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The potential risk of human exposure to tick-borne infection by Borrelia burgdorferi sensu lato, Anaplasma phagocytophilum, and Babesia microti in selected recreational areas of the Poprad Landscape Park in southern Poland

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Abstract

Introduction and Objective. Ticks (Acari:Ixodida) are dangerous ectoparasites and, at the same time, vectors and/or resevoirs of many pathogens, among others Borrelia burgdorferi sensu lato, Anaplasma phagocytophilum and Babesia microti. These ethiological agents of Lyme borreliosis, anaplasmosis and babesiosis are transferred to humans mainly by ticks during feeding. The aim of this study was to estimate the potential risk of human exposure to tick borne infection of B. burgdorferi s.l., A. phagocytophilum and B. microti in selected areas of Poprad Landscape Park in southern Poland [PLP].
Materials and Method. Ixodes ricinus ticks were collected from vegetation by the flagging method. Under a stereoscopic microscope, specimens were determined to the species and developmental stage. In total, DNA was isolated from 363 ticks. To detect B. burgdorferi s.l,.two pairs of primers specific to the flagelline gene were used. In turn, to detect A. phagocytophilum and B. microti, two pairs of primers specific to the 16S rRNA gene fragment and 18S rRNA gene fragment were used, respectively. The amplification products were separated electrophoretically in 2% ethidium bromide stained agarose gels, and visualized under ultra violet light.
Results. Generally, pathogens were observed in 19.6% of ticks. Borrelia burgdorferi sensu lato was detected in 11.8% of studied ticks. In turn, A. phagocytophlium and B. microti were presented, respectively, in 0.3% and 7.4% of examined I. ricinus.
Conclusions. The study indicated a potentially high risk of human exposure to infection with tick-borne pathogens, mainly B. burgdorferi s.l. and B. microti, in the areas of PLP. In turn, the presence of A. phagocytophilum in lower percentage was shown in the studied ticks

Key words

Anaplasma phagocytophilum, Babesia microti, Ixodes ricinus, Borrelia burgdorferi sensu lato, tick-borne pathogens, Poprad Landscape Park

INTRODUCTION

The area of southern Poland has a large number of attractive places in which to spend free time close to nature, both for residents and tourists from all parts of Poland and worldwide. There are 6 national parks, eleven landscape parks, and 10 protected landscape areas within the region. Many tourist trails, bicycle paths, recreation areas, a large number of rivers and forests create perfect conditions for exploring these areas. Tourist traffic in the Poprad Landscape Park (PLP), especially in the season from early spring to late autumn, coincides with the highest seasonal activity of ticks which are looking for hosts. The area of the Province of Lesser Poland has been monitored for over 60 years in terms of the occurrence and activity of ticks (Acari: Ixodida), especially *Ixodes ricinus* [1–5]. Moreover, 13 species of ticks have been registered in the Lesser Poland region, from the *Argasidae* family: *Argas polonicus*, *Carios vespertilionis*, from the *Ixodidae* family: *Ixodes trianguliceps*, *Ixodes arboricola*, *Ixodes crenulatus*,

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Ixodes hexagonus, *Ixodes lividus*, *Ixodes rugicollis*, *Ixodes simplex*, *Ixodes vespertilionis*, and *Ixodes apronophorus*, from the family A*mblyommidae*: *Dermacentor reticulatus* [5–8].

Barcice (49°31′48″N, 20°38′50″E) and Barcice Dolne (49°31′39″N, 20°39′06″E) are summer resorts located in the Lesser Poland Province, in the commune of Stary Sącz, in the Nowy Sącz county, 79 kilometers south-east of Kraków, the regional capital. The resorts are located in the Poprad Landscape Park, in the Poprad Valley (Fig 1). A certain pressure is put on the occurrence of ticks on tourist trails and the regions with conditions for the development of agritourism. However, knowledge about the occurrence of ticks in protected areas, national and landscape parks in Poland, is not fully documented. Among the 19 species of ticks, the common elements of the Polish tick fauna, *I. ricinus* is still the most popular [6, 7, 9], and plays the most important role as a reservoir and carrier of pathogens of all transmission diseases registered in Poland, which results in the number of tick-borne diseases among people in Poland.

