Exp Appl Acarol (2016) 68:127–137
DOI 10.1007/s10493-015-9988-y

Prevalence of pathogenic bacteria in *Ixodes ricinus* ticks in Central Bohemia

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Received: 2 June 2015 / Accepted: 31 October 2015 / Published online: 26 November 2015

Abstract Bacteria associated with the tick *Ixodes ricinus* were assessed in specimens unattached or attached to the skin of cats, dogs and humans, collected in the Czech Republic. The bacteria were detected by PCR in 97 of 142 pooled samples including 204 ticks, i.e. 1–7 ticks per sample, collected at the same time from one host. A fragment of the bacterial 16S rRNA gene was amplified, cloned and sequenced from 32 randomly selected samples. The most frequent sequences were those related to *Candidatus* Midichloria midichlori (71 % of cloned sequences), followed by *Diplorickettsia* (13 %), *Spiroplasma* (3 %), *Rickettsia* (3 %), *Pasteurella* (3 %), *Morganella* (3 %), *Pseudomonas* (2 %), *Bacillus* (1 %), *Methylobacterium* (1 %) and *Phyllobacterium* (1 %). The phylogenetic analysis of *Spiroplasma* 16S rRNA gene sequences showed two groups related to *Spiroplasma eriocheiris* and *Spiroplasma melliferum*, respectively. Using group-specific primers, the following potentially pathogenic bacteria were detected: *Borellia* (in 20 % of the 142 samples), *Rickettsia* (12 %), *Spiroplasma* (5 %), *Diplorickettsia* (5 %) and *Anaplasma* (2 %). In total, 68 % of *I. ricinus* samples (97/142) contained detectable bacteria and 13 % contained two or more putative pathogenic groups. The prevalence of tick-borne bacteria was similar to the observations in other European countries.

Keywords Tick · Bacteria · Central Bohemia · *Ixodes ricinus* · Pathogens

Introduction

Ixodes ricinus is the most prevalent and widely distributed tick species in both natural and urban areas of Central Europe (Venclikova et al. 2014a, b). The ticks easily transmit pathogenic microorganisms by biting large animals and humans. Risk of infection with the

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tick-borne pathogens in Central Europe is common since 15 % of *I. ricinus* are infected with at least one group of pathogenic microorganisms (Pangracova et al. 2013). Consequently, the microorganisms associated with *I. ricinus* have been intensively studied using PCR methods (Sparagano et al. 1999) combined with real-time PCR quantification (Fenollar and Raoult 2004; Mediannikov and Fenollar 2014) and next-generation sequencing (Vayssier-Taussat et al. 2013; Qiu et al. 2014). Microorganisms described in *I. ricinus* include human pathogenic bacteria (*Anaplasma phagocytophilum*, *Bartonella henselae*, *Borrelia burgdorferi* sensu lato, *Coxiella burnetii*, *Candidatus* Neoehrlichia mikurensis, *Rickettsia helvetica*, *Francisella tularensis*) and eukaryotic parasites (*Babesia microti*, *Babesia divergens*, *Babesia venatorum*) (Hai et al. 2014; Michelet et al. 2014; Rizzoli et al. 2014). Other bacteria with unknown or suspected pathogenicity for humans included *Arsenophonus nasoniae*, *Spiroplasma ixodeti*, *Candidatus* Midicholoria mitochondrii, *Wolbachia pipientis* and *Ehrlichia muris* (Subramanian et al. 2012a). Suspected pathogenic bacteria *Pasteurella pneumotropica* (Stojek and Dutkiewicz 2004) and non-pathogenic bacteria of the genera *Micrococcus*, *Bacillus*, *Paenibacillus*, *Oceanobacillus*, *Staphylococcus*, *Arhrobacter*, *Corynebacterium*, and *Dietzia* were cultivated from *I. ricinus* homogenates (Rudolf et al. 2009; Egyed and Makrai 2014). Although numbers of pathogenic bacteria are known to be transmitted by ticks, the infestation largely differs from one site to another, and thus the spectrum of yet described transmitted bacteria is incomplete.

