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Tick-borne pathogens in *Dermacentor reticulatus* collected from dogs in eastern Poland

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Abstract
In recent years, the distribution of *Dermacentor reticulatus* ticks has expanded into new territories in many European countries, including Poland, with increased population densities in areas of their regular occurrence. The spread of *D. reticulatus* enhances the risk of exposure of domestic animals and their owners to tick-borne diseases. The objective of this study was to assess the prevalence of infection of *D. reticulatus* ticks feeding on dogs with the pathogens *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum*. The study material comprised 152 *D. reticulatus* ticks collected from dogs in the northeastern part of Lublin Province (eastern Poland). A ready-made AmpliSens® TBEV, *B.burgdorferi sl*, *A.phagocytophilum*, *E.chaffeensis*/*E.muris*-FRT PCR kit was used for qualitative detection and differentiation of tick-borne infections. The assessment of the degree of infection of the analyzed ticks with the two pathogens revealed that 9.2% (14/152) of the examined ticks were infected with one of the pathogens. No co-infections with the pathogens were detected in any of the ticks. The highest specific percentage of infections (8.6%, 13/152) was associated with *A. phagocytophilum*. The presence of *B. burgdorferi* s.l. was detected in only one of the examined ticks (0.7%). The spread of *D. reticulatus* to new territories and the increase in population density in areas of their regular occurrence implies the need for further studies of the prevalence of pathogens with medical and veterinary importance in order to assess the risk of tick-borne diseases.

Keywords *Dermacentor reticulatus* · *Borrelia burgdorferi* · *Anaplasma phagocytophilum* · Dogs · Poland

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Introduction

Dermacentor reticulatus (Fabricius) is the second (after *Ixodes ricinus*) most important reservoir and vector of infectious diseases in Europe. In comparison with *I. ricinus*, the role of this species in the risk of infection by transmitted pathogens has insufficiently been recognized (Grochowska et al. 2020).

Dermacentor reticulatus is characterized by a wide host range, a high reproduction rate, a rapid (typically annual) developmental cycle, and high rates of survival in adverse conditions. The high adaptability of *D. reticulatus* has been confirmed by new data on its occurrence range (Földvári et al. 2016). In recent years, the distribution of these ticks has expanded into new territories in many European countries, including Poland, with increased population densities in areas of their regular occurrence Bullová et al. 2009; Dautel et al. 2006; Földvári et al. 2016; Karbowiak 2014; Kiewra and Czułowska 2013; Kubiak et al. 2018; Mierzejewska et al. 2015, 2016; Naminа et al. 2019; Nowak 2011; Paulauskas et al. 2015; Rubel et al. 2016; Sreter et al. 2005; Široký at al. 2011; Zając et al. 2020b).

In contrast to *I. ricinus*, the occurrence range of *D. reticulatus* does not concern the entire area of Poland. Before the 1990s, *D. reticulatus* localities were found mainly in the northeastern part of the country. The regions between the Vistula River and the western border of the country were considered free from *D. reticulatus*. These areas were part of the gap in the geographical range of *D. reticulatus* in central Europe. It divided the *D. reticulatus* population into the western and eastern macroregions. However, an expansion of *D. reticulatus* has been observed in Poland for over 2 decades. Currently, the eastern population of this species covers areas from the eastern border of the country to central Poland and spreads further west. In turn, the western population is expanding eastwards (Kiewra and Czułowska 2013; Król et al. 2016; Kubiak et al. 2018; Mierzejewska et al. 2015a, 2016; Nowak 2011; Opalińska et al. 2016; Zając et al. 2020b). The expansion of *D. reticulatus* is so intense that, in some regions of eastern and central Poland, this species dominates over *I. ricinus* (Mierzejewska et al. 2015b; Pańczuk et al. 2021; Zając et al. 2020a, 2021; Zygner and Wędrychowicz 2006).

