RESEARCH ARTICLE

Broad-range survey of vector-borne pathogens and tick host identification of *Ixodes ricinus* from Southern Czech Republic

Vaclav Honig[1,2,3,*,†], Heather E. Carolan[4], Zuzana Vavruskova[1,2], Christian Massire[4], Michael R. Mosel[4], Christopher D. Crowder[4], Megan A. Rounds[4], David J. Ecker[4], Daniel Ruzek[1,3], Libor Grubhoffer[1,2], Benjamin J. Luft[5] and Mark W. Eshoo[4]

[1]Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Branisovska 31, 370 05, Ceske Budejovice, Czech Republic, [2]Faculty of Science, University of South Bohemia, 370 05, Ceske Budejovice, Czech Republic, [3]Department of Virology, Veterinary Research Institute, Hudcova 70, Brno, Czech Republic, [4]Ibis Biosciences Inc., Abbott Laboratories, 2251 Faraday Ave, Ste 150, Carlsbad, CA 92008, USA and [5]Department of Medicine, State University of New York, Stony Brook, NY 11794-8166, USA

*Corresponding author: Laboratory of Arbovirology, Institute of Parasitology, Biology Centre, Czech Academy of Sciences, Branisovska 31, 370 05, Ceske Budejovice, Czech Republic. Tel: +420387775463; E-mail: honig@paru.cas.cz
One sentence summary: Prevalence of multiple human and veterinary pathogens was estimated in host-seeking nymphal *Ixodes ricinus* ticks, and their previous vertebrate host was determined.
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ABSTRACT

Ixodes ricinus ticks are vectors of numerous human and animal pathogens. They are host generalists able to feed on more than 300 vertebrate species. The prevalence of tick-borne pathogens is influenced by host–vector–pathogen interactions that results in spatial distribution of infection risk. Broad-range polymerase chain reaction electrospray ionization mass spectrometry (PCR/ESI-MS) was used to analyze 435 *I. ricinus* nymphs from four localities in the south of the Czech Republic for the species identification of tick-borne pathogens. *Borrelia burgdorferi* sensu lato spirochetes were the most common pathogen detected in the ticks; 21% of ticks were positive for a single genospecies and 2% were co-infected with two genospecies. Other tick-borne pathogens detected included *Rickettsia helvetica* (3.9%), *R. monacensis* (0.2%), *Anaplasma phagocytophilum* (2.8%), *Babesia venatorum* (0.9%), and *Ba. microti* (0.5%). The vertebrate host of the ticks was determined using PCR followed by reverse line blot hybridization from the tick's blood-meal remnants. The host was identified for 61% of ticks. DNA of two hosts was detected in 16% of samples with successful host identification. The majority of ticks had fed on artiodactyls (50.7%) followed by rodents (28.6%) and birds (7.8%). Other host species were wild boar, deer, squirrels, field mice and voles.

Keywords: tick; *Ixodes ricinus*; PCR-ESI/MS; *Borrelia*; host; Lyme borreliosis; *Babesia*; *Anaplasma*; *Rickettsia*

INTRODUCTION

Ticks harbor a wide variety of viruses, bacteria and protozoans, some of which can be transmitted to vertebrate hosts, including humans, and cause disease (Parola and Raoult 2001; Charrel *et al.* 2004). *Ixodes ricinus* is the most widespread human biting tick in Europe. It is the vector of multiple pathogens including the *Borrelia burgdorferi* sensu lato complex, which cause Lyme borreliosis; the intracellular bacteria *Anaplasma phagocytophilum*, which cause granulocytic anaplasmosis (Woldehiwet 2006); several species of *Rickettsia* (Karbowiak *et al.* 2016); and protozoan parasites of the genus *Babesia* (Hunfeld and Brade 2004; Yabsley and Shock 2013). In nature, ticks transmit these pathogens between species of vertebrate blood-meal hosts. Humans and some species of animals are the accidental, and frequently 'dead end', hosts. The species of the tick blood-meal hosts differ in their abundance, in the level of tick infestation, and in the efficiency of pathogen gain, amplification and transmission (Perez *et al.* 2012; Geller *et al.* 2013; Hofmeester *et al.* 2016). Therefore, the knowledge of the impact of a particular host species on pathogen circulation is of substantial importance for the estimation of infection risk and preventive measures design.

Ixodes ricinus is a host generalist able to feed on more than 300 vertebrate species including rodents (Kozuch *et al.* 1967; Matuschka, Richter and Spielman 1991; Estrada-Pena *et al.* 2005; Perez *et al.* 2012), birds (Estrada-Pena *et al.* 2005; Geller *et al.* 2013; Lommano *et al.* 2014), insectivores (Kozuch *et al.* 1967; Matuschka, Richter and Spielman 1991; Perez *et al.* 2012), artiodactyls (Kiffner *et al.* 2010; Kjelland *et al.* 2011) and reptiles (Matuschka, Richter and Spielman 1991; Majlathova *et al.* 2006). This behavior makes it difficult to decipher the complex system of zoonotic pathogen circulation among the populations of hosts and tick vectors. Host trapping methods frequently focus only on selected species and are influenced by differences in trapping effort and efficiency. Therefore, the contribution of the individual species to the overall pool of tick hosts is not easy to assess from this type of data. Molecular biology methods, which allow host identification in questing ticks, have potential to overcome these obstacles. These techniques are primarily based on polymerase chain reaction (PCR) amplification of host DNA from blood remnants in the questing tick using universal primers. Host species identification is performed by the means of reverse hybridization (reverse line blotting) (Kirstein and Gray 1996; Gray *et al.* 1999; Pichon *et al.* 2003; Humair *et al.* 2007), restriction fragment polymorphism analyses (Kirstein and Gray 1996; Wodecka, Rymaszewska and Skotarczak 2013), or sequencing (Gariepy *et al.* 2012). Currently available DNA-based methods differ in the target nucleotide sequence as well as in the spectrum of hosts they are able to detect and identify at the taxonomic level (Estrada-Pena *et al.* 2005; Pichon *et al.* 2005; Humair *et al.* 2007; Kent 2009). DNA-based host identification methods are generally able to identify the host in approximately 30%–60% of the samples (Pichon *et al.* 2005; 2006, Humair *et al.* 2007; Allan *et al.* 2010; Wodecka, Rymaszewska and Skotarczak 2013; Léger *et al.* 2015). In this work, we identified host DNA from *I. ricinus* blood remnants by a reverse line blotting procedure described by Humair *et al.* (2007) using species-, genus-, and a group of species-specific probes described by Moran Cadenas *et al.* (2007).

To survey the prevalence of tick-borne pathogens in *I. ricinus* ticks, we used a broad-range PCR electrospray ionization mass spectrometry (PCR/ESI-MS) assay designed to detect a wide-range of tick-borne organisms. We have previously applied this technique for the detection of vector-borne pathogens such as *Borrelia, Ehrlichia,* Powassan virus, *Babesia* spp. and canine heartworm from ticks and/or clinical specimens (Crowder *et al.* 2010b, 2012; Eshoo *et al.* 2010, 2012, 2014). In addition, this PCR/ESI-MS technique was used to detect *Borrelia* DNA in ticks used for the xenodiagnoses of patients with Lyme disease (Marques *et al.* 2014). Following a multilocus broad-range PCR, automated electrospray ionization mass spectrometry is used to determine the masses of the PCR amplicons. From these masses, basecount signatures (i.e. the number of A's, G's, C's, and T's) are determined for each primer pair, which are then matched to a database to identify the microorganism or microorganisms present in the sample (Ecker *et al.* 2006a). This technique identifies pathogens, including those involved in co-infections, genotypes pathogens, and can identify new genetic variants. For example, we previously demonstrated our ability to distinguish *B. burgdorferi* genotypes, even when present in mixtures of genotypes (Crowder *et al.* 2010a), and we also identified a novel and widespread *Anaplasmataceae* species in a survey of *I. pacificus* ticks in California using our PCR/ESI-MS method (Eshoo *et al.* 2015). This same approach can be used to detect and identify a wide range of vector-borne pathogens in a single test (Eshoo *et al.* 2014).