The aim of the study was to describe bacteria associated to *I. ricinus* ticks collected from different hosts in urban area near Prague. We focused on the identification of bacterial pathogens using routine PCR and nested PCR approach with an increased detection sensitivity and specificity (Kim et al. 2013).

Materials and methods

Ticks

Ixodes ricinus ticks were collected by volunteers in Tuchomerice (50°7′58″N, 14°16′47″E; Central Bohemia, the Czech Republic) attached on cats (6 individuals) and dogs (5 individuals) and unattached on skin of dogs, cats and the volunteers (dog and cat owners) from March 2013 to September 2014, the animals were inspected once per week. A total of 204 ticks were collected including 31 nymphs, 10 males, 70 females, and 93 enlarged females. The collected ticks were placed into Eppendorf tubes and immediately transferred to the laboratory. The ticks were stored in a fridge at 4 °C for up to 4 days before DNA extractions. The ticks were checked under dissection microscope to identify the species and pooled from the same animal and sampling date, resulting in 142 samples (1–7 ticks per sample).

DNA extraction

Total DNA was extracted from all 142 tick samples. Before the extraction, the ticks were washed twice in 96 % ethanol for 30 min, followed by three washes in phosphate buffered saline (PBST, containing 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, and 0.05 % Tween® 20). The washing procedure reduced the contamination of body surface by bacteria, as observed in mites (Kopecky et al. 2014). The sample in a total

Table 1 The list of primers and reaction conditions used in the study

Target	Gen		Primers (5′–3′)	AT °C	Size (bp)	References
Bacteria	16S RNA	F27	AGAGTTTGATYMTGGC	50	1400	Barbieri et al. (2001)
		R1492	GYTACCTTGTTACGACTT			
Pasteurella multocida	tox A	tox AF	CTTAGATGAGCGACAAGG	55	846	Lichtensteiger et al. (1996)
		tox AR	GAATGCCACACCTCTATAG			
Anaplasma phagocytophilum (sensu lato complex)	16SRNA	5Ana-out	CAAGTCGAACGGATTATTC	56	753	Kim et al. (2013)
		3Ana-out	AGATAAAAAATCCCCACATTC			
		5Ana-in	GTCGAACGGATTATTCTTTATAGC	64	516	Kim et al. (2013)
		3Ana-out	GGCATTTCACCTTTAACTTACCG			
Borrelia burgdorferi (sensu lato complex)	16SRNA	5Bor-out	GTCAAACGGGATGTAGCAATAC	64	811	Kim et al. (2013)
		3Bor-out	CACACTTAACACGTTAGCTTCG			
		5Bor-in	ATTCAGTGGCGAACGGGTG	66	469	Kim et al. (2013)
		3Bor-in	AACAACGCTCGCCCCTTAC			
Bartonella spp.	gltA	BhCS871.p	GGGGACCAGCTCATGGTGG	56	379	Norman et al. (1995)
		BhCS1137.n	AATGCAAAAAGAACAGTAAACA			
	ribC	BARTON-1	TAACCGATATTGGTTGTGTTGAAG	45	585	Johnson et al. (2003)
		BARTON-2	TAAAGCTAGAAAGTCTGGCAACATAACG			
Spiroplasma spp.	16SRNA	BS1	AAGTCGAACGGGGTGCTT	57	975	Meeus et al. (2012)
		BS976	TGCACCACCTGTCTCAATGT			
Rickettsia spp.	16SRNA	Rick_1	GGACTAATTGGGGTTTGCTC	55	1352	Hoy and Jeyaprakash (2005)
		Rick_2	GCTGCCTCTTGCGTTAGCT			
Diplorickettsia spp.	16SRNA	MstrDip633F	CGACTCGAGTGAGACAGAGG	58	510	Ishii et al. (2013)
		MstrDip1143R	AAGTTCCCAGCTTGACCTGG			

volume 250 μL PBST was homogenized using plastic pestle in Eppendorf tube. Total DNA was extracted using Wizard® Genomic DNA Purification kit (Promega, Madison WI, USA) according to manufacturer's instructions. The extracted DNA was stored in a freezer at −20 °C before the analyses.

