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Research Note

Study of the 49 kDa excretory-secretory protein gene of *Trichinella nativa* and *Trichinella spiralis*

B. L. ZHENG[1,2], L. H. XIAO[3], X. R. WANG[4], D. M. LI[2], Y. X. LU[2], Y. ZHANG[4], Q. B. YAN[2], M. X. SONG[2*]

[1]Zhengzhou College of Animal Husbandry and Engineering, Bioengineering Department, Zhengzhou, 450011, China; [2]Section of Parasitology, Department of Preventive Veterinary Medicine, College of Veterinary Medicine, Northeast Agricultural University, Harbin, 150030, China; [3]Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Bldg 22, Rm14,4770 Buford Highway, Chamblee, GA 30341. USA; [4]National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, 150001, China, E-mail: *mingxinsong@yahoo.com*;

Summary

To study the function of the 49 kDa excretory-secretory (ES) protein gene (P49) of *Trichinella*, the genes was amplified by RT-PCR from RNA of *Trichinella spiralis* and *Trichinella nativa* and several Chinese *Trichinella* isolates of domestic animals, and sequenced after being cloned. The amplified products of these parasites produced bands of about 950 bp. The 97.2 % to 100 % nucleotides identity and 94.3 % to 100 % identity of deduced amino acids among P49 gene of these *Trichinella* strains showed the close relationship of these parasites. The P49 gene of *T. nativa* was cloned into the BamHI site of the prokaryotic expression vector pET-30a, and the recombinant vector was expressed. The expressed product was 40.8 kDa in size. In Western blot analysis, the expressed product was reactive to sera of mice infected with *T. nativa*, *T. spiralis* and their Chinese geographical strains.

Key words: *Trichinella nativa*; *Trichinella spiralis*; 49kDa ES protein; gene sequence; western blot analysis

Introduction

Trichinella excretory-secretory (ES) proteins are produced by stichocytes of living worms (Despommier & Muller, 1976; Gamble & Graham, 1984; Silberstein & Despommier, 1984). It was shown in previous studies (Gamble & Graham, 1984; Yan *et al.*, 1997) that three ES proteins with molecular weights of 45 kDa, 49 kDa and 53 kDa predominate in *Trichinella spiralis* (*T. spiralis*). Larval *T. spiralis* ES proteins and the antigens from ES components purified by monoclonal antibodies are frequently used in the immunological diagnosis of trichinellosis using ELISA, western blot, immunochemical and immunoprecipitation assays (Gamble *et al.*, 1983; Su *et al.*, 1991a,b; Turčeková *et al.*, 1997; Sofronic-Milosavljevic *et al.*, 2001). Larval ES proteins of *T. spiralis* can also induce strong protective immune responses (Silberstein & Despommier, 1984; Gamble *et al.*, 1986; Dea-Ayuela & Bolas-Fernandez, 1999), and the protection induced by them is stronger than that conferred by other *T. spiralis* antigens (Li & Yuan, 1989).

Because of the wide geographical distribution and broad host range that typify the genus *Trichinella*, characterizations of the parasites at biological, immunological and biochemical levels have been active fields of research on trichinellosis (Zarlenga & La Rosa, 2000). This is also important to the control of trichinellosis. Immunogenicity of *Trichinella* ES antigens is increasingly studied by researchers, and several genes coding for *Trichinella* ES antigens have been identified and cloned (Zarlenga & Gamble, 1990; Vassilatis *et al.*, 1992; Yao *et al.*, 1997; Nagano *et al.*, 2001). One of the antigens, the 49 kDa ES protein of *T. spiralis*, has been cloned and sequenced, and used in serological diagnosis of swine trichinellosis (Su & Prestwood, 1991a; Yan *et al.*, 1997).

