



Detection of canine *Schistosoma japonicum* infection using recombinant thioredoxin peroxidase-1 and tandem repeat proteins

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ABSTRACT. Humans and dogs live very close together and share various pathogens causing zoonotic parasitoses like schistosomiasis. A previous population genetics study done for schistosomes in the Philippines suggested that there is a high transmission level of *Schistosoma japonicum* among humans and dogs proving that the latter are important reservoirs for this zoonotic parasite. A more sensitive and specific test detecting schistosome infection in dogs will therefore strengthen the zoonotic surveillance, which might help in the possible elimination of this ancient disease. In this study, recombinant thioredoxin peroxidase-1 (SjTPx-1) and tandem repeat proteins (Sj1TR, Sj2TR, Sj4TR, Sj7TR) previously tested on human and water buffalo samples were used to assess its diagnostic applicability to dogs. Fifty-nine dog serum and stool samples were collected in the schistosomiasis-endemic municipalities of Calatrava, Negros Occidental and Catarman, Northern Samar in the Philippines and examined using the ELISA as compared to microscopy and fecal sample-based PCR. Samples positive for *Babesia gibsoni* and *Dirofilaria immitis* were also used to check for cross-reaction. Results showed that SjTPx-1 (80% sensitivity, 92.3% specificity) and Sj7TR (73.3% sensitivity, 92.3% specificity) have good potentials for diagnosing *S. japonicum* infection in dogs. These diagnostic antigens will therefore improve the surveillance in the transmission of the parasites from dogs to humans.

KEY WORDS: diagnosis, recombinant antigen, *Schistosoma japonicum*, veterinary public health, zoonotic schistosomiasis

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Domesticated animals are known to significantly contribute to the transmission of zoonotic schistosomiasis caused by *Schistosoma japonicum* [11, 18]. In several studies done in different endemic areas, cats, dogs, pigs, cattle and water buffaloes were found to be potential hosts for *S. japonicum* [6, 7, 19]. Among these animals, cattle and water buffaloes are the ones usually implicated as important reservoir hosts for schistosomiasis [14, 24]. However, a population genetics study done on *S. japonicum* revealed that there was a high level of transmission between humans and dogs indicating the importance of dogs as reservoir hosts of the parasite [21]. In addition, dogs are also known to have the highest number of schistosome eggs shed per female worm per day as compared to other animals like cattle and water buffaloes [13]. Experimental dogs have been shown to establish worm infection after a single exposure at a rate of 50 to 77% [13]. Dogs, therefore, should also be considered as an important source of human schistosomiasis and as such, should become an integral part of animal surveillance.

Animal surveillance is not generally done for schistosomiasis in endemic areas which might hinder the elimination of this parasitic disease. Identification of schistosome-infected animals like dogs requires a more accurate and precise diagnostic test.

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Improvement of current techniques should be done to increase the sensitivity and specificity of animal surveillance. One of the strategies that can be used is by developing serological tests through the use of recombinant proteins.

Diagnostic recombinant proteins have only been tested for humans, cattle and water buffaloes but not for dogs. In our previous studies, 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 [2] and water buffalo sera [1]. Results of these studies showed that SjTPx-1 was a good diagnostic antigen for both while Sj7TR was better for humans and Sj1TR for water buffaloes. In this study, we examined the immunodiagnostic potentials of these recombinant proteins in detecting schistosome infection in dogs using enzyme-linked immunosorbent assay (ELISA). The diagnostic potential of these recombinant proteins in ELISA were also compared with fecal sample-based PCR assay and the conventional Soluble Egg Antigen (SEA)-ELISA.