Dermacentor reticulatus have a wide host range, as >60 wild and domestic animal species have been identified as hosts for the three active developmental stages (larvae, nymphs, and adults). This tick species is referred to as burrow-questing non-nidicolous, i.e., the larval and nymph stages are associated with the burrows of their hosts. Larvae and nymphs usually ingest blood from the same host, usually a small mammal. Typical hosts for *D. reticulatus* larvae are voles, mice, hedgehogs, shrews, moles, hares, and rabbits (birds are occasional hosts). In turn, in addition to the larval hosts, nymphs feed on weasels, polecats, cervids, goats, and dogs. Adult stages have a wider range of hosts, e.g., a variety of cervid species, wild boars, foxes, wolves, hedgehogs, hares, and rabbits from the wild fauna. Domestic animals, mainly dogs, horses, donkeys, cattle, sheep, goats, and pigs, are equally important and sometimes dominant tick hosts in cities or agricultural areas. Domestic animals are infested almost exclusively by adult ticks. Exceptionally, animals that explore burrows may be attacked by juvenile *D. reticulatus* stages (Földvári et al. 2016; Mierzejewska et al. 2015b; Nowak-Chmura 2013; Paziewska et al. 2010; Pfäffle et al. 2015).

Studies of the species composition of ticks feeding on dogs in Poland report a more frequent presence of *D. reticulatus* than *I. ricinus*. *Dermacentor reticulatus* were reported to dominate (64.6%) among ticks collected from dogs near Warsaw (central Poland) in

2003–2005 (Zygner and Wędrychowicz 2006). Even greater dominance of this species (86.1%) was observed in ticks collected from dogs in the Mazovia and Mazuria regions (central and northern Poland) in 2012–2013 (Mierzejewska et al. 2015b). As shown by an 8-year study (2009–2016) conducted in the urban agglomeration of Olsztyn (northern Poland), although *I. ricinus* (60.1%) dominated over *D. reticulatus* (39.7%), there was a gradual increase in the prevalence of the latter species in the subsequent years of the study (in 2016, *D. reticulatus* accounted for 57.9% of ticks collected from dogs) (Michalski 2019). In a 3-year study (2017–2019) carried out in the northeastern part of Lublin Province (eastern Poland), *D. reticulatus* accounted for 55.5% of all collected specimens (Pańczuk et al. 2021). The high percentage of *D. reticulatus* ticks infesting dogs implies the necessity to investigate the prevalence of pathogens with medical and veterinary importance in order to assess the risk of tick-borne diseases. As both *I. ricinus* and *D. reticulatus* infest dogs in their co-occurrence range, there may be a higher probability of co-infection with several pathogens and, consequently, a more severe or atypical course of diseases complicating the diagnosis and therapy.

Dermacentor reticulatus are involved in the transmission of pathogens with medical and veterinary importance. Undoubtedly, the most important pathogen transmitted by these ticks to animals is the protozoan *Babesia canis* (Földvári et al. 2016), and canine babesiosis is one of the most dangerous infectious diseases of dogs in endemic areas. Besides *B. canis*, genetic material of other pathogens has also been detected in *D. reticulatus*, e.g. bacteria of the genus *Rickettsia*, *A. phagocytophilum*, *Borrelia burgdorferi* s.l., *Francisella tularensis*, or TBEV (Ben and Lozynskyi 2019; Biernat et al. 2014; Bonnet et al. 2013; Dzięgiel et al. 2014; Karbowiak et al. 2014; Mierzejewska et al. 2015a; Namiņa et al. 2019; Reye et al. 2013; Roczeń-Karczmarz et al. 2018; Rybářová and Široký 2017; Schreiber et al. 2014; Szczotko et al. 2019; Tomanović et al. 2013; Wójcik-Fatla et al. 2011, 2015; Zając et al. 2017). Two of these tick-borne pathogens, *A. phagocytophilum* and *B. burgdorferi* s.l., have now been reported in dogs in nearly all European countries, including Poland (Krämer et al. 2014). Studies on seroprevalence in European dogs have reported that 3–57% of dogs were carriers of *A. phagocytophilum* (Sainz et al. 2015). In Poland, analyses of 3,094 samples of serum collected from dogs from all 16 Polish provinces showed the presence of anti-*A. phagocytophilum* antibodies in 12.3% of dogs, and the presence of anti-*B. burgdorferi* antibodies in 3.8% of dogs. The study demonstrated nationwide occurrence of *A. phagocytophilum* and *B. burgdorferi* s.l. in the studied population of dogs. The highest percentages of dogs (>20%) infected with *A. phagocytophilum* were reported in Lesser Poland, Silesia and Łódź Provinces (southern and central regions of Poland). For *B. burgdorferi* s.l., the highest prevalence (>10%) was noted in dogs from Łódź Province (central Poland) (Krämer et al. 2014). In Europe, *I. ricinus* is a known vector of *A. phagocytophilum* (Sainz et al. 2015) and *B. burgdorferi* s.l. (Skotarczak 2002), but these pathogens have also been detected in other tick species, e.g., *D. reticulatus* (Ben and Lozynskyi 2019; Bonnet et al. 2013; Dzięgiel et al. 2014; Karbowiak et al. 2014; Michalski et al. 2020; Mierzejewska et al. 2015a; Rar et al. 2005; Reye et al. 2013; Roczeń-Karczmarz et al. 2018; Rybářová and Široký 2017; Szczotko et al. 2019; Zając et al. 2017).

