

Molecular Survey on *Leishmania Infantum* Infection in Red Foxes (*Vulpes Vulpes*) From Romania

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ABSTRACT

Leishmania spp. (Kinetoplastida, Trypanosomatidae) are parasitic protozoans transmitted by sand flies to animals and humans. In Europe, the predominant species is *Leishmania infantum*, the agent of canine leishmaniasis (CanL) and of visceral (VL) and cutaneous (CL) forms in humans. Despite being regarded as a non-endemic country, recent data from Romania have shown the occurrence of autochthonous cases of human and canine infections. The aim of this study was to investigate the occurrence of *L. infantum* in red foxes (*Vulpes vulpes*) from Romania. Between December 2016 and April 2017, 514 foxes originating from ten counties of Romania were evaluated and individual conjunctival swab samples were tested for the presence of *L. infantum* DNA by means of real-time PCR. All samples were negative. However, considering the new national epidemiological context, the importance of the red fox as sentinel and its potential role in the epidemiology of this disease in Romania should not be disregarded in the future.

Keywords: *Leishmania*, CanL, *Vulpes vulpes*, Romania

INTRODUCTION

Leishmania spp. (Kinetoplastida, Trypanosomatidae) are parasitic protozoans transmitted by female sand flies to animals and humans (Schönian *et al.*, 2010). In Europe, three species have been reported so far: *L. infantum*, which is the predominant species and the agent of canine leishmaniasis (CanL) and of visceral (VL) and cutaneous (CL) forms in humans (Otranto and Dantas-Torres, 2013); anthroponotic *L. tropica* and *L. donovani*, which have a focal distribution, occasionally causing VL and CL in Greece and Cyprus (Mazeris *et al.*, 2010).

CanL is regarded as an endemic disease along the Mediterranean coast, from Portugal to Turkey (Maia and Cardoso, 2015). However, an expanding trend has recently been noted, with cases being reported in northwestern Italy, as well as in other Central European countries (Maroli

et al., 2008; Schönian *et al.*, 2010), due to the spreading of sand fly populations, the extension of their breeding seasons and the shortening of their larval development, as a consequence of climate change (Killick-Kendrick, 1996; Desjeux, 2001). Furthermore, the traveling and temporary or permanent relocation of dogs together with their owners may also facilitate the spreading of the disease throughout Europe (Maia and Cardoso, 2015).

In Romania, between 1969 and 2013, all cases of canine or human infection were imported (Dumitrache *et al.*, 2016). Recently, after more than 80 years of absence of clinical CanL, an autochthonous case was described in 2014, in a dog from Vâlcea County (Mircean *et al.*, 2014) and a focal serological prevalence of 8.7% was subsequently established in apparently healthy dogs originating from the same area (Dumitrache

et al., 2016). Furthermore, *L. infantum* infection was recently confirmed by PCR screening also in a golden jackal originating from Romania (Mitková *et al.*, 2017).

Romania holds an important wildlife-domestic animal interface. Among wild carnivore species, the red fox (*Vulpes vulpes*) is the most widely distributed at a national level, with an estimated population of more than 63.000 specimens in 2013, according to the Romanian Ministry of Environment. As for many wildlife species, red foxes may also play a role in the epidemiology of CanL (Millán *et al.*, 2014) and no recent studies regarding the national distribution of the infection nor its sand fly vectors are currently available from Romania, the aim of the present study was to investigate the molecular occurrence of *L. infantum* in red foxes from Romania.

MATERIALS AND METHODS

Between December 2016 and April 2017, a total of 514 red foxes originating from ten counties of Romania were evaluated. All the animals were legally hunted and collected by the National Sanitary Veterinary Authority during the rabies monitoring program. From each examined fox, conjunctival secretions were collected using a sterile swab. All swabs were labeled and stored at -20°C until further processing. Genomic DNA was extracted individually from each swab, following suspension in 300 µl 1x PBS, using a commercial kit (Isolate II Genomic DNA Kit, BIOLINE, UK) according to the manufacturer's instructions. The DNA samples were processed by means of real-time PCR amplification of the kinetoplast minicircle DNA of *L. infantum*, using the LEISH-1/LEISH-2 primer pair and TaqMan-MGB probe, as previously described (Francino *et al.*, 2006).

