Trichinella spp. infection in European polecats (Mustela putorius Linnaeus, 1758) from Romania

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Summary
The European polecat (Mustela putorius Linnaeus, 1758) is in decline in Romania, often living near human settlements, from mountains to lowlands. They feed on a wide variety of small animals, including rodents, such as mice or rats. The occurrence of this parasite in polecats from Romania was mentioned only once in 1991, but the parasite species was not confirmed by molecular biology. The study aimed to investigate the occurrence of Trichinella spp. in European polecats from Romania and to identify the parasite species by molecular tools. A total of 75 wild European polecats were examined by trichinoscopy and artificial digestion. For species determination, the positive muscle samples and the larvae recovered from artificial digestion were collected for DNA isolation and further processed by means of Multiplex PCR. Only two polecats from southern Romania tested positive for Trichinella spp. infection. During trichinoscopy examination, 48 (in a polecat from Giurgiu County) and 78 (in a polecat from Ialomița County) cysts were found in the tested (56 samples/animal) tissue samples. Artificial digestion revealed infection with 2466 larvae/100 g of muscle in the polecat from Ialomița and 254/100 g in the polecat from Giurgiu. The Multiplex PCR indicated the occurrence of Trichinella spiralis in the polecat from Giurgiu and a co-infection with T. spiralis and T. britovi in the polecat from Ialomița. The current study confirms through molecular biology, the occurrence of T. spiralis and T. britovi, as well as the occurrence of co-infection with these two Trichinella species in European polecats from Romania.

Keywords: European polecats; Trichinella spp.; trichinoscopy; artificial digestion; multiplex PCR

Introduction
Nematodes of the genus Trichinella are among the most widespread muscular parasites of predatory, scavenger, and omnivorous animals (Campbell, 1988). Trichinella species are primarily parasites of wildlife, but they can also be found in domestic animals, which represent the main source of infection for humans. Wild carnivores and rodents can act as a source of infection with
Trichinella spp. also for domestic animals (Pozio & Zarfenga, 2005). The infection develops after the ingestion of raw meat, harboring the infective larvae (Pozio, 2007). In several regions of Europe, a wide variety of carnivores (badgers, bears, lynx, polecats, and wolves) were reported to be infected with Trichinella spp., but due to low population levels of these hosts, their ecological implication in the sylvatic cycle was considered of marginal importance (Pozio, 1998). In Romania, Trichinella spp. were detected in several wild species, such as red foxes (Vulpes vulpes), wolves (Canis lupus), wild boars (Sus scrofa), brown bears (Ursus arctos), and European wildcats (Felis silvestris) (Blaga et al., 2009 a,b). The prevalence rate of T. britovi infection in wild boars from Romania was 57.3 %, which is significantly higher than the prevalence rate obtained for bears, (9.3 %) (Nicorescu et al., 2015). Regarding carnivores from Romania, the smallest prevalence rate of T. britovi infection was found in red foxes (5.6 %), followed by European wildcats (20 %), and wolves (31 %) (Blaga et al., 2009a).

The European polecat’s preferred habitat type is variable and includes riparian vegetation, watercourses, grasslands, pastures, human settlements, woodlands, agricultural lands, and in some cases pine forests (Birks & Kitchener 1999; Virgòs, 2003; Baghli al., 2005; Mestre et al., 2007). They feed on a wide variety of small animals, including rodents, such as mice or rats (Lode, 2011). The recent data shows that in Romania, the European polecat population is in decline (Croose et al., 2018). Some authors consider that in Europe, mustelids should also be considered when analyzing the presence of Trichinella spp. infection (Hurníková et al., 2009).

Considering that previous studies reported Trichinella spp. in several wild animal species, including mustelids from Romania, our research aimed to investigate the occurrence of these parasites in European polecats and to identify the involved Trichinella species.

Materials and methods

Study area

Romania is located in Eastern Europe, in the northern part of the Balkan Peninsula (Trusca & Alecu, 2005). Between 2016 and 2020, 75 (60 adults, 15 juveniles; 54 males, 21 females) wild European polecats (Mustela putorius) were examined by complete parasitological necropsy. The collected animals were found dead (road kills) or legally hunted (31 March – 15 September) from nine different counties of Romania. A large number of polecats were examined because of their wide distribution in Romania and their presence near human settlements that exposes them to Trichinella spp. infections. The counties from where the animals originated are the following: Arad, Brașov, Constanța, Brăila, Călărași, Ialomița, Giurgiu, Teleorman, and Olt (Fig. 1).

Trichinoscopy and artificial digestion

For analyzing the presence of Trichinella spp., 56 oat kernel sized pieces of diaphragm (n=20), foreleg muscles (n=18), and posterior leg muscles (n=18) were collected from each animal and tested by trichinoscopy, and examined under a light microscope using 40× magnification (Blaga et al., 2009a). Five muscle samples from each trichinoscopy-positive animal were collected in two 1.5 ml tubes with 70 % ethanol (one/animal). To collect the larvae for species identification, artificial digestion was done from all samples (in a total of 100g of muscle from each animal, meaning diaphragm, foreleg muscles, and posterior leg muscles) according to Gamble et al. (2000). Afterward, the detected larvae were collected using a micropipette in two 1.5 ml tubes (one/animal) with 70 % ethanol.