The aim of the study was to assess the prevalence of infection of *D. reticulatus* ticks feeding on dogs with the pathogens *B. burgdorferi* s.l. and *A. phagocytophilum*.

Materials and methods

Study area

The study was carried out in the northeastern part of Lublin Province (eastern Poland). The northern and northeastern regions of Lublin Province are characterized by the highest percentage of grasslands in the entire area. A significant percentage of land in this area is also covered by fallow, wasteland, and forest patches. The mosaic character of the landscape provides *D. reticulatus* populations with favorable conditions. Forest areas are associated with the presence of hosts for adult ticks, whereas rodents, i.e., hosts for juvenile stages, inhabit grasslands and wastelands. The area of Lublin Province is characterized by a high density of *D. reticulatus* populations. As reported by Zając et al. (2020b) in a study conducted in 2019, the mean number of ticks collected in Lublin Province amounted to 96.8 specimens/100 m^2, with the highest density noted in the northern part of the province.

In the present study, dogs were examined as hosts or carriers of ticks mainly in the following localities: Biała Podlaska (52°01'56"N, 23°06'59"E), Janów Podlaski (52°11'38"N, 23°12'43"E), Konstantynów (52°12'28"N, 23°05'07"E), Mokre (51°51'01"N, 23°04'38"E), Łęgi (52°10'03"N, 23°28'11"E), Porosiuki (52°01'00"N, 23°03'28"E), Zakalinki (52°12'55"N, 23°02'55"E), Bereza (51°56'14"N, 22°46'39"E), Małaszewicze (52°01'33"N, 23°31'51"E), Styrzyniec (52°01'23"N, 22°59'35"E), and Janówka (51°58'08"N, 23°04'56"E).

Tick collection

The study material comprised 152 *D. reticulatus* ticks (71 females, 81 males) collected from 55 dogs in 2018–2020. Seventy-eight ticks (51.3%) were collected from stray dogs coming from shelters, whereas the remaining 74 ticks (48.7%) were collected from owned dogs. The ticks were collected on a yearly basis. Maximum nine ticks collected from one dog were used for the analyses (average number of ticks per dog: 2.76). Ticks attached to dog's skin and those present on the coat were collected by dogs' keepers and delivered to the laboratory. All ticks analyzed were non-engorged. The species, sex, and developmental stage were identified based on morphological traits with the use of an identification key (Nowak-Chmura 2013). The ticks were stored individually in Eppendorf tubes in 70% ethanol at 6 °C.

