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SHORT REPORT
Prevalence of *Anaplasma phagocytophilum* infection in European wild boar (*Sus scrofa*) populations from Transylvania, Romania

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SUMMARY

Between 2007–2008 and 2010–2012, 870 organ samples were collected from wild boars in 16 Transylvanian counties. *Anaplasma phagocytophilum* DNA was identified using a nested PCR protocol that amplifies a fragment of the 16S rRNA gene. Prevalence was compared between sampling periods and counties using Fisher's exact test. In total, 39 (4·48%) samples tested positive, with significantly higher values recorded in the second period, caused by an increased infection rate in boars from Sibiu county. Positive cases tended to concentrate in the central part of the country. During the second sampling period, *A. phagocytophilum* was detected in two additional counties, suggesting a spatial spreading of the pathogen. The results confirm that Transylvanian wild boars are naturally infected with *A. phagocytophilum*, thus raising awarness concerning a potential zoonotic cycle. This is the first study to evaluate spatial and temporal variations of *A. phagocytophilum* distribution in wild boar populations from Transylvania.

Key words: *Anaplasma phagocytophilum*, emerging infections, molecular epidemiology, wild boar, Romania.

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is an obligate intracellular tick-borne pathogen that infects a wide range of hosts including humans and wild and domestic animals [1]. Formally named *Ehrlichia phagocytophila* or *E. equi, A. phagocytophilum* is the causative agent of tick-borne fever in ruminants, equine and canine granulocytic anaplasmosis [1, 2], and also of human granulocytic anaplasmosis, considered an emerging tick-borne infectious disease [3] with an increasing incidence [4, 5]. The pathogen is transmitted by *Ixodes* spp. ticks; in Europe, the main vector species is represented by *I. ricinus* [6, 7].

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For most tick-borne diseases, the pathogen is maintaned in well established cycles, with natural hosts usually not manifesting signs of disease. Outbreaks appear when new hosts, such as humans and domestic animals are introduced in this tick-pathogen cycle [7]. Wild animals, especially ungulates, are good hosts for ticks and can represent the natural reservoir for many tick-borne microorganisms [8]. The role of cervids as reservoir hosts for *A. phagocytophilum* has been previously established [9]. Since wild boars (*Sus scrofa*) also act as hosts, although to a lesser extent [10], for parasitic stages of *I. ricinus*, it can be assumed that they may also represent reservoirs, similarly to cervids, at least in some regions [8].

In Transylvania, wild boar is one of the major wild ungulate game species increasingly managed for hunting purposes. As a consequence of their relatively high numbers and the frequent overlap in human and wild

boar activity areas during hunting or inadvertent feeding of the animals in crops, the risk of human exposure to ticks infected with various pathogens harboured by wild boars has to be taken into consideration.

To the best of our knowledge, until now, no large-scale study has been conducted in Romania concerning the prevalence of *A. phagocytophilum* in free-living wild animals in general and wild boars in particular, even if the pathogen was identified in questing *I. ricinus* ticks [11] and exposure was confirmed by serological testing in dogs [12] and horses (T. Kiss *et al.*, unpublished observations). The aim of this study was to evaluate spatial and temporal variations of *A. phagocytophilum* distribution in wild boar populations from Transylvania, during two separate periods.

Between 2007–2008 and 2010–2012, respectively, a total of 870 organ samples were obtained from wild boars in 16 counties. These samples comprised fragments of spleen, liver, and kidney, and were provided by the Directorates of Veterinary Health and Food Safety of each county. During transport, the samples were kept at <0 °C, and after that, stored at −80 °C.

For each animal, after a preliminary mechanical disruption, 125 µl of mixed tissue – including spleen, liver and kidney – were processed for DNA extraction using G-DEX™ IIC Genomic DNA Extraction (iNtRON Biotechnology, Korea) commercial kit, according to the manufacturer's instructions. The eluted genetic material was stored at −20 °C until analysis.

