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# *Anaplasma* infections in ticks and reservoir host from Slovakia

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ABSTRACT

*Anaplasma phagocytophilum* is a worldwide distributed bacterium with a significant medical and veterinary importance. It grows within the phagosome of infected neutrophils and is responsible for human granulocytic anaplasmosis (HGA), tick-borne fever (TBF) of small ruminants and cattle, canine and equine granulocytic anaplasmosis, but infects also a great variety of wildlife species. Wild ungulates and rodents are considered reservoirs of infection in natural foci. The objective of this study was to determine the spectrum of animal species involved in the circulation of *A. phagocytophilum* in Slovakia and to analyze the variability of obtained nucleotide sequences, in order to determine whether genotypes from Slovakia cluster according to host-species or geographical location.

Several animal species and vector ticks were screened for the presence of members of the family Anaplasmataceae using PCR based methods. Additional data on the molecular evidence of *Anaplasma ovis* and *Candidatus* Neoehrlichia mikurensis are presented. These pathogens were detected in tested sheep flocks and rodents with the mean infection rates of 8.16% and 10.75%, respectively. *A. phagocytophilum* was genotyped by 16S rRNA and *groEL* gene sequencing. Bacterial DNA was confirmed in questing ixodid ticks, in domesticated canine, wild rodents and several species of wild ungulates.

In European isolates, 16S rRNA gene does not seem to be an appropriate locus for the analyses of heterogeneity as it is too conservative. Similarly, 16S rRNA isolates from our study did not reveal any polymorphisms. All isolates were identical in overlapped region and showed identity with sequences from ticks, horses or ruminants previously isolated elsewhere in the world. On the other hand, the groESL heat shock operon is widely used for determination of diversity and the analyses have already revealed considerable degree of heterogeneity.

Tested ungulates were infected with *A. phagocytophilum* to a considerable extent. High proportions of red and roe deer tested positive and the rates of infection reached over 60.0%. GroEL sequences from canine, wild ungulates and ticks from Slovakia clustered within a clade together with isolates from horses, humans, wild ungulates and ticks from Slovakia or elsewhere in the world. Sequences from rodents clustered apart from those obtained from wild ungulates, ticks and humans. These results suggest that European rodents do not harbour *A. phagocytophilum* strains with strong zoonotic potential such as those from United States.



## 1. Introduction

*Anaplasma phagocytophilum* is a Gram-negative intracellular bacterium causing febrile disease of humans (human granulocytic anaplasmosis – HGA) and animals (pasture fever, equine and canine granulocytic anaplasmosis) (Dumler et al., 2001). The principle vectors of this rickettsial pathogen are ticks from the *Ixodes ricinus* complex. Transovarial transmission in ixodid ticks has not yet been confirmed; therefore the vertebrate hosts are crucial for the maintenance and circulation of pathogen in enzootic foci. Bacteria multiply in a broad range of hosts, especially in small rodents and wild ruminants which are discussed main reservoirs (Alberdi et al., 2000; Liz et al., 2002; Petrovec et al., 2003; Hulínska et al., 2004; Polin et al., 2004; Smetanová et al., 2006; de la Fuente et al., 2008; Bown et al., 2008; Štefanidesová et al. 2008). Based on analyses of several genetic markers (16S rRNA, *groESL*, *ankA*, *msp*), the existence of intraspecific heterogeneity has been recorded within *A. phagocytophilum*. Complex of closely related strains shows differences in vector and host preference,

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geographical distribution, pathogenicity and severity of clinical manifestations (Massung et al., 2006; Stuen et al., 2002; Stuen, 2007; Carpi et al., 2009). Significant differences were detected in the epidemiology of granulocytic anaplasmosis (GA) in Europe and North America. Whereas in the USA GA belongs among the most common tick-borne diseases, it remains relatively rare in Europe (Strle, 2004).

In the United States, the circulation of two distinct variants of *A. phagocytophilum* was confirmed on the basis of revealed differences in 16S rRNA (Massung et al., 2002). Similarly, de la Fuente et al. (2005c) characterized two European monophyletic groups based on analysis of *msp4* gene. One group consisted of strains from humans, dogs and horses and second one comprised strains from wild ruminants. They did not find *A. phagocytophilum* in tested rodents. In Europe, the situation seems to be rather different compared to the United States and the ecology and circulation of distinct ecotypes are poorly understood. Analyses of 16S rRNA did not confirm the unambiguous association of two genotypes with rodents and ruminants, as it has been reported in the USA. In European ungulates both variants have been recorded. Mostly those not associated with human infections, but also strains closely related to human granulocytic anaplasmosis (HGA) derived from red deer in Slovenia (Petrovec et al., 2002). Studies from England outlined the possibility of co-existence of two distinct subpopulations of *A. phagocytophilum* circulating in separate enzootic cycles, one involving deer and *I. ricinus* ticks and the other involving field voles (*Microtus agrestis*) and nidicolous *Ixodes trianguliceps* ticks (Bown et al., 2008).

