Coexistence of tick-borne pathogens in game animals and ticks in western Poland

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ABSTRACT: Molecular studies enabling the recognition of the role of game and ticks in the circulation of pathogens transmitted by ticks and detection of coinfections in order to estimate a risk which a contact with tissues of roe deer, red deer and wild boar from north-western Poland brings were the aim of this research. DNA isolated from the blood and spleen of game and from *Ixodes ricinus* were the study materials. The results shows that *Capreolus capreolus* and *Cervus elaphus* play an important role in the life cycle of *Anaplasma phagocytophilum*, two *Bartonella* species, *Theileria* and *Babesia* spp. Whereas in the isolates obtained from 50 representatives of *Sus scrofa*, the DNA of only one pathogen, *A. phagocytophilum* occurred. 63.5% of 74 PCR+ isolates from *Capreolus capreolus* showed a double coinfection and three isolates – triple. In the tissues of *Cervus elaphus*, the coinfections were triple in 38% of individuals, double in 40%, single in 84%.

Keywords: game animals; *Borrelia*; *Anaplasma*; *Bartonella*; *Babesia*; *Theileria*; *Ixodes ricinus*; PCR

Many microorganisms, pathogenic to animals and humans, are transmitted with participation of arthropods which take part in a passive transport of pathogens as vectors and additionally they constitute their natural reservoir. Among arthropods taking part in the transmission of pathogenic microorganisms, ticks play the great part. On the basis of computer studies Vorou et al. (2007) identified 15 different pathogens causing so-called emerging diseases in Europe from 2000 to 2006; *Borrelia burgdorferi, Anaplasma phagocytophilum* are mentioned at the first place and they are transmitted by ticks. The role of ticks is also considered in the transmission of bacteria from the genus *Bartonella* (Chang et al., 2000; Skotarczak and Adamska, 2005). Vertebrates being the competent reservoir of *Borrelia burgdorferi* have been known thanks to the studies of the reservoir of pathogens transmitted by ticks; however, this subject-matter is still little known in reference to *A. phagocytophilum*. In the USA, it is known that the agents of human granulocytic anaplasmosis, Lyme disease and human babesiosis occur together in the vector tick and rodents. Thus, the risk of infection is geographically the same.

Our studies of *Ixodes ricinus*, ticks collected from vegetation in forested localities in Szczecin and north-western Poland, carried out by PCR method, revealed the presence of DNA of *B. burgdorferi* s.l. (from 0.3 to 15.7%) in all developmental stages in the next few years (Wodecka, 2003). The studies of *I. ricinus* collected from the same localities revealed the presence of DNA of *Babesia microti* and *B. divergens* and an agent of human granulocytic anaplasmosis (*A. phagocytophilum*), single infections of ticks at the first place and then, double and triple coinfections (Skotarczak et al., 2002, 2003).

Results of our preliminary studies of roe deer and red deer on the presence of DNA of *Bartonella, Anaplasma, Theileria, Babesia* and *B. burgdorferi* have shown that the animals are significant reservoirs of four out of the five above-mentioned pathogens but they do not play such a role in the endemic area for *Borrelia* (Skotarczak and Adamska, 2005; Sawczuk et al., 2008). The results of studies carried out on the blood of forest rodents and birds and

ticks infesting them, living in middle-west Poland, have indicated that there are species among them able to maintain *Borrelia* (Wodecka 2003; Michalik et al., 2005; Skotarczak et al., 2006).

The aim of this study was a comprehensive molecular analysis that enables to recognize the role of wildlife animals in the maintenance and circulation of tick-borne pathogens and ticks as the vectors of these pathogens. Moreover, the aim was a detection of possible coinfections in order to estimate a risk arising from a contact with tissues of wild animals that were shot in the area of Wkrzanska Forest surrounding Szczecin (north-western Poland).

MATERIAL AND METHODS

Blood samples of 1 ml and spleen samples of about 1 cm^3 in volume were collected from 138 individuals of European roe deer (*Capreolus capreolus*), from 50 red deer (*Cervus elaphus*) and from 50 wild boars (*Sus scrofa*), 238 samples in total. 320 ticks belonging to one species, *I. ricinus*, were collected from all infested animals. They were removed from skin with tweezers and put into separate labelled test-tubes. 200 individuals of *I. ricinus* were collected from the vegetation in the shooting area with the use of flannel flags.

