

Original papers

The risk of exposure to *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, *Babesia* sp. and co-infections in *Ixodes ricinus* ticks on the territory of Niepołomice Forest (southern Poland)

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ABSTRACT. Niepołomice Forest is located about 20 kilometers east of Cracow (Małopolska province, southern Poland). Its natural and touristic values, as well as wide range of hosts occurring within indicate this to be an area of high risk of exposure to *Ixodes ricinus* and tick-borne diseases it transfers. *I. ricinus* is a common species in Poland and Europe. Its seasonal activity begins in Poland in the early spring, and ends with late autumn. A total number of 129 specimens of *I. ricinus* was collected by flagging in Niepołomice Forest. DNA was isolated by ammonia method from 30 randomly-selected individuals. PCR was used to detect tick-borne pathogens with primers specific for *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato and *Babesia* sp. Molecular studies confirmed the presence of all three pathogens in *I. ricinus*. *A. phagocytophilum* was found in 76.7%, *Babesia* sp., 60%, *B. burgdorferi* s. l., in 3.3% of studied ticks. *A. phagocytophilum* co-infection with *Babesia s*p., was found in 46.7% of the specimens. A co-infection of all three tested pathogens was recorded in one case (3.3%). In Poland the problem of tick-borne diseases is a growing issue, therefore people residing in southern Polish touristic areas should be informed about the prevention and protection against ticks.

Key words: ticks, *Ixodes ricinus*, *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Babesia* sp., co-infection, Niepołomice Forest, Poland

Introduction

The medical, veterinary and economic importance of ticks is the result of their parasitism and role as reservoirs and vectors of pathogens dangerous to humans and animals. Epidemiologically the most important species of tick in Europe is *Ixodes ricinus* (Linnaeus, 1758), whose distribution area covers almost the whole continent. It is the most common species of tick in Poland and Central Europe. Its anatomical, physiological and ecological features make this species an important link in the chain of transmission of epidemiological diseases, thus sustaining natural tick-borne disease outbreak spots. *I. ricinus* is the vector of several zoonoses in Europe, including: tick-borne encephalitis, Lyme borreliosis, anaplasmosis (granulocytic ehrlichiosis, tick-bite fever), babesiosis, tularemia, Louping ill virus and others [1]. Furthermore it can potentially cause tick paralysis, although no such case has been registered in Poland, yet [2]. In Poland *I. ricinus* is active from early spring till late autumn, it is also a parasite with no specific host requirements and attacks all terrestrial vertebrates including people.

Niepołomice Forest is located about 20 kilometers east of Cracow (Małopolska province, southern Poland). Its natural and touristic values, as well as wide range of hosts occurring within, indicate this to be an area of high risk of exposure to *Ixodes ricinus* and tick-borne diseases transferred by this tick. This large forest complex is not only a place of recreation for the inhabitants of the southern Poland, it has also certain historical value. Some sites located within its area are dedicated to soldiers who died in 1914 and 1939. Furthermore it is a scientific base for Polish researchers observing diverse flora and fauna, a location for legal hunting as well as a breeding centre of European bison (*Bison bonasus*).

Previous studies have confirmed the presence of the following tick species: *I. ricinus* [3–5], *Ixodes arboricola* Schulze et Schlottke, 1929 [6,7], *Ixodes trianguliceps* Birula, 1895 [8], *Dermacentor reticulatus* (Fabricius, 1794) [9].

The aim of this study was to estimate the prevalence of *I. ricinus* and examine the co-occurrence detection of certain tick-borne pathogens in an area as extensively used for tourism as Niepołomice Forest in southern Poland.

Materials and Methods

The area of studies and tick collection. Ticks were collected in the spring on 31 March 2011 in Niepołomice Forest (DA44 UTM), (Małopolska province, southern Poland) (Fig. 1). It is a large forest complex situated in the western part of the Sandomierz Valley, adjacent to Cracow, one of the largest cities in Poland. The total area of Niepołomice Forest covers approximately 110 km^2 of diverse environments with slight slopes. It is surrounded from each side with marshy and wet meadows, with considerable number of water reservoirs. Mountains and open valleys corridor are located in its proximity. Two rivers act as borders, the Vistula to the east and the Raba to the south, and separated and deforested wetlands lie to the north. About 11 tourist trails as well as those for horse riding, biking and education run through Niepołomice Forest, which are directly bound with nature and the environment of the Forest.

