Communication

First Identification and Molecular characterization of Trichinella britovi in Pine marten (Martes martes) in Romania

Ana Maria Marin , Ovidiu Alexandru Mederle * , Gianluca Marucci , Dan Cornel Popovici , Narcisa Mederle

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Article

First Identification and Molecular Characterization of *Trichinella britovi* in Pine Marten (*Martes martes*) in Romania

Ana-Maria Marin [1], Ovidiu-Alexandru Mederle [2,*], Gianluca Marucci [3], Dan-Cornel Popovici [4] and Narcisa Mederle [1]

[1] Faculty of Veterinary Medicine, University of Life Sciences "King Michael I" from Timisoara, 300645 Timisoara, Romania
[2] University of Medicine and Pharmacy "Victor Babes" from Timisoara, 300041, No. 2 Piața Eftimie Murgu, Timisoara, Romania
[3] Department of Infectious Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome 00161, Italy
[4] Transilvania University Brasov, Forestry Faculty, 500123, No. 1 Sirul Beethoven, Brasov, Romania
* Correspondence: mederle.ovidiu@umft.ro, Tel.: +40722343722

Abstract: Trichinella spp. are etiological zoonotic agents spread throughout the world affecting mammals, birds, and reptiles, involved in the evolution of the two cycles, domestic and sylvatic. The pine marten (*Martes martes*) is a mammal carnivorous nocturnal from the family *Mustelidae*. In Romania, this host is widespread in all the forests of the country. The pine marten has an extremely voracious appetite, feeding on fruit but also on a variety of small animals, including rodents such as mice or rats. The aim of the study was identification and molecular characterization of *Trichinella* larvae isolated from the muscles of tree martens from different counties of Romania. The muscle samples were examined by artificial digestion, and the larvae were processed by multiplex PCR. *Trichinella britovi*, a species frequently identified in wild carnivores in temperate zones, has been confirmed. Although *T. britovi* has been reported in Romania to several host animals, this study confirms for the first time its presence in the pine marten, a result which undoubtedly underlines the fact that the parasitic reserve in the sylvatic environment is maintained at a consistent level, and the knowledge of these vector hosts remains a permanent concern of the specialists.

Keywords: *T. britovi*; pine marten; Romania

1. Introduction

A large group of mammals, the mustelids have dominantly conquered the northern hemisphere, but they also have representatives in South America or the south of the African continent, developing specialized adaptations, typical of the habitat in which they live (arboreal, aquatic, etc.) [1].

In Romania, there are many species of mustelids, including the badger (*Meles meles*), the otter (*Mustela lutreola*), the polecat (*Mustela putorius*), the weasel (*Mustela nivalis*), the stone marten (*Martes foina*) and the pine marten (*Martes martes*) [2].

The pine marten is present in all areas with forest vegetation, being found in high densities in deciduous or mixed forests in the lowland and hill areas. An extremely active species, the tree marten does not shy away from leaving the canopy of trees and venturing on the ground even over kilometres in search of prey [3].

The food and habitat need as well as the interrelationships it has with other animals in the trophic pyramid support the reservoir role and the involvement of this sylvatic animal in the transmission of various etiological agents, especially those with zoonotic potential (*Trichinella* spp.) [1].

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Trichinella spp. are the etiological agents of a zoonosis that affects humans, caused by the consumption of raw or undercooked meat of animals infested with the larvae of these zoonotic nematodes [4]. Trichinellosis is a parasitic disease that affects mammals, birds, carnivorous and omnivorous reptiles. The disease is widespread everywhere except Antarctica [5].

Currently, 13 taxa are described in the *Trichinella* genus, namely the encapsulated species *T. spiralis, T. nativa, T. britovi, T. murrelli, T. nelsoni, T. patagoniensis, T. chanchalensis* and *Trichinella* genotypes T6, T8 and T9, exclusively for mammals. Non-encapsulated species are *T. pseudospiralis, T. papuae* and *T. zimbabwensis*, which infest mammals and birds or mammals and reptiles [4].

In Romania, trichinellosis is a zoonosis with a high level of infestation, and inadequate consumption of pork, but also of game meat (wild boar, bear) represents the major way of human infestation [6, 7].

In Europe, mustelids (badgers, beech marten, etc.) and other carnivores (bears, lynxes, wolves, etc.) represent a source of infestation with larvae of *Trichinella* spp. and have a role in the ecology of sylvatic trichinellosis, although the red fox and the raccoon dog are considered the most important reservoir hosts [8].