PCR and cloning

The presence of bacterial DNA was verified by amplification of the 16S rRNA gene fragment with universal bacterial primers (Table 1) (Barbieri et al. 2001). The samples positive for bacterial 16S rRNA gene were screened by primers (Table 1) specific for the genera *Anaplasma*, *Borrelia*, *Bartonella*, *Pasteurella* and *Spiroplasma* (Norman et al. 1995; Lichtensteiger et al. 1996; Johnson et al. 2003; Kim et al. 2013). Among them, the detection of *Anaplasma* and *Borrelia* included nested PCR (Kim et al. 2013). Amplifications were performed in C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). A total volume of 25 μL PCR reaction mixture contained final concentration of 200 μM dNTPs, 3 mM $MgCl_2$; forward and reverse primers (100 nM each), 0.5 unit *Taq* polymerase (all Promega) and 50–300 ng template DNA including tick genomic DNA (see Table 1 for PCR conditions). The resulting PCR products were visualized by agarose gel electrophoresis.

Randomly selected 32 PCR amplicons from universal bacterial primers and all obtained amplicons from the genus specific primers were purified with Wizard® SV Gel and PCR product clean-up system Kit (Promega) and cloned using pGEM®-T Easy Vector (Promega). Selected clones were sequenced by Macrogen (Seoul, South Korea).

Sequence processing

The cloned sequences were assembled with CodonCode Aligner, version 1.5.2 (CodonCode, Dedham, MA, USA). The sequences cloned from genus-specific amplicons were analyzed using BLAST search against the GenBank database. From the 32 randomly chosen bacterial 16S rRNA gene amplicons, 344 sequences were obtained and analyzed using the operational taxonomic units defined at 97 % similarity level (OTU_{97}). The consensus sequences were compared to the sequences in GenBank using BLAST search.

Statistical analyses

The prevalence of pathogenic bacteria was recalculated as a percentage in the total of 142 pooled samples. The Spearman correlation was used to analyze a relationship between the numbers of individual tick stages in pooled samples and presence or absence of bacteria. To compare the occurrence of *Borrelia* in the samples, the Chi square test was performed. The numbers of positive and negative samples were analyzed with the type of host (i.e. dog, cat, volunteer-owner of the dogs and cats, and unattached ticks) as an independent variable.

Phylogenetic analysis of *Spiroplasma* clones

The alignment of *Spiroplasma* partial 16S rRNA gene sequences was performed using SILVA Incremental Aligner v.1.2.11 (Pruesse et al. 2012). The reference sequences from the GenBank were included according to Henning et al. (2006) and Lo et al. (2013) with

the sequence of *Esherichia coli* (Acc. No. U00096) as an outgroup. For the analysis of phylogenetic relationships, the best-fit model of nucleotide substitution was selected using jModelTest 2 software (Guindon and Gascuel 2003; Darriba et al. 2012). Based on the selection, model GTR with a proportion of invariable sites (+I) and gamma distribution in four rate categories (+G) was employed to infer phylogeny by Bayesian analysis using PhyloBayes-MPI, v.1.4e (Lartillot et al. 2009) and maximum likelihood analysis in PhyML v.3.0 (Guindon et al. 2010). The resulting phylograms were finalized using MEGA 6 (Tamura et al. 2007).

Results and discussion

Bacterial DNA was detected by PCR amplification with universal bacterial primers in 97 of 142 pooled samples including 204 ticks (68 %). Using the genus-specific primers (Table 1), the following genera were detected: *Borrelia* (in 20 % of 142 samples), *Rickettsia* (12 %), *Spiroplasma* (5 %), *Diplorickettsia* (5 %), and *Anaplasma* (2 %). Among the analyzed samples, 18 (13 %) contained two pathogenic bacterial genera. The PCR detection was confirmed by cloning and sequencing of the amplification product. *Bartonella* and *Pasteurella* were not found in any of the samples. Detection of *Borrelia* was correlated to the proportion of nymphs and detection of *Diplorickettsia* to the proportion of males in the pooled samples (Table 2).

