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Original article

Tick-borne pathogens and their reservoir hosts in northern Italy

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ABSTRACT

The aim of this study was to determine the occurrence of *Anaplasma phagocytophilum*, *Rickettsia* spp., *Babesia* spp., and *Candidatus* Neoehrlichia mikurensis in *Ixodes* spp. ticks removed from wildlife, domestic animals and humans in the Province of Trento (northern Italy) in order to better understand their ecology and provide public health professionals with an updated list of pathogens which should be considered during their diagnostic procedures after a tick bite.

During 2011–2012, 848 feeding ticks at all life stages (adults, nymphs and larvae) from various hosts (wild ungulates, birds and rodents; domestic sheep, dogs and humans) were collected. The highest prevalences of *A. phagocytophilum* and *Rickettsia* spp. were detected in adult and nymphal tick stages feeding on wild ungulates (11.4% prevalence for both pathogens), while the *Babesia* spp. prevailed in nymphal and larval ticks feeding on wild birds (7.7%). A wide spectrum of tick-borne agents was present in larval ticks: those detached from wild ungulates were positive for *A. phagocytophilum*, *B. venatorum*, *R. helvetica*, *R. monacensis* and *R. raoultii*, while those removed from wild rodents were positive for *B. venatorum*, *R. helvetica*, *R. monacensis* and *Ca.* N. mikurensis, and ticks from wild birds carried *A. phagocytophilum*, *B. venatorum*, *B. capreoli* and *R. helvetica*.

This study provides evidence of circulation of five tick-borne pathogens not reported in this region before, specifically *R. raoultii*, *R. monacensis*, *B. venatorum*, *B. capreoli* and *B. microti*. Furthermore, it discusses the epidemiological role of the animal species from which the ticks were collected highlighting the needs for more experimental studies especially for those pathogens where transovarial transmission in ticks has been demonstrated.

1. Introduction

In Europe, one of the most common tick species of both medical and veterinary importance is *Ixodes ricinus*, a competent vector of a series of zoonotic agents including, among others, viruses (the agent of tick-borne encephalitis, TBE), bacteria (the agent of Lyme borreliosis, LB), and protozoa (causing, for example, human babesiosis) (Hubálek and Rudolf, 2012; Michelet et al., 2014; Socolovschi et al., 2009a). However, a number of other microorganisms, including rickettsiae, *Babesia* spp., *A. phagocytophilum* and *Candidatus* Neoehrlichia mikurensis, have recently been identified in this tick species (Carpi et al., 2011; Hodžić et al., 2015; Vayssier-Taussat et al., 2013).

Rickettsiae of the spotted fever group are Gram-negative bacteria comprising more than 31 different species (Merhej et al., 2014). Considered non-pathogenic for decades, many of these are now being considered a threat to human health, including *R. monacensis*, *R. slovaca* and *R. helvetica* (Nilsson et al., 2011; Parola et al., 2013; Socolovschi et al., 2009b). *Rickettsiae* are also efficiently transmitted vertically within the tick population by the transovarial route; therefore, their persistence in foci does not exclusively depend on the reservoir hosts (Burri et al., 2014; Sprong et al., 2009).

Babesia spp. are protozoan blood parasites with more than 100 described species. The most common infections in humans are caused by *B. divergens* in Europe and *B. microti* in the USA (Yabsley and Shock, 2012). *B. divergens* is known to cause babesiosis in cattle as well as in sheep, deer and humans (Chauvin et al., 2002; Hildebrandt et al., 2013; Langton et al., 2003; L'Hostis and Chauvin, 1999). Infection caused by *B. venatorum* is accompanied by similar clinical signs, but results in a

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milder course in comparison with infections caused by *B. divergens* (Hunfeld et al., 2008). There is no evidence that *B. venatorum* causes chronic disease, but in immunocompromised patients the infection can be prolonged (Häselbarth et al., 2007).

Although originally considered a pathogen of veterinary relevance causing tick-borne fever in livestock, dogs and horses (Stuen et al., 2013), *A. phagocytophilum* is the causative agent of human granulocytic anaplasmosis (HGA), with a subclinical to fatal course of infection (Chen et al., 1994). In Europe, the occurrence of clinical human cases is sporadic in comparison to the number of cases reported in the USA (Blanco and Oteo, 2002). Intraspecific genetic variability of *A. phagocytophilum* plays an important role in its ecology and pathogenicity. In Europe, two distinct enzootic cycles involving different vector and host species of this pathogen have been recognized (Baráková et al., 2014; Blaňarová et al., 2014; Bown et al., 2009; Burri et al., 2014).

Candidatus Neoehrlichia mikurensis is a Gram-negative bacterium from the Anaplasmataceae family that was described for the first time in 2004 in Japan (Kawahara et al., 2004). This agent had already been detected previously in Europe in questing *I. ricinus* ticks and ticks detached from asymptomatic patients from the Netherlands, Slovakia and Italy (Brouqui et al., 2003; Schouls et al., 1999; Špitalská and Kocianová, 2002). Pathogenicity of this bacterium was unclear until recently, when it was detected in immunocompromised patients with febrile illness and sepsis, as well as in immunocompetent humans (von Loewenich et al., 2010; Welc-Faleciak et al., 2014; Welinder-Olsson et al., 2010). Due to the recent discovery of this pathogen, knowledge of its ecology is still at a preliminary stage. According to the latest field studies, rodents have an important role in the maintenance of *Ca.* N. mikurensis based on its presence in rodent blood and/or in ticks that had fed on them (Burri et al., 2014; Obiegala et al., 2014).

