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Prevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in *Ixodes ricinus* collected from dogs in eastern Poland

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Abstract

Introduction: *Ixodes ricinus* ticks are an important vector and reservoir of pathogenic microorganisms causing dangerous infectious diseases in humans and animals. The presence of ticks in urban greenery is a particularly important public health concern due to the potential for humans and companion animals to be exposed to tick-borne diseases there. The study assessed the prevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* infection in *I. ricinus* ticks feeding on dogs. **Material and Methods:** The study consisted in analyses of *I. ricinus* ticks collected in 2018–2020 from owned and stray dogs in the north-eastern part of Lubelskie province (eastern Poland). An AmpliSens PCR kit was used for qualitative detection and differentiation of tick-borne infections. **Results:** Infections of *B. burgdorferi* and *A. phagocytophilum* were detected in 10.9% and 12.9% of the examined ticks, respectively. One tick (0.7%) was co-infected by both pathogens. Infection with *B. burgdorferi* was significantly more highly prevalent in ticks collected from the owned dogs than from the strays (18.7% and 2.8%, respectively), whereas the prevalence of *A. phagocytophilum* was similar in both groups (12.0% and 13.9%, respectively). **Conclusion:** The co-infection observed in the study suggests the possibility of simultaneous infection by both pathogens from a single tick bite. The presence of pathogens in ticks collected from dogs is a factor in assessing infection risk not only to companion animals but also to their owners, who are in close contact with their dogs and visit the same green areas recreationally.

Keywords: ticks, tick-borne infections, canine borreliosis, anaplasmosis, eastern Poland.

Introduction

Ixodes ricinus is the most widespread tick species in Europe. Its geographic range has expanded considerably in recent decades. The species can now be found in areas and habitats located further north and at higher altitudes than several decades ago (5, 10, 12). These ticks prefer habitats with deciduous and mixed (or less often coniferous) forests with a thick litter layer. As a result of changes in land use and wildlife management, ticks can also be found in urban and suburban areas in many European countries (9, 19, 24, 26, 36). In urban habitats, small, medium-sized and larger mammals (roe deer and wild boar), birds, and companion animals (dogs and cats) contribute to the maintenance of tick populations and are reservoirs of tick-borne pathogens. The presence of ticks in urban greenery is of particular concern in respect of public health, due to the potential exposure of visiting humans and companion animals to tick-borne diseases (26) and the commonality of major tick-borne infectious diseases to humans, dogs and cats. *Ixodes ricinus* is one of the most important vectors and reservoirs of the pathogens which cause dangerous infectious diseases in humans and animals (11). Borreliosis, anaplasmosis, ehrlichiosis and rickettsiosis are the most important bacterial diseases transmitted by *I. ricinus* (30), the first of these being the most common vector-borne disease in the northern hemisphere (16). Spirochetes from the complex *Borrelia burgdorferi* sensu lato (s.l.) transmitted by ticks are the aetiological agents of the disease. Although most dogs exposed to *Borrelia* infections remain clinically asymptomatic (4, 18), cases of clinical canine borreliosis have been reported in almost all European countries, Poland being no exception (17). Canine borreliosis most often has an arthritic form, manifesting as inflammation in the extremities, usually of the carpal or tarsal joints. These symptoms are accompanied by malaise (fever, lack of appetite and fatigue) and lameness developing after a few days. Myocarditis is rarely diagnosed in canine borreliosis; however, the renal form of the disease and neurological dysfunctions may appear in older dogs (29). *Anaplasma phagocytophilum* is the aetiological agent of human granulocytic anaplasmosis (1). Granulocytic anaplasmosis has been diagnosed in various species of wild and domestic animals, including dogs (34). The clinical spectrum of the disease ranges from subclinical and self-limiting to subacute, chronic or severe disease in immunocompromised patients. The clinical signs of the disease vary in severity but are usually non-specific, *e.g.* fever, lethargy and anorexia (2), the first two of these being the most common clinical signs in infected dogs, and ones appearing after an incubation period of 1–2 weeks (3). Most dogs naturally infected by *A. phagocytophilum* will probably remain healthy (3). The concomitant occurrence of anti-*A. phagocytophilum* and anti-*B. burgdorferi* antibodies was observed in two healthy dogs (2/100) from the Lubelskie province and two healthy dogs (2/100) from the Mazowieckie province (Poland) (6). Since *A. phagocytophilum* is transmitted by the same *Ixodes* species as *B. burgdorferi* and is maintained in sylvatic cycles with the same rodent reservoirs, co-infections by these pathogens are possible, which may result in mutual enhancement of the pathogenicity of these microorganisms (21).

