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ORIGINAL ARTICLES

Co-Infection and Genetic Diversity of Tick-Borne Pathogens in Roe Deer from Poland

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Abstract

Wild species are essential hosts for maintaining *Ixodes* ticks and the tick-borne diseases. The aim of our study was to estimate the prevalence, the rate of co-infection with *Babesia, Bartonella*, and *Anaplasma phagocytophilum*, and the molecular diversity of tick-borne pathogens in roe deer in Poland. Almost half of the tested samples provided evidence of infection with at least 1 species. *A. phagocytophilum* (37.3%) was the most common and *Bartonella* (13.4%) the rarest infection. A total of 18.3% of all positive samples from roe deer were infected with at least 2 pathogens, and one-third of those were co-infected with *A. phagocytophilum*, *Bartonella*, and *Babesia* species. On the basis of multilocus molecular studies we conclude that: (1) Two different genetic variants of *A. phagocytophilum*, zoonotic and nonzoonotic, are widely distributed in Polish roe deer population; (2) the roe deer is the host for zoonotic *Babesia* (*Bab. venatorum*, *Bab. divergens*), closely related or identical with strains/species found in humans; (3) our *Bab. capreoli* and *Bab. divergens* isolates differed from reported genotypes at 2 conserved base positions, *i.e.*, positions 631 and 663; and (4) this is the first description of *Bart. schoenbuchensis* infections in roe deer in Poland. We present 1 of the first complex epidemiological studies on the prevalence of *Babesia, Bartonella*, and *A. phagocytophilum* in naturally infected populations of roe deer. These game animals clearly have an important role as reservoir hosts of tick-borne pathogens, but the pathogenicity and zoonotic potential of the parasite genotypes hosted by roe deer requires further detailed investigation.

Key Words: Roe deer—*Babesia*—*Anaplasma*—*Bartonella*—Genetic diversity—Co-infection.

Introduction

The transmission of infectious diseases between wild animals and humans is now an issue of major interest for scientists. It is believed that more than 70% of human emerging infectious diseases (EID) reported between 1940 and 2004 have their origin in wildlife, and vector-borne diseases are believed to have been responsible for almost 30% of EID events in the last decades (Jones et al. 2008). Therefore, it is crucial to identify the most important wild host species that serve as major reservoirs of infections acquired from, and transmitted by, ticks to humans and other animals.

In central Europe, the roe deer (*Capreolus capreolus*) is one of the most important hosts for adult *Ixodes ricinus* ticks, which act as vectors for several microbial pathogens (Kiffner et al. 2011). The local density of this vector species depends on many factors, including climatic conditions (mainly temperature and humidity), and also on the abundance of suitable warm-blooded vertebrate hosts (Randolph 2004). For example, the presence of wild ungulate species, such as roe deer, has been shown to be essential in maintaining and amplifying tick populations and, consequently, the tick-borne diseases vectored by these ticks (Carpi et al. 2008, Pugliese et al. 2008). Furthermore, both the numbers of infected ticks and the tick-borne encephalitis infections diagnosed in human patients are positively correlated with the density of local roe deer populations (Hudson et al. 2001, Rizzoli et al. 2009). The role of roe deer, as reservoir hosts of tick-borne pathogens and as the source of infections for *I. ricinus* nymphs and females, requires deeper investigation,

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because there is still a lack of complex epidemiological studies exploring the processes regulating the prevalence of tick-borne diseases in wild cervid populations.

It is already well established that roe deer constitute the main reservoir for the *Anaplasma phagocytophilum,* the agent of human anaplasmosis, and for *Babesia venatorum* (EU1) infection, previously recognized as an agent of human babesiosis in Europe (Herwaldt et al. 2003). Recently, the presence of *Bab.* divergens and a different *Bab. Divergens*–like organism was reported in wild cervids (roe deer and red deer) (Duh et al. 2005, Cancrini et al. 2008, Tampieri et al. 2008), but the exact systematic positions of these isolates are still being evaluated (Zintl et al. 2011).

A. phagocytophilum is not transmitted transovarially in ticks (Ogden et al. 1998), and therefore *A. phagocytophilum* is thought to be maintained mainly in reservoir hosts. Asymptomatic *Babesia* spp. infections in humans, especially infections with *Bab. microti*, may persist for months or even years and can lead to nonzoonotic transfer of cases of babesiosis during blood transfusion (Leiby 2011, Siński et al. 2011), posing a particular threat for immunocompromised individuals. Both in animals and humans, infections with *A. phagocytophilum* and *Babesia* spp. can manifest with a varied range of symptoms, from totally asymptomatic or very mild infections, through chronic and latent nonspecific manifestations, to acute life-threatening infections (Blanco and Oteo 2002, Vannier and Krause 2012). Humans are invariably accidental hosts in these cases, having acquired the pathogens through tick bites during inadvertent contact with these vectors in the natural environment, through blood transfusions, or by direct contact with deer blood, *i.e*., while hunting, especially during field dressing of game animals with bare hands.

Bacteria of the genus *Bartonella* are increasingly being recognized as important human pathogens and are responsible for a wide range of clinical manifestations, including Carrion's disease or trench fever. Lice and flies are thought to be their main vectors. Additionally *I. ricinus* may likely act as a vector of these bacteria, although the vector competence of ticks for *Bartonella* transmission is still discussed (Telford 3rd et al. 2010, Reis et al. 2011). Humans are believed to be competent hosts for only 2 species, *Bart. quintana* and *Bart. bacilliformis* (Dehio and Sander 1999), but the majority of human infections are due to zoonotic *Bartonella* species. These incidental infections may display various clinical manifestations, *i.e*., cat scratch disease (*Bart. henselae*, *Bart. clarridgeiae*), neuroretinitis (*Bart. grahamii,* isolated from rodents), endocarditis (*Bart. vinsonii* subsp. *berkhoffi* isolated from dogs) or myocarditis (*Bart. washoensis* from ground squirrels). Recently, 2 *Bartonella* species, *Bart. schoenbuchensis* and *Bart. capreoli*, have been detected in roe deer (Dehio et al. 2001, Bermond et al. 2002), but so far little is known about the risk of human infection with cervid-specific *Bartonella* species. No data exist on the prevalence of these bacteria in naturally infected host populations, nor on their genetic diversity and co-existence with other tick-borne disease pathogens.

