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ORIGINAL PAPER

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PREVALENCE OF TICK-BORNE PATHOGENS AT VARIOUS WORKPLACES IN FOREST EXPLOITATION ENVIRONMENT

WYSTĘPOWANIE PATOGENÓW PRZENOSZONYCH PRZEZ KLESZCZE NA RÓŻNYCH STANOWISKACH PRACY W ŚRODOWISKU EKSPLOATACJI LASU

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Abstract

Background: The objective of the study was the evaluation of the infection of ticks with pathogenic microorganisms at various workplaces (timber acquisition, forest growing, forest cultivation, forest protection). **Material and Methods:** Eight hundred sixty one *Ixodes ricinus* ticks collected from 4 workplaces were examined for the presence of *Borrelia burgdorferi*, *Anaplasma phagocytophilum* and *Babesia microti* by polymerase chain reaction (PCR). Then, a comparative analysis of the relative density and infection of ticks at individual workplaces was done. In the statistical analysis, Chi^2 test, and Pearson's test for correlation were applied. **Results:** The differences in infection (15.9–50%) of ticks with *B. burgdorferi* between the examined workplaces were highly significant, with the highest percentage observed at forest growing. The percentages of infection of ticks with *A. phagocytophilum* at individual workplaces ranged from 1.1–3.7%, and differences were statistically insignificant. The percentages of infections of ticks with *Babesia microti* at individual workplaces fluctuated from 3.6–4.4% and differences were also insignificant. Co-infections of ticks with 2 or 3 pathogens were rare. **Conclusions:** Co-infections with *B. burgdorferi* and *B. microti* showed a significant relationship with the workplaces, while those with *B. burgdorferi* and *A. phagocytophilum* did not show such a dependence. No significant positive correlation was found between the relative density of ticks and the frequencies of infections with *B. burgdorferi*, *A. phagocytophilum* and *B. microti*. Med Pr 2014;65(5):575–581

Key words: *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia burgdorferi*, *Ixodes ricinus* ticks, forestry, workplaces

Streszczenie

Wstęp: Celem badań była ocena zakażenia kleszczy występujących na różnych miejscach i stanowiskach pracy w leśnictwie (uzyskiwanie drewna, hodowla lasu, uprawa lasu, ochrona lasu) patogenami przenoszonymi przez kleszcze. **Materiał i metody:** Z 4 miejsc pracy zebrano 861 kleszczy *Ixodes ricinus*, które zbadano na obecność *Borrelia burgdorferi*, *Anaplasma phagocytophilum* i *Babesia microti* przy pomocy łańcuchowej reakcji polimerazy (polymerase chain reaction – PCR). Następnie przeprowadzono analizę porównawczą względnej gęstości kleszczy i ich zakażenia w poszczególnych miejscach pracy. W analizie statystycznej zastosowano test Chi^2 i test korelacji Pearsona. **Wyniki:** Różnice w zakażeniach kleszczy *B. burgdorferi* (15,9–50%) zebranych z różnych miejsc pracy były statystycznie wysoce istotne, a najwyższe zakażenie kleszczy zaobserwowano w miejscu hodowli lasu. Odsetki zakażeń kleszczy *A. phagocytophilum* i *B. microti* na poszczególnych miejscach pracy wahały się odpowiednio od 1,1% do 3,7% i od 3,6% do 4,4%, a różnice były nieistotne statystycznie. Współzakażenia kleszczy 2 lub 3 patogenami występowały rzadko. **Wnioski:** Wykazano istotną zależność koinfekcji *B. burgdorferi* i *B. microti* od stanowiska pracy, natomiast nie stwierdzono jej dla współwystępowania *B. burgdorferi* i *A. phagocytophilum*. Nie wykazano korelacji między względną aktywnością a zakażeniem kleszczy *B. burgdorferi*, *A. phagocytophilum* i *B. microti*. Med. Pr. 2014;65(5):575–581

