

Serodiagnosis of trichinellosis by ELISA using recombinant nudix hydrolase of *Trichinella spiralis*

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Abstract. *Trichinella spiralis* nudix hydrolase (TsNd) gene encoding a 46 kDa protein was expressed in *Escherichia coli* and the potential of recombinant TsNd protein (rTsNd) as an antigen for the serodiagnosis of trichinellosis was investigated by ELISA and compared with those of ELISA with *T. spiralis* muscle larval excretory–secretory (ES) antigens. The sensitivity of both ELISA was 100% (30/30), for the detection of anti-*Trichinella* IgG antibodies in sera of the experimentally infected mice, and the specificity of rTsNd-ELISA and ES-ELISA was 100% (54/54) and 98% (53/54), respectively ($P > 0.05$). Serum anti-*Trichinella* antibodies were firstly detected by rTsNd-ELISA at 14 days post infection (dpi), then continued to increase with a detection rate of 100% at 36 dpi. The anti-*Trichinella* antibody levels at different times after infection were statistically different ($P < 0.05$). The results showed that the rTsNd might be a potential candidate antigen for specific serodiagnosis of trichinellosis. But, it needs to be further evaluated with sera of the patients with trichinellosis and other helminthiasis.

INTRODUCTION

Trichinellosis is a serious parasitic zoonosis acquired by eating raw or undercooked meat contaminated with infective larvae of the nematode genus *Trichinella* (Murrell & Pozio, 2011). *Trichinella* infection has been documented in 66 countries of the world, and is considered as an emerging/re-emerging disease (Cui *et al.*, 2006). From 2004 to 2009, 15 outbreaks of human trichinellosis, consisting of 1 387 cases and 4 deaths, were reported in China (Cui *et al.*, 2011b). Trichinellosis is not only a public health hazard but also an economic problem in porcine animal production and food safety (Cui *et al.*, 2013a, Dorny *et al.*, 2009). However, the clinical diagnosis of trichinellosis is rather difficult, because its clinical manifestations are nonspecific. The enzyme-linked immunosorbent assay (ELISA) using excretory–secretory (ES)

antigens of *T. spiralis* muscle larvae is the most commonly used serological method for diagnosis of trichinellosis recommended by the International Commission on Trichinellosis (ICT) (Gamble *et al.*, 2004, Gottstein *et al.*, 2009), but the *T. spiralis* ES antigens had a cross reaction with sera of patients with other parasitic diseases (e.g., paragonimiasis, schistosomiasis, clonorchiasis, cysticercosis, anisakiasis and so on) (Yera *et al.*, 2003). When the synthetic tyvelose antigens were used in serodiagnosis of trichinellosis, the cross-reaction may occur with cases of anisakiasis, schistosomiasis, etc. (Bruschi *et al.*, 2001, Dea-Ayuela *et al.*, 2001). Hence, there is an urgent need to develop the new specific antigens for serodiagnosis of trichinellosis.

In our previous study, *T. spiralis* nudix hydrolase (TsNd) binding to normal mouse intestinal epithelial cells (IECs) were identified by screening a T7 phage display

cDNA library from *T. spiralis* intestinal infective larvae (IIL) (Ren *et al.*, 2013). The full-length cDNA sequence of TsNd gene (GenBank accession No. EU263318.1) encoding a 46 kDa protein from *T. spiralis* IIL was cloned and expressed in our laboratory. Expression of TsNd gene was observed at all *T. spiralis* different developmental stages, rTsNd had the enzymatic active of nudix hydrolase. Western blot analysis showed that anti-rTsNd serum obviously recognized the native TsNd protein in crude antigens of IIL, adult worms and newborn larvae, and ES antigens of ML; an immunolocalization analysis identified TsNd in the cuticle and stichocytes of the parasite, indicating that TsNd is one component of both the crude and ES proteins from *T. spiralis* (Cui *et al.*, 2013b, Long *et al.*, 2014).