In Poland, the distribution and public health relevance of tick-borne pathogen infections and co-infections in wild cervids has been relatively neglected when compared with other mammalian species (Siński et al. 2006, Welc-Falęciak et al. 2008, Welc-Falęciak et al. 2009) and remains to be fully defined. Relatively few studies have investigated the occurrence of tick-borne pathogens in roe deer, and little is known about the existence of co-infections and the genetic diversity of *A. phagocytophilum, Babesia* spp., and *Bartonella* spp. in these mammals (Rymaszewska 2008, Sawczuk et al. 2005, Michalik et al. 2012). It is worth noting that roe deer are actually the most widely distributed game species in Central Europe, and are hunted in large numbers. In Poland, roe deer are found throughout the country, and with approximately 830 000 individuals (data for 2011, Agricultural Property Agency, Directorate General of the State Forests and the Polish Hunting Association) represents the most numerous game mammals. In the hunting season, hunters and forestry workers are exposed to direct contact with animal blood as well as tick infestation, creating obvious opportunities for direct transmission of tick-borne disease pathogens. Therefore, in view of the relative paucity of information on the role of roe deer in the maintenance of tick-borne diseases, the main aim of our study was to assess the potential of these game animals as reservoirs of zoonotic tick-borne disease using complex multilocus molecular assays. Accordingly, in this paper we estimated the prevalence and rate of co-infection of *Babesia* spp., *Bartonella* spp., and *A. phagocytophilum* in roe deer and demonstrated the molecular diversity of tick-borne pathogens in this host species.

Materials And Methods

Blood samples

Blood samples were collected from 67 roe deer (52 females, 15 males) harvested during the seasonal cull in several provinces of Poland. The most representative samples (77%) were obtained during the fall 2010 ($n=36$) and the winter 2011 ($n=16$) in 3 districts (Murowana Goślina, Margonin, Sieraków) of the Wielkopolskie province, in west-central Poland. The remaining blood samples ($n=15$) were collected during the seasonal cull from east-central Poland (Lubuskie, Mazowieckie, and Warmińsko-Mazurskie provinces). From each animal, 3 mL of whole blood was collected into 0.001 M EDTA. Genomic DNA was extracted from whole blood using a DNAeasy Blood and Tissue Kit (Qiagen, Crawley, UK) and stored at -20°C.

PCR analysis

Primers and cycling conditions used in this study are listed in Table 1. Detection and genotyping of *A. phagocytophilum* and *Bartonella* spp. were performed by amplification and sequencing of 2 loci. For this, 2 sets of primers for the *groESL* heat shock operon and *16S rRNA* gene for *A. phagocytophilum* or the β-subunit of the RNA polymerase gene (*rpoB*) and a fragment of the gene encoding the enzyme citrate synthase (*gltA*) for *Bartonella* spp. were applied. *Babesia* spp. were detected and identified using GR2/GF2 primers targeting the fragment of 18S rDNA. Genotyping of *Babesia* isolates from positive animals was done by amplification with CRYPTO R and CRYPTO F primers and sequencing of a long 18S rRNA gene fragment (Table 1). Reactions were performed in a final volume of 20 μL and contained 0.33 mM deoxyribonucleotide triphosphates (dNTPs; Eurobio, Lille, France), 2 mM $MgCl_2$, 1 × PCR buffer, 1 U Taq polymerase (Fermentas), 1 μM of each primer, and 5 μL of extracted DNA sample. *Bab. divergens* DNA extracted from a roe deer spleen, *Bab. microti* King

Table 1. Nucleotide Sequences and Annealing Temperature of the Primers Used for Polymerase Chain Reaction

Species	*Gene*	*Primer*	*Sequence 5′→3′*	*Fragment size (bp)*	*Reference*
A. phagocytophilum	*groESL*	HS1	TGGGCTGGTA(A/C)TGAAAT	1350	Sumner et al. (1997)
		HS6	CCICCIGGIACIA(C/T)ACCTTC		
		HS43 (nested)	AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC	480	
		HS45 (nested)	ACTTCACG(C/T)(C/T)TCATAGAC		
	16S rRNA	ge3a	CACATGCAAGTCGAACGGATTATTC	932	Massung et al. (1998)
		ge10r	TTCCGTTAAGAAGGATCTAATCTCC		
		ge9f	AACGGATTATTCTTTATAGCTTGCT	546	
		ge2	GGCAGTATTAAAAGCAGCTCCAGG		
Bartonella	*gltA*	BhCS.781p	GGGGACCAGCTCATGGTGG	380	Norman et al. (1995)
		BhCS.1137n	AATGCAAAAAGAACAGTAAACA		
	rpoB	rpoR	CGCATTATGGTCGTATTTGTCC	333	Paziewska et al. (2011)
		rpoF	GCACGATT(C/T)GCATCATCATTTTCC		
Babesia	*18S rRNA*	GR2	CCAAAGACTTTGATTTCTCTC	559	Bonnet et al. (2007)
		GF2	G(C/T)(C/T)TTGTAATTGGAATGATGG		
	18S rRNA	CRYPTO R	GAATGATCCTTCCGCAGGTTCACCTAC	1 727	Herwaldt et al. (2003)
		CRYPTO F	AACCTGGTTGATCCTGCCAGTAGTCAT		

College strain DNA isolated from BALB/c mice blood, and *Bart. grahamii* DNA obtained from bacterial cultures initiated with samples from free-living rodents were used as positive controls. *A. phagocytophilum* DNA isolated from blood of infected European bison (*Bison bonasus*) was used as positive control. Negative controls were performed in the absence of template DNA. Amplicons were visualized with Midori Green stain (Nippon Genetics Europe GmbH) following electrophoresis in 2% agarose gels. Amplicons were purified using the Axygen Clean-up purification kit (Axygen, USA) and sequenced by a private company (Genomed S.A., Poland) in both directions.

Phylogenetic analysis

DNA sequence alignments and phylogenetic analysis were conducted using MEGA version 5.0 (Tamura et al. 2011). Phylogenetic trees were created using alignments performed with the Kimura-2 parameter algorithm as a distance method and Neighbor Joining (NJ) as the tree construction method. For comparison, sequences of *Babesia, Bartonella*, and *A. phagocytophilum* species/strains obtained from GenBank (www.ncbi.nlm.nih.gov) were implemented in the sequence alignment. The stability of inferred phylogenies was assessed by bootstrap analysis of 1000 randomly generated sample trees.

