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Hard Ticks Infesting Dogs in Hungary and their Infection with *Babesia* and *Borrelia* Species

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Abstract

A survey was carried out in Hungary to investigate the occurrence of hard tick species (Acari: Ixodidae) collected from dogs and *Borrelia* and *Babesia* spp. detected in them. In total, 1,424 ticks were removed from 477 dogs appearing for clinical consultation in veterinary practices and clinics countrywide. *Ixodes ricinus* and *Dermacentor reticulatus* were the most common species occurring in most of the studied areas. Females of these two species were selected for molecular analyses. One to twelve specimens were used in each sample for DNA extraction. Polymerase chain reactions were performed with BSL-F/BSL-R primers for detecting *Borrelia* spp. in *I. ricinus* and with PIRO-A1/PIRO-B primers to amplify *Babesia* spp. DNA in *D. reticulatus*. Randomly selected PCR products were sequenced to identify the pathogens' species or subspecies. DNA of *Borrelia* spp. could be detected in six (5.6%) from 108 *I. ricinus* samples and 43 (29.9%) from 144 *D. reticulatus* samples were PCR-positive for *Babesia* spp. Sequencing revealed the highest similarity with *Borrelia afzelii*, *Borrelia garinii* and *Babesia canis canis*, respectively. *Babesia* and *Borrelia* spp. were identified in ticks with molecular methods for the first time in Hungary, and a high prevalence of *B. canis canis* in *D. reticulatus* females collected from dogs was detected.

Introduction

Ticks (Acari: Ixodidae) are of considerable medical and veterinary interest worldwide because of the wide range of pathogens they can transmit. In Europe, emerging tick-transmitted canine diseases (e.g. babesiosis, borreliosis and anaplasmosis) have drawn both public and scientific attention to these arthropods (Shaw *et al.* 2001, Beugnet 2002, Camacho *et al.* 2003, Criado-Fornelio *et al.* 2003a, b, Jensen *et al.* 2007).

Studies examining the hard tick infestation of dogs have been published from the mid 1960s in Europe. The 16 species of five genera reported to feed on dogs in various European countries are listed in Tab. 1. Concerning occurrence, veterinary and zoonotic importance, *Ixodes ricinus*, *Rhipicephalus sanguineus* and *Dermacentor reticulatus* are the most important species infesting dogs in Europe (Shaw *et al.* 2001, Beugnet 2002). Babos (1964) mentioned seven tick species that can infest dogs in Hungary, of which *D. reticulatus* has been found to be the local vector of *Babesia canis* (Janisch 1986). Because canine babesiosis has been a severe and frequent disease in the country (Csikós *et al.* 2001, Földvári *et al.* 2005b), it is crucial to study the geographical and seasonal distribution of this tick species in particular. Hornok and Farkas (2005) recently reported the first occurrence of *R. sanguineus* on dogs in Hungary, which was imported accidentally.

Cases of autochtonous large *Babesia* infections of dogs have been reported from many European countries (Beugnet 2002, Holm *et al.* 2006). In Hungarian dogs, only *Babesia canis canis* has been detected (Földvári *et al.* 2005b). Recent studies using molecular methods showed that in France, Slovenia and Spain, where *R. sanguineus* and *D. reticulatus* coexist, both *Babesia canis canis* and *Babesia canis vogeli* were detected (Cacciò *et al.* 2002, Criado-Fornelio *et al.* 2003b, Duh *et al.* 2004). Small canine piroplasms also occur in Europe. *Babesia gibsoni* has been identified by Criado-Fornelio *et al.* (2003a) for the first time in European dogs. *Theileria annae,* described by Zahler *et al.* (2000), was found amongst Spanish dogs with a high frequency (Camacho *et al.* 2001). *Theileria equi* and *Theileria annulata* are probably also capable of infecting dogs, since they were detected in symptomatic animals (Criado-Fornelio *et al.* 2003b, Criado *et al.* 2006).