Słowa kluczowe: *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia burgdorferi*, kleszcze *Ixodes ricinus*, leśnictwo, miejsca pracy

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INTRODUCTION

Among the biological agents causing occupational zoonoses, those transmitted by ticks (bacteria, viruses, protozoa) are of great importance because they cause dangerous infections in humans, often described as 'emerging zoonoses' (1–4). Ticks (*Ixodida*) are known as vectors transmitting microbial pathogens from animals to humans, and the diseases caused by these microbes constitute a serious epidemiological problem, especially in the environment of forest exploitation and at agricultural work (4–6).

In Europe, the common tick – *Ixodes ricinus* (3,4,7,8) is the primary vector transmitting following pathogens: spirochetes *Borrelia burgdorferi* which belongs to the pathogenic agents obligatorily transmitted by ticks, causing a multisystem disease – borreliosis (Lyme disease), tick-borne encephalitis viruses, rickettsiae *Anaplasma phagocytophilum* causing human granulocytic anaplasmosis, and protozoa of the genus *Babesia* being the cause of babesiosis in humans (1,2,4).

Lyme borreliosis is the most common vector-borne disease both in Europe and North America. According to the Central Register of Occupational Diseases kept at the Institute of Occupational Medicine in Łódź, in 2011 in Poland, 649 cases of occupational infectious and invasive diseases were registered, including 474 cases of infectious or parasitic diseases in the sector of agriculture, forestry, hunting and fishery. In these occupational groups, the most frequent disease was borreliosis, it accounted for 94.3% of the total cases (9). In the years 2009–2011, the total number of occupational infectious and parasitic diseases in forestry amounted to 1041 cases, among which borreliosis constituted 99.1%, i.e., 1032 cases (10).

Borrelia burgdorferi are characterised by a very complicated genetic structure which enables them to effectively adapt to the human organism and to avoid immune response through the strategy of 'stealth pathology,' which covers, among other things, the immunosuppressive effect of *B. burgdorferi*, its development in various tissues, secretion of harmful substances, and antigenic variability (11). The taxonomy of *B. burgdorferi* is based on genetic criteria; hence, in the scientific literature, the term '*Borrelia burgdorferi* sensu lato' is used to describe a collective species which comprises a number of genospecies, of which *Borrelia burgdorferi* sensu stricto, *Borrelia afzelii* and *Borrelia garinii* occur most frequently in Europe and cause arthritis, skin infections and neural infections (4,12,13).

The agent of human granulocytic anaplasmosis (HGA) is the obligate intracellular bacterium *Anaplasma phagocytophilum* classified into rickettsia. The risk of *A. phagocytophilum* infections in groups of occupational risk, including clinical (also asymptomatic) cases of HGA after tick bites has been described in several reports (4,14,15). The course of *A. phagocytophilum* infection is variable, from asymptomatic forms, through mildly symptomatic (flu-like), to very severe forms ending in death, especially in the elderly, and in patients with autoimmune diseases or decreased immunity (14,15).

In recent years, attention has been paid to infections caused by protozoa of the genus *Babesia* (*Apicomplexa*, *Piroplasmida*). These protozoa form a very abundant group of intraerythrocytic parasites which cause babesiosis. The course of the disease depends, among others, on the immune status of a patient, and the protozoan species. Since 1956, in Europe, more than 30 cases of babesiosis in humans have been described. In Poland, only 7 cases of human babesiosis were reported, including 5 probable cases in forestry workers, and 1 case of co-infection with *Borrelia burgdorferi* (6,16). Babesiosis, as one of the tick-borne diseases in humans, may be of importance for blood banking (17).

The occurrence of mixed infections (co-infections) transmitted by *Ixodes ricinus* ticks is a phenomenon of great epidemiological importance, which may aggravate the course of tick-borne diseases. In an individual tick, co-infections with viruses, bacteria, and protozoa may form some type of micropopulations (parasitocenoses), typical for ecologically different areas (2,8,18).