Statistical analysis

The frequency distribution of infracommunity species richness was tested for goodness-of-fit to the positive binomial distribution (assumption of the null model is a regular distribution), the Poisson distribution (assumption of the null model is a random distribution), and the null model of Janovy et al. (1995) (assumption of the null model is that, in the absence of associations and interactions between species, the frequency distribution of infracommunity species richness is predicted by prevalence values). Goodness-of-fit in each case was tested by chi-squared analysis. The degree of aggregation in species richness was calculated by the index of dispersion (I; the variance to mean ratio) and the index of discrepancy (D) as described by Poulin (1993) (a value of 0 indicates an even distribution of counts across all hosts and a value of 1 indicates all pathogen genera aggregated in a single host).

Nucleotide sequences accession numbers

New nucleotide sequences were deposited in GenBank with the accession numbers JQ965530-31 and JQ955734-35 (*16S rRNA* and *groESL* genes of *A. phagocytophilum*, respectively), JQ929916 and JQ929918 (*18S rRNA* gene of *Bab. divergens* and *Bab. capreoli*, respectively), and JQ929915 and JQ955736 (*gltA* gene of *Bart. capreoli* and *Bart. schoenbuchensis,* respectively).

Results

In all, 67 roe deer were sampled. Overall, 6 species of tick-borne disease pathogens belonging to 3 genera were detected in blood samples, and half of them (35 out of 67) tested positively for at least 1 species. *A. phagocytophilum* was the most common infection and *Bartonella* spp. the rarest. The overall mean number of genera of pathogens (*Anaplasma* sp., *Babesia* sp., and *Bartonella* sp.) per host was 0.830 ± 0.106 (standard error of the mean, SEM), with a variance to mean ratio of 1.11. Significant differences in overall prevalence of tick-borne disease pathogens between males and females roe deer were noted (females 63.5%, males 13.3%; Fisher exact test $p = 0.0002$).

A. phagocytophilum *infections*

This was the most prevalent pathogen in our study; 37.3% (25/67) of blood samples tested positively for *A. phagocytophilum*. The 546-bp fragment of the *16S rRNA* gene and the 480-bp fragment of the *groESL* heat shock operon were further analyzed for 20 isolates. The nucleotide identity/similarity of the sequenced *16S rDNA* fragments was very high (99.8–100%). Twelve of 20 sequences were identical, representing genetic variant A. Only 8 isolates could be distinguished on

Table 2. Heat Shock Operon *groESL* and *16S rRNA* Gene Variants of *A. phagocytophilum* in Roe Deer from Different Districts of Poland

Gene	*No. of isolates*	*No. of samples*	*Site of study*	*Genetic variant*
16S rRNA	10	12, 13, 17, **18, 33**, 34, 41, 45, 48, 49	North-central Poland (Wielkopolskie)	A
	2	61, 63	East-central Poland (Mazowieckie, Podlaskie)	A
	8	2, 27, 36, 38, 39, 44, 54, **55**	Wielkopolskie	B
groESL	9	12, 13, 17, 34, 41, 45, 48, 49, **55**	North-central Poland (Wielkopolskie)	C
	2	61, 63	East-central Poland (Mazowieckie, Podlaskie)	C
	9	2, **18,** 27, **33,** 36, 38, 39, 44, 54	North-central Poland (Wielkopolskie)	D

Isolates common for genetic variants A (*16S rRNA*) and C (*groESL*) or B (*16S rRNA*) and D (*groESL*) are shown in bold.

the basis of substitution at position 175 (C→A) in a variable region near the 5′ end of the *16S rRNA* gene, representing genetic variant B (Tables 2 and 3A). Isolates belonging to variant A were found in samples collected all over the area of Poland. Isolates of variant B were found only in blood samples from the Wielkopolskie district. Isolates from variants A and B showed 99.6–99.8% homology with the partial nucleotide sequence of *A. phagocytophilum* obtained from *I. ricinus* in Lithuana (JN181069) and Belarus (HQ629914) as well as roe deer from the Czech Republic (EU839847). Additionally, the *16S rRNA* sequence of variant B differed from the human pathogenic strains isolated in Slovenia, Poland and Italy by only 2 nucleotides: G and A in positions 76 and 84, respectively (Table 3A).

The partial *groESL* gene fragments (480 bp) were sequenced for all positive samples ($n=20$) because the *16S rRNA* gene is too conserved for analysis of genetic heterogeneity. The level of homology between isolates was also very high (99.8–100%). Sequence analysis demonstrated nucleotide substitution at position 398 (G→A) (Table 3B) and allowed identification of 2 genetic variants. Genetic variant C included 11 isolates from samples collected in the Wielkopolskie, Podlaskie, and Mazowieckie districts (Table 2). Variant D was composed of 9 sequences found only in samples from the Wielkopolskie district. Scrutiny of the phylogenetic tree, based on the partial *groESL* operon sequences, showed that isolates from variant C were closely related to other European isolates, mainly from *I. ricinus* ticks and roe deer (Fig. 1). Isolates belonging to variant D clustered with *A. phagocytophilum* pathogenic for human and domestic animals in Europe, as well as in North America, and differed from them only by 1 nucleotide in positions 167 or 401 (Table 3).

Table 3. Polymorphism in the Fragment of the *16S rRNA* Gene (A) and *groESL* Heat Shock Operon (B) in *A. phagocytophilum* Isolates from Roe Deer and Human Pathogenic Strains (Sequences Published in GenBank)

A

Gene	*Strain/genetic variant*	*No. of isolate*	*Nucleotide positions 5′→3′*[a] 76	84	165	175	328	*GenBank accession number*	*Host*	*Country*	*Reference*
16S rRNA	A	12	**G**	**A**	A	**A**	A	JQ965530	Roe deer	Poland	This study
	B	8	**G**	**A**	A	**C**	A	JQ965531	Roe deer	Poland	This study
	A. phagocytophilum strains pathogenic for human		A	G	A	C	T	JN107802	Human	Poland	Welc-Falęciak et al. (2010)
			A	G	A	C	A	AY833407	Human	Poland	Grzeszczuk et al. (2006)
			A	G	A	C	A	GU236658	Human	Slovenia	Scharf et al. (2011)
			A	G	A	C	A	DQ029028	Human	Italy	de la Fuente et al. (2005)
			—	—	G	C	A	GU908492	Human	China	Li et al. (2010)[b]
			A	G	A	C	A	GU236664	Human	USA	Scharf et al. (2011)

[a]The number corresponds to the positions of nucleotide substitutions relative to the sequence of the complete *16S rRNA* gene of *A. phagocytophilum* strain HZ (NC_007797). Base substitutions are shown in bold.
[b]Unpublished, sequence deposited in GenBank.

