver, spleen and brain. Furthermore, the large number of eggs produced might cause a more widespread contamination of the environment (Mahmoud, 2001).

3. Serodiagnosis

Serodiagnostic methods use serum samples to detect potential pathogenic infection. Several distinct serodiagnostic assays have been evaluated in the detection of different parasitic infections, including *S. japonicum* infection. Serodiagnostic assays can be divided into two main categories based on the target molecule: antigen-based and antibody-based serodiagnostic assays.

In an antigen-based detection assay, specific antibodies are raised against a target antigen. This antigen is "captured" by the antibodies which can in turn be detected enzymatically (Schmidt *et al.,* 2012). The advantage of detecting parasite-derived antigens is the ability to indicate the true infection status as well as the response to chemotherapy (Zhou *et al.,* 2010). The two most studied and commonly targeted circulating antigens are the circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) of *S. mansoni*. Currently, the only field applicable antigen-based test for schistosomiasis is a point-of-care-test (POCT) based on the CCA of *S. mansoni* (van Dam *et al.,* 2004). This test has been successfully applied in numerous endemic areas in Africa and South America where *S. mansoni* and its closely related species *S. haematobium* are present. However, this test had low sensitivity and specificity in the detection of Asian schistosomiasis, where infection is mainly caused by *S. japonicum* (van Dam *et al.,* 2015). This drawback has led to the evaluation of further potential antigen targets from the excretory and secretory products of *S. japonicum* (Gao *et al.,* 2016; Ren *et al.,* 2017).

In contrast to antigen-based tests, antibody-based serodiagnostic assays detect specific antibodies against a particular component of the parasite. This method mostly utilizes crude antigens derived from the parasite. The use of soluble egg antigen (SEA) or adult worm antigen (AWA) has been proven to be sensitive in the diagnosis of *S.*

japonicum infection (Weerakon *et al.,* 2015). However, the use of crude antigens can lead to cross-reaction with other trematode species and geohelminths infections, making it irreproducible in most cases (Yu *et al.,* 2007; Zhou *et al.,* 2010). It is preferable to perform the detection of antibodies using a specific purified antigen derived from the parasite itself as a target (Zhou *et al.,* 2010). Angeles *et al.,* 2011, 2012 identified TPx-1 as a good antigen for the antibody-based detection of *S. japonicum* infection in humans and water buffaloes.

The benefit of using purified recombinant antigens—as opposed to crude antigens—in the mass screening of humans and animals has made it a useful tool for evaluating the prevalence in regions with low endemicity in which patients are known to have a low burden of infection (Tsang *et al.,* 1997; Al-Sherbiny *et al.,* 1999). In addition, the test can also be suitable in areas in which infection has reemerged after an apparently effective control program (Ross *et al.,* 2001). Antibody detection is useful in cases involving patients suffering from acute infection when no eggs are detected in their urine or feces.

The detection of parasite circulating antigens provides informative results, as it can indicate the true status of infection and the response to curative treatment. Thus, a better treatment regimen and a more accurate estimation of the disease prevalence in endemic areas is expected. In addition, a highly specific and sensitive antibody-based serodiagnosis would be a valuable tool in the assessment and screening of individuals coming from endemic areas and for monitoring travelers potentially exposed to the disease. The benefits identified for both tests may complement the weakness of each other in terms of monitoring disease transmission. A recent study performed by Cai *et al.* (2014) supports the use of both tests in order to maximize their diagnostic potential.

Table 1. Comparison between antigen and antibody-based serodiagnostic assays.

Antigen-based serodiagnostic assay		Antibody-based serodiagnostic assay	
Advantage	Disadvantage	Advantage	Disadvantage
Can identify active infection	Antigens may fluctuate causing false negatives	Highly sensitive	Cannot distinguish ongoing and past infections in general
Can identify response to cure	Low sensitivity in areas with low endemicity	Can be applied in areas with low endemicity	Cannot identify response to cure in general

4. Objectives of the present study

The general objective of the present study was to evaluate potential antigen targets that could be used as biomarkers of *S. japonicum* infection. Identifying good antigens for both antigen and antibody-based diagnostic assays would allow for the more accurate diagnosis of *S. japonicum* infection and would address the pitfalls of the diagnostic methods that are currently available. It is envisioned that this would help in the surveillance efforts that are in place in endemic areas. The determination of the true status of infection as well as past exposure could result in the more efficient administration of mass drug administration (MDA). Thus, the specific objectives of the present study were: (1) to evaluate the potential application of *S. japonicum* thioredoxin peroxidase-1 as an antigen target for the antigen-based diagnosis of *S. japonicum* infection; and (2) to determine the diagnostic value of *S. japonicum* cathepsin B as an early biomarker of infection in an antibody-based format.

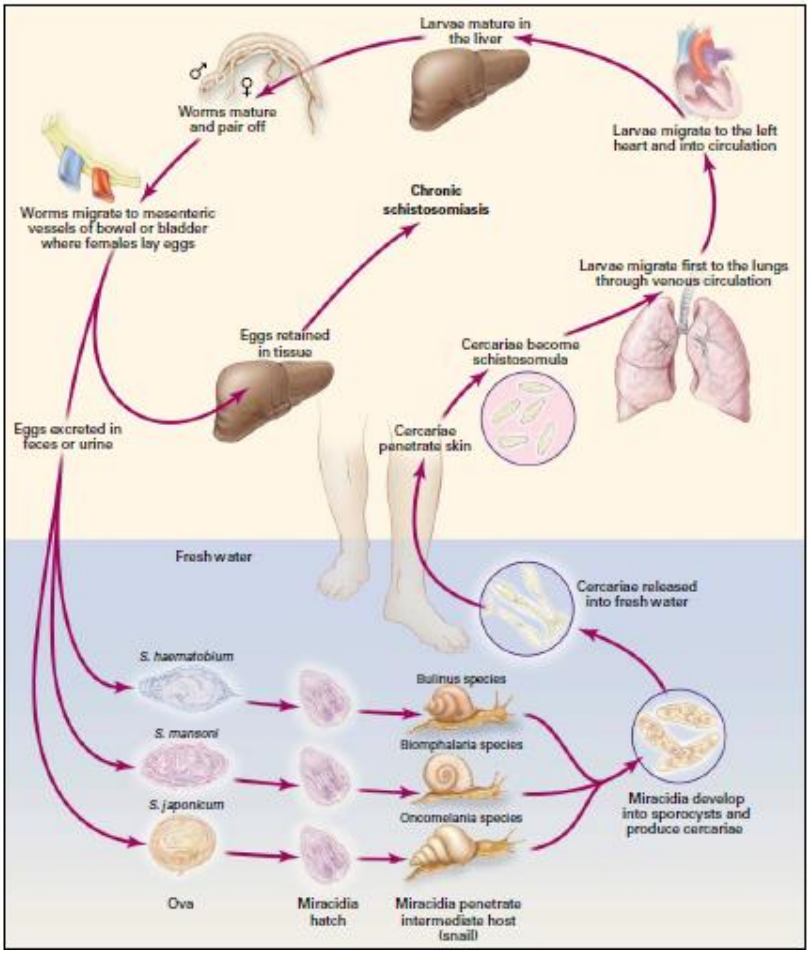

Figure 1. The life cycle of *Schistosoma* spp. (Ross *et al.,* 2002)

Chapter I. Evaluation of *Schistosoma japonicum* thioredoxin peroxidase-1 as a potential circulating antigen target for the diagnosis of Asian schistosomiasis

1.1 Introduction

Schistosomiasis is an NTD that has been reported in 78 countries worldwide; at least 240 million people require preventive chemotherapy for the disease (WHO, 2017). At the present time, Asian schistosomiasis caused by *S. japonicum* remains a public health threat in China, the Philippines and parts of Indonesia. Several decades of employing numerous control measures, including mass drug administration (MDA) with praziquantel, have resulted in a decrease in the prevalence in endemic areas (Leonardo *et al.,* 2002; Ross *et al.,* 2013; Satrija *et al.,* 2015). Stool microscopy remains the only confirmatory test for *S. japonicum* infection. Although highly specific, this test is labor intensive and requires trained personnel; furthermore, this method currently shows decreased sensitivity due to a decrease in the intensity of infection (Lier *et al.,* 2009).

Mass screening of individuals by antibody-based serological assays has been shown to have better sensitivity in comparison to traditional stool examination, making it a promising alternative for diagnosing schistosomiasis (Alarcón de Noya *et al.,* 2007). However, antibodies are known to persist for several years, even after a cure; thus, such methods might not be able to differentiate between past and present infection (Gomes *et al.,* 2014). On the contrary, an antigen-based diagnosis can definitely detect present infection. Since circulating antigens are released by living parasites, the decrease in the

number of adult worms upon treatment would result in a decrease in the antigen levels in the blood of the host, indicating the response to drug therapy (Gomes *et al.,* 2014).

At present, CCA POCT has been developed based on the CCA of *S. mansoni* (van Dam *et al.,* 2015). This test has been successfully applied in numerous endemic areas in Africa (Ochodo *et al.,* 2015). Conversely, this does not remain true in Asian schistosomiasis where the POCT has been evaluated in a proof-of-concept study (van Dam *et al.,* 2015). The current need for a specific antigen-based serodiagnosis has led to the evaluation of several antigen targets from the excretory and secretory products of the parasite, which have been shown to be potential diagnostic biomarkers for schistosomiasis (Gao *et al.,* 2016; Ren *et al.,* 2017; Sangfuang *et al.,* 2016).

The present study evaluated the applicability of *S. japonicum* thioredoxin peroxidase-1 (SjTPx-1) as a biomarker for Asian schistosomiasis. SjTPx-1 has multiple biological functions; however, its main function is as a key enzyme that combats reactive oxygen species (Kumagai *et al.,* 2006). Immunohistochemistry demonstrated the presence of SjTPx-1 in all life stages of *S. japonicum*, as well as its extensive distribution on the surface tegument of the parasite and the tissues surrounding the eggs in the liver (Kumagai *et al.,* 2006; Mulvenna *et al.,* 2010a). Additionally, this enzyme was recently identified as a component of the excretory and secretory products of the adult worm (Gao *et al.,* 2016; Liu *et al.,* 2009). Thus, SjTPx-1 is a promising candidate for an antigen-based diagnosis.

Our previous studies on the use of SjTPx-1 as a biomarker for an antibody based ELISA showed good diagnostic capability in humans and water buffaloes (Angeles *et al.,* 2011; Angeles *et al.,* 2012). Although the optical density (OD) values of an enzyme-linked immunosorbent assay (ELISA) declined to negative levels after treatment

(unpublished data), the detection of both SjTPx-1 (as an antigen) and its specific antibody may help to confirm the active stage of infection in patients and parasite-infected animals. Thus, the present study evaluated the potential of using SjTPx-1 as a target antigen for the diagnosis of *S. japonicum* infection. Here, a double-antibody sandwich ELISA (DAS-ELISA) was used to detect the presence of this antigen in serum samples from experimentally infected animals and patients with stool-confirmed infection from an endemic area in the Philippines.

1.2 Materials and methods

Ethics statement. The collection of serum samples from the patients in Sorsogon, the Philippines was carried out in compliance with the ethical clearance sought from the University of the Philippines, Manila (clearance no: UPM REB 2011-098). The animal experiments in the present study were carried out in compliance with the guidelines for animal experimentation at Dokkyo Medical University and Obihiro University of Agriculture and Veterinary Medicine (permission numbers: 0006 and 28-30).

