Tropical Biomedicine 36(3): 792–802 (2019)

Primary assessment of a *T. spiralis* putative serine protease for early serological detection of experimental trichinellosis

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Received 18 June 2018; received in revised form 15 April 2019; accepted 19 April 2019

Abstract. A putative serine protease of *T. spiralis* (TsSP) was expressed in *Escherichia coli* and its potential as a diagnostic antigen was primarily assessed in this study. Anti-*Trichinella* IgG in serum samples from *T. spiralis* different animal hosts (mice, rats, pigs and rabbits) were detected on Western blot analysis with rTsSP. Anti-*Trichinella* antibodies were detected in 100% (30/30) of experimentally infected mice by rTsSP-ELISA. Cross-reactions of rTsSP-ELISA were not found with sera from mice infected with other parasites (*S. erinaceieuropaei*, *S. japonicum*, *C. sinensis*, *A. cantonensis* and *T. gondii*) and sera from normal mice. There was no statistical difference in antibody detection rate among mice infected with the encapsulated *Trichinella* species (*T. spiralis*, *T. nativa*, *T. britovi*, and *T. nelsoni*) (P>0.05). The results of rTsSP-ELISA showed that serum specific antibody IgG in mice infected with 100 or 500 *T. spiralis* muscle larvae (ML) were detectable early at 7-8 dpi, but not detected by ML ES antigen-ELISA prior to 10-12 dpi. Specific anti-*Trichinella* IgG was detected in 100% (18/18) of infected pigs by rTsSP-ELISA and ES-ELISA, but no specific antibodies was not detected in 20 conventionally raised normal pigs by two antigens. The results showed the rTsSP had the potential for early serodiagnosis of animal *Trichinella* infection, however it requires to be assayed with early infection sera of swine infected with *Trichinella* and other parasites.

INTRODUCTION

Trichinellosis is a worldwide foodborne zoonosis resulted from the parasitic nematode *Trichinella*, which is infectious to a variety of hosts including mice, rat, rabbit, pig and humans (Pozio, 2007). Human trichinellosis is caused mainly by eating the products made of raw or half-cooked pork (Sequeira *et al*., 2016). *Trichinella spiralis* is the major pathogen of human trichinellosis (Wang *et al*., 2012b). During 2004-2009, fifteen outbreaks of human trichinellosis were recorded in mainland China and the pork products are the major infectious source of trichinellosis (Cui *et al*., 2011a; Cui *et al*., 2011b). A survey revealed that the prevalence of *Trichinella* infection in small farm pigs of central China was 0.61-3.79% from 2010 to 2015 (Cui *et al*., 2013; Jiang *et al*., 2016). Therefore, *Trichinella* infection has a serious hazard on public health and an economic impact on food safety of animal meat (Bai *et al*., 2017; Rostami *et al*., 2017).

The clinical diagnosis of *Trichinella* infection in various animals before slaughter is not easily established because its clinical manifestations are often apparent and non-specific (Dupouy-Camet *et al*., 2002). Serological test before slaughter is a useful measures for control of this zoonotic parasite. At present, ELISA and Western blotting using

T. spiralis muscle larvae (ML) excretory-secretory (ES) antigens is the frequently used method for serological diagnosis and monitoring for *Trichinella* infection in various animals and humans, and they are also the serological test recommended by FAO/WHO/OIE and International Commission on Trichinellosis (ICT) (Gamble *et al.*, 2004; Dupouy-Camet and Bruschi, 2007). But, the main drawback of detecting anti-*Trichinella* IgG against the ML ES antigens is the false negative in early phase of *Trichinella* infection (Cui *et al.*, 2015a). There is an evident 4–7 window period between the larval infectivity and positive serology in lightly *Trichinella*-infected pigs (Gamble and Patrascu, 1996; Nockler *et al.*, 2005). However, the pre-encapsulated *T. spiralis* larvae at 14–18 days post infection (dpi) have already possessed the infectious to the new host (Despommier, 1983; Jiang *et al.*, 2012). The delay in the generation of specific antibodies is possibly because the major ML ES antigenic epitopes are the ML stage-specific and not recognized by specific early anti-*Trichinella* IgG against intestinal infective larvae (IIL) and adult worms (AW) in the process of intestinal *Trichinella* infection (Liu *et al.*, 2016b; Tang *et al.*, 2015). Detection of circulating antigens (CAg) or DNA appears to be an early diagnostic method for *Trichinella* infection, but their levels in serum and fecal samples are generally low and the persistence is transient (Li *et al.*, 2010; Liu *et al.*, 2017; Wang *et al.*, 2012a). Hence, determination of *Trichinella* CAg or DNA wasn't widely applied to the diagnosis of *Trichinella* infection.

