Experimental Parasitology 131 (2012) 148–152

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Molecular similarities and differences between *Trichinella* spp., isolated from canine skeletal muscle in Zacatecas, Mexico

Luz Ofelia Franco Sandoval [a], María de Lourdes Caballero García [a], Gabriela Rebeles Hernández [b], Maria Alejandra Moreno García [b], Enedina Jiménez Cardoso [a,*]

[a] Children's Hospital of Mexico Federico Gomez, Dr. Márquez No. 162, CP 06720, Mexico, DF, Mexico
[b] Center for Experimental Biology of UAZ, Autonomous University of Zacatecas, Zacatecas, Mexico

ARTICLE INFO

Article history:
Received 16 December 2011
Received in revised form 22 February 2012
Accepted 7 March 2012
Available online 16 March 2012

Keywords:
PCR
5SRNAr
IsRNAr
Trichinella spp.
Canine

ABSTRACT

Four different isolates of *Trichinella* spp. (Z1, Z2, Z3, and Z4) obtained from the skeletal muscle of street dogs in the state of Zacatecas, Mexico were serial passaged in Wistar rats; infective larvae from the skeletal muscle of the rats were collected and frozen in liquid nitrogen. After centrifugation, DNA was extracted and the *5SRNAr* and *IsRNAr* genes were amplified. The isolates were identified by the size of the amplified products from the *5SRNAr* and *IsRNAr* genes (750 and 290 bp, respectively). The amplicons obtained by PCR were sequenced, aligned, and compared to the reference strain *Trichinella spiralis* MSUS/MEX/91//EM isolated from pigs.

Based on our results, we determined that the *Trichinella* isolates from canine (Z1–Z4) belonged to the *T. spiralis* species and had 83% identity with the reference strain. The phylogenetic tree constructed from the sequences showed differences between the isolates from pig and dog. These genetic differences may be related to the immune response of the host or the pathogenicity of the isolates. Therefore, these findings have important epidemiological and public health implications.

1. Introduction

Trichinellosis is a zoonotic disease that occurs worldwide and is caused by the nematode *Trichinella* genus, which consists of eight species and four genotypes (*Trichinella spiralis*, *Trichinella nativa*, *Trichinella murrelli*, *Trichinella britovi*, *Trichinella nelsoni*, *Trichinella pseudospiralis*, *Trichinella papue*, *Trichinella zimbabwensis*, *T-6*, *T-8*, *T-9* and *T-12*) (Krivokapich et al., 2000). *T. spiralis* is cosmopolitan and has spread in nature among a large number of carnivores, human, and other incidental hosts (Murrell et al., 2000). In Mexico, particularly in Zacatecas, 758 cases with trichinellosis have been reported between 1952 and 1997. Most of the outbreaks occurred after consumption of improperly cooked pork meat (Ortega-Pierres et al., 2000). From 1970 to 1990, eight cases of trichinellosis in children were reported, which were diagnosed based on epidemiological and clinical histories as well as laboratory tests. Most cases occurred in children between the ages of 10 and 14 years who were from poor socio-economic areas (Cabral et al., 1990).

One animal species that is infected by *T. spiralis* is the dog, which can eat meat containing viable larvae and subsequently develop trichinellosis. Infection is associated with meat and other by products from pigs, which is a common source of infection for humans. Infection in dogs is often due to consumption of food scraps, others animals, and meat from clandestine slaughterhouses.

In 1978, *T. spiralis* was reported in dogs from the city of Laguna de Carretero, State of Zacatecas, which was during the same period of time when the first outbreak in humans occurred. Eighteen persons became infected and six died, with a fatality rate and an infection rate of 100% (Berumen et al., 2002).

Several molecular markers have been used to identify *Trichinella* spp., but detection of ribosomal DNA (DNAr) is currently the most widely used method, since the *5S* gene of nematodes is highly conserved and the amplified fragment has been well characterized in different species of *Trichinella* (Liu et al., 1996). In addition, detection of the *IsRNAr* gene allows for the identification of different species of this parasite (Gasser et al., 2005).

The aims of this study were to identify genetic differences among four isolates of *Trichinella* spp. (Z1–Z4) isolated from street dogs in the state of Zacatecas. In addition, using the reference strain *T. spiralis* MSUS/MEX/91/CM, we determined the sequences of *5S* ribosomal DNA intergenic spacer region (*5SRNAr*) and mitochondrial large-subunit ribosomal RNA (*IsRNAr*) gene in order to identify differences between species and construct a phylogenetic tree.