Objectives

In the available literature, there is a lack of reports concerning the risk of infection with tick-borne pathogens at individual sites and workplaces in the environment of forest exploitation. Hence, the objective of our study was the evaluation of the infection of ticks with various pathogens at the following workplaces: timber acquisition, forest growing, forest cultivation, forest protection.

The aims of this study were the following:

- to evaluate the occurrence of tick-borne pathogens, namely *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia microti*, in questing *I. ricinus* ticks and the exposure of forest exploitation employees to potential infections caused by these pathogens,
- to perform a comparative analysis of infections and co-infections of ticks at individual workplaces,
- to carry out a comparative evaluation of the density and infection of ticks at individual workplaces.

MATERIAL AND METHODS

Ticks (males, females and nymphs of *Ixodes ricinus*) were collected from vegetation using the flagging method at the 4 workplaces characterised below (i.e., forest growing, acquisition of timber, forest cultivation, and forest protection) in 1 randomly selected forestry management in the Lublin province (eastern Poland), where the density of *I. ricinus* has been previously determined (25).

A. Acquisition of timber – i.e., felling trees, timber trimming, measurement, classification, transport; workplaces: lumberjack saw operators, forest workers, foresters, sub-foresters, supervising engineers. Acquisition of timber took place in the mid part of a mixed forest with the predominance of deciduous trees, comprising: oak, hornbeam, maple, ash, elm, pine. The forest is characterized by rich undergrowth and light insolation.
B. Forest growing (work activities in forest tree nurseries) – i.e., watering of nursery plants, misting, spraying, weeding, fertilizing with compost; workplaces: forest workers, foresters, sub-foresters. It was located on an isolated glade within a mixed forest with the prevalence of coniferous trees, comprising: pine, hornbeam and birch. The area is characterized by very poor undergrowth.
C. Forest cultivation – i.e., work activities associated with the care of young pine forests (i.e., weeding, thinning, replanting, elimination of undesirable species; workplaces: forest workers, senior forest engineers, assistant forest engineers, and forest inspectors). The young pine forest is located at the boundary of a mixed forest and a farm area, and characterized by rich undergrowth and abundant insolation.
D. Forest protection (pest control, counting forest stand and protected animals, patrolling stands); workplaces: forest rangers, foresters, sub-foresters, forest service employees. The area is located within a mixed forest with the predominance/frequent presence of beech and fir. The area is characterized by poor undergrowth, as compared to areas A and C, and by light insolation.

Relative ticks' density was determined by means of a combined method of a single sample and area sampling. A forest area of approximately 100 m^2 was brushed with a flag. The number of ticks collected by 1 person during 1 hour on such a delineated area was considered as the relative density of ticks at a given site/workplace of a forestry employee (19).

Isolation of DNA from ticks

Deoxyribonucleic acid (DNA) from ticks was isolated using the alkaline hydrolysis method with ammonium solution according to Rijpkema et al. (12).

Polymerase chain reaction (PCR and nested PCR) for the amplification of *Borrelia burgdorferi* s.l. genospecies

Ticks were examined for the presence of individual pathogens by means of polymerase chain reaction (PCR). The *fla* gene fragment of *Borrelia burgdorferi* sensu lato (s.l.) was amplified. All positive PCR products/amplicons, showing the presence of *B. burgdorferi* s.l., were further tested for the presence of 3 pathogenic genospecies of *B. burgdorferi* using nested-PCR and the following pairs of primers: BB1/BB2 for *B. burgdorferi* sensu stricto (s.s.), BA1/BA2 for *B. afzelii* and BG1/BG3 for *B. garinii* (Eurogentec, Seraing, Belgium), as described by Wodecka and Skotarczak (20).

