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ORIGINAL PAPER

The Prevalence of *Anaplasma phagocytophilum* in Questing *Ixodes ricinus* Ticks in SW Poland

DOROTA KIEWRA[1]*, GRZEGORZ ZALEŚNY[2] and ALEKSANDRA CZUŁOWSKA[1]

[1] Department of Microbial Ecology and Environmental Protection, Institute of Genetics and Microbiology, University of Wroclaw
[2] Department of Invertebrate Systematics and Ecology, Institute of Biology, Wroclaw University of Environmental and Life Sciences

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Abstract

Ticks constitute important vectors of human and animal pathogens. Besides the Lyme borreliosis and tick-borne encephalitis, other pathogens such as *Babesia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum*, are of increasing public health interest. In Poland, as in other European countries, *Ixodes ricinus*, the most prevalent tick species responsible for the majority of tick bites in humans, is the main vector of *A. phagocytophilum.* The aim of the study was to estimate the infection level of *I. ricinus* with *A. phagocytophilum* in selected districts, not previously surveyed for the presence of this agent. Sampling of questing ticks was performed in 12 forested sites, located in four districts (Legnica, Milicz, Lubań, and Oława) in SW Poland. Altogether, 792 ticks (151 females, 101 males, and 540 nymphs) representing *I. ricinus* were checked for the presence of *A. phagocytophilum.* The average infection level was 4.3%, with higher rate reported for adult ticks. The highest percentage of infected adults was observed in Milicz (17.4%) and the lowest in Oława (6.8%). The abundance of questing *I. ricinus* in all examined sites as well as the infection with *A. phagocytophilum* indicate for the first time the risk for HGA transmission in SW Poland.

Key words: *Anaplasma phagocytophilum, Ixodes ricinus*, Poland

Introduction

The geographical range of tick-borne diseases (TBD), identification of new pathogens, as well as the increase of TBD incidence makes TBD a serious public health problem. Besides Lyme borreliosis and tick-borne encephalitis, which are monitored in Poland, other TBD caused by *Babesia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum*, are of increasing public health interest.

Anaplasma phagocytophilum, a small gram-negative obligate intracellular alfaproteobacteria, is a tick-borne rickettsial bacterium that replicates in mammalian granulocytes but also in the salivary gland and midgut cells of ticks (Rikihisa, 2011). It causes human granulocytic anaplasmosis (HGA), previously known as human granulocytic ehrlichiosis (HGE), but also the disease in horses, dogs, sheep, and cats (Rar and Golovljova, 2011; Severo *et al*., 2012). HGA is an emerging disease with varying symptoms, from asymptomatic seroconversion to non-specific symptoms like fever, malaise, myalgia and headache, up to fatal disease (Dumler *et al*., 2005; Zwoliński *et al*., 2007; Rar and Golovljova 2011). The majority of confirmed HGA cases, including the first case of HGA recorded in the early 1990s (Rikihisa, 2011), have been reported from the USA. In Europe, the prevalence of HGA infection is significantly lower, although human cases have been accounted for the majority of European countries, including Poland (Tylewska-Wierzbanowska *et al*., 2001; Grzeszczuk *et al*., 2009; Rikihisa, 2011).

Anaplasma phagocytophilum DNA has been detected in several species of hard ticks (*Ixodes scapularis, I. pacificus, I. spinipalpis, I. ricinus, I. persulcatus, I. trianguliceps, I. ventalloi, I. ovatus, Dermacentor silvarum*) in the United States, Europe, and Asia (Rar and Golovljova 2011; Rikihisa, 2011). In Poland, but also all over Europe, *I. ricinus*, the most prevalent tick species responsible for the majority of tick bites in humans, is the main vector of *A. phagocytophilum. I. ricinus* has a three-host live cycle: larvae and nymphs feed mostly on small to medium-sized animals, and adults prefer large-sized mammals. Ticks (larvae, nymphs, or adults) acquire *A. phagocytophilum* from infected hosts

* Corresponding author: D. Kiewra, Department of Microbial Ecology and Environmental Protection, Institute of Genetics and Microbiology, University of Wroclaw; **e-mail: dorota.kiewra@microb.uni.wroc.pl**

through a blood meal, and they can maintain the bacterium through subsequent moultings (transstadial passage), but not by transovarial transmission (Rikihisa, 2011). In Europe, a wide range of mammalian species is infected with *A. phagocytophilum* (Rar and Golovljova, 2011). However, it is worth noting that the zoonosis potential and host infectivity of *A. phagocytophilum* depend on genetic variations of strain, due to the host tropism of this bacterium (Severo *et al.*, 2012). Thus, humans are susceptible to limited strains only. The wild host reservoir for strains that infect humans (Ap-ha) is poorly known (Massung *et al.*, 2002; Rikihisa, 2011; Michalik *et al.*, 2012).

The aim of the study was to estimate the infection level of *I. ricinus* with *A. phagocytophilum* in selected districts of Lower Silesia, SW Poland, not hitherto surveyed for the presence of this agent.