The sylvatic cycle presents some relevant particularities in trichinellosis, where emphasized the risk of human infestation: directly, through the consumption of game meat (especially wild boar) insufficiently cooked, but also indirectly by the transmission of *Trichinella* strains from wild animals to domestic animals (pigs raised in the yard) [9].

In Romania, there are no reports that support the possible consumption of meat from wild carnivores, but they appear as invasive species and suitable hosts for *Trichinella* spp. [6, 7, 10].

In Romania, no infestation of pine marten with larvae of *Trichinella* spp. has been reported [6] but respiratory tract parasites have been identified in this host, namely *Crenosoma vulpis* and *C. petrowi* [11].

The present study followed the identification and molecular characterization of *Trichinella* larvae isolated from the muscles tissue of pine martens from the counties of Romania.

2. Materials and Methods

2.1. The target hosts

The pine marten is a nocturnal carnivorous mammal belonging to *Mustelidae* family, with reddish-brown fur and down that fades to yellow, a yellowish-white neck patch, and a black tail. It is widespread in Europe (including Romania and the Republic of Moldova), Asia Minor, northern Iraq and Iran, the Caucasus, and Western Siberia [3]. In Romania it is widespread in all the forests of the country, it is more frequently found in the mountains, up to the limit of the forest vegetation; but it is also found in the plains in the forests. A hunter par excellence with predominantly arboreal activity, the pine marten has an extremely voracious appetite, feeding on fruit but also on a variety of small animals, including rodents such as mice or rats [1]. The characteristics of habitat, food, and biology make it easier to understand the involvement of this sylvatic animal in the maintenance and transmissibility of various pathogens, especially parasites with zoonotic potential, such as the nematode *Trichinella* spp.

2.2. Diagnostic procedures

The research was carried out over a period of 2 years, on a total of 12 tree martens (four males, respectively eight females), aged between 1 - and 2 years. The collected animals were found dead (road killed) or legally hunted (September 15-March 31) in five counties of Romania: Timiș, Arad, Caraș-Severin, Vâlcea, and Olt (Figure 1). The animals were examined at the Parasitic Diseases Clinic of the Faculty of Veterinary Medicine Timisoara/ULST.

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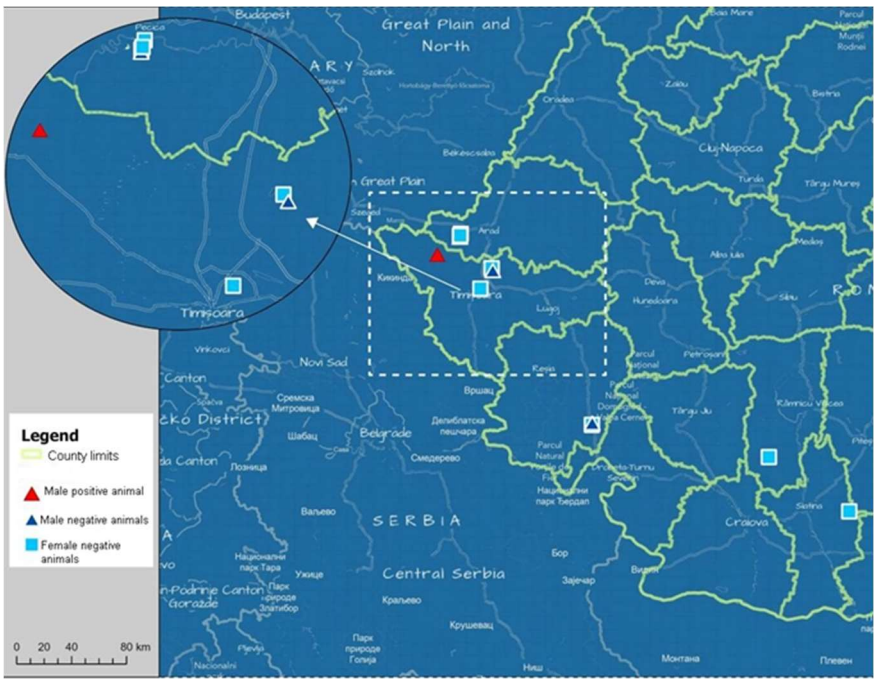

Figure 1. Map showing the geographical areas where pine marten carcasses were collected.

About 30 g of muscle from the diaphragm and foreleg muscles were harvested from each animal and tested for the presence of *Trichinella* spp. larvae by the artificial digestion method according to the Commission Regulation (EC) no. 1375/2015 [12]. After artificial digestion, larvae were collected, counted, stored in 96% ethanol, and sent to the European Reference Laboratory for Parasites (EURLP) (Rome, Italy) for species identification by multiplex PCR [13].