* Corresponding author. Fax: +55 55884019.
E-mail addresses: jimenezce@yahoo.com.mx, enedina@servidor.unam.mx (E.J. Cardoso).

0014-4894/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exppara.2012.03.004

2. Materials and methods

2.1. Artificial digestion

Isolated *Trichinella* spp. Z1, Z2, Z3, and Z4 isolates obtained from muscle of street dogs in the city of Zacatecas, Mexico as well as the *T. spiralis* MSUS/MEX/91/CM reference strain were sequentially inoculated in Wistar rats. Thirty days after infection, the rats were sacrificed to isolate larvae from the skeletal muscles (L1). The larvae were digested with 1% pepsin–HCl at 37 °C for 3 h with constant agitation. They were then collected by sedimentation using a Baermann funnel, washed with 1× phosphate-buffered saline (PBS), and stored at −70 °C until assayed (Pozio and Zarlenga, 2005).

2.2. Extraction of DNA and amplification of the 5SRNAr and IsRNAr genes

Five hundred larvae frozen in N_2 were lysed and homogenized with 0.1 mg/mL proteinase K and 0.5% SDS solution at 50 °C overnight. The samples were then sequentially mixed with phenol, phenol–chloroform–isoamílico alcohol, and chloroform–isoamilico alcohol to isolate the DNA (Sambrook et al., 2000). The suspension was centrifuged for 20 min at 5000*g* at 4 °C and the DNA pellet was resuspended in 50 μL of double-distilled water. The concentration and quality of DNA were determined by measuring absorbance at 260 and 280 nm and the samples were stored at −20 °C for future use.

The intergenic region of the *5SRNAr* gene was amplified by the method previously described (De Bruyne et al., 2005). The master mix contained 8 pmol/μL of each primer (3′-GCGAATTCTTGGATCG-GAGACGGCCTG-5′ and 3′-GCTCTAGACGAGATGTCGTGCTTTCAACG-5′), 200 μM of dNTPs, 1× PCR buffer (100 mM Tris–HCl pH 8.3; 500 mM KCl), 1.5 mm $MgCl_2$, 1.5 U/μL Taq DNA polymerase, and 900 ng of genomic DNA in a final volume of 25 μL. The PCR cycling conditions were as follows: one cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 40 s. A final extension cycle was performed at 72 °C for 15 min. The method used to amplify the *IsRNAr* gene has been previously described (Gasser et al., 2005). The reaction was performed in 50 μL containing the following components: 10 pmol/μL of each primer (3′-GCGATTGAGTTGAACGC-5′ and 3′-GTTCCATGTGAACAGCAGT-5′), 200 μM of dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, 1.5 U/μL Taq DNA polymerase, and 500 ng of genomic DNA. The amplification cycling was as follows: one cycle of 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s. A final extension cycle was performed at 72 °C for 10 min. The products were separated by 1.5% agarose gel electrophoresis at 80 V and stained with 0.5% ethidium bromide.

2.3. Sequencing of the 5SRNAr and IsADNr genes

The purified PCR products (40 ng) were incubated with 3 pmol of sense oligonucleotides and 8 μL Big Dye (Applied Biosystem®) were added in a final volume of 20 μL with water. The amplification program for the *5SRNAr* gene was as follows: one cycle of 96 °C for 2 min followed by 30 cycles of 96 °C for 10 s, 55 °C for 5 s and 60 °C for 4 min. For the *IsRNAr* gene, the cycling program was as follows: one cycle at 96 °C for 4 min, followed by 30 cycles of 96 °C for 10 s, and 70 °C for 4 min. The sequencing reactions were purified on Centri-Sep columns (Applied Biosystem®), dried for 30 min in a SpeedVac®, and hydrated with 15 μL of formamide at 96 °C for 3 min. The samples were processed on an ABI Prism 310 (Applied Biosystem®) at 50 °C for 35 min per reaction (Anderson et al., 1981). The sequences of these genes were analyzed and aligned using the Clustal W, Bioedit, and DNAman bioinformatics programs. Mega5 was used to construct the phylogenetic tree (Altschul et al., 1990) utilizing 1000 replications for bootstrap and with the method that provided maximum parsimony.