Genetic material belonging to *A. phagocytophilum* was identified using the outer primer pair *ge3a* (CACATGCAAGTCGAACGGATTATTC) and *ge10r* (TTCCGTTAAGAAGGATCTAATCTCC), and the inner primer pair *ge9f* (AACGGATTATTCTTTATAGCTTGCT) and *ge2* (GGCAGTATTAAAAGCAGCTCCAGG) in a nested PCR protocol described by Massung *et al.* [13]. The primary step, which amplifies a 932-bp fragment of the 16S rRNA gene, was performed in 50-µl reaction mixture containing 5 µl 10 × DreamTaq Green buffer (Fermentas, Lithuania), 100 mM dNTP mix (Fermentas), 1 mM of each primer (Eurogentec, Belgium), 1·25 U of DreamTaq polymerase (Fermentas) and 4 µl template. The nested PCR amplifies a 546-bp fragment of the same gene, and was performed in 50 µl reactions containing 5 µl DreamTaq Green buffer (Fermentas), 200 mM dNTP (Fermentas), 1 mM of each primer from the second pair (Eurogentec), 1·25 U of DreamTaq polymerase (Fermentas) and 2 µl of the primary PCR product. Both primary and nested amplifications were performed in an iCycler thermocycler (Bio-Rad, USA): an initial 5 min denaturation step at 95 °C was followed by 40 cycles, each comprising 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and a 1 min extension at 72 °C. A single 5-min extension step at 72 °C was performed after the last cycle.

The final PCR products were analysed by UV transillumination after electrophoresis on 1·5% agarose gel (Lonza) and staining with ethidium bromide. Negative controls including double-distilled, sterile water instead of DNA template, were included in each batch. Positive control consisted of DNA extracted from equine neutrophil culture infected with *A. phagocytophilum*.

Statistical analysis of the results was performed using *R* 2.12.0 for Windows (The R Foundation for Statistical Computing, Austria). Fisher's exact test was used to assess the differences between prevalences. Values of $P<0{\cdot}05$ were considered statistically significant.

The global prevalence for *A. phagocytophilum* infection in Transylvanian wild boar populations for the entire study period was 4·48% (39/870). Between 2007 and 2008, 10/435 (2·29%) samples originating from six counties tested positive for the presence of *A. phagocytophilum* (Fig. 1). No significant differences were observed between counties. During the second sampling period (2010–2012), *A. phagocytophilum* was identified in significantly more ($P=0{\cdot}006$) wild boars, with 29/435 (6·67%) testing positive. Significantly higher prevalences were recorded in the counties of Sibiu, Hunedoara, Harghita and Maramureş (Fig. 1). The number of samples from each county, along with prevalence and *P* values for significant differences, are listed in Table 1. Although *A. phagocytophilum* was found in two additional counties (Arad, Maramureş), located in the northwestern part of Transylvania during the second sampling period, positive samples originated mainly from the central part of the country during both sampling periods.

We identified *A. phagocytophilum* DNA in 4·48% of the wild boar samples collected between 2007 and 2012. The significant difference between the two sampling periods is the consequence of a high infection rate detected in Sibiu county between 2010 and 2012. Because serum samples collected from horses in this county during 2011 also showed higher seropositivity compared to samples originating from other counties (T. Kiss *et al.*, unpublished observations), we hypothesized that a local increase in pathogen

