

## DETECTABILITY OF TICK-BORNE AGENTS DNA IN THE BLOOD OF DOGS, UNDERGOING TREATMENT FOR BORRELIOSIS\*

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Wodecka B, Rymaszewska A, Sawczuk M, Skotarczak B: Detectability of tick-borne agents DNA in the blood of dogs, undergoing treatment for borreliosis. *Ann Agric Environ Med* 2009, **16**, 9–14.

**Abstract:** In the wake of controversies surrounding the usefulness of PCR in the diagnostics of borreliosis, the aim of the presented study was to monitor the presence of *B. burgdorferi* s.l. in dogs with clinical borreliosis in the course of relevant treatment. The monitoring was based on detecting borrelia's DNA before- (study I), during- (study II), and after completion of the therapy (study III). In addition, to rule out possible co-infections, the dogs' blood was examined for the presence of anaplasma, babesia and rickettsia. Blood samples taken from 11 dogs, with clinically detected borreliosis, were used for obtaining DNA for PCR. Positive results of PCR, with primers complementary to the *fla* gene, indicating the presence of DNA of *B. burgdorferi* s.l., were noted, in study I, in the blood of 7 dogs (63.6% dogs), in study II in 3 dogs, while in study III all blood samples were negative. In 6 out of 7 PCR+, the first study was carried out during week 1. Therefore, the PCR method is useful for monitoring early canine infections with spirochetes *B. burgdorferi* s.l. In all positive samples, subjected to PCR-RFLP, it was the case of a single genospecies, i.e. *B. burgdorferi* sensu stricto. Studies for the presence of DNA of *Babesia* sp., as well as DNA of *Rickettsia helvetica*, were negative in all samples. *Anaplasma phagocytophilum* DNA was detected in the blood of a single dog, and only in study I. The same dog also proved positive for the presence of borrelia DNA. Co-occurrence of both pathogens did not disturb the clinical picture of borreliosis, and the administered treatment was also effective for the mixed infection.

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**Key words:** dogs, tick-borne agents, PCR, PCR-RFLP, borreliosis, anaplasmosis.

### INTRODUCTION

Progressive development of molecular biology methods enables prompt detection of pathogens in humans as well as in domestic- and farm animals. It also allows examination of potential vectors of those pathogens and surveying their reservoirs. Currently, a total of 6 tick-borne pathogens of humans and animals have been detected in Poland, known to be transmitted by the common European tick, *Ixodes ricinus* (i.e. *Borrelia burgdorferi* s.l., *Babesia microti*, *Babesia divergens*, *Anaplasma phagocytophilum*, *Rickettsia* sp., and *Bartonella* sp.) [31, 32, 34, 42]. Lyme borreliosis is caused by spirochetes representing the collective species of *Borrelia burgdorferi* sensu lato, transmitted

by ticks of the genus *Ixodes*, and affecting numerous vertebrates because of its wide host specificity.

Under natural conditions, the clinical form of borreliosis can be diagnosed in species representing non-sylvatic habitats, i.e. humans, dogs, cats, and cows, but this problem becomes increasingly important for dogs and humans [1, 19, 21, 22, 23, 24, 40]. While human borreliosis has been extensively studied, very little is still known about canine borreliosis. In dogs, this disease assumes, most often, arthritic form, affecting joints of limbs, most frequently those of carpus or tarsus. One or two joints become

\*A part of the results were presented at the scientific conference "Mastering of effective methods for detection of animal-borne diseases and food poisoning, and the assessment of relevant threats" Puławy, 13 May 2005.

swollen and groin- and prescapular lymph nodes enlarge. The above symptoms are associated with malaise (expressed as elevated body temperature, loss of appetite, and fatigue) and lameness ensuing within a few days. Myocarditis rarely develops in the course of canine borreliosis; however, in older dogs a renal form and neurological dysfunction appear [4, 5, 9, 11, 16].

