EVALUATION OF RECOMBINANT SERINE PROTEASE INHIBITOR FROM *TRICHINELLA SPIRALIS* FOR IMMUNODIAGNOSIS OF SWINE TRICHINOSIS

Supaporn Nuamtanong, Paron Dekumyoy and Poom Adisakwattana

Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. Serine protease inhibitors, known as serpins, are mainly expressed in newborn and early-stage *Trichinella spiralis* larvae, suggesting that *T. spiralis* serpin (TsSERP) could be used as antigen for the immunodiagnosis of swine trichinosis. We produced His-tagged recombinant TsSERP (rTsSERP) in *Escherichia coli* and purified it using a Co^{2+}-affinity column. Western blot (WB) and enzyme-linked immunosorbent assay (ELISA) were performed to determine *T. spiralis*-infected swine sera samples ($n = 5$), negative controls ($n = 26$), and other parasite-infected samples ($n = 83$). WB showed that *T. spiralis*-infected sera initially reacted with rTsSERP at day 6 post-infection (dpi), and more strongly in late infection (62 and 84 dpi). However, other parasite-infected sera also elicited cross-reactivity to rTsSERP. On the other hand, indirect ELISA showed that TsSERP was an appropriate antigen for detecting late (>60 dpi) but not early infection. No cross-reaction was observed with other parasite-infected sera. Sensitivity and specificity of TsSERP-ELISA at 62 dpi was 80% and 100%, respectively, and at 84 dpi 100% and 100%, respectively. These preliminary results show that TsSERP-ELISA method is suitable for the diagnosis of swine trichinosis, and could become the standard test for diagnosis of trichinosis in several hosts, including humans.

Keywords: *Trichinella spiralis*, serine protease inhibitor, western blot, enzyme-linked immunosorbent assay, swine/pig

INTRODUCTION

Trichinella spiralis, a parasitic nematode, causes trichinosis in a wide variety of vertebrate hosts. Infection occurs when host ingests raw or undercooked meat containing infective larvae. Most cases of human trichinosis are acquired after ingestion of *T. spiralis*-infected domesticated or wild animals (Gottstein *et al*, 2009). In order to eliminate the disease, control programs have been established globally, especially for farmed pigs, which must be tested prior to import and export (van Knapen, 2000; Pyburn *et al*, 2005).

Swine trichinosis can be diagnosed by direct and indirect methods. For direct detection, tissue compression and digestion is simple and cost-effective, but has low sensitivity (van Knapen *et al*, 1981; Gamble, 1998). Consequently, immuno-

Correspondence: Dr Poom Adisakwattana, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Ratchathewi, Bangkok 10400, Thailand.
Tel: +66 (0) 2643 5600
E-mail: tmpad@mahidol.ac.th

logical techniques have been developed in the last few decades to detect circulating anti-*Trichinella* antibodies, including latex agglutination (Innella and Redner, 1959), western blotting (WB) (Gruden-Movsesijan *et al*, 2002; Frey *et al*, 2009; Nöckler *et al*, 2009), indirect immunofluorescent (IIF) assay (Issa and el Serougi, 1998), and enzyme-linked immunosorbent assay (ELISA) (Smith, 1987; Moller *et al*, 2005; Korinkova *et al*, 2008; Moskwa *et al*, 2009; Nöckler *et al*, 2009). Along with correct immuno-detection techniques, the type of antigens used in immunodiagnosis also plays a significant role. Crude L3 extracts (CLEs) show high cross-reactivity with other parasitic infections (Au *et al*, 1983; De-la-Rosa *et al*, 1995). Excretory-secretory (ES) products of muscle larvae have been used as antigens to detect swine trichinosis yielding higher specificity than CLEs (Gamble *et al*, 1983, 1988; Smith, 1987; Mahannop *et al*, 1992; Korinkova *et al*, 2008). However, the results are strongly dependent on the quality of ES products, which requires proper methods for cultivating the parasites. Alternatively, recombinant antigens, such as 49 and 53 kDa proteins, have been utilized in order to improve reliability of the assay (Su *et al*, 1991; Jung *et al*, 2007).