Fig.1. Localization of the Niepolomice Forest in Poland

The area is protected by six nature reserves, including the strictly controlled and only partially open forest, floral and aquatic reserves. Two Nature 2000 areas, which are examples of modern forms of nature protection within the European Union are also present. Their task is to protect natural habitats, plant and animal species regarded as important on the European scale. In addition, the „Żubrowisko" European Bison Breeding Centre is located in the Forest. Wild game management is also well developed, and hunting is legal [10].

Questing ticks collection was performed during their spring peak of activity in the area accessible for walks. The method applied for this purpose was dragging. Grass and lower sections of shrubs (up to 1.5 m in height) were swept with flannel flag measuring 100×80 cm to which questing ticks become easily attached to. Subsequently they were preserved in 70% ethanol. In the laboratory, the developmental stage, genus and species of the tick specimens were determined according to Siuda using a stereomicroscope [8] and they were preserved for the further molecular studies.

DNA isolation and detection of *Borrelia burgdorferi* s. l., *Anaplasma phagocytophilum* and *Babesia* sp. in ticks by Polymerase Chain Reaction. DNA was isolated from randomly selected tick females (n=30) collected on the territory of Niepołomice Forest. Ticks were removed from alcohol and air dried on a filter paper. Subsequently, each tick was individually immersed in 100 µl of 0.7 M NH_4OH and crushed mechanically. The samples were boiled in a heating block at 100°C for 15 min. Furthermore the caps were opened and the samples were boiled for 10 min. in order to remove ammonia. Finally, DNA concentration was measured in a nano-spectrophotometer (Implen, Germany).

For the detection of *A. phagocytophilum* a commercial kit containing buffer, synthetic

Table 1. Number of the active *Ixodes ricinus* ticks on the examined locations in the territory of Niepołomice Forest (southern Poland)

Collection site	**Number of ticks**				**Total**
	females	**males**	**nymphs**	**larvae**	
1	22	27	24	0	73
2	14	11	8	0	33
3	11	7	5	0	23
Total	47	45	37	0	129

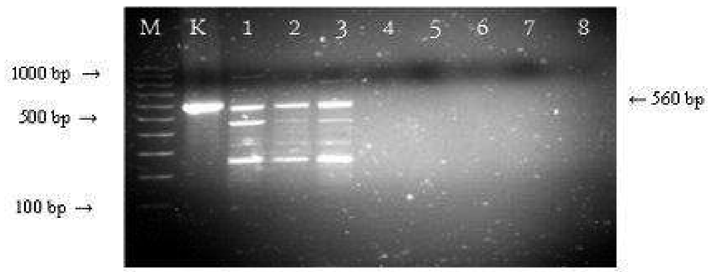

Fig. 2. The products of amplification of 18S rDNA gene fragment for *Babesia* sp., the size of positive samples was 560 base pairs [bp]. Explanations: M – marker (Blirt S.A., Poland); K – *Babesia* sp., positive control (Blirt S.A., Poland); 1–3 – positive samples; 4–8 – negative samples.

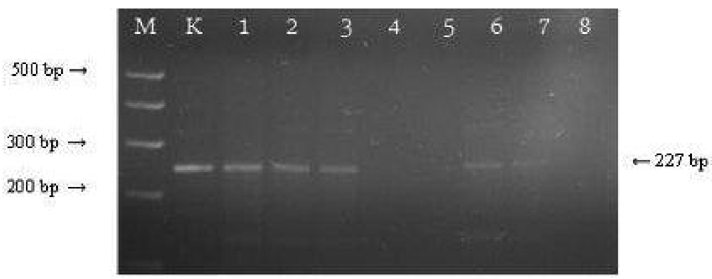

Fig. 3. The products of amplification of 16S rDNA gene fragment for *Anaplasma phagocytophilum,* the size of positive samples was 227 base pairs [bp]. Explanations: M – marker (Blirt S.A., Poland); K – *A. phagocytophilum* positive control (Blirt S.A., Poland); 1,2,3,6,7 – positive samples; 4,5,8 – negative samples.