DNA from the collected material was isolated with a commercial MasterPure^{TM} DNA Purification Kit (Epicentre, USA) according to the producer's instructions. The isolated DNA was suspended in the TE buffer and was kept at –70°C till the time of analysis.

The choice of genetic markers and primers complementary to them was made on the basis of the analysis of literature data and preliminary results of our own studies (Table 1).

DNA of *A. phagocytophilum* was detected with the use of PCR, with primers complementary to *msp2* gene (Table 1). Then, in order to sequence the obtained amplicons, the nested PCR with pairs of primers HS1, HS6 and HS43, HS45 complementary to sequences of the heat shock operon *groESL* of *A. phagocytophilum* was additionally applied (Table 1). The composition of reaction mixture for PCR and the reaction conditions were the same as described by Massung and Slater (2003).

JEN1F and B1623R primers specific to *Bartonella* spp. were applied (Table 1). The time and thermal profile were described earlier (Skotarczak and Adamska, 2005). Primers TH-FOR – TH-REV specific to *Babesia* and *Theileria* spp. (Table 1), amplifying 633 and 656 bp fragments, respectively, were applied (Sawczuk et al., 2008).

Nested PCR was applied for the detection of DNA of *Borrelia burgdorferi* s.l. using two primer sets (Table 1.) described earlier (Skotarczak et al., 2005).

All PCR amplifications were carried out in T-gradient (Biometra, Germany) thermal cycler and Peltier Thermal Cycler 200 (MJ Research Inc., USA). PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide. The results of PCR were viewed under UV light and were archived in computer storage using BioCapt software (Vilber Lourmat, France).

PCR products were randomly chosen for sequencing performed in the Sequencing and Oligonucleotides Synthesis Laboratory of Biochemistry and Biophysics Institute of the Polish Academy of Sciences in Warsaw.

The analysis of genetic similarity was carried out on the basis of DNAMAN program (Lynnon Biosoft, Canada). The obtained sequences of the same fragment of genome were compared with each other and with other homologous sequences available in the GenBank.

The non-parametric chi-square test was used to test differences in the occurrence of *I. ricinus* developmental stages and in the prevalence rates of pathogens in the samples. Differences were regarded significant when $P < 0.05$. STATISTICA 6.1 program (Statsoft, Polska) was used for analysis.

RESULTS

I. ricinus females constituted 72.9% of the collected ticks (232 individuals), males – 11.9% (38 individuals) and nymphs – 15.1% (50 individuals). The statistical analysis showed the significant differences in the number of females and males as well as females and nymphs of *I. ricinus* infesting animals, whereas the difference in the number of males and nymphs was not significant.

All females and nymphs were fully engorged with blood. The percentage of infested individuals among roe deer was 44.2% (61/138), the infestation of *C. elaphus* was 40% (20/50), *S. scrofa* 6% (3/50). The comparison of the infestation level of roe deer and red deer showed a lack of significance, whereas the differences in infestation levels between roe deer and wild boars or red deer and wild boars showed to be significant.

Table 1. Primers used for the amplification of DNA of *A. phagocytophilum, Bartonella* spp., *B. burgdorferi* s.l., *Babesia* spp. and *Theileria* spp.