Recent studies have showed that the IIL and AW antigens were early exposed to host's immune system and trigged the generation of specific early antibodies against intestinal worms (Xu *et al.*, 2017; Yang J *et al.*, 2015). Serum anti-*T. spiralis* IgG in experimentally infected mice as well as swine was detected by the native or recombinant antigens of IIL or AW as soon as 8-15 dpi (Sun *et al.*, 2015a; Sun *et al.*, 2015b; Tang *et al.*, 2015). Therefore, the intestinal worms of *T. spiralis* might contain the molecular markers as early diagnostic antigens (Wang *et al.*, 2017b). Moreover, a putative serine protease of *T. spiralis* (TsSP, GenBank accession no. ABY60762) had been screened and identified in IIL and AW ES proteins by immunoproteomics with early infection sera (Liu *et al.*, 2016a; Liu *et al.*, 2016b; Wang *et al.*, 2017a), and the TsSP gene had a high expresion level in the IIL relative to the ML (Liu *et al.*, 2015b). The full-length sequences of TsSP gene were cloned and expressed, and the recombinant TsSP (rTsSP) protein was also purified in our department (Sun *et al.*, 2018b). The objective of this study was to evalute the potential of the rTsSP as a diagnostic marker for early serological detection of *Trichinella* infection in animals.

MATERIALS AND METHODS

Parasites

T. spiralis isolate (T1, ISS534) used in this experiment was collected from a domestic swine in central China. Other *Trichinella* species are as follows: *T. nativa* (T2, ISS10), *T. britovi* (T3, ISS100), *T. nelsoni* (T7, ISS29), and *T. pseudospiralis* (T4, ISS13), which were gifted from International *Trichinella* Reference Centre (ITRC; Rome, Italy) (Li *et al.*, 2011). All *Trichinella* species were maintained in Kunming mice by serial passages in our department.

Experimental animals

Female, 6 weeks old BALB/c mice, Wistar rats, and New Zealand white rabbits were obtained from the Henan Provincial Experimental Animal Center (Zhengzhou, China). Chinese Ninxiang domestic pigs were provided by the Institute of Parasitic Diseases of Hunan, China. All the animal experiments were approved by the Zhengzhou University Life Science Ethics Committee.

Experimental infection and collection of serum samples

Ten mice were orally inoculated with 100 or 500 ML of *T. spiralis*. About 100 μl of blood was collected from tail of the infected mice every other day during 2–28 dpi (Cui *et al.*, 2015b; Liu *et al.*, 2013). Infection sera were prepared from mice infected with 300 ML of five *Trichinella* species (*T. spiralis*, *T.*

nativa, *T. britovi*, *T. nelsoni* and *T. pseudospiralis*) at 35 dpi. Serum samples from mice experimentally infected with *Spirometra mansoni* spargana were collected at 30 dpi in our laboratory. Other serum samples of mice infected with *Angiostrongylus cantonensis*, *Clonorchis sinensis*, *Schistosoma japanicum*, and *Toxoplasma gondii* were presented by colleagues of other Chinese universities.

Rats and rabbits were experimentally inoculated per os with 3000 *T. spiralis* ML, respectively and infection sera were obtained at 35 dpi. Pigs were infected per os with 5000 *T. spiralis* ML, and swine infection sera were obtained at 70 dpi. All serum samples were conserved at -80°C until use.

Preparation of ML ES products

ML were collected by artificial digestion of the infected mouse carcasses at 42 dpi (Gamble *et al.*, 2000; Li *et al.*, 2010). The ES products from *T. spiralis* ML were prepared as reported (Wang *et al.*, 2013a; Yang W *et al.*, 2015).

