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The occurrence of three tick-borne pathogens in *Ixodes ricinus* ticks collected from the area of the Kraków-Częstochowa Upland (Southern Poland)

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**ABSTRACT**

The common tick *Ixodes ricinus* is the main vector of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in Europe. The aim of this study was to estimate the potential risk of exposure to common tick-borne diseases in the recreational areas of the Kraków-Częstochowa Upland, Poland with particular emphasis on the city of Częstochowa. The DNA from 459 *I. ricinus* ticks was used to detect *B. burgdorferi* s. l., *A. phagocytophilum* and *B. microti* by PCR and nested PCR methods. Generally, infectious agents were found in 26.3% of all the examined ticks: *B. microti* was found in 23.3%, and *A. phagocytophilum* in 2.4% of the ticks. The protozoan was found mainly in females and nymphs, while *A. phagocytophilum* was detected mainly in adults. The co-existence of *B. microti* and *A. phagocytophilum* was found in 2.1% of the examined ticks. The presence of *B. burgdorferi* s. l. was not revealed in the examined material. The conducted studies demonstrate the high potential risk of exposure of tourists and pilgrims to *B. microti*, and a low risk of exposure to *A. phagocytophilum* and *B. burgdorferi* s.l. in the examined areas of the Kraków-Częstochowa Upland. Furthermore, the possible co-existence of *A. phagocytophilum* and *B. microti* in 3 individual ticks means that infection by multiple infectious agents is possible. The obtained results highlight the need to conduct further research on tick-borne pathogens in this region of Poland.

**Keywords** *Borrelia burgdorferi* sensu lato; *Babesia microti*; *Anaplasma phagocytophilum*; tourism; exposure risk

**Introduction**

Ticks (Acari: Ixodida) are commonly known parasites of animals and humans. In Europe, the main vector of *Borrelia burgdorferi* sensu lato, *Babesia microti* and *Anaplasma phagocytophilum* is the common tick *Ixodes ricinus* (Balmelli and Pifarretti 1995; Blanco and Oteo 2002; Kiewra and Lonc 2004; Nowak-Chmura 2013; Yabsley and Shock 2013). These pathogens are etiological agents of borreliosis, human babesiosis and human granulocytic anaplasmosis, respectively (Spielman 1976; Burgdorfer et al. 1982; Maeda et al. 1987). Lyme disease counts among the most dangerous tick-borne diseases. There are three main clinical symptoms of this disease: chronic arthritis, neuroborreliosis and acrodematitis chronica atrophicans (ACA) (Balmelli and Pifarretti 1995). In Poland, the first case of this polymorphous disease in...
humans was described in West Pomerania (Januszkiewicz and Kieda 1987). The rickettsiae A. phagocytophilum causes human granulocytic anaplasmosis (Oehme et al. 2002; Grzeszczuk et al. 2004). The first case of this disease in Europe was recorded in Slovenia (Petrovec et al. 2001). In Poland, the first case of human anaplasmosis was reported in 2001 (Tylewska-Wierzbanowska et al. 2001). The symptoms of this disease are flu-like: fever, myalgias, arthralgias and headache (Blanco and Oteo 2002). Babesiosis in Europe was described for the first time in Yugoslavia (Boustani and Gelfand 1996). The initial symptoms of this disease are often under-recognised and confused with a cold or flu. Up to 9 weeks after the infection, we can observe non-specific symptoms such as headaches, muscle aches, fever, chills or general fatigue (Rożej-Bielicka et al. 2015). Humans can be infected by these pathogens by a tick bite if they encounter the common vector Ixodes ricinus in meadows, forests, parks and gardens (Nowak-Chmura 2013).

The Kraków-Częstochowa Upland is one of the most attractive tourist regions in Poland. This region is situated between two towns – Kraków and Częstochowa (Figure 1), popular recreational places with a lot of tourist attractions. This is particularly true for the city of Częstochowa, which is a well-known European pilgrimage city. In this city, there are a lot of parks where citizens and tourists spend their free time. The most popular, especially among pilgrims, is Jasna Góra Park located under the Jasna Góra Monastery. Work and recreation in the area of the Kraków-Częstochowa Upland can be associated with an increased risk of human exposure (forestry workers, tourists, citizens) to ticks. However, we still know little about the potential exposure risk for the transmission of tick-borne pathogens within these densely inhabited regions. So far, studies on tick fauna have only been conducted in some parts of this region. During these studies, only I. ricinus was found, suggesting that I. ricinus is the most common tick species in Poland. In addition, these faunistic studies confirmed that the tick density in the studied areas depends on the humidity conditions and air temperature (Siuda et al. 1992; Nowak et al. 2009; Solarz et al. 2007, 2010; Nowak-Chmura 2013).