Pathogen	Genetic marker	Sequences of primers	Length of amplicons	References
A. phagocytophilum	*msp2*	MSP2-3F: 5'–CCAGCGTTTAGCAAGATAAGAG–3' MSP2-3R: 5'–GMCCAGTAACAWCATCATAAGC–3'	334 bp	Massung and Slater, 2003
	groESL	PCR: HS1 5'–TGGGCTGGTAMTGAAAT–3' HS6 5'–CCCCGGACAYACCTTC–3'	1 343 bp	Massung and Slater, 2003
		Nested PCR: HS43 5'–ATWGCWAARGAAGCATAGTC–3' HS45 5'–ACTTCACGYYTCATAGAC–3'	480 bp	
Bartonella spp.	ITS	JEN1F: 5'–CTCTTTCTTCAGATGATGATCC–3' B1623R: 5'–AACCRACTGAGCTACAAGCC– 3'	155–290 bp	Maillard et al., 2004
Theileria spp.	18S rDNA	THFOR: 5'-TGACACAGGGAGGTAGTGA-3' THREV: 5'-TCAGCCTTGCGACCATACT- 3'	656 bp	Sawczuk et al., 2008
Babesia spp.	18S rDNA	THFOR: 5'-TGACACAGGGAGGTAGTGA-3' THREV: 5'-TCAGCCTTGCGACCATACT- 3'	633 bp	Sawczuk et al., 2008
Borrelia burgdorferi sensu lato	*fla*	PCR: 132F: 5'-TGGTATGGGAGTTTCTGG-3' 905R: 5'-TCTGTCATTGTAGCATCTTT-3'	774 bp	Wodecka, 2007
		Nested PCR: 220F: 5'-CAGACAACAGAGGGAAAT-3' 823R: 5'-TCAAGTCTATTTTGGAAAGCACC-3'	604 bp	

Two hundred individuals of *I. ricinus*: 19 females (9.5%), 12 males (6%) and 169 nymphs (84.5%) were collected from vegetation in the shooting area of the observed animals. The statistical analysis showed the significance of differences in the number of collected adults and nymphs of *I. ricinus*, whereas the difference in the number of males and females was not significant.

Table 2 shows the occurrence of DNA of *B. burgdorferi* s.l., *A. phagocytophilum*, *Bartonella* spp., *Babesia* spp. and *Theileria* spp. in *C. capreolus, C. elaphus* and *S. scrofa*. The occurrence of DNA of *B. burgdorferi* s.l., *A. phagocytophilum*, *Bartonella* spp., *Babesia* spp. and *Theileria* spp. in *I. ricinus* is shown in Table 3.

The statistical analysis showed that the difference in the prevalence of DNA of *A. phagocytophilum* between ticks collected from roe deer and from red deer was not significant, likewise the difference in the prevalence of DNA of *Babesia* and *Theileria*. The difference in the prevalence of DNA of *Borrelia* between ticks collected from roe deer and from vegetation was significant, whereas the differences in the prevalence of DNA of *Anaplasma*, *Babesia*

Table 2. Occurrence of DNA of *A. phagocytophilum* (An), *B. burgdorferi* s.l. (Bb), *Bartonella* spp. (Br), *Babesia* spp. (Bab), *Theileria* spp. (Th) in tissues of *C. capreolus*, *C. elaphus*, *S. scrofa*

Agent	*n*	An *n* (%)	Bb *n* (%)	Br *n* (%)	Th *n* (%)	Bab *n* (%)	An+Br *n* (%)	An+Bab *n* (%)	An+Th *n* (%)	Br+Bab *n* (%)	Br+Th *n* (%)	An+Br+Th *n* (%)	An+Br+Bab *n* (%)
C. capreolus	138	74 (53.6)	2 (1.4)	59 (42.7)	34 (24.6)	42 (30.4)	5 (3.6)	23 (16.6)	17 (12.3)	16 (11.6)	2 (1.4)	2 (1.4)	–
C. elaphus	50	34 (68)	–	22 (44)	42 (84)	1 (2)	1 (2)	–	14 (28)	–	2 (4)	15 (30)	1 (2)
S. scrofa	50	3 (6)	–	–	–	–	–	–	–	–	–	–	–

and *Theileria* between ticks infesting animals and ticks collected from vegetation were not significant. Significant differences in the occurrence of DNA of *Theileria* and *Babesia* were noted with regard to the investigated tissues of roe deer and red deer. Significant differences between roe deer and red deer (24.6% and 84%, respectively) were found in the occurrence of DNA of *Theileria* as well as DNA of *Babesia* (30.4% and 2%, respectively). Within both investigated species, there were not any significant differences in the frequency of occurrence of *Theileria* and *Babesia* DNA in roe deer, while in red deer these differences were significant (84% and 2%, respectively).