B

Gene	*Strain/genetic variant*	*No. of isolates*	*Nucleotide positions 5′→3′*[b] 167	398	401	*GenBank accession number*	*Host*	*Country*	*Reference*
groESL	C	11	G	**G**	A	JQ955735	Roe deer	Poland	This study
	D	9	G	**A**	A	JQ955734	Roe deer	Poland	This study
	A. phagocytophilum strains pathogenic for human		A	A	A	AF033101	Human	Slovenia	Petrovec et al. (1999)
			G	A	G	JF494839	Human	USA	Rejmanek et al. (2012)
			G	A	G	EF473207	Human	China	Zhang et al. (2007)[b]

[a]The number corresponds to the positions of nucleotide substitutions relative to the sequence of the *groESL* heat shock operon of the human pathogenic *A. phagocytophilum* strain (U96728). Base substitutions are shown in bold.
[b]Unpublished, sequence deposited in GenBank.

FIG. 1. Phylogenetic tree of the *A. phagocytophilum* isolates studied in the current work and chosen isolates from GenBank based on the fragment of the *groESL* heat shock operon. Numbers at the nodes of the tree indicate bootstrap values (1000 replicates, only bootstrap values >70% are shown). The nucleotide sequence of *A. platys* was used as an outgroup. Our isolates are marked in bold.

In spite of nucleotide substitutions in the *groESL* heat shock operon sequence, the deduced amino acid sequences did not change for any of the studied isolates. Combining the results of the molecular analysis of these 2 loci (*groESL* and *16S rRNA*), 2 genogroups were obtained: (1) 10 isolates representing genetic variant A on the basis of *16S rRNA* and variant C on basis of the *groESL* operon are probably nonzoonotic strains; and (2) 7 isolates representing variant B (*16S rDNA*) and variant D (*groESL*) are closely related to *A. phagocythophilum* strains pathogenic for human and domestic animals (Table 2, Fig. 1). Three isolates displayed contrasting results when genotyped at these 2 loci. The *A. phagocytophilum* isolate from roe deer no. 55 was classified as variant B accordingly to the *16S rRNA* sequence, but represented variant C according to the *groESL* gene fragment (Table 2). Two isolates, nos. 18 and 33, were classified as variants A accordingly to *16S rRNA* sequence but represented variant D accordingly to the *groESL* gene fragment (Table 2).

Babesia *spp. infections*

DNA from *Babesia* spp. was detected in 18 out of 67 animals (26.9%). Sequence analysis of the 1100-bp fragment of 18S rDNA obtained from 12 isolates showed the presence of 3 different *Babesia* species. The most prevalent species was *Bab. capreoli* (8/12, 66.7%) identical with *Bab. capreoli* F10 (FJ944828) originally isolated from roe deer in France (Fig. 2) and differing by 1 nucleotide (T → A) at position 620 from *Bab. capreoli* CVD5 (Table 4). Two out of 12 isolates (16.6%) showed 100% homology with *Bab. venatorum* (EU1) (HQ830266) derived from roe deer blood in France and were identical with *Bab. venatorum* (EU1) isolated from human in Europe (Fig. 2). The last 2 isolates were identified as *Bab. divergens* (2/12, 16.6%). These isolates showed 99.8% similarity to *Bab. divergens* (GQ304525) derived from red deer in France and clustered with other *Bab. divergens* isolates pathogenic for humans and cattle and differed from them only at 2 nucleotides positions, 54 and 148 (Table 4, Fig. 2). These 2 isolates differed from the *Bab. capreoli* and *Bab.* divergens–like isolates from Slovenia by 2 nucleotides at positions 631 and 663, supporting their identification as *Bab. divergens* (Table 4).

Bartonella *infections*

DNA from the genus *Bartonella* was found in 13.4% of the animals (9/67). Molecular analysis was based on the 333-bp

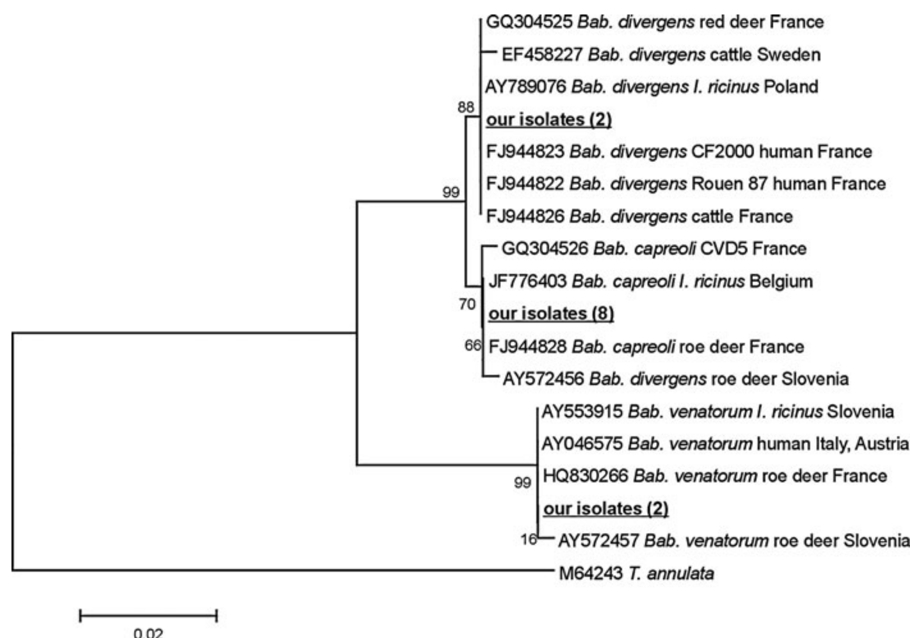

FIG. 2. Phylogenetic tree of *Babesia* isolates studied in the current work and chosen isolates from GenBank based on the *18S rRNA* gene fragment. Numbers at the nodes of the tree indicate bootstrap values (1000 replicates). The nucleotide sequence of *T. annulata* was used as anoutgroup. Our isolates are marked in bold.

