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First record of wild boar infected with *Trichinella pseudospiralis* in Poland

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Abstract

Introduction: The paper describes identification of *Trichinella* species isolated from wild boars (*Sus scrofa*) in the most popular hunting region of the West Pomeranian Province of Poland. **Material and Methods:** The *Trichinella* larvae were identified by digestion method. For species identification of the larvae, multiplex PCR was used according to the European Reference Laboratory for Parasites Multiplex PCR protocol. The results were confirmed by molecular amplification of 5S rDNA gene and sequence analysis. **Results:** Prevalence of 0.54% *Trichinella*–positive wild boars in the West Pomeranian Province was recorded. Examination of the larvae showed the occurrence of *T. spiralis* in 79 %, *T. britovi* in 16.5 %, mixed infection with *T. spiralis/T. britovi* in 3.5%, and *T. pseudospiralis* in 1.0% of the boars. **Conclusion:** This is the first record of wild boar infected with non-encapsulated larvae of *T. pseudospiralis* in Poland. The species is very difficult to determine, especially using trichinoscopic method. The discovery of the larvae in the animals which may be intended for human consumption confirms that digestion technique should be the only method used for the inspection of meat, especially that from wild boars..

Keywords: wild boars, *Trichinella pseudospiralis*, West Pomeranian Province, Poland.

Introduction

Trichinellosis still constitutes an epidemiological problem, both in Poland and in other countries of the European Union. Despite application of new rules concerning post-mortem inspection, every year new cases of trichinellosis are recorded in human. The disease is caused by internal nematodes *Trichinella* belonging to the family Trichinellidae. So far, nine species of *Trichinella* have been classified: *T. spiralis*, *T. britovi*, *T. nativa*, *T. nelsoni*, *T. murrelli*, *T. zimbabwensis*, *T. papuae*, *T. pseudospiralis, T. patagoniensis*, and three genotypes: T6, T8, and T9. In Europe, *T. spiralis* and *T. britovi* occur most often, but cases of infections with *T. nativa* and *T. pseudospiralis* are recorded with increasing frequency, especially in population of wildlife animals. The reservoir of the parasite includes about 150 species of animals, mainly carnivores and omnivores; however, herbivores can also be a vector of *Trichinella* (20). *Trichinella* occurs both in wildlife and in synanthropic environment. The transfer of the parasite from one environment to another can occur under favourable conditions. Natural forest areas have suitable conditions for the spread of this parasite, with wild boars (*Sus scrofa*) and red foxes (*Vulpes vulpes*) as the most important vectors. Transmission of the parasite occurs when a susceptible host ingests muscle tissue containing live *Trichinella* larvae. Due to the high risk for humans, wild boar meat represents the main source of infection (25). Based on the data obtained from the National Institute of Public Health - National Institute of Hygiene, Poland, 956 cases of human trichinellosis were reported in Poland in 2000–2012 (25). Parasitological examination for the presence of *Trichinella* larvae in wild boar meat is a part of routine post-mortem examination of carcasses. In Poland, more than 100,000 wild boars are harvested yearly, and most of them come from the West Pomeranian Province, where the presence of *Trichinella* among wild boars in

Poland is the highest. Till now, two species of *Trichinella* were detected in Polish wild boar population: *T. spiralis* and *T. britovi* (2). *T. pseudospiralis* was reported once in the red fox (14), but up to now it has never been detected in wild boars. This non-encapsulated species is very difficult to detect using the compressor method. Since the Veterinary Inspection Services in the West Pomeranian Province have introduced the digestion method to routine examination of wild boar meat, the chances to identify this parasite have largely increased.

The aim of the presented study was to identify the *Trichinella* species circulating in wild boar population in the West Pomeranian Province of Poland.

Material and Methods

Samples were collected from the West Pomeranian Province in 2012. Diaphragm muscles from 16 737 wild boars were examined by digestion method (29). For species identification of larvae, the following samples from 91 infected wild boars were provided: 71 wild boar muscle tissues (provided frozen) and 20 isolates of *Trichinella* larvae (preserved in 96% ethyl alcohol). Muscle samples were examined by artificial digestion (50 g of muscle tissue from each animal) according to the EU Regulation 2075/2005, Annex I, Chapter III for parasites larvae isolation and for evaluation of infection intensity. Intensity of parasite invasion was defined as the number of larvae per gram of meat sample. The larvae obtained in digestion method were preserved in 96% ethyl alcohol and stored at -20ºC until DNA extraction. The samples of the second type (larval isolates) were examined under a stereomicroscope by skilled personnel to confirm their identity as *Trichinella* larvae. The larvae were collected from the samples for further DNA extraction.