Lyme borreliosis is caused by tick-transmitted *Borrelia burgdorferi* sensu lato spirochetes. *Borrelia* seropositivity is common amongst dogs because many of them carry a persistent infection for life and only a fraction of infected animals (~5%) enters the disease status (Beugnet 2002). *Borrelia afzelii*, *B. burgdorferi* sensu stricto and *Borrelia garinii* are found commonly in European dogs (Speck *et al.* 2001, Skotorczak and Wodecka 2005), and mixed infection with four species (*B. burgdorferi* s.s., *B. afzelii, B. garinii* and *Borrelia valaisiana*) may also occur (Hovius *et al.* 1998). The pathogenic significance of the above-mentioned European *Borrelia* spp. has not been determined in dogs (Speck *et al.* 2007). Kapiller *et al.* (1995) reported first on the seropositivity of dogs for *B. burgdorferi* s.l. in Hungary, however, there has been no other data on the occurrence and identity of spirochetes causing canine borreliosis in the country.

The goal of this study is to extend our previous surveys (Farkas and Földvári 2001, Földvári and Farkas 2005) on the seasonal and geographical occurrence of tick species infesting dogs, and to clarify to what extent *D. reticulatus* is widespread in Hungary. Since ticks included in our study may harbour pathogens and can also contain infected blood of the host, we attempted to detect and identify *Babesia* and *Borrelia* spp. in them.

Valid name[a]	Reference
Ixodes canisuga Johnston, 1849	Babos (1964), Gothe *et al.* (1977), Liebisch *et al.* (1984), Ogden *et al.* (2000), Földvári and Farkas (2005)
Ixodes ricinus (Linnaeus, 1758)	Babos (1964), Liebisch *et al.* (1984), Grandes (1986), Beichel *et al.* (1996), Papadopoulos *et al.* (1996), Ogden *et al.* (2000), Földvári and Farkas (2005)
Ixodes hexagonus Leach, 1815	Liebisch *et al.* (1984), Beichel *et al.* (1996), Papadopoulos *et al.* (1996), Ogden *et al.* (2000), Földvári and Farkas (2005)
Haemaphysalis inermis Birula, 1895	Babos (1964)
Haemaphysalis concinna Koch, 1844	Babos (1964), Liebisch *et al.* (1984), Földvári and Farkas (2005)
Haemaphysalis punctata Canestrini and Fanzago, 1878	Papadopoulos *et al.* (1996), Ogden *et al.* (2000)
Haemaphysalis parva Neumann, 1897	Babos (1964)
Rhipicephalus bursa Canestrini and Fanzago, 1878	Grandes (1986), Papadopoulos *et al.* (1996)
Rhipicephalus pusillus Gil Collado, 1938	Grandes (1986)
Rhipicephalus turanicus Pomerantsev, 1936	Grandes (1986), Papadopoulos *et al.* (1996), Papazahariadou *et al.* (2003)
Rhipicephalus sanguineus (Latreille, 1806)	Liebisch *et al.* (1984), Grandes (1986), Papadopoulos *et al.* (1996), Papazahariadou *et al.* (2003)
Dermacentor reticulatus (Fabricius, 1794)	Liebisch *et al.* (1984), Ogden *et al.* (2000), Babos (1964), Földvári and Farkas (2005)
Dermacentor marginatus (Sulzer, 1776)	Liebisch *et al.* (1984), Babos (1964), Földvári and Farkas (2005)
Hyalomma aegyptium (Linnaeus, 1758)	Keirans (1984), Hillyard (1996)
Hyalomma marginatum marginatum Koch, 1844	Grandes (1986)
Hyalomma marginatum rufipes Koch, 1844	Keirans (1984)

Tab. 1 Hard tick species infesting dogs in Europe

[a] Camicas *et al.* 1998, Horak *et al.* 2002

Materials and methods

Ticks were collected from dogs appearing for clinical consultation in veterinary practices and clinics in different geographical regions of the country between January 2004 and March 2007. Specimens removed from each animal were stored in individually labelled tubes containing 70 % ethanol. Date and geographical location of infestation were recorded. Ticks were identified under a stereomicroscope using standard keys of Babos (1964), Hillyard (1996) and Estrada-Peña *et al.* (2004).