3. Results

Approximately 20,000 larvae L1/mL were isolated from Z1 to Z4 isolates as well as the reference strain. DNA concentrations ranged from 400 to 600 ng/μL with a purity range of 1.7–2.0. The larvae integrity was assessed by 1.5% agarose gel electrophoresis.

The amplification of the *5SRNAr* gene is shown in Fig. 1. Of note, a 750 bp fragment was amplified from the *T. spiralis* reference strain as well as isolates Z1, Z2, Z3, and Z4, which indicated that there were no differences in the length of the amplified region or in species that have encapsulated larvae. Fig. 2 shows amplification of the *IsRNAr* gene. Similar to the *5DNAr* gene, the amplified region of the *IsRNAr* gene from the reference strain as well as the Z1, Z2, Z3, and Z4 isolates was the same.

We next sequenced the amplified fragments from the *5SRNAr* and *IsRNAr* genes of the reference strain and isolates (Fig. 2). The sequence identity of *5SRNAr* between Z2 and Z4 isolates was

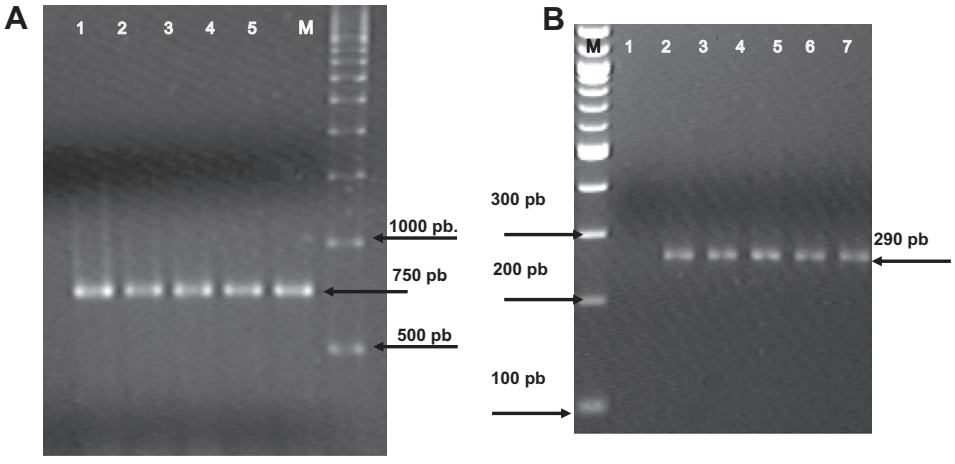

Fig. 1. (A) *5SRNAr* gene amplification. Agarose gel electrophoresis shows amplification of a 750 bp fragment. Lane 1: reference strain *Trichinella spiralis*; lane 2: Z1; lane 3: Z2; lane 4: Z3; and lane 5: Z4; M: 1 kb molecular weight marker. (B) *IsRNAr* gene amplification. Lane 1: negative control; lane 2: 290 bp fragment of *Trichinella spiralis*; lane 3: Z1; lane 4: Z2; lane 5: Z3; and lane 6: Z4 isolate. M: 100 bp molecular weight marker.