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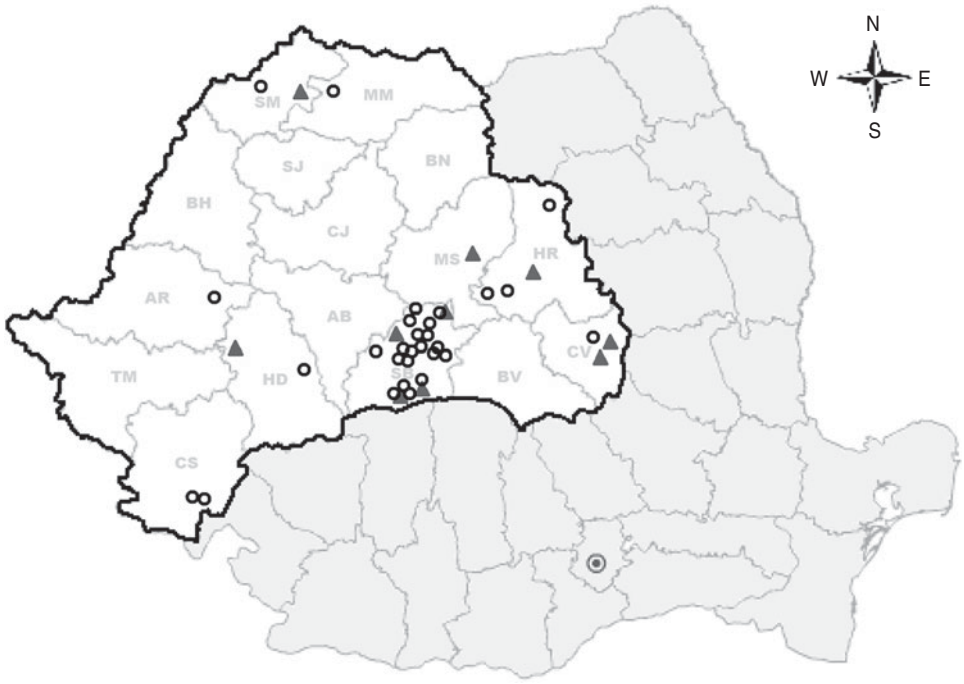

Fig. 1. Geographical distribution of wild boar samples positive for *A. phagocytophilum* DNA, collected between 2007 and 2008 (▲) and 2010–2012 (●). AB, Alba; AR, Arad; BH, Bihor; BN, Bistriţa Năsăud; BV, Braşov; CJ, Cluj; CS, Caraş Severin; CV, Covasna; HD, Hunedoara; HR, Harghita; MM, Maramureş; MS, Mureş; SB, Sibiu; SJ, Sălaj; SM, Satu Mare; TM, Timişoara.

prevalence was the cause of this positivity surge. If we ignore the unusually high value recorded in Sibiu county, the prevalence is equal for the two sampling periods, suggesting that the pathogen has established a stable cycle in nature. Even if the number of cases does not seem to increase in time, spatial spreading is suggested by the identification of positive boars in two additional counties during the second sampling period. There is little or no significant difference between counties regarding prevalence, except the few ones mentioned in Table 1, although cases appear to be concentrated in the central part of the country (Fig. 1). This is in accordance with observations made by Mircean *et al.* [12], during the evaluation of antibody prevalence against *A. phagocytophilum* in dogs.

Active *A. phagocytophilum* infection, translated through identification of the pathogen in blood and tissue samples, has been previously reported in wild boars, generally at rates similar to those found in the present study. Strašek Smrdel *et al.* [3] detected 3·5–4·4% positive samples in wild boars shot in Slovenia; in the Czech Republic, 4·3% of the tested boar samples yielded positive results [9], while in western Poland, Skotarczak *et al.* [10] identified a prevalence of 6% in blood and spleen samples. On the other hand, Michalik *et al.* [14] reported a 12% prevalence of *A. phagocytophilum* in wild boar in Poland. Petrovec *et al.* [15] found DNA of the pathogen in 14·3% of wild boar shot in the Czech Republic. Other authors [16, 17] failed to detect any positive samples in boars from Austria, and northern Spain.

Hulinská *et al.* [9] found that prevalence of *A. phagocytophilum* infection was greater in cervids (*Capreolus capreolus, Dama dama, Cervus elaphus*), hares (*Lepus europaeus*) and rodents (*Myodes glareolus, Apodemus flavicollis*), which are considered reservoirs, ranging from 12·5% to 15%, compared to foxes (*V. vulpes*) and boars, with values of 4–4·3%. Higher prevalence in roe (*C. capreolus*) and red deer (*Cervus elaphus*), reaching 53·6% and 68%, respectively, was observed in western Poland [10]. In Italy, 16·6% of spleen samples collected from foxes were positive for *A. phagocytophilum* [6].