Serum samples. Serum from a Japanese white rabbit (Icl strain; Ichikawa-ya, Chiba, Japan) percutaneously infected with 2,000 cercariae was collected at 11 weeks after cercarial challenge. In addition, serum samples from ten experimentally infected ICR mice (Jcl strain; Clea Inc., Tokyo, Japan) percutaneously infected with 40 cercariae were collected at 8 weeks after cercarial challenge. A total of 417 adult worm pairs were recovered from the infected rabbit and 2-6 adult worm pairs (average=5) were recovered from the infected mice. For the human clinical cases, serum samples from 10 individuals were collected when the stool samples were collected for microscopic examination of *S. japonicum* infection. These samples were collected from Sorsogon,

the Philippines. Serum samples from 7 non-endemic volunteers from the United States (US) were used as negative controls.

Cloning, expression and purification of recombinant SjTPx-1. Cloning, expression and purification of recombinant SjTPx-1 (rSjTPx-1) were carried out as previously described with some modifications (Angeles *et al.,* 2011). Briefly, first-strand synthesis of complementary DNA (cDNA) was performed using PrimeScript™ (Takara Bio. Inc., Otsu, Japan). The following primers were used for the polymerase chain reaction (PCR) amplification of the double-strand DNA: forward, 5'-GC GGA TCC ATG GTA CTG ATT CCA AAT-3'; reverse, 5'-GC CTC GAG TAA TCA GTG ATT CAC TTT-3' (with *Bam* HI/*Xho* I as their restriction sites [underlined]). The DNA fragment was cloned into pET-28a (+) vector (Novagen, Madison, WI, USA) for the expression of the recombinant protein with 6xHis-tag on the N terminal in *Escherichia coli* (BL21) cells (Takara Bio, Inc.). rSjPTx-1 was purified with an affinity chromatography nickel nitrilotriacetic acid (Ni-NTA) protein purification kit (Qiagen, Hilden, Germany) and dialyzed against cold phosphate buffered saline (PBS) (pH 7.4) prior to use. The recombinant protein was quantified using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). The purity of rSjTPx-1 was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Cloning, expression and purification of recombinant mouse Prx-1. Total RNA was extracted from 50 mg of mouse liver tissue by homogenization in 1 ml TRIzol® reagent (Life Technologies, Inc., Frederic, MD, U.S.A.). The complete coding sequence of mouse Prx-1 (MmPrx-1), which is the mouse orthologue for SjTPx-1 was PCR-amplified from the cDNA synthesized using PrimeScript™, as previously described (Lee *et al.,* 1999). The following primers were used for the polymerase chain reaction (PCR) amplification of the double-strand DNA: forward, 5'-GC GGA TCC

ATG TCT TCA GGA AAT GC-3'; reverse, 5'-GC AAG CTT TCA CTT CTG CTT AGA GAA AT-3' (with *Bam* HI/*Hin* dIII as their restriction sites [underlined]). The PCR product was cloned into a pET-28a (+) vector. Prior to the transformation of *E. coli* (BL21) cells for the expression of the recombinant protein, sequencing was performed to confirm its identity with the database-deposited sequence. Recombinant MmPrx-1 (rMmPrx-1) was expressed and purified as previously described (Angeles *et al.,* 2011). The protein concentration was measured using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific) while the purity of rMmPrx-1 was assessed by SDS-PAGE.

Production of capture and detection antibodies. Hyperimmune rabbit serum against rSjTPx-1 was produced by Wako pure chemicals (Wako Inc., Osaka, Japan). Briefly, 300 mg of rSjTPx-1 was injected subcutaneously into an 8-week old Japanese white rabbit. Booster injections were administered twice at two-week intervals and the total serum was collected at 12 weeks post-immunization. IgG from the rabbit serum was purified by affinity chromatography using a MAbTrap™ antibody purification kit (GE Healthcare Bio-Sciences KK, Tokyo, Japan). Eluted IgG was desalted using a PD-10 sepharose column (GE Healthcare) with PBS (pH 7.4). The IgG concentration (polyclonal antibody: pAb) at 280 nm was spectrophotometrically measured using a NanoDrop 2000® (Thermo Scientific, Wilmington, DE, USA), while the purity of pAb was assessed by SDS-PAGE.

For the production of monoclonal antibodies (mAbs) an 8-week old female BALB/c mouse was immunized with 100 µg of rSjTPx-1 with the adjuvant TiterMax Gold® (Sigma-Aldrich, St. Louis, MO, USA). Two booster subcutaneous injections were given at two-week intervals. The final booster of 50 µg of antigen in PBS was intravenously administered three days prior to the harvesting of the spleen. Splenocytes

were fused with SP2/0 myeloma cells and selection was performed in methylcellulose semi-solid media, as previously described (Davis *et al.,* 1982; Hakimi *et al.,* 2015). Hybridomas secreting mAbs were screened for their reactivity against rSjTPx-1 by an indirect ELISA and further confirmed by Western blotting. The isotypes of the mAbs were determined using an IsoStrip™ mouse mAb isotyping kit (Roche Diagnostics, Mannheim, Germany). Three hybridoma clones were selected for their stability and amount of secreted antibodies. These clones were expanded and IgG from the culture supernatant was purified using a MAbTrap™ purification kit and desalted in a PD-10 desalting column. The concentrations of the IgGs (mAbs) at 280 nm were determined by a NanoDrop 2000® spectrophotometer and their purity was evaluated by SDS-PAGE.

Western blotting. The specificity of mAbs against SjTpx-1 was determined by reacting them with rSjTPx-1, rMmPrx-1 and recombinant human Prx-1 (rHsPrx-1) (purchased from Sino Biological Inc., Beijing, China) in Western blotting, as previously described with some modifications (Angeles *et al.,* 2013). Briefly, 200 ng of the recombinant proteins were resolved by SDS-PAGE and the polypeptides were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the mAbs and the antigen-antibody reactions were detected by secondary antibody against mouse IgG conjugated with horseradish peroxidase (HRP) (GE Healthcare). The enzymatic activity was visualized by chemiluminescence using SuperSignal™ (Thermo Fisher Scientific) and an ImageQuant LAS 500 chemiluminescence detection machine (GE Electric, Tokyo, Japan). The reactivity of the antibodies against native SjTPx-1 was also evaluated by Western blotting. Adult worm lysate was prepared from 15 *S. japonicum* adult worms, as previously described (Angeles *et al.,* 2013) and resolved by SDS-PAGE. The polypeptides were transferred onto a PVDF membrane and reacted with rabbit pAb and mouse mAb in a separate

reaction chamber. The antigen-antibody reaction was detected by either HRP-conjugated anti-rabbit IgG or anti-mouse IgG antibodies. The enzymatic activity was visualized by chemiluminescence, as described previously.

DAS-ELISA. The optimal concentrations of capture and detection antibodies were determined by checkerboard titration. Each well of a 96-well polystyrene plate (Thermo Fisher Scientific) was coated with 100 µl of 8, 4, 2 and 1 µg/ml of rabbit pAb as a capture antibody in carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. The wells were washed three times with PBS to remove unbound antibodies. The wells were then blocked by 5% skim milk in PBS at 37°C for 1 h and washed three times with PBS. Then, 100 µl of rSjTPx-1 diluted in PBS at a concentration of 2 µg/ml was delivered into the wells as a positive control while PBS was used as a negative control. The wells were incubated for 1.5 h at 37°C then washed four times with PBS. One hundred microliters of detection antibody (anti-SjTPx1 mouse mAb) was applied to each well at concentrations of 8, 4, 2 and 1 µg/ml, respectively. The wells were incubated for 2 h at 37°C and washed to remove unbound antibodies. Then, 100 µl of HRP conjugated anti-mouse IgG antibody at a dilution of 1:10,000 was added to the wells. The wells were incubated for 1 h at 37°C then washed three times to remove any unbound antibodies. For the enzymatic reaction, 100 µl of tetramethylbenzidine (TMB) (KPL Inc., Gaithesburg, MD, USA) was added to the wells and the reaction was allowed to proceed for 8 min. The reaction was stopped by adding 50 µl of 1 M phosphoric acid to the wells. The absorbance at 450 nm was measured with a microplate reader (Corona electric, Ibaraki, Japan). The optimal concentrations of capture pAb and detection mAb were determined as the combinations that yielded the highest positive to negative ratio. Following the optimization of the antibody concentrations, different concentrations of SjTPx-1 antigen were tested with a DAS-ELISA in order to determine the smallest

amount of antigen that could be detected. The detection limit for SjTPx-1 by the DAS-ELISA was determined with the recombinant protein at concentrations of 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1}, 10^{-2} and 10^{-3} ng/ml in PBS. Serum samples collected from experimentally infected animals and stool-confirmed patients were tested with the DAS-ELISA. The cut-off value for the patient samples was computed as the mean OD reading of the negative controls (n = 7) plus 2 times the standard deviation (SD) of the mean OD readings. Prior to the application of the DAS-ELISA, all serum samples were pretreated with trichloroacetic acid (TCA), as previously described (De Jonge *et al*., 1987).

Statistical analysis. Student's *t*-test was performed to determine the statistical significance of the results. The GraphPad Prism software program (v 5.00, GraphPad Software Inc., La Jolla, CA, USA) was used to perform the statistical analyses.

1.3 Results

Cloning, expression and purification of recombinant SjTPx-1 and MmPrx-1 in *E. coli*. The complete coding sequence of SjTPx-1 and MmPrx-1 was amplified by a PCR from the cDNA prepared from adult worms and mouse liver respectively. The complete coding sequence of SjTPx-1 was ~555 bp in size while the coding sequence for MmPrx-1 was ~600 bp (data not shown). After TA cloning and sequencing, their identity with the database-deposited sequences were confirmed (Sjp_0095720 and AAH86648.1). rSjTPx-1 and rMmPrx-1 were expressed in *E. coli* BL21 strain and were purified (Fig. 2A and 2B). The amino acid (AA) sequence of SjTPx-1 and that of mammalian orthologous Prx-1 showed considerable identity. The AA sequence of *S. japonicum* TPx-1 shared 61.95% and 63.58% identity with mouse Prx-1 and human Prx-1, respectively (Fig. 5).

The production of capture and detection antibodies. Rabbit pAb and mouse mAbs against rSjTPx-1 were produced. For mAbs, a total of seven hybridoma clones were developed and the IgG sub types were identified (Table 2). Based on the stability and antibody production ability, three clones (A1F7, A1F11 and A1G7) were chosen. Among these clones, A1F7 was further selected to be used as a detection mAb in the DAS-ELISA because of its high secretion of mAb (data not shown). The purity of the antibodies was assessed by SDS-PAGE (data not shown).

Specificity of the antibodies. Equal amounts of rSjTPx-1, rMmPrx-1 and rHsPrx-1 were resolved by SDS-PAGE and transferred onto PVDF membranes for Western blotting. The equal loading of samples was confirmed by anti 6x-his tag mAb (Fig. 3A); the bands seen at 50 kDa were the dimeric form typical of a 2-Cys Prx-1. The specificities of the three mAbs against rSjTPx-1 were verified (Fig. 3B, C and D). Likewise the reactivity of mAb A1F7 and pAb against the native SjTPx-1 was also confirmed (Fig. 3E).

Optimization and evaluation of the DAS-ELISA for SjTPx-1. The optimal concentrations of the capture pAb and detection mAb were determined by checkerboard titration (Table 3). The optimal concentrations of the pAb and mAb were 8 µg/ml and 1 µg/ml mAb when 100 µl of antibody solution per well was used for the DAS-ELISA. The lowest concentration of rSjTPx-1 that could be detected by this combination was 1 ng/ml (Fig. 4A). The OD value recorded in the DAS-ELISA for experimentally-infected rabbit serum was significantly higher ($P \leq 0.001$) than that of the normal rabbit serum (Fig. 4B). Likewise, the OD values recorded for the serum samples from the experimentally infected mice were significantly higher ($P \leq 0.001$) than those of normal uninfected samples (Fig. 4C). The testing of the stool microscopy-positive individuals

revealed that DAS-ELISA could detect circulating SjTPx-1 in 4 of 10 serum samples (Fig. 4D).