RESULTS AND DISCUSSION

All of the 514 examined samples were negative for *L. infantum* DNA. With concern to leishmaniasis, Romania is generally regarded as a non-endemic country, with a low risk (Ready, 2010). However, in the past century, between 1912 and 1955, 27 autochthonous human cases have been documented from southern Romania. In the area where human cases were diagnosed; several studies investigating dog population were performed. Thus, in 1967, in a study performed in Dolj County, the prevalence of infection was of

1.2%, while in another survey performed in 1968 in Caraş-Severin County, it was 2.2% (Mircean *et al.*, 2014). All dogs were apparently healthy. Between 1969 and 2013, no other autochthonous cases were reported. More recent investigations found a serological prevalence of 2.9% in apparently healthy dog population originating from Bucharest area (Hamel *et al.*, 2012). In Râmnicu Vâlcea (city in Vâlcea County), where a clinical case of CanL was described (Mircean *et al.*, 2014), a total of 12 apparently healthy dogs (representing 15% of all tested animals) were positive, either by means of serological testing (n=7, 8.7%) or by real-time PCR (n=8, 10%), raising public health concerns (Dumitrache *et al.*, 2016).

Generally, the domestic dog is regarded as the main reservoir of *L. infantum*, due to several factors, including the susceptibility to infection, the great ability to transmit the parasite to the phlebotomine vectors and last but not least, its proximity to humans (Ready, 2010; Dantas-Torres *et al.*, 2014). However, cases of *Leishmania* infection were confirmed also in several species of wild carnivores in Europe (as reviewed by Millán *et al.*, 2014), including canids (golden jackal - *Canis aureus*, grey wolf - *C. lupus*, red fox - *Vulpes vulpes*), felids (Iberian lynx - *Lynx pardinus*, European wild cat - *Felis silvestris*), mustelids (polecat - *Mustela putorius*, European mink - *M. lutreola*, weasels - *Mustela* spp., stone marten - *Martes foina*, pine marten - *M. martes*, European badger - *Meles meles*), viverrids (common genet - *Genetta genetta*) and herpestids (Egyptian mongoose - *Herpestes ichneumon*). Furthermore, the grey wolf and the red fox may also display clinical signs of the disease (Millán *et al.*, 2014).

Recent molecular surveys of foxes originating from endemic countries have shown a prevalence of *Leishmania* infection of 9% in southern France (Davoust *et al.*, 2014), 1.3% in northern, central and southern Portugal (Cardoso *et al.*, 2015), 59.5% in West Fthiotida prefecture in Greece (Karayiannis *et al.*, 2015) and 20.8% in southern Italy (Piantedosi *et al.*, 2016). With regards to the targeted samples, the highest prevalence was recorded in splenic tissue (Davoust *et al.*, 2014; Karayiannis *et al.*, 2015; Piantedosi *et al.*, 2016), while most blood samples (Davoust *et al.*, 2014; Karayiannis *et al.*, 2015) and all skin samples (Davoust *et al.*, 2014) were negative. This raises a question regarding the actual ability of the fox to efficiently transmit

the parasite to the phlebotomine vectors. In crab-eating foxes (*Cerdocyon thous*) it was shown that animals harboring a sub-clinical infection were non-infectious to the vector (Piantedosi *et al.*, 2016). However, transmission from red foxes to sand flies has not been documented so far. Clinical signs suggestive for leishmaniasis were observed only in Greece, in 20 out of 28 positive foxes. However, as the recorded clinical signs were not strictly specific for the disease, they could also be attributed to other conditions, such as nutritional deficiencies and the occurrence of other parasitic diseases (Karayiannis *et al.*, 2015). Similarly, in southern Italy, a frequent finding compatible with leishmaniasis in positive foxes was weight loss, which could more likely be attributed to the concurrent presence of lung and intestinal worms (Piantedosi *et al.*, 2016).

The prevalence of infection in foxes was lower compared to dogs living in the same study areas, indicating a lower exposure risk, but highlighting their importance as sentinel hosts (Davoust *et al.*, 2014; Cardoso *et al.*, 2015; Karayiannis *et al.*, 2015; Piantedosi *et al.*, 2016).

Among wild carnivores, red foxes are among the most widely distributed species worldwide and the most adapted to synanthropic ecosystems (Duscher *et al.*, 2014). Therefore, despite our negative results, in the context of geographical spreading of CanL, the importance of the red fox as sentinel and its potential role in the epidemiology of this disease in Romania should not be disregarded in the future and further research should be carried out.

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