

COINCIDENCE OF THREE PATHOGENS (*BORRELIA BURGdorFERI* SENSU LATO, *ANAPLASMA PHAGOCYTOPHILUM* AND *BABESIA MICROTI*) IN *IXODES RICINUS* TICKS IN THE LUBLIN MACROREGION

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Abstract: Ticks are very important vectors of pathogenic microorganisms (viruses, bacteria, protozoans), which may induce serious contagious diseases in humans and in farm animals. The aim of the study was to determine the coincidence of 3 pathogens: *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in *Ixodes ricinus* ticks in south-eastern Poland and to estimate the degree of infection with each of the examined pathogens depending on the developmental stage of ticks (nymph, female, male). The study material were 1,620 *Ixodes ricinus* ticks collected at 5 sites in the Lublin macroregion, showing the presence of various forest biotopes. The PCR method was used to identify DNA for *B. burgdorferi* and *A. phagocytophilum*, and the nested-PCR – for *B. microti*. In 1,368 (84.44%) of the 1,620 examined ticks no infections were found. Single infections were noted in 217 ticks (13.4%) and coinfections were detected in 35 specimens (2.16%). The most common was the coincidence of *A. phagocytophilum* with *B. microti* (17 infected specimens, 1.05% of the total number). A similar result was obtained for the coincidence of *B. burgdorferi* s. l. with *A. phagocytophilum* (15 infected specimens, 0.93% of the total number). Only 2 cases of the coinfection of *B. burgdorferi* s. l. with *B. microti*, which equals 0.12% of the total number, were found. Infection with all 3 pathogens was identified in only 1 female tick (0.06% of the total number).

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INTRODUCTION

Ticks are very important vectors of pathogenic microorganisms (viruses, bacteria, protozoans), which may induce serious contagious diseases in humans and in farm animals. The coinfection of ticks with several various pathogens increases the probability of infecting the host with more than one microorganism. In Central Europe including Poland, the main vector of germs is the common tick – *Ixodes*

ricinus, which may be infected simultaneously with, e.g. the tick-borne encephalitis and meningitis virus, *Borrelia burgdorferi*, *Anaplasma phagocytophilum* or *Babesia microti* [13, 14, 21, 33]. Studies on the frequency of coinfection of ticks with various pathogens in a determined area may facilitate the prognosis of infections coincidence in humans, which is significant for the correct diagnostics and prophylaxis of tick-borne diseases.

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The aim of this study was to determine the coincidence of 3 pathogens: *Borrelia burgdorferi* sensu lato (s. l.), *Anaplasma phagocytophilum* and *Babesia microti* in *Ixodes ricinus* ticks in the Lublin macroregion, and to estimate the degree of infection with each of the examined pathogens depending on the developmental stage of ticks (nymph, female, male).

MATERIALS AND METHODS

Collection of ticks and biotopes characteristics. The study materials were 1,620 *Ixodes ricinus* ticks collected in south-eastern Poland on the territory of Lublin macroregion, from the following 5 forest districts, characterized by the presence of various biotopes: • Dąbrowa, the suburban recreational area near city of Lublin, harbouring dry upland forests; • Zwierzyniec, situated on the area of Roztocze Highlands, harbouring dry highland forests; • Parczew, harbouring wet lakeland forests; • Puławy, harbouring dry upland forests; • Pojezierze Łęczyńsko-Włodawskie, harbouring wet lakeland forests. The ticks were collected in 3 subsequent vegetative seasons from April to October in 2004–2006. They were collected with the commonly used method of flagging over lower vegetation and litter, along the paths and edges of deciduous and mixed forests. Collected ticks were placed in glass tubes with 70% ethanol for further investigation.

Isolation of DNA from ticks. DNA was isolated from ticks – from every adult specimen separately and from nymphs in pools of 5 – using the ammonium method [24]. The obtained lysates were stored at -20°C until further studies.

PCR assay. The PCR method was used to identify DNA for *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, and the nested-PCR – for *Babesia microti*.

