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Comparison of the occurrence of tick-borne diseases in ticks collected from vegetation and animals in the same area[1)]

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Comparison of the occurrence of tick-borne diseases in ticks collected from vegetation and animals in the same area

Summary

The aim of this study was to compare the prevalence of selected pathogens in ticks taken from cats and dogs and from vegetation in urban settlements. A study was conducted to estimate the distribution of Anaplasma phagocytophilum and Borrelia burgdorferi in adult Ixodes ricinus (236), Dermacentor reticulatus (237) and Ixodes hexagonus (3) ticks collected from animals in veterinary clinics (184) and from vegetation in urban settlements (292). The most numerous ticks collected from animals were Ixodes ricinus (73.9%), followed by Dermacentor reticulatus (24.5%) and Ixodes hexagonus (1.6%). A total of 65.8% of the ticks collected from vegetation were Dermacentor reticulatus and 30% were Ixodes ricinus. The arthropods removed from the animals were most commonly located around the neck (48.1%) and in the mouth area (17.1%). All ticks were analyzed by molecular techniques. The percentages of ticks positive for Borrelia burgdorferi and Anaplasma phagocytophilum among those collected from animals differed from the corresponding rates for ticks taken from vegetation in the same area. Anaplasma phagocytophilum was more common in ticks collected from vegetation (N = 137, or 47.20%) than in those from animals (N = 12, or 6.6%). Borrelia burgdorferi, as well, was more common in ticks collected from the vegetation (N = 96, or 32.9%) than in those from animals (N = 19, or 10.5%). The DNA of A. phagocytophilum and B. burgdorferi were detected in 30.4% and 22.8% of D. reticulatus ticks, respectively, and in 32.6% and 25.4% of I. ricinus ticks, respectively. The DNA of A. phagocytophilum was also found in one Ixodes hexagonus tick. Single infections were noted in 69 I. ricinus ticks, 56 D. reticulatus ticks and 1 I. hexagonus tick. Coinfections of A. phagocytophilum with B. burgdorferi were detected in 33 (14.0%) I. ricinus ticks and in 29 (12.24%) D. reticulatus ticks. Infected companion animals can form a reservoir for human tick-transmitted infectious agents. The monitoring of the pathogens transmitted by ticks is an important tool in preventing and combating infections transmitted to humans and animals.

Keywords: Ixodes ricinus, Dermacentor reticulatus, Ixodes hexagonus, Anaplasma phagocytophilum, Borrelia burgdorferi, pets

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Ticks, as hematophages, are a disease transmission vector for both humans and animals. They play an important role in the natural transmission of disease and in transferring pathogens between hosts. The spread of ticks is favoured by a number of ecological, physiological and molecular features. These include high adaptability to changing environmental conditions, climate, the large number of host species, population density in a given area, and the ability to transmit pathogens transovarially (from one generation to the next via the female ovaries) and trans-stadially (from stage to stage: from larvae to nymphs and to adults). The migration of the hosts to geographically distant areas thus aids the spread of ticks and the pathogens transmitted by them (29, 44). The pathogens most often occur in forest ecosystems, in wild animals that constitute a natural source of infection. Ticks are carried away from their natural environment due to the migration of their hosts on which they parasitize, the development of tourism, and frequent trips of the owners of companion animals. In this way, the spread of ticks into new territories contributes to the appearance of new disease entities in areas where they had not previously occurred (42, 36).

In Poland, the most numerous ticks are those from the genus *Ixodes*: *Ixodes ricinus* and *Dermacentor reticulatus*. The former is one of the most important arthropods in the epidemiology of transmission diseases and is considered the most important tick vector in Europe. In Poland, the pathogens most frequently transmitted by *I. ricinus* ticks are *Borrelia burgdorferi s.l.* and *Anaplasma phagocytophilum*, protozoa from the genus *Babesia* and the tick-borne encephalitis virus (KZM) (Flaviviridae) (47, 54, 53). *D. reticulatus* is the second most important vector of many transmissible diseases, and the pathogens most frequently transmitted by these ticks are *Babesia* spp., *Borrelia burgdorferi* s.l., *Anaplasma marginale*, *Rickettsia* spp., and the tick-borne encephalitis virus (KZM) (Flaviviridae) (4, 21, 46). *Ixodes hexagonus* is rarely found in Poland, probably because it is a typically a nest-dwelling species. This tick is involved in the transmission of pathogens that are dangerous to humans and animals, including tick-borne encephalitis, *Borrelia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum* (23, 29, 40).

Pathogens transmitted by ticks are an important problem in both human and veterinary medicine, with tick-borne diseases being a major threat for humans and animals. The aim of this study was to determine the prevalence of selected pathogens in ticks collected from cats and dogs living in urban habitats and from vegetation in urban settlements.

