International Journal for Parasitology 42 (2012) 903–910

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

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

Trichinella patagoniensis n. sp. (Nematoda), a new encapsulated species infecting carnivorous mammals in South America ☆

Silvio J. Krivokapich [a,*], Edoardo Pozio [b], Graciana M. Gatti [a], Cinthia L. Gonzalez Prous [a], Mabel Ribicich [c], Gianluca Marucci [b], Giuseppe La Rosa [b], Viviana Confalonieri [d]

[a] *Departamento de Parasitología, Instituto Nacional de Enfermedades Infecciosas, Administración Nacional de Laboratorios e Institutos de Salud, "Dr. Carlos G. Malbrán", Av. Vélez Sarsfield 563, 1281 Buenos Aires, Argentina*
[b] *Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*
[c] *Cátedra de Parasitología y Enfermedades Parasitarias, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Av. Chorroarín 280, C1427CWO Buenos Aires, Argentina*
[d] *Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pab. II, Intendente Guiraldes y Costanera Norte, 1428 Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 4 June 2012
Received in revised form 19 July 2012
Accepted 20 July 2012
Available online 17 August 2012

Keywords:
Nematoda
Taxonomy
Ribosomal DNA
Puma concolor
Argentina
Trichinella patagoniensis
New species

ABSTRACT

Until a few years ago, *Trichinella spiralis* was the only taxon of the genus *Trichinella* detected in both domestic and wild animals of South America. Recently, a new genotype, named *Trichinella* T12, was identified in cougars (*Puma concolor*) from Argentina, on the basis of molecular studies using mitochondrial and nuclear ribosomal markers. In the present study, cross-breeding experiments indicated that *Trichinella* T12 is reproductively isolated from all other encapsulated *Trichinella* spp. and suggested that it is biologically more similar to *Trichinella britovi* and *Trichinella murrelli* than to the other encapsulated species/genotypes. Biological assays revealed that the reproductive capacity index of *Trichinella* T12 was ~4 and >2000 times lower than those of *T. spiralis* in mice and rats, respectively. The reproductive capacity index of *Trichinella* T12 in domestic pigs ranged from 0.0 to 0.05. Larvae parasitising the muscles of carnivores were infective to mice after freezing at −5 °C for 3 months, but they lost infectivity after freezing at −18 °C for 1 week. The region within the rDNA, known as the expansion segment V, showed a unique sequence which differs from those of all other known *Trichinella* spp./genotypes. The biological, geographical and molecular data support the classification of the genotype *Trichinella* T12 as a new species widespread in the Neotropical region, for which we propose the name *Trichinella patagoniensis* n. sp.

1. Introduction

Nematodes of the genus *Trichinella* are zoonotic parasites with a cosmopolitan distribution. Two main clades have been identified in this genus: the encapsulated clade with five species (*Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi*, *Trichinella murrelli* and *Trichinella nelsoni*) and four genotypes (*Trichinella* T6, *Trichinella* T8, *Trichinella* T9 and *Trichinella* T12) infecting only mammals; and the non-encapsulated clade with one species (*Trichinella pseudospiralis*) infecting mammals and birds and two species (*Trichinella papuae* and *Trichinella zimbabwensis*) infecting mammals and reptiles (Pozio et al., 2009). Until recently, only *T. spiralis* had been identified in domestic and wild animals in South America (Pozio, 2000; Krivokapich et al., 2006; Ribicich et al., 2010). This parasite is of European origin, as indicated by microsatellite studies (Rosenthal et al., 2008; La Rosa et al., 2012). Larvae of a *Trichinella* sp. isolated from a cougar (*Puma concolor*) in Argentina in 2004 showed the mitochondrial cytochrome *c*-oxidase (cox-1) gene and the nuclear 5S ribosomal intergenic spacer region (5S-ISR) to be different in sequence from those of the other *Trichinella* taxa, and was thus named *Trichinella* T12 (Krivokapich et al., 2008).

In the present paper, we describe the morphological, biological and molecular characteristics of three isolates of the *Trichinella* T12 genotype from cougars. Reproductive and molecular data provide support for the recognition of these isolates as a separate species within the genus *Trichinella*.

