



Molecular detection of *Anaplasma phagocytophilum* in roe deer (*Capreolus capreolus*) in eastern Poland

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Abstract

Wild ungulates may serve as reservoirs for particular tick-borne pathogens (TBP) of importance for public and animal health. The aim of the study was to investigate, for the first time, the occurrence of *Anaplasma phagocytophilum* (AP) in a large population of roe deer in eastern Poland. Spleen samples from 424 roe deer (*Capreolus capreolus*) were collected during the 2018–2019 hunting season. Genetic screening for AP was carried out by PCR amplification of a fragment of the 16S rRNA and groEL genes. Twenty-six of the 424 spleen samples (6.13%) tested positive both for 16S rRNA and groEL. Phylogenetic analysis confirmed the existence of a specific roe deer ecotype of AP in eastern Poland. Despite the low prevalence of AP in roe deer populations in the study area, these animals may act as anaplasmosis reservoirs for ticks. Based on the zoonotic potential of granulocytic anaplasmosis, it seems advisable to introduce continuous monitoring of this infection among domestic, farm and wild animals.

Key words

Anaplasma phagocytophilum, roe deer, Vector-borne disease, Poland

INTRODUCTION

Tick-borne diseases (TBD) are of increasing importance in veterinary and human medicine [1]. The number of pathogens transmitted by ticks is superior to that of any other arthropod and, in Europe and other temperate zones, are considered the most important arthropod zoonotic vector [2–4].

TBD affect humans and animals, constituting a diversified group of diseases that involve a vast amount of pathogens. However, among these, infections caused by *Anaplasma* spp., *Babesia/Theileria* spp., and *Borrelia* spp. have a noticeable impact. The different species in the genus *Anaplasma* are a cause of disease in different animals [5], and infections with AP are an emerging human pathogen in Europe and the USA [6, 7]. *Anaplasma phagocytophilum* is also the most prevalent tick-transmitted animal pathogen [7], responsible for granulocytic anaplasmosis, a disease characterized by fever, weakness, anorexia, thrombocytopenia and occasional lameness in dogs and horses. Ticks and wildlife in general are the main reservoirs of these tick-borne pathogens of human and veterinary interest [8]. Wildlife may play a significant role in the transmission and maintenance of this disease, either acting as reservoirs or amplifying hosts for human or domestic animals [8]. Granulocytic anaplasmosis is transmitted to humans mainly through ticks, but studies by Bakken et al. [9] indicate that direct contact with the blood of animals infected with AP may also result in the development of the disease. The authors of the study described the occurrence of HGA in people working in the processing of deer meat.

The study by Jahfari et al. [10] showed that domestic animals such as horses, dogs and cats, as well as wild animals, such as red deer (*Cervus elaphus*), wild boars (*Sus scrofa*), red foxes (*Vulpes vulpes*) and hedgehogs (*Erinaceus* spp.), all harbour AP strains with zoonotic potential related to human strains, while roe deer (*Capreolus capreolus*), rodents and birds seem to carry genetically distant strains

Therefore it is important to characterize the role in the epidemiology of wildlife species in this disease in order to adequately evaluate the potential risks and to design suitable strategies for control.

There is little information about the presence of AP in wildlife in eastern Poland [11–16], thus the aim of this study was to determine the presence of *A. phagocytophilum* in roe deer in this part of the country.

MATERIALS AND METHOD

The study was conducted during the 2018–2019 hunting season. Spleen samples (n = 424) were taken from roe deer (*Capreolus capreolus*) in two provinces: Lublin and Subcarpathia (Tab. 1). The climate of the respective provinces

Table 1. Geographical distribution of provinces where spleen samples were collected

Provinces	Location	Altitude (above sea level)
Lublin	Situated mainly in the Vistula and Bug Rivers	Highest point – 311 m
Subcarpathia	Located in the south-eastern corner of the country, characterised by its diverse natural environment	Highest point – 1,346 m

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is mostly continental: average temperature – 7–8 °C, average annual precipitation – 500–1200 mm, and vegetation period – 180–220 days.

Upon request, necropsy was performed by veterinary practitioners or hunters, and the spleens were collected immediately and submitted for laboratory testing. Samples were stored at -20 °C until analysis.

DNA extractions from the spleen samples for molecular testing were performed using a commercial DNA Genomic kit (A&A Biotechnology Gdańsk, Poland), according to the manufacturer's instructions. All samples were screened for the presence of AP DNA with a polymerase chain reaction (PCR) targeting a 16S rRNA AP gene fragment with a size of 932 bp, using the primers: ge3a (CACATGCAAGTTCGAACGGATTATTC) and ge10r (TTCCGTTAAGAAGGATCTAATCTCC). An initial 5 min. denaturation at 95 °C was followed by 40 cycles, each consisting of a 30 s denaturation at 94 °C, a 30 s annealing at 55 °C, and a 1 min extension at 72 °C. A single 5 min. extension at 72 °C followed the last cycle. Nested amplifications used 1 µl of the primary PCR product as the template in a total volume of 50 µl. The primers ge9f (AACGGATTATTCTTTATAGCTTGCT) and ge2 (GGCAGTATTAAAAGCAGCTCCAGG) were used in the reaction. They enabled the amplification of the product with a size of 546 bp. Nested cycling conditions were as described for the primary amplification, except that 30 cycles were used [17].