PCR assay for the detection of *Anaplasma phagocytophilum*

Indentification of DNA of *Anaplasma phagocytophilum* was carried out according to Massung et al. (21) based on the results of PCR with the use of HER 521 and HER 747 primers specific for the 16S rRNA gene sequence, and nested PCR, where the msp4 fragment was identified.

PCR assay for the detection of *Babesia microti*

In order to identify *Babesia microti*, a 2-stage/step amplification of the 18S rRNA gene fragment was performed. In the first reaction amplification, the Bab1 and Bab4 primers were applied, whereas in the nested PCR – the Bab2 and Bab3 primer pair was used. Amplifications were performed according to Pershing et al. (22).

Sequence analysis

The reaction products showing the presence of DNA of individual pathogens were subjected to sequencing on the Ambi Prism 310 Genetic Analyzer (Applied Biosystem, USA) using Ambi Prism Big Dye Terminator Cell Sequencing (Applied Biosystem) kits. The results of sequencing were compared with the sequences published in the Gene Bank database using the Blast tool (USA).

Statistical analysis was performed with Chi^2 test, Pearson's test for correlation, using Statistica for Windows 6.0 package (Statsoft, Tulsa, Oklahoma, USA).

RESULTS

Infection of ticks with *Borrelia burgdorferi* s.l.

In total, DNA of *B. burgdorferi* s.l. was detected in 204 (23.7%) out of the 861 tested ticks. The highest prevalence of infections was noted at the workplace of forest cultivation whereas the lowest at the site of timber acquisition. At the 2 remaining workplaces (forest cultivation and forest protection), the prevalence of infections was similar (Table 1). Differences between workplaces in infection rate of ticks with *B. burgdorferi* were statistically significant ($Chi^2 = 31.891$, df = 3, $p < 0.001$). The infection rate determined at forest growing (50%) was significantly higher (t-test, $p < 0.001$), as compared to the mean infection rate (22.8%) for other workplaces. The percentage of the genospecies *B. burgdorferi* s.s. constituted 70.1% of all of the infections (single and co-infections with other *B. burgdorferi* s.l. genospecies) of *I. ricinus* ticks, while for *B. afzelii* this percentage was 44.2%, and for *B. garinii* 53.9% (Table 2).

Table 2. Total occurrence of *Borrelia burgdorferi* sensu lato genospecies in infected ticks (single and mixed infections)
Tabela 2. Występowanie genogatunków *Borrelia burgdorferi* sensu lato u kleszczy (zakażenia pojedyncze i mieszane)

Genospecies Genogatunek	Positive with *B.b.* s.l. vs. infected (total) Kleszcze z *B.b.* s.l. vs zakażone (ogółem) [n (%)]
Borrelia burgdorferi s.s.	143/204 (70.1)
Borrelia garinii	110/204 (53.9)
Borrelia afzelii	90/204 (44.2)

s.s. – *sensu stricto*, *B.B.* s.l. – *Borrelia burgdorferi* sensu lato.

Infection of ticks with *Anaplasma phagocytophilum*

As presented in Table 1, the total prevalence of *A. phagocytophilum* infection in questing ticks was 2.7%. The highest number of ticks infected with *A. phagocytophilum* was observed at the sites of forest protection and forest growing, while at the workplaces of forest cultivation and timber acquisition this percentage was lower. Differences in the infection rate of ticks with this pathogen between workplaces were statistically insignificant ($Chi^2 = 1.633$, df = 3, $p = 0.652$).

The sequence analysis of positive samples confirmed that the amplified product showed 100% homology with the known *A. phagocytophilum* sequences; accession number JN1 81069 (*Anaplasma phagocytophilum* strain LT.Glt8 16S ribosomal RNA gene).