fragment of the β-subunit of RNA polymerase (*rpoB*) and on the 380-bp fragment of the gene encoding the enzyme citrate synthase (*gltA*). Eight isolates were identical and showed a high level of *rpoB* and *gltA* sequence homology (>99%) with *Bart. schoenbuchensis* strain R1 from roe deer from Germany (nucleotide substitution at position 712 [T→C] in fragment of *gltA* gene; Table 5, Fig. 3). One isolate was closely related (>99% homology) to *Bart. capreoli* (isolated from a French roe deer) with a nucleotide substitution at position 736 (A→G) (Table 5, Fig. 3). The observed *gltA* sequence diversity affected the deduced amino acid sequences. Genetic diversity at position 712 or 736 in the *gltA* nucleotide sequences of *Bart. schoenbuchensis* or *Bart. capreoli* involved substitution of a Pro residue for Ser at positions 233 or a Val for Ile at position 241 in amino acid sequences of citrate synthase, respectively (the numbers correspond to the positions of the amino acids substituted relative to the corresponding amino acid sequences of *Bart. schoenbuchensis* strain R6, CAB95650).

Infracommunity species richness and co-infection analysis

The analysis of co-infection was performed at the level of genera (*Babesia, Bartonella, Anaplasma*) because it was not possible to identify to the species level all positive samples. A total of 19.4% (13/67) of all positive roe deer yielded at least 2

Table 4. Polymorphism in the Fragment of *18S rRNA* Gene in *Bab. divergens* and *Bab. capreoli* Isolates from Roe Deer, Red Deer, Cattle, Ticks, and Human Pathogenic Strains (Sequences Published in GenBank)

Species/strain	*Host*	*Country*	*GenBank accession number*	*Nucleotide positions 5′→3′*[a]								*Reference*
				54	*148*	*373*	*620*	*631*	*663*	*804*	*824*	
Bab. divergens Rouen 87 clone 5	Human	France	FJ944822	C	C	A	T	A	A	G	G	Malandrin et al. (2010)
Bab. divergens RD54	Roe deer	Poland	JQ929916	**T**	**T**	A	T	A	A	G	G	This study
Bab. divergens CVD7	Red deer	France	GQ304525	C	C	A	T	A	A	G	G	Jouglin et al. (2009)[b]
Bab. divergens	*I. ricinus*	Poland	AY789076	C	C	A	T	A	A	G	G	Pieniazek et al. (2006)
Bab. divergens B_di08	Cattle	Sweden	EF458227	C	C	A	T	A	A	G	**A**	Vogl et al. (2007)[b]
Bab. divergens C139	Cattle	France	FJ944826	C	C	A	T	A	A	G	G	Malandrin et al. (2010)
Bab. divergens CF2000	Human	France	FJ944823	C	C	A	T	A	A	G	G	Malandrin et al. (2010)
Bab. divergens-like	Roe deer	Slovenia	AY572456	C	C	**G**	T	**G**	**T**	**A**	G	Duh et al. (2005)
Bab. capreoli RD33	Roe deer	Poland	JQ929918	C	C	A	T	**G**	**T**	G	G	This study
Bab. capreoli F10	Roe deer	France	FJ944828	C	C	A	T	**G**	**T**	G	G	Malandrin et al. (2010)
Bab. capreoli CVD5	Roe deer	France	GQ304526	C	C	A	**A**	**G**	**T**	G	G	Jouglin et al. (2009)[b]

[a]The number corresponds to the positions of nucleotide substitutions relative to the sequence of the*18S rRNA* gene of the human pathogenic *Bab. divergens* strain Rouen 87 clone 5. Base substitutions are shown in bold.
[b]Unpublished, sequence deposited only in GenBank.

Table 5. Polymorphism in the Fragment of the *gltA* Gene in *Bart. schoenbuchensis* and *Bart. capreoli* Isolates from Roe Deer (Sequences Published in GenBank)

Species/strain	Host	Country	GenBank accession number	Nucleotide positions 5′→3′[a]														Reference
				681	711	712	732	736	807	837	859	861	900	915	916	930	948	
Bart. capreoli	Roe deer	France	AF293392	A	C	T	A	A	T	C	C	C	A	T	C	C	C	Bermond et al. (2002)
Bart. capreoli RD27	Roe deer	Poland	JQ929915	A	C	T	A	**G**	T	C	C	C	A	T	C	C	C	This study
Bart. schoenbuchensis R1	Roe deer	Germany	AJ278183	A	T	T	G	A	C	C	C	A	G	C	T	C	T	Dehio et al. (2001)
Bart. schoenbuchensis R3	Roe deer	Germany	AJ278184	G	T	T	A	A	C	C	T	A	G	C	T	T	T	Dehio et al. (2001)
Bart. schoenbuchensis R4	Roe deer	Germany	AJ278185	G	T	T	A	A	C	T	T	A	G	C	T	T	T	Dehio et al. (2001)
Bart. schoenbuchensis R6	Roe deer	Germany	AJ278186	A	C	T	A	A	T	C	C	C	A	T	C	C	C	Dehio et al. (2001)
Bart. schoenbuchensis RD48	Roe deer	Poland	JQ955736	A	T	**C**	G	A	C	C	C	A	G	C	T	C	—	This study

[a]The number corresponds to the positions of nucleotide substitutions respect to the sequence *gltA* gene of *Bart. schoenbuchensis* strain R6. Base substitutions in sequences obtained in this study are shown in bold.

pathogens, and one-third of those (4/13) were co-infected with *A. phagocytophilum*, *Bartonella*, and *Babesia* species. The genotyping results of the agents responsible for 10 co-infections are presented in Table 6. Among 10 combinations, only 1 case of co-infection with 2 potentially human pathogenic (zoonotic) species/strains was detected: *A. phagocytophilum* variant D and *Bab. divergens* infection (accompanied by *B. schoenbuchensis*). The observed distribution of infracommunity species richness did not conform to the normal, positive and negative binomial nor to the Poisson distributions (Fig. 4). It was significantly different ($\chi^2=7.8$, degrees of freedom [df] $=3$, $p=0.05$) from that predicted by the null model for interactions of parasite species in an assemblage (Janovy et al. 1995). Fewer single infections and significantly more cases of co-infection with 2 or 3 pathogens were observed in comparison to the values predicted by prevalence (Fig. 4).