Molecular detection of piroplasms was performed from *D. reticulatus* females. Depending on the size and origin of specimens, one whole or a half tick, or pooled ticks (comprising of two to six specimens from the same dog or area per sample) were used. After being washed in detergent, distilled water and then in phosphate buffered saline (PBS), both individual and pooled samples were homogenised in 100 µl PBS with sterilised small scissors in a microcentrifuge tube. DNA was isolated using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, preceded by an overnight digestion in Proteinase K. Piroplasm-specific polymerase chain reaction (PCR) with the primers PIRO-A1/PIRO-B, visualisation and sequencing of PCR products were performed as described in Földvári *et al.* (2005b).
For detection of *Borrelia* spp., one to twelve *I. ricinus* females per sample were used. Selection of specimens and DNA extraction were performed as described for *D. reticulatus*. *Borrelia*-specific PCR with primers BSL-F/BSL-R, visualisation and sequencing of PCR products were made as described in Földvári *et al.* (2005a).
PCR products were selected randomly for sequencing. Obtained sequences were checked with Chromas v.1.45 and compared to sequence data available from GenBank®, using the BLAST 2.2.15. program (http://www.ncbi.nlm.nih.gov/BLAST/). New sequences were submitted to GenBank® database.

For mapping of our data the R-environment (R Development Core Team 2006) was used based on a PostgreSQL-PostGIS database system (http://postgis.refractions.net/docs/). The connection of these two tools was built by RpostGIS package (Solymosi *et al.* 2006). A map of Hungary divided into 150 local administrative units was used.

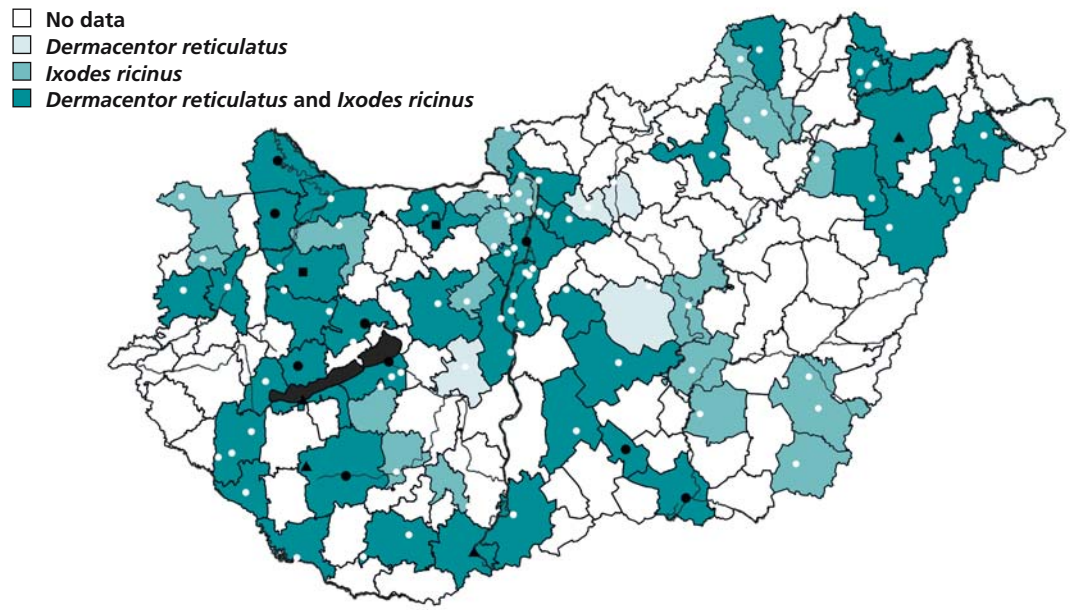

Fig. 1 Geographical distribution of *I. ricinus* and *D. reticulatus* collected from dogs. Data include 37 collecting sites from Földvári and Farkas (2005) and 56 locations from the present study.
Place of tick infestation is shown by white spots, black spots indicate sites where *Babesia* spp., black triangles where *Borrelia* spp. and black squares where both pathogens were detected in ticks by PCR.