Material and methods

Study area and tick collection. The study was conducted in south-eastern Poland in the years 2015-2017. The study included 476 ticks (*Ixodes ricinus*, *Dermacentor reticulatus* and *Ixodes hexagonus*), (*Acari: Ixodidae*). The ticks were examined in two groups: those collected from the environment and those found on animals brought into the veterinary clinics. Ticks originating from the environment were collected during the spring and autumn periods of their activity. They were collected by the flagging method from typically urban areas highly frequented by animals and their owners, such as walking routes and city parks. Grass, shrubs and bushes (up to 1.5 m in height) were swept with a white flannel flag (1 × 1 m). The material collected was preserved in plastic sample-tubes. The second group of ticks were collected from cats and dogs (each tick came from a different animal) in several veterinary clinics in Lublin. The parasites were removed from animals with tweezers, described (cat/dog host, age, sex, breed, and location on the host) and placed in an Eppendorf tube. In the laboratory, all ticks were placed in 70% ethyl alcohol. Next, the developmental stage, genus and species of each tick were determined (43) using the Cell light microscope system software from Olympus and then preserved for further molecular studies.

DNA isolation and detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi.* DNA was isolated from 476 *Ixodes ricinus*, *Dermacentor reticulatus*, and *Ixodes hexagonus* ticks collected from vegetation (292) and from cats and dogs (184). The ticks were removed from ethanol storage and crushed. DNA isolation was performed using a Genomic Mini kit (A&A Biotechnology, Poland), according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR). PCR reactions were carried out to test for the presence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum*. The amplifications were performed using an MJ Research PTC-200 DNA Engine (BioRad, USA). Each PCR reaction was carried out in a 25 µl reaction volume containing 12.5 µl of DreamTaq Green PCR Master Mix (ThermoFisher Scientific, USA), 0.6 µl of 10 µM each of primer (DNA Sequencing and Synthesis Service of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw, Poland.), 3 µl of matrix DNA and 8.3 µl of nuclease-free water supplied for the PCR Master Mix.

Detection of *Borrelia burgdorferi* was carried out using the primers FL6 5′-TTCAGGGTCTCAAGCGTCTTGGACT-3′ and FL7 5′-GCATTTTCAATTTTAGCAAGTGATG-3′, which amplify a product of 276 bp for sequencing the flagellin flaB gene (35). The reactions were performed under the following conditions: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 2 minutes, extension at 72°C for 3 minutes, followed by a final extension at 72°C for 3 minutes.

To identify *Anaplasma phagocytophilum*, the following primers were used: EHR 521: 5′-TGTAGGCGGTTCGGTAAGTTAAAG-3′ and EHR 747: 5′-GCACTCATCGTTTACAGCGTG-3′, which amplify a product of 274 bp for the 16S rRNA gene (54). The reactions were performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 40 cycles with denaturation at 94°C for 45 seconds, annealing at 37°C for 45 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 5 minutes.

The PCR products were subjected to electrophoresis in 2% agarose gels for the detection of *A. phagocytophilum* and in 1% agarose gels for *B. burgdorferi*; the gels were stained with ethidium bromide and visualized under ultraviolet light.

Statistical analysis. The statistical analysis of parasite occurrence related to the environmental origin involved a chi-square test (including the results of the Z Fisher test with Bonferroni correction for significance level) and two types of nominal correlation depending on the number of categories of the variables (V Cramer and Phi). All analyses were performed using the PS Imago software package (IBM SPSS

Statistics Version 23). The chi-square test and the Z Fisher test were used for precise determination of statistically significant differences between the presence of each parasite associated with an independent variable – the environment. Cramer's phi correlation was used to demonstrate a relationship between the variables and their strength. A p value of ≤ 0.05 was considered significant.

Results and discussion

The detection of pathogens in ticks, as definitive hosts, and in animals, as intermediate hosts, is an important tool in understanding the transmission of pathogens in nature. In this study we analyzed *Ixodes ricinus*, *Dermacentor reticulatus* and *Ixodes hexagonus* ticks for the presence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum*.

Overall, a total of 476 ticks were collected, 184 of which originated from animals (65 from dogs, 119 from cats) and 292 from vegetation. There are statistically significant differences in the numbers of *Dermacentor reticulatus* (n = 45) and *Ixodes ricinus* (n = 136) collected from the animals ($Chi^2 = 45.75$, df = 1, $p < 0.001$). Our studies show that the most common ticks in the animals were *Ixodes ricinus* (73.9%), followed by *Dermacentor reticulatus* (24.5%) and *Ixodes hexagonus* (1.6%). With regard to the ticks collected from vegetation, 65.8% were *Dermacentor reticulatus* and 30% were *Ixodes ricinus*. Most of the ticks removed from animals were located in the neck area (48.1%). Ticks were also removed from the area of the mouth (17.1%), paws (14.9%), abdomen (10.5%) and back (9.4%). The high proportion of ticks found on the head is confirmed by other studies (6).