2. Materials and methods

2.1. Parasite origin and collection

Larvae of *Trichinella* spp. were collected from the striated muscles of four cougars hunted in Argentina: (i) near the locality of Trapalcó (Rio Negro province, 66°59′W, 39°34′S) in 2004. Only

☆ New nucleotide sequence data reported in this paper are available in the GenBank database under Accession codes JF260985–JF2601000 and HE819395.

* Corresponding author. Tel./fax: +54 11 43017437.
E-mail address: silkri@anlis.gov.ar (S.J. Krivokapich).

0020-7519/$36.00 © 2012 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ijpara.2012.07.009

dead larvae (worm burden in the hind limb muscles, one larva/g) were collected (isolate code ISS1826), preserved in absolute ethanol, and then identified by sequencing of the cox-1 gene and the 5S-ISR as a new genotype, *Trichinella* T12 (Krivokapich et al., 2008); (ii) near the locality of El Calafate (Santa Cruz province, 72°16′W, 50°20′S; five larvae/g in the intercostal muscles) in 2008. This *Trichinella* isolate (code ISS2311) is maintained by serial passage in laboratory mice at the International *Trichinella* Reference Centre (ITRC), Rome, Italy; (iii) from the district of La Paz (Catamarca province, 65° 4′W, 29°17′S) in 2009. Only dead larvae (worm burden in the tongue, 6.6 larvae/g) were collected (isolate code ISS3559) and preserved in absolute ethanol; and (iv) from the district of La Paz (Catamarca province, 65°4′W, 29°17′S; nine larvae/g in the tongue) in 2009. This isolate was identified as *T. spiralis* (code ISS3558) and was used for comparative biological study. *Trichinella* larvae from natural and laboratory hosts were obtained by the standard artificial digestion method (Gamble et al., 2000).

2.2. PCR

Genomic DNA was extracted from single muscle larvae following a previously published protocol (Krivokapich et al., 2006). DNA from single larvae from the isolates ISS2311, ISS3558 and ISS3559 was subjected to multiplex PCR for identification (Zarlenga et al., 1999; Pozio and La Rosa, 2010). For the sequence analysis of the cox-1 and 5S-ISR markers, DNA was isolated from a pool of muscle larvae and then amplified by PCR according to published protocols (Nagano et al., 1999; Rombout et al., 2001). The region within the lsrDNA, known as the expansion segment V (ESV), was amplified using the primer pair TS10177-F (5′-TAAGAAAACGGCGAAAGC-3′) and TS10177-R (5′-AACCCCACAGAGAGATTAAAG-3′). PCR amplifications were performed in 30 μL of a standard reaction mix (Qiagen, Germany), 10 pmol of each primer and 10 μL out of 100 μL of a DNA sample purified from a single larva. The amplification was carried out for 37 cycles as follows: 95 °C for 30 s, 56 °C for 60, and 72 °C for 30 s, plus a pre-step at 95 °C for 5 min and a post-step at 72 °C for 5 min. Twelve larvae from each of the *Trichinella* isolates ISS2311 and ISS3559 were tested individually. The PCRs were performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA).

2.3. Nucleotide sequencing and sequence analysis

The PCR products from the ESV region of the 12 larvae from the *Trichinella* isolates ISS2311 and ISS3559 were sequenced in both directions using the same primers as those employed for the amplification. The amplification products of the cox-1 and 5S-ISR of the two isolates (ISS2311, ISS3559) were cloned using a Topo TA Cloning Kit into a pCR 2.1-TOPO vector (Invitrogen, Brazil). Three positive clones from each of the two markers (cox-1, 5S-ISR) were sequenced in both directions, using universal M13 forward and reverse primers, by an ABI PRISM 3100-Avant sequencer (Applied Biosystems). Sequences were analysed with the Chromas Lite 2.0 (Technelysium Pty Ltd, Australia) software. Multiple sequence alignments, representing each of the three markers, were performed using ClustalW (Thompson et al., 1994) using the default settings. DNA sequences for cox 1, ESV and 5S-ISR were deposited in GenBank under the Accession Nos. JF260987–JF260992, HE819395 and JF260995–JF261000, respectively (Table 1).

2.4. Phylogenetic analysis

A total of 21 and 20 sequences were used for the phylogenetic analysis of cox-1 and 5S-ISR genes, respectively, corresponding to all of the *Trichinella* taxa known to date and the new *Trichinella* isolates reported herein (Table 1). Sequences of the nematode *Enterobius vermicularis,* of the family Oxyuridae, were used as an outgroup for comparison (GenBank Accession Nos. EU281143 for cox-1 and U65496 for 5S-ISR).