Discussion

The results of our complex study, based on molecular analysis of several loci, revealed an interesting and unexpected diversity of tick-borne disease pathogens among roe deer populations from east-central and west-central Poland, providing the first detailed analysis of existing co-infections associated with this important European cervid species. Moreover, this is the first study, in which 3 genera of infectious agents were concurrently investigated in roe deer. Despite a limited number of tested animals, the high prevalence of pathogens enabled detailed molecular analysis based on several loci. Our genotyping showed that animals harbored more often the pathogen species/strains specific for this game animal (*A. phagocytophilum* variants A and C, *Bab. capreoli*, *Bart. capreoli*, *Bart. schoenbuchensis*) than the species/strains of public health significance (*A. phagocythophilum* variants B and D, *Bab. divergens, Bab. venatorum* [EU1]).

Previous molecular studies have shown a relatively high prevalence of tick-borne pathogens in naturally infected roe deer populations. The most common infection in the current study was *A. phagocytophilum*, with a prevalence of 37%. In Europe, it is believed that cervids are the main reservoir hosts of these bacteria, having been found commonly in roe deer in Spain (18%; de la Fuente et al. 2008), Switzerland (18%; Liz et al. 2002), Slovenia (85%; Petrovec et al. 2002), Slovakia (50%; Stefanidesova et al. 2008), and Austria (43%; Polin et al. 2004). In Poland, prevalence of this species has been reported to range from 9% to 38% in different regions of the country (Adamska and Skotarczak 2007, Michalik et al. 2009, Hapunik et al. 2011). In agreement with data published previously (von Loewenich et al. 2003), sequence analysis of the 2 loci in the current work revealed 2 distinct genetic lineages of *A. phagocytophilum*: (1) genetic variants detected in humans, ticks, dogs, horses, sheep, and red deer from Europe and the United States that are believed to be pathogenic; and (2) genetic variants isolated from ticks and roe deer in Europe that are probably nonzoonotic strains (Portillo et al. 2011, Katargina et al. 2012, Michalik et al. 2012). Recent studies have suggested that these different *A. phagocytophilum* variants have adopted different host tropisms (Petrovec et al. 2002, 2003) and that they show distinct but differing pathogenicity (Massung et al. 2003).

The results of previous studies support our finding of equal distribution of zoonotic and nonzoonotic *A. phagocytophilum*

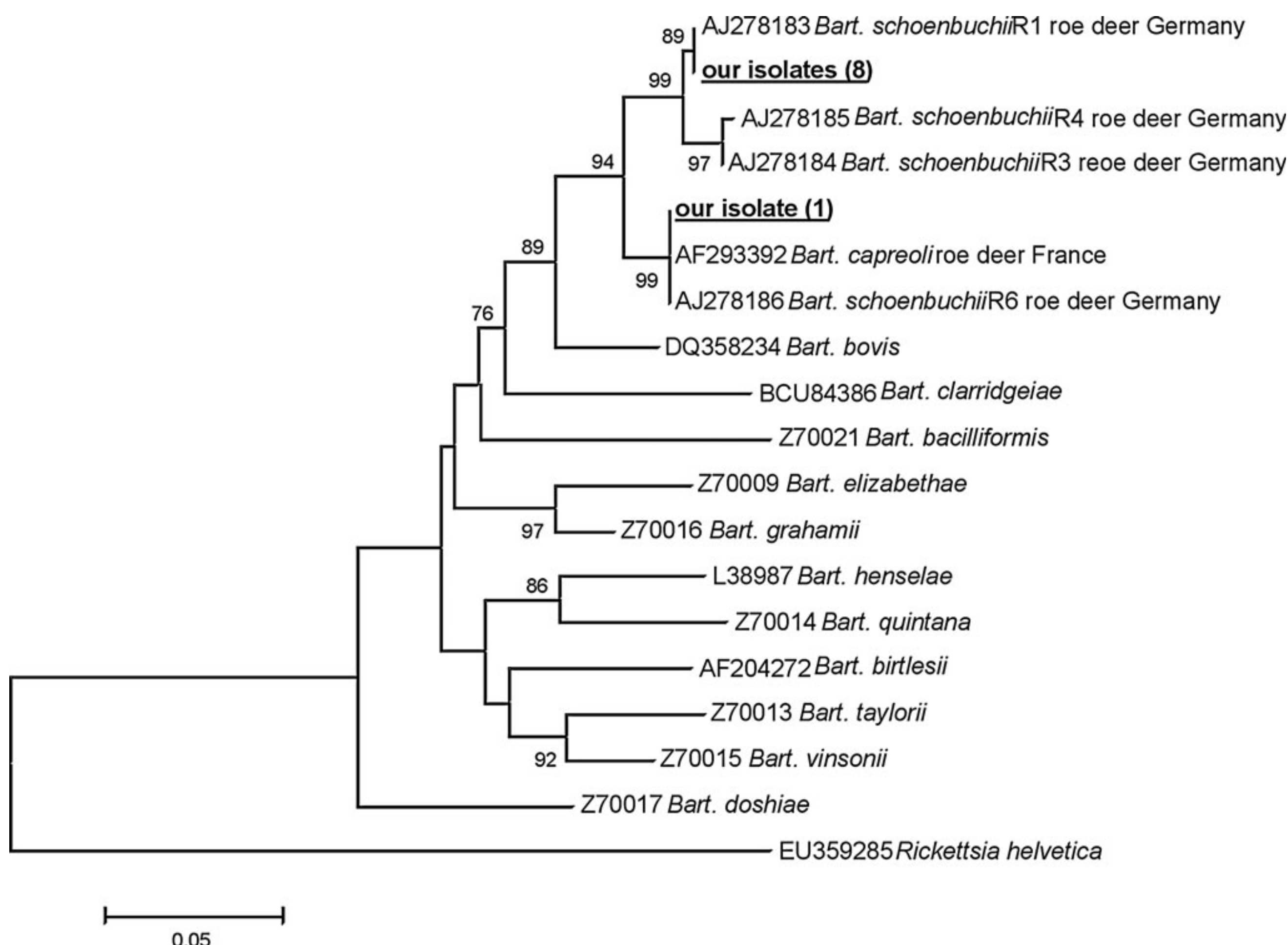

FIG. 3. Phylogenetic tree of *Bartonella* isolates studied in the current work and chosen isolates from GenBank based on the *gltA* gene fragment. Numbers at the nodes of the tree indicate bootstrap values (1000 replicates; only bootstrap values >70% are shown). The nucleotide sequence of *R. helvetica* was used as an outgroup. Our isolates are marked in bold.