***Borrelia burgdorferi* sensu lato DNA identification.** *Borrelia burgdorferi* sensu lato (s. l.) identification was carried out using a pair of primers (Eurogentec, Seraing, Belgium): Fla1 (5' AGA GCA ACT TAC AGA CGA AAT TAA T 3') and Fla2 (5' CAA GTC TAT TTT GGA AAG CAC CTA A 3'). The reaction mixture (20 µl) contained 0.5 U (0.25 µl) of polymerase (DyNAzyme™ II DNA Polymerase, Finnzymes Oy, Espoo, Finland), 2 µl of the reaction buffer 10× diluted (containing: 10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂, 50 mM KCl and 0.1% Triton X-100), 0.5 µl 2 mM dNTPs (final concentration 0.05 mM) (Fermentas, Vilnius, Lithuania), 400 pmol (0.8 µl) of each Fla1 and Fla2 primer (Eurogentec, Seraing, Belgium), 13.65 µl of redistilled water and 2 µl of matrix DNA from tick isolates [37].

DNA amplification consisted of the initial denaturation (3 min at 95°C) and 35 cycles; each of them included the proper denaturation (30 sec at 94°C), primers annealing (45

sec at 54°C), elongation (45 sec at 72°C), and the final elongation (7 min at 72°C).

DNA amplification products were detected in 2% agarose gel (BASICA, LE, Prona, EEC) diluted in TBE buffer, pH 8.3. After electrophoresis in the standard conditions and staining in ethidium bromide, the 482 bp-long products were read under UV light.

The positive control was strain *B. burgdorferi* s. l. B0-148c/2 (obtained by courtesy of Dr. habil. Joanna Stańczak, Interdisciplinary Institute of Maritime and Tropical Medicine, Medical University of Gdańsk, Poland). The negative control, instead of matrix DNA, was double-distilled water (DDW).

***Babesia microti* DNA identification.** 18S rRNA coding gene for a small ribosome subunit was used as a genetic marker to detect *B. microti* DNA. The PCR-nested method was used with two pairs of primers (Eurogentec, Seraing, Belgium): Bab1 (5'-CTT AGT ATA AGS TTT TAT ACA GC-3') and Bab4 (5'-ATA GGT CAG AAA CTT GAA TGA TAC A-3'), as well as Bab2 (5'-GTT ATA GTT TAT TTG ATG TTC GTT T-3') and Bab3 (5'-AAG CCA TGC GAT TCG CTA AT-3') [22].

The reaction mixture (25 µl) contained 0.625 U (0.3125 µl) of polymerase (DyNAzyme™ II DNA Polymerase, Finnzymes Oy, Espoo, Finland), 2.5 µl of the reaction buffer 10× diluted (containing: 10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂, 50 mM KCl and 0.1% Triton X-100), 0.625 µl 2 mM dNTPs (final concentration 0.05 mM), 1 µl of each of the primers 10 µM (final concentration 0.4 µM), 17.0625 µl of redistilled water and 2.5 µl of matrix DNA from tick isolates [33]. DNA amplification consisted of the initial denaturation (1 min at 94°C) and 35 cycles; each of them included the proper denaturation (1 min at 94°C), primers annealing (1 min at 60°C), elongation (2 min at 72°C), and the final elongation (7 min at 72°C). Electrophoresis was performed in 2% agarose gels in the standard conditions. The gels were stained in ethidium bromide and read under UV light. 238 bp electrophoresis strips were considered positive.

The positive control was DNA isolated from the blood of mice experimentally infected with *B. microti* – the King's 67 BALB/c mouse-adapted strain (obtained by courtesy of professor Edward Siński, Parasitology Department, University of Warsaw, Poland). The negative control was 2.5 µl DDW corresponding to the amount of DNA used in the reaction.

Bab2 and Bab3 primers were used in reamplification. 25 µl of the reaction mixture contained the same amounts of polymerase, dNTPs solution, primers and buffer as in the first amplification. The matrix was 1 µl of the primary amplificant, 10× diluted with redistilled water. Reamplified were only those samples which brought a positive result in the first reaction. The time-temperature profile of the reaction was identical with the previous one, with the exception of the amplification time, reduced to 30 cycles.

Table 1. Numbers of *Ixodes ricinus* ticks multiply infected with pathogens: *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in the studied forest districts of the Lublin macroregion.