Products of 16S rRNA gene amplification with universal bacterial primers from 32 randomly selected samples including attached or unattached ticks from cat and dog hosts were cloned and sequenced. The resulting 344 partial 16S rRNA gene sequences formed 12 OTU_{97} (Table 3). The most abundant OTU_{97} Nr. 1 related to *Candidatus* Midichloria mitochondrii, included 71 % of all sequences, followed by OTU_{97} Nr. 2 related to *Diplorickettsia massiliensis* (13 % of sequences). Both OTUs were found in ticks sampled from all hosts The remaining OTUs were found in one of the hosts only. The OTUs were identified as *Spiroplasma*, *Rickettsia*, *Pasteurella*, *Pseudomonas*, *Morganella*, *Bacillus*, *Methylobacterium* and *Phyllobacterium* (Table 3). Representatives of *Borelia* and

Table 2 The Spearman correlation between the proportion of individual *Ixodes ricinus* developmental stages in pooled samples and the presence or absence of bacteria identified by the specific primers

Variables	Bor	Ana	Spir	Dip	Rick
nymphs	**0.221**	−0.054	0.083	−0.084	−0.138
males	0.079	−0.050	−0.072	**0.224**	0.085
females	−0.137	−0.103	−0.147	−0.183	−0.107
en. females	−0.072	0.030	0.043	0.106	0.034
Bor		0.143	0.113	0.166	0.114
Ana	0.161		**0.696**	**0.410**	**0.388**
Spir	0.271	**<0.0001**		**0.259**	**0.332**
Dip	0.104	**<0.0001**	**0.011**		**0.395**
Rick	0.267	**<0.0001**	**0.001**	**<0.0001**	

The Spearman correlation coefficients are presented in the upper part, below are the p values. The numbers in bolds indicate the significant correlation coefficient. enF—enlarged female, Bor—*Borrelia*, Ana—*Anaplasma*, Spir—*Spiroplasma*, Dip—*Diplorickettsia*, Rick—*Rickettsia*

Anaplasma, which were detected by the genus-specific primers, were not found among the clones.

The results of the present study are based on pooled samples. The observed prevalence of individual groups of bacteria were in the range of data from the studies when the ticks were investigated individually. The observed prevalence of samples positive for *Borrelia* was comparable to the previously published data for European dog samples ranging from 2 to 22 % (Claerebout et al. 2013). In the Czech Republic, the occurrence of *Borrelia* was 14 % in a city park and 15 % in a natural ecosystem near Ostrava (Venclikova et al. 2014a), while 26 % of infested ticks were reported in Austria (Glatz et al. 2014). The distribution of *Borrelia* was not influenced by the host (cats or dogs, and unattached on skin of animals) (Chi square $_{(3,\ 95)} = 5.9$; $P = 0.12$).

The prevalence of *Anaplasma* was within a broad range from 1 to 18 % of infested samples reported in other studies (Hildebrandt et al. 2010; Aureli et al. 2012; Claerebout et al. 2013; Glatz et al. 2014; Kiewra et al. 2014; Venclikova et al. 2014b). *Pasteurella pneumotropica/haemolytica*, a potential infectious agent causing pasteurellosis in humans and animals exposed to tick bites, was previously isolated from the ticks collected in the woodlands (Stojek and Dutkiewicz 2004). Although the obtained 16S rRNA sequences showed high similarity (99 %) to *Pasteurella*, amplification of *toxA* gene failed to confirm its presence (Lichtensteiger et al. 1996).

Candidatus Midichloria mitochondrii (*Rickettsiales*, Candidatus *Midichloriaceae*) are intracellular bacteria associated with *I. ricinus* and characterized by the capacity of multiplying inside the mitochondria. They were recently suggested as vector-borne agents with a potential of infecting mammalian hosts (Mariconti et al. 2012; Bazzocchi et al. 2013). Due to their presence in mitochondria of the ticks, it is not surprising that 71 % of cloned 16S rRNA gene sequences belonged to this group. The prevalence of this bacterium was

Table 3 The identified bacterial taxa in the selected samples of *Ixodes ricinus* based on amplification and sequencing of the 16S rRNA gene fragment (344 sequences) from 32 randomly selected pooled samples