variants. For example, almost half of the genotyped *A. phagocytophilum* isolates from ticks in Germany were closely related to strains that caused granulocytic anaplasmosis/ehrlichiosis in human and animals in Europe or the United States (von Loewenich et al. 2003). The distribution of zoonotic and nonzoonotic variants in roe deer is very similar also in our studies (41% vs. 59%, respectively), but this issue certainly needs more scientific attention and further

Table 6. Co-Infection with *A. phagocytophilum*, *Babesia* spp., and *Bartonella* spp. in Roe Deer

		Species/genetic variants of pathogen		
No. of host	District	A. phagocytophilum/*genetic variant* (groESL)	Babesia	Bartonella
RD 2	Wielkopolskie	***A. phagocytophilum*/D**	*Babesia* sp.	—
RD 13	Wielkopolskie	*A. phagocytophilum*/C	*Bab. capreoli*	—
RD 27	Wielkopolskie	***A. phagocytophilum*/D**	*Bab. capreoli*	*Bart. schoenbuchensis*
RD 29	Wielkopolskie	*A. phagocytophilum*/nd	*Bab. capreoli*	*Bart. schoenbuchensis*
RD 33	Wielkopolskie	***A. phagocytophilum*/D**	*Bab. capreoli*	*Bart. schoenbuchensis*
RD 34	Wielkopolskie	*A. phagocytophilum*/C	*Bab. capreoli*	—
RD 36	Wielkopolskie	***A. phagocytophilum*/D**	—	*Bart. schoenbuchensis*
RD 39	Wielkopolskie	***A. phagocytophilum*/D**	*Babesia* sp.	—
RD 45	Wielkopolskie	*A. phagocytophilum*/C	*Bab. capreoli*	—
RD 48	Wielkopolskie	*A. phagocytophilum*/C	—	*Bart. schoenbuchensis*
RD 49	Wielkopolskie	*A. phagocytophilum*/C	—	*Bart. schoenbuchensis*
RD 54	Wielkopolskie	***A. phagocytophilum*/D**	***Bab. divergens***	*Bart. schoenbuchensis*
RD 61	Mazowieckie	*A. phagocytophilum*/C	***Bab. venatorum* (EU1)**	—

Species or genetic variants pathogenic for human are shown in bold.

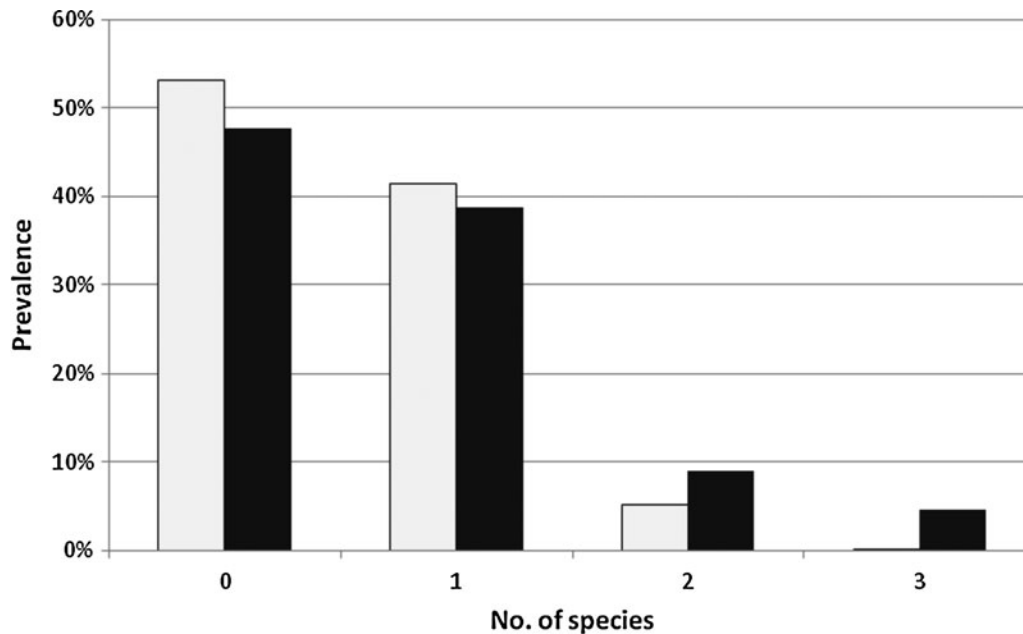

FIG. 4. Frequency distribution of infracommunity species richness. The observed data are in the filled columns and those predicted by the null model of Janovy et al. (1995) are in the open columns. Full explanations and statistical analysis are given in the text.

epidemiological studies. Additionally, we identified 3 *A. phagocytophilum* isolates that constituted contrasting variants, depending on the molecular locus used for genotyping. This may suggest the existence of recombination between zoonotic strains inducing human disease and nonzoonotic strains, as, for example, reported earlier in genotyping *Bartonella* from rodents (Paziewska et al. 2011). Overall genetic diversity in the *groESL* and *16S rRNA* gene sequences is likely a result of bacterial population diversity within the marker region and probably does not affect the function/coded protein or mechanisms of pathogenesis among zoonotic isolates.

