

First report of *Trichinella pseudospiralis* in Poland, in red foxes (*Vulpes vulpes*)

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Abstract

Nematode worms of the genus *Trichinella* are one of the most widespread zoonotic pathogens. Natural transmission between hosts can only occur through the ingestion of infected meat. To date, two *Trichinella* species are known to be etiological agents of disease among domestic animals and wildlife in Poland: *T. spiralis* and *T. britovi*. In the last decades, since the administration of an oral vaccination against rabies, the red fox population in Poland has increased exponentially. The study area covers the Nowy Targ region: a mountainous area (585–1138 m above the sea) in southern Poland. Of 24 red foxes examined in the study, four were infected with *Trichinella* isolates: three were identified as *T. britovi* and one as *T. pseudospiralis*. The muscle of red foxes infected with *T. britovi* harboured 2.75, 3.11, 4.4 LPG and with *T. pseudospiralis* 0.36 LPG. *Trichinella* larvae were identified at species level by genomic and mitochondrial multiplex PCR, the products of which were sequenced for comparison with other sequences available in GenBank. The sequences obtained from the Polish *T. pseudospiralis* isolate, deposited in GenBank under the accession numbers JQ809660.1 and JQ809661.1, matched sequences already published in GenBank. Sequence comparison showed a 100% match with the large subunit ribosomal RNA gene of *T. pseudospiralis* isolate ISS 013, and a 96–95% match with those of *T. pseudospiralis* isolates ISS 141 and ISS 470. This is the first report of the identification of *T. pseudospiralis* larvae from red fox in Poland.

Keywords

Trichinella pseudospiralis, red foxes, Poland, mitochondrial multiplex PCR, sequencing

Introduction

Since Owen first described *Trichinella* as a human pathogen in 1835, the number of organisms, and the number of species, comprising this genus has grown dramatically. This etiological agent of human trichinellosis shows worldwide distribution in both domestic and sylvatic animals. Four species with different biological characteristics have been described in European Union (EU) countries: *T. spiralis*, *T. britovi*, *T. nativa* and *T. pseudospiralis* (Hurníková *et al.* 2005; Malakauskas *et al.* 2007; Merialdi *et al.* 2011; Remonti *et al.* 2005; Pozio and Murrel 2006; Pozio *et al.* 2009).

To date, two *Trichinella* species have been recognised as the etiological agents of epidemiology among domestic and wildlife animals in Poland: *T. britovi* is the dominant species in red foxes and *T. spiralis* is the dominant species in wild boars and domestic pigs. It was found to be highly prevalent

in wild animals, most of which were carnivores, e.g. foxes and wolves, but omnivores, such as badgers and martens, were also affected (Cabaj *et al.* 2000, 2004; Moskwa *et al.* 2012).

Trichinella pseudospiralis is the only species which is able to infect birds (Pozio and Murrel 2006), however, in the last decade, this species has been detected much more frequently in the other predators in the sylvatic and human ecosystems. In Europe, *T. pseudospiralis* has been identified in domestic pigs and rats (Hurníková *et al.* 2005, Széll *et al.* 2012), wild boars (Hurníková and Dubinský 2009, Merialdi *et al.* 2011, Pozio *et al.* 2004, Széll *et al.* 2012) and lynx (Pozio *et al.* 2004). Although the red fox (*V. vulpes*) is considered one of the most important sylvatic reservoirs of *Trichinella* spp. in Europe (Cabaj *et al.* 2000, Cuperlovic *et al.* 2005, Marinculic *et al.* 2001, Olteanu 2001), there are only three reports of *T. pseudospiralis* infection in this species. In Lithuania,

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a mixed infection of *T. pseudospiralis* with *T. britovi* has been noticed in one of 1000 red foxes, and only one larva of *T. pseudospiralis* was detected (Malakauskas 2002). In Hungary, *T. pseudospiralis* larvae were found in one of 2,116 examined red foxes (Széll *et al.* 2008).