Based on this previous work, the objective of the present study was to determine the potential risk of exposure of tourists and pilgrims to tick-borne infections. We therefore screened I. ricinus ticks from the selected recreational areas of the Kraków-Częstochowa Upland, with a particular emphasis on the recreational areas of Częstochowa, for the presence of B. burgdorferi s.l., A. phagocytophilum and B. microti.

**Materials and methods**

To the study on the occurrence of tick-borne pathogens, I. ricinus ticks from the collection of the Department of Parasitology, Medical University of Silesia in Katowice, Poland were used. The ticks were previously collected by flagging (Pet’ko et al. 1997) during the spring and autumn peaks in their activity in 2013-2014. Ticks came from the selected recreational areas of Klucze, Jaroszowiec, Pazurek, Rabszty, Ogrodzieniec, Mirów, Jasna Góra Park in Czestochowa (the common name for two closely situated little parks – the Staszie Park, and the 3rd of May Park, located close to the Jasna Góra Monastery), Aniołowski Forest in Częstochowa, Lisiniec Park in Częstochowa (Figures 1 and 2).

**Evaluation of the environmental impact severity**

The DNA was isolated from whole ticks by the ammonia method (Guy and Stanek 1991). The ticks were removed from alcohol and dried on filter paper. They were then placed in a sterile plastic tube with 100µl 0.7 M NH₂OH. Next, ticks were crushed mechanically. The samples were boiled at 100°C for 15 minutes in a TB-941U thermobloc (JWElectronic, Poland). Then, the caps were opened and samples were boiled at 100°C for 10 minutes to remove the ammonia. Next, the samples were centrifuged at 12 000 rotations per minute [rpm] for 5 minutes in a microcentrifuge (Hettig, Germany). After centrifugation, the supernatant was transferred to a new plastic tube. The concentration of DNA was measured spectrophotometrically in the
PEARL nanophotometer (Implen, Germany). DNA was stored at -20°C for further analysis.

*Borrelia burgdorferi* s. l. in ticks was detected using the Fla1/Fla2 primer pair, specific for the flagelline gene (Wodecka and Skotarczak 2000). The amplification mixture was as follows: 200 ng of the DNA, 2 µL of the reaction buffer diluted 10x, 0.5 µL of 2 mM dNTPs, 0.8 µL of each primer and 0.1 µL of *Taq* polymerase (ThermoScientific, USA). The sample was topped up with molecular grade water to the total volume of 20 µL. The PCR conditions were as follows: the initial denaturation (3 min at 95 °C), the proper denaturation (30 sec at 94 °C), primer annealing (45 sec at 54 °C), elongation (45 sec at 72 °C), and the final elongation (7 min at 72 °C). A total of 35 cycles was performed. The positive control of the DNA of *Borrelia afzelii* was isolated from culture and was obtained by courtesy of dr Angelina Wójcik-Fatla, PhD from The Institute of Agricultural Medicine in Lublin. To detect *B. microti* in ticks, two primer pairs – Bab1/Bab4 and Bab2/Bab3 – specific to the 18S rRNA gene of the small ribosome subunit were used (Pershing et al. 1992). The first PCR conditions were
as follows: the initial denaturation (1 min at 94 °C), the proper denaturation (1 min at 94 °C), primer annealing (1 min at 60 °C), elongation (2 min at 72 °C) and the final elongation (7 min at 72 °C); 35 cycles were performed. The amplification mixture of the first PCR was as follows: 200 ng of the DNA, 2.5 µL of the reaction buffer diluted 10x, 1 µL of 2 mM dNTPs, 1 µL of each primer and 0.1 µL of Taq polymerase (ThermoScientific, USA). The sample was topped up with molecular grade water to the total volume of 25 µL. The conditions of the second PCR were the same as the first, except that 30 cycles were performed. The amplification mixture of the second PCR was as follows: 1 µL of the first amplification product, 2.5 µL of the reaction buffer diluted 10x, 1 µL of 2 mM dNTPs, 1 µL of each primer and 0.1 µL of Taq polymerase (ThermoScientific, USA). The sample was again topped up with molecular grade water to the total volume of 25 µL. The positive control of B. microti was obtained courtesy of dr Angelina Wójcik-Fatla, PhD from The Institute of Agricultural Medicine in Lublin.