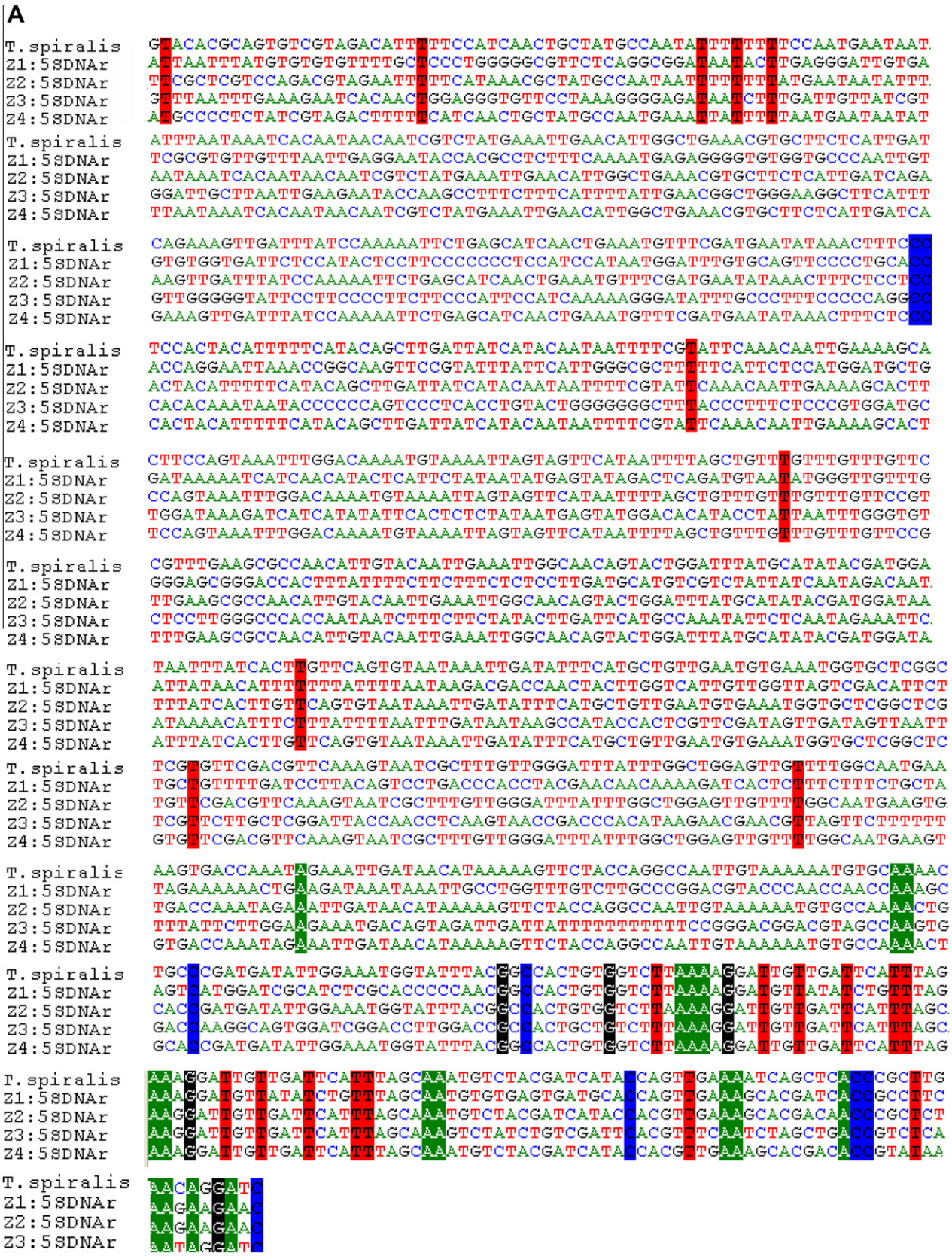

Fig. 2. Sequence alignment of the reference strain and Z1, Z2, Z3, and Z4 isolates. (A) *5SRNAr* gene and (B) *lsRNAr* gene sequences.

approximately 97%, whereas the identity between Z1 and Z3 isolates was 46%. Similarly, the sequence identity of *lsDNAr* was approximately 85% between Z1 and Z3, and 80% between Z2 and Z4. Importantly, the phylogenetic tree showed differences between