The red fox population in Poland has increased exponentially during the last decades since the administration of an oral vaccination against rabies. According to the Agricultural Property Agency, Directorate Generale of the State Forests and the Polish Hunting Association, the number of red foxes reached 211,000 in 2011 (Central Statistical Office, 2011; http://www.stat.gov.pl/cps/rde/xbcr/gus/rl_lesnictwo_2011.pdf).

The aim of this work was to detect and identify *Trichinella* spp. in the red foxes from the Nowy Targ region in Poland.

Materials and methods

Fox sampling

The study area covers the Nowy Targ region (49°28' latitude North and 20°01' longitude East), a mountainous area 585–1138 m above sea level in southern Poland (Fig. 1).



Fig. 1. Map of Poland showing the origin (◆) of the red foxes infected with *T. britovi* and *T. pseudospiralis*

A total of 24 red foxes were examined for the presence of *Trichinella* spp. Sampling was performed in collaboration with the Voivodship Veterinary Inspectorate, Regional Veterinary Laboratory in Kraków. The animals were hunted within rabies vaccination camping in the beginning of 2012.

Sample digestion

The muscle sample was taken from the lower part of the forelegs. The muscles from each individual fox were tested for *Trichinella* spp. using pepsin digestion according to EC Regulation No. 2075/2005 (European Commission 2005). Digested samples were washed three times in H₂O and any larvae present were counted and stored in 95% ethyl alcohol until molecular species identification. The larvae were identified as *Trichinella* based on gross morphology. Finally, the larvae were counted and the number of larvae per gram of muscle tissue (LPG) was calculated.

Species identification

Trichinella larvae were identified at species level by multiplex PCR according to Zarlenga *et al.* (1999). Muscle larvae of *T. spiralis* (ISS003), *T. nativa* (ISS042), *T. britovi* (ISS002), and *T. pseudospiralis* (ISS013) reference strains were used as controls.

The amplified genomic DNA samples were analysed on 2% agarose gels stained with GelRed (Biotium) in TAE buffer, at 100 V. The gels were visualised and analysed under UV light using the KODAK 1D™ Electrophoresis Documentation and Analysis System.

Additionally, for genetic typing, single larvae were tested also by a mitochondrial multiplex polymerase chain reaction (mitochondrial multiplex PCR) as described by Blaga *et al.* (2009). PCR products underwent electrophoresis at 100 V on 2% agarose gels stained with GelRed (Biotium) in TAE buffer. Gels were visualised and analysed under UV light using the KODAK 1D™ Electrophoresis Documentation and Analysis System.

A segment of approximately 276 bp obtained by mitochondrial multiplex PCR for *T. pseudospiralis* was excised from the agarose gel and purified using the NucleoSpin Extract II (Macherey-Nagel, Germany), according to the manufacturer's instructions.

DNA was sequenced in the forward and reverse directions by Microsynth AG Switzerland, the company offering a sequencing service. The sequence chromatograms were edited using Vector NTI Advance™, version 10 (Invitrogen, USA). BLAST searches were performed (<http://www.ncbi.nlm.nih.gov>) in order to compare the sequences with those in the public database.

Results

Of the 24 examined animals, four were infected with *Trichinella* larvae. The foxes harboured 2.75, 3.11, 4.4 LPG and 0.36 LPG, respectively.

The multiplex PCR (Zarlenga *et al.* 1999) identified specific patterns for *T. britovi* (127 bp plus 253 bp) in 3 of the foxes and for *T. pseudospiralis* (310 bp) in the fourth fox. Figure 2