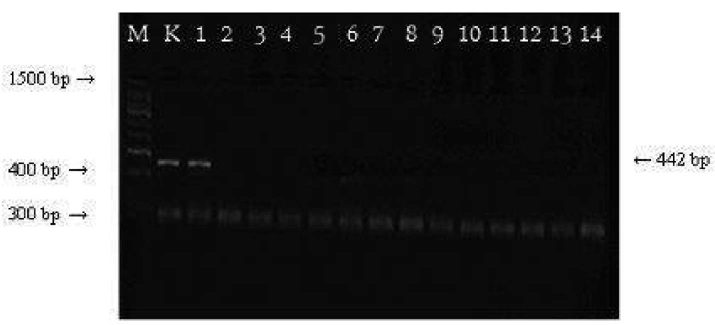

Fig. 4. The amplification products of flagelline gene fragment for *Borrelia burgdorferi* s. l., size of 442 base pairs [bp]. Explanations: M – molecular weight standard (Promega, USA); K – *B. burgdorferi* s. l. positive control; 1 – positive sample; 2–14 – negative samples.

nucleotides, polymerase and primers specific for the fragment of 16S rDNA gene (Blirt S.A., Poland) was used. PCR conditions were described in the instruction which was included within this kit.

For the detection of *Babesia* sp., similar commercial kit was used, which contained primers specific for the fragment of 18S rDNA gene, as well as buffer, synthetic nucleotides and polymerase (Blirt S.A., Poland). PCR conditions were described in the instruction which was included within this kit.

For detection of *B. burgdorferi* sensu lato the pair of specific primers: BFL1 (GCT CAA TAT AAC CAA ATG CAC ATG) and BFL2 (CAA GTC TAT TTT GGA AAG CAC CTA A) for the fragment of *fla* gene was used. Samples were initially denaturated for 2 min at 94°C. Subsequent cycles were at: 94°C for 30 sec. (denaturation), 58°C for 60 sec. (annealing) and 72°C for 60 sec (elongation). Total number of 40 cycles was performed. The final elongation was performed at 72°C for 10 min.

Electrophoresis for 1,5 h at 150V on 2% agarose gels was applied for the analysis of PCR products. DNA bands were stained with ethidium bromide and visualized by UV transillumination in the apparatus Omega 10 (UltraLum, USA). The presence of the specific products of 227 base pairs [bp] (*A. phagocytophilum*), 560 bp (*Babesia* sp.) and 442 bp (*B. burgdorferi* s. l.) was considered as a positive result.

Results

The total number of 129 ticks was collected from the following three sites in Przyborów on the territory of Niepołomice Forest:

1. Przyborów – east of the ranger's lodge.
2. Przyborów – south of the ranger's lodge.
3. Przyborów, about 2 km west of the ranger's lodge in the area „August's Oak".

All these localities were distributed irregularly, the large part of the collection was dominated by adult ticks. Sites 1 and 2 were located along the

Table 2. Prevalence of *Anaplasma phagocytophilum*, *Babesia* sp. and *Borrelia burgdorferi* s. l. in *Ixodes ricinus* female ticks collected in the Niepołomice Forest (southern Poland)

Collection site	Number of pathogens in *Ixodes ricinus* ticks						
	Number of examined ticks	single pathogen			co-existence		lack of pathogens
		A. phagocytophilum	*Babesia* sp.	*B. burgdorferi* s. l.	*A. phagocytophilum* + *Babesia* sp.	*A. phagocytophilum* + *Babesia* sp. + *B. burgdorferi* s. l.	
1	10 (100%)	7 (70%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (30%)
2	10 (100%)	1 (10%)	0 (0%)	0 (0%)	7 (70%)	1 (10%)	1 (10%)
3	10 (100%)	0 (0%)	3 (30%)	0 (0%)	7 (70%)	0 (0%)	0 (0%)
Total	30 (100%)	8 (26.6%)	3 (10%)	0 (0%)	14 (46.7%)	1 (3.3%)	4 (13.3%)

„Royal Route", which has once been used for hunting by the Polish kings since times of Casimir the Great. Nowadays, it is a paved avenue, with high pine trees on the sides forming a sort of walls resembling gorge, the pines are further gradually replaced with oaks aged over 200 years with addition of alders and birches. On the site 3, ticks were collected about 1 km from the historic place „August's Oak", planted to commemorate the Polish king Augustus II of Saxony, who used to hunt here in 1730. It is a deciduous forest area following the „Royal Route" with the shrine of St. Hubert and crossroads near the Przyborów ranger's lodge located nearby.