1.4 Discussion

The present study evaluated the potential application of SjTPx-1 as an antigen marker for *S. japonicum* infection. Several excretory and secretory antigens have been evaluated as potential biomarkers of *Schistosoma* spp. infection (Cesari *et al.,* 1987; Gao *et al.,* 2016; Ren *et al.,* 2017; Sulbarán *et al.,* 2010). Among these, the detection of CCA in *S. mansoni* has been successfully applied as a field-friendly POCT (van Dam *et al.,* 2004). The advantage of detecting excretory and secretory antigens is that it is beneficial for indicating active infection, the effects of chemotherapy, and disease severity (Gomes *et al.,* 2014). Here, serum was used as a biological sample for the detection of the target antigen. Although the currently available antigen-based POCT for schistosomiasis uses urine as a sample, previous studies on circulating antigens have shown that the use of serum samples for antigen detection yielded superior specificity in comparison to urine samples (Al-Sherbiny *et al.,* 1999; Polman *et al.,* 1995; van Leishout *et al.,* 1995). This might be attributed to the fact that since adult worms inhabit the mesenteric veins, any secreted products derived from the worm are carried through the blood circulation first, prior to other bodily fluids (including urine). It should also be noted that in comparison to CCA, the target antigen (SjTPx-1) has not yet been proven to be present in the urine of the infected host, which leaves an avenue for further studies.

SjTPx-1, a component of a repertoire of proteins excreted and secreted by the adult worm was cloned and expressed as a histidine-tagged fusion protein. This recombinant antigen was used to produce specific antibodies in the DAS-ELISA format: a rabbit pAb (as the capture antibody) and a mouse mAb (as the detection

antibody). Because of the ubiquitous nature of TPx-1, Western blotting was performed to ensure the specificity of the mAb used as the detection antibody to the parasite TPx-1 and to verify that it did not react to the mammalian orthologues represented by recombinant mouse Prx-1 and human Prx-1. Furthermore, these pAb and mAbs were shown to recognize native SjTPx-1 derived from the adult worm, indicating that the recombinant SjTPx-1 is antigenically similar to the native protein. Taken together, these results suggest that both pAb and mAbs produced against rSjTPx-1 were suitable capture and detection antibodies for the DAS-ELISA. Checkerboard titration revealed that the optimal concentrations of the capture pAb and detection mAb were 8 µg/ml and 1 µg/ml, respectively. The serial dilution of the recombinant antigen showed that at least 1 ng/ml of the antigen can be detected by this method. The detection limit of this method is much lower than that of CCA (at 5 ng/ml) and is comparable to the detection limit established for CAA in the diagnosis of *S. mansoni* and *S. haematobium* infection (both 1.1 ng/ml) but not for *S. japonicum* infection (Fillié *et al.,* 1994; Nogueira-Queiroz *et al.,* 1986). Several circulating antigens with detection capabilities comparable to or even higher than our own have also been reported as potential antigens for the diagnosis of Asian schistosomiasis (Fu and Carter, 1990; Gao *et al.,* 2016; Lei *et al.,* 2009; Nibbeling *et al.,* 1988).

The DAS-ELISA was able to demonstrate the presence of the target antigen in all infected rabbit and mouse sera, indicating that SjTPx-1 could be used as a diagnostic biomarker for schistosomiasis in experimentally infected animals. In order to determine the diagnostic potential of SjTPx-1 in human clinical samples, 10 stool-confirmed human serum samples were evaluated, 4 of which had detectable serum levels of the target antigen. This result suggests that the target antigen is present and detectable in the patient serum. The stages of infection in which this antigen can be readily detected

in the serum should be investigated, because in other antigen detection methods, inherent factors—including fluctuation in antigen secretion—have been shown to potentially affect the detectability of the target antigen (Polman *et al.,* 1998). When rSjTPx-1 was used as an antigen in an indirect ELISA, all of the tested serum samples were determined to be positive (data not shown). However, no correlation was found between the circulating antigen level and the antibody level in those serum samples (data not shown). The correlation between the antigen level and the antibody level, which may be useful in evaluating the infection status, should also be investigated in future studies.

As schistosomiasis-endemic countries in Asia, including China, the Philippines and Indonesia, are promoting the elimination of this disease (Olveda *et al.,* 2014; Satrija *et al.,* 2015; Wang *et al.,* 2016), the WHO further intensified its commitment to eliminate NTD by increasing access to preventive chemotherapy for schistosomiasis; their current goal is at least 75% global coverage in 2020. Currently, the POCT is the only available antigen-based serological diagnostic assay for schistosomiasis. The POCT, which is based on *S. mansoni* CCA, has been shown to have suboptimal sensitivity in the detection of *S. japonicum* infection (van Dam *et al.,* 2015; van Dam *et al.,* 2004). The continuous use of the laborious microscopy-based diagnostic test, which shows low sensitivity, may potentially interrupt the efforts put into the elimination of this disease. The development of a test that can identify the true infection status based on parasite-derived molecules will be very useful for screening and for the evaluation of the control measures being implemented for Asian schistosomiasis. The determination of active infection through the detection of circulating cell-free parasite DNA in the serum and urine samples from patients has also been reported (Kato-Hayashi *et al.,* 2015). However, the detection of the DNA is PCR-based, and requires

expensive equipment, which may not be affordable in the rural clinical setting (Weerakon *et al.,* 2015). In contrast, the detection of a circulating antigen using a POCT may be advantageous if it can be introduced in the field and in the rural clinical setting.

In the present study, SjTPx-1 was evaluated and confirmed to be detectable in the serum of experimentally infected animals and human clinical cases. However, additional clarification in relation to the antigen dynamics is required. Points that should be clarified include the identification of peak secretion post-infection and the response with regard to antigenemia after treatment. Furthermore, a greater number of clinical samples should be tested in order to determine the applicability in field setting. The application of the test for urine samples as well as serum samples should also be addressed in a future study. The results of the present study pave the way for the potential use of SjTPx-1 as a candidate circulating antigen for the diagnosis of *S. japonicum* infection.

1.5 Summary

Asian schistosomiasis caused by *S. japonicum* is a serious zoonotic disease that is endemic in China, the Philippines and parts of Indonesia. In the endemic areas, MDA resulted in a decline in the severity and intensity of the disease. The low intensity of the infection limits the use of current parasitological methods in the diagnosis of schistosomiasis. The detection of circulating parasite antigens might provide a more informative result, as it may indicate the true infection status. In the present study, SjTPx-1, a 22 kDa secreted antioxidant enzyme expressed throughout the life stages of the parasite, was evaluated for its potential use as a biomarker for *S. japonicum* infection. Rabbit pAb and mouse mAbs were raised against rSjTPx-1. The antibodies produced against rSjTPx-1 were confirmed to detect the native SjTPx-1 in crude adult worm lysate. Likewise, the specific binding of mAbs to parasite TPx-1 and not to

mammalian orthologues was also confirmed. The DAS-ELISA developed in the present study was able to detect at least 1 ng/m*l* of rSjTPx-1. In addition, this method was able to detect the antigen from all serum samples of an experimentally infected rabbit and experimentally infected mice. The diagnostic potential of SjTPx-1 in human clinical samples was also evaluated, and 4 of the 10 stool-confirmed serum samples were found to have detectable levels of the antigen. The results suggest the potential application of SjTPx-1 as a biomarker for *S. japonicum* infection.

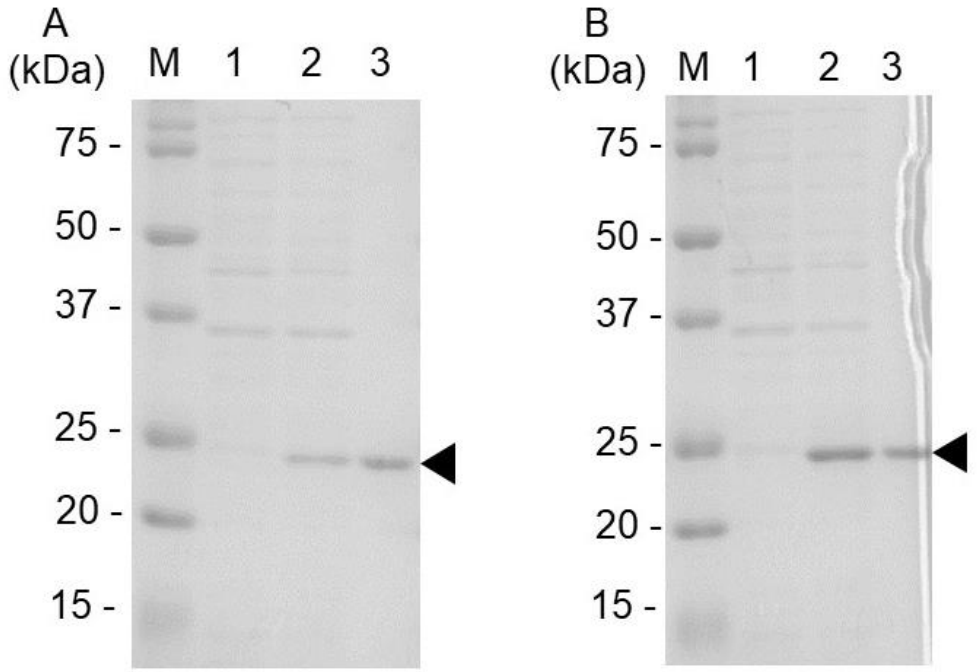

Figure 2. The expression and purification of rSjTPx-1 (A) and rMmPrx-1 (B) The bands corresponding to the proteins are indicated with arrows (◄). *M*, Molecular weight marker. *Lane 1*, cell lysate prior to IPTG induction. *Lane 2*, cell lysate after IPTG induction. *Lane 3*, purified recombinant protein.

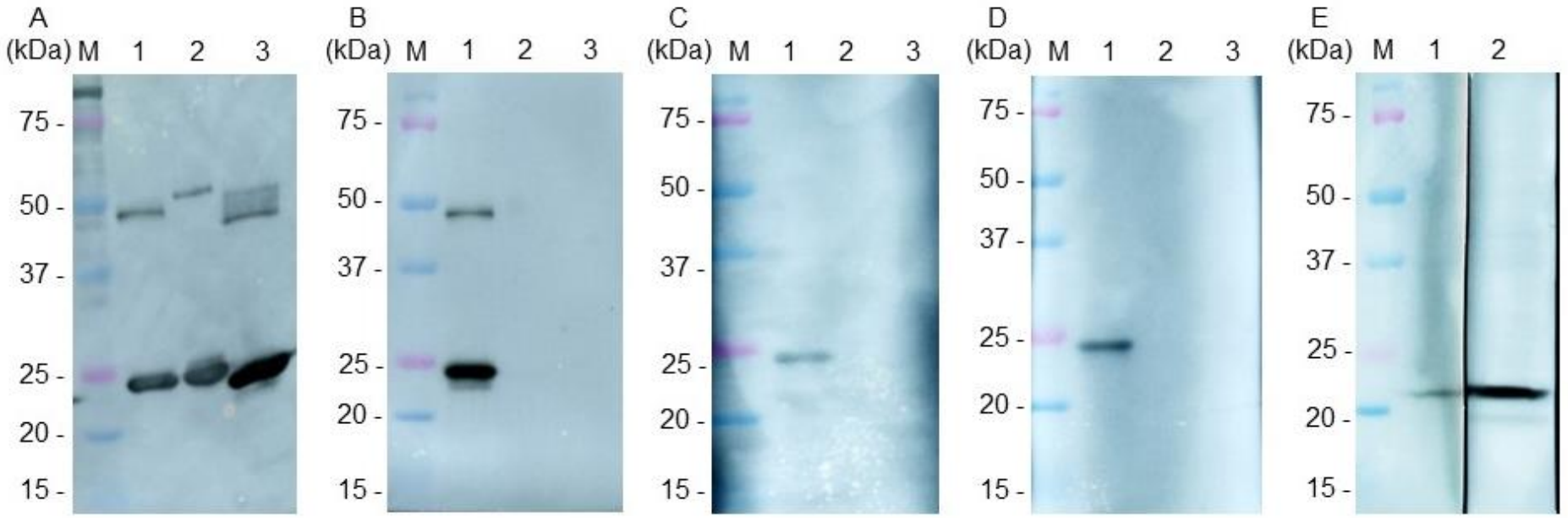

Figure 3**.** Western blotting showing the reaction of specific antibodies to antigens. (A to D) *M*, Molecular weight marker. *Lane 1*, His tagged recombinant SjTPx-1. *Lane 2*, His tagged recombinant MmPrx-1. *Lane 3*, His tagged recombinant HsPrx-1. (E) *M*, Molecular weight marker. *Lane 1 and Lane 2*, *S. japonicum* adult worm lysate. (A) The positive reactions of all His tagged rSjTPx-1, rMmPrx-1 and rHsPrx-1 to anti 6x-his tag antibody. (B to D) The specific reactions of mAbA1F7, A1F11 and A1G7 to SjTPx-1 but not to the mammalian orthologue Prx-1, respectively. (E) The recognition of the native SjTPx-1 as 22 kDa polypeptide by both anti SjTPx-1 mAb (mAbA1F7; lane 1) and pAb (lane 2) from adult worm lysate.