Results

A total of 1,424 ticks, including 1,367 (96%) adults and 57 (4%) nymphs were removed from 477 dogs. Intensity of infestation ranged from one to 129 specimens per dog. Most of the adults (1,050, 76.8%) were semi-engorged or fully engorged females. Amongst the adults, the most common species was *I. ricinus* (779; 57%) followed by *D. reticulatus* (563; 41.2%), *Haemaphysalis concinna* (15; 1.1%), *Ixodes hexagonus* (5), *Ixodes acuminatus* (4) and *Ixodes canisuga* (1). Nymphs of *Haemaphysalis* spp. (23; 40.4%), *I. ricinus* (22; 38.6%), *I. canisuga* (10; 17.5%) and *I. hexagonus* (2; 3.5%) were found. Geographical distribution of the two most common species including 56 locations from the present study and 37 locations from our previous data (Földvári and Farkas 2005) can be seen in Fig. 1. Both *I. ricinus* and *D. reticulatus* occurred in 42 (64.6%) from 65 local administrative units where ticks were collected. Only *I. ricinus* or *D. reticulatus*-infested dogs in 19 and four of these units, respectively. Specimens of this two species occurred in each month with activity peaks in spring and autumn (Fig. 2).

For molecular analysis, 144 samples comprising of 181/393 (46.1%) *D. reticulatus* females were selected. *Babesia* DNA was amplified from 43 (29.9%) samples, eleven of which originated from Budapest and 32 from ten other locations (Fig. 1). The five selected PCR products' sequences showed 100% homology to one another or differed by one to three nucleotides. BLAST search in GenBank® revealed the highest similarity (99.8 to 100%) with 18S rDNA partial sequence of *B. canis canis* (Tab. 2).

For molecular analysis, 108 samples (comprising of 168/638 (26.3%) *I. ricinus* females were selected.