Almost all studies of *Trichinella* ES proteins have been done with *T. spiralis*. *T. spiralis* and *Trichinella nativa* (*T. nativa*) are related gene types (La Rosa *et al.*, 1992; Pozio

* Corresponding author

et al., 1992a). Although there are no significant differences in morphology by scanning electron microscopy (Lichtenfels *et al.*, 1983), the two gene types have different host specificity (Smith, 1985; Song *et al.*, 1998; Kapel, 2000; Oivanen *et al.*, 2002). Moreover, *T. spiralis* and *T. nativa* have different biological and biochemical characteristics (Pozio *et al.*, 1992b; Song *et al.*, 1994). A recent study showed that *T. spiralis* and *T. nativa* induced distinguishable histopathologic and humoral responses in raccoon dogs, there are also significant differences in the shapes and structures of the encapsulated cysts of these two parasites (Sukura *et al.*, 2002). Therefore, heterogeneity may exist in the gene coding for their ES proteins, and the antigenicity of ES proteins could be different between *T. spiralis* and *T. nativa.* If the antigenicity of ES proteins differed between the two species, then the larval *T.spiralis* ES antigens would not be suitable for the diagnosis of all cases of trichinellosis in wild carnivores, as multiple species can be involved (Pozio *et al.*, 1992b).

The traditional way to obtain the ES proteins has been to isolate muscle-stage larvae by pepsin-HCl digestion of mouse tissues or adult worms from the intestines of infected mice followed by culturing the worms in vitro (Gamble *et al.*, 1983; Li & Yuan, 1989). This method is time-consuming and difficult to use to produce concentrated protein. With the development of recombinant technology, now it is possible to obtain pure larval ES proteins of *T. spiralis* by cloning and expression in prokaryotic or eukaryotic cells (Su *et al.*, 1991b; Yan *et al.*, 1997). Compared with crude ES proteins, the recombinant proteins have better diagnostic specificity because of the purity of antigen. In this study, we characterized the 49 kDa ES antigen (P49) genes of six *Trichinella* strains and expressed the protein of *T. nativa* in prokaryotic cells. The results of the study should be useful in the development of new serological tools and in vaccine studies.

Materials and Methods

Propagation and collection of Trichinella muscle larvae

Five groups of five healthy adult Chinese Kunming mice (purchased from the Experimental Animal Center of Harbin Veterinary Research Institute, Harbin, China) were used in this study. Two hundred living larvae suspended in water were inoculated orally to each mouse. Muscle-stage larvae of *T. nativa* (isolate code: ISS10; original host: Polar bear of Norway), *T. spiralis* (isolate code: ISS3; original host: domestic pig of Poland), *Trichinella* strain of Chinese dog-origin (TSCD, Wuchang county of Heilongjiang province of China), *Trichinella* strain of Chinese pig-origin (TSCP, Hailun county of Heilongjiang province of China), *Trichinella* strain of Chinese cat-origin (TSCC, Sunwu county of Heilongjiang province of China) were isolated by pepsin-HCl digestion (Li & Yuan, 1989) on the 40th day post infection.

Extraction of total RNA and RT-PCR amplification of target genes

About 500 muscle-stage larvae of each strain were ground in a glass grinder in a suspension of TRIZOL (Invitrogen, Carlsbad, California) after being washed with diethyl pyrocarbonate (DEPC) (Sigma, Saint Louis, Missouri)-treated water. The mixture was then transferred into 1.5 mL Eppendorf tubes, and the total RNA was extracted using TRIZOL and the manufacturer-suggested procedures. The P49 gene was amplified by RT-PCR using TaKaRa Ex Taq. The primers used in PCR were designed according to the published P49 sequence of T. spiralis (Su *et al.*, 1991b, GenBank accession number M64242): 5′—GCGAATTCATGGCTACTGATGATACAGAATGGTT —3′ (forward), and 5′— GCGGATCCTTCTATTAGCTG TATGGGC —3′ (reverse). The conditions for PCR were as follows: 1 cycle at 95ºC for 5 min; 20 cycles of 94ºC for 1 min, 48ºC for 1 min, and 72ºC for 2 min; 25 cycles at 94ºC for 1 min, 51ºC for 1 min, and 72ºC for 2 min; and 1 cycle at 72ºC for 8 min.