The recombinant TsSP protein

The full-length cDNA sequence of TsSP gene was cloned, and the recombinant plasmid PQE-80L/TsSP was transformed into *Escherichia coli* BL21 (DE3) (Novagen, USA). Expression of rTsSP was induced for 4 h at 30°C with 0.5 mM IPTG and characterized (Sun *et al.*, 2018b). The rTsSP was purified by using a Ni-NTA His-tag affinity kit (Novagen). The purified rTsSP consisted of a 45.2 kDa protein and the His-tag at N-terminus. Using anti-rTsSP serum the native TsSP proteins were identified in ES products or crude somatic proteins of *T. spiralis* ML, IIL and AW on Western blot analysis. An immuno-localization analysis revealed the TsSP was distributed in the cuticle and stichocyte of this nematode.

Western blotting analysis

The rTsSP protein was separated at 120 V for 2.5 h on 12% acrylamide gels (Wang *et al.*, 2013b). Duplicate gels were stained or used to be transferred onto the membranes (Merck Millipore, USA). The membrane was incised into 5 mm-strips. Each strip was blocked at 37°C for 2 h in 5% skim milk in TBS-0.1% Tween-20 (TBST), and then incubated at 4°C overnight with 1:100 dilutions of different infection sera. After wash again, the strips were reacted at 37°C for 1 h with HRP-conjugated anti-pig (mouse, rat, rabbit) IgG (1:5000 dilution), and finally colored with 3, 32-diaminobenzidine tetrahydrochloride substrate (DAB, Sigma, USA) (Li *et al.*, 2015; Xu *et al.*, 2017).

Detection of serum anti-*Trichinella* IgG antibodies by rTsSP-ELISA

Optimal dilution of antigens and reagents was firstly assayed with checkerboard titration. Indirect ELISA was performed by using the rTsSP or ML ES products as coating antigens (Liu *et al.*, 2015a; Long *et al.*, 2015). Briefly, an ELISA plate (Nunc, Denmark) was coated overnight at 4°C with 2.0 µg/ml rTsSP or 2.5 µg/ml ML ES products. After being blocked at 37°C for 2 h with 5% skim milk in PBST, the following reagents were sequentially added and incubated at 37°C for 1 h: (1) different dilutions of mouse serum (1:100) or swine serum (1:200) in PBST, and (2) 1: 5000 dilutions of anti-mouse (or swine) IgG-HRP conjugate (Sigma, USA). The color was developed with a substrate o-phenylenediamine dihydrochloride (OPD; Sigma, USA). Optical density (OD) value at 490 nm was assayed with a microplate reader (Tecan, Switzerland) and all the OD values are the blank-corrected. All serum samples were performed in duplicate. The ratio <2.1 of serum samples to be tested/ negative sample were believed as negative, and ≥ 2.1 as positive (Cui *et al.*, 2011a; Wang *et al.*, 2014). The cut-off value of rTsSP-ELISA and ES-ELISA for detection of mouse sera was 0.202 and 0.21, and for swine sera was 0.31 and 0.21, respectively.

Statistical analysis

Statistical analysis of the data was conducted with SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL). The OD value of ELISA was expressed as mean values ± SD. Chi-square test and repeated measures of analysis of variance (ANOVA) were utilized to determine

differences between two groups and the differences of anti-*Trichinella* IgG levels at various time intervals after infection. Difference at $P<0.05$ was believed as statistical significance.

RESULTS

Assess of anti-*Trichinella* IgG of different hosts by Western blotting with rTsSP

Western blotting results showed the specific antibodies in serum samples of different hosts (mice, rats, pigs, and rabbits) infected with *T. spiralis* could be recognized by the rTsSP (Figure 1), but rabbit infection serum was weakly reacted with the rTsSP. Anti-*Trichinella* IgG in sera of mice infected with other species of encapsulated *Trichinella* (T2, T3 and T7) was also detected by rTsSP on Western blotting. Nonetheless, serum from infected mice with non-encapsulated *T. pseudospiralis* could not be recognized by the rTsSP.

Determination of specific antibodies in infected mice with various *Trichinella* species by rTsSP-ELISA

Serum anti-*Trichinella* IgG of infected mice with various *Trichinella* species were also measured by rTsSP-ELISA and ES-ELISA (Table 1). Anti-*Trichinella* antibody positive rate by using two antigens had no overt difference in infected mice with four encapsulated *Trichinella* species ($\chi^2_{T2}=3.2$, $P>0.05$; $\chi^2_{T7}=3.333$, $P>0.05$). However, the ES-ELISA detection (90%) of mice infected with non-encapsulated *T. pseudospiralis* was statistically higher than those of rTsSP-ELISA ($\chi^2=12.239$, $P<0.0001$). The results testified