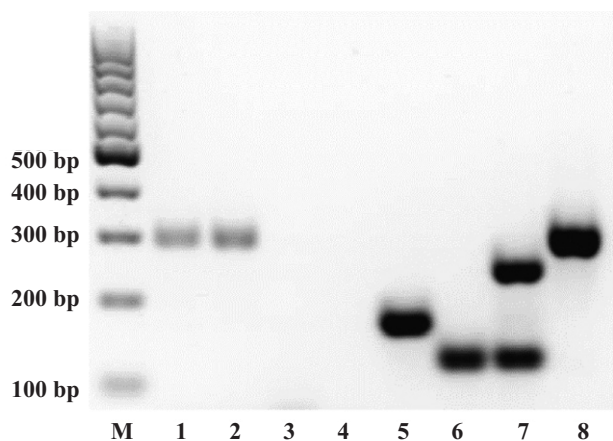


Fig. 2. Agarose gel separation of multiplex PCR products using genomic DNA from: line 1–2 – *T. pseudospiralis* from red fox; lines 3–4 – negative controls; line 5 – *T. spiralis* reference strain; line 6 – *T. nativa* reference strain; line 7 – *T. britovi* reference strain; line 8 – *T. pseudospiralis* reference strain; line M – molecular size marker (GeneRuler 100 bp ladder, Fermentas)

show the agarose gel separation of multiplex PCR products using genomic DNA from *T. pseudospiralis* isolate. In addition, the presence of *T. pseudospiralis* in one examined sample was confirmed by mitochondrial multiplex PCR which identified specific patterns for *T. pseudospiralis* (276 bp) (Fig. 3).

The products of mitochondrial PCR of two separate larvae of *T. pseudospiralis* isolate have been sequenced and deposited in GenBank under accession numbers JQ809660.1 and Q809661.1.

The sequences obtained from *T. pseudospiralis* larvae matched sequences already published in GenBank, showing 100% similarity with the large subunit ribosomal RNA gene of *T. pseudospiralis* isolate ISS 013 (accession number JQ809662.1), and 96–95% similarity with the large subunit ribosomal RNA gene of *T. pseudospiralis* isolates ISS 141 and ISS 470 (accession numbers EF51724.1 and EF51723.1, respectively).

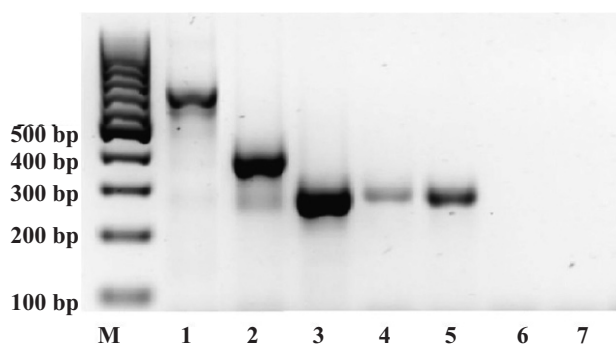


Fig. 3. Agarose gel electrophoresis of mitochondrial multiplex PCR products: line 1 – *T. spiralis* reference strain ISS 003, line 2 – *T. britovi* reference strain ISS 002, line 3 – *T. pseudospiralis* reference strain ISS 013; line 4–5 – *T. pseudospiralis* isolate from red fox; lines 6–7 – negative controls; line M – molecular size marker (GeneRuler 100bp ladder, Fermentas)

Discussion

The elimination of rabies *via* a nationwide vaccination program is likely to have major impacts on the dynamics of red fox populations. An increase in fox numbers and population densities has been found in the majority of European countries participating in this program (Chautan *et al.* 2000, Kauhala *et al.* 2006, Pannwitz *et al.* 2010, Vos 2003). The fox population has increased by 3-fold to more than 10-fold depending on the region of Poland (Goszczyński *et al.* 2008, Panek and Bresiński 2002). The results of longitudinal observations (1998–2011) conducted by the Research Station of the Polish Hunting Association in Czempin reveal a substantial increase in the red fox population from 47,700 in 1991 up to 201,200 in 2011 (data unpublished, personal communication): an increase of 321.7%.