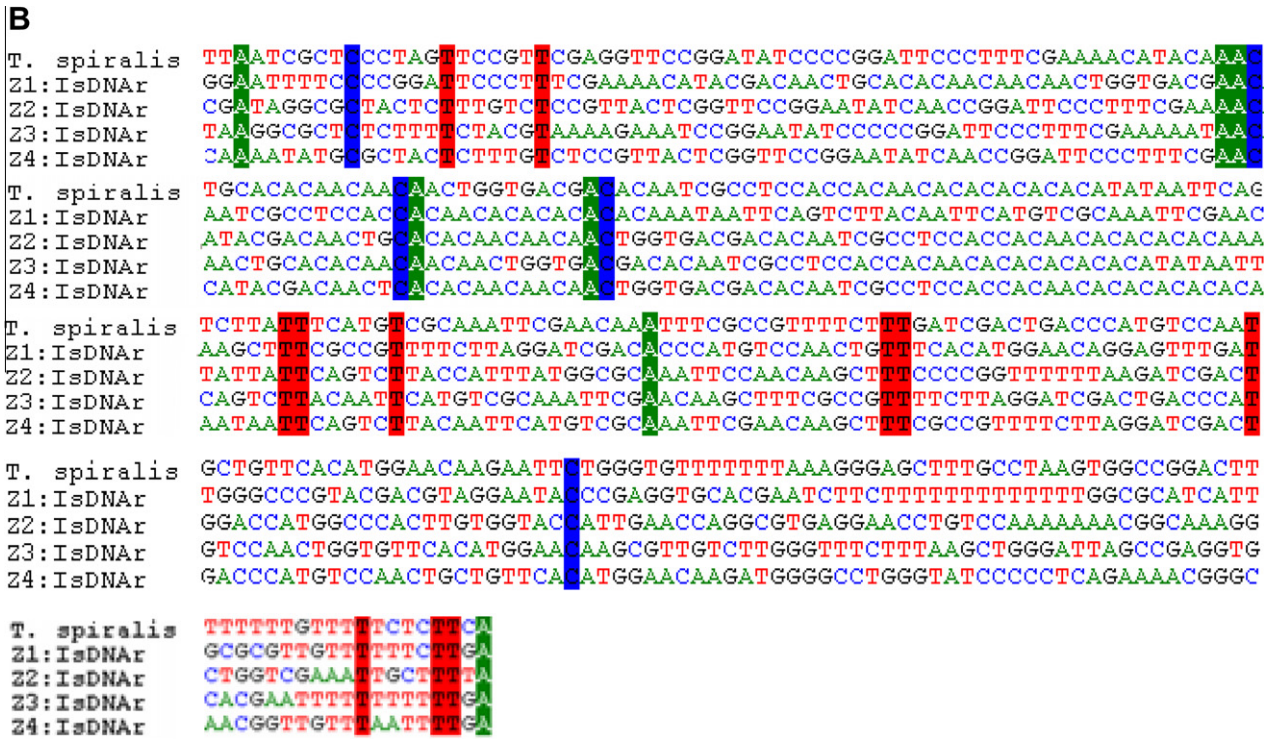

Fig. 2 (*continued*)

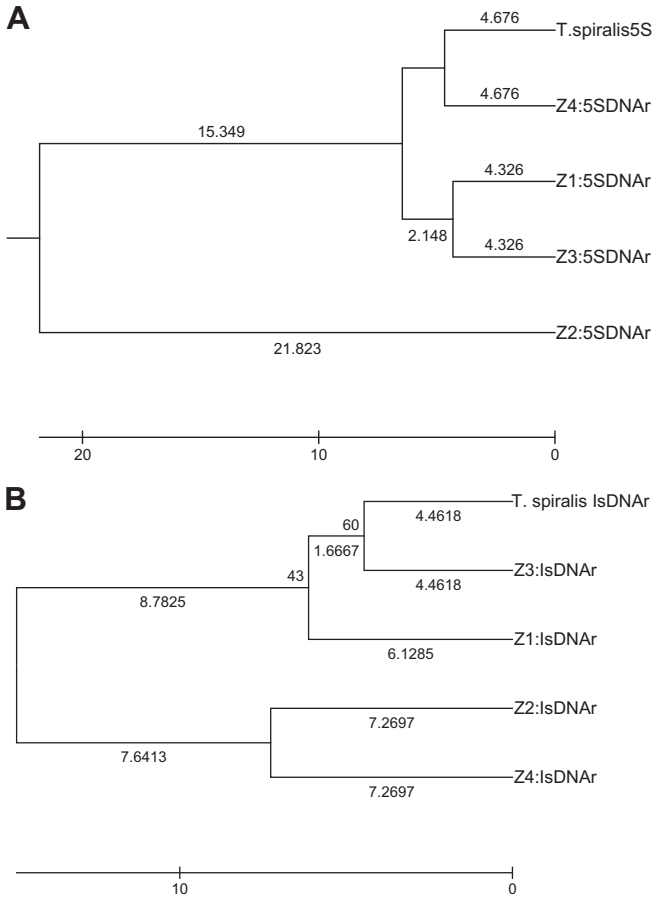

Fig. 3. Phylogenetic trees using the alignments for the (A) *5SRNAr* gene and (B) *IsRNAr* gene. The trees were generated based on 1000 replications for bootstrapping using the maximum parsimony method.

canine *Trichinella* isolates and the reference MSUS/MEX/91/CM strain isolated from pork (Fig. 3).