For this reason, the aim of this study was to estimate the potential risk of human exposure to tick-borne infection of *B. burgdorferi* s.l., *A. phagocytophilum* and *B. microti* on the selected areas of the Poprad Landscape Park.

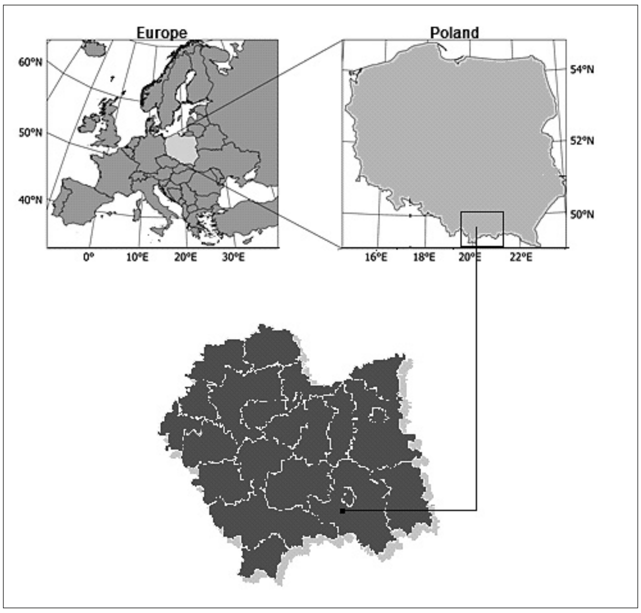

Figure 1. Location of the studied area in Europe and in Poland.

MATERIALS AND METHOD

Ticks were collected from the 6 selected areas of the PLP (3 in 2018 and 3 in 2019) by flagging and placed in plastic tubes containing 70% ethyl alcohol. In 2018, ticks were collected from areas I-III, and in 2019 from areas IV-VI (Figs. 2–3). Next, specimens were identified to the species and developmental stages under a stereoscopic microscope, using keys by Siuda [5] and Nowak-Chmura [7].

DNA was isolated from 363 *I. ricinus* ticks – 139 females, 141 males and 83 nymphs, by the ammonia method [10]. The concentration of DNA was measured spectrophotometrically in a nanospectrophotometer PEARL (Implen, Germany) at the 260/280 nm wave length. Next, DNA was frozen and stored at -20^{O}C for further analysis.

Pathogens in ticks were detected by the nested PCR method. For the detection of *B. burgdorferi* s. l. in ticks the 2 pair of primers specific to the flagelline gene were used [11]. In turn, to detect *A. phagocytophilum* and *B. microti*, the 2 pairs of primers specific to the 16S rRNA gene and 18S rRNA gene were used, respectively [12, 13]. The amplification products were then separated electrophoretically in 2% ethidium bromide stained gels, and visualized under ultra violet light and photographed by an OMEGA 10 device (UltraLum, USA). The obtained results were analyzed by TotalLab software (TotalLab, UK).

To verify the important differences in the number of infected and uninfected ticks in different comparison groups, the χ^2 test (Chi-square test) was used. In order to verify the significance of differences in the number of tick infections with the tested pathogens in different comparative groups, the Yates correction to the χ^2 test (Chi-square test with Yates correction) was used. Calculations were conducted using STATISTICA software.