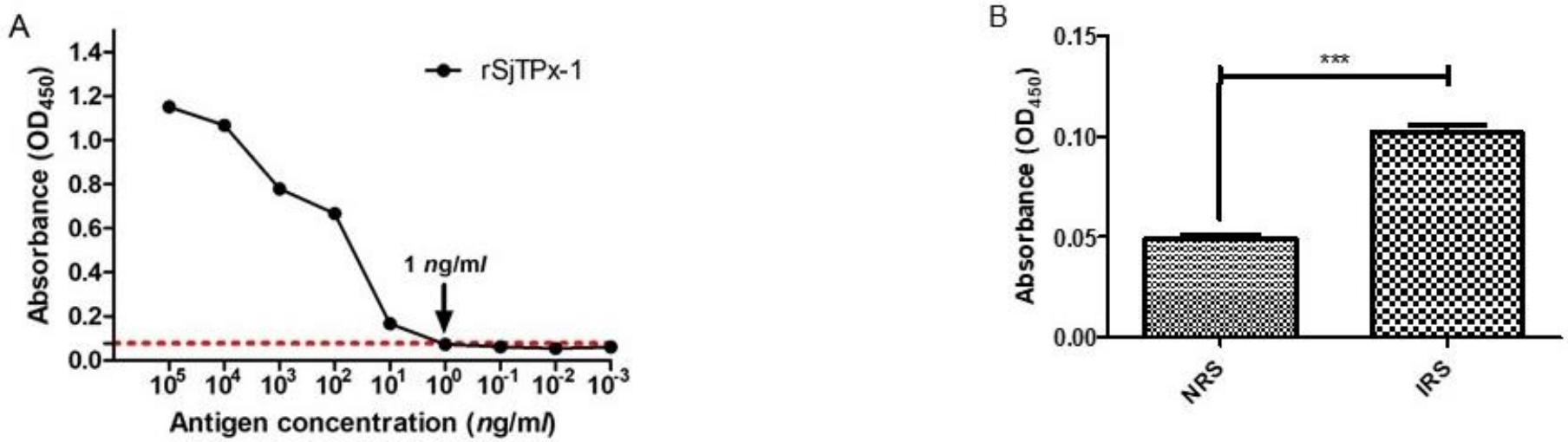

Figure 4. The optimization and performance of the DAS-ELISA. (A) The minimum antigen concentration detected by the system. The broken line shows the cut-off point and the dots indicate the OD450 reading of the recombinant SjTPx-1 at each dilution factor. The detection limit was determined to be 1 ng/ml. (B) The testing of normal rabbit serum (NRS) and experimentally infected rabbit serum (IRS). Error bars represent the SD. Asterisks indicate a statistically significant difference ($P \leq 0.001$) between NRS and IRS in the mean OD450 of triplicate readings.

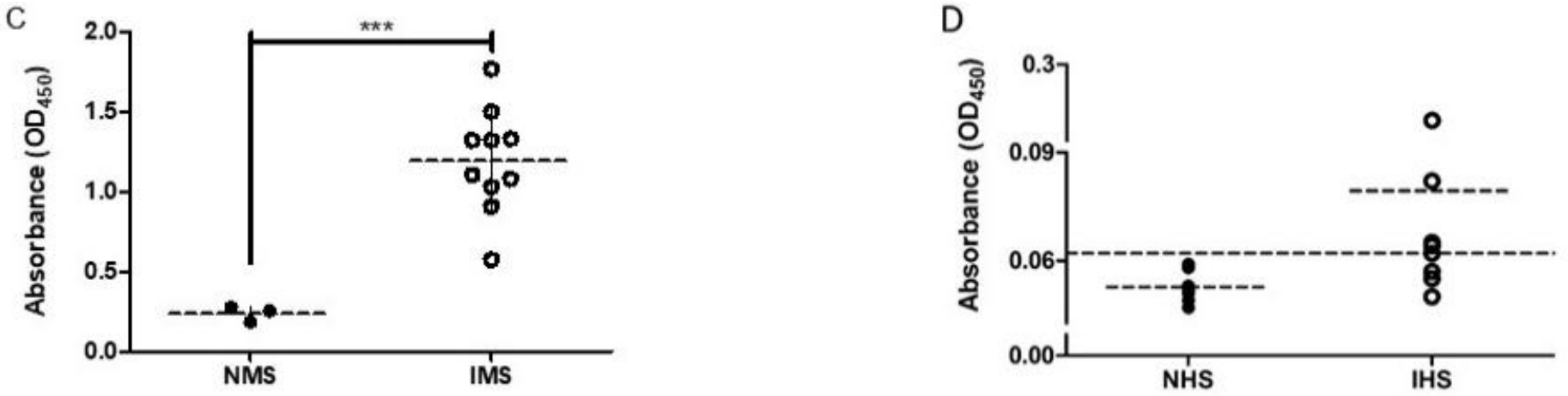

Figure 4. (C) The testing of normal mouse sera (n = 3) (NMS) and experimentally infected mouse sera (n = 10) (IMS). The short dotted lines in each group show the mean absorbance reading between normal and infected mouse sera. Asterisks indicate a statistically significant difference ($P \leq 0.001$) between NMS and IMS in the mean OD450 of triplicate readings. (D) The testing of human clinical cases (n = 10). Dotted lines across the graph signify the cut-off point. The short dotted line in each group shows the mean absorbance reading for normal (NHS) and infected human sera (IHS). This graph shows that 4 of the 10 samples were shown to have detectable antigen levels.

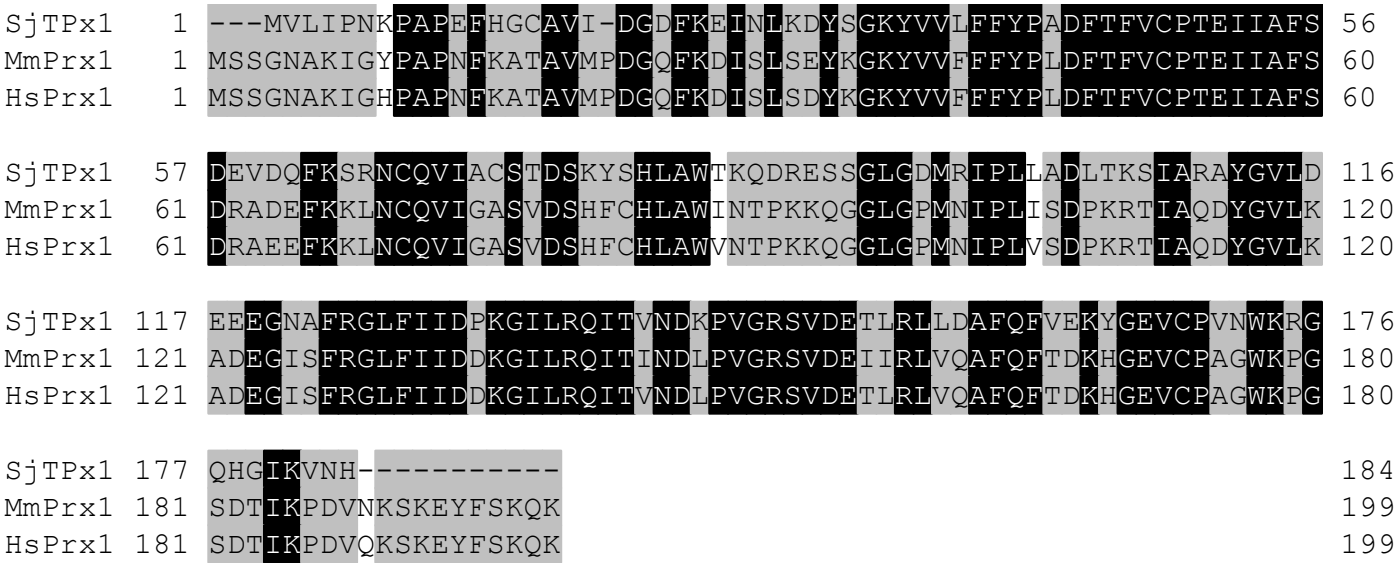

Figure 5. The amino acid sequence alignment of SjTPx-1, MmPrx-1 and HsPrx-1. The complete coding sequence of SjTPx-1 was retrieved online from GeneDB (http://www.genedb.org/) with the accession no. Sjp_0095720. The sequences of Prx-1 from selected mammalian hosts were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). Mouse, MmPrx-1 (accession no. AAH86648.1) and human, HsPrx-1 (accession no. AAH21683.1). These sequences were aligned using the GENETYX® software program (ver. 12 GENETYX, Tokyo, Japan). Identical residues are highlighted in black and similar residues are highlighted in gray.

Table 2. mAb isotyping.

Isotyping of the mAbs produced by hybridoma clones was performed to determine their subtype and to investigate the presence of kappa light chain. All mAbs produced were of the IgG subtype.

mAb clone	Isotype
A1F3	IgG 2b, Kappa light chain
A1C2	IgG 2b, Kappa light chain
A1F7	IgG 2b, Kappa light chain
A1F6	IgG 2b, Kappa light chain
A1B12	IgG 2b, Kappa light chain
A1G7	IgG1, Kappa light chain
A1F11	IgG 2a, Kappa light chain

Table 3. Checkerboard titration absorbance readings.

