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Original article

Anaplasma phagocytophilum in questing *Ixodes ricinus* ticks from Romania

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ABSTRACT

Granulocytic anaplasmosis is a common vector-borne disease of humans and animals with natural transmission cycle that involves tick vectors, among which *Ixodes ricinus* is the most important. The present paper reports the prevalence and geographical distribution of *A. phagocytophilum* in 10,438 questing *Ixodes ricinus* ticks collected at 113 locations from 40 counties of Romania. The unfed ticks were examined for the presence of *A. phagocytophilum* by PCR targeting a portion of ankA gene. The overall prevalence of infection was 3.42%, with local prevalences ranging between 0.29% and 22.45%, with an average prevalence of 5.39% in the infected localities. The infection with *A. phagocytophilum* was detected in 72 out of 113 localities and in 34 out of 40 counties. The highest prevalence was recorded in females followed by males and nymphs. The results and the distribution model have shown a large distribution of *A. phagocytophilum*, covering Romania's entire territory. This study is the first large scale survey of the presence of *A. phagocytophilum* in questing *I. ricinus* ticks from Romania.

Introduction

Anaplasma phagocytophilum, is a small, pleomorphic, Gram-negative, obligate intracellular bacterial organism that typically infects granulocytes of mammals (Chen et al., 1994; Dumler et al., 2001). The disease is known as tick-borne fever (TBF) in ruminants, human granulocytic anaplasmosis (HGA) in people, canine granulocytic anaplasmosis (CGA) in dogs and equine granulocytic anaplasmosis (EGA) in horses (Dumler et al., 2001).

Infections with *A. phagocytophilum* were found in animals and humans from most parts of the northern hemisphere. It was detected in North America (USA and Canada) and in almost all countries of Europe (Strle, 2004; Teglas and Foley, 2006). From Asia, it was detected in Turkey, Russia, China, Korea and Japan (Aktas et al., 2011; Cao et al., 2000; Ohashi et al., 2005; Kim et al., 2006). Reports of *A. phagocytophilum* or closely related strains from Africa and South America are occasional (Inokuma et al., 2005; André et al., 2012, 2014). There is no reliable data available about its occurrence in South America and Australia.

Ticks of the genus *Ixodes* are the main vectors for *A. phagocytophilum* (Stuen et al., 2013). In Europe, the most important vector is *Ixodes ricinus* (Strle, 2004). This tick is widespread and has a very broad host spectrum, being also the dominant tick in Romania, including on humans (Briciu et al., 2011; Mihalca et al., 2012b,c).

Despite the common presence of *A. phagocytophilum* in Europe, its first molecular identification in Romania was in 2012, when Păduraru et al. (2012) identified it in *I. ricinus* collected from roe deer (*Capreolus capreolus*) and goats in 2 sites in eastern Romania. Subsequently, several other reports on its presence or prevalence were published in dogs (Hamel et al., 2012; Mircean et al., 2012) and wild boars (*Sus scrofa*) (Kiss et al., 2014), as well as in ticks collected from livestock (Ioniţă et al., 2013), hedgehogs (*Erinaceus roumanicus*) (Dumitrache et al., 2013), tortoises (*Testudo graeca ibera*) (Paştiu et al., 2012) and birds (Mărcuţan et al., 2014). Despite the climatic and habitat heterogeneity of Romania (Doniţă et al., 2005), the prevalence and distribution of *A. phagocytophilum* in questing ticks has never been evaluated so far. Although *I. ricinus* has a wide distribution across Romania (Mihalca et al., 2012c), the reservoir hosts such as wild ruminants, wild boars, rodents

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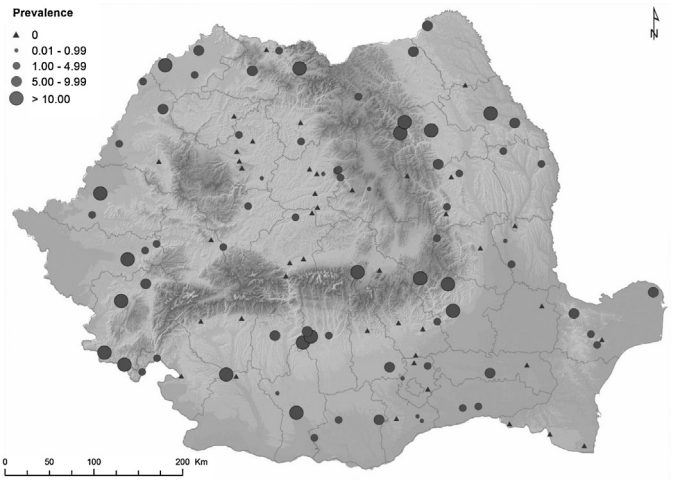

Fig. 1. Prevalence of *A. phagocytophilum* in questing *I. ricinus* ticks from Romania.

and insectivores (Stuen et al., 2013) may have a variable density and distribution in Romania (Wagner, 1974, 1976). With this view, the aims of our study were to evaluate the prevalence of *A. phagocytophilum* in questing *I. ricinus* ticks from multiple locations distributed throughout Romania and to correlate its presence with different ecological features.