Figure 2. Sites sampled for ticks in the selected areas of Poprad Landscape Park. A – Area I; B – Area II, C – Area III, D – Area IV, E – Area V, F – Area VI

RESULTS

In total, pathogens were detected in 19.6% of studied ticks. The spirochaetes *B. burgdorferi* s.l., were shown in 11.8 % of studied *I. ricinus*. In turn, *B. microti* in 7.4% of studied ticks was stated. Rickettsiae *A. phagocytophilum* was detected in only 0.3% of ticks. The highest percentage of ticks infected with *B. burgdorferi* s.l. were found in areas IV (27.5%) and V (21.1%), with the lowest percentage of ticks infected by this spirochaete in area I (4.9%) was shown. The highest number of ticks infected with *B. microti* was found in the area I (16,7%) and the lowest in areas III and V. None of the tested ticks from the area IV were infected with this protozoan. *A. phagocytophilum* was detected only in a single female collected in area III (Tab. 1). No co-existence was found of these 3 pathogens in the studied ticks.

B. burgdorferi s.l. and *B. micorti* were found in all developmental stages of the studied *I. ricinus* ticks. The highest number of *B. burgdorferi* s.l. was discovered among

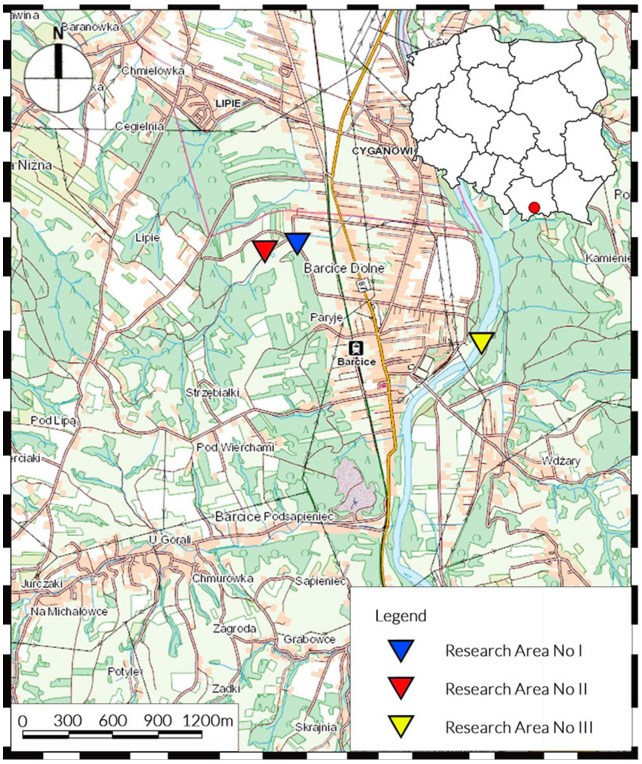

Figure 3. Localities of the 3 studied areas in 2018 within the Poprad Landscape Park.
Area I – A meadow surrounded by a deciduous forest, where there is a combat shelter. Geographic coordinates: 49°32′00.4″N 20°38′33.3″E.
Area II – A meadow along a popular walking trail, surrounded by a deciduous forest, mostly birch. Geographic coordinates: 49°31′58.3″N 20°38′25.5″E.
Area III – A meadow next to a pine forest, near the Poprad River. Geographic coordinates: 49°31′34.9″N 20°39′41.5″E.