Table 1
GenBank accession numbers of cytochrome *c*-oxidase subunit I (cox-1) gene and 5S rDNA intergenic spacer region (5S-ISR) gene sequences of all *Trichinella* taxa used for the phylogenetic analysis in this study.

Trichinella taxa	Isolate[a]	GenBank accession number for cox-1	Isolate[a]	GenBank accession number for 5S-ISR
Trichinella spiralis	ISS248	DQ007890[b]	NA	AY009946[c]
Trichinella nativa	ISS70	DQ007891[b]	NA	AY009944[c]
Trichinella britovi	ISS271	DQ007892[b]	NA	AY009943[c]
Trichinella pseudospiralis	ISS13	DQ007893[b]	NA	AY009950[c]
Trichinella murrelli	ISS470	DQ007894[b]	NA	AY009947[c]
Trichinella T6	ISS40	DQ007895[b]	NA	AY009948[c]
Trichinella nelsoni	ISS37	DQ007896[b]	NA	AY009945[c]
Trichinella T8	ISS149	DQ007897[b]	NA	AY009949[c]
Trichinella T9	ISS408	DQ007898[b]	NA	NA
Trichinella papuae	ISS572	DQ007899[b]	ISS 572	AY845861
Trichinella zimbabwensis	ISS1029	DQ007900[b]	ISS1029	AY845862
Trichinella T12	ISS1826	EU161657[d]	ISS1826	EF694983[d]
Trichinella T12	ISS1826	JF260985[e]	ISS1826	JF260993[e]
Trichinella T12	ISS1826	JF260986[e]	ISS1826	JF260994[e]
Trichinella T12	ISS2311	JF260987[e]	ISS2311	JF260995[e]
Trichinella T12	ISS2311	JF260988[e]	ISS2311	JF260996[e]
Trichinella T12	ISS2311	JF260989[e]	ISS2311	JF260997[e]
Trichinella T12	ISS3559	JF260990[e]	ISS3559	JF26098[e]
Trichinella T12	ISS3559	JF260991[e]	ISS3559	JF26099[e]
Trichinella T12	ISS3559	JF260992[e]	ISS3559	JF261000[e]

NA: isolate and/or sequence data not available in GenBank.
[a] Isolate code of the International Trichinella Reference Centre, Rome, Italy (www.iss.it/site/Trichinella/index.asp).
[b] Zarlenga et al. (2006).
[c] Rombout et al. (2001).
[d] Krivokapich et al. (2008).
[e] Present work.

The nucleotide divergence was based on the proportion of nucleotide sites at which the compared sequences were different (*p*-distance); the analysis was conducted using the program MEGA v. 4.0.1 (Tamura et al., 2007). Neighbour-Joining (NJ) trees were constructed from matrices of genetic distances in MEGA v. 4.0.1 (Tamura et al., 2007).

2.5. Morphological study

Muscle larvae and adults of isolate ISS2311 were fixed in 70% ethanol. Measurements were taken and averaged from: (i) 30 male and 30 female muscle larvae recovered from the fifth passage in mice, obtained by artificial digestion of the carcass of a CF-1 mouse that had been infected for 35 days; and (ii) 30 male and 30 female adult worms recovered from the gut of a CF-1 mouse 6 days p.i., which had been infected with 1000 larvae from the fifth passage in mice. The following morphometric variables were examined in muscle larvae: total body length, width at mid-body, length of oesophagus, length of stichosome, length of genital primordium, length of rectum, and distance from posterior margin of genital primordium to posterior extremity of body. Morphometric variables taken from adults were: total body length, width at mid-body, length of oesophagus, length of stichosome, length of uterus, length of ovary, length of testis, and length and width of copulatory appendages. These measurements were compared with those reported for other species of *Trichinella* in the literature (Dick, 1983; Pozio et al., 1992a; Murrell et al., 2000; Pozio and La Rosa, 2000).

2.6. Cross-breeding experiments

Muscle larvae were sorted by sex (Pozio et al., 1999) and five mice (CD1 females of 25 g each) were inoculated by gavage