			8 μg/m*l* pAb		Ave	SD	P/N ratio		4 μg/m*l* pAb		Ave	SD	P/N ratio
8 μg/m*l* mAb	+	0.840	0.851	0.865	0.852	0.013	18.257	0.754	0.812	0.777	0.781	0.029	11.657
	-	0.048	0.046	0.046	0.047	0.001		0.114	0.044	0.043	0.067	0.041	
4 μg/m*l* mAb	+	0.856	0.839	0.784	0.826	0.038	17.964	0.759	0.809	0.776	0.781	0.025	10.559
	-	0.046	0.045	0.047	0.046	0.001		0.064	0.114	0.044	0.074	0.036	
2 μg/m*l* mAb	+	0.900	0.894	0.875	0.890	0.013	17.444	0.750	0.726	0.692	0.723	0.029	16.550
	-	0.049	0.045	0.059	0.051	0.007		0.044	0.044	0.043	0.044	0.001	
1 μg/m*l* mAb	+	0.914	0.870	0.860	**0.881**	0.029	**18.490**	0.863	0.823	0.849	0.845	0.020	17.727
	-	0.049	0.047	0.047	**0.048**	0.001		0.048	0.047	0.048	0.048	0.001	
			2 μg/m*l* pAb		Ave	SD	P/N ratio		1 μg/m*l* pAb		Ave	SD	P/N ratio
8 μg/m*l* mAb	+	0.623	0.649	0.613	0.628	0.019	14.173	0.316	0.332	0.310	0.319	0.011	7.426
	-	0.045	0.043	0.045	0.044	0.001		0.041	0.044	0.044	0.043	0.002	
4 μg/m*l* mAb	+	0.651	0.575	0.617	0.614	0.038	15.231	0.307	0.318	0.337	0.321	0.015	7.074
	-	0.042	0.040	0.039	0.040	0.002		0.040	0.053	0.043	0.045	0.007	
2 μg/m*l* mAb	+	0.673	0.638	0.564	0.625	0.056	15.369	0.312	0.357	0.373	0.347	0.032	8.756
	-	0.040	0.039	0.043	0.041	0.002		0.038	0.039	0.042	0.040	0.002	
1 μg/m*l* mAb	+	0.652	0.684	0.712	0.683	0.030	16.787	0.354	0.311	0.333	0.333	0.022	7.984
	-	0.041	0.041	0.040	0.041	0.001		0.041	0.039	0.045	0.042	0.003	

The OD readings of checkerboard titration with varying concentrations of capture and detection antibodies including computation of the positive/negative ratio for the optimum antibody concentration. The numbers in bold typeface indicate the OD values and the positive/negative (P/N) ratio recorded between the optimal concentrations of the capture pAb and detection mAb (8 µg/ml pAb and 1 µg/ml mAb, respectively).

Absorbance was measured at OD_{450} nm.

Chapter II. *Schistosoma japonicum* cathepsin B as an early diagnostic antigen for Asian schistosomiasis

2.1 Introduction

Schistosomiasis is an NTD that causes chronic debilitating illness and which affects millions of people globally (Chitsulo *et al.,* 2000). In tropical countries, the socioeconomic and public health impact of the disease is second only to malaria (CDC, 2012). *S. japonicum*, the most prevalent species in Asia, is found in China, the Philippines, and some parts of Indonesia (Chitsulo *et al.,* 2000). Due to the huge number of eggs excreted by the female worm (3500 eggs/female/day), *S. japonicum* is considered to be the most pathogenic among the schistosomes that are infective to humans (Mahmoud, 2001). Recent reports of the emergence of new endemic foci attributed to the migration of infected individuals or animals indicate that schistosomes pose a serious threat to public health (Leonardo *et al.,* 2015; Patz *et al.,* 2000). The lack of an effective screening method for humans and animals from endemic areas could lead to the introduction of the disease in non-endemic areas.

Pathologic lesions associated with *S. japonicum* infection mainly occur due to the deposition of eggs in various vital organs, including the liver and spleen (Mahmoud, 2001). The early diagnosis of infection may prove useful—especially in travelers potentially exposed to *S. japonicum* infection, where the prompt treatment will definitely result in a positive outcome by killing the worms prior to egg deposition in vital organs and tissues (Karcher *et al.,* 2008). By developing a diagnostic method capable of early detection, disease progression can be avoided.

The cercaria represents the infective stage of *S. japonicum*. This stage is particularly important in the development of a diagnostic method that can be applied

early in the disease course, because antigens related to this stage are exposed to the host immune system at an early stage. Thus, antibodies against the antigens of the cercaria can be detected early in the course of infection (Zhou *et al.,* 2010). Soluble cercarial antigen (SCA) was previously reported to be a good early diagnostic antigen. However, due to the nature of SCA being a crude antigen, there are difficulties associated with its mass production for field application, and cross-reaction with other parasitic infections has been observed (Zhou *et al.,* 2010). *S. japonicum* cathepsin B (SjCatB) is a protease expressed throughout all life stages of the parasite. The highest level of expression was observed in the infective cercaria stage in which it is utilized by the parasite to penetrate the intact skin of the host, making it an ideal target for vaccine and drug development (Liu *et al.,* 2014; Curwen *et al.,* 2003; Dvorak *et al.,* 2008). Although *S. mansoni* cathepsin B has been evaluated for the serological diagnosis of *S. japonicum* infection (Ruppel *et al.,* 1987b), the use of SjCatB as an authentic antigen for *S. japonicum* may provide a more sensitive and specific diagnostic target.

In the present study, recombinant SjCatB (rSjCatB) was produced and applied as an antigen for an indirect ELISA. In the present study, SjCatB showed satisfactory sensitivity and specificity in the identification of experimentally infected mice and human cases with minimal cross-reactivity against serum samples collected from humans with other parasitic infections.

2.2 Materials and methods

Ethics statement. The collection of serum samples from patients in Sorsogon, the Philippines were carried out in compliance with the ethical clearance sought from the University of the Philippines, Manila (clearance no: UPM REB 2011-098). The animal experiments performed in the present study were carried out in compliance with

the guidelines for animal experimentation of Obihiro University of Agriculture and Veterinary Medicine (permission number: 29-52).

Serum samples. Female, 8-week old ICR mice (Clea Inc.) were used for the experimental infection. The animals were infected with the parasite by percutaneous challenge with 30 cercaria. To determine the diagnostic value of SjCatB in the experimental animals, serum samples from the mice (n = 20) that had been infected with the parasite were collected at >8 weeks post-infection. The serum samples that were used as negative controls were collected from normal uninfected mice (n = 20). To observe the antibody response against SjCatB in the course of infection, serum samples were collected from the experimentally infected animals (n = 4) at 2, 4, 6 and 8 weeks after cercarial challenge. The serum samples that were used as the negative controls were collected from normal uninfected mice (n = 2) at the same time. Serum samples from patients (n = 30) who had been confirmed by stool microscopy and a stool PCR for *S. japonicum* infection (Angeles *et al.,* 2012) were collected from Sorsogon, the Philippines. Serum samples that were used as the negative controls were collected from US volunteers (n = 30) who had not previously visited *S. japonicum* endemic areas. To check the cross-reactivity of the antigen with other parasitic infections, archived sera from humans infected with *Trichuris trichiuria* (n = 1), *Paragonimus westermanii* (n = 11), *Opistorchis viverrini* (n = 8) and *Entamoeba histolytica* (n = 10) were used.

Recombinant protein preparation. The complete coding sequence of SjCatB was retrieved from the *S. japonicum* genome database with the accession number X70968.1. Total RNA was extracted from 50 mg of frozen adult worm by homogenizing it in 1 ml of TRIzol® reagent (Life Technologies, Inc.). cDNA was synthesized using PrimeScript™ (Takara Bio. Inc.). The double stranded full length coding sequence was amplified by a PCR with the following primers: 5'-GC GGA TCC

ATG TTG AAA ATC GCA GTT-3' (underline indicates the *Bam* HI site) and 5'-GC CTC GAG TTA GGT TTT TAT AAG TCC AG-3' (underline indicates the *Xho* I site). The PCR product was cloned into pCR 2.1-TOPO vector (Invitrogen). The identity of the cloned sequence with the sequence deposited in the database was confirmed by sequencing using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The coding sequence was then double-digested with *Bam* HI and *Xho* I and was subcloned into pET-28a (+) vector (Novagen). The recombinant protein with 6xHis-tag on the N terminal was expressed using *E. coli* (BL21) cells (Takara Bio) grown in Luria Bertani broth (Sigma-Aldrich, St. Louis, MO, USA) with kanamycin (50 μg/ml). The rSjCatB was purified with affinity chromatography using an Ni-NTA protein purification kit (Qiagen) under denaturing conditions, and then was dialyzed against cold PBS (pH 7.4) prior to use. The recombinant protein was quantified using a Pierce™ BCA protein assay kit (Thermo Scientific) and its purity was confirmed by SDS-PAGE.

Western blotting. Two hundred nanograms of rSjCatB was resolved by SDS-PAGE and the polypeptides were transferred onto a PVDF membrane. The membrane strips were blocked with 5% skim milk in PBS overnight at 4°C with constant shaking. Excess blocking buffer was removed by washing twice with PBS containing 0.05% Tween 20 (PBS-T). The strips were incubated with antibody or serum samples in separate reaction chambers for 1 h at room temperature with constant shaking. The mAb against 6xHis-tag (Thermo Fisher Scientific) was diluted 500 times using 1% bovine serum albumin with PBS-T (1%-BSA-PBS-T) while the mouse and rabbit sera were diluted 400 times with PBS-T. Human serum was diluted 200 times with 1%-BSA-PBS-T. After the incubation, the strips were washed three times with PBS-T and incubated with secondary antibody for either 1 h against mouse IgG, rabbit IgG or human IgG conjugated with HRP (GE Healthcare). The antigen-antibody reaction was

visualized by chemiluminescence using SuperSignal™ (ThermoFisher Scientific) and a chemiluminescent detection machine ImageQuant LAS 500 (GE Electric).

ELISA. Each well of a 96-well polystyrene plate (Thermo Fisher scientific) was coated with 200 ng of rSjCatB in 100 µl of carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. The wells were washed three times with PBS to remove unbound antigens. The wells were blocked with 1%-BSA-PBS-T for 5 min. The mouse sera were diluted 200 times while the human sera were diluted 400 times in 1%-BSA-PBS-T. For each sample, 100 µl of the diluted serum sample was delivered to each well in triplicate. The wells were incubated for 1 h at 37°C and were subsequently washed three times using PBS. One hundred microliters of the secondary antibody, either HRP-conjugated anti-mouse IgG (diluted at 1:10,000) or HRP-conjugated anti-human IgG (diluted at 1:20,000), was added to each well. The wells were incubated for 1 h at 37°C and were subsequently washed three times with PBS. The antigen-antibody reaction was detected by adding 100 µl of HRP substrate, TMB (KPL, Inc.) to the wells and the enzymatic reaction was allowed to proceed for 10 min. The reaction was stopped by adding 50 µl of 1 M phosphoric acid and the absorbance at 450 nm was measured using a microplate reader (Corona Electric).

Statistical analysis. The sensitivity and specificity were analyzed using the MedCalc software program (version 16.4.3, MedCalc Software, Ostend, Belgium; https://www.medcalc.org; 2016).

2.3 Results

Cloning and protein expression. The complete coding sequence of SjCatB was amplified by a PCR from cDNA prepared from adult worm RNA, and was cloned into a pCR plasmid vector for sequencing. The coding sequence was 1.045 kbp in size (Fig.

6A and B). The identity with the GenBank deposited sequence (X70968.1) was confirmed (data not shown). The coding sequence was then subcloned into a pET 28a (+) plasmid vector for the recombinant protein expression in *E. coli* with isopropyl-thio-β-D-galactoside (IPTG) induction. The Ni-NTA column-purified recombinant protein was recognized as a single band in SDS-PAGE with a molecular mass of ~39 kDa, which was in good accordance with the size predicted from the SjCatB AA sequence (Fig. 6C). rSjCatB was recognized as a 39 kDa polypeptide with anti-6xHis-tag mAb in Western blotting (Fig. 7). rSjCatB was recognized in serum samples collected from experimentally infected animals and the stool-positive patients but not in normal serum samples (Fig. 7). The results suggested that the recombinant protein was antigenically similar to the native protein.

ELISA. The diagnostic potential of rSjCatB was evaluated in an ELISA with sera collected from experimentally infected mice. Twenty negative serum samples from normal uninfected mice were used to determine the cut-off value (mean OD value plus 2-fold SD), and the value was determined to be 0.08. Using this cut-off value, all 20 experimentally infected mice were determined to be positive (sensitivity = 100%), while 19 of the 20 normal uninfected mice were determined to be negative (specificity = 95.0%) (Fig. 8A and Table 4).

The sera collected from experimentally infected mice at 2, 4, 6 and 8 weeks post-infection were evaluated for the antibody response against rSjCatB in ELISA. From as early as 6 weeks post-infection, sera from 2 of the 4 experimentally infected mice showed OD values higher than the cut-off value of 0.08. Sera from normal uninfected mice did not show OD values higher than the cut-off value (Fig. 8B).