DNA isolation. Five larvae from each sample were separately examined and used for DNA isolation. When a sample contained fewer than five larvae, the whole sample was used for the isolation. The DNA was isolated and purified with the use of DNA IQtm System kit (Promega, USA), according to the protocol of the European Union Reference Laboratory for Parasites (EURLP) "Identification of *Trichinella* muscle stage larvae at the species level by multiplex PCR" (10). Purified DNA samples were stored at -20ºC until used in PCR.

Multiplex PCR. The PCRs were performed according to the EURLP Multiplex PCR protocol (10) in thermocycler TProfessional (Biometra). The PCR products were separated electrophoretically in 1.5% agarose gels and stained with ethidium bromide. DNA bands in gel were visualised under UV light. For every PCR, negative (nuclease free water) and positive (reference *Trichinella* larvae ISS3, ISS2 or ISS13 from EURLP) controls were used.

PCR of 5S rDNA and sequencing. Fifty-six DNA samples were also examined by touchdown PCR to obtain amplification of 5S ribosomal DNA intergenic spacer region. PCR primers for 5S rDNA (forward: 5'GAACACGCAGTGTCGTAGA 3' and reverse 5'CAACGTGGTATGATCGTAGAC 3') were designed based on the consensus sequence of *T. spiralis* (GenBank accession numbers: AY009946.1, U65504.1, EF694983.1), *T. britovi* (GenBank accession numbers: GU325737.1, AY009943.1), *T. nativa* (GenBank accession number: AY009944.1), and *T. peudospiralis* (GenBank accession number: AY009950.1). PCRs were performed in 50 µL volume containing 5 µL of genomic DNA, 1U of Taq polymerase (Fermentas, EU), 3 mM of $MgCl_2$, 200 µM of each dNTPs, and 0.5 µL of 10 mM primers. PCR was performed under the following conditions: denaturation step at 95ºC for 5 min, followed by 10 cycles with denaturation at 95ºC for 30 s, annealing at 55-50ºC for 30 s (temperature was reduced about 0.5ºC step by step), extension at 72ºC for 40 s, followed by 25 cycles with denaturation at 95ºC for 30 s, annealing at 50ºC for 30 s, extension at 72ºC for 40 s. Each stage of amplification was performed by TProfessional thermocycler (Biometra). Electrophoresis was performed in 2% agarose gel with ethidium bromide. DNA bands in gel were visualised under UV light. PCR products were purified using ExoSAP (Affymetrix, UK) according to manufacturer' procedure. Sequencing was performed in thermocycler Applied Biosystem Veriti. The obtained products were separated using genetic analyser (3730xl DNA Analyser, Applied Biosystems).

Sequence analysis and phylogeny. Forward and reverse sequences were aligned and edited manually using the freeware computer programmes ClustalW and Mega5. Subsequently, phylogenetic analysis of consensus sequences was performed using the Neighbour-Joining method with Jukes-Cantor model and was confirmed by Maximum Likelihood method with the Jukes-Cantor model in Mega5 (Fig. 1).

Data obtained from the Veterinary Inspection Service (VIS) were used to create the map with marked places where each *Trichinella*-positive animal was shot down. The map was created in Qgis 7.1 programme (Fig. 2).

Results

Among 16 737 wild boars examined post-mortem, *Trichinella* larvae were found in 91 (0.54%) carcasses. Geographical distribution of the infected wild boars is shown in Fig. 2. The intensity of invasion for 71 muscle samples examined by artificial digestion method varied from 0.02 up to 47 larvae per gram (with mean 15.3 and median 17.4).

Fig. 1. The Neighbour-Joining tree of *Trichinella* species inferred from 5S rDNA inter-gene spacer region sequences. Phylogeny Test - Bootstrap method, Number of Bootstrap Replications -10000, Jukes-Cantor model