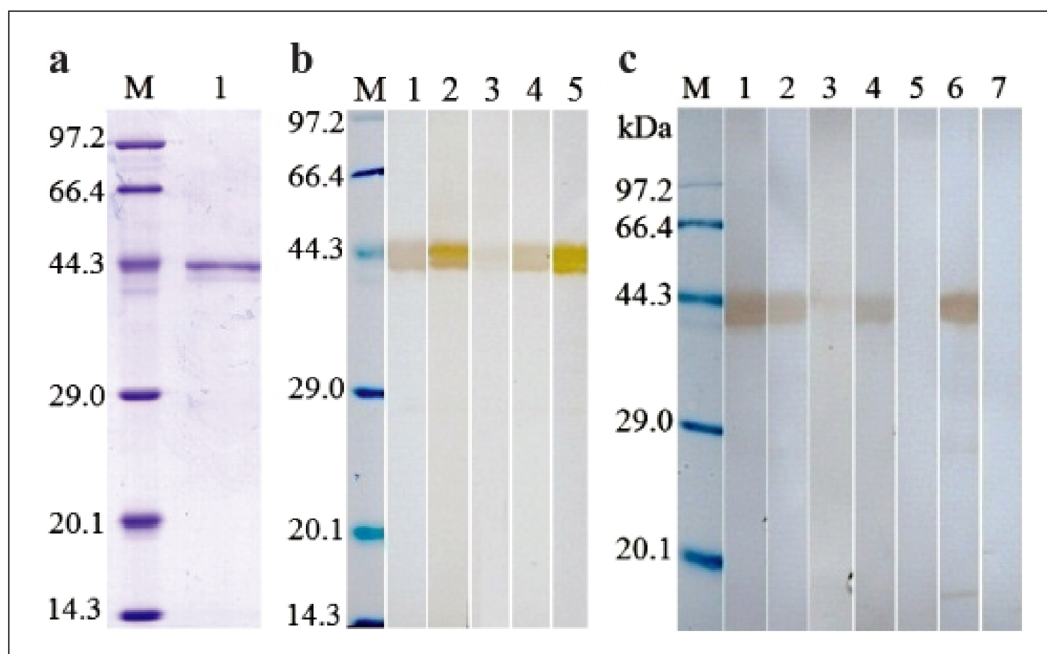

Figure 1. Western blotting for detecting anti-*Trichinella* IgG in different hosts infected with *Trichinella* spp. **(a):** SDS-PAGE analysis of rTsSP**.** M: protein marker; 1: the purified rTsSP. **(b):** Western blotting for detecting anti-*Trichinella* IgG in different hosts infected with *T. spiralis*. The serum samples from mice (lane 1), pig (lane 2), rabbit (lane 3), and rat (lane 4) infected with *T. spiralis* were detected by the rTsSP. Anti-rTsSP serum was used as positive control (lane 5), **(c):** Sera of mice infected with *T. spiralis* (lane 1), *T. nativa* (lane 2), *T. britovi* (lane 3) and *T. nelsoni* (lane 4) were also detected by Western blotting with rTsSP, *T. pseudospiralis*-infected mouse sera (lane 5) were not probed by rTsSP. Anti-rTsSP serum was used as positive control (lane 6), and normal mouse serum as negative control (lane 7). All positive sera were collected from the animals experimentally infected with *Trichinella* spp. and stored at -80°C in our laboratory.

Table 1. Determination of specific antibodies in infected mice with various *Trichinella* species

Sera of mice infected with	No. of serum samples	ELISA with rTsSP antigens		ELISA with ML ES antigens	
		OD value	No. of positive serum samples (%)	OD value	No. of positive serum samples (%)
T. spiralis	30	0.48±0.11	30 (100)	0.80±0.05	30 (100)
T. nativa	24	0.30±0.11	21 (87.5)	0.70±0.13	24 (100)
T. britovi	14	0.41±0.10	14 (100)	0.78±0.08	14 (100)
T. nelsoni	15	0.57±0.06	12 (80)	0.75±0.08	15 (100)
T. pseudospiralis	22	0.19±0.15	9 (40.9)	0.52±0.21	20 (90.9)

Table 2. Identification of rTsSP specificity for detection of anti-*Trichinella* IgG

