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Blood DNA Analysis for *Ehrlichia (Anaplasma) phagocytophila* and *Babesia* spp. of Dogs from Northern Poland

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Abstract

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The most common pathogen transmitted by the tick *Ixodes ricinus* is the spirochete *Borrelia burgdorferi*, rarely *Ehrlichia* bacteria and protozoans from the *Babesia* genus. The aim of this paper was determine if infested to ticks dogs are a reservoir for *E. phagocytophila* and *Babesia* spp. and examine the possibility of coinfection. Canine blood was sampled, part of the material originated from dogs exhibiting symptoms of borreliosis. In an earlier study, the samples were screened for DNA from *B. burgdorferi* sensu lato. In order to screen for *E. phagocytophila* and *Babesia* spp. DNA, a PCR-based method was used with the following primers: EHR521/EHR747 for *Ehrlichia* and FOR1/REV1 for *Babesia*. In 192 samples only two contained *E. phagocytophila* DNA. One of these samples originated from a healthy canine, the other from an individual with symptoms of borreliosis. The examined samples were not positive for *Babesia spp* DNA. Coinfection was not discovered. The low level of *E. phagocytophila* infection may indicate that the domestic dog is not a reservoir for *Ehrlichia* and *Babesia* in Szczecin and northwestern Poland. Moreover, this area does not have populations of the brown dog tick (*Rhipicephalus sanguineus*) or *Dermacentor reticulatus* - both of which are vectors of *E. canis* and *B. canis* and commonly induce ehrlichiosis and babesiosis in canines.

Ehrlichia (Anaplasma) phagocytophila, Babesia spp. d*ogs, PCR*

Infections caused by the spirochete *Borrelia burgdorferi* sensu lato may be accompanied by other microorganisms, such as *Ehrlichia* and *Babesia*. Recently, reports of coinfection with multiple tick-borne organisms in humans and dogs have been published (Hofmeister et al. 1998; Krause 1996, 2002; Kordick et al. 1999; Skotarczak et al. in press).

Ehrlichia canis is a widely distributed species and commonly occurs in canines (McDade 1990; Rikihisa 1991). Other *Ehrlichia* species, infrequently found in dogs, include *E. platys* (Chang and Pan 1996; Sainz et al. 1999), *E. ewingii* (Goodman et al. 2003) and the HGE agent, currently identified with *E. phagocytophila* (Egenvall et al. 2000; Magnarelli et al. 1997, 2001). Aetiological factors in canine babesiosis are comprised of two species: *B. gibsoni* and *B. canis* with 3 subspecies, additionally a third uncommon agent called *B. microti*-like has been documented (Camacho et al. 2002; Zahler et al. 2000). The prevalence of the pathogens is largely dependent on the distribution of their vectors. Tick vectors for *E. canis* (*Rhipicephalus sanguineus)* and *B. canis* (*R. sanguineus* and *Dermacentor reticulatus)* do not inhabit northwestern Poland. However, a common tick species in Poland and northern Europe is *Ixodes ricinus*.

Research involving PCR amplification of pathogen DNA in *I. ricinus* collected from forested localities in Szczecin and northwestern Poland has revealed the presence of *B. burgdorferi* s.l. (from 0.3 to 15.7%) in all life stages of the tick over a period of several years (Skotarczak and Wodecka 1998, 2000). Other studies using ticks from the same localities have informed of finding DNA from *B. microti* and *B. divergens* and also the

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human granulocytic ehrlichiosis agent (*E. phagocytophila*), first as single tick infections (Skotarczak and Cichocka 2001a, 2001b; Skotarczak and Rymaszewska 2001; Skotarczak et al. 2003a) and later as double and triple coinfections (Skotarczak et al. 2002, 2003b). A similar study has shown the presence of the human granulocytic ehrlichiosis agent and also the aetiological factor inducing Lyme borreliosis in *I. ricinus* from North of Poland (Stańczak et al. 2002).