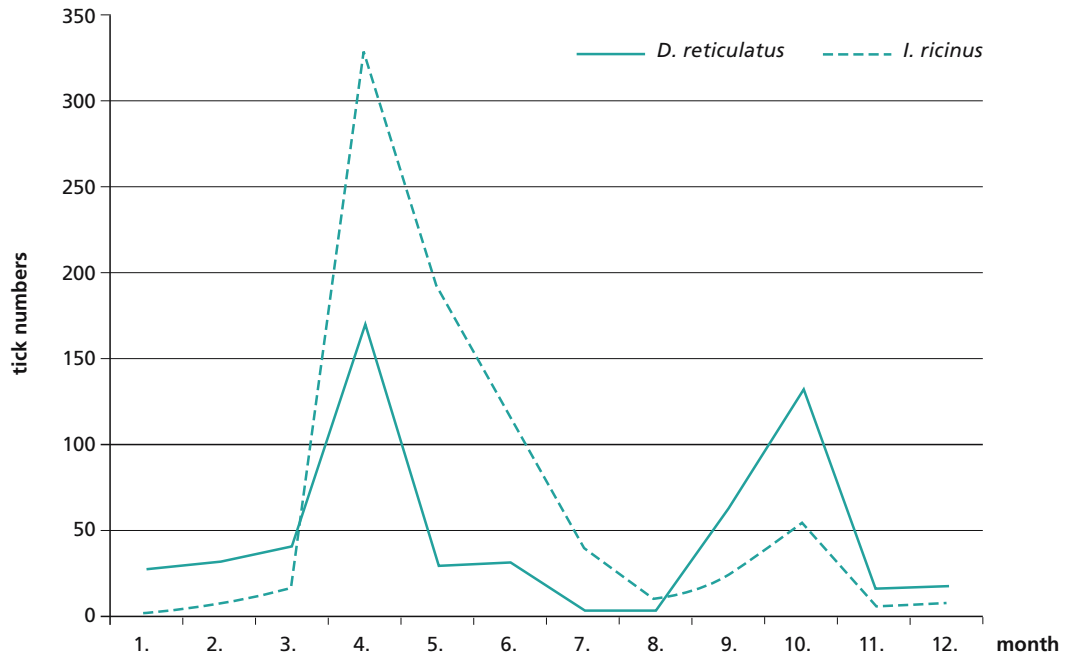

Fig. 2 Seasonal occurrence of *I. ricinus* and *D. reticulatus* adults on dogs according to month of collection

Origin of sample	Accession No.	Sequence length (bp)	Similarity to *B. canis canis* (%)
Budapest	DQ181652	411	99.8
Budapest	DQ181653	412	100
Csorna	DQ181654	391	100
Pápa	DQ181655	413	100
Pápa	DQ181656	415	100

Tab. 2 *Babesia* sequences from *D. reticulatus* females submitted to the GenBank® database

Origin of sample	Accession No.	Sequence length (bp)	Similarity to *Borrelia* species
Balatonfenyves	DQ193521	257	100 % *B. afzelii*
Mohács	DQ193522	254	100 % *B. afzelii*
Pápa	DQ193523	254	100 % *B. garinii*

Tab. 3 *Borrelia* sequences from *I. ricinus* females submitted to the GenBank® database

Six (5.6 %) samples were PCR-positive for *Borrelia* spp. collected in different areas of the country (Fig. 1). BLAST search of the sequences resulted 100 % homology with *B. afzelii* for two samples and 100 % homology with *B. garinii* for the third sequenced PCR product (Tab. 3).

Discussion

In the present study, 1,424 tick specimens were collected from 477 dogs living in different regions of Hungary. The results confirmed our previous data (Farkas and Földvári 2001, Földvári and Farkas 2005) that the most prevalent species on dogs is *I. ricinus* followed by *D. reticulatus*. *Ixodes ricinus* occurred in 61 (94 %) and *D. reticulatus* in 46 (71 %) of 65 local administrative units where ticks were collected. If we compare these results with the data of a recent study (Földvári and Farkas 2005), *D. reticulatus* was found in several new areas including southern, northern, eastern and western parts of the country. In two previously studied counties (Borsod-Abaúj-Zemplén and Szolnok), *D. reticulatus* could not be found before (Földvári and Farkas 2005) but occurred in the present survey. This can be explained by a geographical expansion of this species, which has already been proposed by Meyer-König *et al.* (2001) and Sréter *et al.* (2005). In Germany 30 years ago, this tick species was found only in a few natural foci in the south, however, its distribution has expanded and it is now covering large areas of the country (Heile *et al.* 2006). Dautel *et al.* (2006) recently collected ticks from dogs in the states of Berlin and Brandenburg and found animals infested with *D. reticulatus* from 26 different locations, all previously unknown as endemic sites for this species. In Hun-

gary, the populations of some of its natural hosts like wild boar and deer species increased, while hare, stray dog, stray cat and red fox populations remained at high levels during the last decades (Csányi 2005), therefore, it is possible that this tick species of high adaptability is spreading. On the other hand, increased temperatures due to climate change and shifting agricultural practices may also have an influence on the population dynamics of *D. reticulatus* in every European environment, including Hungary.