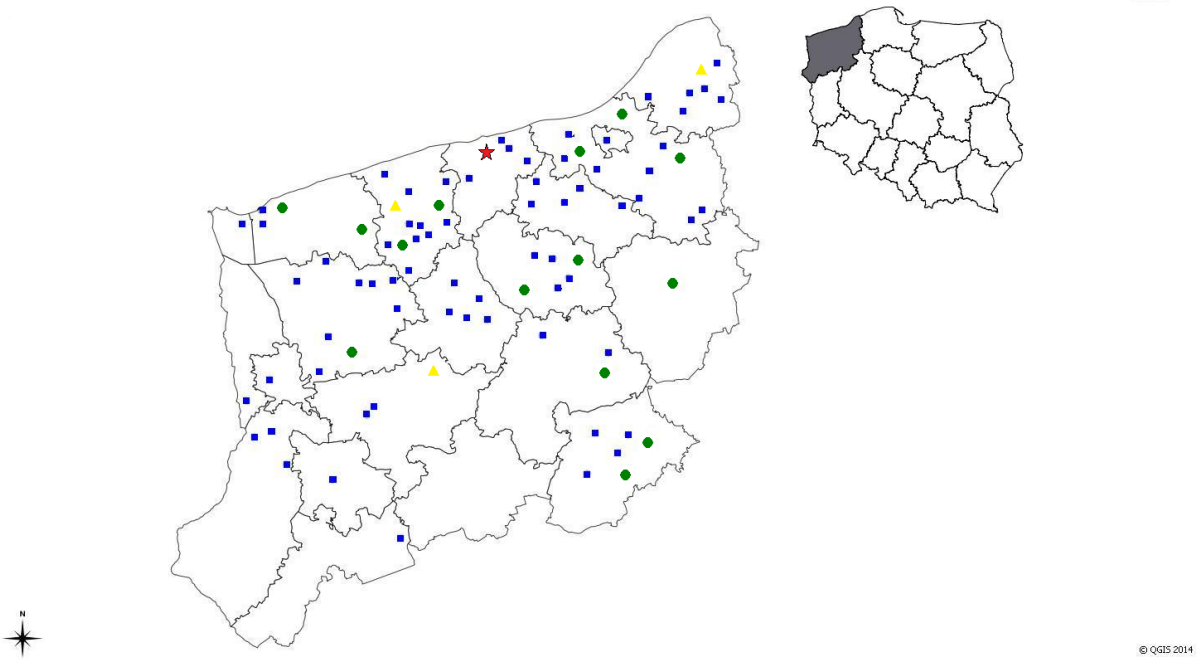

Fig. 2. Geographical distribution of *Trichinella*-positive wild boars hunted in the West Pomeranian Province. Red star – *T. pseudospiralis*, green points – *T. britovi*, blue squares – *T. spiralis*, yellow triangle – mixed infection with *T. spiralis/T. britovi*

Among 91 analysed samples, 88 were positive in multiplex PCR. One band of 173 bp length was observed in 70 samples, and these samples were identified as *T. spiralis*, 14 samples showed bands with the size of 127 and 253 bp and were identified as *T. britovi*. One sample showed a band with 310 bp and was identified as *T. pseudospiralis*. The mixed infection with *T. spiralis/T. britovi* was recognised in three samples. The remaining three isolates were negative in multiplex PCR as the amount of DNA material was insufficient for species identification. Examination of isolated larvae showed the occurrence of *T. spiralis* in 79.5%, *T. britovi* in 16.0%, mixed infection with *T. spiralis/T. britovi* in 3.5%, and *T. pseudospiralis* in 1.0%. The amplification of 5S rDNA resulted in expected products for every examined sample. Fifty-six obtained sequences were deposited in GenBank with the following accession numbers: KJ716690-KJ716745.

The results of alignments of *T. spiralis* 5S rDNA sequences from 51 collected samples demonstrated 100% identity with reference sequence of *T. spiralis* (GenBank access number AY009946). One sequence of *T. spiralis* has one single nucleotide polymorphism (SNP) difference in comparison to the *T. spiralis* AY009946 sequence. All sequences of *T. britovi* were 100% identical with the reference sequence of *T. britovi* (GeneBank access number GU325737.1).

The comparison of 5S rDNA sequence of *T. pseudospiralis* showed also 100% identity with the reference sequence of *T. pseudospiralis* (GenBank access number AY009950). Phylogenetic analysis of the sequencing results of 5S ribosomal intergenic spacer region with the GenBank database confirmed species identification obtained in multiplex PCR.

Discussion

The aim of the presented study was to determine the *Trichinella* species occurring in wild boar populations in the West Pomeranian Province in Poland. This Province is one of the most popular hunting regions, especially for large game animals (Krokosz A., Polish Hunting Association, personal communication, May 2014). In this Province, hunters have harvested the highest number of wild boar carcasses for decades. In 2012, over 16 000 wild boars were hunted. By now, 91 carcasses were recognised as unfit for human consumption by VIS due to the presence of *Trichinella* larvae in muscles. The prevalence of *Trichinella* in wild boar population in the West Pomeranian Province is systematically increasing (7). Post-mortem examination of wild boar carcasses in 2000–2002 showed mean prevalence of trichinellosis as 0.18, in 2003–2005 it was 0.49%, and in 2006–2009 it amounted to 0.64% (5). In this study, the prevalence was estimated as 0.54%. *Trichinella* prevalence in wild boar in other regions of Poland varied from 0.20% to 0.50% in 2000–2011 (29). It is interesting that in Mecklenburg-Western Pomeranian, the German region bordering with West Pomeranian Province, the prevalence of *Trichinella* spp. in wild boar ranged from 0.0027% to 0.0032% between 2002 and 2008. In the same region, a sudden increase in the number of *Trichinella* positive wild boar in comparison to the rest of Germany was observed in 2005 (6).