PCR-16S rRNA positive samples were analysed further with primers targeting a fragment of the groEL gene of AP. EphplgroEL(569)F (ATGGTATGCAGTTTGATCGC), EphplgroEL(1193)R (TCTACTCTGTCTTTGCGTTC), and EphgroEL(1142)R (TTGAGTACAGCAACACCACCGGAA) primers were used in combination to generate a primary and heminested PCR for the selective amplification of 624 and 573 bp of the groEL gene of AP respectively. The PCR was followed by 40 cycles, each consisting of a 30 s denaturation at 94 °C, a 30 s annealing at 55 °C, and a 45 s extension at 72 °C [18].

As positive controls, the DNA of AP was obtained from the National Reference Centre for Borrelia of the Max von Pettenkofer Institute (Munich, Germany). PCR amplification was performed using a programmable thermocycler (Biometra, Göttingen, Germany). The size of each PCR product was evaluated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

Final identification was performed by sequencing of the PCR products. Purification was performed using QIAquick spin columns (Qiagen) and eluted in 50 µl of Tris10 mM, pH 7.6. DNA sequencing was performed on both strands using the same primers employed for PCR at the DNA Sequencing and Synthesis Service of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.

DNA sequences were assembled and edited using SeqMan (DNASTar, Lasergene, Madison, WI, USA) and MegAlign (DNASTar, Lasergene, Madison, WI, USA), with alignments to the published sequences of the 16S rRNA gene of: *A. phagocytophilum* GU183908 (from horses in Poland); *A. centrale*, EF520690 (from cattle in Italy); *A. marginale* AF414876 (origin not known – from Israel); *A. ovis* AY837736 (from mosquitos in Sweden); *A. platys* EU439943 (from dogs in Italy), and with alignments to the published sequences of groEL gene (Tab. 2). Phylogenetic analysis (based on the neighbour-joining algorithm method) of the obtained sequences was performed using MegAlign software.

Table 2. Sequences of AP groEL gene used in the study

Host	Origin	Accession No.
horse	USA	AF172158
horse	USA	AF172159
sheep	NO	AF548386
sheep	GB	U96730
human	USA	U96728
human	USA	AF172159
human	SL	AF033101
Dog	SL	EU381150
<i>I. ricinus</i>	SL	EU246960
<i>I. ricinus</i>	IT	KF031386
<i>I. ricinus</i>	SK	KF383237
<i>I. ricinus</i>	AT	AY220467
<i>I. ricinus</i>	IT	KF031388
<i>I. ricinus</i>	SK	KF383240
<i>I. ricinus</i>	DE	AY281827
Roe deer	PL	DQ779568
Roe deer	PL	JN005747
Roe deer	AT	AY220470
rodent	IT	KF031385
rodent	IT	KF03139-
vole	RUS	KC583431
Eurasian shrew	RUS	HQ630617

AT – Austria; DE – Germany; GB – Great Britain; IT – Italy; NO – Norway; PL – Poland; RUS – Russia; SK – Slovakia; SL – Slovenia.

RESULTS AND DISCUSSION

Twenty-six of the 424 spleen samples tested positive in PCR, with an overall PCR prevalence of 6.13%. The partial 16S rRNA gene was sequenced for all 26 positive PCR products and confirmed as *A. phagocytophilum*. They showed a 99.6–100% identity with the AP GU183908 sequence obtained from horses in Poland during a previous study [19].

All the DNA samples positive for the 16S rRNA gene also proved positive for the groEL gene. All the groEL sequences generated here were 100% identical to the groEL sequences of AP from roe deer in Poland (GenBank: DQ779568) and 100% to groEL sequences of AP extracted from *I. ricinus* in Slovenia (GenBank: EU246960) (Fig. 1). The groEL sequences obtained in the author's study showed the lowest similarity (94.7%) with the groEL sequences of AP from rodents (GeneBank KF031385 and KF031390), whereas their similarity with the groEL sequences of AP from humans (GeneBank AF142159, U96728, and AF033101) was 97.9–98.4%.

The phylogenetic tree for partial groEL genes, including previously published reference samples (Tab. 2), consisted of three main clades. The first clade included ecotypes detected in *I. ricinus* ticks and various vertebrate hosts, such as sheep, horses, dogs and humans. The second clade included only ecotypes detected in roe deer in Poland and Austria, as well as in *I. ricinus* ticks. The third clade included only ecotypes detected in rodents.

The AP sequences obtained in the study were evaluated as meeting the second clade, which is phylogenetically distant from clades 1 and 3. Wildlife is seen to have increasing importance in the maintenance and transmission of TBD.