The diagnostic potential of rSjCatB in an ELISA with sera from human clinical cases was also evaluated. Thirty serum samples from normal healthy US volunteers

were used to determine the cut-off value, which was calculated to be 0.33. Using this cut-off value, 26 of 30 stool-confirmed clinical cases were determined to be positive (sensitivity = 86.7%), while 29 of 30 US volunteers were determined to be negative (specificity = 96.7%) (Fig. 9 and Table 5).

The *T. trichiura*-positive sample showed no cross-reaction with the recombinant antigen. In contrast, 1 of the 11 *P. westermani*-, 3 of the 8 *O. viverrini*- and 1 of the 10 *E. histolytica*-positive samples showed OD values that were slightly higher than the cut-off value. (Fig. 9).

2.4 Discussion

The current diagnostic test for *S. japonicum* infection is based on stool microscopy, and is highly specific and easy to perform (Katz *et al.,* 1972). However, the test is associated with some disadvantages, including its inability to detect early infection or infection with a low parasite burden (Pontes *et al.,* 2003; Yu *et al.,* 2007; Zhu, 2005). To address this, several modifications have been performed in order to increase the sensitivity of the test, including the concentration of eggs in the stool using the formalin-ether concentration technique (FECT) and the collection of stool samples over several consecutive days (Utzinger *et al.,* 2001; Booth *et al.,* 2003; Ridley and Hawgood, 1956). These modifications, however, are deemed too laborious to be applied in large-scale nationwide surveys (Weerakon *et al.,* 2015). Although POCT kits are available for other schistosome species, the performance of these kits was deemed to be unsatisfactory in the diagnosis of *S. japonicum* (van Dam *et al.,* 2015). The performance of the POCT based on *S. mansoni* CCA in the diagnosis is correlated with the intensity of infection; thus, its sensitivity may decrease in cases with a low parasite burden, similar to stool microscopy (Stothard *et al.,* 2006; Tchuem *et al.,* 2012; Legesse *et al.,* 2007). An ELISA that can detect early infection with high sensitivity and

specificity may provide an alternative to stool microscopy and the POCT for cases involving early infection or a low parasite burden.

CatB is a lysosomal cysteine protease found in the gut of *Shistosoma* parasites. The parasites use CatB as a major enzyme for nutrient acquisition through hemoglobin metabolism (Caffrey *et al.,* 2004). CatB is identified in the acetabular gland as its main enzyme in the cercarial stage of *S. japonicum*. The parasite uses the enzyme to dissolve collagen IV, fibronectin and laminin in the extracellular matrix of the host skin during penetration (Dvorak *et al.,* 2008; Curwen *et al.,* 2003). The abundant expression of the enzyme in the cercarial stage of *S. mansoni*—particularly in the cecum and protonephridia—is also reported (El Ridi *et al.,* 2014; Skelly and Shoemaker, 2001). The crucial role of this enzyme in the parasite makes it an ideal target for drug and vaccine development (Smooker *et al.,* 2010; Wasilewski *et al.,* 1996). The early exposure of CatB to the host immune system also implies an early immune response against this enzyme. Hence, this antigen may have an advantage as a potential marker for the early antibody-based diagnosis of infection.

In the present study, an ELISA using rSjCatB showed high sensitivity and specificity in detecting the parasite infections both in experimentally infected mice and in stool-confirmed patients. This potential application of this antigen in the early diagnosis of the infection was also shown, since it correctly detected 2 of 4 experimentally infected mice from as early as 6 weeks post-infection, before the parasite develops into an adult and starts to lay eggs. This finding validates the previous findings related to *S. mekongi* CatB (Sangfuang *et al.,* 2016) and *S. mansoni* CatB, (Ruppel *et al.,* 1985a, b), for which specific antibodies were detected from as early as 4 weeks post-infection. Early detection is beneficial in cases of acute infection, such as Katayama fever, when eggs are not detected in the stool or urine. It is also useful for

identifying new endemic foci where patients have a low level of infection (Tsang *et al.,* 1997; Al-Sherbinny *et al.,* 1999). The earlier diagnosis could provide travelers suspected of exposure with early access to treatment and prevent the introduction of the parasite into an uncontaminated environment. The results of the present study showed that SjCatB has high sensitivity and specificity in the diagnosis of *S. japonicum* infection, even in early stages of infection. Moreover, this antigen has minimal cross-reactivity with other parasitic infections.

The environmental changes brought about by the development of water resources, such as the building of dams, which introduces the intermediate snail hosts, as well as the migration of patients and reservoir animals into other previously non-endemic areas, may give rise to new endemic foci (Patz *et al.,* 2000; Ross *et al.,* 2001). Effective screening of individuals and animals moving from schistosomiasis-endemic areas may prevent the introduction of the disease in non-endemic areas. Thus, a sensitive and specific test that can detect early-stage schistosomiasis infection may have advantage over the traditional diagnostic methods that are currently applied, and may in turn improve elimination programs that are currently set in place.

Taken together, SjCatB, which showed high sensitivity and specificity in the detection of early-stage *S. japonicum* infection may be a promising serodiagnostic antigen. To facilitate its field application, additional experiments with larger numbers of samples from humans and animals are currently under way.

2.5 Summary

In the present study, the diagnostic value of SjCatB as an antigen for the early detection of antibodies against *S. japonicum* infection was evaluated. The full-length coding sequence of SjCatB was retrieved from GenBank and was expressed as rSjCatB in *E. coli* with N-terminal 6xHis-tag. The diagnostic value of rSjCatB in an ELISA was

evaluated using sera collected from experimentally infected mice. The early detection of antibodies against rSjCatB in the ELISA was evaluated with sera collected from experimentally infected mice at 2, 4, 6 and 8 weeks post-infection. Furthermore, the potential of the recombinant antigen in detecting the infection was evaluated with sera collected from individuals who had been diagnosed with *S. japonicum* infection based on the results of stool examinations. In the present study the use of rSjCatB as antigen in an indirect ELISA achieved 100% sensitivity and 95.0% specificity in the detection of *S. japonicum* infection in serum samples collected from experimentally infected mice at >8 weeks post-infection. *S. japonicum* was detected in serum samples of two of four mice from as early as 6 weeks post-infection. When the recombinant antigen was used in an ELISA to detect *S. japonicum* in serum samples from stool-positive human patients, it achieved 86.7% sensitivity and 96.7% specificity. In addition, when serum samples from patients with other parasitic diseases were tested, there was minimal cross-reactivity with rSjCatB. The results of the present study suggest that SjCatB is a promising diagnostic antigen that is capable of the sensitive and specific early detection of *S. japonicum* infection.

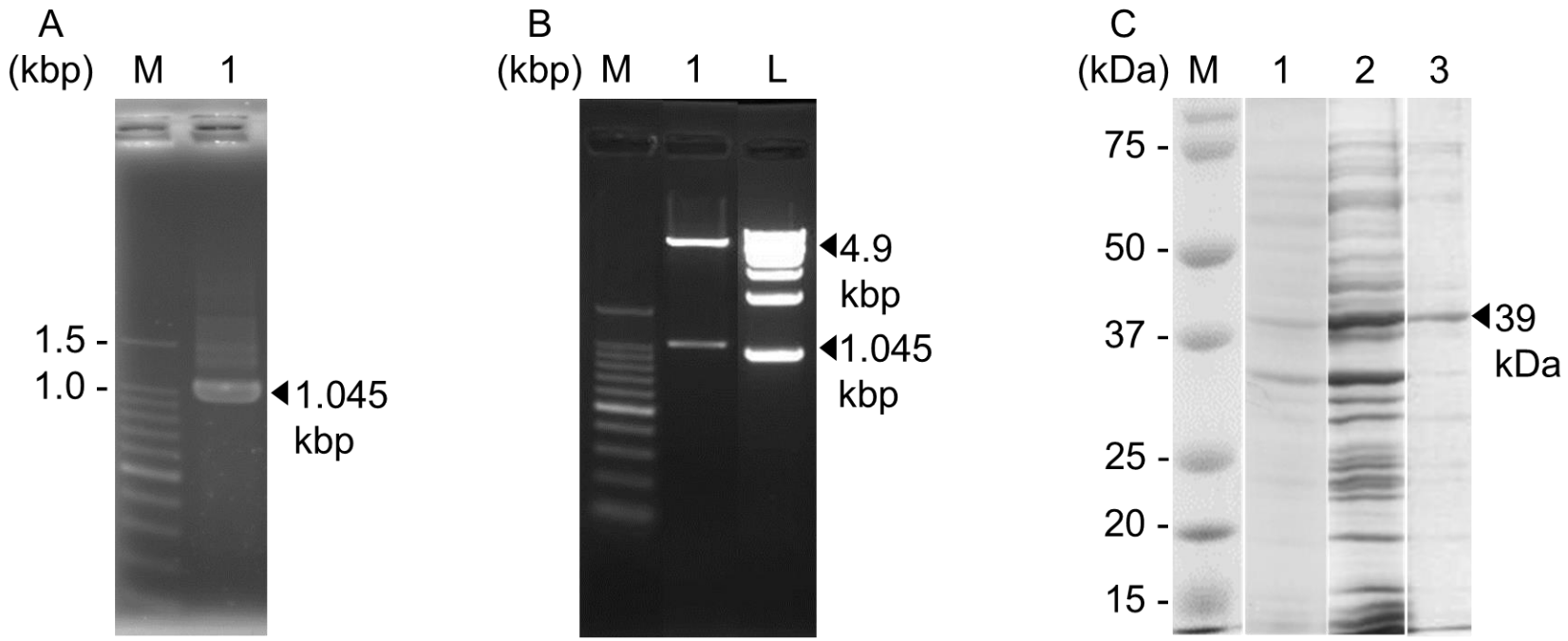

Figure 6. Gel electrophoresis and SDS-PAGE of rSjCatB. (A) The PCR-amplified DNA fragment coding for SjCatB. *Lane 1*, DNA for the SjCatB coding sequence amplified from the *S. japonicum* adult worm cDNA. *M*, Marker. The triangle indicates the DNA fragment with a size of 1.045 kbp. (B) Recombinant plasmids with the SjCatB coding sequence. *Lane 1*, the recombinant plasmid with enzyme digestion. The upper band represents the plasmid DNA and lower band represents DNA for the SjCatB coding sequence. *M and L*, 100bp (*M*) and kbp (*L*) ladder markers. The triangles indicate DNAs of size of 4.9 kbp and 1.045 kbp, respectively. (C) The expression and purification of rSjCatB. *M*, Marker. *Lane 1*, *E. coli* culture before adding IPTG, *Lane 2*, after adding IPTG, and *Lane 3*, after purification with Ni-NTA column chromatography. The triangle indicates the polypeptide of 39 kDa in size.