Sera of mice infected with	No. of serum samples	ELISA with rTsSP antigens		ELISA with ML ES antigens	
		OD value	No. of positive serum samples (%)	OD value	No. of positive serum samples (%)
T. spiralis	30	0.48±0.11	30 (100)	0.80±0.05	30 (100)
Angiostrongylus cantonensis	12	0.05±0.04	0	0.03±0.01	0
Spirometra mansoni	20	0.06±0.04	0	0.15±0.03	0
Schistosoma japonicum	15	0.05±0.04	0	0.14±0.02	0
Clonorchis sinensis	6	0.11±0.05	0	0.14±0.04	0
Toxoplasma gondii	5	0.08±0.05	0	0.14±0.01	0
Normal mice	40	0.10±0.03	0	0.10±0.03	0

that the rTsSP could be believed as useful for serodiagnosis of the infection with the encapsulated *Trichinella* species.

Identification of rTsSP specificity for detection of anti-*Trichinella* IgG

Anti-*Trichinella* antibodies in serum of infected mice with *T. spiralis* and other parasites were measured by rTsSP-ELISA and ES-ELISA (Table 2). The sensitivity and specificity of two antigens for detection of anti-*Trichinella* antibodies was 100%. Both antigens had no cross-reaction with sera from mice infected with other parasites.

Dynamics of serum anti-*Trichinella* IgG of mice infected with different larval dose

In mice with infected 100 ML, anti-*Trichinella* IgG were firstly detected at 8 dpi by rTsSP-ELISA and 12 dpi by ES-ELISA; sero-conversion rate reached 100% at 16 and 22 dpi, respectively (Figure 2a, b). In mice infected with 500 ML, the specific antibodies were firstly detected at 7 dpi by rTsSP-ELISA and 10 dpi by ES-ELISA, and the sero-conversion was up to 100% at 10 and 16 dpi, respectively (Figure 2c, d). It is noteworthy that anti-*Trichinella* antibodies were detectable by rTsSP as soon as 7-8 dpi, but the specific antibodies could not been detected by ES antigens prior to 10-12 dpi. Specific antibody level in two groups of infection dose measured using rTsSP and ES antigens had statistical difference (F_{rTsSP} = 11.436, $P < 0.01$; F_{ES} = 6.731, $P < 0.05$), and the antibody level of two groups at 2-28 days after infection also had statistical significance (F_{rTsSP} = 73.890, $P < 0.0001$; F_{ES} = 70.313, $P < 0.0001$).

Evaluation of rTsSP-ELISA for detection of swine sera and comparison with ES-ELISA

In domestic pigs infected with 5000 ML of *T. spiralis*, serum anti-*Trichinella* IgG were