Studied forest district	Number of pathogens in <i>Ixodes ricinus</i> ticks								Total
	No pathogens	1 pathogen			2 pathogens			3 pathogens	
		<i>Borrelia</i>	<i>Anaplasma</i>	<i>Babesia</i>	<i>Borrelia</i> + <i>Anaplasma</i>	<i>Borrelia</i> + <i>Babesia</i>	<i>Anaplasma</i> + <i>Babesia</i>	<i>Borrelia</i> + <i>Anaplasma</i> + <i>Babesia</i>	
Dąbrowa	503 (90.5%)	24 (4.3%)	4 (0.7%)	21 (3.8%)	0 (0%)	1 (0.2%)	3 (0.5%)	0 (0%)	556 (100%)
Zwierzyniec	220 (85.6%)	11 (4.3%)	18 (7.0%)	1 (0.4%)	4 (1.5%)	0 (0%)	3 (1.2%)	0 (0%)	257 (100%)
Parczew	188 (76.7%)	28 (11.4%)	17 (7.0%)	4 (1.6%)	3 (1.2%)	0 (0%)	5 (2.1%)	0 (0%)	245 (100%)
Puławy	323 (79.0%)	14 (3.4%)	34 (8.3%)	27 (6.6%)	4 (1.0%)	1 (0.25%)	5 (1.2%)	1 (0.25%)	409 (100%)
Pojezierze	134 (87.6%)	3 (2.0%)	7 (4.6%)	4 (2.6%)	4 (2.6%)	0 (0%)	1 (0.6%)	0 (0%)	153 (100%)
Total	1368 (84.44%)	80 (4.94%)	80 (4.94%)	57 (3.52%)	15 (0.93%)	2 (0.12%)	17 (1.05%)	1 (0.06%)	1620 (100%)

The reaction products were detected in 2% agarose gels in the standard electrophoresis conditions. After ethidium bromide staining, the strips were read under UV light. The samples with a 154 bp-long strip were considered positive.

Anaplasma phagocytophilum DNA identification.

The identification of *A. phagocytophilum* DNA was based on the PCR reaction using primers (Eurogentec, Seraing, Belgium): EHR 521 (5'-TGT AGG CGG TTC GGT AAG TTA AAG-3') and EHR 747 (5'-GCA CTC ATC GTT TAC AGC GTG-3') specific for the sequence of 16S rRNA gene, coding ribosome RNA [19].

The reaction mixture (25 µl) contained 0.65 U (0.325 µl) of polymerase (DyNAzyme™ II DNA Polymerase, Finnzymes Oy, Espoo, Finlandia), 2.5 µl of the reaction buffer 10× diluted (containing: 10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂, 50 mM KCl and 0.1% Triton X-100), 2.5 µl of 2.5 mM dNTPs (final concentration 0.25 mM), 0.5 µl and 10 µM of primers EHR 521 and EHR 747, respectively, 16.175 µl of redistilled water and 2.5 µl of matrix DNA from tick isolates [7].

Amplification covered the initial denaturation at 94°C for 5 min, and 40 cycles; each of them included: the proper denaturation (45 sec at 94°C), primers annealing (45 sec at 60°C), elongation (45 sec at 72°C) and the final elongation for 5 min at 72°C. The amplification products were detected in 2% agarose gel after electrophoresis performed in the standard conditions. The gels were stained in ethidium bromide, and the results were read under UV light. The length of the amplified positive samples was 274 bp. The positive control was the HGE-1 strain cultured on KL-60 cells, which has been previously described [38]. As the negative control DDW was used in the same volume as DNA from tick isolates.

Statistical analysis method. Statistical analysis of the frequency of the studied pathogens in ticks was carried out using the chi-square test.

RESULTS

Incidence of tick-borne coinfections in the studied forest districts of the Lublin macroregion. In 1,368 (84.44%) of 1,620 examined ticks no infections were found. The highest proportion of infections (13.4%) were those with single pathogens (*B. burgdorferi* s. l., *A. phagocytophilum* or *B. microti*). In 80 ticks, i.e. in 4.94% of the total number, *B. burgdorferi* was identified. The same percentage of single infections (4.94%) was found for *A. phagocytophilum*, while slightly fewer ticks (57 specimens, 3.52%) were infected with *B. microti*.