A total number of 30 females (10 from each site) were selected for molecular analyses. Epidemiological studies confirmed the presence of *Anaplasma phagocytophilum*, *Babesia* sp., and *Borrelia burgdorferi* s. l. (Table 1) in *I. ricinus* females. *A. phagocytophilum* was found in 23 out of 30 specimens of examined ticks (76.7%), *Babesia* sp. in 18 (60%) and spirochetes *B. burgdorferi* s. l., only in one (3.3%) (Figs 2–4). Eleven specimens of *I. ricinus* (36.7% of all ticks tested) infected with a single pathogen (*A. phagocytophilum*, *Babesia* sp.) while co-infections of *A. phagocytophilum* with *Babesia* sp., were found in 14 specimens (46.7%). One female (3.3%) was infected with all three pathogens: *A. phagocytophilum*, *Babesia* sp., and *B. burgdorferi* s. l. Only four females (13.3%) were not infected with any of the pathogens (Table 2).

The highest risk of infection has been shown for *A. phagocytophilum* at site number 2, where 9/10 (90%) ticks selected for analysis were infected with the pathogen. However, in the case of *Babesia* sp., a high risk exists in the region of site number 3, where 10/10 (100%) ticks were infected with this protozoan. There were two and three co-infections at sites 2 and 3, in contrast to site number 1, where the ticks were infected with only one pathogen. Triple co-infection (*A. phagocytophilum*, *Babesia* sp., and *B. burgdorferi* s. l.) was detected in one female collected at site No. 2, at the same time it was the only specimen positive for *B. burgodorferi* s. l. spirochetes. The research proved that half of the ticks (50%) used for the study was infected with more than one pathogen.

Disscusion

The castor bean tick, *I. ricinus* is a widespread species in Poland [8] with 165 localities currently described in the Małopolska province itself, and this number is steadily growing. Previous studies have shown that *I. ricinus* occurs throughout the Niepołomice Forest, with 11 sites recorded so far [4].

I. ricinus in Poland is active from early spring till late autumn, but has two activity peaks: in the spring (spring peak) and in late summer and fall (autumn peak). Questing ticks are definitely the most numerous during the spring activity peak, so-called autumn peak may not appear in the years with very hot and dry summer months (July and August) [8,11,12]. Seasonal activity of *I. ricinus* was also examined in detail in the south-eastern Poland, especially in the Małopolska province and did not show any significant changes [13].

I. ricinus is a parasite which quests outside host's nests. It is not too mobile and moves no further than a few meters. It is adapted to forest environment (mixed or deciduous forests, forest meadows, paths, parks, gardens) and appears in opened habitats, but covered with shrubs and bushes. The optimal habitat humidity ranges between 80–100%. This species feeds on mobile, migrating hosts, not tied closely to the designated areas of residence and reproduction. Mammals and birds are crucial in order to sustain durability of tick populations in forest ecosystems, they are both hosts for *I. ricinus* and their natural nutritional base, as well as the source and reservoirs of various pathogens [14].

Large populations of rodents (Rodentia) and insectivorous mammals (Insectivora) exist in Niepołomice Forest, as well as about 2,300 roe deer (*Capreolus capreolus*), 350 red deer (*Cervus elaphus*), 300 wild boars (*Sus scrofa*) and bisons (*Bison bonasus*) in the breeding facility. Sporadically occurring species can also be found, such as moose (*Alces alces*), wolve (*Canis lupus*), and about 175 species of birds [10]. Wildlife studies conducted in other forest areas of Poland clearly indicate that these animals can be reservoirs of tick-borne pathogens such as *Anaplasma* [15–17], *Bartonella* [18,19], *Babesia* and *Theileria* [20,21]. Species that are reservoirs of *B. burgdorferi* s. l. in Europe are mostly small and medium-sized mammals and birds. Large mammals (*C. capreolus, C. elaphus*, *A. alces*, *Dama dama*, *Bos taurus*, *Ovis aries*) are incompetent reservoirs, but they play a significant role in the circulation of this pathogen in nature [22].

