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Original papers

Molecular detecting of piroplasms in feeding and questing *Ixodes ricinus* ticks

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ABSTRACT. The purpose of this study was to detect piroplasms, which are pathogens of veterinary and zoonotic importance in ticks, that were collected from ponies and field vegetation and to determine the role of Shetland ponies as potential reservoir hosts for piroplasms. A total of 1737 feeding and 371 questing *Ixodes ricinus* collected from horses or vegetation were tested for the presence of *Babesia* and *Theileria* DNA. Piroplasm 18S rRNA gene amplification was conducted, and the obtained amplicons were sequenced. *Babesia* DNA was detected in only three ticks (one tick collected from a pony and two collected from vegetation), and all of the obtained sequences had 100% similarity to *B. divergens*. *Theileria* DNA was not present in the examined ticks. Thus, the above results indicate that ponies are probably not essential hosts for the detected species of piroplasms. Piroplasm species typical for horses (*Babesia caballi* and *Theileria equi*) were not detected because *I. ricinus* is not their vector. The low infection rate of *I. ricinus* with *B. divergens* shows that the disease risk for the local horse population and people associated with pony horses is low, but it demonstrates their possible role as a source of human infection in northern Poland.

Key words: feeding and questing *Ixodes ricinus*, *Babesia*, *Theileria*, PCR, sequencing

Introduction

Ixodes ricinus is the most widespread and abundant European tick species that is capable of transmitting numerous pathogens of both medical and veterinary importance, which are considered agents of emerging human diseases. Among these pathogens are viruses, bacteria and protozoans, but the most frequently described is *Borrelia burgdorferi*, a bacterium that causes Lyme disease. Less frequently, some species of protozoans are reported.

The Sporozoa class (phylum Apicomplexa) is a large group of parasitic protozoans, including organisms such as piroplasms, coccidia, gregarines, and haemogregarines [1]. Among the piroplasms, *I. ricinus* ticks can transmit protozoans of the *Babesia* genus, such as *Babesia divergens, B. microti, Theileria* spp., or the newly described *B. venatorum* (sp. EU1) [2–4].

Babesiosis is caused by intraerythrocytic apicomplexan parasites that belong to the genus *Babesia* and is mainly transmitted by tick vectors to a variety of vertebrate hosts, including wild and domestic animals and humans [5,6]. Numerous human cases have been reported throughout Europe, primarily in splenectomized patients. Clinical infection continues to be rare in Europe, with approximately 40 acquired cases to date, but it has a 42% mortality rate [7]. Infection is most often caused by tick bites but also occurs via blood transfusion from asymptomatic *B. microti*-infected donors. Described and detected cases in Poland were transmitted from tropical countries and diagnosed as co-infections with other tick-borne agents [8]. Recently, the *B. microti* infection was described in patients in northeastern Poland who have non-specific symptoms but were bitten by ticks [9].

In Europe, *B. divergens*, *B. microti* [10,11] and *B. venatorum* (also known as *Babesia* EU1) [12,13] have been reported in *I. ricinus* ticks from various countries. However, equine piroplasmosis, a disease of Equidae, including horses, donkeys, mules, and zebras, is caused by either *B. caballi* or *Theileria equi* [14]. These species are present mainly in

tropical and subtropical areas and are spread by ticks of the *Dermacentor, Hyalomma* and *Rhipicephalus* genera [15]. However, equine piroplasms may occur in European countries such as Germany, Switzerland [16,17] or the Netherlands [18]. In Central Poland [19], illness was observed in three horses that exhibited symptoms of fever, ataxia, mucus membrane paleness, haematuria and thrombocytopenia. PCR performed on blood samples revealed the presence of DNA from *Babes - ia/Theileria* spp., with a 95.6–97.5% sequence similarity to *Babesia equi*. In the study of equine from southern Spain, the presence of genetic material of equine piroplasms has been demonstrated in 20% of the examined animals, although all of the animals were asymptomatic and apparently healthy [20]. Usually, in endemic areas, infected horses are apparently healthy and do not exhibit any clinical symptoms, so it is often difficult to recognize the infection [21]. Nevertheless, infected horses may act as *B. caballi* transporters for several years or *T. equi* infection carriers for the rest of their lives, serving as reservoirs for ticks [22].