Infection of ticks with *Babesia microti*

In total, 35 (4.1%) out of the 861 ticks were tested positive for the presence of *Babesia microti*. At all of the

Table 1. Prevalence of tick-borne pathogens and relative tick density at various workplaces
Tabela 1. Występowanie patogenów w kleszczach i względna gęstość kleszczy na różnych stanowiskach

Workplace Miejsce pracy	Ticks (relative density) Kleszcze (względna gęstość)	Ticks infected by pathogens vs. examined Kleszcze zakażone vs badane [n (%)]					Examined ticks (total) Kleszcze badane (ogółem) [n (%)]
		infections (total) zakażenia (ogółem)			co-infections współzakażenia		
		B. burgdorferi s.l.	*B. microti*	*A. phagocytophilum*	*B. burgdorferi* s.l. + *B. microti*	*B. burgdorferi* s.l. + *A. phagocytophilum*	
Acquisition of timber / / Uzyskiwanie drewna	42.0	29/182 (15.9)	8/182 (4.4)	2/182 (1.1)	1/182 (0.6)	0/182 (0.0)	182 (100)
Forest growing / / Hodowla lasu	8.0	14/28 (50.0)	1/28 (3.6)	1/28 (3.6)	0/28 (0.0)	1/28 (3.6)	28 (100)
Forest cultivation / / Uprawa lasu	35.0	78/326 (23.9)	12/326 (3.7)	8/326 (2.5)	6/326 (1.8)	4/326 (1.2)	326 (100)
Forest protection / / Ochrona lasu	37.0	83/325 (25.5)	14/325 (4.3)	12/325 (3.7)	7/325 (2.1)	1/325 (0.3)	325 (100)
Total / Ogółem	122.0	204/861* (23.7)	35/861 (4.1)	23/861 (2.7)	14/861** (1.6)	6/861 (0.7)	861 (100)

s.l. – *sensu lato*.
* Differences between 4 workplaces were statistically significant / Różnice między 4 stanowiskami były statystycznie istotne: $Chi^2 = 31.891$, df = 3, $p < 0.001$.
** Differences between 4 workplaces were statistically significant / Różnice między 4 stanowiskami były statystycznie istotne: $Chi^2 = 10.57$, df = 3, $p = 0.015$.

workplaces the percentage of infections of ticks was similar and ranged from 3.6% to 4.4% (Table 1). Differences in infection rate of ticks with this pathogen at individual workplaces were statistically insignificant ($Chi^2 = 0.131$, df = 3, p = 0.988).

The sequence analysis of positive samples confirmed 100% homology with *B. microti* sequences; accession number – EU 882727 (*Babesia microti* 18S ribosomal RNA gene).

Co-occurrence of *Borrelia burgdorferi*, *Anaplasma phagocytophilum* and *Babesia microti*

Co-infection with *B. burgdorferi* and *A. phagocytophilum* was observed in 6 (0.7%) out of the 861 examined ticks (Table 1.). The statistical analysis of co-infections with *B. burgdorferi* and *A. phagocytophilum*, performed using Chi^2 test, showed that differences between A–D workplaces were insignificant ($Chi^2 = 6.00$, df = 3, p = 0.112).

The percentage of co-infection with *B. burgdorferi* and *B. microti* in the 861 examined ticks was 1.6%. In the case of co-infections with *B. burgdorferi* and *B. microti*, differences between A–D workplaces were significant ($Chi^2 = 10.57$, df = 3, p = 0.015).

In the present study, neither co-infections with *Anaplasma phagocytophilum* and *Babesia microti*, nor co-infections with 3 pathogens, i.e., *Borrelia burgdorferi*, *Anaplasma phagocytophilum* and *Babesia microti* were found in *Ixodes ricinus* ticks.

Correlation between relative density of ticks and infection rates at individual workplaces

No significant positive correlation was found between the relative density of ticks and infection rates with *B. burgdorferi*, *A. phagocytophilum*, and *B. microti*. The correlation between relative density of ticks and infection rate with *B. burgdorferi* was significant (R = –0.988, p = 0.012), but negative. No significant relationship was found between the relative density and infection rates of ticks with *A. phagocytophilum* (R = –0.595, p = 0.405) and *B. microti* (R = 0.758, p = 0.242).