These results appear to support the theory that cervids, and possibly rodents, are the main reservoir species for *A. phagocytophilum.* Transcriptomics studies revealed that while susceptible to *A. phagocytophilum*, pigs are capable of controlling the infection through activation of innate immune responses to promote phagocytosis and autophagy. This control presumably pushes infection levels below the PCR

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Table 1. *Distribution of wild boar positive for A. phagocytophilum according to sampling period and county*

County	2007–2008 Prevalence ($n=435$), *n* (%)	2010–2012 Prevalence ($n=435$), *n* (%)
Alba	0/29 (0)	0/12 (0)
Arad	0/19 (0)	1/15 (6·66)
Bihor	0/3 (0)	0/39 (0)
Bistriţa Năsăud	0/8 (0)	0/30 (0)
Braşov	0/10 (0)	0/13 (0)
Cluj	0/27 (0)	0/32 (0)
Covasna	2/62 (3·22)	1/33 (3·03)
Hunedoara	1/33 (3·03)	1/11 (9·09)***
Harghita	1/51 (1·96)	3/27 (11·11)**
Maramureş	0/11 (0)	1/11 (9·09)***
Mureş	1/32 (3·12)	0/34 (0)
Sibiu	4/101 (3·96)	19/72 (26·39)*
Satu Mare	1/43 (2·33)	1/32 (3·12)
Timiş	0/6 (0)	0/35 (0)
Caraş Severin		2/23 (8·69)
Sălaj		0/16 (0)

* $P=0·0004$, ** $P=0·02$, *** $P=0·04$.

detection threshold, or even clears it, and may account for the consistently lower prevalence values reported in boars compared to cervids. Therefore, several authors believe that low prevalences found in wild boar populations in Europe confirm the diminished importance of this species as a reservoir [8, 10, 18].

Interestingly, sequencing of the *groESL* gene revealed that genetic variants of *A. phagocytophilum* identified from wild boars in the Czech Republic [15], Slovenia [3] and Poland [14] were identical to those implicated in cases of human granulocytic anaplasmosis, suggesting that these animals may still play an important role in the transmission cycle of this pathogen by constituting a source of zoonotic infection. All these data serve to confirm only that the role of wild boars as potential reservoirs for *A. phagocytophilum* is far from being elucidated.

Even if their reservoir capacity remains debatable, because of their relatively high numbers, ease of access to tissue samples, and presumably rapid clearance of the pathogen, wild boar would represent a good choice as sentinels, by providing a sensitive environmental risk indicator of the changes in the epidemiology of *A. phagocytophilum*. This would be especially true for strains known to be pathogenic for humans, but their utility as such remains to be evaluated.

Massung *et al.* [13] tested the specificity of their PCR protocol by trying to amplify genetic material from related bacterial species, and by sequencing the PCR products. They found that all amplicons were identical to either the 16S rRNA gene sequence of the agent of human granulocytic ehrlichiosis, or to a pathogen named *Ehrlichia* sp. at the time, isolated from white-tailed deer in Wisconsin. Dumler *et al.* [1] proved, by the means of phylogenetic analysis of the 16S rRNA gene and *groESL* sequences, that both are actually variants of the same species, *A. phagocytophilum.* Therefore, we can reasonably assume that the positive samples of this study do contain genetic material from *A. phagocytophilum*. Nevertheless, for a definitive confirmation, sequencing of the amplicons is under way, and along with the identification of circulating strains, has the potential to shed some light on the circulation of the pathogen in central and eastern Europe. The present study did not set out to differentiate genetic variants of *A. phagocytophilum*. To our knowledge, there are no reported sequences of *A. phagocytophilum* isolated from Romanian patients or free-living or domestic animals, thus a comparison between genetic variants infecting wild boars and humans or other species is not possible.

Conducted on a reasonable number of samples, this study ascertained the presence of *A. phagocytophilum* in wild boar, and presents its molecular epidemiology in the region of Transylvania, suggesting a slow spatial spreading of the pathogen. The results indicate that the risk of contracting granulocytic anaplasmosis should be considered for humans, especially those belonging to risk cathegories such as forestry workers, or hunters, and that future studies should aim to confirm and assess this risk.

In conclusion, the present study confirms that Transylvanian wild boar are naturally infected with *A. phagocytophilum*, thus raising awarness concerning a potential zoonotic cycle. By gaining a first glimpse on the molecular epidemiology of *A. phagocytophilum* in Transylvania, it becomes obvious that concerted efforts of veterinarians and medical practitioners will be necessary in order to assess the risk represented by this pathogen in Romania.

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DECLARATION OF INTEREST

None.

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