3. Results

One out of the four pine marten specimens collected from Timiș County (lat. 46.009390, long. 20,846470) resulted positive to the presence of *Trichinella* spp. larvae. The larval burden was estimated to be 1.12 larvae per gram (LPG) of muscle tissue.

Ten *Trichinella* larvae have been individually tested by multiplex PCR and identified as *T. britovi* (Figure S1).

4. Discussion

Comparing the results of this study with the literature from the country and abroad it can be stated that it is the first identification of the nematode *T. britovi* in the pine marten in Romania.

The European reservoir for *Trichinella* spp. is represented by wildlife, wild animals being the most important source of infection for the domestic pig, which is the main source of infection for other animals (e.g., horses), but especially for humans [14].

An ecological model of the parasitic system of *Trichinella* spp., based on predation, necrophagy, and cannibalism, as the main ecological factors, is provided by Varlamova A. et al. [15]. The red foxes play a major role in the accumulation and distribution of larvae and in maintaining a stable circulation of natural trichinellosis outbreaks. On a secondary level are the wolf, the raccoon dog, the badger, the pine marten, and the stone marten. Domestic carnivores can be infected by wild predators [14].

A monitoring program of the sensitive host species (domestic pigs), but also of the wild fauna in the monitored region, as well as the evaluation of the factors (habitat characteristics, of the wild host) that favour the circulation of *Trichinella* spp. in nature is fundamental in assessing the risk for domestic animals [14].

In Europe, *T. britovi* and *T. spiralis* are the most prevalent species isolated from wildlife. *T. britovi* is more widespread than *T. spiralis*, with a different prevalence depending on the carnivore family,

with the exception of mustelids in which only *T. britovi* was identified [14]. The same authors also support the existence of a domestic cycle maintained by *T. britovi,* in countries where pigs are raised in backyards, and the nematode can easily be transmitted from wild to domestic animals (Bulgaria, Croatia, Estonia, France, Italy, Macedonia, Poland, Romania, Spain, and Ukraine) [14].

T. britovi parasite wild carnivorous mammals of the families *Canidae, Felidae, Mustelidae, Ursidae,* and *Viverridae*, living in the temperate regions of Europe, Western and Northern Asia, and West Africa [9]. In Europe, *T. britovi* was detected in 89% and 38% respectively of *Trichinella* isolates from carnivores and wild boars [14]. *T. britovi* can also affect domestic pig populations and is the second species of *Trichinella* that can affect human health [16, 17].

The identification of *T britovi* in wild carnivores living in the vicinity of rural localities may represent a way of transmission of this zoonosis to species of wild fauna of hunting interest (wild boar), and, through it, to humans [6, 10, 18].

T. britovi is a cold-resistant species; T. *britovi* larvae can survive frozen in carnivore muscle for up to one year and in pig muscle for up to three weeks compared to *T. spiralis* larvae that do not survive more than a few hours or a few days [9, 19].

A species with a pronounced arboreal character, the pine marten dominates its sylvatic habitat where it frequently feeds on numerous species of mammals (squirrel, woodchuck), birds (blackbird, wood pigeon, collared pigeon), reptiles or amphibians, but undertakes numerous incursions outside the forest ecosystem in order to supplement food requirements and establish new hunting territories [1]**.** Thus, it can reach the vicinity of human settlements, animal farms, and grain warehouses, where in addition to rats or mice, it also feeds on domestic birds (chickens, ducks, turkeys), causing economic damage and representing a potential danger of infestation [2].

In Europe, *T. britovi* has been reported in numerous sylvatic hosts: fox [14, 20], jackal [14, 21], raccoon dog [14, 22], wolf [14, 23], wild cat [14, 24], lynx [14, 25], badger [14, 26], the stone marten [14, 27], European otter [14], European beaver [28], wild boar [14, 29, 30] and the brown bear [14, 31].

Therefore, the occurrence of *T. britovi* in wildlife in Europe is reported in a significant number of hosts, but information is scanty when we talk about the pine marten.

In Serbia, Klun I. et al. examined 469 wild animals including the pine marten, and the *Trichinella* species identified were *T. britovi* and *T. spiralis* [32].

In Lithuania, Senutaitė J. and Grikienienė J. identified *Trichinella* spp. in the muscles tissue of wild animals, with a high prevalence of infection in the fox, the raccoon dog, and the pine marten [33]. Similarly, an extensive study performed in Bulgaria in wild animals, have been identified the presence of *Trichinella* spp. in the 26 host animals' muscles tissue, with a high prevalence in wolves, pine martens, and foxes [34].