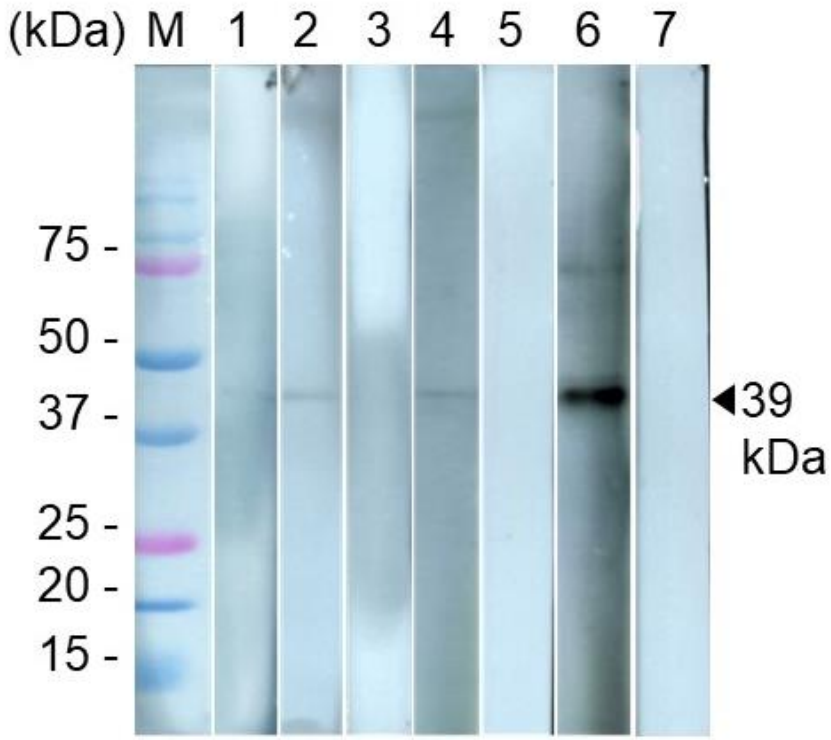

Figure 7. The recognition of rSjCatB on Western blotting. *M*, Marker. *Lane 1*, anti-6xHis-tag mAb. *Lane 2*, serum collected from an experimentally infected rabbit. *Lane 4*, serum collected from an experimentally infected mouse. *Lane 6*, serum collected from a stool-positive patient. *Lane 3*, *5 and 7*, rabbit, mouse and human negative control sera.

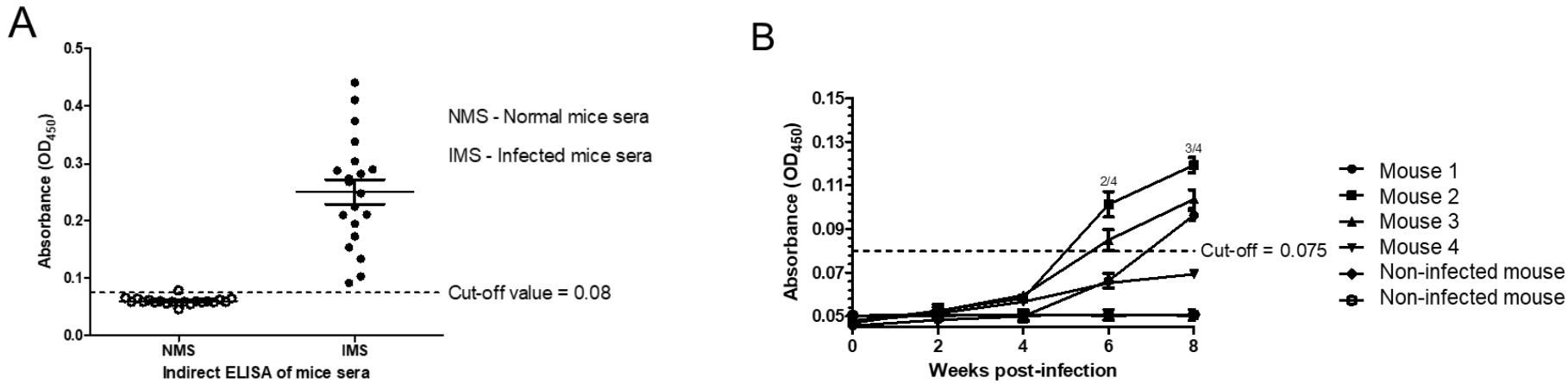

Figure 8. The ELISA results with rSjCatB. (A) The results with mouse sera. NMS: Serum samples collected from normal uninfected mice (n = 20). IMS: Serum samples collected from mice experimentally infected with the parasite (n = 20). The dotted-line across the graph indicates the cut-off value. The thin-solid line in each group shows the mean absorbance, while the thick-solid lines represent the SD. (B) The results of the early diagnosis. Each symbol shows the mean absorbance of the triplicate readings for the serum samples collected from normal (n = 2) and parasite-infected mice (n = 4) at 2, 4, 6 and 8 weeks post-infection. The dotted-line across the graph indicates the cut-off value.

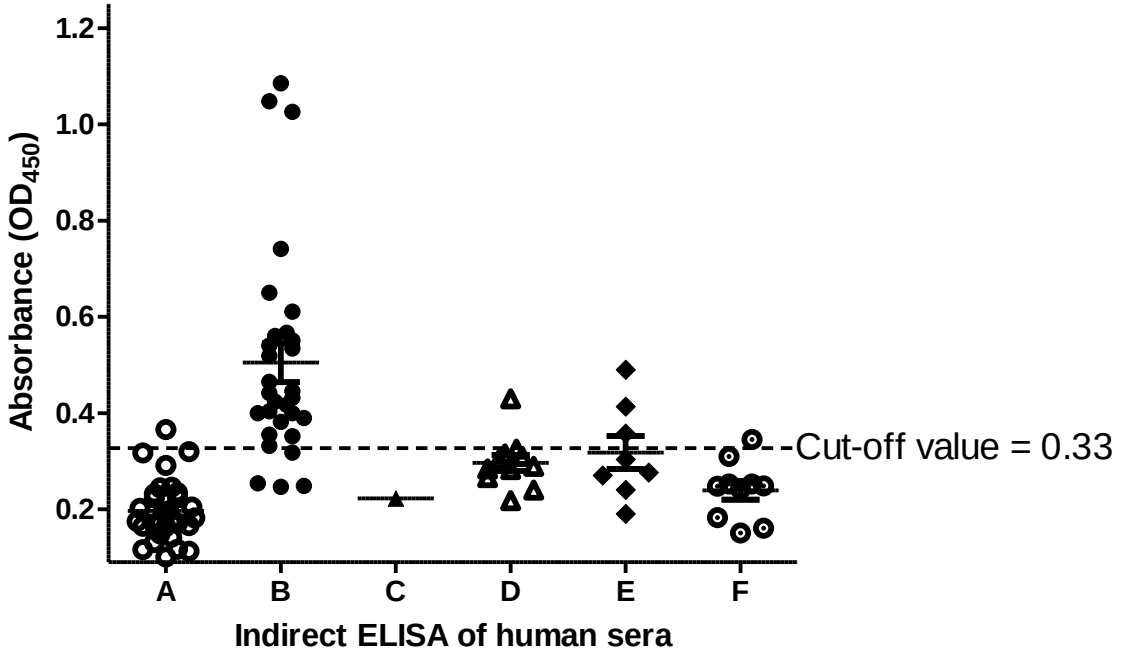

Figure 9. The results with human sera. A: Negative serum samples from US volunteers (*n* = 30). B: Serum samples from *S. japonicum* stool-positive patients (*n* = 30). C: Serum-positive for *T. trichiura* (*n* = 1). D: Serum-positive for *P. westermani* (*n* = 11). E: Serum-positive for *O. viverrini* (*n* = 8). F: Serum-positive for *E. histolytica* (*n* = 10). The dotted-line across the graph indicates the cut-off value. The thin-solid line in each group shows the mean absorbance.

Table 4.
Calculation of the diagnostic value of the indirect ELISA for rSjCatB with experimentally-infected mouse sera.

Indirect ELISA results	Disease status		Total
	Present	Absent	
Positive	20^{a}	1^{c}	21
Negative	0^{b}	19^{d}	19
Total	20^{a+b}	20^{c+d}	40

Sensitivity (a/a+b) = 100%; Specificity (d/c+d) = 95.0%

Table 5.
Calculation of the diagnostic value of the indirect ELISA for rSjCatB with patient sera.

Indirect ELISA results	Disease status		Total
	Present	Absent	
Positive	26	1	27
Negative	4	29	33
Total	30	23	60

Sensitivity (a/a+b) = 86.7%; Specificity (d/c+d) = 96.7%

General Discussion

The aim of the present study was to develop antigen- and antibody-based serodiagnostic assays for the detection of *S. japonicum* infection. An antigen-based diagnostic test would be advantageous because it would be able to determine the true status of infection and the response to chemotherapy. The first chapter of the present study focused on the antigen-based detection of *S. japonicum* infection by evaluating the circulating antigen SjTPx-1 as a potential biomarker. Currently, the only available antigen-based test for schistosomiasis is a POCT based on the CCA of *S. mansoni*; this test has been observed to have sub-optimal sensitivity in the detection of Asian schistosomiasis (van Dam *et al.,* 2004; van Dam *et al.,* 2015). Thus, the lack of a reliable antigen-based diagnostic test for *S. japonicum* infection has led to the evaluation of numerous antigen targets from the excretory and secretory protein of *S. japonicum* (Gao *et al.,* 2016; Ren *et al.,* 2017). However, none has been applied for field surveillance or the diagnosis of disease in the clinical setting. Thus, the development of an antigen-based diagnostic test is urgently needed.

Previous studies showed that SjTPx-1 was a good diagnostic antigen in an antibody-based indirect ELISA in humans and water buffaloes (Angeles *et al.,* 2011; Angeles *et al.,* 2012). Recent evidence shows that SjTPx-1 is also a component of the excretory and secretory products of the adult worm (Kumagai *et al.,* 2006; Gao *et al.,* 2016). Thus, chapter 1 aimed to evaluate the potential use SjTPx-1 as a target antigen in a DAS-ELISA. The results of the present study showed that the target antigen is detectable in both experimentally-infected animals and the patients. The development of a test that can identify the true status of infection based on parasite derived molecules will be very useful in the effective screening and evaluation of the control measures being implemented for *S. japonicum* infection.

The second chapter of the present study aimed to evaluate the applicability of using SjCatB as an antigen for the early detection of *S. japonicum* infection. Early detection is beneficial in cases of acute infection, such as Katayama fever, during which eggs are not detected in the stool or urine, as well as in identifying areas of low endemicity where patients have low levels of infection (Tsang *et al.,* 1997; Al-Sherbinny *et al.,* 1999). The implication of an earlier diagnosis could provide patients suspected of exposure, such as travelers, useful precautionary measures, and facilitate the early provision of treatment to prevent the pathologies associated with egg deposition, as well as preventing the introduction of the parasite into the environment. The microscopic examination of stool samples is the gold standard for the diagnosis of schistosomiasis. However, years of MDA using praziquantel has decreased the sensitivity of this test. In addition, a reliable diagnosis by stool microscopy can only be achieved at 8-10 weeks post-infection, when the worm attains full maturity and starts laying eggs. At this time, thousands of eggs might have been laid, which can accidentally be deposited in several vital organs, where they cause serious damage.

CatB is a lysosomal cysteine protease found in the gut of *Shistosoma* parasites. This protease, which is found in the acetabular gland, is its main enzyme in the cercarial stage of *S. japonicum.* The parasite uses CatB to penetrate the intact skin of the host (Dvorak *et al.,* 2008; Curwen *et al.,* 2003). The early exposure of CatB to the host immune system suggests an early immune response against this enzyme. Hence, this antigen may have an advantage as a potential marker that can be applied in the antibody-based diagnosis of infection, and which can be used to diagnose the infection at an early stage. In the present study, SjCatB was cloned and expressed as a 6xHis-tag fusion protein. The results of the present study show that with the use of rSjCatB antigen, *S. japonicum* infection can be detected at an earlier stage in comparison to stool

microscopy. This result validates the recent findings of a study by Sangfuang *et al.,* (2016) on *S. mekongi* CatB and the findings of a previous study by Ruppel *et al.,* (1985a, b) on *S. mansoni* CatB, which reported that circulating antibodies against the antigen were detected earlier than the egg-laying stage (8-10 weeks post-infection).

The use of rSjCatB in the detection of *S. japonicum* infection by an indirect ELISA in experimentally infected mice achieved 100% sensitivity and and 95.0% specificity. In human samples, the sensitivity was 86.7% and the specificity was 96.7%, with minimal cross-reaction when serum samples from patients infected with other parasites were tested. This result suggests that rSjCatB is potentially a good target antigen for the detection of *S. japonicum* infection.

