SHORT COMMUNICATION

First Molecular Evidence of Zoonotic Bacteria in Ticks in Bosnia and Herzegovina

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Summary

In Bosnia and Herzegovina, the tick fauna is very diverse, but data on the occurrence of zoonotic tick-borne bacteria are lacking. Thus, the aim of this study was to investigate the presence of Borrelia burgdorferi sensu lato, Anaplasma phagocytophilum, ‘Candidatus Neoehrlichia mikurensis’, spotted fever group (SFG) rickettsiae and Francisella tularensis in questing ticks. In 19 (21.8%) of 87 ticks (Ixodes ricinus, n = 30; Dermacentor reticulatus, n = 54; D. marginatus, n = 3) collected by flagging the vegetation at the collection site in the Glamoc Municipality (south-western Bosnia and Herzegovina), Rickettsia monacensis (1.1%), R. helvetica (5.7%), R. raoultii (5.7%), R. slovaca (8.0%), A. phagocytophilum (1.1%) and F. tularensis subsp. holarctica (1.1%) were detected and identified by molecular methods. None of the tested ticks were positive for B. burgdorferi s.l. and ‘Candidatus N. mikurensis’, and co-infection of R. slovaca and F. tularensis subsp. holarctica was detected in only one D. marginatus (1.1%). This study reports the occurrence of emerging zoonotic bacteria in ticks from Bosnia and Herzegovina for the first time, indicating a public health threat to humans. Therefore, physicians and practitioners should be aware of the presence of these tick-borne bacteria, especially when they are faced with acute febrile illnesses after tick exposure.

Introduction

In Bosnia and Herzegovina, Ixodes ricinus and Dermacentor marginatus are the most common and widely distributed tick species, whereas D. reticulatus has more focal occurrence (Omeragić, 2011). These tick species are principal vectors of many viral, bacterial and protozoan pathogens of medical and veterinary importance (Reye et al., 2013). In the past three decades, the interest of the scientific community for tick-borne diseases has increased with the emergence and identification of B. burgdorferi sensu lato (s.l.) as a causative agent of Lyme disease in humans in 1982. Spotted fever group (SFG) rickettsiae are mainly transmitted by ticks, which act both as vectors and as reservoirs, and they are the most common pathogens detected in I. ricinus and D. reticulatus in Europe (Reye et al., 2013). These obligate intracellular bacteria are able to cause mild to severe diseases in humans (Parola and Raoult, 2001). Other tick-borne bacteria, A. phagocytophilum, ‘Candidatus Neoehrlichia mikurensis’ and F. tularensis are also zoonotic pathogens, causing human granulocytic anaplasmosis, neoehrlichiosis and tularaemia, respectively (Parola and Raoult, 2001; Lommano et al., 2012; Reye et al., 2013). All these are considered to be emerging pathogens (Parola and Raoult, 2001; Lommano et al., 2012). To our knowledge, only a few clinical cases of Lyme borreliosis and tularaemia (Dautović-Krkić et al., 2008; Hukić et al., 2010; Arapović et al., 2014) have been reported in the country, but there are no any data available on human infections caused by other tick-borne bacteria. The main reason may be an underdiagnosing due to the lack of knowledge and awareness among physicians and practitioners. Furthermore, information on the occurrence of zoonotic bacteria in ticks in Bosnia and Herzegovina is lacking, and thus, this study aimed to investigate the presence of abovementioned pathogens in questing ticks by PCR and sequencing.
Methods

In June 2015, questing ticks were collected from pastures and wooded habitats by flagging the vegetation, near to human settlements and military training camp, in the Glamoč Municipality (44°02′44″N, 16°50′54″E; elevation 936 m), south-western Bosnia and Herzegovina (Fig. 1). Two 200-m²-sized transects were selected within the collection site. The sampled area is characterized by crops, pastures for sheep flocks and cattle herds and coniferous forest with small lawns and shrubs, and it was previously known for high tick abundance and relatively high diversity of tick species (Omeragić, 2011). All collected ticks were morphologically identified to species level and sexed by using standard taxonomic keys (Estrada-Peña et al., 2004) and stored at −20°C until further procedure. Prior to DNA extraction, ticks were washed in 3% H₂O₂ and 70% ethanol, rinsed with distilled water and dried on sterile filter paper. Each tick was then crushed and homogenized individually in 100 μl of DEPC water and 3 mm steel beads, using a TissueLyser II (Qiagen, Hilden, Germany). After centrifugation (3000 g/10 s), the supernatant was collected and DNA was extracted with the peqGOLD TriFast Isolation System (Peqlab, Germany) following the manufacturer’s instructions. The presence of B. burgdorferi s.l., A. phagocytophilum, ‘Candidatus N. mikurensis’, SFG rickettsiae and F. tularensis DNA in ticks was tested using PCR protocols published elsewhere (Regnery et al., 1991; Roux et al., 1996; Sjöstedt et al., 1997; Liebsch et al., 1998; Brown et al., 2001). Amplified PCR products were visualized by agarose gel electrophoresis, purified and directly sequenced at a commercial company (LGC Genomics, Germany). Confidence interval (95% CI) for the prevalence rate was computed with SPSS 20.0 statistical software.