It is probable that the anti-rabies vaccination program is not the only reason for this increase. Easy adaptation to new habitats like suburban or urban areas, and the use of abundant food such as human garbage are also possible causes (Contesse *et al.* 2004; Gloor *et al.* 2001; Goldyn *et al.* 2003; König 2008; Tryjanowski 2000). The increase in fox population density has been found to correlate with the numbers of breeding dens located outside forests (Goszczyński *et al.* 2008).

The estimated prevalence of *Trichinella* spp. in red foxes in EU countries ranges from 0.001% in Denmark (Enemark *et al.* 2000) to 6.4% in Poland (Cabaj *et al.* 2005). In Poland, the prevalence of trichinellosis in red foxes varies according to environmental conditions and region (Ramisz *et al.* 1998). In north-western Poland, *Trichinella* larvae were found in 4.25% of 395 examined foxes, which contrasts with south-western Poland (Lower Silesia and Opole District), where no examined foxes were found to be infected with *Trichinella* spp. The results of the study performed in central and north-eastern Poland revealed 5.4% and 6.3% of infected red foxes. The results of longitudinal observations published by Ramisz *et al.* (2011) reveal the prevalence of trichinellosis in red foxes in the Western Pomerania region show the tendency of increased significantly from 3.4% in 2000 to 5.4% in 2009. Until now, only three reports of *T. pseudospiralis* infection in red foxes have been published: two as a mixed infection with *T. britovi* (Malakauskas 2002) and one as an infection of a single nematode species (Széll *et al.* 2008). It must be noted that the detected prevalence was very low. In Lithuania, only one *T. pseudospiralis* larva was detected in one of 1,000 red foxes. In Hungary, *T. pseudospiralis* larvae were found in one of 2,116 examined red foxes. The findings in a wild boar and red fox infected with *T. britovi* and *T. pseudospiralis* in Slovakia (Hurníková and Dubinský 2009) are interesting from an epidemiological point of view; both infected animals originated from the same region where an outbreak caused by *T. pseudospiralis* occurred in a pig farm in 2004 (Hurníková *et al.* 2005).

The *T. pseudospiralis*-infected red fox identified in this study was found in southern Poland, in a mountain region,

where the higher moisture and lower temperatures favour the survival of muscle larvae in the host carrion for a longer period of time (Pozio 1998). This location is only 159 km from a *T. pseudospiralis* domestic focus in Michalovce, Slovakia (Hurníková *et al.* 2005) and 181 km from a *T. pseudospiralis* sylvatic focus in Szabolcs-Szatmár-Bereg in Hungary (Széll *et al.* 2008). The next nearest *T. pseudospiralis* sylvatic focuses are located about 395 km away in the village of Medgyesbodzás-Gábortelep in Hungary and 436 km away in the village of Kapelna (Donji Miholjac district) (Beck *et al.* 2009). A non-encapsulated *Trichinella* focus was also found near the north-west border with Poland (Zirchow, Usedom island in Macklenburg-Western Pomerania) about 637 km from Nowy Targ (Nöckler *et al.* 2006). In addition, non-encapsulated species of *Trichinella* were found in domestic pigs and synanthropic rats in Slovakia, in red foxes and wild boars in Hungary, in domestic pigs in Croatia and in wild boars in Germany.

Hurníková *et al.* (2005) and Széll *et al.* (2008) indicate that infected birds represent a potential risk for the spread of the parasite over large areas, and can be responsible for multiple incidences of *T. pseudospiralis* infection. As *T. pseudospiralis* focuses have been noticed at significant points where bird migration routes across Europe cross, it has been speculated that *T. pseudospiralis* is transmitted this way; however, it is possible that the parasite was previously established in the wildlife of this region (Hurníková and Dubinský 2009). Nöckler *et al.* (2006) suggest that changes in the population densities of wild boars or omnivorous birds, such as corvids and seagulls, through Europe may be an effect of the growing number of *T. pseudospiralis* findings. The presence of infected rats and red foxes in domestic focuses suggests the important role of both species in nematode transmission (Hurníková *et al.* 2005, Hurníková and Dubinský 2009). It is possible that the widespread transmission of *T. pseudospiralis* may be the result of the migration of larger, long-lived carnivores: these would establish new areas of sylvatic transmission and provide a means of transmission by predation upon infected animals.

