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Detection and prevalence of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks in eastern Poland

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Abstract

Introduction and Objective. *Anaplasma phagocytophilum* are tick-borne bacteria affecting both human and animal health. The aim of the study was to examine the prevalence of *A. phagocytophilum* in questing *I. ricinus* ticks collected in Lublin Province, eastern Poland.

Materials and Method. Ticks were collected by the flagging method. Total DNA from ticks was extracted by boiling in ammonium hydroxide. Detection of *A. phagocytophilum* was performed by amplifying a fragment of the 16S rDNA gene.

Results. Overall, 626 *I. ricinus* ticks were tested for the presence of A. phagocytophilum DNA. The prevalence of the pathogenic bacteria was 1.28%. The occurrence of *A. phagocytophilum* among adults was 1.8%, whereas none of the collected *I. ricinus* nymphs were infected.

Conclusions. The study revealed the presence of *A. phagocytophilum* in *I. ricinus* in eastern Poland, which constitutes a potential health risk for residents, tourists, forestry, and agricultural workers.

Key words

Poland, *Anaplasma phagocytophilum*, ticks, *Ixodes ricinus*

INTRODUCTION AND OBJECTIVE

Ixodes ricinus is one of the most common tick species in Europe. The species is a vector and reservoir of viruses (tick-borne encephalitis virus), bacteria (*Borrelia burgdorferi* sensu lato), and protozoa (*Babesia*), some of which are pathogenic to humans and animals [1]. From a veterinary and public health perspective, *Anaplasma phagocytophilum* is among the most important tick-borne microorganisms vectored by *I. ricinus*. *A. phagocytophilum* is a Gram-negative, obligate intracellular bacterium of the family *Anaplasmataceae*, capable of infecting many mammalian hosts. The reservoir of the bacteria is primarily large game (including deer, roe deer, moose) and rodents (mice, shrews, voles). It is the etiological agent of human granulocytic anaplasmosis (HGA), tick-borne fever (TBF) in ruminants, equine granulocytic anaplasmosis (EGA) in horses and canine granulocytic anaplasmosis (CGA) [2]. The attachment time of infected ticks required for effective transmission of *A. phagocytophilum* bacteria ranges from 24 – 48 hours; incubation period: 5 – 14 days after the tick bite. The disease usually has a mild, self-limiting course, but in some cases, it may be severe and even fatal [3, 4].

The aim of the study was to examine the prevalence of *A. phagocytophilum* in questing *I. ricinus* ticks collected in Lublin Province, eastern Poland.

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MATERIALS AND METHOD

Between 2011 – 2012, ticks were collected from vegetation by the flagging method in two localities in Lublin province: Dąbrowa and Wilków, both study sites located in natural rural areas. Ticks were collected from the edges of mixed forests, bordered by grassy and shrubby meadows, with access roads. Species and developmental stages of ticks were identified using a taxonomic key [5]. Total DNA from ticks was extracted according to Rijpkema et al. [6]. All ticks from Dąbrowa and adults from Wilków were tested separately, while nymphs from Wilków were pooled into groups containing five individuals. Ticks were placed in disposable bags, crushed, and boiled for 20 min in 0.7M ammonium hydroxide: 100 µl for adults and nymphs tested in pools, and 50 µl for nymphs tested individually. The tubes were then left open at 100 °C for 10 min to allow ammonia to evaporate [6]. The extracts were stored at -20 °C until use. Detection of bacteria was performed in all samples using a nested PCR protocol with two sets of primers: ge3a/ge10 and ge9/ge2, specific for *A. phagocytophilum* 16S rDNA fragment amplification according to Massung et al. [7], with the modification described by Zając et al. [8]. Both reactions were performed in a volume of 25 µl, using the following reaction components: 0.25 µl (1.25 U) Taq Polymerase (Qiagen, Germany), 2.5 µl 10xPCR buffer containing 15 mM $MgCl_2$ (Qiagen, Germany), 2.5 µl 2 mM dNTP (final concentration 0.2 mM) (Thermo Scientific, USA), 1.25 µl of 10 µM ge3a/ge10 primers in the first reaction, or 0.5 µl 10 µM ge9/ge2 for nested-PCR (Eurogentec, Belgium), 2.5 µl of isolated DNA in the first reaction or 1 µl of amplification product from the first reaction for the second round of amplification, and nuclease-free water (Ambion, USA). The

amplification programme included an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

For the second round of amplification, all cycling conditions were the same, with the exception of 30 cycles. The PCR products were separated electrophoretically in 2% agarose gels, stained in ethidium bromide (2 µg/ml), and visualised and photographed under UV light. The size of the amplified DNA fragment was 546 bp. The positive control was DNA isolated from HGA-1 strain-infected HL60 cells fixed on diagnostic slides (Focus Diagnostic, USA). Ncelase-free water was used as a negative control. Differences in *A. phagocytophilum* prevalence between developmental stages and study sites were analysed using the Fisher Exact Test with online calculator [9]. A P-value of <0.05 was considered statistically significant.