Cloning, sequencing and analysis of target genes

PCR products were purified with the Gel Extraction Mini Kit (Watson Biotechnologies, Inc., Shanghai, China). The purified PCR products together with restriction sites for cloning purposes were cloned into the pMD18-T Vector (TaKaRa Biotechnology, Dalian, China), and the recombinant plasmids were transformed into Escherichia coli (E. coli) JM-109 strain (TaKaRa Biotechnology). Recombinant plasmids were identified by PCR and restriction analysis. The positive recombinant plasmids were sequenced by Bioasia Biotechnology Co., Ltd. (Shanghai, China). The sequences were assembled and analyzed together with the published sequence (M64242) of the P49 gene of *T. spiralis*. The deduced amino acids of each sequence were also compared and analyzed using DNAStar software (DNA Star, Inc., Madison, Wisconsin).

Prokaryotic expression of P49 of T. nativa

The expression vector pET-30a (provided by the National Key Laboratory of Veterinary Biotechnology, Harbin, China) was treated with BamHI and 0.01U/μl alkaline phosphatase (TaKaRa Biotechnology). The P49 gene with an added initiator ATG encoding *T. nativa* was digested from the clone vector with BamHI and cloned into the BamHI site of pET-30a. Recombinant plasmid was transformed into E. coli BL-21 cells (provided by the National Key Laboratory of Veterinary Biotechnology, Harbin, China), and positive clones were identified by PCR and restriction analysis. The expression of recombinant protein was induced by adding isopropylthio-β-D-galactoside (IPTG, TaKaRa Biotechnology) to a final concentration of 1 mM and further cultivation at 37ºC for 3 h. The recombinant protein was identified by 12 % SDS-PAGE and then examined by commassie blue-staining. Recombinant protein was also identified by the use of the scanner JX-330 (Sharp Corporation, Japan).

Preparation of sera

Sera were collected after the eyeballs were picked off on

the 40th day after mice were infected by *T. nativa*, *T. spiralis*, TSCD, TSCP, TSCC. Control serum was obtained from a normal mouse.

Western blot analysis

Recombinant protein was electrophoresed on 12 % SDS-PAGE, and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was cut into strips after staining with Ponceau S (Shanghai Chemical Reagent Corporation, Shanghai, China). Five sera from mice infected with different *Trichinella* strains and one normal mouse serum at 1:100 dilution were used to react with the recombinant protein. Goat anti-mouse IgG labeled with horseradish peroxidase (Sino-America Biotechnology Co., Luoyang, China) was used as the secondary antibodies in Western blot analysis.

Results

PCR amplification and cloning of target gene

The genes coding for the 49 kDa ES protein of five *Trichinella* strains were cloned, which produced the expected bands about 950 bp except for TSCC (data not shown). These target products were cloned and identified by PCR and restriction analysis (data not shown). The positive clones were sequenced.

Sequencing analysis of P49 genes

DNA sequences were seen for the open reading frame of the gene coding for the 49 kDa ES protein of four *Trichinella* strains. They were about 950bp in length, and encoded 315 amino acids. Sequence comparisons with DNA Star software showed that they all had high homology to the previously identified gene P49 of *T. spiralis* (GenBank accession number M64242), and the target gene from TSCD was identical to the gene P49 of *T. nativa*. There was 97.2 % to 100 % nucleotide identity and 94.3 % to 100% identity of deduced amino acids among P49 genes of the four *Trichinella* strains (Tables 1 and 2). The P49 genes and deduced amino acid sequences were used to produce a dendrogram with two main clusters represented by *T. nativa* and *T. spiralis* (Fig. 1) using DNAStar software. TSCD and TSCP, the two Chinese *Trichinella* isolates of domestic animals seem to belong to *T. nativa*. There were 22 nucleotide and 15 deduced amino acid differences in the P49 gene between *T. nativa* and *T. spiralis*, with 97.7 % and 95.2 % of identity, respectively.