MATERIALS AND METHODS

Fecal and serum collection

Fecal and serum samples were taken from 59 dogs in two schistosomiasis endemic municipalities in the Philippines namely Calatrava, Negros Occidental (n=43) and Catarman, Northern Samar (n=16). Fecal samples were collected and placed in code-labeled cups and stored with 10% neutralized formalin until processing. Using the formalin-ether concentration technique and Kato-thick technique, microscopy was initially done on the stool samples revealing three *S. japonicum* egg-positive samples. Sera from 31 dogs found in non-endemic area of Hokkaido, Japan were collected for the calculation of the cut-off values for ELISA. Dog serum samples collected in Japan tested positive for *Babesia gibsoni* (n=8) using blood PCR and for *Dirofilaria immitis* (n=10) using serum antigen test were also evaluated in this study to check whether cross-reaction might occur between these parasites and the schistosome antigens used. These two parasites have also been reported among dogs in the Philippines [8, 22, 25]. However, other dog-infecting trematodes such as the one causing canine schistosomiasis in the North America, *Heterobilharzia americana*, [15] are not found in the Philippines. These dogs were without any risk of contracting *S. japonicum* infection. All the owners of the dogs were informed about the study and gave consent for use of their dogs in this study. The present study was done according to the ethical guidelines for epidemiological studies provided by the ethical committees from Obihiro University of Agriculture and Veterinary Medicine (Permit No. 23-153 and No. 26-31).

Fecal sample-based PCR

Fecal sample-based PCR served as the standard test in this study. DNA extraction was done using QIAamp DNA Stool Mini Kit (QIAGEN Inc., Valencia, CA, U.S.A.) according to the manufacturer's protocol. Fecal DNA was also collected from a dog in schistosomiasis-free Obihiro, Hokkaido, Japan to serve as negative control. PCR was done on the fecal samples using a previously used primer set based on *S. japonicum* mitochondrial DNA sequence [10]. The primer pair 5'-GCC GTT ACG CTT AGA GCG-3' (forward) and 5'-CAT CCA AGC CGA TTA CCC-3' (reverse) amplified a region from the *cox2* gene at position 12878 to position 13120 within *nad6* with a size of 242 bp. A reaction mixture with a total volume of 20 μ l was prepared containing 2 μ l of PCR buffer, 0.6 μ l of 1.5 mM MgCl₂, 1.6 μ l of 2.5 mM dNTP, 0.4 μ l of each 20 pmol/ μ l primer, 0.2 μ l of 5 U/ μ l Taq DNA polymerase (Takara, Otsu, Japan) and 2 μ l of the DNA template. Using Veriti 96 Well Thermal Cycler (Applied Biosystems, Carlsbad, CA, U.S.A.). The PCR conditions used included an initial denaturation of 10 min at 95°C, followed by 45 cycles of 94°C for 30 sec denaturation, 63°C for 1 min annealing, 72°C for 1 1/2 min extension, and finished with a final extension of 72°C for 10 min. The PCR products were separated using electrophoresis in 1.5% agarose gel and then stained with ethidium bromide for visualization. PCR reactions were performed in triplicates for every fecal sample and a sample is regarded as PCR positive when at least one of the reactions showed the band corresponding to the target 242 bp. Stool DNA from a non-infected dog was used as negative control and *S. japonicum* DNA template extracted using Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, U.S.A.) [16] served as the positive control.

Recombinant antigen ELISA

After grouping the samples into PCR positives and PCR negatives, ELISA was then performed using the crude SEA and the recombinant proteins. For the SEA-ELISA, the crude antigen [4] and the recombinant proteins including SjTPx-1 and the four tandem repeat proteins (TRPs) [2] were prepared as previously described. In brief, cloning of SjTPx-1 was done using *S. japonicum* Yamanashi strain adult worm cDNA while the 4 TRPs' nucleotides coding a partial tandem repeat domain were synthesized by GenScript U.S.A. Inc. (Piscataway, NJ, U.S.A.). The ELISA was done as previously instructed [1] except that HRP-conjugated goat anti-canine IgG (ThermoScientific, Rockford, IL, U.S.A.) was used as the secondary antibody with 1:10,000 dilution. All the tests were done in triplicates and mean values were calculated. The cut-off value was calculated as the mean absorbance value of the 31 negative controls plus 3 standard deviations.

Statistical analysis

Sensitivity, specificity and predictive values were calculated to check the validity of the ELISA assays using the recombinant proteins against the stool PCR which as the reference standard test. Kappa value was used to estimate the agreement between the antigens and stool PCR [23].