Experimental

Materials and Methods

Tick collection. Sampling of questing ticks was performed in 12 forested sites, located in four districts in SW Poland: Lubań (sites: L1, L2, L3), Milicz (M1, M2, M3), Legnica (Leg1, Leg2, Leg3) and Oława (O1, O2, O3). Ticks were collected by flagging method during spring peak of tick activity, from April to June 2011, at least once a month in each site. Collected ticks were placed in plastic tubes and kept in the refrigerator. The identification of species and life instar was carried out in stereomicroscope, based on the key provided by Siuda (1993).

Detection of *A. phagocytophilum* in *I. ricinus* ticks. DNA for PCR was extracted from *I. ricinus* by lysis in ammonium hydroxide (Rijkpema, 1996). The isolates from nymphs and adults were used for the detection of *A. phagocytophilum*. Due to the predominance of nymphs in the population, the PCR reactions for nymphs were carried out in pools (5 specimens per pool), which increased the representation of this particular instar and reduced the costs of analysis. Thus, for the calculation of the level of infection in nymphs minimal infection rate (MIR) was used (calculated on the assumption that in each positive pool of tested nymphs only one was infected). The procedure was carried out for 30 samples from each of the 12 monitored sites. To estimate the presence of *A. phagocytophilum* the diagnostic kit (PK24N DNA Gdańsk) was used. The detection was based on amplification of 16S rDNA by nested PCR reaction. The reaction was performed in a reaction volume of 50 µl containing 42 µl Master Mix PCR-OUT for first and PCR-IN for second amplification, 5 µl dNTPs mixture, 1 µl DNA polymerase *TaqNova*, and 2 µl of the processed tick sample or 2 µl PCR product for nested-PCR. The positive control contained *Anaplasma* DNA from a diagnostic kit, whereas the negative control contained sterile water. The amplification was performed in a BioRad T100™ Thermal Cycler. The amplification included initial denaturation for 2 min at 95°C, subjected to 40 cycles (for a second reaction 30 cycles) of 30 s denaturation at 94°C, 30 s for the annealing reaction at 55°C, 60 s extension at 72°C and final extension for 5 min at 72°C. The separation of the nested PCR products was carried out on a 1.5% agarose gel with the addition of ethidium bromide in the TBE buffer. Product of 546 bp was regarded as a positive result.

Statistical analysis. The results were analysed using STATISTICA v.10 software. The χ^2 test, Kruskal-Wallis ANOVA test and Mann-Witheney test were applied. Probability at $p < 0.05$ was regarded as significant.

Results

In total, 2507 host-seeking ticks (148 larvae, 1314 nymphs, 478 females, and 567 males) identified as *Ixodes ricinus* were collected in four districts. Ticks were present in each of 12 sites. However, the tick density varied between districts ($H = 235.2$; $p < 0.01$) with the highest prevalence in Lubań (Table I). Altogether, 792 *I. ricinus* ticks (151 females, 101 males, and 540 nymphs) were tested for the presence of *A. phagocytophilum*. Infected ticks were found in all districts. The average infection rate was 4.3%. The infection rate in adults was higher than in nymphs ($\chi^2 = 37.093$; $p < 0.001$). The minimum infection rate was 1.3% in nymphs and 10.7% in adults (Table II). The highest level of nymph infection was recorded in Legnica district (3.1%), while no infected nymphs were found in Lubań district. The highest rate of infected adults was found in Milicz (17.4%) and the lowest one in Oława (6.8%). However, the differences beetwen districts were not statistically significant, both for nymphs and adults ($\chi^2 = 4{,}321$; $p = 0{,}229$, $\chi^2 = 3.547$; $p = 0.314$, respectively). A higher infection rate was observed in females in comparison to males (15.2% and 3.9% respectively,

Table I
The average density of *I. ricinus* in four districts in south-western Poland from April to June 2011.

District	The average number of *I. ricinus* ticks collected by one person in 30 min			
	n	♀	♂	Total
Lubań	26.5	4.8	4.4	35.7
Milicz	10.2	4.8	7.2	22.2
Legnica	3.4	4.9	5.0	13.3
Oława	4.0	4.1	3.7	11.8

Table II
Anaplasma phagocytophilum infected *I. ricinus* ticks in four districts in south-western Poland in 2011.

District	*A. phagocytophilum* infected *I. ricinus* ticks							
	nymphs*		females		males		females & males	
	number of tested	positive (%)	number of tested	positive (%)	number of tested	positive (%)	number of tested	positive (%)
Lubań	170	0	37	10.8	19	5.2	56	8.9
Milicz	220	1.8	23	26.1	23	8.7	46	17.4
Legnica	65	3.1	47	17.0	30	3.3	77	11.7
Oława	85	1.2	44	11.4	29	0	73	6.8
Total	**540**	**1.3**	**151**	**15.2**	**101**	**3.9**	**252**	**10.7**

* nymphs were tested in pools consisting of five specimens

$\chi^2 = 8.037$; $p = 0.004$). The highest level of infection in females was found in Milicz and in Legnica districts (26.1% and 17.0% respevtively), and the lowest one (10.8%) – in Lubań district. The infection level in males varied between 0 (Oława district) and 8.7% (Milicz district). There were no statistically significant differences between the density of adult ticks in districts, and the level of their infection ($Z = -0.282$; $p = 0.778$)