The study conducted by Dzięgiel *et al.* (6) in 400 healthy dogs showed that tick control was important as a protective factor against *A. phagocytophilum* and *B. burgdorferi*, while the breed (pure) was a risk factor for *B. burgdorferi* infection. In Europe, the prevalence of *B. burgdorferi* spirochetes in *I. ricinus* ticks varies considerably. A meta-analysis of European studies from 1984–2003 revealed a 13.7% prevalence of *B. burgdorferi* s.l. (25). A later pan-European meta-analysis based on data published in 2010–2016 showed the presence of *B. burgdorferi* s.l. in 14,134 (12.3%) of the 115,028 examined ticks (33). In terms of European regions, the highest *B. burgdorferi* prevalence was found in Central Europe (19.3%). In this region, the data for the meta-analysis were provided by Austria, the Czech Republic, Germany, Hungary, Slovakia, Switzerland and Poland (33). While the meta-analyses did not reveal an upward trend in the prevalence of *B. burgdorferi* infection in *I. ricinus* ticks (25, 33), this does not exclude possible changes in the prevalence in individual regions. A study of *I. ricinus* ticks collected from vegetation in Lublin province (eastern Poland), the north-eastern part of which was the research area in the present study, reported a significant increase in *B. burgdorferi* s.l. infection rates. Two periods separated by a five-year interval were analysed in that study. The incidence of *B. burgdorferi* s.l. infection was 6.0% in 2008–2009 and 15.3% in 2013–2014 (37). The increase in the prevalence observed in the Lublin region most likely has a focal character. As emphasised by the authors, the prevalence of tick infections recorded at the end of the study is similar to the European mean value and is not alarming, but the high rate of the increase in this parameter may indicate a potential risk. One of the possible causes of the rise in the infection rate suggested by the authors is the growing recreational activity in the analysed area and the presence there of greater numbers of humans and companion animals, *i.e.* potential tick hosts (37). Given the great human and veterinary medical importance of the *I. ricinus* tick problem, the ever-higher abundance of these ticks in urban and suburban areas, and the close contact between humans and dogs, it was regarded as worthwhile to make an attempt to assess the prevalence of infection of *I. ricinus* ticks feeding on dogs with *B. burgdorferi* s.l. and *A. phagocytophilum*.

Material and Methods

Study area. The study was carried out in Biała Podlaska county (51°58′20″N, 23°9′12″E) located in the north-eastern part of Lubelskie province. The northern and north-eastern regions of Lubelskie are characterised by the highest percentage of grassland cover in the entire region. A large percentage of the land in this area is also fallows, wasteland and island forests (38). In 2018, the forest cover in Biała Podlaska county was 27.6%, compared with 23.4% in Lubelskie province as a whole (32). Ticks were collected from host or carrier dogs in Łęgi (52°10′03″N, 23°28′11″E), Biała Podlaska (52°01′56″N, 23°06′59″E), Porosiuki (52°01′00″N, 23°03′28″E), Mokre (51°51′01″N, 23°04′38″E) and Janówka (51°58′08″N, 23°04′56″E).

Tick collection. The study involved 147 adult *Ixodes ricinus* ticks (81 females and 66 males) collected from dogs in 2018–2020. Ticks attached to the skin or present on the coat of the dogs were collected once a year. Almost half of the specimens, 72 ticks (49.0%), were collected from 33 homeless dogs kept in a shelter. The other 75 ticks (51.0%) were collected from 7 owned dogs. Ticks were collected twice from two owned dogs and six times from one owned dog after a walk in different locations in the study area, none of which were engorged. Among the 147 collected ticks, 33 (22.4%) specimens were engorged: 30 of them from 21 shelter dogs (3 or fewer engorged ticks per dog) and 3 from 2 owned dogs (with no recurrent infestation). The ticks were collected by the dogs' owners and delivered to the laboratory. The tick species, sex and developmental stage were identified in accordance with the key developed by Nowak-Chmura (23). The ticks were kept individually in Eppendorf tubes in 70% ethanol at 6°C.