Coinfections were detected in 35 cases (2.16%). The most common (17 infected specimens) was the coincidence of *A. phagocytophilum* with *B. microti* (1.05% of the total number). A similar result was obtained for the coincidence of *B. burgdorferi* sensu lato with *A. phagocytophilum* (15 infected specimens, 0.93% of the total number). Only 2 cases of the coinfection of *B. burgdorferi* s. l. with *B. microti*, which equals 0.12% of the total number, were found. Infection with all three pathogens was identified in only one female tick (0.06% of the total number). As assessed by chi-square test, the coincidence of *A. phagocytophilum* with *B. microti* and coincidence of *B. burgdorferi* sensu lato with *A. phagocytophilum* were significant ($p < 0.001$), while coincidence of *B. burgdorferi* s. l. with *B. microti* was not significant ($p > 0.05$).

Most coinfections were found in Puławy area, where various types of coincidence were discovered in 11 out of 409 ticks (2.7%). In almost half of the cases (5 cases, 1.2%), the coincidence of *A. phagocytophilum* with *B. microti* was identified. The fewest coinfections were found in Dąbrowa (4 cases out of 556 ticks, 0.7%), where the coincidence of *A. phagocytophilum* with *B. microti* (0.5%) prevailed. No coinfections of *B. burgdorferi* s. l. and *B. microti* were found in Zwierzyniec, Parczew and Pojezierze; similarly, no coinfection of *B. burgdorferi* s. l. and *A. phagocytophilum* was detected in Dąbrowa (Tab. 1, Figs 1, 2, 3).

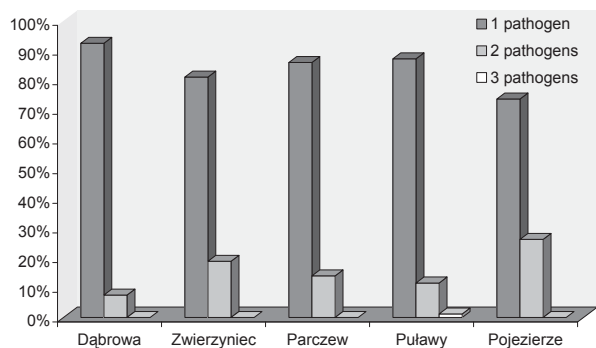


Figure 1. Frequency of *Ixodes ricinus* tick infections with three pathogens in Lublin macroregion (positive findings).

Frequency of coinfections in subsequent developmental stages of *Ixodes ricinus* ticks. Considering the developmental stage of *Ixodes ricinus* tick, nymphs were the least infected with the studied pathogens: in 94.8% of nymphs none of them was identified. 78.7% of male ticks were found to be infection-free, while 68.5% of females were not infected with any of the studied microorganisms. Most of 35 ticks with coinfections were females (14), slightly fewer were males (12), and the fewest – nymphs (9). In nymphs, infection with *B. burgdorferi* s. l. and *B. microti* prevailed (1.8% and 1.3%, respectively). Only slightly lower was the percentage of infections with *A. phagocytophilum* (1.0%). The highest number of coinfections was found for *A. phagocytophilum* and *B. microti*, which constituted 0.7% of the total number of examined nymphs.

In the case of male ticks, the percentage of specimens infected with single pathogens was similar and amounted to 6.4% for infections with *B. burgdorferi* s. l. and *B. microti*, and 5.3% for *A. phagocytophilum*. With the exception of two cases, all coinfections in this group were related to the coincidence of *A. phagocytophilum* with *B. microti*, and constituted 2.6% of the total number of examined males.