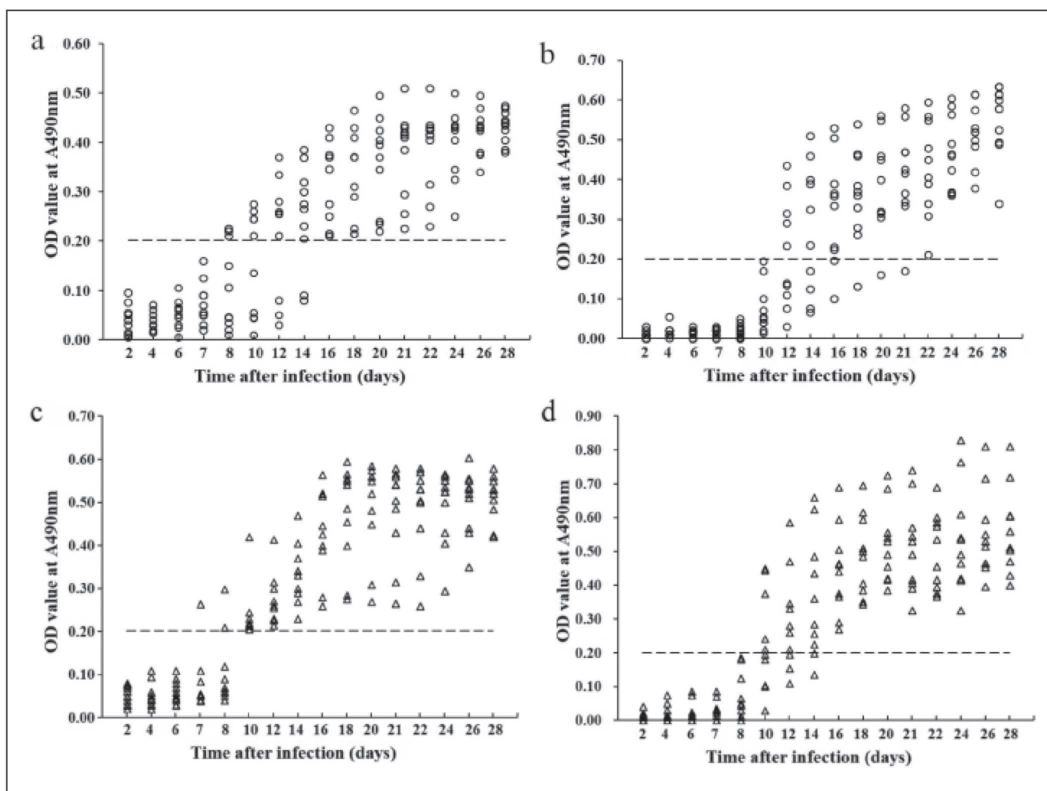

Figure 2. Anti-*Trichinella* IgG kinetics in serum of infected mice infected with 100 ML(a,b) and 500 ML (c,d). **a** and **c:** rTsSP-ELISA; **b** and **d:** ES-ELISA. The dotted line represent the cutoff values of ELISA.

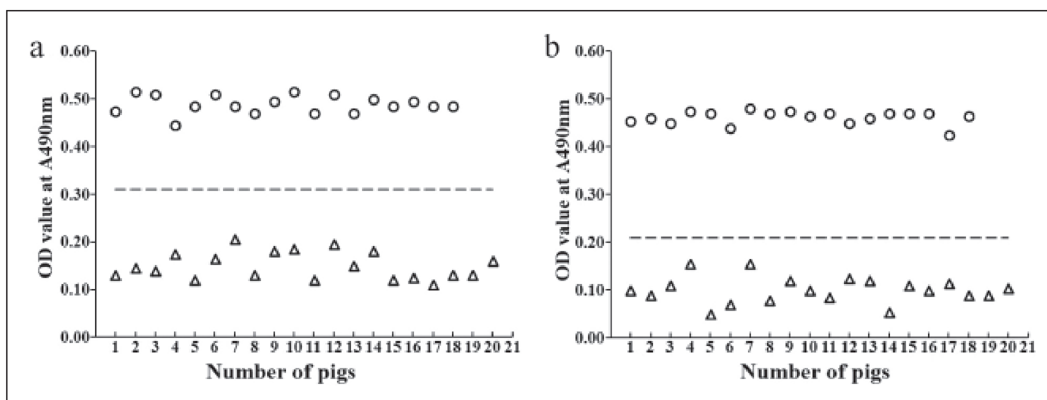

Figure 3. The OD values of rTsSP-ELISA (a) and ES-ELISA (b) for measuring anti-*Trichinella* IgG in pig experimentally infected with 5000 *T. spiralis* ML (o) and conventionally raised normal pigs (Δ).

detected in 100% (18/18) of infected pigs by rTsSP-ELISA and ES-ELISA (Figure 3), but none of specific antibodies were not detected in 20 conventionally raised normal pigs by two antigens.

DISCUSSION

After the *Trichinella*-infected meat is ingested, the ML is activated into the IIL by intestinal contents or bile. The IIL penetrated

into intestinal epithelial cells where the IIL develop to adult worms after they molt four times. Then the adults lodge in intestinal mucosa of mice and rats for 10–20 days (Campbell, 1983). The interaction between the parasitic nematode and host occurred at early intestinal infection. The ES antigens from IIL and AW were primarily exposed to the immune system and elicited the generation of early anti-*Trichinella* antibodies (Liu *et al.*, 2016a; Liu *et al.*, 2016b; Yang J *et al.*, 2015). Our previous studies showed that specific antibody IgG in infected mice could be detected by ES antigens of IIL or AW early to 8 dpi (Sun *et al.*, 2015a; Sun *et al.*, 2015b). Nevertheless, preparing ES antigen needs the collection of the nematodes from infected animals, which is actually inconvenient because of the labor, time and cost. Recombinant antigens are an alternative to native ES antigens as they can be *in vitro* generated in large quantities and may be applied as a diagnostic antigen in a standardized ELISA for diagnosis of *Trichinella* infection (Cui *et al.*, 2015a; Jung *et al.*, 2007; Zocevic *et al.*, 2014). Hence, development of recombinant *Trichinella* antigens expressed at early enteral infection stage will increase the sensitivity of serodiagnosis of *Trichinella* infection (Bien *et al.*, 2015; Wang *et al.*, 2017a).