T. spiralis serine protease inhibitor, TsSERP, is a secretory protein produced by infective-stage larvae (Nagano *et al*, 2001). It is expressed abundantly in newborn and muscle larvae at day 18 post-infection (dpi). Previous studies have suggested that TsSERP reacts with *T. spiralis*-infected mouse serum at month 2 post-infection (Nagano *et al*, 2001). In a related species, filarial serpin elicits strong immune responses in both cellular and humoral immunity (Zang *et al*, 2000). These findings suggest that TsSERP might also be a good candidate for diagnosis of trichinosis in swine and other hosts.

The aim of this study was to evaluate the potential of TsSERP for use in serodiagnosis of swine trichinosis. Plasmid containing TsSERP cDNA was constructed and heterologously expressed in a prokaryotic system. The purified recombinant protein was evaluated for its ability to diagnose swine trichinosis by WB and ELISA.

MATERIALS AND METHODS

Parasites

Muscle-stage *T. spiralis* larvae were prepared by digestion in order to release larvae from the striated muscles of ICR mice as previously described (Gamble, 1996). About 100 *T. spiralis* larvae were infected orally into 6-8 weeks-old female ICR mice and maintained for 2 months in the Laboratory of Animal Science Center, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand in accordance with the guidelines of the Faculty of Tropical Medicine Animal Care and Use Committee (FTM-ACUC 004/2004).

Production of recombinant TsSERP

Muscle-stage *T. spiralis* larvae were washed several times with 0.01 M phosphate-buffered saline pH 7.4 (PBS) and stored at -70ºC until used. Total RNA isolation was performed by homogenizing parasites in TriZol reagent (Invitrogen, Carlsbad, CA) and extracting according to the manufacturer's instructions.

RNA (5 g) of muscle-stage *T. spiralis* was used to generate 1st strand cDNA as follows. RNA was mixed with 0.5 mM of each dNTP (Fermentas, Glen Burnie, MD) and 100 pmol of oligo dT primer. The mixture was heated at 65ºC for 5 minutes and rapidly cooled on ice. Then, 1x Reverse Transcriptase buffer and 200 U RevertAid

MMuLV reverse transcriptase (Fermentas) were added and the solution incubated at 42ºC for 1 hour before terminating by heating at 70ºC for 5 minutes. Double-stranded cDNA was synthesized by PCR in a reaction volume of 25 l containing 1x Taq buffer, 0.2 mM of each dNTP, 1 M TsSERP forward and reverse primers (see below), 2 mM $MgCl_2$, and 1 U *Taq* polymerase (Fermentas). Thermal cycling was performed at 94ºC for 5 minutes; followed by 30 cycles of 94ºC for 1 minute, 52ºC for 1 minute, 72ºC for 1 minute, and a final heating at 72ºC for 5 minutes.

Primers were designed from the mRNA sequence (accession no. AF231948) of *T. spiralis* TsSERP obtained from NCBI database (http://www.ncbi.nlm.nih.gov) for heterologous expression of the full-length recombinant protein *Escherichia coli* expression system. The forward and reverse primer was 5′-GGGCATGCATGGAAACAGAAATTGCAAAAC and 5′-CCCTGCAGTTAATTACCAGAAAAACGTCCAAT, respectively, incorporating *Bam*HI and *Xho*I restriction sites at the 5′ terminus (underlined).

TsSERP cDNA was purified by agarose gel-electrophoresis using a gel purification kit (Geneaid, New Taipei City, Taiwan), and inserted into pQE30 expression vector (Qiagen, Hilden, Germany) at *Bam*HI (Fermentas) and *Xho*I (Fermentas) cloning sites. Recombinant plasmid was used to transform competent *E. coli* M15 by heat-shock technique, and transformants were selected by 100 g/ml ampicillin and 25 g/ml kanamycin, and the correct insert was confirmed by DNA sequencing (AIT biotech, Singapore).