The results of studies of sera and the detection of spirochete DNA in canine blood show that dogs naturally exposed to ticks in Szczecin and neighboring areas, endemic for *I. ricinus* and borrelias, are a reservoir for *B. burgdorferi* s.l. (Skotarczak and Wodecka 2003; Skotarczak et al. in press). In the present study, the same population of dogs was been screened for the presence of *E. phagocytophila* and *Babesia* spp. DNA in order to investigate the possibility of coinfection and to evaluate the status of dogs from northwestern Poland as a potential reservoir for these pathogens.

Materials and Methods

Blood samples were taken from 192 dogs naturally exposed to *I. ricinus* ticks. Canine borreliosis was suspected by veterinarians in 92 of them. Remaining100 individuals of different breeds were from Szczecin's dog shelter and were healthy. The age (from 0.5 to 14 years), sex and breed of the individuals was noted, and also infestation by ticks in the past and present. Blood used in the serological study and PCR's was sampled immediately after periods of pronounced activity of ticks, i.e. from June to the middle of July and from September to the middle of October.

DNA was isolated from blood samples using the QIAamp® DNA Mini Kit (Qiagen, Germany) according to the attached protocol. In order to amplify a fragment of the 16S rRNA gene of *E. phagocytophila*, the primers EHR521 and EHR747 were used, which give a product of 247 bp (Guy et al. 1998). As a positive control we used HGE agent DNA from a culture of HL 60 cells, MRL Diagnostics (Stańczak et al. 2002). PCR amplifications involved an initial denaturation step at 94 °C for 2 min; 35 cycles of denaturation at 94 °C - 30 seconds, primer annealment 64 °C - 45 s, extension at 72 °C -30 s, final extension at 72 °C -5 min. Reagents used in the reactions were from Fermentas (Lithuania).

In order to detect protozoans from the *Babesia* genus by the PCR method we used the primers FOR1 (5'TGT-CTT-AAA-GAT-TAA-GCC-ATG-CAT-GT3') and REV1 (3'CTT-CTT-TTA-AGT-GAT-AAG-GTT-CAC-AA5') which amplify a fragment of the 18S rRNA gene and are widely specific for the genus with a product of 1700 bp. PCR amplifications involved an initial denaturation step at 94 °C for 3 min; 35 cycles of denaturation at 94 °C - 45 s, primer annealment 65°C - 45 s (FOR1 and REV1), elongation at 72 °C - 1 min and 30 s, final elongation at 72 °C -7 min. The product of the first reaction was diluted 20 × and reamplified.

PCR's also incorporated negative and positive controls. Reactions were performed in a T-gradient (Biometria, Germany) thermocycler, reactions for each sample were repeated. The products were separated in 2% (HGE agent) or 1.5% agarose (*Babesia* spp.) (ICN, USA) with ethidium bromide (Sigma-Aldrich, Germany) at 80 V for 1.5 h. In order to evaluate the size of the amplified fragments, a molecular mass marker (Polgen) with bands between 501 and 110 bp was used for the HGE agent or SmartLadder, Bioline, Germany for the *Babesia* spp. The PCR products were visualized in UV light and computer-archived with the BioCapt program (Vilber Lourmat, France) which analyses the image from the transilluminator. A positive result in the PCR's was inferred by the presence of bands of the appropriate molecular mass.

Results and Discussion

In 192 blood samples only two (1.0 %) were found to be positive by PCR for *E. phagocytophila* DNA. One of the infected samples came from a healthy dog, the other from a dog with symptoms of borreliosis. The two infected individuals did not have *B. burgdorferi* s. l. DNA in their blood. The presence of *Babesia* protozoans DNA was not detected in none of 192 blood samples.

Both humans and dogs infected with multiple tick-borne agents can experience a wide range of clinical manifestations (Farwell et al. 1982; Boustani and Gelfand 1996; Harrus et al. 1997). However, the consequences of co-infection have not been well established in either, compared with infection by one agent only (Kordick et al. 1999).

Ehrlichiosis is a disease affecting many bodily systems and can have a mild or severe course, partly determined by the species of *Ehrlichia* and type of infected blood cells. The state of the immunological system of the host is of utmost importance. Serious infections

result in long-term complications, mainly haemorrhage and secondary infections, often leading to death.