In the present work, we found that *B. burgdorferi* sensu lato group were the most common microorganisms detected in the *I. ricinus* nymphal ticks in the Southern Czech Republic. Of 435 specimens, 100 (23%) were positive for *B. burgdorferi*. In 91% of the positive detections, we observed a single genospecies, whereas 9% of these were simultaneously infected with two genospecies. Other microorganisms detected included *A. phagocytophilum, Babesia* spp. and *Rickettsia* spp. Identification of the previous host was successful in 61% of the *I. ricinus* nymphs with artiodactyls and rodents being the most prevalent host species.

MATERIALS AND METHODS

Tick collection and nucleic acid extractions

A total of 435 *I. ricinus* nymphs were collected by flagging in mixed and deciduous forests at four sites in South Bohemia region of the Czech Republic during the summer of 2010 (Fig. 1). A total of 153 ticks were collected from locality Zavadilka (48°58′47.32″N, 14°25′47.41″E; 415 m above the sea level) between 28 May 2010 and 8 June 2010. This sampling site is in a peri-urban mixed forest near recreational and residential housing areas of the city of Ceske Budejovice. In the tree stratum, *Picea abies, Pinus sylvestris, Quercus* spp. prevail, accompanied by *Tilia cordata, Populus tremula, Corylus avellana*. The density of undergrowth and height of litter layer are variable. On 8 August 2010, 89 ticks were collected from Netolice (49°2′28.29″N, 14°10′41.90″E; 470 m a. s. l.). The habitat is slightly sloped with orientation toward the south and is markedly drier compared to the other collection sites. Deciduous trees predominate (*T. cordata, Quercus* spp., *B. pendula, Fraxinus excelsior, Fagus sylvatica*), although formations of *P. abies* are also present. The undergrowth consists mainly of grass species (*Calamagrostis epigejos, Dactylis glomerata, Poa annua*) and herbs (*Holcus mollis, Veronica officinalis*). On 8 June 2010, 100 ticks were collected from Blatná (49°26′42.54″N, 13°52′56.57″E; 480 m a. s. l.). The terrain is flat and covered by variegated vegetation of an ecotonous character. Vegetation cover consists of deciduous trees (*Acer platanoides, P. tremula, C. avellana, Salix caprea, Q. petraea, Carpinus betulus, Prunus avium*). In the undergrowth, the grass species (*Poa nemorosa, Festuca ovina, C. epigejos, D. glomerata*) together with *Dryopteris filix-mas, V. officinalis* and *Lamium* spp. are observed. On 10 June 2010, 93 ticks were collected from Dačice
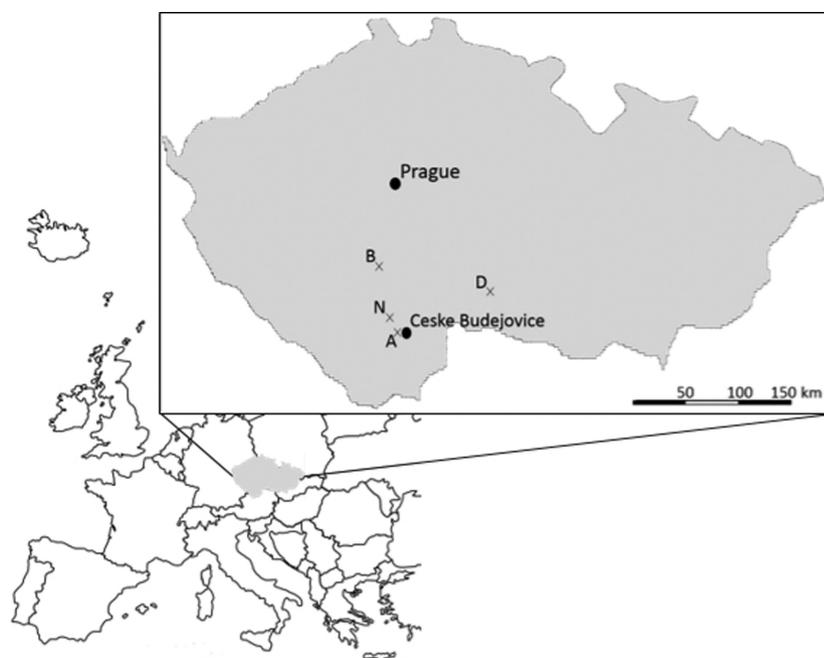
Figure 1. Tick sampling sites located in the southern part of the Czech Republic. A, Zavadilka; B, Blatná; D, Dačice; N, Netolice.

(49°5′56.90″N, 15°26′24.06″E; 610 m a. s. l.). This sampling site is characterized by dense shrubby vegetation of deciduous tree species (*Q. robur* mainly) with minor representation of *P. abies*. The herbaceous stratum is also dense and rich in species (*Stellaria holostea*, *Lathyrus vernus*, *Pulmonaria officinalis*, *Hypericum perforatum*, *Campanula trachelium, Rubus idaeus*). Sample homogenization and total nucleic acids extraction was performed using a previously described Qiagen column-based protocol, with the substitution of the Qiagen DNeasy column (Qiagen, Valencia, CA, USA) for the Qiagen Virus Minelute columns (Crowder *et al.* 2010b). Nucleic acids were extracted from the ticks at the University of South Bohemia, and the extracts shipped overnight at room temperature in screw capped tubes to Ibis Biosciences for analysis on the PCR/ESI-MS system using the broad-range vector-borne microorganism detection assay. The extracts were stabilized prior to shipping using either RNAstable (Biomatrica, San Diego, CA, USA) according to the manufacturer's instructions or with 1–2 μL/sample SUPERase-in (Invitrogen, Carlsbad, CA, USA).

Identification and characterization of tick-borne pathogens by PCR/ESI-MS

Detection of tick-borne pathogens was performed using a PCR/ESI-MS assay using nine broad-range PCR primer pairs designed to amplify genomic material from bacterial and protozoan vector-borne organisms (Table 1). Primer pairs, BCT3517 and INV4855 were employed in a single multiplexed reaction. Primer pairs BCT2328, BCT3511, INV4443, BCT3514, BCT1083, BCT3570 and BCT3575 were employed in individual singleplex PCR reactions (Eshoo *et al.* 2015). Internal positive controls made from cloned synthetic DNA (BlueHeron Biotechnology, Bothell, WA, USA) were included in each PCR reaction at 20 copies per reaction. The internal controls were designed to be identical to the expected amplicon for one of the primer pairs in the reaction with the exception of a 5-base pair deletion to enable the control to be distinguished from the target-derived amplicon.

PCR was performed in a 50 μL reaction volume with 10 μL nucleic acid extract in a reaction mix as previously described (Crowder *et al.* 2010a). PCR cycling conditions were the same as those reported previously (Eshoo *et al.* 2007). PCR amplicons were analyzed by ESI-MS, and base composition analyses were performed on a research-use-only ESI-MS instrument (Abbott Molecular, Des Plaines, IL, USA) as previously described (Ecker *et al.* 2005, 2006b, 2010; Crowder *et al.* 2010a, 2012; Eshoo *et al.* 2010; Rounds *et al.* 2012).

The *Babesia venatorum* PCR/ESI-MS signature was confirmed by Sanger sequencing of a 613-nt portion of the 18S rDNA, the ITS1 and ITS2 regions (~550 and ~400 nt, respectively), and a 333-nt segment of the *HSP70* gene as previously described (Blaschitz *et al.* 2008). All sequencing PCR reactions were performed using Platinum Taq High Fidelity (Invitrogen) in Platinum Taq buffer with 200 μM of each dNTP, 2 mM MgSO$_4$ and 250 nM of each primer. Sequencing PCR reactions were cycled with the following conditions: 95°C for 2 min; 8 cycles of 95°C for 15 s, 50°C for 45 s (increasing 0.6°C per cycle) and 68°C for 90 s; 37 cycles of 95°C for 15 s, 60°C for 15 s, and 68°C for 60 s; followed by 4 min at 68°C. For samples requiring cloning, PCR products were ligated and cloned using the ZeroBlunt TOPO PCR Cloning kit (Invitrogen) according to the manufacturer's instructions. Individual clones were purified and sequenced by SeqWright (Houston, TX, USA) using SP6 and T7 promoter primers.