There are statistically significant differences in the occurrence of *Anaplasma phagocytophilum* ($Chi^2 = 84.99$, df = 1, $p < 0.001$) and *Borrelia burgdorferi* ($Chi^2 = 30.41$, df = 1, $p < 0.001$) depending on the origin of the ticks. *Anaplasma phagocytophilum* was more common in ticks originating from vegetation (n = 137, or 47.20%) than in those from animals (n = 12, or 6.6%). *Anaplasma phagocytophilum* was found in one *Ixodes hexagonus* tick, but due to the low numbers of this tick, the results were not taken into account in statistical calculations. *Borrelia burgdorferi* was more common in ticks from the vegetation (n = 96, or 32.9%) than in ticks from animals (n = 19, or 10.5%).

Single infections were noted in 69 *I. ricinus*, in 56 *D. reticulatus* and in 1 *I. hexagonus* ticks. Coinfections of *Anaplasma phagocytophilum* with *Borrelia burgdorferi* were detected in 33 (14.0%) *Ixodes ricinus* ticks and in 29 (12.24%) *Dermacentor reticulatus* ticks. The prevalence of *A. phagocytophilum* and *B. burgdorferi* in adult *I. ricinus*, *D. reticulatus* and *I. hexagonus* ticks collected from animals and vegetation is shown in Table 1.

I. ricinus is widespread in Poland, and its numbers are steadily growing (Siuda et al. 1995). The risk of infestation with *D. reticulatus* is also high in the study area, because eastern and central Poland is inhabited by populations of this tick (21, 22). Recreational areas in Poland abound in *I. ricinus* (1, 50, 51, 55), and to a lesser extent in *D. reticulatus* (3, 24, 55). Both *I. ricinus* (10, 12, 20, 49) and *D. reticulatus* ticks (19, 19, 32) can be found in Europe.

In our study, the majority of ticks collected from walking areas in the Lublin districts belonged to the *D. reticulatus* species, while the ticks collected from animals were mostly from the *I. ricinus* species. Very similar results for ticks collected from pets were obtained in other studies in Poland (24) and in Belgium (6). Other authors in Poland (27, 57) found the largest number of *D. reticulatus* ticks on animals, especially dogs. Both species differ in terms of geographic range, seasonal and daily activity peaks and type of habitat (15, 17, 30). Nevertheless, both species occur in most housing estates and walking routes in the Lublin region. Most *I. ricinus* were collected in alleys and housing estates, places where short vegetation passes into dense shrubs

Tab. 1. Prevalence of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in adult *Ixodes ricinus*, *Dermacentro reticulatus* and *Ixodes hexagonus* ticks collected from animals in urban areas

Location of tick collection	Pathogen	*D. reticulatus*	*I. ricinus*	*I. hexagonus*
Vegetation	*B. burgdorferi*	51	44	0
	A. phagocytophilum	72	65	0
Cats	*B. burgdorferi*	2	6	0
	A. phagocytophilum	0	6	0
Dogs	*B. burgdorferi*	1	10	0
	A. phagocytophilum	0	6	1

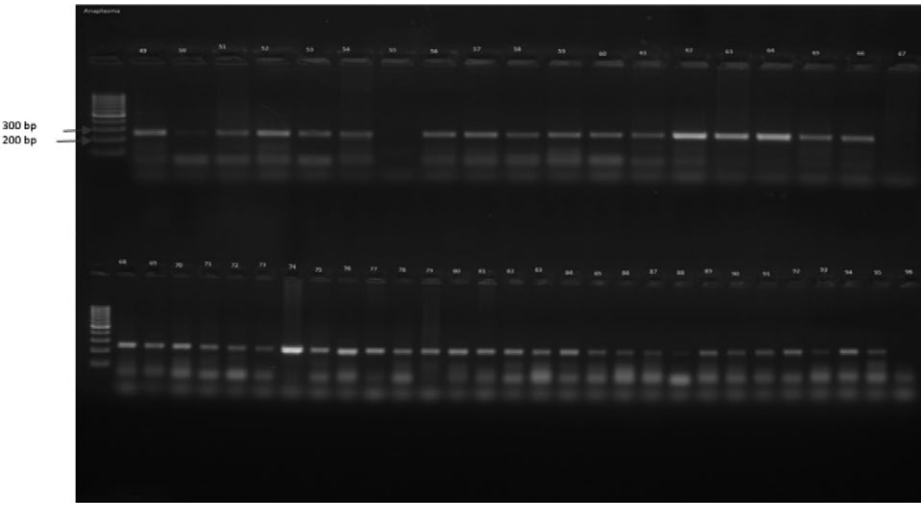

Fig. 1. Gel electrophoresis of PCR products in a 2% agarose gel using *Anaplasma phagocytophilum* specific 16S rRNA gene region primers (274 pb PCR product). Lines from 49 to 58 – *D. reticulatus*, and from 59 to 70 – *I. ricinus*