In Slovakia, an assessment of *Trichinella* spp. infection mentions pine martens as the most affected hosts with a much higher prevalence than foxes. This epidemiological aspect reinforces the synanthropic behaviour of these animals and, finally, their role in the epidemiology of trichinellosis [35, 36].

Epidemiological inquiries carried out in Latvia revealed a high prevalence of *Trichinella* spp. infestation in sylvatic carnivorous mammals, which highlights that they are good indicators for assessing the risk of infestation with this nematode. The predominant species was *T. britovi* isolated even from the muscles of the pine marten [37-39].

We remain in Latvia, where the results of a study compared to the situation in Lithuania (Kaunas region) claim that *Martes* species are frequent natural reservoirs for *Trichinella* zoonotic agents, in both countries. The stone marten was more susceptible to infestation compared to the pine marten, and the prevalence of *Trichinella* infestation in Latvia was higher compared to Lithuania. The widespread species in Latvia and Lithuania (Kaunas region) was *T. britovi* [40, 41].

Moskwa B. et al. identified for the first time in Poland larvae of *T. britovi* in the pine marten in 2012 [26].

The identification of *T. spiralis* in raccoon and pine marten in Poland reinforces the claim that new invasive species of carnivorous mammals become reservoirs for *Trichinella* spp. and are responsible for maintaining the infection in food chains, including game and domestic animals

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intended for human consumption [42]. Four years later, Cybulska A. et al. isolated species *T. britovi* from the muscles tissue of martens, with a maximum intensity of larval distribution in the tongue muscles [43].

In the central Abruzzi region of Italy, Badagliacca P. et al. (2016) identified *T. britovi* in six wildlife hosts: pine martens, stone martens, wolves, foxes, wild cats, and wild boars [27].

In the Scandinavian countries, *T. nativa*, T. *britovi*, and *T. spiralis* were identified in the muscles of the pine marten, the badger, the raccoon dog, and the mink [44], but also of the wild boars and lynx [45].

The American marten (*Martes americana*) from Quebec (Canada) was parasitized with the species *T. spiralis* [46].

In Romania, *Trichinella* infestation is present and widespread throughout the country, affecting wild animals significantly [6]. In Romania, two species circulate: *T. spiralis* and *T. britovi*. Animals affected by this parasitism are fox, golden jackal, mink, wolf, wild cat, lynx, stone marten, badger, ermine, polecat, bear, and wild boar. *T. britovi* is the most widespread species in the sylvatic cycle in Romania [6, 7, 47-54].

The fact that in the present study, *T. britovi* was identified in Romania in the pine marten, along with other host species (fox, jackal, wolf, wild cat, lynx, badger, stone marten, ermine, polecat, bear, wild boar) further confirms once the hypothesis that these host species represent an important vector of trichinellosis transmission in the sylvatic environment with direct effects on species of hunting interest, in particular, wild boar and bear, with the risk of human infestation.

In European countries, cases of human trichinellosis as a result of infestation with *T. britovi* through the consumption of wild boar meat have been reported in Serbia [55-57], Portugal [58], Italy [59-63], Spain and Sweden [64, 65], Greece [66], Iran and Turkey [67], France [68-70], Slovakia [71, 72]. Even more, human infestations with *T. britovi* through the consumption of other wild and domestic animals' meat have been reported: jackal meat [73], horse meat [62], domestic dog meat [74], and domestic pig meat in Spain [75, 76], Argentina [77], Bulgaria [78], Sardinia [79], Corsica [80] and Slovakia [74].

In Romania, no cases of human trichinellosis with *T. britovi* have been reported, only with *T. spiralis* [81].

The pine marten fulfils the role of a true vector of transmission of parasites (especially zoonotic ones) being a transit element between the wild ecosystem and the peripheral domestic one like the wild boar is.

The identification of the *T. britovi* species in a new host in Romania, the pine marten, undoubtedly underlines the fact that the parasitic reserve in the sylvatic environment is maintained at a consistent level, and the knowledge of these vector hosts is a permanent concern of the specialists.

5. Conclusions

Analysing the data obtained from the literature, the present study reports the infestation with *T. britovi* in the pine marten for the first time in Romania.

Supplementary Materials: Figure S1. Capillary electrophoresis run of the multiplex PCR on *Trichinella* larvae collected from the pine marten. A1 to A10 larvae isolated from the pine marten; A11 *T. spiralis* positive control; A12 *T. britovi* positive control; B1 negative control; B2 size marker.

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