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Anaplasma phagocytophilum in Multiple Tissue Samples of Wild Carnivores in Romania

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ABSTRACT: Wild vertebrate hosts can serve as reservoirs or amplification hosts for tick-borne pathogens (TBPs). Wild carnivores due to their large size have an increased risk for harboring large numbers of ticks. Moreover, their large home ranges and long lives may increase the risk of exposure to ticks and TBPs. Wild carnivores therefore may be good sentinel species with which to monitor the distribution of TBPs. We aimed to evaluate the presence of rickettsial DNA in wild carnivores and to compare its presence in different types of samples. In total, 95 wild carnivores from nine species, originating from 17 counties of Romania collected during 2014–18, were included in the study. From each animal, DNA was extracted from multiple tissue samples, including blood clot, heart, liver, lungs, spleen, kidney, lymph node, and bone marrow, and screened for the presence of rickettsial pathogen DNA (*Anaplasma phagocytophilum*, *Ehrlichia canis*, and *Rickettsia* spp.). Samples from 10 animals from six species (*Canis aureus*, *Ursus arctos*, *Canis lupus*, *Felis sylvestris*, *Lutra lutra*, and *Martes foina*) were found to be positive for *A. phagocytophilum*. The most frequently positive sample was the spleen. No animal was positive for *Ehrlichia* spp. or *Rickettsia* spp. Wild carnivores may be involved in the ecoepidemiology of *A. phagocytophilum* by maintaining the infection in synanthropic environments.

Key words: Anaplasma phagocytophilum, DNA, tick-borne diseases, wild carnivores.

Wildlife may play a major role in the transmission and maintenance of zoonotic agents, with most emerging infectious diseases being of wildlife origin (Jones et al. 2008). However, knowledge of the pathogens that naturally occur in wild animals, and their potential to spread to humans and domestic animals, is still scarce, especially for tick-borne pathogens (Tomassone et al. 2018). In Europe, tick-borne rickettsial infections include zoonotic diseases such as spotted fever group (SFG) rickettsioses, anaplasmoses, and ehrlichiosis (Parola 2004).

There are studies that suggest the involvement of vertebrate hosts in the epidemiology of SFG *Rickettsia* spp. (Tomassone et al. 2018). Among the wild carnivores, *Rickettsia* spp. DNA has previously been detected in red foxes (*Vulpes vulpes*) in Switzerland and Lithuania (Hofmann-Lehmann et al. 2016; Sakalauskas et al. 2019). Despite rickettsial DNA detection in wild carnivores, in a study from Spain, *Rickettsia massiliae*–positive ticks were collected from *Rickettsia*-negative carnivores (Millán et al. 2016), raising uncertainties regarding their reservoir role. Nevertheless, the biologic and social features of wild carnivores (large size, large home range, and long life span) suggest a possible importance as hosts for ticks and tick-borne pathogens, as these aspects allow cofeeding by different tick species, which may be an important transmission mechanism in perpetuating rickettsiae in nature. Moreover, high tick aggregation levels on a given individual may favor *Rickettsia* maintenance in tick populations (Tomassone et al. 2018).

Ehrlichia canis, a species with zoonotic potential, has been detected in red foxes, gray wolves (*Canis lupus*), raccoons (*Procyon lotor*), and Eurasian otters (*Lutra lutra*) in Italy, Portugal, and Spain (Cardoso et al. 2015; Millán et al. 2016; Santoro et al. 2017; Criado-Fornelio et al. 2018).

Compared to other rickettsial agents, *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis, seems to be more common in wild carnivores. It has been detected in red foxes, brown bears (*Ursus arctos*), gray wolf, raccoon dogs (*Nyctereutes procyonoides*), European polecat (*Mustela putorius*), golden jackals (*Canis aureus*), European badger (*Meles meles*), raccoons, and stone martens (*Martes foina*) in several European countries (Víchová et al. 2010; Ebani et al. 2011; Härtwig et al. 2014; Tolnai et al. 2015; Hofmann-Lehmann et al. 2016; Jaarsma et al. 2019; Battisti et al. 2020). Although the role of wild carnivores as reservoir hosts in Europe is uncertain, some species such as raccoon dogs and red foxes are considered to be capable of maintaining *A. phagocytophilum* in nature (Härtwig et al. 2014). *Anaplasma platys*, a related pathogen with a suggested zoonotic potential (Maggi et al. 2013), has been detected in red foxes from Portugal (Cardoso et al. 2015).