and trees. Also, tall uncropped grass favoured the occurrence of *I. ricinus*. The vicinity of the Bystrzyca River and the Zemborzycki Reservoir favoured the occurrence of *D. reticulatus*. In our study, out of 184 ticks collected from animals, only 3 were *I. hexagonus*. In Europe, ticks of this species are most often found on hedgehogs (8, 11, 34), but they are also found on companion animals (24, 27, 28, 31, 45).

The main vector of *A. phagocytophilum* is the *I. ricinus* tick. The literature mentions few cases of *A. phagocytophilum* transmission by *D. reticulatus* (22, 52, 56). Our study indicates a high risk of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in the study areas. This study showed high rates of *A. phagocytophilum* infection in *D. reticulatus* and *I. ricinus* ticks. The percentages of *I. ricinus* ticks infected with *A. phagocytophilum* and *B. burgdorferi* were very high: 32.6% and 25.4%, respectively. *Ixodes ricinus* was within the range of infections with *Borrelia* (3.1-40.0%) and *Anaplasma* (1.0-35.0%) in other European countries (5, 6, 10), although in Poland our results indicate the highest level for *Borrelia* (5.6-23.5%) and *Anaplasma* (1.7-19.1%) (9, 13, 24, 39, 48, 51) in urban areas and on walking paths. *Anaplasma phagocytophilum* is found rarely in *D. reticulatus* ticks in Poland and other European countries (1.1-25.36%) (22, 56), whereas *B. burgdorferi* occurs more often and with lower infection rates (0.6--2.7%) (9, 24, 37). In our study, *D. reticulatus* ticks were highly infected with *A. phagocytophilum* (30.4%) and *B. burgdorferi* (22.8%). Our study shows that *A. phagocytophilum* in *D. reticulatus* ticks occurred only in specimens collected from vegetation, and that the rates of infection with both pathogens for *I. ricinus* and *D. reticulatus* ticks were at a similar, high level. This may result from the fact that the primary source of infection occurs in urban settlements, where infected intermediate hosts are likely to occur.

The percentages of ticks collected from animals and positive for *Borrelia burgdorferi* and *Anaplasma phagocytophilum* were 10.5% and 6.6%, respectively. These rates were within the range of infection for *Borrelia* (0.3-22.5%) and *Anaplasma* (1.6-19.5%) found in other European countries (2, 6, 24, 28, 57).

The presence of *B. burgdorferi* and *A. phagocytophilum* in ticks can be determined by the activity of the ticks' hosts inhabiting the biotope, whose occurrence is influenced by a multiple of biotic and abiotic factors (14, 26, 41). The reservoir hosts for *B. burgdorferi* in urban settings are rodents, hedgehogs, foxes, squirrels, hares, birds and deer (14, 16, 25, 38). The typical reservoir hosts for *A. phagocytophilum* are red deer, roe deer, bank voles and wood mice (21, 52). The natural hosts for *I. ricinus*, which are found in forests (Cervidae, Carnivores), most probably also act as reservoirs for *A. phagocytophilum* (51). However, the role of pets should also be taken into account. The development of settlements and their expansion into wasteland, in the vicinity of forests and rivers, increases the access to vectors. Economic and civilizational development often takes place at the expense of the natural environment. Many species of animals settle and increase in numbers in an uncontrolled manner (7).

There was no significant difference between *I. ricinus* and *D. reticulatus* in the proportion of ticks that contained DNA from both pathogens. All multiple infections were detected in adult *I. ricinus* and *D. reticulatus* ticks, suggesting that they may have fed on the same reservoir hosts. The study of the frequency of coinfection in ticks in a specific area can be used as a tool to help diagnose diseases in humans and animals. In our study, the frequency of coinfection with *A. phagocytophilum* and *B. burgdorferi* was high in both *I. ricinus* and *D. reticulatus*. Other authors have also observed the coincidence of *B. burgdorferi* with *A. phagocytophilum* (33, 54). Such results show that there is a high risk of simultaneous transmission of both pathogens during a single tick bite.

This study shows that there is a high risk of contracting Lyme disease and anaplasmosis in the urban environment, which poses a threat to human and animal populations. It has been shown that, next to *I. ricinus*, *D. reticulatus* is the second most important vector involved in the transfer of *A. phagocytophilum*. Inhabitants and health professionals in cities should be more aware of the risk of Lyme disease and anaplasmosis in green areas or other recreational areas where infected ticks occur.

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