The high red fox population densities may be associated with an increased level of scavenging and cannibalism, which are the major routes of *Trichinella* transmission (Pozio *et al.* 1996, Remonti *et al.* 2005). In addition, wild boars can be responsible for the spread of *Trichinella* via ingestion of the carcasses of red foxes or infected small mammals (Antolová *et al.* 2006).

The route of *T. pseudospiralis* infection in pigs remains unknown. However, consumption of meat from severely infected animal tissue, possibly from a garbage dump in the vicinity of the farm, would seem to be the most likely explanation (Beck *et al.* 2009).

The sequences obtained from the large subunit ribosomal RNA gene of the Polish *T. pseudospiralis* isolate originating from the red fox matched with sequences already published in GenBank (<http://www.ncbi.nlm.nih.gov/genbank>). In particular, a 100% match was found with the large subunit ribosomal

RNA gene of *T. pseudospiralis* isolate ISS 013 (accession number JQ809662.1) and a 96–95% match with the large subunit ribosomal RNA gene of *T. pseudospiralis* isolates ISS 141 and ISS 470 (accession numbers EF51724.1 and EF51723.1, respectively).

Isolate ISS 013 was obtained from a raccoon (*Procyon lotor*) from the Caucasus, Russia (42°30' latitude North and 44°44' longitude East) and has been designated as the reference strain for *T. pseudospiralis*, Palearctic region. Isolate ISS 141 was obtained from a tiger cat (*Dasyurus maculatus*) from Tasmania, Australia (41°20'9" latitude South and 146°37'14" longitude East) and has been designated as the reference strain for *T. pseudospiralis*, Australian region. Isolate ISS 470, the designated reference strain for *T. pseudospiralis* Nearctic region, was obtained from a black vulture (*Coragyps atratus*) in Alabama, USA (32°5' latitude North and 86°47' longitude West) (www.iss/site/Trichinella/script/read.asp).

All told, four geographically disparate isolates, ISS 013, ISS 141, ISS 470 and the Polish *T. pseudospiralis* isolate originating from the red fox, closely match the large subunit ribosomal RNA gene. These results correspond well with the dendrogram constructed by the unweighted pair group analysis of *Trichinella* species/genotype by La Rosa *et al.* (2003). Among the non-encapsulated species, two groups can be observed, one of which is the non-encapsulated species infecting mammals and birds.

Based on the available data of *Trichinella* isolates (www.iss/site/Trichinella/script/read.asp), it was not possible to perform a full analysis of the similarity between *T. pseudospiralis* isolates originating from such European Countries as Slovakia (the host: domestic pig, ISS 1432; the host: brown rat, ISS 1460; the host: domestic cat, ISS 1461), Hungary (the host: red fox, ISS 1837; the host: wild boar, ISS 3178), Germany (the host: wild boar, ISS 1816, ISS 1817, ISS 1911, ISS 2246, ISS 2247, ISS 2758, ISS 3343, ISS3637, ISS 3871, ISS 3872) or Czech Republic (host: wild boar, ISS 3501 and ISS 3502) and Croatia. The DNA sequences of these *Trichinella* isolates were not sequenced and deposited in GenBank.

These results further confirm the tendency of increasingly frequent detection of non-encapsulated species of *T. pseudospiralis* in Europe, first noted by Nöckler *et al.* (2006). The detection of a non-encapsulated parasite species infecting both birds and mammals in domestic and sylvatic cycles strongly confirms the circulation of the parasite among domestic and wildlife animals. As a consequence of the very low prevalence of *T. pseudospiralis* found in this study, future investigations of this nature will need a larger population sample size in order to allow a valid interpretation of the results.

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