DNA analysis. AmpliSens TBEV, *B.burgdorferi* s.l., *A.phagocytophilum*, *E.chaffeensis*/*E.muris*-FRT (Ecoli Dx, Bratislava, Slovak Republic) for specific detection of a fragment of the 16S ribosomal RNA gene in *B. burgdorferi* s.l. and a fragment of the merozoite surface protein 2 gene in *A. phagocytophilum* was used in the study. The analyses consisted of several steps. The first step was based on the use of the RIBO-prep kit (Ecoli Dx, Bratislava, Slovak Republic) for isolation of genetic material from tick tissues. The next step involved the reverse transcription reaction, which allowed the synthesis of complementary DNA (cDNA) with the use of the REVERTA-L reagent kit (Ecoli Dx, Bratislava, Slovak Republic). The samples prepared in this way were directly subjected to a real-time PCR reaction using the TBEV, *B. burgdorferi* s.l., *A. phagocytophilum*, *Ehrlichia chaffeensis*/*E. muris*-FRT PCR kit (Ecoli Dx, Bratislava, Slovak Republic), which contained the following reagents: PCR-mix-1-FRT tick-borne encephalitis virus (TBEV), *A. phagocytophilum*, *E. chaffeensis/ E. m*uris, PCR-mix-1-FRT *B. burgdorferi* s.l./internal control (IC), RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), cDNA TBEV, *B. burgdorferi* s.l., *A. phagocytophilum*, *E. chaffeensis/E. muris* positive control, DNA buffer and IC. The real-time PCR reaction was performed in a Rotor Gene Q 2 Plex HRM thermal cycler (Qiagen, Hilden, Germany). The specificity of the apparatus, which is equipped with two fluorescence detection channels, was sufficient for identifying a smaller number of tick-borne pathogens than that facilitated by the kit. The reaction also included three checkpoints for each fluorescence channel: a positive control of amplification, a negative control of extraction (C−), and a negative control of amplification. The following amplification procedure was used: 1 cycle at 95°C for 15 min; 5 consecutive cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 15 s; and 40 consecutive cycles at 95°C for 10 s, 56°C for 30 s and 72°C for 15 s. The test used in this study has high specificity confirmed in laboratory clinical studies. The primers and probes were checked for potential homology to all sequences deposited in GenBank using comparative sequence analysis.

The χ^2 test was used to test the significance of differences in the number of infected and non-infected ticks between the different groups. The statistical analysis was performed in the STATISTICA 13.0 program (StatSoft) at a significance level of P-value < 0.05.

Results

Borrelia burgdorferi s.l. infection was detected in 10.9% (16/147) and *A. phagocytophilum* in 12.9% (19/147) of the *I. ricinus* ticks (Fig. 1). One male tick (0.7%) was co-infected by both pathogens and was a specimen collected from an owned dog after a walk in Łęgi. Infection by at least one of the pathogens was detected in 23.1% (34/147) of the ticks (Table 1). The presence of *B. burgdorferi* was detected significantly more frequently (P-value = 0.0103) in male ticks (18.2%) than in females (4.9%). The prevalence of *A. phagocytophilum* infection in the male and female specimens was similar, with a 1.5% greater prevalence in females (13.6% compared with 12.1%). Most of the ticks were not engorged at the time of collection (29/34). Each engorged tick (5/34) was collected from a different homeless dog kept in the shelter. Of those five engorged ticks, one was infected by *B. burgdorferi* and four by *A. phagocytophilum*. *Borrelia burgdorferi* infection was significantly more frequent (P-value = 0.0020) in ticks collected from the owned dogs (18.7%) than from the shelter dogs (2.8%). In contrast, the prevalence of *A. phagocytophilum* infection was similar in both groups (12.0% and 13.9%, respectively).