Borrelia genotyping

All *Borrelia*-positive specimens were genotyped by a previously described multilocus PCR/ESI-MS *Borrelia* genotyping assay, which targets eight loci in the *Borrelia* genome to enable genotype assignment (Crowder *et al.* 2010a). *Borrelia garinii* identification requires amplification of only seven primers as it is frequently not detectable with primer pair BCT3514. For *Borrelia* species with fewer than 20 genomes/PCR reaction as quantified by the PCR/ESI-MS instrument, the nucleic acid extracts were treated with an isothermal amplification prior to genotyping as previously described (Eshoo *et al.* 2012).

Table 1. PCR/MS-ESI and DNA sequencing primers and targets.

Primer pair	Primer ID	Primer sequence	Target	Target clade/genus	Purpose	Reference
BCT2328	BCT5602F / BCT5603R	TGAGGGTTTTATGCTTAAAGTTGGTTTTATTGGTT / TGATTCGATCATACGAGACATTAAAACTGAG	asd	*F. tularensis*	PCR/ESI-MS	(Crowder et al. 2012)
INV4855	INV10812F / INV10813R	TGAGAGAAATCGTACACATTCAAGCGGG / TCCATGTTCGTCGGAGATGACTTCCCA	*β-tubulin*	All *Babesia* spp.	PCR/ESI-MS	(Crowder et al. 2012)
INV4443	INV10034F / INV10035R	TGGCGAAATTACCGAATCCTGACAC / TCCAGACTTGCCCTCCAATTGTA	18S rRNA	All *Babesia* spp.	PCR/ESI-MS	(Crowder et al. 2012)
BCT3511	BCT8229F / BCT8230R	TGCATTTGAAAGCTTGGCATTGCC / TCATTTTAGCACTTCCTCCAGCAGAATC	*gyrB*	All Spirochetes	PCR/ESI-MS	(Crowder et al. 2012)
BCT3514	BCT8235F / BCT8236R	TTTGTACCACAAAGGAATGGGA / TGCGAGCTCTATATGCCCCAT	*rpoC*	All Spirochetes	PCR/ESI-MS	(Crowder et al. 2012)
BCT3517	BCT8241F / BCT8242R	TGCTGAAGAGCTTGGAATGCA / TACAGCAAATTGCTTGATCTTGATTTGC	*flagellin*	All Spirochetes	PCR/ESI-MS	(Crowder et al. 2012)
BCT1083	BCT2764F / BCT2763R	TAAGAGCGCACCGGTAAGTTGG / TCAAGCGGATCTACCCGCATTACAA	*RNaseP*	All *Rickettsia* spp.	PCR/ESI-MS	(Crowder et al. 2012)
BCT3570	BCT8336F / BCT8337R	TGCATGCAGATCATGAACAGAATGC / TCCACCATGAGCTGGTCCCCA	*gltA*	Alphaproteobacteria	PCR/ESI-MS	(Crowder et al. 2012)
BCT3575	BCT8346F / BCT8347R	TGCATCACTTGGTTGATGATAAGATACATGC / TCACCAAAACGGCTGACCACCAAA	*rpoB*	Alphaproteobacteria	PCR/ESI-MS	(Crowder et al. 2012)
Ap-msp4	F / R	M13F-ICAGTMTGYGCYTGCTCCCT / M13R-CCTTAIYTGAAMISIAATCTTGCTCC	*msp4*	*Anaplasma* spp.	Sequencing	This study
Ap-groEL	F / R	M13F-GAIAIIACTGAYGGTATGCAGTTTG / M13R-CYAIMGIYTCYYTMAGYTTTTTCCTT	GroEL	*Anaplasma* spp.	Sequencing	This study
18S rRNA-M13	F / R	M13F-GACTAGGGATTGGAGGTC / M13R-GAATAAATTCACCGGATCACTC	18S rRNA	*Babesia* spp.	Sequencing	(Blaschitz et al. 2008)
ITS1-M13	F / R	M13F-CGAGTGATCCGGTGAATTATTC / M13R-CCTTCATCGTTGTGTGAGCC	ITS1	*Babesia* spp.	Sequencing	(Blaschitz et al. 2008)
ITS2-M13	F / R	M13F-GGCTCACACAACGATGAAGG / M13R-CTCGCCGTTACTAAGGGAATC	ITS2	*Babesia* spp.	Sequencing	(Blaschitz et al. 2008)
HSP70-M13	F / R	M13F-GCTATTGGTATTGACTTGGG / M13R-CCTTCATCTTGATAAGGACC	*hsp70*	*Babesia* spp.	Sequencing	(Blaschitz et al. 2008)
Bb-16S	F / R	M13F-CGCTGGCAGTGCGTCTTAAG / M13R-GCGTCAGTCTTGACCCAGAAGTTC	16S rRNA	*B. burgdorferi* s. l.	Sequencing	This study
Bb-ITS	F / R	M13F-CTGCGAGTTCGCGGGAGA / M13R-TCCTAGGCATTCACCATA	ITS	*B. burgdorferi* s. l.	Sequencing	This study
rrs-rrIA IGS	F / R	GTATGTTTAGTGAGGGGGTG / GGATCATAGCTCAGGTGGTTAG	IGS Outer	*B. miyamotoi*	Sequencing	(Bunikis et al. 2004)
rrs-rrIA IGS-M13	F / R	M13F-AGGGGGGTGAAGTCGTAACAAG / M13R-GTCTGATAAACCTGAGGTCGGA	IGS Inner	*B. miyamotoi*	Sequencing	(Bunikis et al. 2004)
M13	F / R	CCCAGTCACGACGTTGTAAAACG / AGCGGATAACAATTTCACACAGG	M13		Sequencing	(Eshoo et al. 2007)

Tick host identification

Identification of the host DNA from blood remnants in the tick was carried out using reverse line blotting according to Humair *et al.* (2007). Briefly, a portion of 12S rDNA was amplified using a universal pair of primers with the reverse primer labeled on the 5′ end with biotin. PCR was performed in 25-μL reactions using PPP Premix (Top-Bio, Prague, Czech Republic), 0.4 μM primer 12S-6F (CAAACTGGGATTAGATACC) and 0.4 μM primer B-12S-9R (biotin-AGAACAGGCTCCTCTAG) (Humair *et al.* 2007), and 10 μL of tick extractions as source of template DNA brought to volume with PCR-grade water. The amplification was carried out according to the cycling profile previously reported (Humair *et al.* 2007). Subsequently, the PCR products were hybridized to species-, genus- and group of species-specific probes described previously (Moran Cadenas *et al.* 2007); *Rattus rattus* and *Neomys anomalus* probes were not used. The probes were bound to Biodyne C membranes and hybridized with the labeled PCR products. After washing and binding of streptavidin-linked horseradish peroxidase, the hybridized PCR products were visualized on a sensitive film using chemiluminescence (Humair *et al.* 2007).

Reverse line blotting is a highly sensitive technique that requires multiple steps and hence tends to be sensitive to cross-contamination among the samples, external contamination from environment, and surface contamination of the ticks. The possibility of contamination can never be fully excluded, but measures were taken to minimize the risk and monitor possible false positive results. Pre-PCR and post-PCR processes were spatially separated and were performed at different times. All nucleic acid extractions performed in a biohazard box (class II), and all PCR mastermixes were prepared in a PCR box. All surfaces were treated before and after work with UV light (20 min), DNA remover solution (Minerva Biolabs, Berlin Germany), and were subsequently washed with dH$_2$O. Filtered tips were used for pipetting. In each set of samples, a negative extraction control (a blank sample without a tick) was included that entered the analysis on the level of sample homogenization (i.e. prior to nucleic acid extraction). Thus, the negative controls were subjected to all the components (except the tick) and all steps of the process from sample homogenization to analysis.

Statistics

The data were analyzed using χ^2 or Fisher´s exact test in case of low frequencies (GraphPad Software, Inc., San Diego, California, USA, version 5.04). Differences with $P < 0.05$ were considered significant.