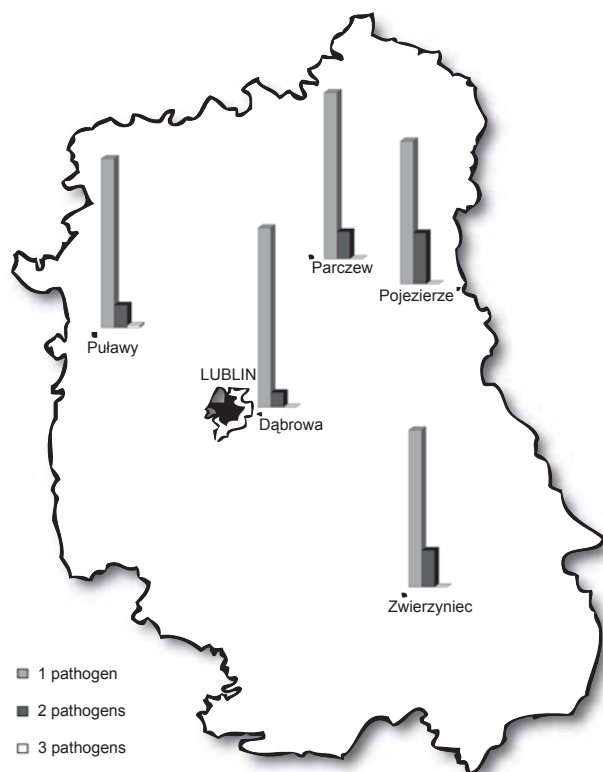


Figure 3. Distribution of the *Ixodes ricinus* ticks infection with three pathogens in Lublin macroregion (positive findings).

The main pathogens occurring singly in female ticks were *A. phagocytophilum* (12.7% of all females) and *B. burgdorferi* s. l. (10.1%). *B. microti* was found in less than half of them (5.4%). In contrary to males, in females more coinfections of *B. burgdorferi* s. l. with *A. phagocytophilum* were detected; they occurred in 11 specimens, which amounted to 2.7% of all females. Total proportions of coinfections in females, males and nymphs were 3.3%, 3.2% and 1.1%, respectively (Tab. 2, Fig. 4).

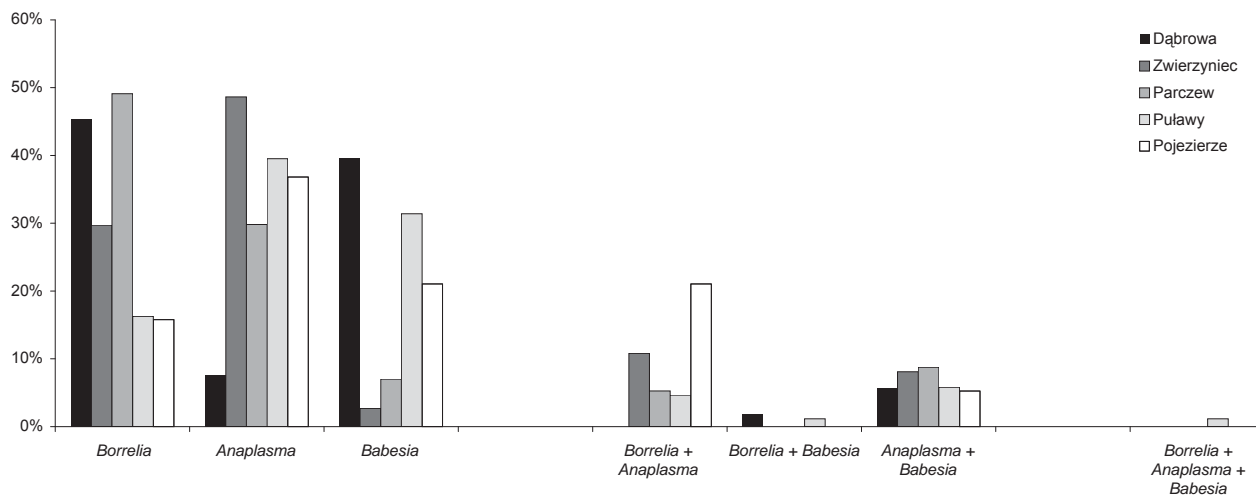


Figure 2. Frequency of *Ixodes ricinus* tick infections with single pathogens and of coinfections in Lublin macroregion (positive findings). *Borrelia* – *Borrelia burgdorferi* sensu lato; *Anaplasma* – *Anaplasma phagocytophilum*; *Babesia* – *Babesia microti*.