Expression of the recombinant protein was induced with 1 mM isopropyl-thiogalactoside (IPTG) (Fermentas) for 4 hours. Bacteria were harvested by centrifugation at 6,000*g* at 4ºC for 30 minutes, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole) and disrupted by sonication (Heat Systems, Farmingdale, NY). Supernatant from centrifugation at 12,000*g*, 4ºC for 30 minutes was stored at -70ºC until used. For purification, bacterial lysates were incubated with Talon Co^{2+} resin (Clontech, Mountain View, CA) at 25ºC for 1 hour with agitation. rTsSERP was eluted from resin with an imidazole gradient from 20 to 250 mM. Fractions were analyzed using 12% SDS-PAGE under reducing condition (Laemmli, 1970), and stained with Coomassie brilliant blue G-250 (USB Corp, Cleveland, OH). Fractions containing rTsSERP were pooled and dialyzed against PBS for 16-18 hours at 4ºC. Protein concentration was determined using a Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, Barrington, IL).

Swine sera

T. spiralis- and other parasite-infected pig sera were provided by the Parasitology Section, Department of Livestock Development, National Institute of Animal Health (NIAH), Bangkok, Thailand. All cases were confirmed by microscopic examination (Table 1). The negative control group was categorized as healthy, without helminth eggs, larvae, or protozoa in stool or *T. spiralis* larvae in muscle. *T. spiralis*-infected sera (positive control) were prepared by oral inoculation of 1,000 muscle-stage *T. spiralis* into 5 healthy pigs with no history of parasitic infection, and animals were bled before infection [0 day post-infection (dpi)] and on 6, 12, 28, 62, and 84 dpi.

Western blot analysis

rTsSERP separated by 12% SDS-PAGE under reducing condition was blotted

Table 1
Parasite-infected pig sera and diagnosis criteria.

Disease	No. of cases	Diagnosis[a]
Trichinosis	5	(-) SE, (+) TC
Ascariasis	3	(+) SE, (-) TC
GI nematode infection	4	(+) SE, (-) TC
Trichuriasis	12	(+) SE, (-) TC
Balantidiasis	1	(+) SE, (-) TC
Coccidiasis	29	(+) SE, (-) TC
Cryptosporidiasis	1	(+) SE, (-) TC
Ascariasis, GI nematode infection	3	(+) SE, (-) TC
Coccidiasis, GI nematode infection	9	(+) SE, (-) TC
Coccidiasis, strongyloidiasis	2	(+) SE, (-) TC
Coccidiasis, trichuriasis	10	(+) SE, (-) TC
Coccidiasis, cryptosporidiasis	2	(+) SE, (-) TC
Coccidiasis, ascariasis, GI nematode infection	4	(+) SE, (-) TC
Coccidiasis, strongyloidiasis, GI nematode infection	2	(+) SE, (-) TC
Coccidiasis, strongyloidiasis, GI nematode infection, cryptosporidisis	1	(+) SE, (-) TC
Negative control	26	(-) SE, (-) TC
Total	114	

[a] SE, stool examination; TC, tissue compression.

onto polyvinylidene difluoride (PVDF) membrane. Western blot was performed as previously described, with some modification (Adisakwattana *et al*, 2007). In brief, membrane was incubated with blocking solution [5% skimmed milk dissolved in PBS containing 0.05% Tween 20 (Sigma, St Louis, MO)] and incubated with (1:200 dilution) pig serum. Then, goat anti-porcine IgG (H+L) antibodies, conjugated with horse radish peroxidase (HRP) (Southern Biotech, Birmingham, AL) (1:1,000 dilution) were added to the membrane and incubated for 1 hour at 25ºC. Finally, bands were visualized by adding colorimetric substrate, 2,6 dichlorophenol indophenol (Sigma).

ELISA

One hundred l aliquots of rTsSERP (2.5 g/ml of 0.05 M carbonate buffer pH 9.6) were coated into each well of a microtiter plate and incubated at 37ºC for 16-18 hours. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST), and incubated with 100 ml of blocking solution [0.5% bovine serum albumin (BSA), 0.2% gelatin in PBST] at 37ºC for 1 hour. Then, pig serum (1:200 dilution) was added to each well and incubated at room temperature (RT) for 1 hour, followed by goat anti-porcine IgG (H+L) antibodies conjugated with HRP (Southern Biotech) (1:5,000 dilution), and further incubated at RT for 1 hour. A colorimetric reaction was developed with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma) diluted in citrate buffer, pH 4.5 at 37ºC for 30 minutes. Reaction was terminated by adding 1% SDS and absorbance at 405