RESULTS

Tick collections and broad-range detection PCR/ESI-MS

A total of 435 *I. ricinus* nymphs were collected during a 10-week period over the summer of 2010 from four sites in the South Bohemia region of the Czech Republic. Tick extracts were analyzed using a broad-range vector-borne assay designed to detect and identify to the species level a wide range of vector-borne pathogens and other microorganisms, notably spirochetes (including all *Borrelia* species), *Anaplasma* spp., *Ehrlichia* sp., *Rickettsia* spp., *Franciscella* sp., *Babesia* spp. and tick species-specific endosymbionts.

The *B. burgdorferi* sensu lato group was the most common group of organisms found in the ticks with 100 of the 435 (23%) ticks positive for this pathogen. Infections by a single genospecies were detected in 91 of these 100 (91%) ticks, whereas nine (9%) of the *B. burgdorferi*-positive ticks were simultaneously infected with two genospecies (Table 2). The most prevalent *B. burgdorferi* genospecies (multiple infections included) was *B. afzelii* (38/109 or 34.9% of all *B. burgdorferi* s.l. positive identifications), followed by *B. burgdorferi* s.s. (32/109 or 29.4%) and *B. garinii* (23/109 or 21.1%). *Borrelia valaisiana* was observed in 8 of 109 (7.3%) of identifications and was not found at study site Dačice. *Borrelia lusitaniae* was found at only one site, Netolice, and accounted for eight (7.3%) of identifications overall. The relapsing fever-like spirochete, *B. miyamotoi*, was detected in 9 of the 435 (2.1%) ticks (Table 2).

In addition to the *Borrelia* species mentioned above, several other organisms were found in the ticks. *Anaplasma phagocytophilum* was detected at all four tick collection sites and was observed in 12 of 435 (2.8%) ticks (Table 2). Two variants of *A. phagocytophilum* were detected; both shared the same basecount for primer pair BCT3575 (A22 G33 C20 T27), but differed by a single nucleotide polymorphism in the region amplified by primer pair BCT3570 (A23 G39 C27 T36 and A24 G38 C27 T36).

Babesia spp. were found in 7 of 435 (1.6%) *I. ricinus* nymphs. *Babesia microti* was detected once at Netolice and once at Zavadilka with primer pairs INV4855 and INV4443, producing the expected basecounts of A29 G22 C15 T20 and A45 G32 C25 T28, respectively. A different basecount signature was found in four ticks, one each from Blatná and Dačice and two from Zavadilka with these same primers, producing novel basecounts of A26 G24 C20 T16 and A44 G32 C26 T27, respectively. By sequencing of the 18S, ITS1, ITS2 and *HSP70* loci from one of the representative positive specimens, this signature was determined to represent *Ba. venatorum* (formerly called *Babesia* sp. EU1). The 18S rDNA sequence exhibited identity of 611 or 613 nucleotides with *Babesia* sp. '*venatorum*' isolate DP-1569 (accession number JX042320.1), and the ITS1 sequence was identical to that of *Babesia* sp. '*venatorum*' (accession number HM113372.1). The 408-nt ITS2 and 33-nt *HSP70* sequences from our *Ba. venatorum* were identical to accession numbers EU185802.1 and EU185813.1 from a previously described *Babesia* sp. GoA3. However, no ITS2 or *HSP70* sequences exist in GenBank for *Ba. venatorum*, nor do any 18S rDNA or ITS1 sequences exist for *Babesia* sp. GoA3. Given the close match of the 18S rDNA and the ITS region to *Ba. venatorum*, our *Babesia* signature is considered to be that of the widely reported *Ba. venatorum*.

Rickettsia spp. were found in 18 of 435 (4.1%) of the tick samples. Two species of *Rickettsia* were detected: *R. helvetica* and *R. monacensis*. The former was observed at all four sites in 17 (3.9%) of ticks (Table 2); the basecounts for primer pairs BCT1083 and BCT3570 (A41 G33 C30 T31 and A29 G30 C29 T37, respectively) matched those found in German ticks (Eshoo *et al.* 2014). We also detected *R. monacensis* (BCT1083: A42 G33 C24 T30; BCT3570: A28 G30 C32 T35) in one tick from Zavadilka (Table 2). None of the samples tested were positive for *Franciscella tularensis*. No significant differences were found in the prevalence rate of the individual pathogens among the sampling sites.

Borrelia genotyping

The *B. burgdorferi* s.l. positive specimens were further characterized using a previously described PCR/ESI-MS based *Borrelia* genotyping assay (Crowder *et al.* 2010a). As the nymph extracts contained a limited amount of *Borrelia* DNA, most *Borrelia* positive specimens were subjected to a nested isothermal amplification to increase the amount of material available for genotyping (Eshoo *et al.* 2012). Of the *B. burgdorferi* sensu stricto (s.s.)-positive

Table 2. Identification of microorganisms in nymphal *Ixodes ricinus* ticks using PCR/ESI-MS.

		Site				
		Blatná	Dačice	Netolice	Zavadilka	Overall
	Number of ticks	100	93	89	153	435
	Pathogen					
Single infections	*Anaplasma phagocytophilum*	3	0	2	4	9
	Babesia microti	0	0	1	0	1
	Babesia venatorum	0	1	0	2	3
	Babesia sp. unknown	0	0	1	0	1
	Borrelia afzelii	4	4	5	19	32
	Borrelia burgdorferi s.s.	10	8	2	6	26
	Borrelia garinii	7	2	3	4	16
	Borrelia lusitaniae	0	0	7	0	7
	Borrelia valaisiana	2	0	1	0	3
	Borrelia miyamotoi	2	2	0	2	6
	Rickettsia monacensis	0	0	0	1	1
	Rickettsia helvetica	4	1	3	4	12
Co-infections	*B. burgdorferi* s.s. and *B. afzelii*	0	3	0	0	3
	B. garinii and *B. valaisiana*	1	0	1	2	4
	B. lusitaniae and *B. valaisiana*	0	0	1	0	1
	B. miyamotoi and *B. garinii*	0	0	0	1	1
	B. miyamotoi and *A. phagocytophilum*	0	0	0	1	1
	B. burgdorferi s.s. and *R. helvetica*	0	0	0	1	1
	B. afzelii and *R. helvetica*	1	0	0	0	1
	B. garinii and *R. helvetica*	0	0	1	0	1
	B. garinii and *Babesia venatorum*	1	0	0	0	1
	B. afzelii and *Babesia microti*	0	0	0	1	1
	B. burgdorferi s.s. and *B. afzelii* and *R. helvetica*	1	0	0	0	1
	B. burg. s.s. and *B. miyamotoi* and *A. phagocytophilum*	0	1	0	0	1
	A. phagocytophilum and *R. helvetica*	0	0	0	1	1
	Total infected	36	22	28	49	135
	% infected	36.0%	23.7%	31.5%	32.0%	31.0%

ticks, there were two genotypes that differed from the majority of the detections (Fig. 2). The first variant, found in two ticks from Dačice, had a unique basecount at primer pair BCT3515 (A15 G23 C15 T15). The second variant, found in five ticks from Blatná and one from Dačice, had a unique basecount at primer pair BCT3517 (A40 G25 C24 T32).

Co-infections

In the 435 ticks tested, 18 (4.1%) contained more than one organism (including different *B. burgdorferi* s.l. genospecies) (Table 2). Of these, 16 (89% of co-infections) harbored two organisms and two (11%) harbored three. The most frequent co-infection was two *B. burgdorferi* s.l. genospecies (50.0%), followed by a *B. burgdorferi* s.l. genospecies and *R. helvetica* (22%).

Tick host identification

Aliquots of samples analyzed by PCR/ESI-MS were subjected to host identification by reverse line blotting. From the total number of 435 samples of questing *I. ricinus* nymphs, identification of the previous host was successful in 266 samples (61.1%). In 42 (15.8%) of cases, DNA of two different hosts was identified, resulting in a total of 308 host identifications. The differences in identification success rate among the localities (range 56.0% to 64.5%) were not statistically significant (Table 3). Artiodactyls (50.7%) and rodents (28.6%) were the most prevalent host species, while birds, small predatory mammals, and insectivores were less frequently a source of blood-meal for larval

ticks (Table 3). Most of the hosts were identified only to the group level. *Sus scrofa* (105/156 or 67.3%) and *Capreolus capreolus* (2/156 or 1.3%) were detected among artiodactyl host species. *Sciurus vulgaris* (10/119 or 8.4%) was the most frequently identified rodent species followed by *Apodemus* spp. (5/119 or 4.2%), *Microtus* spp. (5/119 or 4.2%) and *Myodes* (*Clethrionomys*) *glareolus* (Carleton *et al.* 2014) (1/119 or 0.8%). The only avian species-specific identification was achieved by a single probe specific for *Turdus* spp. and *Parus* spp. host DNA (3/24 or 12.5%). *Mustella* spp. (8/8) and *Neomys* sp. (1/1) were detected among predatory mammals and insectivores, respectively.