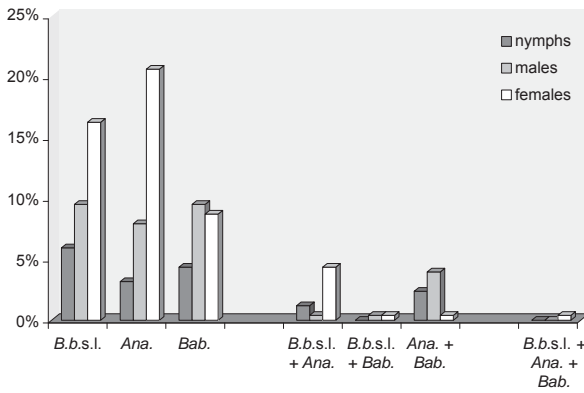


Figure 4. Frequency of *Ixodes ricinus* infections in subsequent developmental stages with 3 pathogens and their coinfections. *B.b.s.l.* – *Borrelia burgdorferi* sensu lato; *Ana.* – *Anaplasma phagocytophilum*; *Bab.* – *Babesia microti*.

DISCUSSION

To date, there have been few reports on tick coinfections with various pathogens in Poland. Thus, there is a need to conduct further studies in this field in order to precisely determine the risk of multiple infections in different parts of the country.

The percentage of *Ixodes ricinus* tick coinfections with *B. burgdorferi* s. l. and *B. microti* reported in our study is slightly lower than that found in the research conducted in the environs of Szczecin, where this kind of coinfection was found in 12 specimens (0.6%). This is also a higher than in our study (4.7%) percentage of positive results in tests for *B. microti* (6.2%) [28]. A later research in Western Pomerania shows that double coinfections constituted 3.2% of the total number of ticks. In 3 specimens (1.1%) 3 pathogens were found. The percentages higher than in our study may be due to a different number of ticks examined for the presence of pathogens (3 times more specimens in our study than in that conducted in Szczecin). This is also related to a higher proportion of ticks infected with the spirochetes *Borrelia burgdorferi* s. l. (16.7%) and the protozoa *B. microti* (13.3%) [29].

Such high indices were not confirmed by studies on tick hosts: birds and dogs. In *Ixodes ricinus* ticks collected from

birds no pathogen was identified [31]. Similarly, in the blood of dogs *Babesia* spp. and coinfection with *A. phagocytophilum* were not found [30]. Considerably higher percentages of double coinfections (10.6%) have been obtained in other studies conducted in Western Pomerania. As many as 25 out of 32 identified coinfections were those with *B. burgdorferi* s. l. and *A. phagocytophilum* (8.3% of the total number of ticks). Only in 6 specimens (2.0%) the coincidence of *A. phagocytophilum* and *B. microti* was found. Triple infections were not detected; however, also in this case, the number of 303 examined ticks was considerably lower [33]. A high percentage of double infections with *B. burgdorferi* s. l. and *A. phagocytophilum* was also reported in earlier studies by Stańczak *et al.* [32]. Among 424 specimens, 21 ticks (5%) had both pathogens, while as many as 19.2% ticks were infected with *A. phagocytophilum* and 11.6% with *B. burgdorferi* s. l.

In Germany, the coincidence of *B. burgdorferi* s. l. with *A. phagocytophilum* was found to reach the level of 0.7–0.8%, which is comparable to the percentage found in the Lublin region (1.0%). The level of tick infections with *B. burgdorferi* s. l. turned out to be almost twice as high (11.1%) as in our study (6.0%), while the percentage of tick infections with *A. phagocytophilum* was considerably lower (2.3%) than in the Lublin region (7.0%). In Moldova, coinfections with *A. phagocytophilum* and *B. burgdorferi* s. l. were 2.5% of 198 examined ticks [13]. In France, the percentage of coincidence, additionally including *Rickettsia* spp., reached even 24.5% in nymphs and 13% in female ticks [9]. However, a low percentage of double tick infections with *B. burgdorferi* s. l. and *B. microti* (one specimen, 1%) was found there [8], similarly to the results in the Lublin region (2 specimens, 0.1%). In the Netherlands, the mean proportion of coinfections with *Borrelia* and *Anaplasma* was 1.6%, while it increased to reach even 3% in ticks collected in dense oak forests [36]. Much earlier studies in the Netherlands show that 4.1% of ticks might be infected in such a way [26]. According to more recent publications, the reported percentage is much lower, ca. 2.0% [27].