The aim of this study was to evaluate the potential application of rTsNd for serodiagnosis of experimental trichinellosis by ELISA, and its sensitivity and specificity were compared with those of ELISA with ES antigen of *T. spiralis* muscle larvae.

MATERIALS AND METHODS

Parasites and experimental animals

T. spiralis isolate (T1, ISS534) used in this study was obtained from a domestic pig in Nanyang city of Henan Province, China. The reference *Trichinella* isolates used in this study were *T. nativa* (T2, ISS10), *T. britovi* (T3, ISS100), *T. pseudospiralis* (T4, ISS13) and *T. nelsoni* (T7, ISS29), which were obtained from International *Trichinella* Reference Centre (ITRC; Rome, Italy). All of the *Trichinella* isolates were maintained by serial passages in Kunming mice in our laboratory. Specific pathogen free (SPF) female BALB/c mice aged 6 weeks were purchased from the Experimental Animal Center of Henan province (Zhengzhou, China). All procedures of animal experiment of this study were approved by the Life Science Ethics Committee of Zhengzhou University.

Serum samples

Mouse infection sera were obtained from BALB/c mice infected with 300 larvae of *T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis* and *T. nelsoni* at 42 days post infection (dpi). Serum samples of mice infected with three spargana of *Spirometra erinacei* were collected at 30 dpi in our department. Serum samples of mice infected with *Toxoplasma gondii* were gifted by Prof. GR Yin of Department of Parasitology, Shanxi Medical University.

Ten female BALB/c mice were orally infected with *T. spiralis* (500 larvae/mouse), and about 50 µl of tail blood was collected on alternate days during 2-42 dpi (Cui *et al.*, 2015, Wang *et al.*, 2012). All the serum samples were stored at -80°C until used.

Collection of muscle larvae and preparation of ES antigens

T. spiralis muscle larvae were recovered from the infected mice at 42 dpi by artificial digestion as described previously (Gamble *et al.*, 2000, Li *et al.*, 2010). The ES antigens of the muscle larvae were prepared as previously described (Wang *et al.*, 2011). Briefly, after being washed thoroughly in sterile saline and serum-free RPMI-1640 medium supplemented with 100 U/ml penicillin and 100 U/ml streptomycin, the larvae were incubated in the same medium at 5 000 worms per milliliter at 37°C in 5% CO₂ for 18 h. After incubation, the media containing the ES proteins were poured into 50-ml conical tubes and the larvae were allowed to settle for 20 min. The supernatant containing the ES products was filtered through a 0.2 µm membrane. The ES products were dialyzed and then lyophilized by a vacuum concentration and freeze-drying (Heto Mxi-Dry-Lyo, Denmark). The protein concentration (2.5 µg/ml) was determined by the Bradford assay (Bradford, 1976).

The recombinant TsNd protein

The rTsNd protein was expressed in an *E. coli* expression system and characterized in our laboratory. The rTsNd protein was obtained after expression and purification.

Anti-rTsNd serum recognized the native TsNd protein in crude antigens and ES antigens of muscle larvae. An immunolocalization found that TsNd in the cuticle and stichocytes of the parasite, demonstrating the TsNd protein was from the ES products of *T. spiralis* muscle larvae (Long *et al.*, 2014).

ELISA for detection of anti-*Trichinella* antibodies

ELISA was performed as previously described (Cui *et al.*, 2011a). In brief, 96-well ELISA plates (Corning, USA) were coated with purified rTsNd (1 µg/ml) or ES antigens (2.5 µg/ml) in 100 µl of bicarbonate buffer (pH 9.6) at 4°C overnight. After being blocked in PBS-0.1% Tween 20 (PBST) containing 5% skimmed milk at 37°C for 2 h, the following reagents were sequentially added and incubated at 37°C for 1 h: (1) mouse sera diluted at 1:100 in PBST, and (2) goat anti-mouse IgG (Sigma, USA) diluted at 1:5000. The reactions were detected by addition of the substrate o-phenylenediamine dihydrochloride (OPD; Sigma, USA) plus H₂O₂ and stopped with 50 µl/well of 2M H₂SO₄. Optical density (OD) values at 490 nm were measured with a microplate reader (TECAN, Austria). All samples were run in duplicate. The ratio <2.1 of samples to be tested/negative sample OD values of the samples to be tested divided by OD of the negative, S/N < 2.1) were regarded as negative, and S/N ≥2.1 as positive (Wang *et al.*, 2014). The cut-off values of rTsNd-ELISA and ES-ELISA for detection of mouse sera were 0.27 and 0.21, respectively.