The composition of the tick host fauna differed among the individual sampling sites. Locality Netolice presented with a statistically significant higher portion of rodent blood-fed ticks (Fisher exact test; P < 0.01) and a relatively lower frequency of artiodactyl host species (Fisher exact test; P < 0.01) than other sites. Ticks sampled in locality Dačice had significantly more frequently fed on birds than ticks from the Zavadilka study site (Fisher exact test; P < 0.01); differences from other localities were not statistically significant (Table 3).

Tick hosts and pathogens

From the 266 ticks with only one host species identified, 72 carried at least one pathogen (including four samples with two pathogens). The prevalence rate of *B. burgdorferi* s.l. was 23.9% in rodent-fed ticks, 26.1% in ticks fed on artiodactyl blood and 16.7% in bird-fed ticks (Table 4). The differences in pathogen prevalence rate among ticks fed on different groups of hosts

Genos pecies	Genotype	BCT3514	BCT3515	BCT3516	BCT3517	BCT3518	BCT3519	BCT3520	BCT3511	Czech Sample ID
Borrelia burgdorferi sensu stricto	14	A45G37C14T45	A15G22C15T16	A38G28C23T39	A37G29C24T31	A39G21C14T36	A42G30C19T44	A58G23C15T45	A36G30C14T36	B58†, B60, B65, B72, D31, D67, N1
	16	A45G37C14T45	A15G22C15T16	A38G28C23T39	A37G29C24T31	A37G23C14T36	A42G30C19T44	A58G23C15T45	A36G30C14T36	A56, A75, A121, N21
	21	A45G37C14T45	A15G22C15T16	A39G27C23T39	A40G25C24T32	A38G22C14T36	A39G32C19T45	A58G22C15T46	A36G30C14T36	B50†, B56, B63, B69, D65
	84	NoDetect	A15G22C15T16	A39G27C23T39	A40G25C24T32	A39G21C14T36	A39G32C19T45	A58G22C15T46	A36G30C14T36	B11
	85	NoDetect	A15G23C15T15	A39G27C23T39	A39G27C24T31	A37G23C14T36	A39G32C19T45	A58G22C15T46	A36G30C14T36	D16
	86	A45G37C14T45	A15G23C15T15	A39G27C23T39	NoDetect	A37G23C14T36	A42G30C19T44	A58G22C15T46	A36G30C14T36	D44†
Borrelia afzelii	1	A44G39C15T43	A15G22C17T14	A39G25C20T44	A39G27C24T31	A35G25C18T35	A41G30C20T44	A58G22C15T46	A35G31C15T35	A11
	2	A44G39C15T43	A15G22C17T14	A39G25C20T44	A39G27C24T31	A35G25C18T35	A41G30C21T43	A58G22C14T47	A35G31C15T35	A99, A119, N52
	3	A44G39C15T43	A15G22C17T14	A39G25C20T44	A37G22C18T36		A41G30C21T43	A58G22C14T47	A35G31C15T35	D12*
	4	A44G39C15T43	A15G22C17T14	A39G25C20T44	A39G27C24T31	A36G23C16T35	A41G30C21T43	A58G22C14T47	A35G31C15T35	A26*, A59, A90*, D12*, N20*, N83
	6	A44G39C15T43	A15G22C17T14	A39G25C20T44	A39G27C24T31	A36G24C18T35	A41G30C21T43	A58G22C14T47	A35G31C15T35	A01, A18, A26*, A29, A37, A90*, B21, B51, B62, B74, D49†, D56*, D60, N99*
	7	A44G39C15T43	A15G22C17T14	A39G25C20T44	A39G27C24T31	A35G25C17T36	A41G30C21T43	A58G22C14T47	A35G31C15T35	A34, A36, A65, A96, D56*, N09
	10	A44G39C15T43	A15G22C17T14	A38G26C20T44	A39G27C24T31	A36G24C15T35	A41G30C21T43	A58G22C14T47	A35G31C15T35	D19
	11	A44G39C15T43	A15G22C17T14	A39G25C20T44	A39G27C24T31	A36G24C15T35	A39G32C23T41	A58G22C14T47	A35G31C15T35	N20*
	12	A44G39C15T43	A15G22C17T14	A39G25C20T44	A39G27C25T30	A35G25C18T35	A41G30C20T44	A59G21C15T46	A35G31C15T35	A124
B. garinii	1	A48G35C13T45	A14G23C16T15	A38G26C22T42	A34G20C17T33	A34G20C16T34	A41G31C16T47	A58G22C15T46	A36G30C18T32	A118, N08*
	3	NoDetect	A14G23C16T15	A38G26C22T42	A40G26C25T30	A34G20C16T34	A41G31C16T47	A59G21C15T46	A36G30C17T33	B30, B93*
	5	A48G35C13T45	A14G23C16T15	A38G26C22T42	A39G27C25T30	A34G20C16T34	A41G31C16T47	A58G22C15T46	A36G30C18T32	B05
	6	NoDetect	A14G23C16T15	A38G26C22T42	A39G27C25T30	A33G21C16T34	A41G31C16T47	A59G21C15T46	A36G30C18T32	A21, B70, B89*†
	7	NoDetect	A14G23C16T15	A38G26C22T42	A39G27C25T30	A34G20C16T34	A41G31C17T46	A59G21C15T46	A36G30C19T31	A68
	8	NoDetect	A14G23C16T15	A38G26C22T42	A39G27C25T30	A34G20C16T34	A41G31C16T47	A59G21C15T46	A36G30C17T33	A115*, B93*, N41*
	10	NoDetect	A14G23C16T15	A38G26C22T42	A40G26C25T30	A34G20C16T34	A41G31C16T47	A59G21C15T46	A36G30C18T32	B89*†
	12	NoDetect	A14G23C16T15	A38G26C22T42	A40G26C25T30	A34G20C17T33	A41G31C16T47	A59G21C15T46	A36G30C18T32	A115*
	13	A48G35C13T45	A14G23C16T15	A38G26C22T42	A37G28C26T30	A34G20C17T33	A36G30C18T47	A58G22C15T46	A34G32C20T30	N08*
	14	A48G35C13T45	A14G23C16T15	A38G26C22T42	A37G28C26T30	A34G20C16T34	A41G30C16T48	A58G22C15T46	A36G30C18T32	N41*
	15	NoDetect	A14G23C16T15	A38G26C22T42	A39G27C25T30	A33G20C16T35	A41G31C16T47	A59G21C15T46	A36G30C18T32	B84, D80
	16	NoDetect	A14G22C16T16	A38G25C23T41	A39G27C26T29	A34G19C15T36	A42G32C16T47	A58G22C14T47	A36G30C19T31	A22†, A129†
	17	A49G34C13T45	A14G23C16T15	A38G26C22T42	A39G27C25T30	A33G21C16T34	A41G31C16T47	A58G22C15T46	A36G30C18T32	B67
	18	A48G35C13T45	A14G23C15T16	A38G26C22T42	A39G27C27T28	A34G20C15T35	A41G31C16T47	A58G22C15T46	A36G30C18T32	B87
	19	A44G39C14T44	A14G23C16T15	A38G26C22T42	A39G27C25T30	A34G20C17T33	A41G31C16T47	A58G22C15T46	A36G30C18T32	D94
	20	A48G35C13T45	A14G23C16T15	A38G26C22T42	A39G27C27T28	A34G20C16T34	A41G31C16T47	A58G22C15T46	A36G30C18T32	B37*
	21	A48G35C13T45	A14G23C15T16	A38G26C22T42	A39G27C25T30	A34G20C16T34	A41G31C16T47	A58G22C15T46	A36G30C18T32	B37*
B. valaisiana	3	A44G39C14T44	A15G22C16T15	A38G26C21T43	A39G27C26T29	A35G20C15T34	A42G29C17T47	A58G22C14T47	A34G32C17T33	B92
	4	A44G39C14T44	A15G22C16T15	A37G27C22T42	A39G27C26T29	A35G23C14T32	A41G30C17T47	A58G22C14T47	A34G32C17T33	A22†, N30
B. lusitaniae	2	A43G40C15T43	A15G22C17T14	A37G28C22T41	A39G27C23T32	A35G23C14T35	A41G29C19T46	A58G22C15T46	A34G32C16T34	N85*, N88, N93*
	3	A43G40C15T43	A15G22C17T14	A37G28C22T41	A39G27C23T32	A35G22C14T36	A41G29C19T46	A58G22C15T46	A34G32C17T33	N74, N93*
	4	A43G40C15T43	A15G22C17T14	A37G28C22T41	A39G27C23T32	A35G22C14T36	A41G29C19T46	A58G22C15T46	A34G32C16T34	N85*
European relapsing fever *Borrelia*	1	NoDetect	A13G22C15T18	NoDetect	A41G30C23T27	A37G23C14T36	A41G29C19T46	A52G29C13T47	A36G32C13T35	A06†, A110, A129†, A136, B29, B40, D44†, D66†, D100