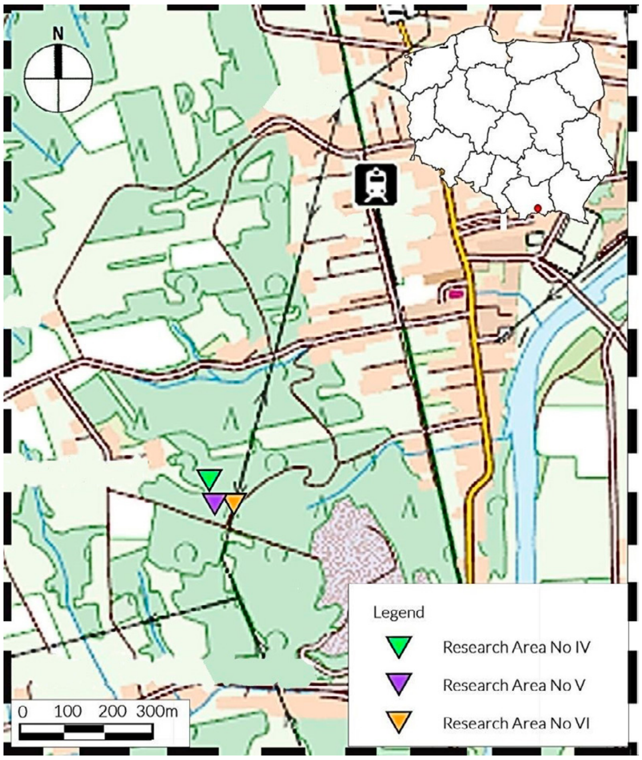

Figure 4. Localities of the 3 studied areas in 2019 within the Poprad Landscape Park.
Area IV – A meadow surrounded by a deciduous forest, where there is a high metal cross, visited by local processions. Geographic coordinates: 49°31′12.1″N 20°38′33.1″E.
Area V – A path covered with grass leading through a deciduous forest – mostly birch and hornbeam. Geographic coordinates: 49°31′10.7″N 20°38′34.0″E.
Area VI – A meadow surrounded by a deciduous forest. Geographic coordinates: 49°31′10.0″N 20°38′36.1″E.

females (20%), and the lowest among males (2%). The majority of females infected with this pathogen were in Area IV (30.8%), and the smallest number in Area I (11.4%). Infected males were found only in Areas IV and V (20% and 10%). In Areas I-III and VI, there were no males with this pathogen. 11% of males were infected with *B. microti* and only 5% of females. The largest number of infected males was found in Area I (17.6%), whereas in IV and VI, no males were carriers of the above-mentioned pathogen. In females, this pathogen was detected only in Areas I and VI (13.6% and 6.7%). This pathogen was not detected in females in other areas (Tab. 1).

Results of the χ^2 test – despite large differences in the number of ticks collected in individual study areas (from N=35 on study plot VI and N=102 on study plot I) – did not show any statistically significant differences ($p<.05$) between the proportions of the number of infected and uninfected ticks in all tested pairs of study fields. This proved the homogeneity of study areas (Tab. 2).

The number of tick infections in individual research areas was characterized by high variability and ranged from 3 – 11 in the case of *B. burgdorferi* s.l., and from 0 – 17 in the case of *B. microti*. Generally, the number of ticks infected with *B. burgdorferi* s.l., was higher than the number of ticks infected with *B. microti*. In 4 study areas, the number of ticks infected with *B. burgdorferi* s.l., was higher than the number of ticks infected with *B. microti*. In one plot the number was the same (plot VI), and in the other plot, more ticks were infected with *B. microti* than with *B. burgdorferi* s.l. (Tab. 1).

To sum up, the number of mature ticks and nymphs of infected ticks was lower than the number of uninfected ticks at these stages of development in all study areas (Tab. 1) The proportions of the number of infected and uninfected ticks in terms of 2 developmental stages (nymphs and mature ticks) did not show statistically significant differences, χ^2 (1, N=363)=.099; p-value=.753. The situation was different with regard to the number of infected and uninfected ticks in terms of the gender of mature individuals. In this case, there was a statistically significant difference ($p<.01$) between females and males – comparably, there were more females and fewer males, χ^2 (1, N=280)=7.029; p-value=.008.

Generally, there were more tick infections caused by *B. burgdorferi* s.l., than by *B. microti* in nymphs, adults (both females and males combined). On the other hand, in the case of males, the number of ticks infected with *B. microti* was higher than the number of ticks infected with *B. burgdorferi* s.l.

Statistical analysis also did not indicate any statistically significant differences in the number of ticks infected with *B. burgdorferi* s.l., and *B. microti* between nymphs and mature individuals (female and male together), χ^2 (1, N=70)=.795; p-value=.373. Highly statistically significant differences ($p<.001$) were revealed in the comparison between the gender of mature individuals, χ^2(1, N=53)=19.638, p-value<.0001, where statistically significantly more males than females were infected with *B. microti* and more females than males were infected with *B. burgdorferi* s.l.