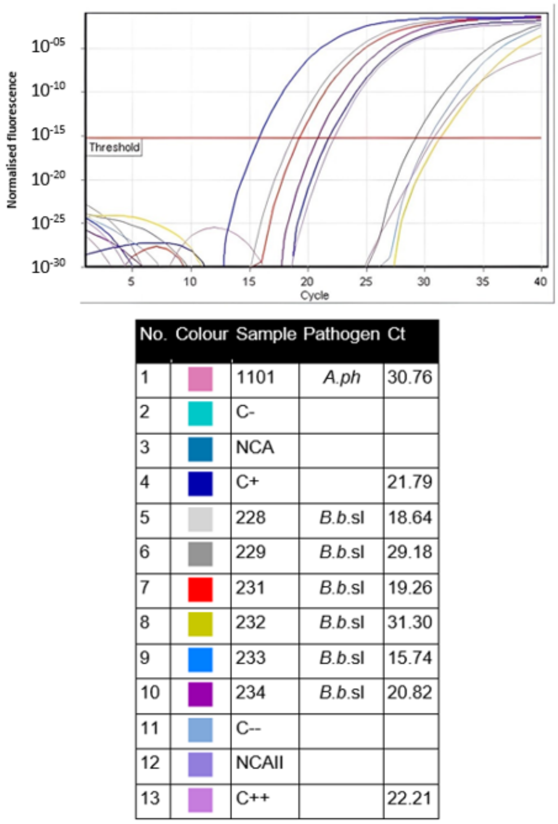

Fig. 1. Examples of PCR results positive for complimentary DNA of *Anaplasma phagocytophilum* (*A.ph.*) and *Borrelia burgdorferi* sensu lato (*B.b.* s.l.). C- – negative extraction control; NCA – negative amplification control; C+ – positive amplification control; C-- – positive amplification control and internal control; NCAII – negative amplification control and internal control; C++ – positive amplification control and internal control; Ct – threshold cycle

Table 1. *Borrelia burgdorferi* sensu lato (s.l.) and *Anaplasma phagocytophilum* infection in *Ixodes ricinus* ticks collected from dogs in the north-eastern part of Lubelskie province, eastern Poland

		Total number (%) of collected ticks	Number (%) of positive ticks for each pathogen		Total number (%) of infected ticks
			B. burgdorferi s.l.	*A. phagocytophilum*	
Ticks	Female	81 (55.1)	4 (4.9)	11 (13.6)	15 (18.5)
	Male	66 (44.9)	12 (18.2)	8 (12.1)	19 (28.8)*
	Engorged	33 (22.4)	1 (3.0)	4 (12.1)	5 (15.2)
	Non-engorged	114 (77.6)	15 (13.2)	15 (13.2)	29 (25.4)*
Dogs	Stray	72 (49.0)	2 (2.8)	10 (13.9)	12 (16.7)
	Owned	75 (51.0)	14 (18.7)	9 (12.0)	22 (29.3)*
Total		147 (100.0)	16 (10.9)	19 (12.9)	34 (23.1)*

* – the infected tick total is lower by one than the sum of the per-pathogen infected tick numbers because *A. phagocytophilum* and *B. burgdorferi* s.l. were present simultaneously in one of the ticks

Discussion

Companion animals are more susceptible to contact with ticks than humans are, as they tend to spend more time outdoors at a closer distance to the ground and vegetation and have a coat which ticks attach to easily (31). Investigations of the prevalence of pathogen infection in ticks infesting domestic animals allow assessment of the risk of tick-borne diseases in these animals and of the potential risk of infection posed to animal owners. Dogs can carry infected ticks from the natural environment to human habitats, as dogs and humans share the same living space and visit the same outdoor areas (7). Studies on *I. ricinus* ticks collected from domestic animals in different European countries showed a varied prevalence of *B. burgdorferi* and *A. phagocytophilum* infections. A higher percentage of infections with *B. burgdorferi* than with *A. phagocytophilum* was reported from Germany (11.6% and 6.5%, respectively) (28) and the Netherlands (7.2% and 1.6%, respectively) (22). In contrast, a higher prevalence of *A. phagocytophilum* infection was observed in ticks collected from dogs and cats in Belgium. The presence of *A. phagocytophilum* and *B. burgdorferi* was detected in 19.0% (127/668) and 11.1% (83/745) of *I. ricinus* ticks, respectively (4). A higher prevalence of *A. phagocytophilum* was also reported in studies conducted by Geurden *et al.* (8) on *I. ricinus* ticks collected from dogs in Hungary, France, Belgium and Italy, where the 15% of ticks infected by these bacteria was remarkably higher than the 1% of ticks infected by *B. burgdorferi*. Among the analysed countries, Hungary alone provided an instance of *B. burgdorferi* infection. This country also had the highest level of *A. phagocytophilum* infection in ticks at 20%, France with a 14% rate having the next highest. The pathogen was not detected in ticks collected in Belgium or Italy (8). The low prevalence or absence of infections reported in these studies, especially in the case of *B. burgdorferi*, may be associated with the small number of ticks analysed in each of these countries.

Within Poland, the presence of *B. burgdorferi* spirochetes in *I. ricinus* collected from dogs also varied greatly. It ranged from 0%, as shown in studies on domestic dogs from Zakopane in southern Poland (the Polish town at the highest altitude) (13) to 35.7% reported in studies on dogs admitted to veterinary clinics in the city of Olsztyn in north-eastern Poland (20). The prevalence of these pathogens was estimated at 6.2% in studies conducted in the Warsaw agglomeration in central Poland (39) and 22.5% in analyses of ticks collected from dogs and cats in the Wrocław agglomeration in south-western Poland (14). These investigations did not reveal statistically significant differences in the infection prevalence between ticks collected from dogs and those collected from cats.