The first epidemiological study on *I. ricinus* in Niepołomice Forest did not detect any pathogens [3], whereas the further research proved the presence of *B. burgdorferi* s. l. in *I. ricinus* [5]. More recent studies have shown that in various places of the Małopolska province, which is an area where Niepołomice Forest is situated, the prevalence of infection of *I. ricinus* with *B. burgdorferii* s. l. ranges from 0.0 to 66.6% [23,24]. This present study performed in Niepołomice Forest confirms for the first time the presence in southern Poland of *A. phagocytophilum* and *Babesia* sp. in *I. ricinus* and co-infection of these two (*A. phagocytophilum*, *Babesia* sp.), as well as triple infection of pathogens (*A. phagocytophilum*, *Babesia* sp. and *B. burgdorferi* s. l.). This proves that there are no safe places in Poland in terms of exposure to ticks and the diseases they carry.

The medical issue of co-infection, the presence of at least two different pathogens in the same tick, has been described and interpreted in many studies. While single infections in ticks are commonly recognized, co-infections of two, three or more microorganisms are rarely reported. The present study shows the co-infection of *A. phagocytophilum* and *Babesia* sp., in 46.7% (14/30 ticks) and co-infection of *A. phagocytophilum*, *Babesia* sp., and *B. burgdorferi* s. l. in 3.3% (1/30 ticks).

The reports on co-infections in Poland most frequently concern *I. ricinus*, and usually are caused by two different pathogens: *A. phagocytophilum* and *B. burgdorferi* s. l. [25,26], *B. burgdorferi* s. l. and *B. microti* [25–27] or *A. phagocytophilum* and *B. microti* [25,26]. Similar to the following results on co-infections in *I. ricinus* were found in northern Poland, 6 ticks (2.0%) were infected with *A. phagocytophilum* and *B. microti* [25]. Studies in the Lublin macro-region also showed co-infection of *A. phagocytophilum* and *B. microti* in 17 ticks (1.05%) [26]. Triple co-infection (*A. phagocytophilum*, *B. microti*, *B. burgdorferi* s. l.) in *I. ricinus* female (0.25%) has been found at the same time in the Lublin macro-region [26].

Comparing the results of other European authors, it can be concluded that the phenomenon of co-infection with different species of pathogens in *I. ricinus* is prevalent [25,28–34]. Internal factors, such as biology and density of ticks, feeding period, transstadial and transovarial transmissions, and external factors, such as environmental factors, climate, tourism, that affect the competence of ticks (Ixodidae) in the transmission of pathogens are strong enough to maintain the hot zones of tick-borne diseases in the wild [11,32].

Tick infection with various pathogens increases the likelihood of infection of the host with more than one pathogen. Human co-infection with different tick-borne pathogens through the bite of a tick infected with multiple pathogens, or multiple bites of ticks infected with a single pathogen, certainly makes it difficult to diagnose the patient and has an impact on the clinical course of mixed infections, which is often associated with stronger symptoms. The medical problem is not fully understood and needs further investigation [35,36].

In Poland, the number of incidences of tick-borne diseases is of increasing concern. Most cases are observed in the northern Polish regions (Podlaskie province, Warmia-Mazuria province) [25,37–40]. The results of the study indicate that a high risk of exposure to ticks and tick-borne diseases exist in southern Poland. *A. phagocytophilum*, *Babesia* sp. and *B. burgdorferi* s. l. pose a serious threat to people living in the Niepołomice

Forest. Local inhabitants and tourists should be better informed of the need to protect against ticks, and the consequences of these parasites feeding on humans. This is particularly true concerning professional groups closely related to the forest environment.

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