Figure 2. *Borrelia burgdorferi* s.l. genotypes detected among *I. ricinus* ticks from the Czech Republic. Each column represents basecounts of a specific PCR product (primer pairs marked with BCT and number). '*' indicates more than one genotype of particular genospecies present; '†' indicates more than one *B. burgdorferi* s.l. genospecies detected. Sample ID code: A, Zavadilka; B, Blatna, D, Dacice; N, Netolice.

Table 3. Tick hosts by sampling site.

	Zavadilka	Blatna	Dacice	Netolice	Total
Identification success rate	60.8%	56%	64.5%	64%	61.1%
	(93/153)	(56/100)	(60/93)	(57/89)	(266/435)
Rodents	37.6%	31.7%	26.9%	**62.3%	28.6%
	(41/109)	(19/60)	(21/78)	(28/61)	(119/308)
Artiodactyls	54.1%	60%	53.9%	**31.15%	50.7%
	(59/109)	(36/60)	(42/78)	(19/61)	(156/308)
Birds	5.5%*	5.0%	*15.4%	4.9%	7.8%
	(6/109)	(3/60)	(12/78)	(3/61)	(24/308)
Predatory mammals	1.8%	3.3%	3.9%	1.6%	2.6%
	(2/109)	(2/60)	(3/78)	(1/61)	(8/308)
Insectivores	0.9%	0.0%	0.0%	0.0%	0.3%
	(1/109)	(0/60)	(0/78)	(0/61)	(1/308)

Identification success rate is the number of positively identified samples out of the number of ticks tested; proportional representation of groups of host species is the number of identifications of a particular host out of all successful identifications including two hosts identified in one sample. Statistically significant results are indicated by *P < 0.05 and **P < 0.01.

were not statistically significant (Table 5). Ticks infected by *B. afzelii* had fed on rodents or artiodactyls but not on birds. Avian blood-fed ticks were infected either by *B. garinii* (1/2) or a combination of *B. garinii* and *B. valaisiana* (1/2). *Borrelia garinii* and/or *B. valaisiana* infected ticks were found also in rodent or artiodactyl-fed ticks, but with lower frequency. The difference for *B. garinii* but not for *B. valaisiana* was statistically significant (Fisher exact test; P < 0.05). Nymphs positive for DNA of *B. lusitaniae* had fed on rodents (2/21) or artiodactyls (1/30) as larvae. *Rickettsia helvetica* infected ticks contained DNA from all three groups of hosts. *Babesia* spp. were found in nymphs that fed on rodents (*Ba. Microti*, 1/88, *Ba. Venatorum*, 1/88) and artiodactyls (*Ba. venatorum*, 2/;119 *Ba. microti*, 1/;119 *Babesia* sp. unidentified, 1/119). In addition, *A. phagocytophilum*-positive ticks also obtained their blood-meal from rodents (2/88) or artiodactyls (2/119) (Table 4). No statistically significant differences were found in the frequency of occurrence of co-infections by multiple pathogens (including different genospecies of *B. burgdorferi* s.l.) in samples with only one host identified (10/72; 16%) compared to those positive for multiple hosts (2/42; 4.8%).

Table 4. Pathogen prevalence in ticks fed on a specific group of hosts.

	Rodents	Artiodactyls	Birds	Unidentified
Borrelia burgdorferi s.l.	23.9%	26.1%	16.7%	23.1%
	(21/88)	(31/119)	(3/12)	(39/169)
Borrelia miyamotoi	1.1%	0.0%	0.0%	4.1%
	(1/88)	(0/119)	(0/12)	(7/169)
Babesia sp.	2.3%	3.4%	0%	0.6%
	(2/88)	(4/119)	(0/12)	(1/169)
Anaplasma phagocytophilum	2.3%	1.7%	0.0%	4.1%
	(2/88)	(2/119)	(0/12)	(7/169)
Rickettsia sp.	5.7%	4.2%	8.3%	4.1%
	(5/88)	(5/119)	(1/12)	(7/169)

Only samples with a single detected host are included (number of positive samples/number of samples with particular host detected); samples with positive pathogen detection in which the host could not be identified are labeled as unidentified.

Table 5. Representation of genospecies of *Borrelia burgdorferi* s.l. among different groups of *Ixodes ricinus* hosts.

	Rodents	Artiodactyls	Birds
B. burgdorferi s.s.	33.3%	33.3%	0.0%
	(7/21)	(10/30)	(0/2)
B. afzelii	33.3%	33.3%	0.0%
	(7/21)	(10/30)	(0/2)
B. garinii	19.1%	10.0%	50.0%*
	(4/21)	(3/30)	(1/2)
B. valaisiana	0%	6.7%	0.0%
	(0/21)	(2/30)	(0/2)
B. lusitaniae	9.5%	3.3%	0%
	(2/21)	(1/30)	(0/2)
B. garinii + *B. valaisiana*	4.8%	6.7%	50%
	(1/21)	(2/30)	(1/2)
B. burgdorferi s.s. + *B. afzeliiB. afzelii*	0%	3.3%	0%
	(0/21)	(1/30)	(0/2)
B. valaisana + *B. lusitaniae*	0%	3.3%	0%
	(0/21)	(1/30)	(0/2)

Number of positive samples of a genospecies/total number *B. burgdorferi* s.l. positive ticks fed on particular host are reported. '*' indicates that higher frequency of B. garinii occurrence among bird fed ticks compared to other hosts is statistically significant (P < 0.05).

DISCUSSION

In this study, 435 questing nymphal *I. ricinus* ticks were tested using a broad-range assay designed to detect a variety of tick-borne pathogens. The prevalence of *B. burgdorferi* s.l. infected ticks (23%) was higher than average prevalence rates previously reported in nymphal *I. ricinus* in Central Europe (16.7%) (Strnad *et al*. 2017). The difference may be due to the employment in this study of more sensitive molecular biology based methods (PCR/ESI-MS) or to seasonal or geographical differences.

Most of the *B. burgdorferi* s.l. infected ticks were infected by a single genospecies (91%). Most of the multiple infections consisted of *B. garinii* and *B. valaisiana* or *B. burgdorferi* s.s. and *B. afzelii*. Since the questing nymphs likely obtained only a single blood-meal as larvae and since *B. burgdorferi* genospecies show a certain level of host specificity (Kurtenbach *et al*. 1998b, 2002), these combinations of genospecies might have been obtained from a single co-infected host during a single feeding. Co-infections by genospecies associated with different species of hosts (*B. afzelii* and *B. lusitaniae*) were also detected, however. Such findings might be explained by interrupted larval feeding

on one infected host followed by successful feeding on another host infected by a different genospecies of *B. burgdorferi*. Infected and seemingly unfed larvae of *I. ricinus* have been reported in multiple studies (Nazzi *et al*. 2010; Kalmar *et al*. 2013; Tappe *et al*. 2014; van Duijvendijk *et al*. 2016). Detection of DNA of multiple host species in up to 17% of questing nymphal ticks in this study as well as previous studies (Humair *et al*. 2007; Moran Cadenas *et al*. 2007; Collini *et al*. 2016) supports the possibility of interrupted feeding.

