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Original article

Candidatus Neoehrlichia mikurensis and *Anaplasma phagocytophilum* in natural rodent and tick communities in Southern Hungary

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ABSTRACT

The aim of this study was to investigate the natural cycle of the new human pathogenic bacteria *Candidatus* Neoehrlichia mikurensis and *Anaplasma phagocytophilum* in Southern Hungary. We collected rodents with live-traps (2010–2013) and questing ticks with flagging in 2012. Small mammals were euthanized, tissue samples were collected and all the ectoparasites were removed and stored in 70% alcohol. We found relatively low overall prevalence of tick infestation (8%). Samples were analysed for *A. phagocytophilum* and *Candidatus* N. mikurensis with multiplex quantitative real-time PCR targeting a part of major surface protein 2 (msp2) and the heat shock protein groEL genes, respectively. The overall prevalence in tissue samples was 6.6% (skin) and 5.1% (spleen) for *A. phagocytophilum* and 1.7% (skin) and 3.4% (spleen) for *Candidatus* N. mikurensis. *Candidatus* N. mikurensis was only detected in *Apodemus flavicollis* and *Apodemus agrarius*, while *A. phagocytophilum* was found in *A. flavicollis*, *A. agrarius*, *Myodes glareolus*, *Microtus arvalis* and *Mus musculus* samples. Prevalence of *A. phagocytophilum* in skin samples of *A. flavicollis* was significantly higher than prevalence of *N. mikurensis* ($p < 0.05$). Among questing *Ixodes ricinus* ticks we found three (8.8%) individuals (female, male, nymph) infected with *Candidatus* N. mikurensis. Five (3.1%) questing ticks had *A. phagocytophilum* infection (one *I. ricinus* male, two *Dermacentor reticulatus* females and two *Haemaphysalis concinna* females). We found one *I. ricinus* nymph removed from a male *A. flavicollis* with *A. phagocytophilum* infection. Our study provides new data on the occurrence of these pathogens in rodent tissue samples, questing ticks and engorged ticks in Southern Hungary.

Introduction

Rodents are reservoir hosts for several emerging zoonotic pathogens and with other small mammals (insectivores) have important role in the tick life cycle serving as main feeding and maintenance hosts for the developmental stages of various tick species. They also play an important role in the endemic cycles of tick-borne pathogens (e.g. tick-borne encephalitis virus or *Babesia microti*) (Silaghi et al., 2012). Therefore, the health of humans can be seriously impaired by contact with infected rodents or ticks that have previously fed on them. Ticks found on small mammals can be either exophilic or endophilic. Exophilic (or non-nidicolous) species such as *Ixodes ricinus* await a host on the vegetation thus may act as bridge vectors between small mammals and humans in natural or urban habitats (Oliver et al., 2003). Endophilic (or nidicolous) ticks like subadult stages of *Dermacentor reticulatus* or all three stages of *Ixodes trianguliceps* are more specialised regarding their hosts by living in their nests or in their close environment thus may provide stable local niche cycles in rodents' nest for pathogens such as *Anaplasma phagocytophilum* (Bown et al., 2006).

Candidatus Neoehrlichia mikurensis is a coccoid Gram-negative pathogen belonging to the family Anaplasmataceae (Kawahara et al., 2004). It was first detected in the late 1990's in *I. ricinus* in The Netherlands and Italy and later on it was also found in China in a wild Norway rat (*Rattus norvegicus*). It was initially called *Ehrlichia*-like due to a diverging 16S rRNA gene sequence (Schouls et al., 1999). Further findings of the microorganism in rats and *Ixodes ovatus* ticks in Japan and the passaging of the agent in laboratory rats led to its description as the new species *Candidatus*

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Neoehrlichia mikurensis in 2004 (Kawahara et al., 2004). This emerging zoonotic intracellular tick-borne pathogen forms a separate cluster in the family Anaplasmataceae together with the North American *Candidatus* Neoehrlichia lotoris, which has been detected in raccoons (*Procyon lotor*) (Yabsley et al., 2008). In Switzerland, Sweden, Germany, Czech Republic and in China *Candidatus* N. mikurensis was shown to be a human and in Germany as a canine pathogen (Grankvist et al., 2014; Jahfari et al., 2012; Li et al., 2012; Pekova et al., 2011; Silaghi et al., 2012; Tijsse-Klasen et al., 2014). Most of the human patients were immunocompromised due to splenectomy or immunosuppressive therapy and the reported manifestations of neoehrlichiosis were severe. In China, however, *Candidatus* N. mikurensis infection was also reported in immunocompetent patients (Li et al., 2012). *Ixodes ricinus* is most likely the vector in Europe, but the range of reservoir hosts is not fully known. Some studies suggested rodents as potential reservoirs (Jahfari et al., 2012) and recently the reservoir role of *Apodemus* mice (*A. flavicollis*, *A. sylvaticus*) and bank voles (*Myodes glareolus*) has unambiguously been proven in a xenodiagnostic study (Burri et al., 2014).

Several studies have identified DNA of *Candidatus* N. mikurensis in questing or host-attached *I. ricinus* in Europe including Hungary (Derdáková et al., 2014; Hornok et al., 2013; Jahfari et al., 2012). Recently, Northern white-breasted hedgehogs (*Erinaceus roumanicus*) were shown to carry this pathogen in a city park of Budapest (Földvári et al., 2014). However, potential rodent reservoir hosts have thus far not been examined in Hungary. Accordingly, in this study the occurrence of *Candidatus* N. mikurensis was investigated in small mammals, ticks parasitizing them and questing ticks in natural habitats in Southern Hungary.