4. Discussion

Our study found that amplification of a specific segment of the *5SRNAr* gene from the *T. spiralis* reference strain as well as the Z1, Z2, Z3, and Z4 isolates generated a 750 bp fragment, this allowed us to unequivocally identify single encapsulated and non-encapsulated larvae of *Trichinella*; it suggested that these isolates could belong to *T. spiralis, T. murrelli*, or *T. britovi* species, since the same fragment size has been reported in these three strains, because have encapsulated larvae (Liu et al., 1996). However, although *T. britovi* is capable of infecting bears, wolves, jackals, and dogs, it has only been reported in Europe to date. Therefore, based on the geographical distribution and the connection to Zacatecas State, we originally hypothesized that these isolates may be the *T. murrelli* strain (Pozio and La Rosa, 2000). To explore this hypothesis further, we conducted an evaluation of the *IsRNAr* gene, which can distinguish *T. spiralis* from *T. murrelli*. We found that the amplified fragment from this gene matched *T. spiralis* and not *T. murrelli*, since the latter strain exhibits a characteristic 230 bp amplicon, which was not observed in our analysis, we obtained fragments of 290 bp. Therefore, we concluded that the Z1, Z2, Z3, and Z4 isolates belong to the *T. spiralis* species.

Interestingly, we found that the sequenced fragments from the two genes showed differences based on sequence alignment, suggesting that that there were genetic differences between the isolates. These differences can be explained by the role of the host. Our reference strain was obtained from pork, whereas the analyzed isolates were recovered from street dogs. We hypothesize that the different host environments induced a mutation or mutations within these genes of the isolates, which could reflect pathogenic differences between the reference strain and isolates. These differences were also observed in the phylogenetic tree.

The molecular differences between the isolates could result in a discrepancy between structural proteins of each isolate in relation to the host and its immune response. For instance, the nucleotide sequences that were not shared by the different encoded proteins could influence the composition of the antigen excretion-secretion, which would result in different host-immune responses, virulence, and pathogenicity. Notably, these differences could have a marked impact on epidemiological studies (Nagano et al., 2009).

The importance of canine hosts for trichinellosis has been demonstrated not only in Mexico, but also in countries such as China, where presence of the pathogen has been analyzed in canines from 13 provinces. These studies revealed a mean prevalence of 16.2% (range: 1.2–44.8%) in local butcher shops and slaughterhouses. In addition, the prevalence of trichnellosis in the markets was found to be 3.5%, which represents a serious public health problem, particularly in northern China, where cold-resistant *T. nativa* was reported in 37 outbreaks of human trichinellosis between 1964 and 2004 (Wang et al., 2007).

In countries such as Finland, the risk of trichinellosis in domestic dogs was reported to be 19.4%. Infection with *T. nativa* occurs most frequently with multiparasitic *Trichinella* species in street dogs. Importantly, the fact that wild animals are infected increases the possibility of human infection (Oivanen et al., 2002). Epidemiological studies in 15 districts of Estonia reported that 1.0–79.4% of wild and domestic animals are parasitized, and 6–24.5% of these hosts are canines (Jarvis et al., 2001).

Our study found that the morphology of the parasite and PCR amplification of the isolates and control strain were equal to the encapsulated larvae (Vander-Giessen et al., 2005) and had 83% molecular similarity with the control. Therefore, these isolates only differed from the reference strain by the host that they parasitize. It is important to note that we did not use a microsatellite genetic variation technique to amplify the *5SRNAr* gene, because none of the larvae were encapsulated and therefore the species could not be *T. pseudospiralis* (Zarlenga et al., 1996).

In the future, it will be important to study and characterize the excretory-secretory antigens to determine if there are shared proteins between these isolates. In addition, future work will study the infectivity of each strain by inducing trichinellosis in biological models (Dupouy-Camet et al., 1991; Yao et al., 1997). The difference in prevalence and abundance of helmints in both animals found in this study may reflect differences in host ecology and susceptibility.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Anderson, S., Bankier, A.T., Barrell, B.G., De Brujin, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlian, D.O., Roe, B.A., Sanger, F., Schreirer, P.H., Smith, A.J., Staden, R., Young, I.G., 1981. Sequence and organization of the human mitochondrial genome. Nature 290, 457–465.