In the present study of ticks collected from dogs in the north-eastern part of Lubelskie province in eastern Poland, *B. burgdorferi* infection was detected in 10.9% (16/147) of the analysed specimens. This percentage is very similar to the results reported in a study on ticks collected from domestic animals in several veterinary clinics in Lublin in the central part of the province, where 11.8% (16/136) of *I. ricinus* ticks collected from dogs and cats were infected by *B. burgdorferi* (27). The *A. phagocytophilum* infection rate was 12.9% (19/147). Male ticks had *B. burgdorferi* infection significantly more frequently (P-value = 0.0103) than females (18.2% compared with 4.9%). The prevalence of *A. phagocytophilum* infection in the male and female specimens was similar, males being slightly less frequently infected (12.1% compared with 13.6%). In total, infection by at least one of the pathogens was detected in 23.1% (34/147) of the ticks (Table 1). *Borrelia burgdorferi* infection was significantly more frequent (P-value = 0.0020) in ticks collected from the owned dogs (18.7%) than from the homeless animals (2.8%); however, the prevalence of *A. phagocytophilum* infection was similar in both groups (12.0% and 13.9%, respectively). The prevalence of *A. phagocytophilum* infection in *I. ricinus* ticks collected from dogs or dogs and cats in Poland ranged by region of collection from 1.2% to 21.3% (16), the lowest percentage having been reported in ticks from dogs admitted to veterinary clinics in the city of Olsztyn. Noteworthily, a different study showed the highest rates of *B. burgdorferi* infections in ticks collected from dogs (35.7%) in this region of Poland (20). In turn, the highest prevalence of *A. phagocytophilum* infections (21.3%) was reported in a study of ticks collected from dogs and cats in the Wrocław agglomeration in south-western Poland. It is worth noting that the study did not show statistically significant differences between the pathogen positivity rate in ticks infesting cats and in those infecting dogs (15). Low *A. phagocytophilum* infection rates were reported in other studies conducted in Poland. *Anaplasma phagocytophilum* DNA was detected in 2.9% of *I. ricinus* ticks collected from dogs in the Warsaw agglomeration and in 3.4% of ticks infesting domestic dogs and cats from Zakopane in southern Poland (13). In the present analyses of ticks collected from dogs in the north-eastern part of Lubelskie province, 12.9% of the specimens were infected by *A. phagocytophilum*. This result was higher than the value reported from the central part of the province, which showed *A. phagocytophilum* infection in 8.8% (12/136) of *I. ricinus* ticks collected from dogs and cats in several veterinary clinics in Lublin (27). This finding was an interesting difference from the near-parity of *B. burgdorferi* infection rates across the province.

Ticks can serve as a reservoir and vector of more than one pathogen. Co-infections are becoming an increasingly serious clinical problem and a more needful research area because their ecology and pathological mechanisms are still poorly explored in comparison with single-pathogen infections. The best-known co-infection in ticks and humans is that caused by the pathogens of current interest, *A. phagocytophilum* and *B. burgdorferi* s.l. (21, 35). Previous studies in Poland rarely reported the presence of this co-infection in *I. ricinus* ticks infesting domestic animals. No such co-infection was reported in studies from north-eastern (20) or southern Poland (13). However, the present study focused on eastern Poland revealed this co-infection in one *I. ricinus* tick (0.7%). This low prevalence contrasts sharply with the high prevalence of the co-infection reported in a study conducted by Roczeń-Karczmarz *et al.* (27) in south-eastern Poland, *i.e.* a region neighbouring the present study area. The authors examined ticks collected from vegetation and infesting dogs and cats. Co-infection with *A. phagocytophilum* and *B. burgdorferi* was detected in 14.0% of the analysed *I. ricinus* specimens. These results indicate a high risk of simultaneous transmission of both pathogens through a single tick bite and concurrent development of Lyme disease and anaplasmosis.

Conclusion

The results of the present study confirm the presence of *B. burgdorferi* and *A. phagocytophilum* in *I. ricinus* ticks infesting dogs in eastern Poland and indicate the prevalence of infection caused by these pathogens to be similar. The co-infection detected in an examined tick suggests the possibility of simultaneous infection by both pathogens through a single tick bite. With the knowledge of the presence of *B. burgdorferi* and *A. phagocytophilum* in ticks collected from dogs, more accurate assessment is possible of the risk of infection posed to companion animals, and by extension to their owners who are in close contact with their pets and visit the same green areas recreationally.

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