Anaplasma phagocytophilum is an obligate Gram-negative intracellular bacterium. It has been a well known pathogen among the domestic ruminants causing "tick-borne fever" but it is a generalist pathogen and can infect several other land-living vertebrate species (including humans) on the Northern hemisphere where ticks of the *I. ricinus* complex are endemic. Fatal infection cases were reported in sheep, horse, roe deer, dogs and humans. This bacterium infects and colonizes the neutrophils thus the pathogen decreases the number of the useful immune cells often leading to immunodeficiency (Stuen et al., 2013).

Wild ruminants and probably small mammals (rodents and insectivores) play the most important role in the life cycle of *A. phagocytophilum* but other animals (bears, wild boars, foxes, horses, reptiles) can also serve as hosts or possible reservoirs (Földvári et al., 2014; Silaghi et al., 2012; Stuen et al., 2013; Víchová et al., 2010, 2014). In the USA the white-footed mouse (*Peromyscus leucopus*) is considered the major reservoir of this pathogen (Stuen et al., 2013). The bank vole (*My. glareolus*), the yellow-necked mouse (*A. flavicollis*) and the field vole (*Microtus arvalis*) are the candidate rodent reservoirs in Europe (Stuen et al., 2013), but in a xenodiagnostic study the *Apodemus* spp. mice and *My. glareolus* did not infect larvae that had fed on them (Burri et al., 2014). Thus, the exact role of European rodent species in the circulation and maintenance of bacteria is unclear and prevalence rate of *A. phagocytophilum* DNA is low in this group of animals (Stuen et al., 2013). *Anaplasma phagocytophilum* can also be transmitted by ticks to a wide range of domestic ruminants e.g. bovines (cattle, yak), camelids (llama, alpaca), sheep and goats.

In Europe, the increasing geographic range of *I. ricinus* as well as the expansion to higher altitudes opened new regions and heights to this pathogen (Jaenson et al., 2012; Medlock et al., 2013). In Hungary the prevalence of *A. phagocytophilum* infection in rodents (Rigó et al., 2011) and questing ticks (Egyed et al., 2012) was relatively low, but in a recent paper using a more sensitive qPCR the prevalence in hedgehogs (*E. roumanicus*) was high (Földvári et al., 2014).

This study investigates the occurrence of *Candidatus* N. mikurensis and *A. phagocytophilum* DNA in small mammals, ticks parasitizing them and questing ticks in natural habitats in Southern Hungary, with the aim to highlight the role of the rodent species as prospective hosts that contribute to the maintenance of these pathogens in the area.

Materials and methods

Sample collection

Between July 2010 and May 2013, small mammals were live-trapped with 100 modified Sherman-traps (17 × 7 × 8 cm) within the Gemenc area which is a forest covered floodplain near the Danube River, in Southern Hungary (Fig. 1). The total number of trapnights (the sum of the total number of nights each trap was used) was 2200. Traps were set at sunset and checked early the following morning. The species and sex of trapped rodents was identified (Aulagnier et al., 2008) and animals belonging to protected species were then released. All the other rodents were euthanized. The carcasses were checked for ticks and other ectoparasites and samples from spleen and skin were collected. The spleen and skin samples in this study did not originate from the same individuals.

During the trapping in May 2012, ticks were collected with flagging from the vegetation in several different locations within the Gemenc area. Ticks collected from the trapped animals and from the vegetation were stored in 70% ethanol, and were later identified using standard identification keys (Hillyard, 1996; Nosek and Sixl, 1972).

DNA extraction

The DNA was extracted from ticks by alkaline hydrolysis (Guy and Stanek, 1991) before being examined for the presence of the bacteria by polymerase chain reaction. Pool samples were prepared from each 10 larvae originating from the same host individual. The nymphs and adults were processed individually. DNA was isolated from the tissue samples with a modified Miniprep Express Matrix protocol (MP Biomedicals, Santa Ana, USA). We stored the tubes with extracted DNA at −20 °C in the freezer for further analyses.

Polymerase chain reaction

To determine whether the tissue or tick samples contained any pathogens, we used multiplex quantitative-PCR (qPCR) for *A. phagocytophilum* and *Candidatus* N. mikurensis. For *Candidatus* N. mikurensis we targeted GroEL heat shock protein gene, the product length was 102 bp, with forward primer groEL-F2a (5′ CCTTGAAAATATAGCAAGATCAGGTAG 3′) (Jahfari et al., 2012). We used two reverse primers groEL-R2a (5′ CCACCACGTAACTTATTTAGCACTAAAG 3′) and groEL-R2b (5′ CCACCACGTAACTTATTTAGTACTAAAG 3′), with the probe groEL-P2a (5′ CCTCTACTAATTATTGCtGAAGATGTAGAAGGTGAAGC 3′). For *A. phagocytophilum*, we targeted the major surface protein 2 gene with the forward primer apMSP2F (5′ ATGGAAGGTAGTGTTGGTTATGGTATT 3′), reverse primer apMSP2R (5′ TTGGTCTTGAAGCGCTCGTA 3′) and probe apMSP2P (5′ TGGTGCCAGGGTTGAGCTTGAGATTG 3′) (Courtney et al., 2004), resulting in a 77 bp long product. In the analysis of qPCR result we selected the positive samples by two criteria, the shape of curves (compared to positive controls) and CT (threshold cycle) values. Samples were considered positive with CT values below 41 cycles for both *Candidatus* N. mikurensis and for *A. phagocytophilum*. In PCR reactions we used negative controls to verify and exclude any contaminations.

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