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Research paper

Molecular characterization of *Trichinella* species from wild animals in Israel

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ABSTRACT

Trichinellosis is a worldwide disease caused by nematode worms of the genus *Trichinella*, frequently diagnosed in Israel. However, the identity of the Israeli isolates have not been studied. Here we describe the molecular characterization of 58 isolates collected from jackals (*Canis aureus*), wild boar (*Sus scrofa*), foxes (*Vulpes vulpes*) and a wolf (*Canis lupus*) in central and northern Israel. Isolates were analyzed using the multiplex PCR analysis encompassing expansion segment V (ESV) and internal sequence 1 (ITS-1) markers, which identified 52 of the 58 samples. Out of the six unidentified samples, four were successfully identified using extended PCR assays for ESV and ITS-1, developed in this study. Our analysis identified 44 isolates as *T. britovi*, 8 as *T. spiralis*, four mixed infections, and two isolates were not identified. Clonal analysis of the ITS-1 sequences from six isolates confirmed the initial identification of four mixed infections. These results show that the prevalent species in Israel are *T. britovi* and *T. spiralis*, with nearly 7% (4 of 58) incidence of mixed infection.

1. Introduction

Trichinellosis is a widespread parasitic disease caused by nematodes of the *Trichinella* genus, which infect both animals and humans (Pozio, 2007). The first outbreak of human trichinellosis in the modern state of Israel was reported in 1971, where thirty nine people were severely infected following consumption of wild boar meat (Margyah, 1975). The second outbreak was reported in 1992 following consumption of undercooked wild boar meat (Eisenman and Einat, 1992). Since then, an increasing number of human infections have been reported In Israel (Hefer et al., 2004; Marva et al., 2005). Since 1992, annual examination of approximately 1000 wild boars revealed an overall incidence rate that ranged between 1.9% and 4.2% (Alexander Marcovics, unpublished data). Due to the morphological similarity of different *Trichinella* species, their correct identification is challenging, and can be met by using molecular tests (Pozio and Zarlenga, 2005). Such methods include species-specific size difference of PCR products (Zarlenga et al., 1999; Marucci et al., 2009; Rodríguez et al., 2008) or specific marker sequences (Pozio et al., 2002, 2005; Franssen et al., 2015). Although *Trichinella* sp. infections have been frequently diagnosed in Israel during the last 20 years, this study is the first to characterize *Trichinella* species circulating in Israel using both PCR fragment analysis and DNA sequencing.

2. Materials and methods

2.1. Collection and isolation of trichinella larvae from muscle specimens

Trichinella sp. larvae were isolated from carcasses of 58 animals, as follows: 10 (3.57%) out of 280 examined from wild boar (*Sus scrofa*), 45 from jackals (*Canis aureus*), two from foxes (*Vulpes vulpes*), and one from a wolf (*Canis lupus*), out of 430 examined carnivores (a total of 11.16%). The jackals originated from Northern and Central Israel, while all other animals (wild boar, fox, and wolf) originated from Northern Israel. Larvae Isolation was performed from muscle diaphragms (Markovics et al., 1993; Zarlenga et al., 1999) and is described in detail in the Online Supplementary Methods file. Isolated larvae were pooled and numbered according to host sample number.

2.2. DNA extraction, PCR analysis and clone analysis

DNA was extracted from pools of 3 to about 1500 larvae, using the MasterPure kit (Epicentre) according to the manufacturer's instructions. Initial species identification was performed using the multiplex PCR as described by Zarlenga et al. (1999).

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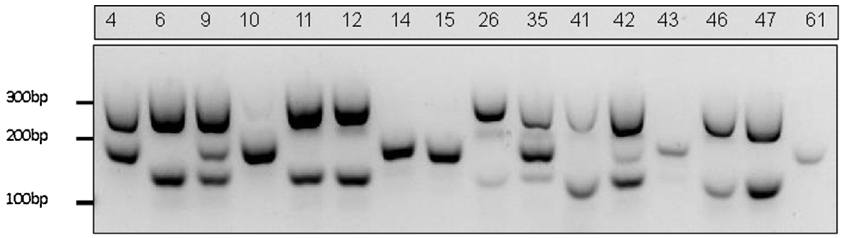

Fig. 1. Identification of *Trichinella* isolates using duplex PCR and extended amplification PCR. Multiplex PCR analysis was performed for ITS-1 and ESV sequences, as described in Section 2.2 and in the Supplementary Methods. Representative samples are shown with their assigned isolate number. Similar PCR examination was performed for the other 42 isolates. Interpretation of the multiplex PCR results is detailed in Section 3. Molecular size is indicated on the left.

The detailed procedure is described in the Supplementary Methods file. In order to sequence the genomic regions flanking the segments amplified by the multiplex PCR, an extended PCR was designed and used. Briefly, two reactions were designed, to amplify a 562bp-long segment of the Internal Transcribed Sequence 1 (ITS-1) region, and a 550–595 bp segment of the Expansion Segment 5 (ESV) region. The primers used were as follows: for the ESV PCR: forward: 5′-AAATGGATAGCGTTTGAGCTCC-3′ and reverse: 5′-GAATCCCTATCTCCGGACAACC-3′. For the ITS-1 PCR: forward:5′-GGCTTCGCGCCGGGAAATTTC-3′ and reverse: 5′-TTTAAACCTGATGCACAACAC-3′. The detailed description of the reaction conditions appears in the Supplementary Methods file. PCR products were purified and sequenced. ITS-1 PCR products that gave ambiguous results in the direct sequencing, or for which a mixed infection was suspected, were examined using clonal analysis, as detailed in the supplementary Methods file. Briefly, the PCR products from the examined isolates were cloned into a bacterial plasmid and 3–5 single clones from each isolate were sequenced.