Table 1. Number of *Ixodes ricinus* males, females and nymphs infected with *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in the studied areas of the Poprad Landscape Park

Studied area	Developmental stage	No. of ticks	No. of uninfected ticks	Number of infected ticks			
				Borrelia burgdorferi sensu lato	*Anaplasma phagocyto-philum*	*Babesia microti*	Total
Area I	Male	51	42	0	0	9	9
	Female	44	33	5	0	6	11
	Nymph	7	5	0	0	2	2
	Total	**102**	**80**	**5**	**0**	**17**	**22**
Area II	Male	28	24	0	0	4	4
	Female	22	16	6	0	0	6
	Nymph	26	25	0	0	1	1
	Total	**76**	**65**	**6**	**0**	**5**	**11**
Area III	Male	32	31	0	0	1	1
	Female	38	27	10	1	0	11
	Nymph	2	2	0	0	0	0
	Total	**72**	**60**	**10**	**1**	**1**	**12**
Area IV	Male	10	8	2	0	0	2
	Female	13	9	4	0	0	4
	Nymph	17	12	5	0	0	5
	Total	**40**	**29**	**11**	**0**	**0**	**11**
Area V	Male	10	8	1	0	1	2
	Female	7	6	1	0	0	1
	Nymph	21	15	6	0	0	6
	Total	**38**	**29**	**8**	**0**	**1**	**9**
Area VI	Male	10	10	0	0	0	0
	Female	15	12	2	0	1	3
	Nymph	10	7	1	0	2	3
	Total	**35**	**29**	**3**	**0**	**3**	**6**
Areas I-VI	Male	141	123	3	0	15	18
	Female	139	103	28	1	7	36
	Nymph	83	66	12	0	5	17
Total [n]		**363**	**292**	**43**	**1**	**27**	**71**
[%]		*100.0*	*80.4*	*11.8*	*0.3*	*7.4*	*19.6*

Area I – meadow surrounded by a deciduous forest, where there is a combat shelter.
Area II – meadow along a popular walking trail, surrounded by a deciduous forest, mostly birch.
Area III – meadow next to a pine forest, near the Poprad River.
Area IV – meadow surrounded by a deciduous forest, where there is a high metal cross, visited by local processions.
Area V – path covered with grass leading through a deciduous forest – mostly birch and hornbeam.
Area VI – meadow surrounded by a deciduous forest.

Table 2. Results of χ^2 tests between number of infected and uninfected ticks representing every pair of studied areas of the PLP

Studied area		p-value					
		I	II	III	IV	V	VI
χ^2	I		.228	.297	.452	.789	.575
	II	1.452		.891	.089	.223	.717
	III	1.087	.019		.119	.277	.804
	IV	.567	2.893	2.434		.700	.285
	V	.072	1.486	1.182	.149		.489
	VI	.314	.132	.061	1.142	.477	

DISCUSSION

The potential risk of tick infestation and acquiring a tick-borne infection may be influenced by the behaviour of individual persons, including the choice of places for rest and recreation. The places where ticks of this species are mostly found are moist habitats, mainly deciduous and mixed forests, bush thickets, and moist pastures. However, they avoid high mountain areas, dry pine forests, coniferous forests, swamps and peat bogs [7]. The percentage of infections with tick borne pathogens depends on the region of Poland. When choosing places for outdoor activities, it is worth knowing the threats that occur in a particular area, which is important not only for residents, but also for tourists who frequently visit the area. It is commonly known, that *I. ricinus* is the most common tick in Poland which often feeds on humans [7, 14]. The results obtained in this work confirmed this fact, because all the ticks collected in the studied areas of the PLP belonged to the species *I. ricinus*. Moreover, in Poland as in the rest of Europe, this tick species is considered one of the most important arthropod species in the epidemiology of the transmission diseases [15, 16]. The main pathogens transmitted by this tick are: spirochaete *B. burgdorferi* s.l., the tick-borne encephalitis virus (TBEV), protozoan *B. microt,i* and the rickettsiae *A. phagocytophilum*.