To detect A. phagocytophilum in ticks two primer pairs – ge3a/ge10r and ge9f/ge2r – specific to the 16S rDNA gene were used (Massung et al. 1998). The conditions of the first PCR were as follows: the initial denaturation (2 min. at 95 °C), the proper denaturation (30 sec at 94 °C), primer annealing (30 sec at 55 °C), elongation (1 min at 72 °C) and the final elongation (5 min at 72 °C). A total of 40 cycles was performed. The amplification mixture of the first PCR was as follows: 200 ng of the DNA, 2.5 µL of the reaction buffer diluted 10x, 1 µL of 2 mM dNTPs, 1 µL of each primer and 0.1 µL of Taq polymerase (EURx, Poland). The sample was topped up with molecular grade water to the total volume of 25 µL. The conditions of the second PCR were the same as described for the first, except that 30 cycles were performed. The amplification mixture of the second PCR was as follows: 1 µL of the first amplification product, 2.5 µL of the reaction buffer diluted 10x, 1 µL of 2 mM dNTPs, 1 µL of each primer and 0.1 µL of Taq polymerase (EURx, Poland). The sample was topped up with molecular grade water to the total volume of 25 µL. The positive control of A. phagocytophilum was obtained courtesy of dr Angelina Wójcik-Fatla, PhD from The Institute of Agricultural Medicine in Lublin.

Amplifications and reamplifications were carried out in a MJ Mini thermal cycler (BioRad, USA). The reaction products were separated electrophoretically in 2% ethidium bromide stained agarose gels at 80 V for 2 hrs, and then were visualized and photographed in the Omega 10 analyser (UltraLum, USA). The expected product sizes were as follows: 482 base pairs [bp] for B. burgdorferi s.l., 238 bp and 154 bp for B. microti, 932 bp and 546 bp for A. phagocytophilum.

The statistical analysis was performed using CSS-Statistica for Windows version 12, with a significance threshold of p-value < 0.05. Results were analyzed using the χ² test. Differences in the prevalence of pathogens in ticks were compared using the Yates corrected χ² test (2 x 2).

Results

A total of 459 I. ricinus ticks was collected, including 178 females, 138 males and 143 nymphs; all were screened for detection of B. burgdorferi s. 1., A. phagocytophilum and B. microti. Pathogens were found in 26.3% of the examined ticks. B. microti was found in 23.3%, and A. phagocytophilum was found in 2.4%. The protozoan was reported mainly in females and nymphs, whereas the rickettsiae was found mainly in males. The co-existence of both infectious agents was shown only in nymphs and males (Table 1). The presence of B. burgdorferi s. 1. was not shown in the examined material.

The highest percentage of ticks infected with B. microti was reported in Rabsztyn (41.4%), Jaroszowice (32.4%), Częstochowa (26.4%), Mirów (24.6%) and Ogrodzieniec (20.5%). It should be stressed that the difference in the percentage of infected ticks was statistically significant between Rabsztyn and Częstochowa (χ² = 5.05; p = 0.025), whereas the difference was insignificant between Rabsztyn and Jaroszowice (χ² = 1.75; p = 0.19). Moreover, differences in the percentage of ticks infected with B. microti were also statistically significant between
Table 1 Number and percentage of developmental stages of ticks infected with *Borrelia burgdorferi* sensu lato, *Babesia microti* and *Anaplasma phagocytophilum* in the studied areas of the Kraków-Częstochowa Upland.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Number of studied specimens</th>
<th>1 pathogen</th>
<th>2 pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Borrelia burgdorferi</em> sensu lato</td>
<td><em>Babesia microti</em></td>
</tr>
<tr>
<td>Female</td>
<td>178</td>
<td>0 (0.0%)</td>
<td>49 (27.5%)</td>
</tr>
<tr>
<td>Male</td>
<td>138</td>
<td>0 (0.0%)</td>
<td>22 (15.9%)</td>
</tr>
<tr>
<td>Nymph</td>
<td>143</td>
<td>0 (0.0%)</td>
<td>36 (25.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>459</td>
<td>0 (0.0%)</td>
<td>107 (23.3%)</td>
</tr>
</tbody>
</table>

Rabsztyn and Mirów or Rabsztyn and Ogrodzieniec ($\chi^2= 5.09$, $p = 0.0241$ and $\chi^2= 8.44$, $p = 0.0037$, respectively). The remaining differences, between Mirów and Jaroszowiec or Częstochowa, between Ogrodzieniec and Jaroszowiec or Częstochowa, and between Mirów and Ogrodzieniec were statistically insignificant ($\chi^2; p > 0.1$ in all cases).