The majority of the *Borrelia*-positive ticks were infected by one of the unequivocally pathogenic genospecies (*B. afzelii*, *B. garinii*, *B. burgdorferi* s.s.) and therefore represent a significant health risk for humans. Moreover, 2% of the ticks, similar to the percentage reported previously (Crowder *et al*. 2014) were infected with *B. miyamotoi*. This bacterium causes mild febrile disease in immunocompetent humans (Platonov *et al*. 2011; Chowdri *et al*. 2013; Krause *et al*. 2013) but serious infections of the central nervous system in immunocompromised patients (Gugliotta *et al*. 2013; Hovius *et al*. 2013). This spirochete was previously detected in European *I. ricinus* ticks with a prevalence ranging from 0% to 3.85% (Gern *et al*. 2010; Richter and Matuschka 2012; Potkonjak *et al*. 2016).

Apart from *Borrelia* species, other human pathogens were detected in the questing nymphs. *Anaplasma phagocytophilum*, a causative agent of human granulocytic anaplasmosis, was found at all the sampling sites with prevalence ranging from 1% to 3%. The prevalence rates in nymphal *I. ricinus* vary significantly among different studies (<0.8% to ~9%), possibly due to differences in study sites and years of sampling (Stuen, Granquist and Silaghi 2013). Despite relatively high seroprevalence in human population, diagnosed human disease cases are still a rare event in Europe. It was suggested previously that this discrepancy is associated with the occurrence of specific strains of the bacterium, which are unable to cause disease in humans, underdiagnosis of the infection, or antibody cross-reactivity (Dumler *et al*. 2005; Silaghi *et al*. 2012).

The prevalence of *Babesia* spp. was 1.6%, which is in concordance with other findings in *I. ricinus* in Central Europe (0.3%–4.1%) (Rudolf *et al*. 2005; Schorn *et al*. 2011; Venclikova *et al*. 2015; Hamsikova *et al*. 2016). *Babesia microti* and *Ba. venatorum* were previously found in *I. ricinus* (Silaghi *et al*. 2012; Venclikova *et al*. 2015; Hamsikova *et al*. 2016). *Babesia venatorum* was reported to cause disease in immunocompromised patients (Hildebrandt, Gray and Hunfeld 2013). In Europe, *Ba. microti* is mostly associated with imported human cases (Hildebrandt, Gray and Hunfeld 2013). Nevertheless, there are also confirmed autochthonous (Hildebrandt *et al*. 2007) and probable autochthonous (Arsuaga *et al*. 2016; Moniuszko-Malinowska *et al*. 2016) infections by European lineages of *Ba. microti*. In one tick in our study a basecount matching *Ba. gibsoni* was detected by one primer pair, but we were not able to confirm the identity by sequencing. Therefore, we refer to this sample as *Babesia* sp. in the results. The presence of *Ba. gibsoni* is unlikely since Mediterranean *Rhipicephalus sanguineus* ticks are considered to be the main vectors.

The spotted fever group *Rickettsia*, *R. monacensis* was detected at a single locality in a single tick, whereas *R. helvetica* was omnipresent. *Rickettsia helvetica* belongs to commonly occurring species in *I. ricinus* ticks (Karbowiak *et al*. 2016). *Rickettsia monacensis* is less prevalent, frequently reported in ticks sampled from birds (Elfving *et al*. 2010; Biernat *et al*. 2016; Mărcuţan *et al*. 2016). Both these *Rickettsia* species are considered generally nonpathogenic and have rarely been identified in association with human disease cases with variable in severity (Fournier *et al*.

2000; Jado *et al.* 2007; Nilsson, Elfving and Påhlson 2010). None of the samples was positive for *F. tularensis*. Although several studies report presence of this bacterium, in *I. ricinus* ticks in Central and Western Europe, the prevalence is reaching only up to 0.2% even in tularemia active foci (Hubalek *et al.* 1996; Gehringer *et al.* 2013). South Bohemia is not a known tularemia endemic region.

Identification of the host in questing exophilic ticks presents a complex methodological challenge, since the blood-meal acquired in the previous developmental stage is already digested and the remnants, which allow host species detection, are usually present in a limited quantity and quality (Kirstein and Gray 1996). Therefore, sensitivity of the identification method is crucial. Multiple molecular biology methods of blood-meal analysis in questing ticks have been developed. Generally, these methods are based on amplification of a multicopy (usually mitochondrial) gene target such as 12S rDNA (Humair *et al.* 2007; Wodecka, Rymaszewska and Skotarczak 2013), 18S rDNA (Pichon *et al.* 2003; Allan *et al.* 2010) or *cytB* (Kirstein and Gray 1996; Gray *et al.* 1999). Techniques differ in the method of analysis (reverse line blotting, sequencing, restriction fragment length polymorphism, real-time PCR coupled with high-resolution melting analysis) and have variable levels of sensitivity and specificity.

In our study, the identification of the tick host was possible in 61% of the samples, which is in the range reported in previous studies (33%–65%) (Gray *et al.* 1999; Pichon *et al.* 2003, 2005; Estrada-Pena *et al.* 2005; Pichon *et al.* 2006; Wodecka, Rymaszewska and Skotarczak 2013; Collini *et al.* 2016) but higher than in studies using the same method of analysis (49%, 43% and 39% as reported in Humair *et al.* 2007; Moran Cadenas *et al.* 2007; Burri *et al.* 2011, respectively). In previous studies, the identification efficiency varied significantly according to the sampling locality (Moran Cadenas *et al.* 2007) and sampling season (Pichon *et al.* 2005), indicating the influence of temperature (Moran Cadenas *et al.* 2007) and/or period post molting (Pichon *et al.* 2005; Léger *et al.* 2015). No statistically significant differences in the identification success rates were observed that correlated with localities or month of collection. The previously reported efficient DNA extraction procedure (Crowder *et al.* 2010b) used here may have led to the higher success rate as previous studies with lower identification rates used an ammonium hydroxide extraction method (Pichon *et al.* 2003, 2005; Estrada-Pena *et al.* 2005; Humair *et al.* 2007; Moran Cadenas *et al.* 2007; Burri *et al.* 2011). It was shown previously by direct comparison, that DNA extraction influences the host identification rate (Allan *et al.* 2010; Collini *et al.* 2016) with differences due to final concentration of the target DNA in the homogenate or eluate, extent of contamination with PCR inhibitors, or extent of damage to the integrity of the DNA during extraction.

As expected, the portion of samples with multiple hosts is higher in those studies with higher host identification rates. In our study, DNA of two host species was detected in 16% of the samples, even though only questing *I. ricinus* nymphs, which presumably fed only once as larvae, were tested. Similar results were reported for questing nymphal *I. ricinus* (Moran Cadenas *et al.* 2007; Collini *et al.* 2015, 2016) and questing nymphal *Amblyomma americanum* (Allan *et al.* 2010). Interrupted feeding and subsequent infestation of a different host species might be an explanation for such results. It was shown previously that ixodid ticks are able to reattach and successfully complete feeding until engorgement after being prematurely detached from the host (Shih and Spielman 1993; Wang, Henbest and Nuttall 1999).

A high host identification rate as well as a high prevalence of multiple host detections could be due to contamination. A positive signal in negative extraction controls was rarely detected in our analysis and was never confirmed in repeated analysis, indicating that contamination of the DNA extract is unlikely. Therefore, we are convinced that the difference in identification success rate is not due to contamination. Further, the extraction method used here seems to produce a more efficient template for host identification (Allan *et al.* 2010). Although we have used a relatively old method (Humair *et al.* 2007) of host identification, a recently presented PCR—high-resolution melting analysis brings very similar results concerning identification success (65% and 55% using Qiagen silica-based extraction kit) and portion of samples with multiple host species detected (24% and 11%) (Collini *et al.* 2015, 2016).