RESULTS

Fecal sample-based PCR

Results showed that out of the 59 dog samples, 15 were found to be PCR positive including the 3 microscopy positive samples. As shown in Fig. 1, samples having the target band of approximately 242 bp were regarded as PCR positive samples whereas those which presented no band were considered as PCR negative samples. The band was also seen in the positive control but not in the negative control.

Recombinant antigen ELISA

ELISA results showed that SEA has the highest number of positive samples in the PCR negative samples with 7 out of 34 samples. Among the recombinant proteins, 3 were positive for SjTPx-1, Sj2TR and Sj7TR, 5 for Sj1TR and 2 for Sj4TR (Fig. 2A). On the other hand, 12 out of the 15 PCR positive samples were positive with SEA and SjTPx-1 (Fig. 2B). As for the TRPs, 11 of the stool PCR positive samples were positive for Sj7TR, 9 for Sj4TR, 8 for Sj1TR and 6 for Sj2TR. All of the 3 microscopy positive samples were also positive for SEA, SjTPx-1 and Sj7TR; and negative for Sj1TR, Sj2TR and Sj4TR. To assess specificity, 10 dog serum samples positive for *D. immitis* (Fig. 2C) and 8 positive for *B. gibsoni* (Fig. 2D) were also used. SEA showed high degree of cross-reaction with both groups of samples. The recombinant proteins SjTPx-1, Sj1TR, Sj4TR and Sj7TR only react with 1 of the *B. gibsoni* positive sera.

Statistical analysis

As shown in Table 1, SjTPx-1 showed the highest agreement with the fecal sample-based PCR based on the kappa values, followed by Sj7TR. The specificity and the positive predictive values of these 2 recombinant proteins were also higher than those of SEA whereas sensitivity is the same for both SjTPx-1 and SEA.

DISCUSSION

This study aims to find a recombinant antigen that can be used in the development of a more sensitive and specific diagnostic tool for zoonotic schistosomiasis in dogs. Results showed that SjTPx-1 and Sj7TR have the highest diagnostic potentials among the five recombinant antigens being evaluated.

Although the sensitivity of SjTPx-1 was comparable to that of the crude SEA, SEA cross-reacts with the *D. immitis* and *B. gibsoni* positive sera giving SjTPx-1 a higher diagnostic edge than SEA when it comes to specificity (92.3%) and positive predictive value (75.0%). Therefore, SjTPx-1 is a good antigen candidate for the development of a diagnostic test that can detect schistosome infection in multiple hosts as it was seen to have good immunodiagnostic potentials for humans [2], water buffaloes [1] and dogs in our previous and current studies.

Furthermore, results of our previous studies showed that Sj7TR has proven to work well with human samples while Sj1TR for water buffaloes. However in this study, the Sj7TR was shown to be better for canine diagnosis than Sj1TR. These differences in the antigenicity of the recombinant proteins used in these studies among the human and animal hosts can be attributed both