Results comparable to those noted in this study were obtained in Italy, where the coincidence of *Borrelia burg-*

Table 2. Numbers of *Ixodes ricinus* ticks multiply infected with pathogens: *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in Lublin macroregion, according to developmental stage.

Developmental stage	Number of pathogens in <i>Ixodes ricinus</i> ticks								Total
	No pathogens	1 pathogen			2 pathogens			3 pathogens	
		<i>Borrelia</i>	<i>Anaplasma</i>	<i>Babesia</i>	<i>Borrelia</i> + <i>Anaplasma</i>	<i>Borrelia</i> + <i>Babesia</i>	<i>Anaplasma</i> + <i>Babesia</i>	<i>Borrelia</i> + <i>Anaplasma</i> + <i>Babesia</i>	
Nymphs	792 (94.8%)	15 (1.8%)	8 (1.0%)	11 (1.3%)	3 (0.4%)	0 (0%)	6 (0.7%)	0 (0%)	835 (100%)
Males	296 (78.7%)	24 (6.4%)	20 (5.3%)	24 (6.4%)	1 (0.3%)	1 (0.3%)	10 (2.6%)	0 (0%)	376 (100%)
Females	280 (68.5%)	41 (10.1%)	52 (12.7%)	22 (5.4%)	11 (2.7%)	1 (0.2%)	1 (0.2%)	1 (0.2%)	409 (100%)
Total	1368 (84.4%)	80 (4.9%)	80 (4.9%)	57 (3.5%)	15 (1.0%)	2 (0.1%)	17 (1.1%)	1 (0.1%)	1620 (100%)

dorferi s. l. with *A. phagocytophilum* was 1.2%, while the coincidence of *B. burgdorferi* with *B. microti* was 0.5% [20]. A higher proportion of coinfections was found in the regions of Italy bordering with Slovenia, where the percentage of coincidence of *B. burgdorferi* with *A. phagocytophilum* reached 4.3% [17]. In both cases, the level of tick infection with *B. burgdorferi* s. l. was higher than that obtained in our study (8.2% and 11.8–45.5%, respectively), while the level of *A. phagocytophilum* infection was lower (4.4% and 1.6%). The data are compatible with the results of tests performed among Italian foresters in whom antibodies to *B. burgdorferi* and *A. phagocytophilum* were simultaneously detected [25].

Studies conducted in Russia show that in *Ixodes persulcatus* ticks coinfections with *B. burgdorferi* s. l. and *B. microti* reached the level of 0.9%, while it was 0.12% in our studies. This research also shows that in *Ixodes ricinus*, *B. microti* infections are closely connected with *B. burgdorferi* s. l. infections, the percentage of which is considerably higher (34.0%) than that obtained in the Lublin region (6.0%) [2]. Later studies [3] on *I. persulcatus* species excluded the coincidence of *B. microti* and *Ehrlichia* spp.

In the United States, coinfections with *B. burgdorferi* s. l. and *A. phagocytophilum* among ticks: *Ixodes scapularis*, *Ixodes pacificus* and *Ixodes ricinus* reach the level of <1% to 6%, depending on the part of the country. A high proportion of such coincidence cases was found only in Westchester County, New York (26%). An equally higher level of double infections was reported for *Borrelia burgdorferi* s. l. and *B. microti* (from 2–19%); this is considerably higher compared to our results (0.12%). The results are also confirmed by tests performed on patients in the eastern part of the USA, where such coincidences were 80% of all coinfections detected in patients. The coincidence of *B. burgdorferi* and *A. phagocytophilum* was identified in 3%–15% of patients [4, 11]. It is believed, however, that these coinfections occur in ticks much more frequently than the coincidence of *B. burgdorferi* and *B. microti* [37], which is also the case in the Lublin region. Other research conducted in New Jersey indicates that *B. burgdorferi* and *Bartonella* spp. are the pathogens most often occurring together (8.4%), while coinfections with other pathogens (*B. microti*, *A. phagocytophilum*) reached a much lower level, from 0.9–1.9% [1, 12].