Multiplex PCR

The collected positive muscle samples (5 samples/animal), and larvae from the two 1.5 ml tubes with 70 % ethanol (one/animal) were subjected to DNA extraction. DNA was extracted from the 5 positive muscle samples/animal, as well as from the pooled larvae (50 larvae/animal) recovered from the artificial digestion, using a commercially available kit (IsoLATE II Genomic DNA Kit, Bioline, London, UK), according to the manufacturer’s instructions. Trichinella species were identified by means of a multiplex PCR reaction able to discriminate 9 species of Trichinella, as previously described in the literature (Zarfenga et al., 1999). The PCR products were visualized by electrophoresis in a 2 % agarose gel stained with RedSafe™ 20000x Nucleic Acid Staining Solution (Chembio, Hertfordshire, UK), and their molecular weight was assessed by comparison to a molecular marker (HyperLadder™ 100 bp, Bioline, London, UK).

Statistical analysis
Prevalence and 95 % Confidence Interval (95 % CI) were calculated using EpiInfo 7 software (CDC, USA).

Ethical Approval and/or Informed Consent

All applicable national and institutional guidelines for the care and use of animals were followed. The examination and collection of dead animals were approved by the bioethetical committee of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Faculty of Veterinary Medicine: number 232 from 23.11.2020.

Results and Discussion

Overall, two European polecats (2.7 %; 95 % CI 0.32 – 9.3 %) were positive for Trichinella spp. by both methods. The animals originated in southern Romania, from Ialomița and Giurgiu counties (Fig. 1). The number of identified cysts varied between the examined muscles in both animals. The polecat from Ialomița county had a total of 78 cysts (diaphragm 15; foreleg 46; posterior leg 17).
and the polecat from Giurgiu county had 48 cysts (diaphragm 13; foreleg 12; posterior leg 23).

Artificial digestion revealed a larval burden of 2466 larvae/100 g of muscle in the polecat from Ialomița and 254/100g in the animal from Giurgiu. The electrophoretic profile of the polecat from Ialomița (infected muscle and larval isolates) included a total of three bands, indicating the co-occurrence of *Trichinella britovi* (~127 and 253 bp respectively) and *Trichinella spiralis* (173 bp). The electrophoretic profile for the polecat from Giurgiu county (infested muscle and larval isolates) included only one band of ~173bp, thus indicating the occurrence of only *T. spiralis* in this second polecat.

The current study revealed that European polecats from Romania can act as reservoirs for both *T. britovi* and *T. spiralis*. The prevalence obtained during the present study was 1.3 % (95 % CI 0.03 – 7.2 %) for *T. britovi* and 2.7 % (95 % CI 0.32 – 9.3 %) for *T. spiralis*, which is more precise than the results reported by similar studies performed in other European countries, but with smaller sample sizes, such as Slovakia, where the number of animals tested was only 3 (one positive) and respectively 9 (3 positive) (Hurníková et al., 2007; Hurníková et al., 2009). In Belorussia, 40 animals (2 positive) were tested (Shimalov et al., 2002), and in Lithuania only 7 (1 positive) (Jaunė & Grikienienė, 2001).

The current study used both, trichinoscopy and artificial digestion, to detect *Trichinella* spp. in European polecats, whereas other studies used exclusively one of these methods.

A good example is the study from Belorussian Polesie, where a total of 40 polecats were examined for *Trichinella* spp. by trichinoscopy. Only two animals were identified as positive for this parasitic infection (Shimalov et al., 2002).

Other studies that used artificial digestion also collected the larvae for species identification by multiplex PCR. In Slovakia, during the hunting season 2005 – 2006, one European polecat was positive for *T. britovi* (Hurníková et al., 2007). In a follow-up study, the multiplex PCR revealed that three polecats were positive for *T. britovi* (Hurníková et al., 2009). The current study also identified the occurrence of *T. spiralis* in European polecats, besides *T. britovi*, and also proved the possibility of a co-infection with these two parasite species.
However, not all studies targeting polecats revealed positive results, such as the study from Poland (Piekarska et al., 2016) and Serbia (Klun et al., 2018). However, in both cases, the number of tested animals was small (Piekarska et al., 2016; Klun et al., 2018).

One of the earliest experimental studies on *Mustela putorius furo* and *T. spiralis* was done in 1982. The experimental study showed that *T. spiralis* can be transmitted from mice to ferrets, with the highest number of larvae located in the diaphragm muscles. The experimental study confirmed that, the same thing can also happen in polecats in the sylvatic fauna (Campbell et al., 1982).

A previous study indicated the presence of *Trichinella* spp. in polecats in the sylvatic fauna (Campbell et al., 1982). The present study confirmed that, the same thing can also happen in polecats in the sylvatic fauna (Campbell et al., 1982). One of the authors (Georgiana Deak) was financially supported by Altius SRL Romania by a grant in order to support and promote research in Romania.

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