The comparison of obtained sequences with the homological ones available in the GenBank showed that sequences of the gene fragment *msp2* and *groESL* operon being 334 bp and 480 bp in length, respectively, are characteristic of *A. phagocytophilum* and sequences of the uncoding fragment of ITS region are characteristic of *Bartonella schoenbuchensis* (317 bp) and *Bartonella bovis* (198 bp).

From the amplification of the partial ssr RNA gene, a 633 bp product for *Babesia* and a 656 bp product for *Theileria* were sequenced. BLAST search revealed that our *Theileria* sequence (DQ520837) is the most similar (99%) to the sequences of *Theileria* sp. 3185/02 (AY421708) and to the sequences of *Theileria capreoli* (AY726011), whereas in the case of the sequence obtained for *Babesia* from samples taken from *C. capreolus* (DQ520838), the highest similarity (99%) was shown with *Babesia capreoli* (AY726009) and *Babesia divergens* (AY098643).

The sequencing of five amplification products of *fla* gene fragments of *B. burgdorferi* s.l., 604 bp in length, gave three types of sequences with high similarity to European strains of *B. garinii* (99%), *B. afzelii* (100%) and *B. valaisiana* (98%). Two sequences belonging to *B. garinii* and two of *B. afzelii* originated from tissues of *C. capreolus* and *I. ricinus* and *B. valaisiana* only from ticks. All sequences were submitted to the GenBank and their accession numbers were obtained: DQ650336 (*B. garinii*), DQ650334 (*B. afzelii*) and DQ650330 (*B. valaisiana*).

Table 3. Occurrence of DNA of *A. phagocytophilum* (An), *B. burgdorferi* s.l. (Bb), *Bartonella* spp. (Br), *Babesia* spp. (Bab), *Theileria* spp. (Th) in *I. ricinus* collected from *C. capreolus*, *C. elaphus*, *S. scrofa* and from vegetation

I. ricinus from	*n*	An *n* (%)	Bb *n* (%)	Br *n* (%)	Th *n* (%)	Bab *n* (%)	An+Bab *n* (%)	An+Th *n* (%)
C. capreolus	224	28 (12.5)	6 (2.6)	6 (2.6)	12 (5.3)	8 (3.6)	1 (0.4)	2 (0.9)
C. elaphus	89	8 (9)	–	–	5 (5.6)	3 (3.4)	1 (1.1)	–
S. scrofa	7	–	–	–	–	–	–	–
Vegetation	200	11 (5.5)	22 (11)	–	8 (4)	6 (3)	1 (0.5)	–

DISCUSSION

The larvae and nymphs of *I. ricinus* usually feed on small rodents while adult ticks on different bigger mammals. Based on the long feeding (from a few hours to more than one week), the geographical spreading happens together with the host's movement. It seems that there is a certain connection between *B. burgdorferi* s.l. species and some vertebrate hosts. We expected to find such a relation in some of the studied animal species but only two out of all studied samples (238), both belonging to *C. capreolus*, showed the presence of DNA of *Borrelia* and it was *B. garinii.* Thus, none of the studied mammals seems to be important in the enzootic cycle of *B. burgdorferi* s.l. in the studied area although 44.2% (61/138) of *C. capreolus* and 40% (20/50) of *C. elaphus* was infested by *I. ricinus*. The fact that the percentage of PCR+ for the presence of DNA of *B. burgdorferi* s.l. in the population of *I. ricinus* collected from game was significantly lower (2.6%) than among individuals collected from vegetation in the shooting area (11%, the difference statistically significant) may be explained by the fact that the components of the complement system are active also in fed ticks. Thus, roe deer and red deer do not seem to have the reservoir competence for *B. burgdorferi* s.l. in the studied area, however, as a very important host of all three developmental stages of the vector they are very important elements of the enzootic cycle of this bacteria and they also enable the survival as well as the reproduction of *I. ricinus*.