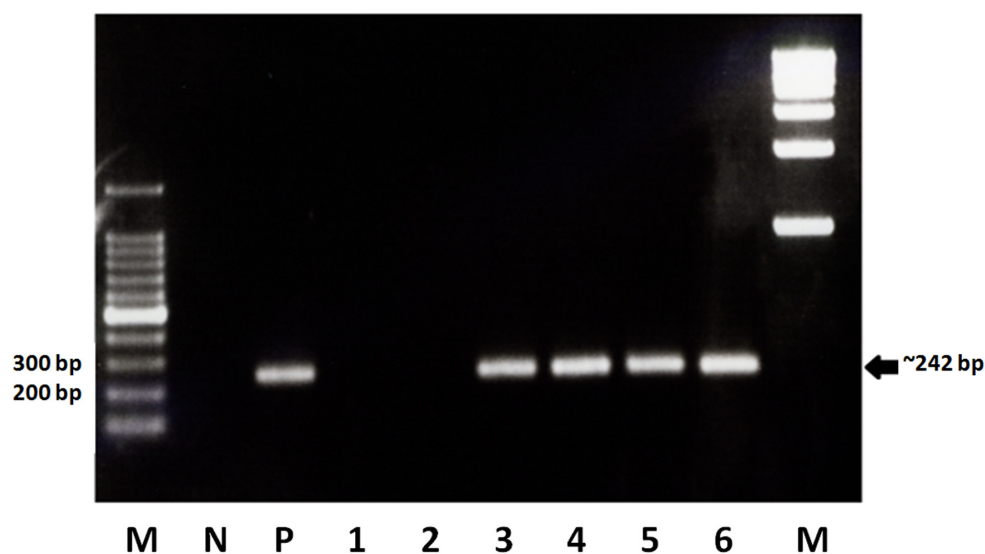


Fig. 1. Gel electrophoresis of the stool PCR for dogs targeting the *Schistosoma japonicum* mitochondrial *cox2-nad6* gene. M, marker. N, negative control: fecal DNA from non-infected dog. P, positive control: *S. japonicum* adult DNA template. Lanes 1–4, *Schistosoma* egg (–) dog stool sample. Lanes 5–6, *Schistosoma* egg (+) dog fecal sample. Positive control, lanes 3 to 6 show positive results with bands at ~242 bp while none is seen on the negative control, lanes 1 and 2.