In this study, anti-*Trichinella* IgG in *T. spiralis*-infected hosts (rats, mice, rabbits and pigs) could be detected on Western blot with the rTsSP. By using rTsSP-ELISA specific antibodies was detected in 100% (30/30) of infected mice. No cross-reactions of rTsSP-ELISA were found with sera from mice infected with other parasites (*A. cantonensis*, *S. erinaceieuropaei*, *S. japonicum*, *C. sinensis* and *T. gondii*), and with normal mouse sera. Besides, no obvious differences of serum antibody positivity were observed in mice infected with the encapsulated *Trichinella* species, demonstrating that serine protease was conserved in the encapsulated *Trichinella* species (Bien *et al.*, 2012; Somboonpatarakun *et al.*, 2018; Zhang *et al.*, 1993). The results of sequence alignment showed that the amino acid sequences of the serine protease from *T. spiralis* had a higher identity with that of other encapsulated *Trichinella* species, e.g., *T. nativa* (90%), *T. britovi* (90%) and *T. nelsoni* (84%) (Sun *et al.*, 2018b). Furthermore, the majority of antigenic epitopes of serine protease recognized by infection sera might be the same to encapsulated *Trichinella* species (Bioreau *et al.*, 1997; Kapel and Gamble, 2000). Our results demonstrated that the recombinant serine protease from *T. spiralis* could also be applied to the diagnosis of *Trichinella* infection resulted from all the encapsulated species (Long *et al.*, 2015; Wang *et al.*, 2015).

The rTsSP-ELISA results revealed that specific antibodies in mice infected with 100 or 500 ML were detectable by rTsSP early at 7-8 dpi, but not detected prior to 10-12 dpi by ML ES antigens. Anti-*Trichinella* IgG level quickly increased from 7 dpi, and seroconversion achieved 100% at 16 and 10 dpi in two groups of infected mice, and sustained to the end of this study (28 dpi). It is likely that the TsSP produced by this nematode entered host's blood circulation at early intestinal phase, elicited an early anti-*Trichinella* IgG response, and then persisted to the muscle stage (Wang *et al.*, 2017a). Our previous study also demonstrated that on IFT and Western blot analysis, the natural TsSP was expressed not only in the early *T. spiralis* stages (IIL at 6 hpi, AW at 3 and 6 dpi, newborn larvae), but also it continues to be expressed in the *T. spiralis* muscle stage (ML at 42 dpi) (Sun *et al.*, 2018a). Other studies revealed that recombinant 53 kDa proteins, rTsDAF-21 and L20h-Ts3 protein could be recognized at 8-15 dpi by mouse or porcine infection sera (Nagano *et al.*, 2008; Yang *et al.*, 2014; Zocevic *et al.*, 2014). Our results indicated the TsSP had potential for early serodiagnosis of animal *Trichinella* infection. Additionally, the rTsSP antigen continues to be recognized by the late infection serum (serum from infected mice, rats and rabbits at 35 dpi, and swine infection serum at 70 dpi), suggesting that it might be valuable for serodiagnosis of the chronic phase of the disease. However, the sensitivity and specificity of the rTsSP need to be evaluated with the early sera of porcine infected with various dose of *Trichinella* and other parasitic helminths.

CONCLUSION

The recombinant *T. spiralis* serine protease was sensitive and specific for the detection of serum anti-*Trichinella* IgG in infected animals, and exhibited the potential as early serodiagnostic antigens for *Trichinella* infection. But, it needs to be assessed with early sera of swine infected with light or moderate dose of *Trichinella* and other helminths.

Acknowledgements. This study was supported by grants of the National Key Research and Development Program of China (2017YFD0501302) and the National Natural Science Foundation of China (81672043 and U1704284). The authors wish to thank Prof. Guo Rong Yin of Shanxi Medical University, Prof. Jia Hui Lei of Tongji Medical College, Huazhong University of Science and Technology, and Prof. Zi Yue Lv of Zhongshan School of Medicine, Sun Yat-Sen University, who presented the serum samples of mice infected with *Toxoplasma gondii*, *Schistosoma japanicum* or *Angiostrongylus cantonensis*, respectively.

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