Roe and red deer have been shown to be hosts for 4 *Babesia* species, including zoonotic *Bab. venatorum* (EU1), *Bab. divergens*, and nonzoonotic *Bab. capreoli* and *Bab. odocoilei*-like organisms (Duh et al. 2005, Sawczuk et al. 2005, Bonnet et al. 2007, Cancrini et al. 2008, Hoby et al. 2009, Zintl et al. 2011). In Europe, the majority of human babesiosis cases have been caused by *B. divergens*, but several human cases of infection with *B. venatorum* have also been reported recently in Germany, Austria, Italy, and Poland (Herwaldt et al. 2003, Häselbarth et al. 2007, Welc-Falęciak et al. 2010). Only 1 case of human *Bab. microti* infection was reported in Europe (Hildebrandt et al. 2007). The prevalence of *Babesia* spp. infections in our study was almost 27%, which is in the range of the values reported for roe deer from other European countries (from 26% for Switzerland to 76% for Slovenia) (Duh et al. 2005, Hoby et al. 2009). On the basis of the *18S rRNA* gene, we have identified 3 different *Babesia* species. Isolation of *Bab. venatorum* in our study confirmed that these animals are reservoir hosts of this relatively "new" pathogen also in Poland, and, to the best of our knowledge, this is the first report of the presence of *Bab. venatorum* in roe deer from Poland.

The presence of *Bab. divergens* in roe deer was confirmed in Slovenia (Duh et al. 2005), Italy (Cancrini et al. 2008, Tampieri et al. 2008), and also in Poland (Sawczuk et al. 2005), but molecular identification was based only on a short fragment (407 bp) of the nuclear small subunit rRNA gene. Differentiation of *Bab. capreoli* from *Bab. divergens* is possible only on the basis of molecular studies and its incapacity to infect gerbils or cattle under laboratory conditions (Gray et al. 1990, Herwaldt et al. 2003) because these species are morphologically indistinguishable from each other (Malandrin et al. 2010). Recent results indicate that *Bab. capreoli* and *Bab. divergens* can be distinguished on the basis of 3 conserved nucleotide differences at positions 631, 663, and 1637 in their *18S rRNA* sequences (Table 4; Malandrin et al. 2010). Although Duh et al. (2005) showed a high level of similarity (99.6%) of their *Babesia* isolate obtained from roe deer to *Bab. divergens* species, molecular analysis of nucleotide substitutions suggests that this isolate is more closely related to *Bab. capreoli* (Table 4).

Identification of *Babesia* species in the present study was based on analysis of a 1100-bp fragment of the *18S rRNA* gene that constitutes more than 60% of the complete gene (1728 bp). Nucleotide substitutions at positions 631 and 663 and phylogenetic relationship strongly suggest that in Poland roe deer are reservoir hosts for nonzoonotic *Bab. capreoli* and zoonotic *Bab. divergens*. Additionally, 2 of our isolates identified as *Bab. divergens* differed from strains known to be pathogenic in humans and cattle by only 2 nucleotides, which supports their potential public health significance. For example, in the case of *Bab. venatorum*, diversity among as many as 31 nucleotides in the 18S rDNA sequence did not affect its pathogenicity (Herwaldt et al. 2003), although we cannot exclude the possibility that 1 or other of the 2 nucleotide changes was critical. In our study, the prevalence of nonzoonotic *Babesia* species was twice as high (67% vs. 33%) as the prevalence of zoonotic ones. As in the case of *A. phagocythophilum*, the distribution of zoonotic/nonzoonotic species in reservoir hosts certainly needs more scientific attention and more extensive epidemiological studies.

In Europe, *Bart. schoenbuchensis* and *Bart. capreoli* have been identified recently and described in the blood of free-living roe deer, but so far there are few available data on the epidemiology of these 2 species in naturally infected hosts (Dehio et al. 2001, Bermond et al. 2002). Only 1 paper has reported on the prevalence of *Bartonella* in roe deer in Poland, in the area of Szczecin, where prevalence was higher than in our study (21% vs. 13%) (Skotarczak and Adamska 2005), but there are no more data available in the public domain. Our results confirmed the presence of *Bart. capreoli* and *Bart. schoenbuchensis* in roe deer, and of these the latter had a higher prevalence. Genetic diversity of the *gltA* nucleotide sequence was probably due to recombination among *Bartonella* strains, as was suggested by Paziewska et al. (2011). Because the observed *gltA* sequence diversity affected the deduced amino acid sequences of this gene product, there is a need for further study of the consequences of these changes for the structure and function of this protein.

This is the first report of *Bart. schoenbuchensis* in Poland. All of our isolates of *Bart. schoenbuchensis* were closely related to *Bart. schoenbuchensis* strain R1, that shows 86.6% sequence similarity (*gltA*) with *Bart. bacilliformis* (Dehio et al. 2001). Given its close relatedness to *Bart. bacilliformis* as well as its considerable heterogeneity, Dehio et al. (2001) speculated that *Bart. schoenbuchensis* may have not only the potential to cause zoonosis (*i.e.*, in hunters and forestry personnel who are exposed to the blood of roe deer), but also may adapt to humans or eventually develop into a human-specific pathogen such as *Bart. baciliformis*.

Recently, co-infections in tick vectors and reservoir hosts have generated a lot of attention because of the growing interest in how they interact with one another (Swanson et al. 2006, Telfer et al. 2010). Naturally occurring co-infections among ticks and free-living hosts are believed to constitute an additional and hitherto undervalued layer of public health risk (Telfer et al. 2010). Although 3 species co-infections were revealed in the current study, and even occurred more often than predicted by prevalence values of individual species, they did not seem to increase the risk of tick-borne diseases in humans, as nonzoonotic species/strains were involved in the great majority of cases. Our study clearly demonstrates that the precise identification of the species and strains involved in co-infection is crucially important for a full assessment of overall risk.

Conclusions

Applying a range of molecular tools, we have conducted 1 of the first epidemiological studies of the prevalence of *Babesia, Bartonella*, and *A. phagocytophilum* in naturally infected populations of roe deer. The relatively high prevalence of tick-borne disease pathogens encountered in our study and the significant proportion of zoonotic species/strains of *A. phagocytophilum* and *Babesia* that we identified underline the role of roe deer as a zoonotic reservoir of these pathogens in Europe. The genotyping results provided novel data on the genetic diversity of the pathogens, and this has been deposited in the GenBank database. Finally, our study of co-infection has emphasized the importance of detailed genotyping for meaningful and comprehensive assessment of the health risk arising from tick-borne diseases harbored by wild roe deer.

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Author Disclosure Statement

No competing financial interests exist.

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