For several years, Lyme boreliosis has been a growing problem for infectious disease clinics, and a matter of intensive research [17]. The total number of ticks infected with *B. burgdorferi* s.l., in southern Poland ranges from 0% in the Kraków-Częstochowa Upland to 62% of ticks infected with this pathogen in the Żywiec Beskids [18, 19]. In the current research, of the 363 ticks tested in Barcice, 11.8% were carriers of this pathogen. In the Niepołomice Forest in Lesser Poland, a similar number of infected ticks was found – of 286 *I. ricinus*, 11.5% were the carrier of *B. burgdorferi* s.l.. A higher percentage of infected ticks was observed in Ojców National Park (25%) and in Lasek Wolski (35.7%) [20]. In turn, in Tarnowskie Góry in the Silesian District, the spirochaete *B. burgdorferi* s.l., was found in 16.5% of the examined ticks [21], while in the area of the Żywiec Landscape Park and its vicinity – 1.7% – 26.92% [22, 23]. In Lower Silesia, western Poland, the percentage of ticks infected with *B. burgdorferi* s.l., was higher than in Barcice, ranging from 14% – 22.5% [24, 25]. In the Lubelskie Province in eastern Poland, the percentage of infected ticks varied from 6% – 24.3% [26, 27]. In northern Poland, however, *B. burgdorferi* s.l., infection among ticks ranged from 0% in the Słowiński National Park to 12.4% in Gdańsk [28, 29].

In the current study, the highest proportion of ticks infected by *B. burgdorferi* s.l. was found in areas IV (27.5%) and V (21.1%), in places covered with grass, surrounded by deciduous forest. A large number of infections may be due to the fact that these places are far from buildings and roads, and are therefore inhabited by many wild animals that are hosts to ticks and play an important role in the transmission of tick-borne diseases. In turn, the lowest percentage of infected ticks with this spirochaete (4.9%) was found in Area No. I, a large meadow surrounded by deciduous forest. The site is located near a railway line and a narrow road used by local residents, the noise of which can deter, and therefore limit, the number of animals living in this area. In areas II and VI, the number of ticks with *B. burgdorferi s.l.* was similar and constituted 7.9% and 8.6%. Area III is a small meadow located next to a pine forest, where 13.9% of the above pathogen was detected in ticks.

The results of the presented study reveal a large variation in the percentage of infection with *B. burgdorferi* s.l., spirochetes in individual populations of *I. ricinus* in tourist and recreational areas in southern Poland, and clearly indicate the circulation of pathogens for the transmission of diseases in nature. Additionally, it is assumed that females transmit the majority of tick-borne diseases, and are therefore one of the most dangerous developmental stages of this tick species. This fact was confirmed by the results concerning *B. burgdorferi* s.l. in Barcice. Infections among females were established at each of the examined sites and predominated in Areas I-IV and VI. The largest number of infected females were found at Area No. IV (30.8%), where the number of infected males (20%) and nymphs (29.4%) was slightly lower. In Areas No. II and III, there were 27.3% and 26.3% infected females, not a single infected male or nymph tick was detected. In other areas, the value of infections among females was similar and ranged from 11.4% – 14.3%. The exception, where a different developmental stage prevailed, was Area No. V, where as many as 28.6% of *B. burgforferi* s.l. were detected among the nymphs. At areas No. I-III, no infections were found among the nymphs. Infected males lived only in Areas No. IV and V, which constitute 20% and 10%, respectively.