In Europe the most widely distributed species of *Ehrlichia* is the HGE agent infecting mostly humans. It belongs to one genogroup with *E. equi* and *E. phagocytophila* and induces granulocytic ehrlichiosis (Rikihisa 1997). In 2001, Dumler and colleagues proposed to combine the mentioned species into one species, *Anaplasma phagocytophila*, on the basis of similarities in the *groESL* gene, the 16S rRNA gene of the small ribosomal subunit and genes coding for surface proteins. The primers used in our study were complementary to the 16S rRNA gene for all three species.

Ehrlichias from the *E. phagocytophila* genogroup were found in canine blood by serological methods (Egenvall et al. 2000; Magnarelli et al. 1997, 2001) and PCR (Breitschwerdt et al. 1998; Greig et al. 1996; Johansson et al. 1995; Kordick et al. 1999)

Clinical diagnosis of canine granulocytic ehrlichiosis (CGE) is difficult. Two distinct clinical syndromes, including chronic, moderate to severe anemia and polyarthritis are associated with CGE (Goldman et al. 1998). Clinical signs are nonspecific and include fever, lethargy, anorexia, vomiting, and diarrhoea.

The dog from the group of healthy individuals, naturally exposed to *I. ricinus*, whose blood contained *E. phagocytophila* (*A. phagocytophila*) DNA did not exhibit symptoms of the active form of this infection. Similarly, the PCR+ individual from the group with diagnosed borreliosis did not exhibit symptoms of ehrlichiosis, only arthritis and a high level of *B. burgdorferi* s. l. antigens characteristic of the former disease. The ehrlichiosis infection was probably in a phase before ehrlichiosis proliferation.

Protozoans from the genus *Babesia* have been found in canine blood by using a PCR-based method with species-specific primers amplifying different fragments of the 16S rRNA and 18S rDNA genes (Zahler et al. 1998; Carret et al. 1999; Camacho et al. 2002). We constructed a pair of genus-specific primers complementary to the 18S rRNA gene. These primers can amplify DNA from *B. canis canis, B. canis vogeli, B. gibsoni, B. divergens, B. microti* (UO9833, AB032434, AF188001 "spanish dog", AF231349 "strain Berlin", AB071177-"strain Munch", AB085191-"strain Hannover"), *B. odocoilei, B. rodhaini* and *Theileria* spp. The negative results for the presence of *Babesia* DNA in canine blood is probably the result of the lack of appropriate vectors for *Babesia* species in the studied area.

DNA *Ehrlichia (Anaplasma) phagocytophila* a DNA *Babesia* spp. *v* krvi psů v severním Polsku

Nejčastější patogeny přenášené klíšťetem *Ixodes ricinus* je spirocheta *Borrelia burgdorferi,* vzácně bakterie *Ehrlichia* a protozoa rodu *Babesia*. Cílem této studie bylo zjistit, zda jsou psi nakažení klíštětem - rezervoárem *E. phagocytophila* a *Babesia* spp. a prověřit možnost infekce oběma patogeny zároveň. Byla testována krev psů, přičemž část vzorků pocházela od psů se symptomy boreliózy. Při studii byla ve vzorcích nejprve zjišťována přítomnost DNA *B. burgdorferi*. Za účelem průkazu DNA *E. phagocytophila* a *Babesia* spp. byla použita metoda PCR s využitím následujících primerů: EHR521/EHR747 pro ehrlichie a FOR1/REV1 pro babesie. Ze 192 vzorků jen dva obsahovaly DNA *E. phagocytophila.* Jeden z těchto vzorků byl odebrán zdravému psovi, ostatní od jedinců se symptomy boreliózy. Ve zkoumaných vzorcích nebyla zjištěna přítomnost DNA *Babesia* spp. Infekce oběma patogeny zároveň nebyla zaznamenána. Nízká prevalence výskytu *E. phagocytophila* může indikovat, že pes domácí není rezervoárem *Ehrlichia* a *Babesia* ve Štětíně a severozápadním Polsku. Navíc se v této oblasti nevyskytují klíšťata *Rhipicephalus sanguineus* či

Dermacentor reticulatus, která jsou vektory *E. canis* a *B. canis* a často způsobují ehrlichiózu a babesiózu psů.

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