DISCUSSION

In the available literature, no reports have been found describing the relationship between the infection of ticks with individual pathogens and the density of ticks occurring at an individual workplace in the environment of forest exploitation; therefore, the results of the present study in this area cannot be directly compared with the results obtained by other researchers. However, some authors, such as Boyard et al. (23) and Estrada-Pena et al. (24), indicate that the risk of exposure to *Borrelia*-infected ticks depends on the properties of the forest environment, such as: the composition of tree species, undergrowth, fragmentation, microclimate. These environmental conditions vary to a great extent between climate zones and countries and depend on forest managements. Some differences between workplaces can also exist, warranting a greater exposure at a given workplace, as compared to others.

Our previous studies concluded, on the basis of the density and infectivity of *Ixodes ricinus* ticks study, that forestry workers performing work at different workplaces are nearly at the same risk of *B. burgdorferi* infections (25). The results of present research suggest greater risk of exposure to *Borrelia*-infected ticks possible at forest growing workplaces, where the wet environment and the presence of reservoir hosts can promote the presence of infected ticks. The present studies indicate that the percentage of *I. ricinus* ticks infected with *B. burgdorferi* was 23.7% of the total number of ticks examined, and was considerably higher than in the previous years (26). This phenomenon might have been due to the increasing numbers of reservoirs and the vector of *B. burgdorferi*. The results of the present studies in this area remain within the scope of results obtained by other scientific centres in Europe (2,7,8,13,27).

In the presented study, no positive correlation between the *Ixodes ricinus* density and the infection with *B. burgdorferi* was found. Such a phenomenon was also observed by Petko et al. (19), Wójcik-Fatla et al. (28) and Nazzi et al. (27). Similarly to 2006, in the presented study, it was confirmed that *B. burgdorferi* s.s. (26) was the dominant genospecies within *B. burgdorferi* s.l., whereas in most other reports from Central Europe, *Borrelia afzelii* and *Borrelia garinii* (2,7,13,24) are the genospecies which occur most frequently.

Earlier studies of the occurrence of *A. phagocytophilum* in ticks, collected from the mid-eastern Poland, conducted by Tomasiewicz et al. (29), showed much higher infections of *I. ricinus* (13.1%), as compared to the present studies. In the studies published by the above-mentioned researchers, only EHr1 and EHr2 primers were used which, as a result of PCR, provide a product which can also amplify other microbes (e.g., *Bartonella* spp.). The percentage of ticks infected with *A. phagocytophilum* observed in the

present studies was within the range of the results found by other authors in Germany (3), Switzerland (1) and Spain (30).

Lack of correlation between the infection of ticks from the south-eastern Poland with *A. phagocytophilum* and their density was demonstrated also by Wójcik-Fatla et al. (28).

The percentage of ticks infected with *Babesia microti* in own studies was 4.1%, and it was higher than that obtained by other European researchers (2,3,8).

Infections of *I. ricinus* caused by more than one pathogen (mostly *B. burgdorferi* and *A. phagocytophilum*) were noted in various European scientific centres (2,8,18).

Our studies have shown that the risk of tick infection with one pathogen does not depend on other pathogens. In contrast, Vaclav et al. (31) suggested that *Anaplasma* can play a role in suppressing the transmission of *Borrelia* to tick vectors.

CONCLUSIONS

1. Employees of forest exploitation, at such workplaces as timber acquisition, forest growing and cultivation, and forest protection are potentially exposed to infections with *Borrelia burgdorferi*, *Anaplasma phagocytophilum* and *Babesia microti*.
2. The infection rate of *Borrelia burgdorferi* in *Ixodes ricinus* ticks depends on the workplace in forest exploitation environment. Low density of ticks at an individual site is no evidence of the low risk of infection with tick-borne pathogens.

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