Discussion

The occurrence of questing *Ixodes ricinus* ticks is a major factor of tick-borne infection in humans. Previous studies on ticks as vectors of pathogens, carried out in Lower Silesia, were focused mainly on the presence of spirochetes of *Borrelia burgdorferi* s.l., regarded as the etiological agent of Lyme borreliosis (Kiewra, 2005; Kiewra *et al.*, 2002, 2006). Lyme borreliosis is still considered the most common tick-borne disease in the Northern Hemisphere (Stanek *et al.*, 2012). However *I. ricinus* can harbour also other pathogens (including bacteria, viruses and protozoa) (Franke *et al.*, 2013). Human granulocytic anaplasmosis (HGA) has been recently recognized as a tick-borne emerging disease, which is spread over USA, Europe and Asia. In Europe, the first confirmed case of HGA was reported in 1997, in Slovenia (Petrovec *et al.*, 1997), whereas the first cases of acute human granulocytic ehrlichiosis were described in 2001 in Poland (Tylewska-Wierzbanowska *et al.*, 2001). It is worth noting, that *A. phagocytophilum* infection can be asymptomatic, and many infections can be unrecognized (Dumbler *et al.*, 2005).

In Poland, questing *I. ricinus* ticks infected with *A. phagocytophilum* were found in north, north-eastern, eastern and central-eastern parts of the country (Stańczak *et al.*, 2004; Grzeszczuk *et al.*, 2002, 2004; Tomasiewicz *et al.*, 2004; Wójcik-Fatla *et al.*, 2009; Sytykiewicz *et al.,* 2012). During present studies, the observed abundance of questing ticks in all examined localities in Lower Silesia, and also the infection of *I. ricinus* with *A. phagocytophilum* in Legnica, Milicz, Lubań, and Oława districts, has revealed for the first time the risk for HGA transmission in SW Poland. The prevalence of infected *I. ricinus* with *A. phagocytophilum* was 4.3%. Similar level of infections, varying between 2% and 6%, depending on the area, were recorded in Lower Saxony, Germany (Templin *et al.*, 2013), whereas the one in urban areas of Kosice, Slovakia reached 2.2% (Vichova *et al.,* 2013), and 2.6% in Belarus (Reye *et al.* 2013). As many as 8.7% questing *I. ricinus* were found to be infected with *A. phagocytophilum* in north-eastern Poland (Grzeszczuk *et al.*, 2004), 8.5% in central-eastern Poland (Sytykiewicz *et al.*, 2012), 13.1% in mid-eastern Poland (Tomasiewicz *et al.*, 2004), 14% in northern Poland (Stańczak *et al.,* 2004). During the present studies no significant differences in infection rates between the districts were observed. However, the infection prevalence can depend on a study area and it may also vary depending on the year of study (Grzeszczuk and Stańczak, 2006a). *A. phagocytophilum* has been found not only in questing ticks but also in ticks removed from human skin. For example, 23.7% *I. ricinus* ticks removed from patients in north-eastern Poland were infected (Grzeszczuk and Stańczak, 2006b). A high prevalence of *A. phagocytophilum* was also detected in engorged ticks from potential reservoir hosts such as roe deer (Overzier *et al.*, 2013). The studies from the territory of Poland have confirmed that roe deer are essential hosts for maintaining *Ixodes* ticks and tick-borne diseases, including *A. phagocytophilum* (Welc-Falęciak *et al.*, 2013).

In our study, the percentage of infected females (15.2%) was almost 4 times higher than in the males (3.9%). Females were found to be more infected than males also in other parts of Poland (Tomasiewicz *et al.*, 2004; Grzeszczuk, 2006; Grzeszczuk *et al.*, 2002, 2004; Stańczak *et al.*, 2004; Sytykiewicz *et al.*, 2012). A significantly lower positivity rate (1.3%) was recorded in nymphs compared to adults. Also other authors

(Tomasiewicz *et al.*, 2004; Grzeszczuk, 2006; Grzeszczuk *et al.*, 2002, 2004; Stańczak *et al.*, 2004; Sytykiewicz *et al.*, 2012) have shown that the nymphs are less infected than adult ticks. The observed tendency may point to the limited role of small mammals in maintaining *A. phagocytophilum* in examined districts, compared to large-sized mammals. In Europe, *A. phagocytophilum* strains associated with rodents are transmitted by *I. trianguliceps*, and not by *I. ricinus* ticks (Pangracova *et al.*, 2013). Thus, the occurrence of *I. ricinus* is not sufficient to rodent infection with *A. phagocytophilum*. However, *I. ricinus* can acquire *A. phagocytophilum* from large-sized mammals like cervids or wild boars, which can play a role as reservoir hosts (Michalik *et al.*, 2009, 2012).

The obtained results confirm that *I. ricinus* ticks infected with *A. phagocytophilum* are widespread in Poland and capable of maintaining the *A. phagocytophilum* circulation in nature. Thus, in diagnosing the tick-borne disease in SW Poland, the possibility of infection with *A. phagocytophilum* should be also taken into account.

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