Molecular identification of pathogens

DNA analysis

A ready-made AmpliSens® TBEV, *B.burgdorferi sl*, *A.phagocytophillum*, *E.chaffeensis*/*E. muris*-FRT PCR kit (InterLabService, Russia) was used for qualitative detection and differentiation of tick-borne infections. The target of the PCR reaction was the cDNA of *B. burgdorferi* s.l. and *A. phagocytophilum*. The detection concerned a fragment of the 16 S RNA gene in the case of *B. burgdorferi* s.l. and a fragment of the msp2 gene in *A. phagocytophilum.*

DNA isolation

DNA was isolated from tick tissues with the use of an AmpliSens RIBO-prep kit. The DNA was stored at 2–8 °C for 24 h or at − 16 °C for a longer time.

DNA amplification

A ready-made kit contained the following reagents: PCR-mix-1-FRT TBEV, *A. ph.*, *E. ch./E.m*., PCR-mix-1-FRT .*B. b. s*.l./IC, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), positive control cDNA TBEV, *B. b. s.l.*, *A. ph.*, *E. ch./E. m*./STI, DNA buffer, and internal control (IC). The Real Time PCR was performed in a Rotor Gene Q 2 Plex HRM thermal cycler. Due to the specificity of the available device, which is equipped with two fluorescence detection channels, the present analyses detected fewer tick-borne pathogens than offered by the kit. Fluorescent signal detection is assigned in the channels for the FAM (Green) and HEX (Yellow) fluorophores respectively for Internal Control (IC) and *B. burgdorferi* s.l. and *A. phagocytophilum*. The reaction also included three checkpoints for each fluorescence channel: positive amplification control (C+), negative control of extraction (C-), and negative control of amplification (NCA). The amplification parameters are shown in Table 1.

Interpretation of results

Results are considered reliable only when the extraction and amplification controls are correct. Negative controls are absent and positive controls take values < 27 Ct value for both pathogens. Clinical samples are considered positive for *A. phagocytophilum*, and *B. burgdorferi* s.l. infection at Ct values < 38 in all detection channels.

The analytical specificity of test AmpliSens® TBEV, *B.burgdorferi sl*, *A.phagocytophillum*, *E.chaffeensis*/*E.muris*-FRT PCR kit is ensured by selection of specific primers and probes as well as by selection of strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequences comparison analysis. The clinical specificity of test was confirmed in laboratory clinical trials and no false-positive results were observed during examination of DNA. Due to the high specificity of the test, we assume the high authenticity of the research results obtained.

Table 1 Real Time PCR amplification parameters

Step	Temperature (°C)	Time	Detection of fluorescence	No. cycles
Hold	95	15 min	-	1
Cycling	95	10 s	-	5
	60	30 s	-	
	72	15 s	-	
Cycling 2	95	10 s	-	40
	56	30 s	FAM/Green, HEX/Yellow	
	72	15 s	-	

Results

The assessment of the degree of infection of the analyzed ticks with the two pathogens (*Borrelia burgdorferii* s.l., *A. phagocytophilum*) revealed that 14 (9.2%) of the 152 examined ticks were infected with one of the pathogens. No co-infections with the pathogens were detected in any of the ticks. The highest percentage of infections was associated with *A. phagocytophilum*. Its presence was detected in 8.6% (13/152) of the examined specimens. The percentage of *A. phagocytophilum* infections was the same in female and male ticks, i.e., 8.5% (6/71) and 8.6% (7/81), respectively. The ticks were collected from nine dogs. Nine ticks were from owned dogs (one tick each from three dogs, two ticks each from another three dogs) and the remaining four ticks were from stray dogs from a shelter (one tick each from two dogs, two ticks from one dog). Apart from the 13 *D. reticulatus* ticks infected with *A. phagocytophilum*, no other tick species was found on the dogs at the time of collection. The presence of *B. burgdorferi* s.l. was detected in only one of the examined ticks (0.7%). This tick was collected from a dog that came from a shelter (Table 2). Two *I. ricinus* ticks were also collected from this dog. Studies revealed that *B. burgdorferi* s.l. and *A. phagocytophilum* were not detected in any of these ticks.