OTU_{97}	GenBank match		Host				N
	Name	ID.	Sim. %	Cat	Dog	Unatt.	
1	*Cand.* Midichloria mitochondrii	NR_074492	99	84	84	75	243
2	*Diplorickettsia massiliensis*	NR_117407	94	1	11	31	43
3	*Spiroplasma*	NR_121794	99	0	12	0	12
4	*Rickettsia*	NR_074483	99	11	0	0	11
5	*Pasteurella*	NR_115138	99	0	0	10	10
6	*Morganella*	NR_102517	99	0	0	9	9
7	*Pseudomonas*	NR_113600	99	8	0	0	8
8	*Bacillus*	NR_074540	99	2	0	0	2
9	*Methylobacterium*	NR_112613	99	2	0	0	2
10	*Phyllobacterium*	NR_043192	99	2	0	0	2
11	*Rhodococcus*	KJ016189	99	1	0	0	1
12	*Proteus*	AB920833	99	0	0	1	1

The closest matches retrieved by Blast search in the GenBank database for the OTU_{97} consensus sequences are presented. Unatt.—unattached on skin of dog and cat, N—total number of cloned sequences within the OTU_{97}

4 % in tick samples collected in Slovakia (Subramanian et al. 2012a). *Diplorickettsia massiliensis* is strictly intracellular and is mainly grouped inside vacuoles of eukaryotic cells (Mediannikov et al. 2010). The bacterium is suscepected human pathogen (Subramanian et al. 2012b). The primers used for detection of *Rickettsia* (Hoy and Jeyaprakash 2005) did not allow a discrimination to the species level. The reported prevalence of *Rickettsia helvetica* was from 11 to 14 % in ticks samples (Hildebrandt et al. 2010;