The objective of this study was to identify epizootiological situation of granulocytic anaplasmosis in Slovakia, to determine the spectrum of animal species involved in the circulation of pathogen and to analyze and characterize the variability of obtained *A. phagocytophilum* DNA sequences, in order to determine whether genotypes from Slovakia cluster according to host-species or geographical location. We also report supplementary data on the presence of *Anaplasma ovis*, an etiologic agent of pasture fever of small ruminants and the recently described tick-borne bacterium *Candidatus* Neoehrlichia mikurensis, with the potential to cause severe diseases in immunocompromised patients. *Ca.* Neoehrlichia mikurensis was discovered in wild rodents and *Ixodes ovatus* ticks in Japan (Kawahara et al., 2004). Since then, it has been identified in ixodid ticks and several rodent species, which may act as reservoir hosts (Schouls et al., 1999; Špitalská et al., 2008; Alekseev et al., 2001; Andersson and Raberg, 2011; Jahfari et al., 2012; Pangrácová et al., 2012; Shpynov, 2012; Vayssier-Taussat et al., 2012), and in blood samples of febrile patients from Sweden, Germany, Switzerland and Czech Republic with a lethal course in one case (Fehr et al., 2010; von Loewenich et al., 2010; Welinder-Olsson et al., 2010).

## 2. Material and methods

### 2.1. Collected samples

#### 2.1.1. I. ricinus ticks

Ticks were collected at the sampling sites continually during 2006–2009. A total of 1075 questing *I. ricinus* ticks were collected by white cloth flag dragging in locations of eastern Slovakia, in a hornbeam deciduous suburban forest park in Košice city (48°43′N, 21°15′E) ($n = 213$), near the Hornád river in Košice city, in the area devastated last year by floods (48°40′N, 21°18′E) ($n = 46$), in the forest habitat of the locality Kavečany in the district of Košice city (48°46′N, 21°12′E) ($n = 108$), in the district of Michalovce city (48°45′N, 21°55′E) ($n = 73$), in the Slovak Karst National Park (48°36′N, 20°52′E) in south-eastern Slovakia ($n = 242$) and 393 ticks were collected at the recreational grounds of Teplý vrch and nearby the water basin Kurinec in the district of Rimavská Sobota city (48°28′N, 20°05′E; 48°20′N, 20°01′E). Collected ticks were preserved in 70% ethanol until DNA isolation.

#### 2.1.2. Dog blood samples

All tested blood and tissue samples of domesticated and wild animals included in study were collected continually from 2006 to 2011.

In total, 137 blood samples of dogs suspected of having non-specific febrile disease with a tick bite history were collected by vet practitioners in the districts of Senica (48°40′N, 17°21′E), Bratislava (48°08′N, 17°06′E), Trenčín (48°53′N, 18°02′E), Liptovský Mikuláš (49°04′N, 19°37′E), Lučenec (48°19′N, 19°39′E), Rimavská Sobota (48°23′N, 20°01′E), and Košice city (48°43′N, 21°15′E). Additionally, 144 blood samples of police and military dogs, previously screened at the Institute of Parasitology for the presence of *Dirofilaria* sp. (Miterpáková et al., 2010), were included in study. Samples were collected in plastic tubes containing anticoagulant (EDTA) and stored at 4 °C until DNA isolation.

#### 2.1.3. Wild carnivores, ungulates and rodents

Samples of tissue were collected from 248 red foxes (*Vulpes vulpes*). Major part of them originated from animals shot by hunters in eastern Slovakia subjected to the post mortem examination of the presence of *Trichinella* spp. and *Echinococcus* spp. (Dubinský et al., 2006; Hurníková and Dubinský, 2009). Samples of blood or tissue (liver, spleen or muscle) were collected from 84 hunter-killed wild boars (*Sus scrofa*), 103 red deer (*Cervus elaphus*), 13 roe deer (*Capreolus capreolus*), and 3 fallow deer (*Dama dama*) from several sites within Slovakia. Moreover, tissue samples of 57 alpine chamois (*Rupicapra r. rupicapra*) from the Slovak Paradise National Park (48°54′N, 20°20′E) were included in study. Altogether 286 samples of blood or tissue (spleen or ear) from rodents were analyzed. Rodents were trapped to live traps in the districts and surroundings of the cities Ružomberok (49°04′N, 19°18′E) ($n = 44$), Rozhanovce (48°45′N, 21°20′E) ($n = 93$), Šebastovce (48°39′N, 21°16′E) ($n = 58$) and at four sampling sites situated within the district of Lučenec city (48°19′N, 19°39′E) ($n = 91$). Trapped individuals belonged to five rodent species: *Apodemus agrarius* 77.27%, *Apodemus flavicollis* 5.24%, *Apodemus uralensis (previously Apodemus microps)* 1.74% *Myodes glareolus* (8.06%) and *Microtus arvalis* (7.69%). Blood samples were stored in plastic tubes containing anticoagulant (EDTA) at 4 °C. Similarly, all obtained tissue samples were preserved in 70% ethanol or frozen at −20 °C in plastic tubes until DNA extraction. Additionally, all samples from rodents were tested for the presence of *Ca.* Neoehrlichia mikurensis.

#### 2.1.4. Ruminants

A total of 178 blood specimens from cattle, sampled at three farms situated within the districts of the cities Prešov (49°00′N, 21°14′E) and Košice (48°43′N, 21°15′E) were screened for the presence of *A. phagocytophilum*. Moreover, 16 blood samples were obtained from a slaughter-house in eastern Slovakia. Except of *A. phagocytophilum*, all blood samples were tested for the presence of *Anaplasma marginale*, an agent responsible for bovine anaplasmosis.

Altogether 147 blood samples from four sheep flocks in central Slovakia, Očová (48°36′N, 19°17′E), Mýto pod Ďumbierom (48°51′N, 19°37′E), Liptovský Mikuláš (49°04′N, 19°36′E) and Veterná Poruba (49°06′N, 19°40′E) were delivered to our lab in plastic tubes with anticoagulant (EDTA). Additionaly, these samples were analysed for the presence of *A. ovis*, an agent responsible for pasture fever of small ruminants. Samples were stored at 4 °C until DNA isolation.