We evaluated the presence of rickettsial agents in wild carnivores from Romania, considering their potential role as sentinel species for tick-borne diseases. In addition, motivated by the variable results in *Anaplasma* spp. detection and the infrequent detection of *Rickettsia* spp. in tissue samples, we also aimed to compare the presence of rickettsial DNA in different sample types.

The wild carnivores included in our study were collected from authorized hunters during the legal hunting period, and from rangers who found dead animals (e.g., road kills) during 2014–18, from 17 counties of Romania (Fig. 1). At necropsy, blood clots, heart, liver, lung, spleen, kidney, lymph node, and bone marrow were sampled for this study. In total, 760 samples were collected from 95 wild carnivores belonging to nine species (golden jackal, grey wolf, European wildcat, European otter, Eurasian lynx [*Lynx lynx*], stone marten, European badger, brown bear, and red fox; Fig. 1).

Genomic DNA was extracted for each tissue sample using the ISOLATE II Genomic DNA Kit (Bioline, Meridian Bioscience, Cincinnati, Ohio, USA), following the manufacturer's instructions. The quality and quantity of genomic DNA were evaluated using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA).

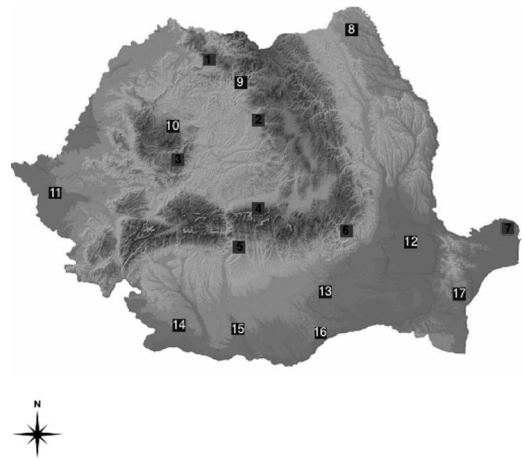

FIGURE 1. Map of Romania showing the geographic origin (counties) of wild carnivores tested in 2014–18 for the presence of rickettsial pathogen DNA. Geographic origins of *Anaplasma phagocytophilum*–positive animals: 1—Maramureş (European wildcat [*Felis silvestris*]), 2—Mureş (grey wolf [*Canis lupus*]), 3—Alba (European otter [*Lutra lutra*]), 4—Braşov (brown bear [*Ursus arctos*]), 5—Vâlcea (golden jackal [*Canis aureus*]), 6—Buzău (golden jackal), 7—Tulcea (golden jackals and stone marten [*Martes foina*]). Geographic origins of *Anaplasma phagocytophilum*–negative animals: 1—Maramureş (European wildcats, European badgers [*Meles meles*], and red foxes [*Vulpes vulpes*]), 2—Mureş (grey wolves), 3—Alba (grey wolves, Eurasian lynxes [*Lynx lynx*], European badger, and red foxes), 6—Buzău (golden jackals, European wildcats, and red foxes), 7—Tulcea (golden jackals, and European otters), 8—Botoşani (golden jackal), 9—Bistriţa-Năsăud (grey wolf), 10—Cluj (European otter and red foxes), 11—Timiş (golden jackals), 12—Brăila (golden jackal), 13—Ilfov (golden jackals), 14—Dolj (golden jackals), 15—Olt (golden jackals), 16—Giurgiu (golden jackals and red fox), 17—Constanta (European wildcat).