Polin et al. (2004) detected DNA of *A. phagocytophilum* in the isolates from the liver of roe deer and red deer shot in Austria and also in *I. ricinus* collected in the animals' shooting areas. A high prevalence of *A. phagocytophilum* DNA in roe deer and red deer was detected in Slovenia and in roe deer coming from Austria and Czech Republic (Petrovec et al., 2003). Our pilot studies showed the presence of *A. phagocytophilum* DNA in the blood and/or tissues of 44.8% out of 29 individuals of *C. capreolus* shot in the forested areas of Szczecin (Skotarczak and Adamska, 2005), therefore it was decided to continue these studies. Among wild ungulates, the participation of European wild boar (*S. scrofa*) has been considered as a potential reservoir of *A. phagocytophilum*. Petrovec et al. (2003) detected DNA of this pathogen in 14.3% out of 63 individuals shot in the Czech Republic. Hulinska et al. (2002) also obtained similar results from wild boars in the Czech Republic, whereas Polin et al. (2004) did not detect DNA of bacteria in any of 179 wild boars shot in Austria. We also found a low prevalence rate in wild boar proving that this game species is not essential in the maintenance of *A. phagocytophilum* in the studied forest environment.

Our results show a significant role of *C. capreolus* (53.6% PCR+) and *C. elaphus* (68% PCR+) in the life cycle of *A. phagocytophilum* in the studied area of Poland. Moreover, in 45 out of 74 PCR+ *C. capreolus*, a double infection was found, the most often with *Babesia*, and in two cases the infection was triple (*Theileria*, *Anaplasma* and *Bartonella*). In *C. elaphus* the coinfections were double in 15 cases (the most often *A. phagocytophilum* with *Theileria*) and triple in 16 cases (the most often *A. phagocytophilum* with *Theileri*a and *Bartonella*). In the isolates obtained from ticks collected from roe deer and red deer, DNA of *Anaplasma* occurred in 12.5% and 9%, significantly higher than in those obtained from *I. ricinus* collected from vegetation (5.5%). Coinfections occurred in five tick samples altogether.

The present studies showed that *C. capreolus* and *C. elaphus* are essential factors in the circulation of *Bartonella* spp. in the forest ecosystem in North-Western Poland, because the rate of infection was above 40% for both species. However, the coinfections in roe deer were not common: double infections with *A. phagocytophilum* and with *Theileria* spp. occurred in 3.6% (Table 2) and 1.4% (Table 2) and triple infections with these pathogens were found in 1.4% (Table 2). In red deer, the coinfection with *A. phagocytophilum* (2%) (Table 2), with *Theileria* (4%) (Table 2) and triple infection (30%) (Table 2) with these pathogens were also found.

The ticks have not been found as a vector of bacteria from the genus *Bartonella* for a long time, however, regarding the ruminants that are infected more often by ticks than by fleas it is thought that they can play a significant role in the transmission of *Bartonella* spp. (Chang et al., 2001). The presence of *Bartonella* spp. has been confirmed in ticks *Ixodes pacificus* in California (Chang et al., 2001) and *Bartonella henselae* – in *I. ricinus* ticks in Italy and Denmark (Schouls et al., 1999; Sanogo et al., 2003, Halos et al., 2005).

Ticks collected from roe deer in forests surrounding the town of Szczecin belonged to one species only, *I. ricinus*. DNA of *Bartonella* was found in six ticks collected from three individuals of *C. capre-*

olus, however, DNA of bacteria was detected in none of these mammals. It should be concluded that these ticks were not infected while feeding on the animals from which they were collected and the infection was not transmitted to them, either (Maillard et al., 2004).

The comparison of obtained DNA sequences from isolates of the game tissues with the homological ones available in the GenBank has shown that sequences of uncoding fragments of ITS region are characteristic of *B. schoenbuchensis* and *B. bovis*, whereas the isolates from *I. ricinus* have been characteristic only of *B. schoenbuchensis*.