The hosts most frequently identified at all but one study site were artiodactyls (31%–60%) followed by rodents (27%–62%). Birds were less frequently identified as hosts, although variations were present among the study sites. The high proportion of artiodactyls is in agreement with findings of other studies employing blood-meal analysis (Pichon *et al.* 2003; Humair *et al.* 2007; Moran Cadenas *et al.* 2007; Burri *et al.* 2011; Wodecka, Rymaszewska and Skotarczak 2013). Large mammals, which are frequently infested by ticks of all stages, are able to support large numbers of ticks (Ruiz-Fons *et al.* 2006; Carpi *et al.* 2008; Kiffner *et al.* 2010; Vor *et al.* 2010). Thus, they may serve as a globally important source of blood-meal despite their relatively lower abundance compared to rodents (Hofmeester *et al.* 2016). In different study sites, different patterns of proportional representation among rodent, artiodactyl and bird species were observed, presumably reflecting the overall representation of the species determined by natural conditions in the particular habitat. Similar results showing high proportion of bird-fed (Estrada-Pena *et al.* 2005; Pichon *et al.* 2005) as well as artiodactyl-fed ticks (Moran Cadenas *et al.* 2007; Wodecka, Rymaszewska and Skotarczak 2013) were reported from different locations in Europe.

Hosts were rarely identified to the species level, although it is known that species of deer, birds, *Apodemus* spp., *Microtus* spp. and *Myodes glareolus* occur in the area of South Bohemia. Since the identification success rate on species level was similar in a study using the same set of probes (Moran Cadenas *et al.* 2007), it seems that the current probes do not cover the real sequence variability of the target species. The probes should be further optimized or new probes added to enable species-level identification of tick hosts. This is especially needed for the artiodactyl probe, which was originally validated using only artiodactyl DNA but not on artiodactyls-fed ticks (Humair *et al.* 2007).

Comparison of the prevalence of tick-borne pathogens among groups of ticks that fed on different hosts shows effectiveness of pathogen transmission from particular host species to feeding ticks. Exclusion of samples with multiple hosts reduced the sample size and statistically significant differences among different host groups were not detected. Surprisingly, a similar proportion of *B. burgdorferi* s.l.-positive samples was found among ticks fed on rodents and artiodactyls, particularly wild boar. Similar results were reported in other studies based on blood-meal analyses (Estrada-Pena *et al.* 2005; Moran Cadenas *et al.* 2007; Wodecka, Rymaszewska and Skotarczak 2013). In contrast, multiple laboratory and field studies indicate that ungulates are generally incompetent as *B. burgdorferi* reservoirs (Matuschka *et al.* 1993; Talleklint and Jaenson 1994; Nelson *et al.* 2000; Kjelland *et al.* 2011). *Borrelia burgdorferi* spirochetes are present in the blood and skin of artiodactyls including wild boar (Pichon *et al.* 2000; Faria *et al.* 2015; Ebani *et al.* 2016; VanBik *et al.* 2017; Zhai *et al.* 2017), and some of the ticks are infected (Kjelland *et al.* 2011; Pacilly *et al.* 2014; Silaghi, Pfister and Overzier 2014), but the prevalence rates in partially fed ticks are markedly

lower compared to questing ticks. Since the probes specific for artiodactyla and wild boar seemed to have appropriate specificity (Humair *et al.* 2007) and similar results were obtained in a study using different approach (Wodecka, Rymaszewska and Skotarczak 2013), it seems improbable that these results are due to false identification of the host. We speculate that part of the difference in the prevalence in questing and fed ticks may be caused by reduced number of spirochetes after feeding, which (together with PCR inhibitors present in the fed tick) could result in lower prevalence rate. After the blood digestion and molting, the spirochetes amplify and are readily detected in the questing ticks (Jacquet *et al.* 2017). An analysis of *B. burgdorferi* s.l. prevalence in fed and unfed larval ticks sampled from multiple host species compared to analysis in questing nymphal ticks from the same locality would address this hypothesis.

The associations between *B. burgdorferi* genospecies and vertebrate hosts reported previously in multiple host trapping and laboratory transmission experiments (Kurtenbach *et al.* 1998a,b; Perez *et al.* 2012) were not completely recapitulated in our study. Nevertheless, the affinity of *B. afzelii* for rodents and of *B. garinii* and *B. valaisiana* for avian hosts was confirmed and is in concordance with conclusions of other blood-meal analysis based studies (Pichon *et al.* 2005; Moran Cadenas *et al.* 2007; Wodecka, Rymaszewska and Skotarczak 2013). In our study, *B. bavariensis* was not differentiated from *B. garinii*, since *B. bavariensis* was not yet established as a separate genospecies at the time of the sample analysis. Therefore, some *B. garinii* identifications in rodent-fed ticks might be in fact be the rodent-associated genospecies *B. bavariensis*. Based on currently available data, the prevalence of *B. bavariensis* is generally low (approximately 4.5% of *B. burgdorferi* infected ticks) among European *I. ricinus* tick populations (Lommano *et al.* 2012; Herrmann, Gern and Voordouw 2013; Glatz *et al.* 2014; Daniel *et al.* 2016) and therefore should not substantially influence the results. In addition, trapping studies also report some exceptions from the general genospecies associations. *Borrelia afzelii* and *B. bavariensis* were identified in bird-fed ticks (including larvae) (Geller *et al.* 2013; Lommano *et al.* 2014), and *B. garinii* and *B. burgdorferi* s.s. were found in lizard-fed ticks (Majlathova *et al.* 2006). *Borrelia garinii* (distinguished from *B. bavariensis*) was detected in rodent tissues (Khanakah *et al.* 2006; Pisanu *et al.* 2014; Hamsikova *et al.* 2017). *Borrelia lusitaniae* was also found in artiodactyl-fed ticks, although lizards are considered the main reservoir host of this *Borrelia* genospecies (Majlathova *et al.* 2006; Richter and Matuschka 2006).

Because of our relatively small sample size, we have limited data on host association of the less frequent tick-borne pathogens and conditional pathogens. In general, our data correspond with current knowledge. *Borrelia miyamotoi* was found in a tick that fed on rodents, which is the most probable reservoir host of this spirochete (Barbour *et al.* 2009; Cosson *et al.* 2014). *Rickettsia helvetica* was detected in ticks that fed on rodent, artiodactyl and avian host species. In concordance with this finding, *R. helvetica* has been detected in the blood of a variety of vertebrate animals (wild boar, deer and rodents) (Karbowiak *et al.* 2016). No specific reservoir host has been identified for this *Rickettsia* species. Apparently, transovarial and transstadial transmission in ticks contributes to the natural circulation of the bacterium (Sprong *et al.* 2009).

Anaplasma phagocytophilum in Europe occurs in four distinct ecotypes and has been detected in a broad range of hosts including rodents and artiodactyls (Silaghi *et al.* 2012; Jahfari *et al.* 2014), as confirmed in our study. We detected *Ba. microti* in a rodent-fed tick, its common host species (Silaghi *et al.* 2012; Hamsikova *et al.* 2016). *Babesia venatorum* was found in artiodactyl- and rodent-

fed ticks in our study, although to date only roe deer have been reported as the host of this *Babesia* species (Duh *et al.* 2005; Bonnet *et al.* 2007; Overzier *et al.* 2013; Andersson *et al.* 2016). Nevertheless, transovarial transmission was reported for *Babesia venatorum* in *I. ricinus* ticks (Bonnet *et al.* 2007), and thus the presence of the pathogen in the tick may not necessarily be the result of an interaction of the tick with the last host.

Molecular methods of blood-meal analysis are still the only available techniques that bring objective, overall assessment of the tick host species spectrum and the proportional representation of different host species. Some of our results are in disagreement with generally accepted opinions on *I. ricinus* ecology and pathogen transmission, in particular, the presence of DNA from multiple hosts in questing nymphs and the unexpected host associations of *B. burgdorferi* s.l. and its particular genospecies. Careful validation of the sensitivity and specificity of the host-identification methods is needed whenever applied in a new environment. A study employing blood-meal analysis of questing nymphs combined with on host tick sampling and pathogen detection should reveal the cause of the discrepancies as well as the true species composition of a particular tick population.

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