New sequences obtained in this work were deposited in GenBank™. The accession numbers for the ESV sequences are **KU374847** to **KU374866**. The ITS1 sequences are numbered: **KU374867** to **KU374887**.

2.3. Sequence analysis

Multiple alignments and similarity calculations were performed using the MUSCLE algorithm (Edgar, 2004) embedded in the Geneious software (Biomatters). Phylogenetic tree construction was performed with the Mega 6 software (Tamura et al., 2013). Maximum likelihood statistical algorithm was used as the construction method, according to the Tamura-Nei model (Tamura and Nei, 1993).

3. Results and discussion

Microscopic examination of digested tissue collected from positive host showed a larvae burden of 1 to 135 larvae per gram of muscle. In order to identify the species of isolated *Trichinella* sp. larvae, multiplex PCR was used, which differentiates between 7 different *Trichinella* species (Pozio et al., 2010). Band patterns resulting from the amplification of Expansion Segment 5 (ESV) and Internal Transcribed Sequence 1 (ITS-1) indicated the presence of *T. britovi*, *T. spiralis*, or both. Representative results of single *T. britovi* or *T. spiralis* band pattern, as well as mixed infection pattern, are shown in Fig. 1. Multiplex PCR Analysis of 58 *Trichinella* isolates from wild animals in various locations in Israel identified 41*T. britovi* and 7*T. spiralis*, as detailed in Table S1. The analysis of four isolates: 4, 9, 35 and 42 suggested that their hosts were infected with both species. Isolates 17, 23, 24, 27, 32 and 48 could not be identified using the multiplex PCR, despite repeated attempts. Each assay was performed with control reactions of *T. britovi* and *T. spiralis* DNA, as size references. Among the mixed infections, isolate 4 repeatedly gave a unique pattern which differed from the patterns described by Pozio et al. (2010), lacking the *T. britovi* ESV 127 bp band (Fig. 1). Isolates 9 and 42 showed a pattern of predominantly *T. britovi* infection, while isolate 35 was predominantly *T. spiralis* (Fig. 1).

The suspected mixed infections in four hosts, as well as the failure to obtain results from six other hosts using the multiplex PCR, prompted the design of an extended PCR assay, to analyze a region corresponding to 594 bp in the *T. spiralis* sequences, and 550 bp in the *T. britovi* ESV sequences, spanning the multiplex PCR amplicon of each region. A Similar analysis was designed to allow examination of a 560 bp section within the ITS1 region. The extended PCR analysis was applied to 20 isolates out of the 58 initially examined: the four that were suspected as mixed infections (isolates 4, 9, 35 and 42), the six that were not identified using the duplex PCR (17, 23, 24, 27, 32 and 48), and 12 samples that were successfully identified using the duplex PCR (isolates 2, 10, 14, 15, 16, 21, 25, 29, 46, 52, 60, 61). Both isolate 9, which was identified as a mixed infection in the duplex PCR analysis, and isolate 17, which was negative in the duplex PCR, were identified as *T. spiralis* in the ESV sequencing (Table S1). Similarly, isolates 27, 32 and 48 that were negative by the multiplex PCR, were identified as *T. britovi* by the ESV sequencing (Fig. 2A, Table S1). Isolates 23 and 24 remained unidentified by both the duplex PCR and the extended PCR.

The ESV sequences were analyzed to confirm the multiplex PCR results and to determine whether there are differences between the newly sequenced isolates and non-Israeli, GenBank™-annotated samples. Analysis was performed using an alignment of the 20 sequenced samples and 10 non-Israeli annotated sequences. As suggested by the duplex PCR analysis (Fig. 1), the *T. spiralis* ESV sequences contained two short stretches (12 bp and 32 bp long) that were absent from the *T. britovi* sequence. The phylogenetic dendrogram inferred from the ESV alignment showed distinct separation between *T. britovi* and *T. spiralis* sequences (Fig. 2A). Within the *T. spiralis* clade, additional clustering was apparent, between samples containing a 10-bp gap and those that do not (Fig. 2A, Fig. S1). All the newly-obtained sequences and 3 of the 10 non-Israeli annotated sequences included in the analysis, contained the 10-bp stretch. Notably, the ESV sequence of isolate 9 was identified as *T. spiralis*, but was located far from the other *T. spiralis* samples in the dendrogram.

Amplification and subsequent sequencing of a 560 bp ITS-1 region spanning the multiplex PCR amplicon of the same 20 isolates that were examined by ESV PCR showed that like the ESV analysis, isolates 17, 27, 32 and 48 were successfully identified, while isolates 23 and 24 remained unidentified (Table S1). The ITS1 sequencing results of larvae pools from six hosts: 4, 9, 10, 17, 35 and 42, were not conclusive, suggesting that these hosts were infected with both species (mixed infections). In order to determine whether larvae of both species were present in a single host, these samples were subjected to clonal analysis, as described in the Supplementary Methods file. The following clones were sequenced from each isolate: isolate 4 – four clones, isolate 9 –three clones, isolate 10 – four clones, isolate 17 – five clones, isolate 35 – four clones and iso-

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