Statistical analysis

All statistical analyses of data were done with SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL). Chi-square test and repeated measures analysis of variance (ANOVA) were used to determine the difference of the sensitivity and specificity of the rTsNd and ES antigens, and the difference between antibody levels at various periods post-infection. *P* < 0.05 was considered statistically significant.

RESULTS

Detection of specific antibodies in mice infected with *Trichinella* and other parasites

The specific anti-*Trichinella* IgG antibodies in serum samples of mice infected with *T. spiralis* and other parasites were determined and the results are shown in Table 1. The sensitivity of rTsNd-ELISA and ES-ELISA for detecting anti-*Trichinella* antibodies was 100.00% (30/30) when the sera of mice experimentally infected with *T. spiralis*. No cross-reactions of rTsNd-ELISA were observed with sera of mice infected *T. gondii*, *Spirometra erinacei* and normal mice. The specificity of both rTsNd-ELISA and ES-ELISA for detecting anti-*Trichinella* antibodies was 100% (54/54) and 98% (53/54), respectively (*P*>0.05).

The specific serum anti-*Trichinella* IgG antibodies in mice infected with other species of *Trichinella* were also assayed by rTsNd-ELISA and ELISA with ES antigens of *T.*

Table 1. Detection of specific anti- *Trichinella* IgG antibodies in serum samples of experimentally infected mice by rTsNd-ELISA and ES-ELISA

Sera of mice infected with	No. of serum samples	rTsNd-ELISA		ES-ELISA	
		OD value ($\bar{x} \pm S$)	No. of positive serum samples (%)	OD value ($\bar{x} \pm S$)	No. of positive serum samples (%)
<i>T. spiralis</i>	30	0.44±0.05	30(100.00)	0.80±0.05	30 (100.00)
<i>Spirometra erinacei</i>	14	0.22±0.03	0 (0.00)	0.16±0.04	1 (7.12)
<i>Toxoplasma gondii</i>	10	0.20±0.03	0 (0.00)	0.14±0.01	0 (0.00)
Normal mice	30	0.13±0.03	0 (0.00)	0.10±0.02	0 (0.00)

Table 2. Detection of serum anti-*Trichinella* IgG antibodies in mice infected with other species of *Trichinella* by ELISA

Sera of mice infected with	No. of serum samples	ELISA with rTsNd antigens		ELISA with ES antigens	
		OD value ($\bar{x} \pm S$)	No. of positive serum samples (%)	OD value ($\bar{x} \pm S$)	No. of positive serum samples (%)
<i>T. spiralis</i>	30	0.44±0.05	40 (100.00)	0.80±0.05	30 (100.00)
<i>T. nativa</i>	30	0.43±0.13	25 (83.33)	0.72±0.13	30 (100.00)
<i>T. britovi</i>	15	0.36±0.15	11 (73.33)	0.79±0.08	15 (100.00)
<i>T. pseudospiralis</i>	22	0.33±0.15	14 (63.64)	0.52±0.21	20 (90.91)
<i>T. nelsoni</i>	16	0.37±0.14	12 (75.00)	0.76±0.08	16 (100.00)

spiralis muscle larvae, and the results are shown in Table 2. The detection rate of anti-*Trichinella* antibodies by rTsNd-ELISA and ES-ELISA was no significant difference among the mice infected with *T. nativa*, *T. britovi* and *T. nelsoni* ($\chi^2_{T_2}=3.20$, $\chi^2_{T_3}=2.25$, $\chi^2_{T_7}=2.25$, $P>0.05$). However, when the serum of mice infected with *T. pseudospiralis* was assayed, the antibody detection rate (63.64%) by rTsNd-ELISA was lower than 90.91% by ES-ELISA ($\chi^2=4.17$, $P<0.05$).