The current study has proved the presence of AP in roe deer in eastern Poland. The presence of these pathogens has also been reported in the blood of wild animals in Europe [13–15, 20–22].

The main reservoir of AP in nature are forest rodents [23] and members of the deer family [24]. Research conducted by Bown et al. [25] in the United Kingdom showed that the period of bacteremia in rodents was short and lasted several weeks, and that the highest frequency of infections with AP was recorded in late summer and autumn. However, infection with AP in deer is long-term and subclinical, and any species of this family may be a reservoir for the bacteriae [7]. The presence of AP has been confirmed in the following: red deer, elk, roe deer and fallow deer [13, 26, 27]. Long-term bacteremia makes the deer a competent reservoir of *Rickettsia* and contributes to the circulation of the pathogen in nature. In wild ruminants the prevalence of AP has been found to vary between regions and countries, with values ranging from 10 – 38% in roe deer, 10 – 51% in red deer, [13, 15, 16, 28, 29] and as high as 98.9% in roe deer infected in Germany [30].

In the current study, the presence of DNA was found only in the spleen of slightly more than 6% of the studied animals. This prevalence seems to be low in comparison to the prevalence of infections in wild cervids from other parts of Europe, as well as from the north-eastern part of Poland where 38.7% of roe deer were infected [12]. In other animal species in eastern Poland, the prevalence of AP also seems to be low, and according to studies by Dzięgiel et al. [15, 31] and Adaszek et al. [13], the DNA of this bacteria was found only in 2.75% of dogs, 14% of fallow deer and 18% of wild bison. The explanation for such variability could depend on the distribution of ticks, and hence with ecoclimatic variation.

Another explanation for the low prevalence of *A. phagocytophilum* in roe deer could be that it was the spleen rather than the whole blood that constituted the material for the study. The choice of spleen as diagnostic material was made on account of the observations by Bown et al. [25], who asserted that the presence of these microbes was more often observed in spleen samples than in the blood. This is due to the late phase of the infection, where the AP passes from the blood vessels to the spleen [15, 25]. However, in research by Kazmirova et al. [32] and Adamska and Skotarczak [26], the DNA of AP in roe deer was detected mainly in the blood, and only occasionally in the spleen. This may indicate that spleen samples are not good material for the isolation of *Anaplasma* DNA

It has also been indicated that wild animals are able to control the infection mainly through the activation of innate immune responses, phagocytosis and autophagy, leading to infection levels below PCR detection or infection clearance, which could also have an impact on the low prevalence of *Anaplasma* in the roe deer in the current study [1, 33, 34]. This study, based for the first time on a large population of roe deer in eastern Poland, shows the presence of AP in these animals. Other *Anaplasma* species such as *A. platys*, *A. centrale*, *A. marginale* has not been detected in roe deer. These species are transmitted by ticks, such as *Rhipicephalus sanguineus* and *Dermacentor auratus* (*A. platys*); *R. annulatus*, *R. decoloratus*, *R. microplus*, *D. andersoni*, *D. variabilis* and *D. occidentalis* (*A. marginale* and *A. centrale*), which do not occur in Poland and might explain the lack of DNA of these bacteriae in the spleen samples of the studied animals [35].

All AP isolates obtained in this study were qualified to a single ecotype, differing from the AP ecotypes found in humans, farm animals and domestic animals, as well as from those isolated from rodents, which may indicate that the roe deer in eastern Poland do not constitute a reservoir host for the human pathogenic strains of AP [36].

Information on the prevalence and geographical distribution of AP infections is essential for the effective planning of control measures. These results suggest that game animals may act as reservoirs for *Anaplasma*, although the zoonotic potential of the AP strains occurring in roe deer is low. Along with the changing climatic conditions and the increasing risk of anaplasmosis in native animals, it seems advisable to introduce continuous monitoring of this infection among domestic, farm and wild animals.

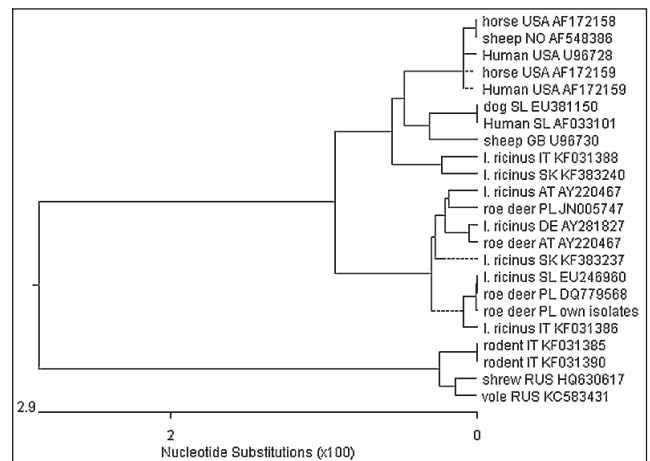


Figure 1. Phylogenetic relationships between AP groEL sequences obtained in the study and deposits in GenBank

Conflicts of Interest

The authors declare that they have no competing interests. The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

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