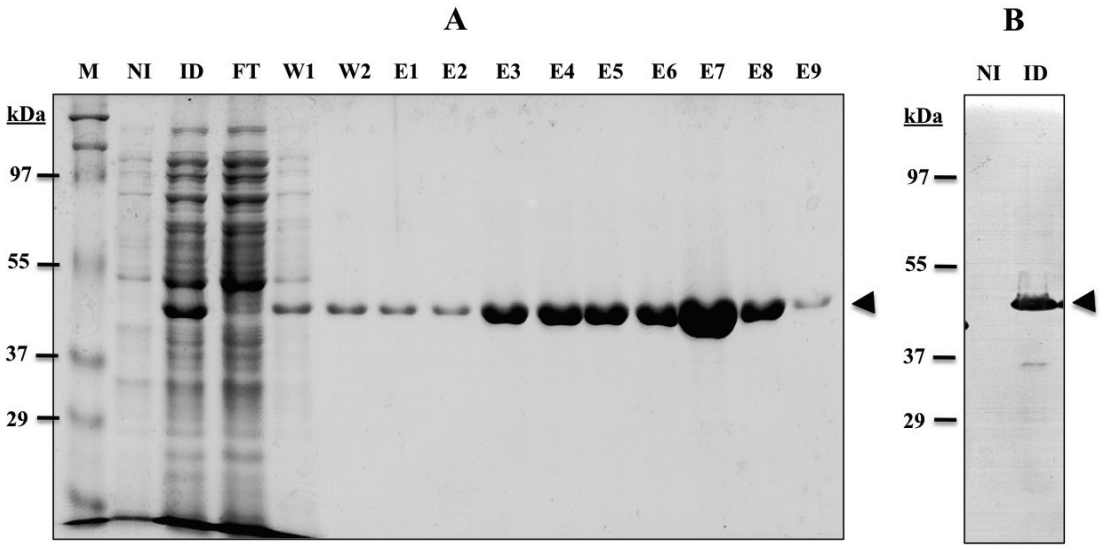

Fig 1–Expression and purification of rTsSERP analyzed by 12% SDS-PAGE (A) and western blotting (B). Recombinant TsSERP was expressed in *E. coli* M15 and purified using Co^{2+} affinity column. All fractions were then analyzed on 12% SDS-PAGE and stained with Coomassie brilliant blue G-250. Purified rTsSERP was blotted onto PVDF membrane and reacted with anti-His tag antibodies. (A) M, molecular weight standard; NI, non IPTG-induced bacterial lysate; ID, IPTG-induced bacterial lysate; FT, flow through from Talon Co^{2+} affinity column; W1-2, flowthrough, E1-9, eluted fractions. (B) NI, non IPTG-induced bacterial lysate; ID, IPTG-induced bacterial lysate. rTsSERP (45 kDa) is indicated by arrowhead.

nm was measured in a microplate reader (Sunrise Basic Tecan, Grödig, Austria). All samples were analyzed in triplicate.

Analysis of sensitivity and specificity of ELISA

Negative and positive *T. spiralis*-infected samples were distinguished by a cut-off value, calculated as the average $OD_{405\ nm}$ of sera from negative control group, plus twice the SD of this group. Specificity and sensitivity were calculated as described previously (Cornelissen *et al*, 2001).

RESULTS

Heterologous expression and characterization of rTsSERP

The cDNA of full-length TsSERP was cloned into pQE30 prokaryote expression vector and heterologously expressed in *E. coli* M15. After affinity purification on Talon resin, a single protein of 45 kDa was obtained as analyzed by 12% SDS-PAGE (Fig 1A), which was reactive with anti-His-tag antibodies by western blot analysis (Fig 1B).

Western blotting analysis

Western blot analysis of rTsSERP with *T. spiralis*-infected mouse (Fig 2A) and pig sera (Fig 2B) exhibited positive reactivity, but not with pre-immune swine serum. Infected swine sera initially detected rTsSERP at 6 dpi, and more strongly at 62 and 84 dpi (Fig 2C). Other parasite-infected sera were used to determine specificity, which showed cross-reaction (Fig 2D). Thus, an indirect ELISA method was developed.