Serum anti-*Trichinella* antibody dynamics in experimentally infected mice

The levels of anti-*Trichinella* antibodies in sera from infected mice at different time points after infection were determined by rTsNd-ELISA and ES-ELISA (Figure 1). Serum anti-*Trichinella* antibodies were firstly detected by rTsNd-ELISA at 14 dpi; then

continued to increase with a detection rate of 100% at 36 dpi. When ES-ELISA was performed, anti-*Trichinella* antibodies were firstly detected at 10 dpi; the antibody positive rate reached 100% at 14dpi (Figure 2). Serum anti-*Trichinella* antibody levels at different time points after infection was statistically significant ($F=105.214$, $P<0.05$); there was also a statistical significance of antibody levels assayed by both ELISA ($F=26.203$, $P<0.05$).

DISCUSSION

The ES antigens of *T. spiralis* muscle larvae is the most commonly used serodiagnostic antigens for trichinellosis recommended by the International Commission on Trichinellosis (ICT) (Gamble *et al.*, 2004,

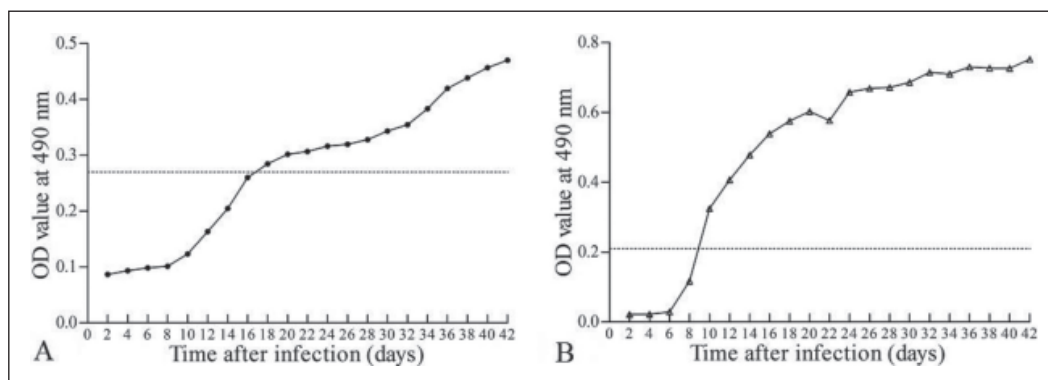


Figure 1. Kinetics of anti-*Trichinella* antibodies in sera of mice infected with *T. spiralis*. Anti-*Trichinella* IgG antibodies were detected by rTsNd-ELISA (A) and ES-ELISA (B), and cut-off value is represented by the dotted line.

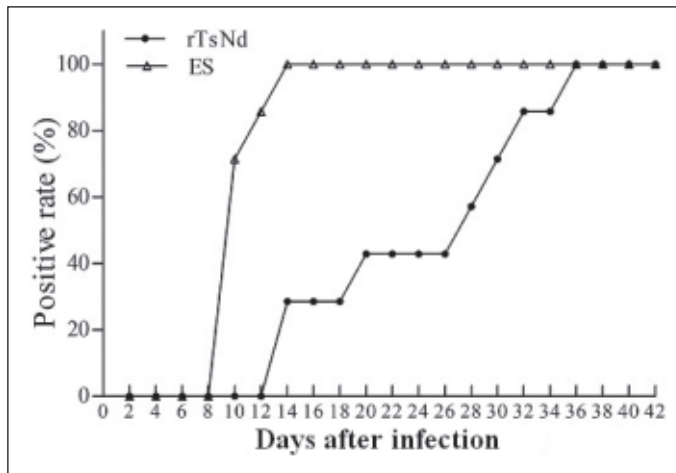


Figure 2. Comparison of detection rate of anti-*Trichinella* IgG antibodies in sera of mice infected with *T. spiralis* at different time intervals post-infection by rTsNd- ELISA and ES-ELISA.