Babesiosis has been an endemic disease amongst dogs in Hungary for many decades (Janisch 1986, Csikós *et al.* 2001, Hornok *et al.* 2006). In a previous study (Földvári *et al.* 2005b), we identified *B. canis canis* as the subspecies responsible for canine babesiosis cases in the country and proved the presence of these piroplasms in north-eastern and south-eastern regions, from where no babesiosis had been reported earlier. The present work is the first molecular survey on *Babesia* infection of *D. reticulatus* specimens. During molecular examination of 144 tick samples originating from 54 dogs, 43 (29.9%) samples were PCR-positive for *Babesia* spp. Because of the pooled samples (one to six specimens were used to each DNA extraction), a minimum prevalence rate of 30% can be calculated for the examined *D. reticulatus* population. Although there is a lack of comparable data from similar studies, this is much higher then the prevalence rates (3.6% and 4.2%) published by Rar *et al.* (2005a) and Rar *et al.* (2005b) for unfed *D. reticulatus*. Our sampling method could also contribute to the high proportion of the *Babesia*-positive ticks, because more *Babesia*-infected animals (possibly carrying the infected tick) might occur amongst dogs taken to the veterinary clinics than in the average population. In the case of ten dogs, PCR-positivity for *Babesia* spp. was also established (G. Földvári unpublished data). We did not find *Babesia*-infected ticks from three of these animals during the present study. These data indicate that a *Babesia*-infected dog does not necessarily harbour infected *D. reticulatus* ticks. The infection of these dogs could have happened earlier or by another tick specimen. A similar conclusion was drawn by a South African study (Horak 1995). However, during experimental infections where a high level of parasitaemia and a long feeding period is provided, piroplasms are taken up by the ticks feeding on the infected dogs (Janisch 1986, Hauschield and Schein 1996). The high frequency and broad geographical distribution of the *Babesia*-positive ticks observed in the present study is an important risk factor for babesiosis of dogs, however, from an epizootiological point of view, further studies are necessary to determine the rate of infection in field-collected populations of *D. reticulatus*.

Six of the 108 samples containing *I. ricinus* females originating from different parts of the country were found to be PCR-positive for *Borrelia* spp. These results provide the first molecular detection of the spirochetes in ticks removed from dogs in Hungary. We found two different species: two sequences were identical to *B. afzelii* and one to *B. garinii*. Both species were described in dogs (Hovius *et al.* 1999) and humans (Brouqui *et al.* 2004). In Hungary, Lakos *et al.* (1991) detected spirochetes in unfed *I. ricinus* specimens, with no species identification. Besides *Borrelia spielmanii, B. burgdorferi* s.s. and *B. afzelii* recently identified from human patients (Földvári *et al.* 2005a), the first molecular evidence for the presence of *B. garinii* in Hungary was provided in this study.

Based on the studied *I. ricinus* population, a minimum prevalence of 5.6% can be given for *Borrelia* spp., which is relatively low compared to similar studies. Hovius *et al.* (1998) determined the prevalence of *Borrelia* spp. by PCR in 138 *I. ricinus* collected from dogs in the Netherlands. *Borrelia burgdorferi* s.l. was present in 20 (14.5%) ticks.

Four species were identified: *B. burgdorferi* s.s. (N = 8), *B. afzelii* (N = 4), *B. garinii* (N = 2) and *B. valaisiana* (N = 2). The same authors found that the prevalence of *B. burgdorferi* s.s. is significantly ($P < 0.05$) lower in semi-engorged than in non-engorged ticks. Gern *et al.* (1996) provided the following possible explanations for this phenomenon: 1) early transmission of the spirochetes from the ticks to the dogs, 2) destruction of the spirochetes in the tick mid-gut and 3) faecal excretion of the spirochetes from the mid-gut. Since the studied *I. ricinus* population of the present survey mainly consisted of semi-engorged and engorged females, we assume that a higher prevalence of spirochetes can be expected in field-collected ticks. We conclude that dogs in Hungary are at a considerable risk for tick infestation, mainly by *I. ricinus* and *D. reticulatus*. High numbers of canine babesiosis cases can also be expected in the future and further investigations are needed to obtain more information on the prevalence and identity of Lyme disease spirochetes in ticks.

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