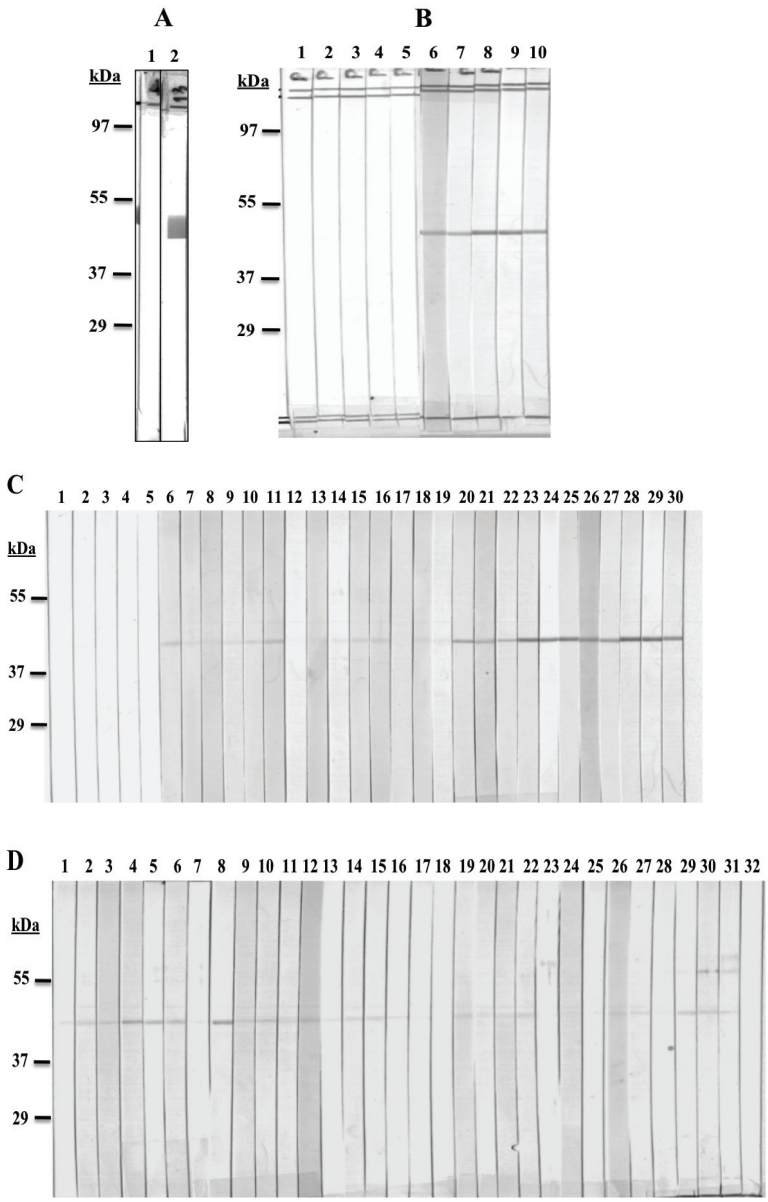

Fig 2–Western blotting analysis of TsSERP with parasite-infected sera. (A) *T. spiralis*-infected mouse serum. Lane 1, pre-immune serum; lane 2, 8 week post-infected serum. (B) *T. spiralis*-infected pig sera. Lanes 1-5, pre-immune sera of 5 pigs; lanes 6-10, 62 days post-infected sera of 5 pigs. Molecular weight of standards is shown on the left. (C) *T. spiralis* infected sera from pigs. Lanes 1-5, pre-infection; lanes 6-10, 6 days post-infection (dpi); lanes 11-15, 12 dpi; lanes 16-20, 28 dpi; lanes 21-25, 62 dpi; lanes 26-30, 84 dpi. (D) Other parasite-infected pig sera. Lanes 1-3, ascariasis; lanes 4-7, GI nematode infection; lanes 8-12, trichuriasis; lane 13, balantidiasis; lanes 14-18, coccidiasis; lane 19, cryptosporidiasis; lanes 20-21, ascariasis/GI nematode infection; lanes 22-23, coccidiasis/cryptosporidiasis; lanes 24-25, coccidiasis/strongyloidiasis; lanes 26-27, coccidiasis/GI nematode infection/strongyloidiasis; lane 28, coccidiasis/GI nematode infection/strongyloidiasis/cryptosporidiasis; lanes 29-32, negative control (healthy pigs).