The ecology of *Ixodes*-transmitted pathogens is often complex and involves several vertebrate hosts. Not only do these hosts play, a role of tick-blood meal providers but in certain cases they also act as reservoirs or amplifiers of pathogens (Burri et al., 2014; Sprong et al., 2009; Yabsley and Shock, 2012). Reservoir capacity, which defines the role of each reservoir host species in maintaining a particular pathogen in nature, varies among species and it is still poorly assessed for a large number of vertebrates, especially wildlife species, since complex laboratory xenodiagnostic experiments must be performed, which are economically and ethically challenging. However, identification of the reservoir hosts and estimate of its reservoir capacity is essential for developing meta-community-based risk assessment models for these emerging tick-borne zoonoses (Estrada-Peña and de la Fuente, 2014; Suzán et al.,2015).

Therefore, the aim of our study was to investigate the occurrence and prevalence of different tick-borne agents (*Rickettsia* spp., *Babesia* spp., *A. phagocytophilum, Ca.* N. mikurensis) in feeding ticks collected from a number of wildlife and domesticated animals in order to better define their epidemiological role, and to estimate their reservoir capacity based on the infection prevalence recorded in feeding larvae. Another aim was to update the list of tick-borne pathogens circulating within the Province of Trento, Italy, and to provide public health professionals with an updated list of pathogens that should be considered during diagnostic procedures.

2. Materials and methods

2.1. Study site

This study was carried out in mixed broad-leaf and coniferous forests in the Valle dei Laghi (from 46°5′N11°6′E southwest to 45°54′N10°52′E) in the Province of Trento (eastern Italian Alps). The Province of Trento is an area with endemic occurrence of several tick-borne infections, including TBE and LB (Rezza et al., 2015; Rizzoli et al., 2011, 2009).

2.2. Sample collection and DNA extraction

Feeding ticks at the adult, nymphal and larval stages were collected during March 2011–June 2013 with sterile tweezers from wild ungulates, rodents, and birds, as well as from domestic sheep, dogs and humans as described previously in Baráková et al. (2014). All animal handling procedures and ethical issues were approved by the Provincial Wildlife Management Committee (authorization n. 595 issued on 04.05.2011). After collection, ticks were placed individually in vials with 70% ethanol and stored at −20 °C until DNA extraction. Ticks were examined microscopically to determine the species and stage; tick species were also confirmed by PCR amplification targeting a fragment of the 16S rRNA gene (Collini et al., 2015).

Ticks were homogenized with a sterile pestle, and DNA from adults and nymphs was extracted using a commercial DNA extraction kit (QIAamp DNA Investigator kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA from larvae was extracted using alkaline hydrolysis as described previously (Guy and Stanek, 1991). More than one negative control was added to each extraction. Lysates were stored at −80 °C prior to use.

2.3. Molecular analysis

In order to verify ticks specie, an approximately 470 bp long fragment of 16S rRNA gene was obtained from each sample and compared with homologous sequences available in GenBank database, using the BLAST (Basic Local Alignment Search Tool) (Collini et al., 2015). *Rickettsia* spp. were identified by amplifying a partial fragment of 17-kDa gene (480 bp) (Reye et al., 2013). *R. helvetica* as well as *R. monacensis/R. tamurae* were confirmed by phylogenetic analysis of 17-kDa gene sequences (not shown) while *R. raoultii* was confirmed by real-time PCR using the specific primers Rraou2850F2, Rraou2956R2 and probe Rrao2896P previously described by Jiang et al. (2012). *R. monacensis* was distinguished from *R. tamurae* by amplifying the ompA gene, using primers, R-ompA-13-R (GCAATTCAAAAAGGTCTTAAA) and R-ompA-554-F (TTTCCTGTAAGTGTTATCTTTG) designed in this study using the software Primer3. *Omp*A sequences of *R. monacensis* and *R. tamurae* were downloaded from NCBI and aligned in MEGA in order to identify the region that distinguishes these two species. For the PCR amplification, a Promega GoTaq® DNA Polymerase protocol was used with 2 µl of DNA by PCR with 1 µl of 10 pmol of each primer (R-ompA-13-R and R-ompA-554-F) in a final volume of 20 µl (1.6 µl of 25 mM $MgCl_2$, 0.4 µl of 10 mM deoxynucleosidetriphosphate, 2 µl of Promega GoTaq® reaction buffer, 0.2 µl of 5 U of Promega GoTaq® DNA Polymerase). Reactions were performed for 35 cycles. After an initial denaturation step of 20 s at 94 °C, each cycle consisted of an annealing step of 20 s at 52 °C followed by an extension stage of 1 min at 72 °C. The program ended by storing the reaction mixtures at 4 °C.

Babesia spp. were detected by amplification of the partial 18S rRNA gene according to Casati et al. (2006) using primers BJ1 and BN2. Screening of *Ca.* N. mikurensis was done by real-time PCR using the following set of primers for the gro*EL* gene; NMikGroEL-F2, NMikGroEL-R1, NMikGroEL-R2 and probe NMikGroEL-P2a-FAM (Jahfari et al., 2012). *A. phagocytophilum* was detected by amplification of a 546-bp long fragment of 16S rRNA gene using ge3a and ge10r for the first round of nested PCR and ge2 and ge9f primers for the second round of PCR, as previously described (Massung et al., 1998). Amplified DNA from all the pathogens was visualized using a BioAnalyzer, Agilent 2100 or a QIAxcel screen gel. Positive PCR products were purified by enzymatic Exosap-IT (USB Corporation) and sequenced in both directions with an ABI BigDye terminator kit (Applied Biosystems, Monza, Italy) and analyzed on an ABI prism 3130 automated sequencer. A 95% confidence interval (CI) was calculated for the prevalence of pathogens. Nucleotide sequences of all 17-kDa, ompA, 18S rRNA, and 16S rRNA were deposited in GenBank (for Assession numbers, see Table 1).

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