We used nested PCR to detect *A. phagocytophilum* and *E. canis* DNA in samples, using specific primers amplifying fragments of the *rrs* gene, while SFG *Rickettsia* DNA detection was performed using a group-specific set of primers amplifying a fragment of the rickettsial *gltA* gene (PCR reactions are in the Supplementary Material). In each PCR

TABLE 1. Species of carnivore, county of origin within Romania, and tissue sample types included in the study (samples collected 2014–18); all eight tissue samples were included from each individual. *Anaplasma phagocytophilum*–positive tissue samples (bold) and number of *A. phagocytophilum*–positive animals are indicated.

Species	Origin[a]	Samples[b]								
		BC	H	LI	LU	S	K	LN	BM	Po./T[c]
***Canis aureus*, golden jackal**	BR, BT, **BZ**, DJ, GR, IL, OT, **TL**, TM, **VL**	**1**	0	**1**	0	**2**	0	0	**2**	5/54
***Canis lupus*, grey wolf**	AB, BN, **MS**	0	0	0	0	**1**	0	0	0	1/12
***Felis silvestris*, European wildcat**	BZ, CT, **MM**	0	0	0	0	**1**	0	0	0	1/8
***Lutra lutra*, European otter**	**AB,** CJ, TL	0	0	0	0	**1**	**1**	0	0	1/6
Lynx lynx, Eurasian lynxes	AB	0	0	0	0	0	0	0	0	0/3
***Martes foina*, stone marten**	**TL**	0	0	0	0	**1**	0	0	0	1/1
Meles meles, European badgers	AB, MM	0	0	0	0	0	0	0	0	0/2
***Ursus arctos*, brown bear**	**BV**	0	0	0	**1**	0	**1**	0	0	1/1
Vulpes vulpes, red fox	AB, BZ, CJ, GR, MM	0	0	0	0	0	0	0	0	0/8
Total		1	0	1	1	6	2	0	2	10/95

[a] The geographic origin of samples (counties of Romania) collected during 2014–18: BR = Brăila; BT = Botoşani; BZ = Buzău; DJ = Dolj; GR = Giurgiu; IL = Ilfov; OT = Olt; TL = Tulcea; TM = Timiş; VL = Vâlcea; AB = Alba; BN = Bistriţa Năsăud; MS = Mureş; CT = Constanta; MM = Maramureş; CJ = Cluj; BV = Braşov.

[b] Tissue samples collected from each animal: BC = blood clot; H = heart; LI = liver; LU = lungs; S = spleen; K = kidney; LN = lymph node; BM = bone marrow.

[c] Po. = positive animals; T = total animals.

reaction set, positive and negative controls were included in order to assess the specificity of the reaction and the possible cross-contamination. Positive controls consisted of DNA extracted from a dog naturally infected with *A. phagocytophilum* and from a castor bean tick (*Ixodes ricinus*) infected with *Rickettsia helvetica*, both confirmed by sequencing. The PCR was carried out using a T100™ Thermal Cycler (Bio-Rad, Hercules, California, USA). Amplicons were visualized by electrophoresis in a 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, California, USA).

All positive PCR samples were sequenced at Macrogen Europe (Amsterdam, the Netherlands), and the obtained sequences were analyzed and compared with those available in GenBank™ by BLAST (National Center for Biotechnology Information, Bethesda, Maryland, USA) analysis.

Statistical analysis was performed using Epi Info™7 software (Centers for Disease Control, Atlanta, Georgia, USA). Pathogen prevalence and 95% confidence interval (CI), and the infection prevalence differentiated by tissue sample, were assessed using the chi-squared test for independence.

Amplification of *E. canis* and *Rickettsia* spp. DNA was negative in all tested samples. *Anaplasma phagocytophilum* was detected with an overall prevalence of 10.53% (10/95, 95% CI 5.16–18.51). In total, 13 positive tissue samples were detected in five golden jackals and in one each of grey wolf, European wildcat, stone marten, Eurasian otter, and brown bear (Table 1). Multiple positive samples were detected in golden jackal, Eurasian otter, and brown bear (Table 2). All samples from Eurasian lynx, red fox, and European badger were negative. The positive animals were collected from seven counties: golden jackals in Tulcea, Buzau, and Vâlcea counties, grey wolf in Mureş county, European wildcat in Maramureş county, stone marten in Tulcea county, Eurasian otter in Alba county, and brown bear in Braşov county (Fig. 1).