Table 2
Sensitivity and specificity of TsSERP- ELISA for detection of parasite-infected pigs

T. spiralis infection	FN	TP	Sensitivity (%)	FP	TN	Specificity (%)
0 dpi	5/5	0/5	0	0/109	109/109	100
6 dpi	5/5	0/5	0	0/109	109/109	100
12 dpi	5/5	0/5	0	0/109	109/109	100
28 dpi	3/5	2/5	40	0/109	109/109	100
62 dpi	1/5	4/5	80	0/109	109/109	100
84 dpi	0/5	5/5	100	0/109	109/109	100

FN, false negative; TP, true positive; FP, false positive; TN, true negative

ELISA analysis

A checkerboard titration was performed to optimize suitable dilutions of primary and secondary antibodies. The optimal dilution for swine sera and conjugated secondary antibodies was 1:200 and 1:5,000, respectively. The cutoff point was determined by plotting the frequency distribution of negative and positive control groups as a scatter plot (Fig 3A). The mean value at $OD_{405\ nm}$ for the negative control group was 0.33 (SD = 0.12); this value plus twice the SD was used as the cutoff point for this study. A sera from pigs in late *T. spiralis* infection (62 and 84 dpi) showed reactivity with OD values > cutoff point, but not in cases at 62 dpi or during early infection (6, 12 and 28 dpi) (Fig 3A).

To determine specificity, sera from other parasite-infected farm pigs were analyzed. No swine sera infected with other parasites cross-reacted to TsSERP at the cutoff value (Fig 3B). These results were used to calculate the sensitivity and specificity of the test. For late *T. spiralis* infection, sensitivity and specificity was 80% and 100%, respectively at 62 dpi and 100% and 100%, respectively at 84 dpi (Table 2).

DISCUSSION

Reliable diagnostic tools for swine trichinosis have been continuously studied and developed. Immunological diagnosis is widely used to detect trichinosis, replacing tissue compression and digestion (Nöckler *et al*, 2000). The advantages of immunological methods are their high sensitivity and cost-effectiveness when analyzing large numbers of samples. However, the accuracy of the tests depends on the type of antigen used. Use of ES products affords high sensitivity and specificity in the detection of *T. spiralis*-infected pigs, but the results rely on the quality of each ES batch (Gamble *et al*, 1988).

In order to improve the immunodetection of this disease, we used recombinant antigen produced by genetic engineering techniques. TsSERP was selected as the candidate, as it is expressed and secreted from *T. spiralis* muscle-stage larvae in early infection (Nagano *et al*, 2001). The recombinant protein was detected by *T. spiralis*-infected mouse serum using western blot analysis and could be detected with sera from infected pigs,