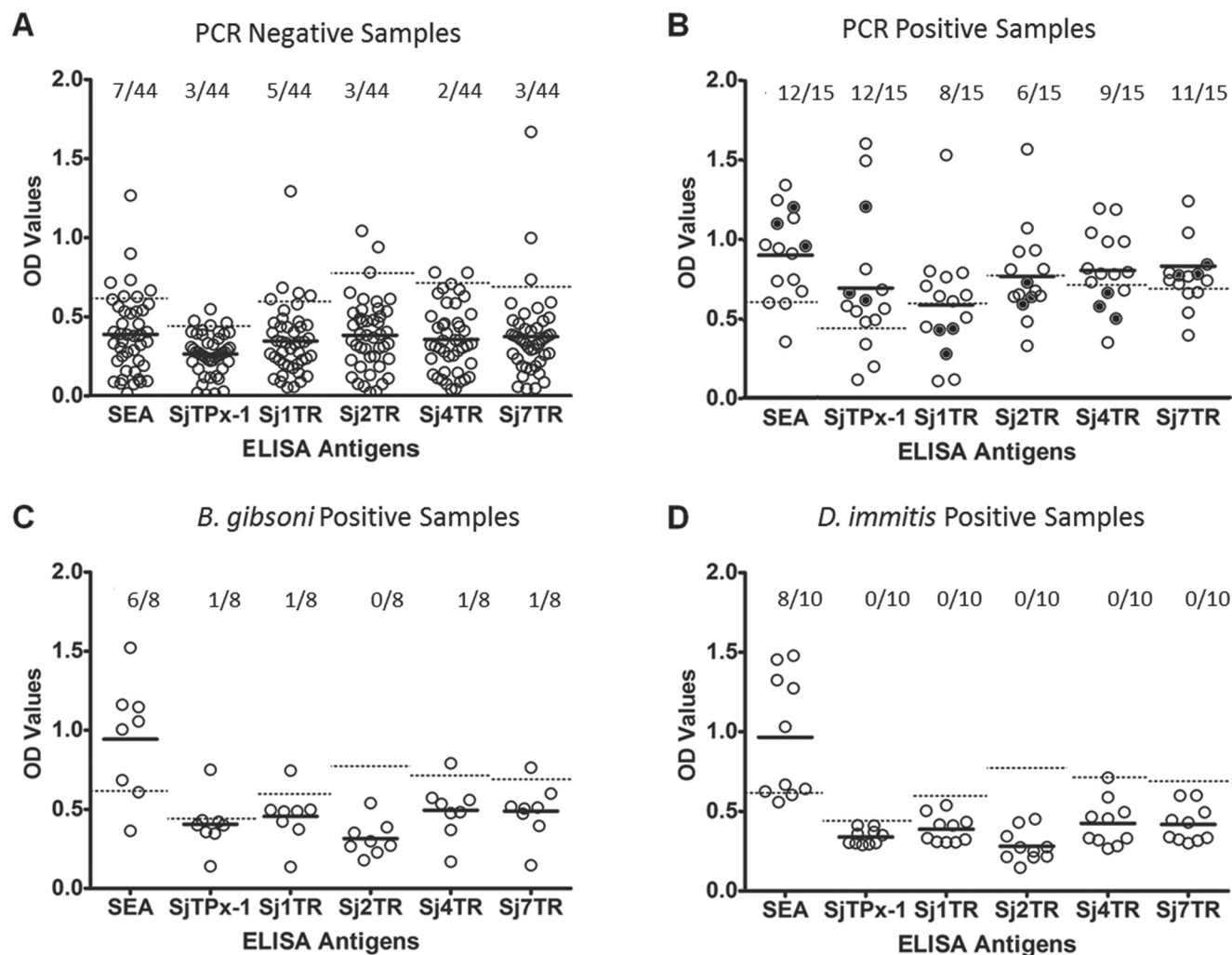


Fig. 2. ELISA results of the PCR-negative, PCR-positive samples and serum positive for *Dirofilaria immitis* and *Babesia gibsoni* using soluble egg antigen (SEA) and the recombinant proteins. The graph shows that a minimal number of PCR negative samples gave positive results in the ELISA with SEA having the highest. (A). SjTPx-1 and Sj7TR have the highest number of positives among the PCR positive samples (B). High percentage of cross-reaction was seen in SEA (C, D). White circles in B correspond to microscopy negative samples (n=12) whereas black circles to microscopy positive samples (n=3). Dotted lines represent the cut-off value while the solid line for the mean optical density (OD) value.

Table 1. Statistical results of soluble egg antigen (SEA) and the recombinant proteins.

| Antigen | Sensitivity (%) | Specificity (%) | *PPV (%) | +NPV (%) | Kappa |
|---------|-----------------|-----------------|----------|----------|-------|
| SEA | 80.0 | 59.6 | 36.4 | 100 | 0.278 |
| SjTPx-1 | 80.0 | 92.3 | 75.0 | 100 | 0.706 |
| Sj1TR | 53.3 | 88.5 | 57.1 | 94.9 | 0.428 |
| Sj2TR | 40.0 | 94.2 | 66.7 | 93.3 | 0.399 |
| Sj4TR | 60.0 | 94.2 | 75.0 | 96.4 | 0.584 |
| Sj7TR | 73.3 | 92.3 | 73.3 | 92.3 | 0.656 |

*PPV, positive predictive value; +NPV, negative predictive value.

to the host and the parasite. This diverse immunological background might be either due to the different survival mechanisms of the schistosome parasite when exposed to the immune system of the host; or to the host's immunological memory such as immunodominance of the primary response to the parasite's pool of antigens [9]. In our previous study, the immunolocalization of Sj7TR was hypothesized to be associated with the developing suckers in the young stages of the parasite expressed from the egg until schistosomula [3]; while Sj1TR is a dentin sialophosphoprotein precursor expressed in the adults [17]. The possibility that the dogs and humans react more with egg antigens such as Sj7TR whereas water buffaloes with the adult schistosome antigens like

Sj1TR could be a good study in the future.

Moreover, Sj7TR being a good diagnostic antigen for both humans and dogs might support the population genetics study done in one schistosomiasis-endemic area in the Philippines using *S. japonicum* microsatellite markers [21]. This previous study concluded that there is a high transmission level between dogs and humans. However, population genetics experiments with a wider coverage of endemic areas for dogs and water buffaloes should be done to really prove that dogs have significantly contributed to human schistosome infection more than the other reservoir hosts.

Dogs have been known to harbor pathogens that are zoonotic in nature. In various studies, they have been implicated as sources of different parasitic diseases such as soil-transmitted helminthiasis such as *Toxocara canis* [12] and *Ancylostoma caninum* [5] as well as tapeworm infection such as *Dipylidium caninum* [20]. Disease transmission is made possible and easier due to the close proximity of the pet dogs to their human owners. This study has therefore emphasized the importance of dogs as contributors to the environmental contamination of schistosome eggs and as silent sources of this parasitic disease to humans. This study also concludes that SjTPx-1 and Sj7TR can be useful for the diagnosis of canine *S. japonicum* infection. However, further assessment for its usefulness in field animal surveillance is needed to determine the full potential of these antigens.

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