*Anaplasma phagocytophilum* was shown mainly in ticks collected in the areas of Rabsztyn (4.8%) and Częstochowa (4.2%). The co-existence of *B. microti* and *A. phagocytophilum* was reported only in 2.1% of the ticks collected in Częstochowa. (Table 2).

Within Częstochowa, ticks were infected mainly with *B. microti* (Jasna Góra Park and Aniołowski Forest). However, *A. phagocytophilum* was found in Jasna Góra Park and Aniołowski Forest. The co-existence of *B. microti* and *A. phagocytophilum* was found only in ticks from the Aniołowski Forest area (Table 2). The difference in the percentage of ticks infected with *B. microti* was statistically significant ($\chi^2= 9.27; p = 0.0023$) between the Jasna Góra Park and Aniołowski Forest, but not between other localities in Częstochowa ($\chi^2; p > 0.1$).

In total, in Częstochowa, pathogens were found in 32.86% of the examined ticks. *B. microti* was found mainly in adults (31.8% of females and 25% of males), whereas *A. phagocytophilum* was mainly found in males and nymphs (5.7% and 4.5%, respectively) (Table 3). None of these values were significantly different ($\chi^2; p > 0.05$).

**Discussion**

So far, studies of tick fauna have only been conducted in the areas of the Kraków-Częstochowa Upland in Poland. These previous faunistc surveys showed a high risk of exposure of humans...
Table 3  Number and percentage of development stages of ticks infected with Babesia microti and Anaplasma phagocytophilum in the studied areas of Częstochowa.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Number of studied specimens</th>
<th>1 pathogen</th>
<th>2 pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Babesia microti</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>44</td>
<td>14 (31.8%)</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>Male</td>
<td>52</td>
<td>13 (25.0%)</td>
<td>3 (5.7%)</td>
</tr>
<tr>
<td>Nymph</td>
<td>44</td>
<td>10 (22.7%)</td>
<td>2 (4.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>37 (26.4%)</td>
<td>6 (4.2%)</td>
</tr>
<tr>
<td>Anaplasma phagocytophilum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>44</td>
<td>14 (31.8%)</td>
<td>1 (2.2%)</td>
</tr>
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</tr>
</tbody>
</table>

and animals to ticks in this region (Siuda et al. 1992; Nowak et al. 2009; Solarz et al. 2007, 2010; Nowak-Chmura 2013). Research on pathogens transmitted by these ticks was not conducted. However, it is commonly known that I. ricinus is the main vector and/or reservoir of B. burgdorferi s.l., B. microti and A. phagocytophilum in other regions of Poland (Nowak-Chmura 2013), highlighting the need for a more indepth study of their presence in the Kraków-Częstochowa Upland area.

From previous work, the highest percentage of ticks infected with B. burgdorferi s. l. was found in the Upper and Lower Silesia province, Mazowieckie, Lubelskie and Western Pomerania province (Kiewra 2014). The studies conducted in areas of southern Poland also showed that the infection prevalence ranged from 4% in the areas of Upper Silesia to up to 62% in areas of Beskid Żywiecki (Pet’ko et al. 2002; Asman et al. 2012). The presence of B. burgdorferi s. l. has not been shown in the present study. The lack of spirochaetes in the studied areas may be caused by the low number of the studied ticks. However, this suggests that the risk of human exposure to these bacteria may be low compared to other regions of Poland. It could also be that infected ticks were missed by our PCR method; indeed, only a single PCR procedure was used rather than a more powerful nested procedure. As Borrelia intensities in questing ticks may vary, it is also possible that ticks infected at low intensities were missed. However, as previous work in Poland used the same protocol (Wójcik-Fatla et al. 2009), our results may suggest that infection risk is still lower in the Kraków-Częstochowa Upland region compared to other regions.