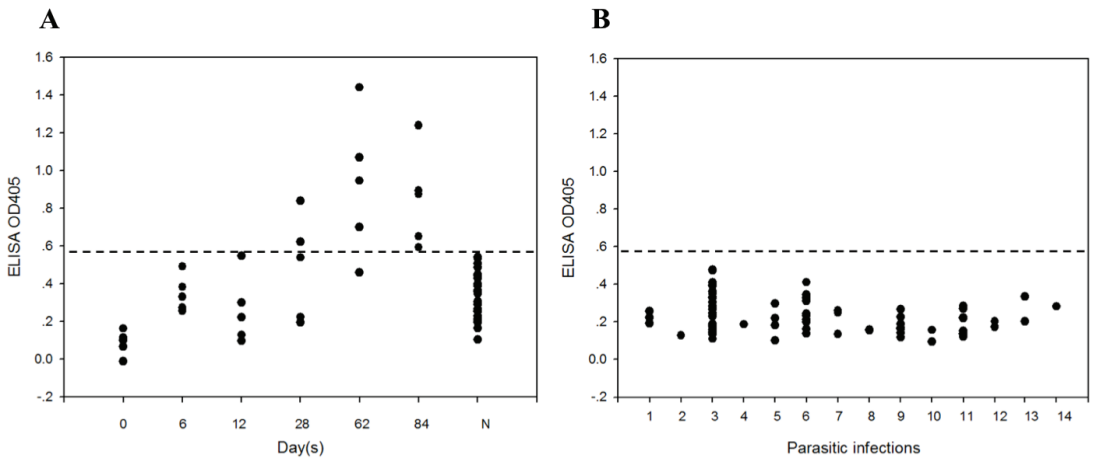

Fig 3–Evaluation of TsSERP-ELISA with sera from farmed non-infected and *T. spiralis*-infected pigs. (A) Analysis of sera obtained from *T. spiralis*-infected sera at different time points (0, 6, 12, 28, 62, and 84 days post-infection) and negative control (N). Dashed line indicates cut-off value of 0.57. (B) Analysis of 83 sera from other parasite-infected pigs. Lane 1, Ascariasis; lane 2, balantidiasis; lane 3, coccidiasis; lane 4, cryptosporidiasis; lane 5, GI nematode infection; lane 6, trichuriasis; lane 7, ascariasis/GI nematode infection; lane 8, coccidiasis/cryptosporidiasis; lane 9, coccidiasis/GI nematode infection; lane 10, coccidiasis/strongyloidiasis; lane 11, coccidiasis/trichuriasis; lane 12, coccidiasis/ascariasis/GI nematode infection; lane 13, coccidiasis/GI nematode infection/strongyloidiasis; lane 14, coccidiasis/GI nematode infection/strongyloidiasis/cryptosporidiasis. Dashed line indicates cut-off value of 0.57.

with similar results to those described by Nagano *et al* (2001). Western blotting analysis of *T. spiralis*-infected pig sera at different stages of infection showed that TsSERP was detected in the late than the early stages of infection. However, pig sera from animals with other parasite-infection also reacted weakly with TsSERP. This cross-reaction with other parasites may be due to recognition of conserved amino acid residues of the serpin family of proteins. Thus use of TsSERP for western blot analysis is inappropriate for immunodiagnosis of *T. spiralis* infection in pigs.

The current study therefore evaluated ELISA instead of WB for diagnosis of trichinosis in pigs. Negative control sera, including other parasite-infected sera, yielded low reactivity (below a cut-off value of $OD_{405\,nm}$ 0.568) against rTsSERP, indicating high specificity of this method. In the positive control group, TsSERP-ELISA was better for detection of late rather early infection. It was expected that TsSERP would stimulate specific antibodies in the early or intermediate phases of infection, as the protein is secreted from hatching of the newborn (Nagano *et al*, 2001). Lower ELISA value in early infection can be explained in terms of parasite life cycle. Harley and Gallicchio (1971) showed that newborn were first produced on day 5 after infection and production peaked on day 9, suggesting that the prepatent period before serological positivity may be approximately 10 days, in agreement

with the negative result of TsSERP-ELISA in early infection (6 and 12 dpi) as shown in our data. However, delayed antibody production against ES of *T. spiralis* was observed by Beiting *et al* (2004) which may be due to the immunosuppressive machinery of *T. spiralis* ES, including serpin. Investigations of Myxoma viral serpin suggested that the protein inhibits both cellular and humoral host immunity thereby preventing elimination (Viswanathan *et al*, 2006, 2009). Nevertheless, ELISA of late infection showed 100 % sensitivity and specificity. However, the number of positive control sera should be increased to confirm sensitivity, and the number of cases of other parasitic infections should be increased, especially different species of *Trichinella* helminths, to confirm specificity.

Previous studies showed that ES and purified TSL-1 are useful in serodiagnosis of trichinosis (Escalante *et al*, 2004; Bolas-Fernandez and Corral Bezara, 2006). However, the antigen preparation and purification procedures are complicated, time consuming, and costly. Use of recombinant antigens in serodiagnosis is rapid and simple, and the quality of antigens in each batch can be controlled. However, the sensitivity and specificity for each antigen need to be evaluated in order to optimize their actual potential. For instance, recombinant 53 kDa protein generated in a baculovirus expression system and used in an indirect ELISA method showed low sensitivity, even in the late stages of swine trichinosis infection (Jung *et al*, 2007).

In summary, we showed that rTsSERP can be used in ELISA in the surveillance of *T. spiralis* infection of farmed pigs, and in epidemiology of this disease among wild boars. TsSERP-ELISA should be established as a diagnostic method for human trichinosis as well.

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