Discussion

Anaplasma phagocytophilum

Anaplasma phagocytophilum is the causative agent of human granulocytic anaplasmosis (HGA) (Bakken and Dumler 2015). The disease has been diagnosed in various species of wild and domestic animals, including dogs (Stuen et al. 2013). In Europe, *I. ricinus* is a known vector of *A. phagocytophilum* (Sainz et al. 2015), but this pathogen has also been detected in other tick species, e.g., *D. reticulatus*. The present assessment of the intensity of pathogen infection of ticks collected from dogs demonstrated the highest prevalence of *A. phagocytophilum*. This pathogen was detected in 8.6% (13/152) of the ticks, which confirmed the risk posed to animals. Similar rates of *A. phagocytophilum* infection were determined in female and male specimens. This is important in terms of the risk of pathogen transmission, as *D. reticulatus* males ingest small amounts of blood repeatedly to initiate spermatogenesis, which indicates that both male and female *D. reticulatus* can be involved in pathogen transmission (Bartosik et al. 2019; Földvári et al. 2016).

Table 2 Rates of infection with *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l. in *Dermacentor reticulatus* ticks removed from dogs

		No. ticks collected	No. (%) positive for each pathogen		Total no. (%) infected ticks
			A. phagocytophilum	*B. burgdorferi* s.l.	
Ticks	Female	71	6 (8.5)	0 (0.0)	6 (8.5)
	Male	81	7 (8.6)	1 (1.2)	8 (9.9)
Dogs	Owned	74	9 (12.2)	0 (0.0)	9 (12,2)
	Stray	78	4 (5.1)	1 (1.3)	5 (6.4)
Total		152	13 (8.6)	1 (0.7)	14 (9.2)

Previous studies on *D. reticulatus* tick populations from Poland showed highly diverging percentages of infections with this pathogen. In studies on *D. reticulatus* ticks infesting dogs in urban areas of northeastern Poland, no *A. phagocytophilum* DNA was detected at all (Michalski et al. 2020). A low infection rate was also observed in ticks collected by flagging in the area of Łęczyńsko-Włodawskie Lakeland (eastern Poland), where the presence of *A. phagocytophilum* was detected in only 1.1% (7/634) of ticks (Zając et al. 2017). In turn, high numbers of ticks infected with *A. phagocytophilum* (30.4%) were reported in a study of *D. reticulatus* ticks collected from vegetation and animals in southeastern Poland. Concurrently, the study did not show the presence of *A. phagocytophilum* in any of the ticks collected from animals (including dogs) (Roczeń-Karczmarz et al. 2018). A high prevalence of *A. phagocytophilum* (32.7%) was detected in *D. reticulatus* isolated from wildlife animals (deer and roe deer) shot during hunting in some districts of Warmia-Mazury Province (north-eastern Poland) (Szczotko et al. 2019).

Low percentages or absence of *A. phagocytophilum* infections were most often observed in countries neighboring Poland. No *A. phagocytophilum* was detected in *D. reticulatus* ticks in studies conducted in Latvia (ticks collected from dogs; Namiņa et al. 2019), Belarus (ticks collected from the vegetation and from cows; Reye et al. 2013), or western Siberia in Russia (ticks collected by flagging; Rar et al. 2005). In turn, the presence of *A. phagocytophilum* DNA was confirmed only in 3.6% (18/500) of ticks tested in a study conducted in the Czech Republic (ticks collected by flagging; Rybářová and Široký 2017). Different results were reported in studies of *D. reticulatus* ticks from western Ukraine, where the *A. phagocytophilum* infection rate was estimated at 15.9% (ticks collected by flagging; Ben and Lozynskyi 2019). Even higher infection prevalence was detected in a study of ticks conducted in the Chernobyl exclusion zone, which reported 25.4% prevalence of *A. phagocytophilum* infection (ticks collected by flagging; Karbowiak et al. 2014).