The studies conducted in northern and north-eastern Poland showed the presence of B. microti in 0.6%-2.3% of ticks (Stańczak et al. 2004; Siński et al. 2006). Comparable results were obtained in central-eastern, eastern and south-eastern Poland. In these areas, the percentage of infected ticks ranged from 3.1% to 3.5% (Wójcik Fatla et al. 2009; Sytykieicz et al. 2012; Kiewra et al. 2014), whereas studies conducted in the areas of southern Poland showed varying levels of ticks infected with B. microti, ranging from 0%-3.9% in ticks collected from people, 35% in ticks collected from the Żywiec Landscape Park to 50.87% in ticks collected from areas of the Tarnogórski district (Asman et al. 2012; Albertyńska et al. 2016; Asman et al. 2014; Asman et al. 2015). The results of the present study are higher than those obtained from northern, eastern, central and south-western Poland and comparable to those of southern Poland. These results may indicate a high risk of exposure of humans to this pathogen in the Kraków-Częstochowa Upland region.

In Europe, the prevalence of ticks infected with A. phagocytophilum ranges from 0.4 to 66.7% (Blanco and Oteo 2002; Smrdel et al. 2010). The studies conducted in southern Poland showed similar prevalence ranges from 2.6% in Lower Silesia, 38.9% in Upper Silesia to even 76.7% in the territory of the Niepolomnice Forest (Cisak et al. 2005; Cuber et al. 2011; Asman et al. 2013; Asman et al. 2014; Kiewra et al. 2014). The studies conducted in eastern Poland by Wójcik-Fatla et al. (2009) showed only 4.9% of ticks infected with A. phagocytophilum. However, studies conducted by Stańczak et al. (2004) in northern Poland, Skotarczak et
al. (2008) in north-western Poland and Tomasiewicz et al. (2004) in mid-eastern Poland, Sytykiewicz et al. (2012) in central eastern Poland showed this rickettsia in 14%, 5.5%, 13.1% and 8.5% of examined ticks, respectively. Studies conducted in northern, mid-eastern and south-eastern Poland showed the pathogen mainly in adult ticks (Stańczak et al. 2004; Tomasiewicz et al. 2004; Cisak et al. 2005). Generally, our findings confirmed that A. phagocytophilum is reported mainly in adult ticks. The exceptions are ticks collected from the recreational areas of Częstochowa, where a higher percentage of nymphs were infected with this pathogen than adult ticks. Overall, these results are within the European average and match results obtained in western, mid-eastern, south-eastern and south-western Poland. However, they are lower than results obtained in recreational areas of southern Poland. Based on the presented results, a low risk of exposure to A. phagocytophilum for tourists and pilgrims in the Kraków-Częstochowa Upland is suggested.

There are known cases of co-existence of two or more pathogens in one tick. However, such a phenomenon usually only occurs in a small percentage of studied ticks (Stańczak et al. 2004; Wójcik Fatla et al. 2009; Asman et al. 2013; Asman et al. 2015). The study conducted by Asman et al. (2012, 2014) in southern Poland showed the co-existence of A. phagocytophilum and B. microti in 0% to 5% of ticks. The studies conducted in other regions of Poland showed the co-existence of these two pathogens ranging from 1.1% in eastern Poland, to 1.8% in central eastern Poland, and up to 10.6% in northern Poland (Stańczak et al. 2004; Wójcik Fatla et al. 2009; Sytykiewicz et al. 2012). Our results are therefore similar to those obtained in southern, eastern and central-eastern Poland, and suggest low levels of co-infections with A. phagocytophilum and B. microti ticks in the areas of the Kraków-Częstochowa Upland.

Conclusions

The conducted study suggests a high potential risk of exposure of humans to B. microti. and a low potential risk for A. phagocytophilum and B. burgdorferi s.l. in the Kraków-Częstochowa Upland area. The co-existence of the studied infectious agents was low, but may lead to difficulties in disease diagnostics. However, the presence of B. microti and A. phagocytophilum in both adults and nymphs confirms that both developmental stages of I. ricinus may be reservoirs and/or vectors for these pathogens. As the apparent lack of B. burgdorferi s.l. in the studied ticks may suggest low prevalence and/or infection intensities of this pathogen in the studied area, further studies using more powerful detection techniques are called for. The obtained results indicate that tourists, pilgrims and other people who visit, or spend their free time in these areas of the Kraków-Częstochowa Upland should use official preventive measures to avoid tick bites.

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