The highest prevalence of *A. phagocytophilum* DNA was detected in spleen tissue (6.32%, 95% CI 2.23–13.24), followed by bone marrow and kidney (2.11%, 95% CI

TABLE 2. *Anaplasma phagocytophilum*–positive wild carnivores from Romania, 2014–18, indicating the tissues that were found to be positive in particular individuals.[a]

Species	BC	H	LI	LU	S	K	LN	BM
Canis aureus 1	+	−	−	−	−	−	−	−
Canis aureus 2	−	−	−	−	+	−	−	−
Canis aureus 3	−	−	−	−	−	−	−	+
Canis aureus 4	−	−	+	−	−	−	−	−
Canis aureus 5	−	−	−	−	+	−	−	+
Canis lupus	−	−	−	−	+	−	−	−
Felis silvestris	−	−	−	−	+	−	−	−
Lutra lutra	−	−	−	−	+	+	−	−
Martes foina	−	−	−	−	+	−	−	−
Ursus arctos	−	−	−	+	−	+	−	−

[a] BC = blood clot; H = heart; LI = liver; LU = lungs; S = spleen; K = kidney; LN = lymph node; BM = bone marrow; − = negative sample; + = positive sample.

0.26–7.4), blood clot, liver, and lung (1.05%, 95% CI 0.03–5.73); *A. phagocytophilum* DNA of this pathogen was detected in the spleen of more than half of the positive animals. Only small numbers of samples were positive, and statistical analysis did not show significant differences between the prevalence differentiated by species, geographic origin, or tissue sample type.

Sequence analysis showed a high similarity (100%) among the sequences obtained from wild carnivores (Supplementary Material Fig. S1) and 99–100% similarity with European *A. phagocytophilum* strains (GenBank nos. CP006618 and JX173651).

Anaplasma phagocytophilum has not previously been reported in European wildcat and European otter. Few previous studies on *A. phagocytophilum* infection in wild carnivores in Europe have clearly specified the tissue samples that were used. In red foxes from Italy, Poland, and Hungary, the specific DNA was detected in spleen tissue (Karbowiak et al. 2009; Ebani et al. 2011; Tolnai et al. 2015). In Czech Republic, one red fox was found to be positive without any indication of the type of tissue sample that was positive (Hulínská et al. 2004). Similarly, in brown bears, the collected tissue was "muscle, liver or spleen," without any data regarding the tissues in which *A. phagocytophilum* DNA was found (Víchová et al. 2010). *Anaplasma phagocytophilum* was also detected in the lungs of red fox and raccoon dog in Germany (Härtwig et al. 2014).

Based on our results and on the high prevalence in red foxes obtained in Italy and Hungary (Ebani et al. 2011; Tolnai et al. 2015), the spleen may be the most appropriate sample type for *A. phagocytophilum* screening. However, since this pathogen may also be detected in other tissues while spleen is negative, a mixture from multiple tissue samples may work better. Based on our results, positive results are less commonly found in heart and lymph node samples.

Several wild carnivores have been suggested as possible reservoir hosts for *A. phagocytophilum* in the US, while in Europe, based on the frequency of its detection, only red foxes are considered to be suitable reservoir hosts (André 2018). The phylogenetic analysis of the *rrs* DNA fragment sequences obtained in our study showed the relatedness with zoonotic strains. However, *rrs* DNA fragment sequence analysis does not have sufficient discriminatory power to classify these strains into biotypes or ecotypes. Based on previous studies, all wild carnivores seem to be infected with potential zoonotic strains belonging to ecotype I (Jaarsma et al. 2019). Thus, their importance in the *A. phagocytophilum* ecoepidemiology should be further investigated. The results of our study together with other relevant published papers indicate that wild carnivores may be involved in the ecoepidemiology of this pathogen by maintaining the infection in synanthropic environments.

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SUPPLEMENTARY MATERIAL

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