By identifying the true status of infection and the response to chemotherapy, prevalence studies will be more accurate in assessing the efficacy of the MDA programs that are currently implemented. Furthermore, the detection of infection at an early stage can significantly improve the treatment outcome and prevent the emergence of new endemic foci, which may eventually lead to the eradication of *S. japonicum*.

General Summary

Schistosomiasis is considered to be the second most devastating human parasitic disease after malaria. It is endemic in 78 countries around the world, including China, the Philippines, and parts of Indonesia. Official estimates show that approximately 240 million people are affected by the parasite, among whom 20 million suffer from severe illness. The parasitic disease caused by *S. japonicum* was discovered in Japan in 1904. The World Health Organization has designated schistosomiasis as a neglected tropical disease and has been promoting its active control. Initially, eggs are passed out in fresh water from the feces of an infected human or animal, which subsequently hatch into a free swimming miracidia. At this stage, the parasite actively seeks to infect the semi-aquatic intermediate snail hosts, for instance, *O. h. hupensis* in China and *O. h. quadrasi* in the Philippines. Inside the snail, the parasite undergoes clonal replication, developing into numerous cercaria in the infective stage of the parasite.

The ability of *S. japonicum* to infect several mammalian species, including domestic animals sets it apart from other *Schistosoma* species that are infective to humans. Animals in close association with humans, such as cattle, water buffaloes, dogs and pigs are the most susceptible to infection. This has made efforts to control the disease particularly complicated. Infection with *S. japonicum* is characterized by marked hepatic portal fibrosis, which leads to ascites. Aberrant eggs may also cross the blood brain barrier causing seizure, as occurs in cerebral schistosomiasis. The huge number of eggs laid by a single female parasite contributes to the higher potential of this disease to spread in the environment and cause pathologies associated with the localization of deposited eggs in vital organs such as the liver, spleen and brain.

The diagnosis of schistosomiasis plays a crucial role in ensuring sound treatment and in the surveillance of the disease, which is targeted for elimination. Prior

to the initiation of treatment, an accurate diagnosis should be made. Re-testing should be performed after the completion of the treatment regimen to confirm the absence of the disease. Over several decades, numerous control measures have been implemented, including MDA with praziquantel, which has resulted in a decrease in the prevalence of schistosomiasis in endemic areas. However, this has also led to a decrease in the sensitivity of stool microscopy, which is the gold standard and the most commonly used diagnostic test for *S. japonicum* infection. In order to compensate for this, stool samples should be collected for three consecutive days, which makes the test more laborious and results in decreased compliance from patients being tested.

The detection of parasite circulating antigens provides informative results, as it can indicate the true infection status. At present, the only available antigen-based test for schistosomiasis is based on the CCA of *S. mansoni.* This test has been successfully applied in numerous endemic areas in Africa. However, in a proof-of-concept study, it was not effective in detecting *S. japonicum* infection. The ongoing need for a reliable *S. japonicum*-specific antigen-based serodiagnostic test has led to the evaluation of several antigen targets from the excretory and secretory products of the parasite, which have been shown to be potential diagnostic biomarkers for schistosomiasis. SjTPx-1 has multiple biological functions, including its main function as a key enzyme that combats reactive oxygen species. Immunohistochemistry demonstrated the presence of SjTPx-1 in all life stages of *S. japonicum*, as well as its extensive distribution on the surface tegument of the parasite, and the tissues surrounding the egg in the liver. Additionally, this enzyme was recently identified as a component of the excretory and secretory products of the adult worm; thus, SjTPx-1 is a promising candidate that may have application in antigen-based serodiagnosis of *S. japonicum* infection.

In the present study, SjTPx-1 was evaluated for its potential use as a target antigen for the antigen-based serodiagnosis of *S. japonicum* infection. Recombinant SjTPx-1 was expressed as a 6xHis-tag fusion protein. Rabbit pAb and mouse mAbs were raised against the recombinant protein. The antibodies produced against the recombinant SjTPx-1 could detect the native form of the antigen in crude adult worm lysate on Western blotting. This confirms that the recombinant antigen produced was antigenically similar to the naturally existing SjTPx-1 from the parasite. Since TPx-1 is conserved throughout the animal kingdom, the specific binding of mAbs to the parasite TPx-1, but not to mammalian orthologues, was also verified. The monoclonal antibodies were highly specific to SjTPx-1; this was demonstrated by a positive reaction to the parasite TPx-1 alone, and not to the mammalian orthologues, represented by MmPrx-1 and HsPrx-1. After the verification of the specific binding of the antibodies that were to be used, a DAS-ELISA was developed. The rabbit pAb was used as a capture antibody while the mouse mAb was used as a detection antibody. This method was able to detect at least 1 ng/ml of rSjTPx-1 antigen, which was comparable to the detection limits observed in previous studies using different circulating antigen targets. In addition, this method was able to detect the antigen from all serum samples from an experimentally infected rabbit and experimentally infected mice at 11 and 8 weeks after cercarial challenge. The diagnostic potential of SjTPx-1 in human clinical samples was also evaluated, in which 4 of 10 serum samples collected from stool-confirmed patients had detectable levels of SjTPx-1. The results suggest that this antigen has the potential to be used as a biomarker for *S. japonicum* infection.

The emergence of new schistosomiasis-endemic foci is a growing concern that is mainly attributed to human and animal migration. An effective screening method that is both sensitive and specific is urgently needed in order to prevent the introduction of

this disease in areas where the snail intermediate host is present. Molecular diagnostic methods, such as indirect ELISAs, are known to be highly sensitive and specific and to have the ability to detect early infection. An early diagnosis is beneficial as it enables patients to receive prompt medical attention, thereby preventing the progression of the disease. In the animal health sector, improved animal quarantine and treatment is expected. Thus, the development of a sensitive and specific method of diagnosis is required.

SjCatB is a protease expressed throughout the life stages of the parasite. This enzyme is highly expressed in the infective cercaria; thus, it is exposed to the host immune system at an early stage. In the present study, the full length coding sequence of SjCatB was cloned and expressed as a fusion protein containing a 6xHis-tag on the N-terminus. This recombinant antigen was tested for its performance in an indirect ELISA. rSjCatB showed 100% sensitivity and 95.0% specificity when the ELISA was used to test serum samples collected from experimentally infected mice at >8 weeks post-infection. From as early as 6 weeks post-infection, 2 of 4 mice were found to be positive in the ELISA. The recombinant antigen achieved 86.7% sensitivity and 96.7% specificity when the ELISA was used to test serum samples collected from patients with stool-confirmed infection. In addition, serum samples collected from humans infected with other parasites showed minimal cross-reactivity with rSjCatB. The results of the present study suggest that SjCatB is a highly sensitive and specific diagnostic antigen that can be applied in the early detection of *S. japonicum* infection. By taking advantage of the strengths of both antigen- and antibody-based serodiagnostic assays, I envision that these tools will improve the surveillance efforts that are used to assess the efficacy of control strategies, potentially leading to the successful elimination of the disease in endemic areas.

要　約

住血吸虫症は，世界 78 ヶ国で流行が認められる寄生虫病で，その被害がマラリア次いで大きい。全世界の 2 億 4000 万人がこの病気に感染し，そのうちの 2000 万人が重篤な症状に苦しんでいる。*Schistosoma japonicum* の感染による日本住血吸虫症は，1904 年に日本で報告され，現在は，中国，フィリピン，およびインドネシアの一部で流行が認められる。世界保健機関（WHO）は，この病気を顧みられない熱帯病に指定して，積極的な対策を推進している。虫卵を含む糞便が中間宿主貝（*Oncomelania hupensis*）が棲息する水系に排泄されると，ふ化したミラシジウムがこの貝に感染して，終宿主への感染源となるセルカリアへと発育する。ウシ，スイギュウ，イヌ，および，ブタなどの家畜も *S. japonicum* の終宿主となり，このことが，日本住血吸虫症の Elimination（排除）を難しくしている。住血吸虫症の患者は，血管内に寄生する成虫が産卵する虫卵が肝臓をはじめとする様々な臓器に栓塞して虫卵結節を形成することで，肝硬変をはじめとする様々な症状を呈する。この病気の診断には，糞便中に排出される虫卵を光学顕微鏡で検出する，セロハン厚層塗抹法が一般に用いられている。一方，治療には Praziquantel（PZQ）が第一選択薬として用いられ，この薬による流行地住民への集団投薬（mass drug administration: MDA）が，住血吸虫症の流行が認められる国と地域での主要な寄生虫病対策になっている。WHO の指導のもと，PZQ による MDA が積極的に推し進められた結果，いくつかの流行地において，この病気の有病率が著しく低下した。これら低度流行地では，セロハン厚層塗抹法に替わる，より高感度・高精度の診断法の導入が必要になっている。そこでこの研究では，これら診断法の開発に応用する寄生虫抗原を検討した。

第一章では，有病期の患者を効率的に診断するため，成虫が患者血液中に分泌する抗原の，抗原検出に基づく血清診断法での有用性を評価した。日本住血吸虫の排泄・分泌抗原（excretory secretory antigen）から，分泌抗原のひとつ，Thioredoxin peroxidase-1（SjTPx-1）を選択して，組換え体 SjTPx-1（rSjTPx-1）を作製した。rSjTPx-1 をウサギおよびマウスに免疫して，それぞれ，抗血清およびモノクローナル抗体を作製した。これらの抗体が，マウスおよびヒトの TPx-1 オルソログ（mPrx および hPrx）と反応しないことを，それぞれの組換え体を抗原として，Western Blotting で確認した。また，これらの抗体が，日本住血吸虫由来の TPx-1 抗原と反応することも，成虫ライセートを抗原として，Western blotting で確認した。SjTPx-1 の標的抗原としての有用性を評価するため，ウサギ抗血清を固相用抗体（捕捉抗体），マウスモノクローナル抗体を検出抗体として，サンドイッチ酵素抗体法（ELISA）を構築した。この ELISA での rSjTPx-1 の検出限界は 1 ng/ml であった。これは，これまでに報告されている，他の分泌抗原を標的とする診断法の検出限界とほぼ同じ値であった。この ELISA を用いて，セルカリアの経皮感染後 11 週および 8 週後のウサギおよびマウス血清から SjTPx-1 の検出を試みたところ，全検体で陽性の結果が得られた。また，この ELISA を用いて，セロハン厚層塗抹法で日本住血吸虫症と診断された有病期の患者血清 10 検体から SjTPx-1 の検出を試みたところ，4 検体が陽性であった。これらの成績から，SjTPx-1 が抗原検出法の標的抗原として有用であることが示唆された。

第二章では，感染初期の患者を診断するため，セルカリアが患者への経皮感染時に分泌する抗原の，抗体検出に基づく血清診断法での有用性を評価した。全発育ステージで発現が認められるタンパク質の中から，セルカリアで特に発現量の多いプロテアーゼ，Cathepsin B（SjCatB）を選択して，組換え体 SjCatB（rSjCatB）を作製した。SjCatB 抗原の有用性を間接 ELISA 法で評価したところ，セルカリアの経皮感染 8 週後のマウス血清に対する rSjCatB の陽性検出の感度は 100%であった。また，正常マウス血清に対する陰性検出の特異性は 95.0%であった。また，実験感染マウスの 4 頭中 2 頭において，セルカリア感染の 6 週後から rSjCatB に対する特異抗体が検出された。セロハン厚層塗抹法で日本住血吸虫症と診断された有病期の患者血清に対する rSjCatB の陽性検出の感度は 86.7%であった。また健康者血清に対する陰性検出の特異性は 96.7%であった。rSjCatB は，調べた日本住血吸虫症以外の寄生虫症患者血清とは，著明な交差反応を示さなかった。これらの成績から，rSjCatB が，日本住血吸虫症を早期に診断する，抗体検出法の標的抗原として有用であることが示唆された。

これらの寄生虫抗原を応用した血清診断法の開発と導入によって，日本住血吸虫症の監視と排除に向けた寄生虫病対策が更に進展することが予想される。

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