The molecular examination of 91 larval samples from wild boars hunted in the West Pomeranian Province showed that *T. spiralis* occurred in 79%, *T. britovi* in 16.5%, mixed infection with *T. spiralis/ T. britovi* in 3.5%, and *T. pseudospiralis* in 1%. The occurrence of *T. spiralis* in 79% of wild boars represents the highest percentage of this species compared to other provinces in Poland. The results of the survey conducted on *Trichinella* isolates collected from all over Poland between 2009 and 2012 indicate *T. spiralis* as dominant species which was found in 75.2% of examined isolates from wild boars, followed by *T. britovi* found in 23.8% of wild boars, and mixed infections with *T. spiralis/T. britovi* found in 1% (2). Research conducted by Moskwa *et al.* (15) also confirmed that *T. spiralis* is the species occurring most often in wild boar population and the recovered larvae were identified as *T. spiralis, T. britovi*, and *T. spiralis/T. britovi* (9/18, 5/18, 1/18 respectively). The occurrence of *T. britovi* in West Pomeranian Province was less common compared to the eastern provinces of Poland and the percentage of wild boars infected by this *Trichinella* species was 16.5. Pannwitz *et al.* (18) examined 20 *Trichinella*-positive wild boars from Mecklenburg-West Pomerania region and demonstrated that 80% of the boars were infected with *T. spiralis*, 15% with *T. pseudospiralis*, and 5% were infected simultaneously with *T. spiralis* and *T. pseudospiralis* (18).

The most interesting is the detection of *T. pseudospiralis* (1%). Larvae of this species are non-encapsulated and their detection by routine trichinoscopic method is very difficult or even impossible. The wild boar infected with *T. pseudospiralis* was hunted near Byszewo in district Kołobrzeg (Fig. 2). This is the first case of *T. pseudospiralis* identification in wild boar population in Poland. In Europe, cases of *T. pseudospiralis* infection in wild boar were previously detected in Finland (17), France (24), Sweden (22), Germany (16), Italy (13), and Hungary (28). This species has been also detected in other mammals, such as red foxes, raccoon dogs, brown rat, and lynx (3, 12, 14, 22). *T. pseudospiralis* can also infect birds; the European cases include: Common buzzard (*Buteo buteo*) (4), rook (*Corvus frugilegus*) (27), tawny owl (*Strix aluco*), and little owl (*Athene noctua*) (21). Although *T. pseudospiralis* can be considered a sylvatic species, the recent discovery of this parasite in domestic pigs in Russia, Slovakia, and Croatia (1, 3, 8, 9, 19, 26) suggests that this parasite can be transmitted to the

domestic environment and should be considered as a potential risk for humans.

The first outbreak of human trichinellosis caused by non-encapsulated larvae was observed in Thailand (with 59 infected and one fatal case), where raw products made from wild boar meat were the source of the infections (11). The second one, with approximately 30 infected individuals, was noted in Kamchatka, Russia, where infection was caused by meat of infected pigs (3). Next human outbreak was triggered by wild boar meat in France, where four people became infected (24).

Epidemiological data show that the prevalence of *T. pseudospiralis* infection is increasing in wild and domestic animals in Europe. As for now, in Poland *T. pseudospiralis* was found only once in red fox in 2012 in the Subcarpathian Province (14), 600 km away from the shooting place of the infected wild boar described in this paper. This species is not common among such vectors as red fox. Examination of over 1600 red foxes from Poland showed the occurrence of *T. britovi, T. spiralis*, and *T. nativa*. No case of *T. pseudospiralis* infection was found in this survey (5).

The origin of *T. pseudospiralis* infection in wild boar from West Pomeranian Province remains unknown due to limited data on the occurrence of this parasite in Poland, but if we take into account the occurrence of *T. pseudospiralis* in 15% of *Trichinella* positive wild boars originating from Mecklenburg – West Pomerania, cross border transmission is very likely (18, 23). West Pomeranian Province is plentiful in wild game and the presence of *T. pseudspiralis* suggests that vectors may exist for wildlife or domestic animals.

Larvae of *T. pseudospiralis* are non-encapsulated and in traditional trichinoscopic examination may remain unnoticed. This confirms the necessity to use digestion method as the only reliable technique for examination of meat, especially from wild boars. The estimated prevalence of *Trichinella* nematode at the level of 0.54% in wild boar population is the highest in decades. The presence of four *Trichinella* species in Polish parasitofauna points to the necessity to continue species identification of *Trichinella* larvae in wild and domestic animals.

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