RESULTS

A total of 626 *I. ricinus* ticks were tested for the presence of *A. phagocytophilum* DNA by nested PCR. The overall prevalence of pathogenic bacteria was 1.28% (Tab. 1). A higher percentage of infected ticks was noted among ticks collected from the Dąbrowa site (1.5% vs. 0.88%), although there was no significant difference in the infection rate between Dąbrowa and Wilków (P= 0.7173). The prevalence of *A. phagocytophilum* was 1.8% in adults, although none of the collected *I. ricinus* nymphs were infected. No statistical significance was observed in the frequency of infection depending on the developmental stage (P=0.1128).

DISCUSSION

Ixodes ricinus ticks serve as the main vector of *A. phagocytophilum* in Europe, where the revalence of the pathogen ranges between <1% – ~20% (reviewed in Stuen et al. 2013) [10]. Studies performed in Poland have confirmed that *A. phagocytophilum* infections occur throughout the country in both *I. ricinus* and *Dermacentor reticulatus* ticks [8, 11]. The occurrence of the bacteria in Poland varies significantly depending on the region, varying from 1.01% in the north-western part (Wolinski National Park) to even 76.7% in the southern part (Niepołomice Forest) [12, 13]. The result obtained in this study from the eastern part of the country is similar to the lower limit of this range. Previous research by Wójcik-Fatla et al. [14] in Lublin province demonstrated a higher percentage of *I. ricinus* ticks infected with *A. phagocytophilum* – 4.94%. Cisak et al. [15] found the pathogen in 2.7% of *I. ricinus* ticks (ranging from 1.1–3.7%) collected from various worksites of forestry workers in the Lublin province. The presence of ticks harbouring the pathogen poses an occupational hazard, especially for forestry workers. Sero-epidemiology studies among individuals with occupational exposure to tick bites reported a high seroprevalence of antibodies against *A. phagocytophilum* in forestry workers (~20%) [16, 17], whereas among the healthy population (blood donors) – 3% and 1.5% for IgM and IgG antibodies, respectively [18].

The presented results showed a higher percentage of infected female (2.64%) *I. ricinus* ticks than males (0.92%). Moreover, no *A. phagocytophilum* were detected in any of the investigated nymphal specimens. Various studies reported that adults are infected more often than nymphs [19, 20, 21, 22]. The lack of infection of nymphs may be due to the limited number of small mammals and/or the low *A. phagocytophilum* infection rate in rodents in the study areas. Consequently, the absence or very low infection rate of nymphs in the environment leads to a relatively low infection rate among adults, which results from the transstadial transmission of the pathogen in the tick population.

The limitation of the study is the lack of sequencing of PCR-positive samples. Sequence analysis of genetic markers would have allowed identification of the strain/genetic variant of the detected pathogen in the tested ticks. *A. phagocytophilum* strains probably differ in their host preference and pathogenicity [19, 21]. Further research on the occurrence of the bacterium is necessary, along with genetic analysis of the detected strains.

CONCLUSION

Ixodes ricinus ticks are common throughout Poland, with one of their highest density areas being recorded in the Lublin Province in eastern Poland [23]. *Ixodes ricinus* ticks are a vector and reservoir of many pathogens that threaten humans and animals. The present study revealed the presence of *A. phagocytophilum* in *I. ricinus* ticks in eastern Poland. Although the results show a low infection rate of *A. phagocytophilum* in questing *I. ricinus* ticks, the presence of ticks infected with the pathogen constitutes a potential health risk for residents, tourists, forestry, and agricultural workers.

Table 1. Prevalence of *Anaplasma phagocytophilum* in questing *Ixodes ricinus* ticks

Sampling area[b]	Adults[a]	Females	Males	Nymphs[a]	Total
	No. of ticks infected/tested (%)				
Dąbrowa	6/264 (2.27)	5/140 (3.57)	1/124 (0.81)	0/135 (0)	6/399 (1.5)
Wilków	2/180 (1.11)	1/87 (1.15)	1/93 (1.08)	0/47 (0)	2/227 (0.88)
Total	8/444 (1.8)	6/227 (2.64)	2/217 (0.92)	0/182 (0)	8/626 (1.28)

Statistically significant at P < 0.05 level; [a] no statistical significance found between developmental stages (P=0.1128); [b] no statistical significance found between sampling areas (P= 0.7173)

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