Borrelia burgdorferi sensu lato

In the present study, only one of the analyzed ticks (0.7%) was infected by *B. burgdorferi* s.l. spirochetes. In previous studies on *D. reticulatus* conducted in Poland, varied levels of *B. burgdorferi* s.l. infections were reported, i.e., from 0.09% (Mierzejewska et al. 2015a) to 22.8% (Roczeń-Karczmarz et al. 2018), as in the case of *A. phagocytophilum.*

Investigations conducted by Michalski et al. (2020) on ticks collected from dogs (northeastern Poland) showed a substantially higher percentage of *B. burgdorferi* s.l. infections than in the present study. The presence of *B. burgdorferi* DNA was detected in 14.1% of analyzed ticks. These results were opposite to the estimated levels of *A. phagocytophilum* infection, as the present study showed that 8.6% of the ticks were infected, whereas no DNA of the pathogen was detected by Michalski et al. (2020). An even higher *B. burgdorferi* infection rate (22.8%) was found in *D. reticulatus* collected from vegetation and animals in southeastern Poland. However, the infection rate in ticks collected from animals was just 6.7% (only three specimens of the 45 ticks collected from the animals—two ticks collected from cats and one tick from a dog were infected by *B. burgdorferi*; Roczeń-Karczmarz et al. 2018).

In studies of ticks collected by flagging in various parts of Lublin Province (eastern Poland), *Borrelia* DNA was detected in only 0.6% of *D. reticulatus* specimens (Dzięgiel et al. 2014). In other studies conducted in this province, *B. burgdorferi* s.l. infection was

detected in 1.6% of the analyzed *D. reticulatus* (Zając et al. 2017). Even lower values were reported in studies on ticks from other areas of Poland, where the prevalence of *B. burgdorferi* s.l. was estimated at 0.09% (1/1107) (Mierzejewska et al. 2015a). A low prevalence or absence of *B. burgdorferi* s.l. in *D. reticulatus* ticks was also noted in other countries. No *B. burgdorferi* s.l. spirochetes were detected in ticks in Latvia (ticks collected from dogs; Namiņa et al. 2019), Serbia (ticks collected by flagging; Tomanović et al. 2013), Germany (Richter et al. 2013), and Great Britain (ticks collected by flagging; Tijsse-Klasen et al. 2013), and their low prevalence was reported from France (1.5%) (ticks collected by flagging; Bonnet et al. 2013), Belarus (2.7%) (ticks collected from vegetation and cows; Reye et al. 2013), and western Siberia in Russia (3.6%) (ticks collected by flagging; Rar et al. 2005). As in the case of the *A. phagocytophilum* infection analyzed here, significantly higher rates of *B. burgdorferi* infection were reported in Ukraine. In studies of ticks collected by flagging in 2009–2014 in western Ukraine, the pathogen was detected in 31.9% of *D. reticulatus* (Ben and Lozynskyi 2019). Despite the higher percentages of *D. reticulatus* infections by *B. burgdorferi*, albeit rarely observed, it has been indicated that this species is unable to serve as a competent vector of *B. burgdorferi* s.l. (Grubhoffer et al. 2005). As demonstrated by Mátlová et al. (1996), in contrast to *I. ricinus* (a competent vector), a gradual decline and loss of *B. burgdorferi* s.l. was noted in *D. reticulatus* shortly after infection. Rudolf and Hubálek (2003) analyzed the impact of extracts from tick salivary glands and midguts on *B. garinii* growth in *in vitro* conditions. It was found that the extract originating from *I. ricinus* exerted a considerable stimulatory effect on the growth of the spirochetes, whereas *D. reticulatus*-derived extracts did not stimulate, but rather inhibited the *in vitro* growth of the pathogen.

Conclusions

The rapid spread of *D. reticulatus* to new territories in many European countries (including Poland) and the increase in population density in areas of their regular occurrence enhance the risk of exposure of domestic animals and their owners to tick-borne diseases. The large number of dog infestations by *D. reticulatus* ticks implies the need for studies of the prevalence of pathogens with medical and veterinary importance in order to assess the risk of tick-borne diseases. The assessment of the degree of infection of the analyzed ticks with the two pathogens revealed the highest prevalence of *A. phagocytophilum*. It is necessary to carry out further investigations of the role of *D. reticulatus* as a vector of pathogens posing a threat to humans and animals.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

No approval of research **ethics committees** was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

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