None of the cited studies confirmed the coincidence of all 3 pathogens in 1 specimen. However, the frequency of various combinations of these pathogens in the United States is considerably higher (<1%–28%) than in Europe (<1%–13%) [35]. In the Lublin region, coinfections in *Ixodes ricinus* ticks were found in 2.2% of the examined specimens.

Tick-borne pathogens are detected, among others, in salivary glands (*A. phagocytophilum*, *Rickettsia* spp.) and in epithelial cells of the intestines (*B. burgdorferi*). In a tick's organism they circulate independently, without influencing one another [34]. Their distribution, usually in

different organs, tissues, or even cell organelles, enables the development of several independent parasitemias in one tick. Specific ecological niches create favourable conditions for multiple coinfections [23]. During pathogens transmission to a host, an interaction between this process and coinfections was discovered. It was shown that in the case of *A. phagocytophilum* and *B. burgdorferi*, infection with one pathogen stimulates acquisition and transmission of another pathogen [16]. Coinfections of this type weaken the immunological response and cause the growth of bacteremia [27].

Experiments on mice simultaneously infected with *B. burgdorferi* and *B. microti* also confirmed the influence of both pathogens on the course of the disease. Doubly infected mice showed considerably more acute symptoms of rheumatoid arthritis than those infected only with *B. burgdorferi* [18]. Coleman *et al.* [6] did not confirm these findings in tests on BALB/c mice, nor did they observe more acute symptoms of babesiosis, concomitant with borreliosis.

Cases of multiple infections with tick-borne pathogens were also found in humans, especially in patients with diagnosed borreliosis. Studies conducted in the United States confirmed infection with more than 1 tick-borne pathogen in 39% of patients, 81% of the infections being coinfections with borreliosis and babesiosis, 9% – borreliosis and ehrlichiosis, 4% – ehrlichiosis and babesiosis. Infections with 3 pathogens were detected in 5% of patients [15]. In Sweden, borreliosis and ehrlichiosis were found in 11% of examined patients [5]. Similarly, in Poland, the coincidence of *B. burgdorferi* s. l. and *A. phagocytophilum* was identified in 10.42% of examined patients, while *B. microti* was not found [10].

Multiple tick-borne infections in humans may produce vague disease symptoms, which hinders a correct diagnosis. It is believed that coinfection with bacteria *Anaplasma* and *Bartonella* or with protozoan *Babesia* in a person with borreliosis may aggravate the course of the disease [4, 35]. On the other hand, multiple infections contribute to the diagnosis of milder forms of the underlying disease [14]. In the case of vague symptoms, knowledge of the place where a person was bitten by a tick and existing endemic foci may be helpful as it enables identification of the pathogen with a much greater probability.

Comparison of the degrees of tick infections with various pathogens in different countries, or even in different parts of the same country, is often difficult because of differences in methodology, biotope character and number of examined ticks. Despite the fact that the comparison of data from different sites is not always successful, the data may be used to estimate the risk of tick-borne diseases in individual European countries and on other continents. Examination of ticks for multiple infections enables the prognosis of such coinfections in humans, which is very important for a correct diagnosis and prophylaxis of tick-borne diseases.



CONCLUSIONS

1. Coinfections with 2 or 3 pathogens occurred in 2.16% of *Ixodes ricinus* ticks collected on the territory of Lublin macroregion (eastern Poland). The most common and statistically significant were coinfections of *Anaplasma phagocytophilum* with *Babesia microti* and *Borrelia burgdorferi* sensu lato with *Anaplasma phagocytophilum*, while those of *Borrelia burgdorferi* sensu lato with *Babesia microti* were rare and not significant.

2. Among developmental stages of *Ixodes ricinus* ticks, coinfections were more common in females and males (3.3% and 3.2% respectively) than in nymphs (1.1%).

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