SDS-PAGE and western blotting analysis of the recombinant protein

With the induction of IPTG, the P49 gene of *T. nativa* was expressed in *E. coli* BL-21. The induced cells were harvested and disrupted by sonication in 20 mM Tris-HCl buffer

Table 1. Sequence comparison in the P49 gene of several *Trichinella* strains

	47*	53	92	118	134	145	147	162	176	289	309	378	387	420	423
P49	t	a	a	t	t	g	t	a	t	c	a	a	t	t	a
T. spiralis	t	a	a	t	t	g	t	a	t	c	a	a	t	t	a
TSCD	a	g	g	a	c	g	c	a	t	t	g	c	t	t	g
T. nativa	a	g	g	a	c	g	c	a	t	t	g	c	t	t	g
TSCP	a	g	g	a	c	g	c	a	c	c	g	c	c	c	a

	459	517	518	540	544	559	577	621	634	638	753	770	794	853	882	*906*
P49	a	a	c	c	c	c	a	t	g	a	c	g	t	a	t	t
T. spiralis	a	g	c	c	c	c	a	t	g	a	c	g	t	a	t	t
TSCD	g	g	a	a	g	a	g	t	a	g	t	a	t	g	t	t
T. nativa	g	g	a	a	g	a	g	t	a	g	t	a	t	g	t	t
TSCP	g	g	a	a	g	a	g	c	a	g	t	a	a	g	c	t

* The starting position begins with the eighth nucleotide of the P49 gene of *T. spiralis* (GenBank accession number M64242). Only the different nucleotides are shown in the table. P49: the published P49 gene sequence of *T. spiralis*. See Materials and Methods for abbreviations.

Table 2 . Sequence comparison of deduced amino acids of the P49 protein among several *Trichinella* strains

	16	18	31	40	45	49	59	126	173	180	182	187	193	212	213	257	265	285
P49	l	k	n	s	v	a	m	l	t	n	q	l	t	a	k	s	v	t
T. spiralis	l	k	n	s	v	a	m	l	a	n	q	l	t	a	k	s	v	t
TSCD	q	r	s	t	a	a	m	f	e	k	e	i	a	t	r	n	v	a
T. nativa	q	r	s	t	a	a	m	f	e	k	e	i	a	t	r	n	v	a
TCP	q	r	s	t	a	a	t	f	e	k	e	i	a	t	r	n	d	a

Note: Only the different deduced amino acids are shown in the table. P49: the published protein sequence of *T. spiralis*(protein ID is AAA30328.1). See Materials and Methods for abbreviations

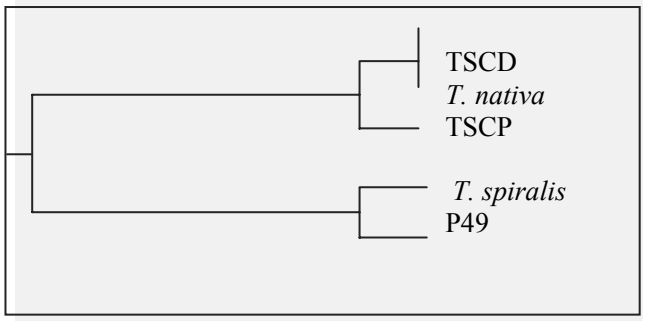

Fig. 1. Dendrogram of several *Trichinella* strains. *Trichinella* strain of Chinese dog-origin (TSCD); *Trichinella* strain of Chinese pig-origin (TSCP)