To summarize, most infections with *B. burgdorferi* s.l. were detected in females (20%), and the least in males (2%). Sytykiewicz et al. [30] and Kubiak et al. [31] also showed that females dominated the percentage of infections, from 23.6% – 43.3%. Similar results were obtained by Kiewra et al. [24] and Spausta et al. [21], where the highest number of infections were among females, 27% – 26.8%, respectively. The above-mentioned studies prove that female *I. ricinus* are one of the main vectors of this pathogen. In turn, babesiosis is a disease caused by protozoa of the genus *Babesia*, transmitted by ticks, which can be asymptomatic, with flu-like symptoms, and a severe course that leads to coma [32]. In the current study, *B. microti* was detected in 7.4% of the tested ticks, while slightly more of this pathogen was discovered in the study byf Asman et al. [33] in the Wolin National Park in north-western Poland. 10.65% of studied *I. ricinus* were infected with this protozoa.

In Barcice, the majority of ticks infected with *B. microti* were detected in Area No. I (16.7%), where infections predominated among nymphs, while in the same area the least *B. burgdorferi* s.l. infections were confirmed, where nymphs were free from this spirochete. This is the only case in which the percentage of *B. microti* infections was higher than that of *B. burgdorferi* s.l.. Statistically in Barcice, the number of ticks infected with *B. burgdorferi* s.l., was higher than the number of ticks infected with *B. microti*. In turn, in Area No. IV, no cases of ticks with *B. microti* were found, and interestingly, the highest number of *B. burgdorferi* s.l. infections were detected at the same site.

The above results may prove that the transmission of various tick-borne diseases does not depend on one another. The results also emphasize the importance of a broad spectrum of tick examination. The fact that a particular area is free or slightly infected with a given pathogen does not mean that it is not a concentration of another, equally dangerous, tick-borne pathogen. Moreover, in the current study, the greatest number of *B. microti* was found in males, where at Area No. I the infection rate was 17.6%, and at sites II and V was similar: 14.3% and 10%. In Area No. III, only one male was infected with this pathogen, and in Areas IV and VI, males were free from *B. microti*. In females, this pathogen was found only in Areas No. I and VI – 13.6% and 6.7%, respectively. Among nymphs, the highest percentage of the pathogen was detected at Areas I and VI, similarly at 28.6% and 20%, and at Area II only 3.8% of infections. In turn, in Areas III – V, nymphs were not infected with the above-mentioned pathogen.

To sum-up, highly statistically significant differences were revealed in the comparison between the gender of mature individuals, where statistics show significantly more males than females are infected with *B. microti,* and more females than males are infected with *B. burgdorferi* s.l.

Anaplasmosis is a systemic infectious disease transmitted by ticks and caused by *A. phagocytophilum* bacteria. The frequency of anaplasmosis and the risk of a more severe course of the disease increases among the elderly population and in those with a weakened immune system. The disease can range from a mild, even asymptomatic course, to a very severe, life-threatening infection with high fever [34]. In Barcice, *A. phagocytophilum* was detected in only 0.3% of all tested ticks. This pathogen was confirmed in only one female tick in Area III, a small meadow located next to a pine forest. A similar percentage of infected ticks was detected in West Pomerania, north-western Poland – 1.01% [33]. More ticks infected with this rickettsia were detected in western Poland – 2.5% [25]. In the Pomeranian Province, the percentage of ticks infected with the above pathogen was 14% [29].

CONCLUSIONS

This study is the first to have been carried out in the area of the Poprad Landscape Park (PLP) of southern Poland. The results obtained indicate the need to monitor places of the natural occurrence of ticks and natural outbreaks of tick-borne diseases. They also indicate a potentially high risk of exposing people to infection with tick-borne pathogens, mainly *B. burgdorferi* s.l., and *B. microti* in the areas of PLP. The zones of national and landscape parks, tourist trails, as well as tourist and recreational places where many people spend time, are especially important. Knowledge about the occurrence of ticks and the risk of human exposure to tick-borne diseases in these areas in Poland is still not sufficiently widespread, and awareness of the inhabitants about the potential threat is also very low. To reduce the risk of people contracting tick-borne diseases in areas where ticks live, especially in forest ecosystems, it is necessary to educate people about the common occurrence of *I. ricinus ticks*. It is important to popularize research on ticks and, more importantly, spread knowledge about prevention methods and the threats posed by tick-borne diseases.

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