The aim of the present study was the molecular detection and identification of pathogenic piroplasms of veterinary and zoonotic importance in feeding (engorged and semi-engorged) and questing *I. ricinus* ticks that were collected from ponies and vegetation in northwestern Poland. This allowed us to determine the role of Shetland ponies as a potential reservoir hosts. All of the ticks had been examined in previous studies to detect and identify species of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum, Rickettsia* spp. [23], and *Toxoplasma gondii* [24].

Materials and Methods

Study site and tick collection from ponies and vegetation. Ticks were collected from 49 individual Shetland ponies, which is a breed of the domestic horse (*Equus caballus*) that is kept mainly for recreational purposes [23]. Fed and semi-fed ticks were collected in March–June and August–November 2010–2012 from the skin of the ponies that were permanently kept as studs. Ticks (n=1737) belonging to the species *Ixodes ricinus* that were collected from horses included females (n=1292), males (n=263), and nymphs (n=182). The questing *I. ricinus* were collected by flagging vegetation in the area surrounding the Imno studs. Among the 371 individuals of host-seeking *I. ricinus* collected from the vegetation, there were 6 females, 10 males, 252 nymphs and 103 larvae. All ticks were maintained at –20°C until DNA extraction.

DNA extraction. DNA extraction from ticks was performed with a phenol-chloroform protocol [25]. DNA samples were stored at –70°C before PCR analyses.

PCR amplification. For the molecular identification of *Babesia* and *Theileria*, a region of the gene encoding 18S rRNA was amplified with the primers ThFOR and ThREV, specific for *Babesia* and *Theileria* genera [4]. Each amplification reaction was carried out in a total volume of 10 µl with the following mixture components: 1 µl of DNA, 1× reaction buffer, 2.5 mM $MgCl_2$, 3 pM each primer, 0.75 nM each nucleotide, and 0.5 U polymerase (Allegro Taq Polymerase, Novazym, Poland). The PCR conditions were as following: an initial denaturation step of 3 minutes at 94°C, followed by 40 cycles consisting of 45 seconds at 94°C, 45 seconds at 65°C and 45 seconds at 72°C. The cycles were followed by a final extension of 3 minutes at 72°C. All analyses were carried out in duplicate. DNA isolates that had previously been obtained from infected ticks were used to detect the presence of *Babesia* and *Theileria* DNA. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

Sequencing. Selected amplicons of the *Babesia* 18S rRNA gene were sequenced (Macrogen, Korea) with the primers ThFOR and ThREV. The obtained sequences were aligned with each other using ClustalW (Mega 5.10 software). They were also initially aligned with homology sequences published in GenBank using BLAST (www.ncbi.nlm.nih.gov) then with ClustalW [26]. The ends of the alignment were trimmed to form blunt ends on all of the sequences. The final alignment contained nucleotides 1 to 634 of *B. divergens*.

Results

Babesia DNA was detected in three (0.14%) ticks, one tick (engorged female) of the 1737 (0.06%) collected from a pony and in two ticks (nymphs) of the 371 (0.54%) collected from vegetation. All of the obtained 634-bp *Babesia* sequences had 100% similarity to both each other and the homologous sequence of *B. divergens* from GenBank (AY789076). The sequences analysed in this study have been deposited in the GenBank

database under the accession numbers KU748894 – KU748896 (*Babesia divergens*).

Discussion

For the first time in Poland, *B. microti* DNA in questing *I. ricinus* was detected in the northwestern part of the country [27–29]. Then, the DNAs of *B. microti, B. divergens* [10], and *Babesia* EU1, later named *B. venatorum* [30], were observed in all tick stages. These results showed the competence of these tick species in serving as vectors and reservoirs. The infection rates ranged from 1.9% to 16.3% of the examined tick populations; however, in the present study, *B. divergens* was detected in only one tick (engorged female) collected from a pony and in two ticks (nymphs) taken from vegetation. *Theileria* DNA and other piroplasms were not present in the investigated ticks. The *Theileria* sp. DNA in *I. ricinus* was identified in our previous studies [4] in northwestern Poland. We also demonstrated that red deer (*Cervus elaphus*) is a competent reservoir of *Theileria* sp. [31]. Molecular characterization of *Theileria* based on large fragments of the 18S rRNA gene containing hypervariable region V4 showed 99.9% similarity to two *Theileria* species: *T. capreolus* and *Theileria* sp. 3185/02 (derived from red deer imported from Germany to Spain). Phylogenetic analysis confirmed that these probes clustered together, which suggests that they might all have the same genotype [31].