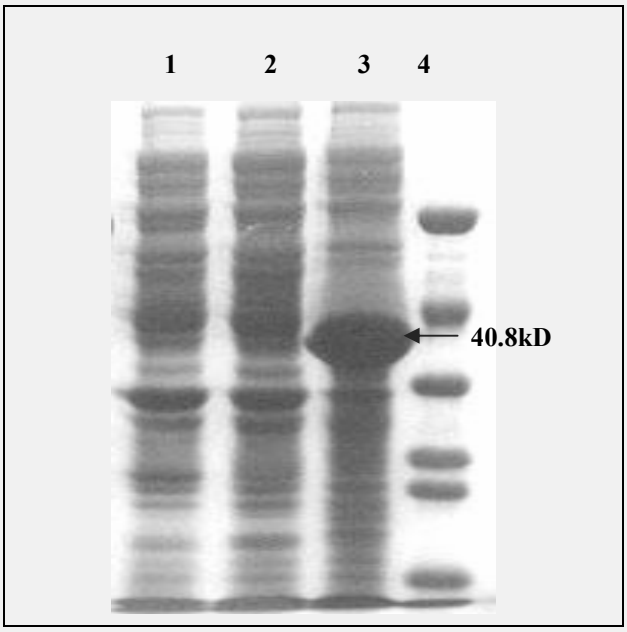

Fig. 2. SDS-PAGE analysis of recombinant 49kDa ES protein of *T. nativa*. Lane 1, total protein of control *E. coli* without IPTG induction; lane 2, total protein of *E. coli* with control plasmid with IPTG induction; lane 3, total protein of *E. coli* with plasmid expressing recombinant 49kDa ES protein of *T. nativa* after IPTG induction; lane 4, protein size markers

(pH 8.0). The expression of the recombinant protein was then analyzed. The recombinant protein migrated at 40.8 kDa - the expected molecular mass (lane 3, Fig. 2), and consisted of 22.8 % of the total cell protein when analyzed with the help of the protein marker and scanner JX-330 (Sharp Corporation, Japan). By contrast, two controls (lane 1 and lane 2, Fig. 2) did not produce the corresponding protein.

In a Western blot analysis, the recombinant protein reacted with all 5 sera from mice that were infected with different *T. nativa* and *T. spiralis* strains (lanes 2 to 5, Fig. 3) but did not react with the serum of normal mouse (lanes 6, Fig. 3). However, only a faint trace was seen in lane 1 with the serum from TSCC infected mice.

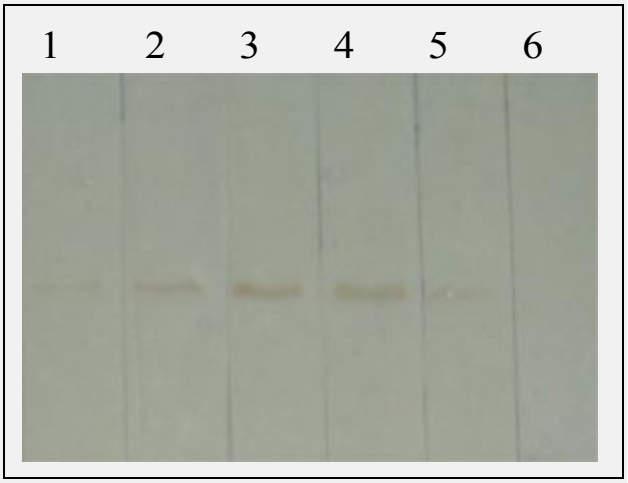

Fig. 3. Western blot analysis of recombinant 49kDa ES protein of *T. nativa*. Strips containing the expressed protein were incubated with mice sera infected with different *Trichinella* strains. Lane 1, serum from mouse infected with *Trichinella* strain of Chinese cat-origin (TSCC); lane 2, serum from mouse infected with TSCP; lane 3, serum from mouse infected with TSCD; lane 4, serum of *T. nativa*-infected mouse; lane 5, serum of *T. spiralis*-infected mouse; lane 6, normal mouse serum