Berumen, T.V., Muñoz, E.J., Moreno, J.M., 2002. Trichinellosis in dogs from in Zacatecas, Mexico. Parasitol. Latinoam. 57, 72–74.

Cabral, S.J., Villacaña, F.H., Fragoso, U.R., Contreras, A., 1990. Perfil epidemiológico de la triquinosis en el Estado de Zacatecas. Salud Pública en México 32 (5), 575–582.

De Bruyne, A., Year, H., Guerhier, F.L., Boireau, P., Dupouy-Camet, J., 2005. Simple species identification of *Trichinella* isolates by amplification and sequencing of the 5S ribosomal DNA intergenic spacer region. Vet. Parasitol. 132, 57–61.

Dupouy-Camet, J., Soule, C., Guillou, J.P., Rouer, E., Lavareda de Souza, S., Ancelle, T., Benarous, R., 1991. Detection of repetitive sequences of *Trichinella spiralis* by polymerase chain reaction in experimentally infected mice. Parasitol. Res. 77, 180–182.

Gasser, R.B., Hu, M., El-Osta, Y.A., Zarlenga, D.S., Pozio, E., 2005. Genetic analysis of *Trichinella* populations by "cold" single-strand conformation polymorphism analysis. Vet. Parasitol. 132, 23–26.

Jarvis, T., Miller, I., Pozio, E., 2001. Epidemiological studies an animal and human trichinellosis in Estonia. Parasite 8, 86–87.

Krivokapich, S.J., Prous, C.L., Gatti, G.M., Confalonieri, V., Molina, V., Matarasso, H., Guarnera, E., 2000. Molecular evidence for a novel encapsulated genotype of *Trichinella* from Patagonia, Argentina. Vet. Parasitol. 156, 234–240.

Liu, L.X., Blaxter, M.L., Shi, A., 1996. The 5S ribosomal RNA intergenic region of parasitic nematodes variation in size and presence of SL1 RNA. Mol. Biochem. Parasitol. 83, 235–239.

Murrell, K.D., Lichtenfels, R.J., Zerlenga, D.S., Pozio, E., 2000. The systematics of the genus *Trichinella* with a key to species. Vet. Parasitol. 93, 293–307.

Nagano, I., Wu, Z., Yakahashi, Y., 2009. Functional genes and proteins of *Trichinella* spp.. Parasitol. Res. 104, 197–207.

Oivanen, L., Kapel, C.M., Pozio, E., La Rosa, G., Mikkonen, T., Sakura, A., 2002. Associations between *Trichinella* species and host species in Finlandia. J. Parasitol. 88, 84–88.

Ortega-Pierres, M.G., Arriaga, C., Yépez-Mulia, L., 2000. Epidemiology of trichinellosis in Mexico, Central and South America. Vet. Parasitol. 93, 201–225.

Pozio, E., La Rosa, G., 2000. *Trichinella murrelli* sp.: etiological agent of sylvatic trichinellosis in temperature areas of North America. J. Parasitol. 86, 134–139.

Pozio, E., Zarlenga, D.S., 2005. Recent advances on the taxonomy, systematic and epidemiology of *Trichinella*. J. Parasitol. 35, 1191–1204.

Sambrook, J., Maniatis, T., Frisch, E.F., 2000. Molecular Cloning a Laboratory Manual, third ed. Cold Spring-Harbour Laboratory, New York.

Vander-Giessen, J.W.B., Fonville, M., Briels, I., Pozio, E., 2005. Phylogenetic analysis of encapsulated and non-encapsulated *Trichinella* species by studying the 5SrDNA tandemly repeated intergenic region. Vet. Parasitol. 132, 51–55.

Wang, Z.Q., Cui, J., Shen, L.J., 2007. The epidemiology of animal trichinellosis in China. Vet. J. 173, 243–244.

Yao, C., Prestwood, A.K., Mc-Graw, R.A., 1997. *Trichinella spiralis* (T1) and *Trichinella* T5: a comparison using animal infectivity and molecular biology techniques. J. Parasitol. 83, 88–95.

Zarlenga, D.S., Aschenbrenner, A.R., Liohtenfels, R.J., 1996. Variations in microsatellite sequences provide evidence for populations differences and multiple ribosomal genes repeats with *Trichinella pseudospiralis*. J. Parasitol. 82, 534–538.