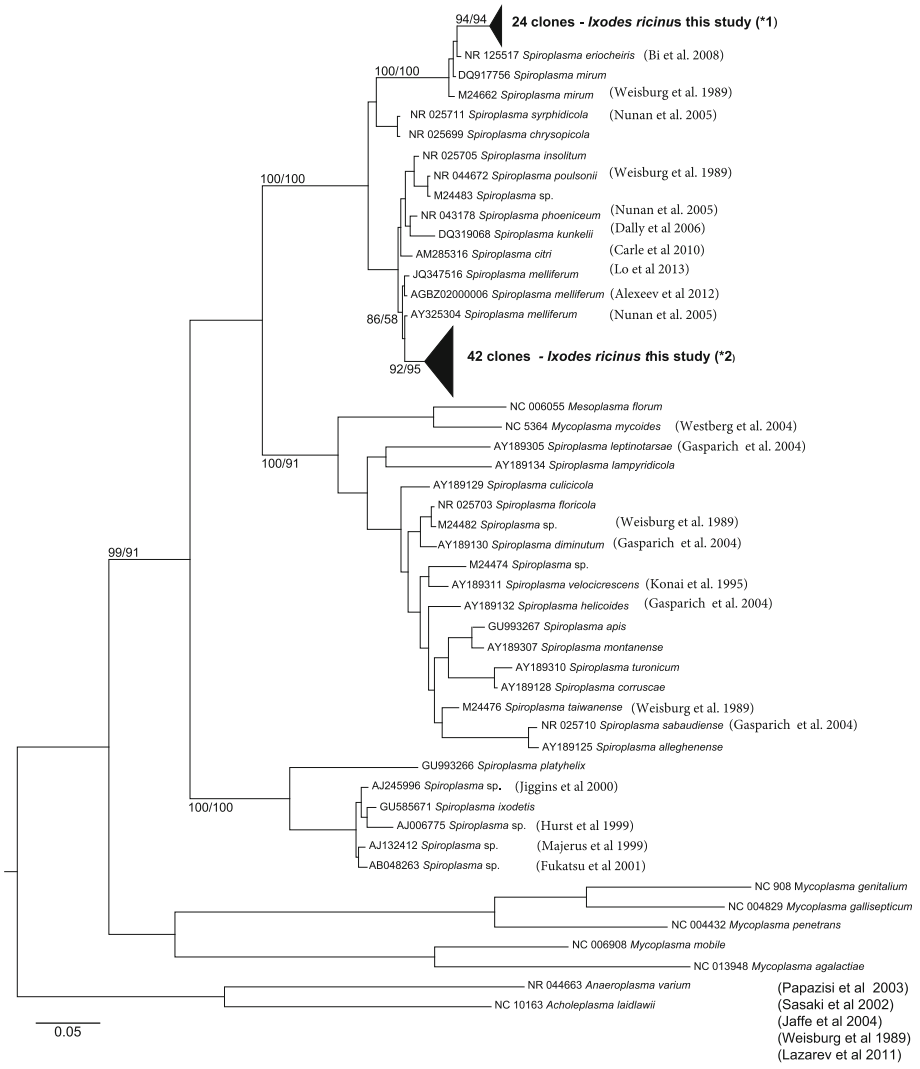

Fig. 1 The phylogeny was inferred by Bayesian analysis of 16S rRNA gene sequence alignment. Branch lengths correspond to mean posterior estimates of evolutionary distances (*scale bar* 0.05). Branch labels indicate the Bayesian posterior probability and supporting bootstrap value from maximum-likelihood analysis for branches with significant support and relevance for clustering of the analyzed sequences. The phylograms were outgrouped using *Escherichia coli* sequence U00096. *1—the sequences from *Ixodes ricinus* collected on April 13, 2014 from dog sample (24 clones), *2—the sequences from *I. ricinus* collected from cats on May 8, 2013 (17 clones) and May 23, 2013 (25 clones)

Subramanian et al. 2012b; Claerebout et al. 2013) corresponding to our results. However, much higher prevalence of *Rickettsia* spp. was reported for the ticks collected in Hamburg, Germany, where 52 % of ticks were infected (May and Strube 2014), while the prevalence of *Rickettsia* was low (3 %) in the samples collected in Ostrava in the Czech Republic (Venclikova et al. 2014b).

Altogether 66 Spiroplasma 16S rRNA sequences were obtained ftom the clones (GenBank Acession Numbers KT983837-KT983902). The presence of *Spiroplasma* was confirmed by specific primers. The phylogenetic placement of the *Spiroplasma* sequences was similar to those previously analyzed by Lo et al. (2013) using previously published sequnces (Alexeev et al. 2012; Bi et al. 2008; Carle et al. 2010; Dally et al. 2006; Fukatsu et al. 2001; Gasparich et al. 2004; Hurst et al. 1999; Jaffe et al. 2004; Jiggins et al. 2000; Konai et al. 1995; Lazarev et al. 2011; Majerus et al. 1999; Meeus et al. 2012; Nunan et al. 2005; Papazisi et al. 2003; Sasaki et al. 2002; Weisburg et al. 1989; Westberg et al. 2004). The analyzed sequences belonged to the cluster Citri-Chrysophicola-Mirum and were separated from cluster Ixodetis (according to Henning et al. 2006; Lo et al. 2013). The *Spiroplasma* sequences cloned from the positive samples formed two clusters. The sequences obtained from the ticks collected on dogs clustered to *Spiroplasma eriocheiris* from crabs (Wang et al. 2011), while those from ticks collected on cats clustered to *Spiroplasma melliferum* (Lo et al. 2013) (Fig. 1). The prevalence of *Spiroplasma* observed here corresponded to the previous report on *Spiroplasma ixodetis* (Subramanian et al. 2012a).

Bartonella was absent in our samples, corresponding to their known low incidence in *I. ricinus*, although we cannot exclude a bias caused by detection method. In north-eastern Italy, *Bartonella* was found in 1 % of samples (Capelli et al. 2012). In the studies from Eastern Europe, *Bartonella* were either not detected at all (Movila et al. 2014) or the observed prevalence of *B. henselae* in *I. ricinus* ticks was 1.7 % (Zajac et al. 2015).

Ixodes ricinus is regarded as an urban tick, which can be distributed by dogs and cats (Uspensky 2014). Our data showed that 68 % of the tested tick samples contained detectable bacteria and 13 % of samples contained at least two pathogenic taxa. These findings correspond to the previous reports of human pathogen infested ticks in Central Europe.

Acknowledgments The study was supported by project RO0415 of the Ministry of Agriculture of the Czech Republic. The authors thank to Martin Markovic for technical help.

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