Gomez-Morales *et al.*, 2008). However, the preparation of ES antigens requires collection of muscle larvae from experimentally infected laboratory animals, which is practically inconvenient in terms of cost, labor, and time. Recombinant proteins are a good alternative to the ES antigens as they can be produced easily in large amounts by using a bacterial expression system and can be used as an antigen in a sensitive, specific, and standardized ELISA for serodiagnosis of trichinellosis (Jung *et al.*, 2007, Zocevic *et al.*, 2014). Therefore, development of sensitive and specific recombinant *Trichinella* antigens will improve the serodiagnosis of this disease (Cui *et al.*, 2015).

In the present study, the sensitivity of rTsNd-ELISA and ES-ELISA for detecting anti-*Trichinella* antibodies in serum samples of experimentally infected mice was 100% (30/30), and the specificity of rTsNd-ELISA and ES-ELISA for detecting the serum of other helminthiasis and normal mice was 100% (54/54) and 98% (53/54), respectively ($P>0.05$). There were no cross-reactions of rTsNd-ELISA that were observed with sera of mice infected with *S. erinacei*, *T. gondii*, and normal mice, suggesting that the rTsNd could be the alternative antigens of ES antigens for diagnosis of trichinellosis. Moreover, there was no significant difference

in the antibody detection rates in sera of mice infected with *T. spiralis*, *T. nativa*, *T. britovi*, and *T. nelsoni* by rTsNd-ELISA and ES-ELISA ($P>0.05$). But, the antibody detection rate (63.64%) of mice infected with *T. pseudospiralis* by rTsNd-ELISA was lower than 90.91% by ES-ELISA ($P<0.05$). The TsNd might expressed at high levels in encapsulated species (such as *T. spiralis*, *T. nativa*, *T. britovi*, and *T. nelsoni*), but at a low level in non-encapsulated species (*T. pseudospiralis*) (Kapel & Gamble, 2000, Zhang *et al.*, 1993), suggesting that the most epitopes of TsNd antigens recognized by mouse infection sera were common to the encapsulated species of *Trichinella* (Bioreau *et al.*, 1997). As to the early serodiagnosis of trichinellosis, the sensitivity of ES antigens was better than those of the rTsNd. The results demonstrated that the rTsNd might be a potential candidate antigen for the early serodiagnosis of trichinellosis, but the sensitivity of the rTsNd antigen should be tested with the serum samples of the patients with trichinellosis. Besides, a large scale validation of specificity of the rTsNd antigen also needs to be further investigated with sera of the patients with other helminthiasis.

The results of rTsNd-ELISA also showed that the specific anti-*Trichinella* IgG antibodies were firstly detected at 14 dpi in infected mice, and continued to grow up to

the end of this experiment (42 dpi). It suggested that the TsNd proteins might be secreted by the parasite into the peripheral blood circulation of host at early infection stage, and induced an early antibody response continuing to the muscle larval stage. Similarly, other some recombinant proteins (such as the recombinant 53 kDa protein, rTs21, rTsDAF-21) were firstly recognized by the sera of mice infected with *T. spiralis* at 8-14 dpi (Nagano *et al.*, 2008, Yang *et al.*, 2014).

In conclusion, the rTsNd was sensitive and specific for detecting anti-*Trichinella* antibodies in serum samples of experimentally infected mice by ELISA, provided a new source of diagnostic antigens, and might be a potential antigen for specific serodiagnosis of trichinellosis. However, it needs to be further investigated with sera of the patients with trichinellosis and other parasitic diseases.

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