Our preliminary studies on the occurrence of *Babesia* spp. DNA in the tissues of game have shown that roe deer is a potential reservoir for these protozoans (unpublished data). Current studies confirmed the important role of European roe deer in the maintenance of *Babesia* spp. in the studied area, however, the isolation of the pathogen from tissues of vertebrates suggests a susceptibility to the infection but it does not prove the reservoir status. Up to 30% (42/138) of the studied *C. capreolus* were infected, when more than a half (54%) was infected additionally with *A. phagocytophilum*. Among the isolates from tissues of 50 individuals of *C. elaphus*, DNA of *Babesia* occurred only in one case in combination with *Theileria* and *A. phagocytophilum.* All studied isolates from tissues of *S. scrofa* were PCR– for *Babesia*. In the isolates from *I. ricinus* collected from vegetation and those infesting roe deer and red deer, the prevalence of DNA of these protozoans was comparably low (about 3%). The isolates from ticks infesting wild boars did not show the presence of *Babesia* as a result of their small number, because the fur of these animals could reduce the infestation effectively. Sequencing of the ssu rDNA fragments showed the highest similarity with *B. capreoli* and *B. divergens*. Currently, morphological, serological and molecular data do not allow to differentiate between these two species (Garcia-Sanmartin et al., 2007).

Theileria spp. make a large group of pathogenic microorganisms parasitising in the mammals' white blood cells and erythrocytes. The infections caused by protozoans cause huge losses (high death rate) among animals that are subjected to diseases. At least a dozen of species pathogenic to a wide spectrum of ruminants can be distinguished. Parasites from the genus *Theileria* occur mainly in the areas of Africa and in the Middle East, in cattle, buffalos and antelopes (Chae et al., 1999; Maxia et al., 1999; Morzaria et al., 2000). Our pilot studies (Sawczuk et al., 2008) and current molecular ones prove the occurrence of species from the genus *Theileria* in the population of roe deer and red deer in the area of the north-western part of Poland. The latter seems to be particularly competent in the maintenance of *Theileria*, because up to 84% of studied isolates were PCR+, 38% showed triple coinfection and 35% double coinfection. Whereas the isolates obtained from ticks infesting these animals showed the presence of *Theileria* DNA at a small percentage (5.3 and 5.6%), not much higher than from *I. ricinus* collected from vegetation in the animals' shooting area (4%). Sequencing of the ssu rDNA revealed the highest similarity to *T. capreoli* isolated from the tissues of red deer from Spain.

The occurrence of coinfections of tick-borne pathogens concerns also farming and domestic animals. Magnarelli et al. (2005) studied the presence of antibodies in cats living in areas infested by *I. scapularis* ticks and exposed to *B. burgdorferi* and *A phagocytophilum.* Fifteen (16%) sera had antibodies against both pathogens, but most cats appeared healthy.

However, what our studies show that not only the exposition to ticks but also potentially the contact with hunted animals may be a source of tick-borne infections and it poses a threat especially to occupational groups having contact with deer. Our earlier studies (Niscigorska et al., 2003) as well as many others concerning forestry workers have shown that this is a high-risk group for tick-borne pathogens. Blood samples of over four thousand forestry workers in the State of Baden-Wuerttemberg, southwestern Germany, were tested for the presence of antibodies against tick-borne agents in various areas (Oehme et al., 2002). The human seroprevalence rates of antibodies to *B. burgdorferi* s.l. ranged from 18% to 52%, and for *Ehrlichia* spp. from 5% to 16% in various counties of the state.

CONCLUSIONS

The studies show that *C. capreolus* and *C. elaphus* can play a very essential role in the life cycle of *A. phagocytophilum*, two species of *Bartonella*, *Babesia divergens* and *Theileria* spp. Whereas *S. scrofa* occurring in the investigated area does not perform such a role. Moreover, in the isolates of DNA obtained from tissues of *C. capreolus* up

to 63.5% of them showed double coinfection and in three isolates even triple infection was identified. In the tissues of *C. elaphus* the infections were triple in up to 38% of individuals, double in 40% and single in 84%. The population of *I. ricinus* in the area of Wkrzanska Forest is a vector for *A. phagocytophilum*, *B. burgdorferi* s.l., *Babesia* spp. and *Theileria* spp., but it does not fundamentally participate in the transmission of the bacterium from the genus *Bartonella*.

These studies are pioneer ones in our country and rare in the world in the range of comprehensive analysis of biology of important pathogens transmitted by ticks. Results of these studies have not only a cognitive value but also they are useful for prophylaxis of tick-borne diseases. They indicate the high level of occupational risk for hunters, forestry workers and other occupational groups having contact with deer and possibility of infection during the tick bite.

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