Results

A total of 87 adult ticks (75 females and 12 males) collected on the vegetation were identified as I. ricinus (n = 30), D. reticulatus (n = 54) and D. marginatus (n = 3). At least one tested pathogen was detected by molecular methods in 21.8% (19/87) of all sampled ticks. PCR for gltA gene of Rickettsia spp. delivered 18 (20.7%) positive samples in the gel electrophoresis, whereas rickettsial ompA gene fragments were detected in only 13 (15%) specimens. The sequences obtained from I. ricinus (n = 6) showed 100% similarity to gltA and ompA sequences of R. monacensis (n = 1) [e.g. GenBank accession nos. KP215387.1 and FJ919640.1] and gltA sequences of R. helvetica (n = 5) [e.g. KP283018.1]. Sequences analyses of five PCR-positive D. reticulatus ticks revealed 100% similarity to gltA [e.g. KP742992.1] and 99–100% similarity to ompA sequences [e.g. HM161792.1] of R. raoultii. The sequences of R. slovaca from D. reticulatus (n = 5) and D. marginatus

![Fig. 1. Map of Bosnia and Herzegovina showing location (Glamoč) where ticks were collected.](image-url)
(n = 2) ticks were 99–100% identical to gltA and ompA sequences available in GenBank [e.g. KJ410267.1 and KF791242.1].

Of 87 ticks analysed, two sequences obtained from PCR-positive *D. reticulatus* and *D. marginatus* showed 100% similarity to 16S rRNA and TUL4 sequences attributed to *A. phagocytophilum* [e.g. KM215233.1] and *F. tularensis* subsp. *holarctica* [e.g. KF607099.1], respectively. A co-infection of *R. slovaca* and *F. tularensis* subsp. *holarctica* was detected in one *D. marginatus* tick (1.1%). None of the tested ticks were positive for *B. burgdorferi* was detected in one tick (0.2%). None of the tested ticks were positive for *B. burgdorferi* subsp. *holartica* [e.g. KF607099.1].

None of the tested ticks were positive for *B. burgdorferi* subsp. *holartica* [e.g. KF607099.1]. Sequences are deposited in GenBank® and are available under accession numbers KT805283-KT805303. Infection rates with detected pathogens in examined ticks are shown in Table 1.

**Discussion**

Ticks play an important role in the transmission of diseases worldwide, mostly due to their great capacity to act as vectors for many pathogens of medical and veterinary importance (Otranto et al., 2014). In the present study, four species of SFG rickettsiae, namely *R. monacensis*, *R. helvetica*, *R. raoultii*, and *R. slovaca*, were identified based on gltA and ompA gene markers. *R. monacensis* and *R. helvetica* were considered to be non-pathogenic for humans, but in 1999 *R. helvetica* was associated with fatal perimyocarditis in two human patients (Nilsson et al., 1999), while *R. monacensis* has recently been recognized as causative agent of Mediterranean spotted fever (MSF)-like illness in three human patients from Spain and Italy (Jado et al., 2007; Madeddu et al., 2012). The most prevalent pathogen in our study was *R. slovaca* (8.0%; 7/87), which was first identified in *D. marginatus* in the former Czechoslovakia in 1968. Since then, it has been found in *D. marginatus* and *D. reticulatus* ticks throughout Europe (Parola et al., 2009). *R. slovaca* and *R. raoultii* were described as human pathogens and agents of tick-borne lymphadenoaphathy (TIBOLA), also known as *Dermacentor*-borne necrosis erythema and lymphadenoaphathy (DEBONEL). However, *R. slovaca* is considered to be more pathogenic than *R. raoultii*, because it has been more frequently associated with human infection (Parola et al., 2009). TIBOLA/DEBONEL is the most prevalent tick-borne rickettsioses in Europe after MSF (Oteo and Portillo, 2012).

The low prevalence of *A. phagocytophilum* and *F. tularensis* subsp. *holarctica* in the present study is in line with the very low incidence of diseases they cause in Eastern Europe (Reye et al., 2013). DNA of *B. burgdorferi* s.l and ‘*Candidatus N. mikurensis*’ was not detected in sampled ticks, most likely due to the small sample size and low prevalence rate of infection in the studied area. All six pathogens detected in this study are zoonotic, and forest

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**Table 1. The prevalence of tick-borne bacteria in questing ticks in Glamoč, south-western Bosnia and Herzegovina**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Male</th>
<th>Female</th>
<th>95% CI</th>
<th>Total infections</th>
<th>Male</th>
<th>Female</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rickettsia monacensis</em></td>
<td>4.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 (0/5)</td>
<td>24.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Rickettsia helvetica</em></td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 (0/5)</td>
<td>24.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Rickettsia raoultii</em></td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 (0/5)</td>
<td>24.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Rickettsia slovaca</em></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 (0/5)</td>
<td>24.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Anaplasma phagocytophilum</em></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 (0/5)</td>
<td>24.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Francisella tularensis</em></td>
<td>9.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 (0/5)</td>
<td>24.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Total positive/total tested</em></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 (0/5)</td>
<td>24.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

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workers, hunters, farmers and military field personnel are particularly at highest risk of ticks bite and contraction of tick-borne diseases in the studied area.

Conclusions
This study reports the presence of emerging zoonotic bacteria in questing ticks from Bosnia and Herzegovina for the first time, indicating a public health threat to humans, and provides a preliminary insight into the diversity and circulation of detected pathogens in the studied area. Also, data presented in this study should increase awareness on the presence of these bacteria among physicians and practitioners, especially when they are faced with acute febrile illnesses after tick exposure. Furthermore, the occurrence of co-infected ticks increases the risk of multiple infections, which may complicate the clinical presentation in humans (Otranto et al., 2014). However, large-scale studies including all geographical regions and consequently more samples are urgently needed to determine the distribution of tick vectors and the pathogens they transmit, and to estimate the risk of infections for humans and animals in certain areas.

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References