Discussion

In recent years, it is now commonly believed that the genus *Trichinella* consists of at least eight gene pools and several genotypes of uncertain taxonomic status (La Rosa *et al.*, 1992; Pozio *et al.*, 1992a; Wu *et al.*, 1998; 1999, Bolas-Fernandez, 2003). New isolates or genotypes have been continually discovered (Pozio *et al.*, 2002; Geerts *et al.*, 2002; La Rosa *et al.*, 2003; Bolas-Fernandez, 2003). The difference between *T. spiralis* and *T. nativa* in the P49 gene was identified in this study. The identity of nucleotide and deduced amino acids in the P49 gene suggested a close relationship between *T. nativa* and *T. spiralis*, which was also supported by the cross-reactivity of P49 antigen in Western blot analysis. The Chinese *Trichinella* strains of domestic animal-origin used in this study, with the exception of TSCC, were identified to be *T. nativa*. The TSCC may be an isolate that is more divergent from *T. spiralis* and *T. nativa*, because it could not be amplified by RT-PCR using primers based on *T. spiralis* sequence, and the serum from TSCC infected mice gave only a faint trace with the recombinant protein in Western blot. A Chinese article indicated that TSCC was related to *T. britovi* (data not shown).

In this study, the gene encoding 49 kDa ES protein of *T. nativa* was acquired and expressed successfully in E. coli. The expressed product reacted with five sera from mice infected with different *Trichinella* strains. The sequence encoding *T. nativa* 49 kDa ES antigen reported here has been deposited in the GenBank database under accession number AY486427. The sequence encoding 49 kDa ES antigen of TSCD in this study was identical to the P49 gene of *T. nativa*, indicating that TSCD was *T. nativa*. This

result was in accord with earlier studies (Song *et al.*, 1994; 1998; Zhu *et al.*, 1998).

The recombinant protein, which consisted of 22.8 % of the total cell protein, indicating that 49 kDa ES protein gene of *T. nativa* could be expressed at a high level in *E. coli*. Prokaryotic expression systems produce protein free of glycosylation whereas native *Trichinella* 49 kDa ES protein are glycoproteins (Denkers *et al.*, 1990; Wisnewski *et al.*, 1993; Yan *et al.*, 1997). In theory, it is possible that the products expressed by *E. coli*, as diagnostic antigen for trichinellosis, may not be recognized by *Trichinella*-infected sera due to the lack of glycosylation. The results of Western blot analyses, however, indicated that the recombinant protein reacted with all five *Trichinella*-infected mice sera used in this study.

Because of the high homology in nucleotide and deduced amino acid sequences of the P49 gene between *T. spiralis* and *T. nativa* in this study, we have to presumed that the antigenicity of the two proteins should not be significantly different, and they both can be used as antigens for the diagnosis of each other. Results of the Western blot analyses supported this idea. The recombinant protein of *T. nativa* could be recognized by either *T. nativa* serum or *T. spiralis* serum. Thus, it is possible that the recombinant ES protein of one *Trichinella* strain can be recognized by other *Trichinella* strains related to either *T. nativa* and *T. spiralis*.

Only the recombinant P49 protein of *T. nativa* was studied here, and we cannot confirm if the same conclusion also applied to other *Trichinella* ES proteins such as 21 kDa, 43 kDa, 45 kDa, and 53 kDa ES proteins. Because of the lack of access to other *Trichinella* isolates, almost all five strains of the parasites studied here were *T. nativa* or *T. spiralis* and their Chinese geographical strains (Zhu *et al.*, 1998; Liu *et al.*, 1998). Consequently, we can only conclude that the recombinant protein in this study can be used to diagnose trichinellosis caused by both *T. nativa* and *T. spiralis*. Whether the recombinant antigen can be recognized by sera from humans or animals with trichinellosis caused by other *Trichinella* isolates, such as *T. nelsoni*, *T. britovi* and *T. pseudospiralis*, remains to be determined.

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