Materials and methods

Tick collection and sampling sites

The tick sampling took place between March and May 2010 and between March and May 2011, using the dragging method (Estrada-Peña, 2001). A random sampling approach was used, as previously described (Kalmár et al., 2013). Grid cells of 10 × 10 km were designed to cover the entire country. Only cells having forests were considered appropriate for sampling (Mihalca et al., 2012c). All the grid cells with negligible forest coverage (<1%) were excluded. As a result of this approach, 2048 out of 2492 cells were used for the design of the sampling areas, representing 82% of the total available grid cells. In the second step, the random selection function available in the ArcGIS software (Hawth's Tools), selected an adequate number of grids covered by forest. Resulting from this evaluation, 164 grid cells were considered for the implementation of the study, representing 8% of the total. For each selected grid, a number of points, from 1 to 3 were randomly distributed using the same GIS tool, as starting points for the flagging transects. Points' placement was restricted to more than 10 m inside the forest. To define the sampling design, the forest spatial data set included in the Corine Land Cover database (Copyright: European Environment Agency/EEA) was used. Points included in the grid cells with a number of collected ticks lower than 30 were automatically excluded from the investigations. As a result, tick collection was performed in 183 localities from all the 41 counties of Romania.

The tick species were morphologically identified based on common features of dichotomous keys (Feider, 1965; Nosek and Sixl, 1972) and stored in 70% ethanol at −20 °C until examination. The collected ticks belonged to 11 species (Mihalca et al., 2012c), but only those identified as *I. ricinus* and from these only nymphs and adults (n = 10,483) were included in this study, resulting in 113 collection sites (localities) (Fig. 1).

DNA extraction

Genomic DNA extraction was performed individually from each tick with ammonium hydroxide (Morán-Cadenas et al., 2007). Ticks were boiled in 300 μl of 1.25% ammonium hydroxide at 100 °C for 30 min, and then cooled. The tubes with the lysate were left open for 30 min at 100 °C for the ammonia to evaporate. In order to assess cross-contamination between extracts, negative controls were used in each extraction procedure. The DNA quantity and purity were assessed on Nanodrop ND-1000 spectrophotometer analyzer (NanoDrop Technologies, Inc., Wilmington, DE, USA), using a representative number of randomly selected samples.

Polymerase chain reaction (PCR)

The PCR was carried out using *Anaplasma phagocytophilum* specific primers LA1/LA6 (forward primer: 5′-GAGAGATGCTTATGGTAAGAC-3′, and a reverse primer: 5′-CGTTCAGCCATCATTGTGAC-3′), amplifying a 444-bp fragment of *ank* A gene (Caturegli et al., 2000; Walls et al., 2000). The amplification was performed as follows: 25 μl reaction mixture containing 4 μl aliquot of isolated DNA, 12.5 μl of Green PCR Master Mix (Rovalab) and 1 μl of each primer (0.01 mM). The PCR was carried out using a T100™ Thermal Cycler (Bio-Rad). The amplification profile consisted of 5 min of initial denaturation at 95 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. In each PCR reaction set (94 samples) a positive and a negative control were included in order to assess the specificity of the reaction and the possible presence of contaminants. Positive controls were kindly provided by IDEXX Germany and consisted of DNA extracted from the blood of a dog naturally infected with *A. phagocytophilum*. The negative control consisted in reaction mix without DNA. In order to confirm the validity of PCR, a representative number of positive samples were randomly selected for sequencing.

Agarose gel electrophoresis

Amplicons were visualized by electrophoresis in a 1.5% agarose gel (1 × TAE, pH 8.0) stained with SYBR® Safe DNA gel stain (Invitrogen).

DNA sequencing

PCR products were purified from gel using the QIAquick PCR Purification Kit (QIAGEN) and then analyzed by sequence analysis (performed at Macrogen Europe, Amsterdam).

Sequences were compared to those available in GenBank™ dataset by Basic Local Alignment Tool (BLAST) analysis.

Phylogenetic tree

The phylogenetic tree was constructed based on Maximum Parsimony method (Felsenstein, 1985). The bootstrap test was carried out according to the Subtree–Pruning–Regrafting (SPR) algorithm (Nei and Kumar, 2000). The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) was calculated. The phylogenetic branches were supported in >50% by bootstrap analysis. Evolutionary analyses were conducted using MEGA6 software (Tamura et al., 2013).