Studies in other European countries also revealed a low prevalence of *Babesia* in questing populations of *I. ricinus* and the same species. In the screening study by Michelet et al. [32] on the occurrence of *Babesia* spp., more than 7 thousand *I. ricinus* nymphs were collected from three European countries. Two species were found in Denmark at a low prevalence: *B. divergens* at 0.1% and *B. venatorum* at two sites at 1.4% and 0.4%, respectively. In German studies of the *Babesia* occurrence in *I. ricinus*, the overall prevalence ranged from 0% to 10.7%, and the *B. divergens* species was dominant [33]. The prevalence of *Babesia* spp. in questing *Ixodes ricinus* ticks from Norway was 0.9%, and the most prevalent of the four detected species was *B. venatorum* [34]. Higher values were obtained in the examined *I. ricinus* populations and ranged from 6.1 to 20.6% in France [35,36], 10% in Slovenia, 9% in southwest Poland [37] and 10% in questing ticks in the Netherlands [38].

Equine piroplasmosis is a disease of Equidae, including horses, donkeys, mules, and zebras, that is caused by one of two protozoan parasites: *T. equi* or *B. caballi*. These parasites are biologically transmitted between hosts via tick vectors. Several ixodid tick species have been identified as either natural or experimental vectors of equine piroplasmosis. *T. equi* is transmitted by 14 species (4 *Dermacentor* sp., 4 *Hyalomma* sp., 5 *Rhiphicephalus* (*Boophilus*) sp., and *Ambyomma cajennense*) and *B. caballi* by 15 species (7 *Dermacentor* sp., 6 *Hyalomma* sp., and 2 *Rhiphicephalus* sp.). *B. caballi* is transmitted transtadially and transovarially by its vectors [39]. *T. equi* is generally transmitted through transstadial and intrastadial transmission [40]. The transovarial transmission of *T. equi* occurs, but its precise role in epidemiology has not been detailed [41].

In presented studies, all of the ticks were collected from ponies belonging to one species, *I. ricinus*. By examining *I. ricinus* ticks engorged with horse's blood, we expected to obtain information about tick infection by *Babesia* and *Theileria* species (*T. equi* and *B. caballi*), which are typical species for equine piroplasm infection. The observed effects indicate that these ponies had probably not been infected by equine piroplasms and that *I. ricinus* is not a vector in the natural endemic cycle of *B. caballi* and *T. qeui.*

The study by Lori et al. [42] aimed to provide molecular evidence on the tick species involved in piroplasm persistence in the central and northern Italian regions. Ticks removed from domestic and wild animals were checked for piroplasmosis to determine the species acting as a reservoir for piroplasms. Molecular diagnostics identified *T. equi* and eight *Babesia* species in 11 ixodid tick species. *T. equi* DNA was detected in *I. ricinus* and *Dermacentor marginatus* and in *Hyalomma marginatum* removed from horses. *B. caballi* was not observed in these species. *I. ricinus* hosted the highest number of species, but horses were mostly parasitized by *Hy. marginatum*. The effects observed in that study confirmed the presence of host-specific ticks in the investigated areas.

The vector relationship is critical to the epidemiology of these piroplasm parasites. To complete their life cycle, these parasites must go through a complex series of developmental stages, including the sexual stage of their tick vectors. Therefore, ticks are the definitive hosts and vectors for these parasites, and the vector relationship is limited to a few competent tick species [14].

However, the presence of a competent tick vector and even infected horses within the same area does not always lead to infection or disease. Many factors must be considered, including the season, climate, competent tick's life cycle and host-specificity [39,43].

The **o**btained results did not show pony involvement in the natural endemic cycle of *B. caballi* and *T. equi.*

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