Canine borreliosis was first described in the 1980s in the USA [14, 17, 18], since when it has been detected in almost all European countries. To assess the relevant epidemiological situation in Poland we studied the presence of DNA and antibodies against *Borrelia burgdorferi* s.l. in dogs naturally exposed to ticks [33, 37, 38, 41]. We studied the immune response of healthy as well as borreliosis-suspected dogs. Moreover, we were detecting DNA of *B. burgdorferi* s.l. genospecies and DNA of *Anaplasma phagocytophilum* in the blood of these dogs [29, 30, 39]. The presented work also includes an attempt to monitor *B. burgdorferi* s.l. infection in the blood of dogs with borreliosis symptoms before-, during-, and after completion of the therapy. In addition, to detect possible co-infections, the blood of these dogs was screened for the presence of the DNA of anaplasma, babesia and rickettsia.

## MATERIAL AND METHODS

DNA for PCR was isolated from the blood samples taken from 11 dogs of different breeds. The dogs were delivered to the Veterinary Clinic, where veterinary surgeons detected the clinical form of borreliosis (study results presented in Table 1). Blood samples (1.5 ml), were collected three times, i.e. before the commencement of treatment (doxycyclinum and ampicylinum) or on day 2 or 3 of the therapy (study I), after three weeks of therapy (study II), and after completion of the therapy (study III). The studies were carried out at the time of the highest activity of ticks, i.e. from April–September 2004 (dogs nr 1–7, Tab. 1) and in the year 2005 (dogs nr 8–11, Tab. 1). The investigated dogs (6 males, 5 females) were aged from 3 to 11 years. According to the information gathered by the veterinarians, all dogs were previously often infected by ticks, in the forested areas surrounding the city of Szczecin. The owners of several dogs provided the date of last contact with a tick (Tab. 1). Information about borreliosis treatment in the past and the date of blood collecting, which agree with the date of first PCR study is also given shown in Table 1.

DNA was isolated from the blood using MasterPure™ DNA Purification Kit (Epicentre, Madison, WI, USA) and stored at  $-70^{\circ}\text{C}$  until analysed.

**Detection of DNA of *B. burgdorferi* s.l. using PCR- and nested PCR techniques.** There were two markers for detection of the DNA of *B. burgdorferi* s.l.: *rrs* gene, encoding rRNA of the small ribosomal subunit, and *fla* gene, encoding flagellar protein, flagellin. The former gene (*rrs*) was detected using primers SC1 and SC2, yielding product

of 325 bp, and developed by Marconi and Garon [23]. The latter gene (*fla*) was detected with the aid of nested PCR technique with 2 pairs of primers developed, by the present authors, using a computer software DNAMAN 5.2.9 (Lynnon Biosoft, Canada). The first reaction was based on primers 132f (5'-TGGTATGGGAGTTTCTGG-3') and 905r (5'-TCTGTCATTGTAGCATCTTT-3') giving a product of 774 bp, while the second reaction involved primers 220f (5'-CAGACAACAGAGGGAAAT-3') and 824 (5'-TCAAGTCTATTTTGGAAAGCACC-3') yielding an amplification product of 605 bp. The 10- $\mu\text{l}$  reaction mixture contained 0.25 U of polymerase Taq DNA (Qiagen, Germany), Tris-HCL (pH 8.7 at  $20^{\circ}\text{C}$ ), KCl,  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 mM  $\text{MgCl}_2$ , 75  $\mu\text{M}$  of each of triphosphate deoxyribonucleotides, and 2 pmol of each of the primer pairs, i.e. SC1 and SC2, 132f and 905r, or 220f and 824 r. In reaction with primers SC1 and SC2, and 132f and 905r, the matrix was 1  $\mu\text{l}$  of DNA suspension isolated from the blood, while in reaction with primers 220f and 824r, the matrix was 1  $\mu\text{l}$  of 10-times diluted post reaction mixture with primers 132f and 905r. The positive control was the DNA of strain Bo-148c/2 of *B. burgdorferi* s.s. (kindly provided by Dr J. Stańczak). The negative control was sterile water. The reaction parameters were as follows: initial denaturation at  $94^{\circ}\text{C}$  for 10 min, 40 cycles involving denaturation at  $94^{\circ}\text{C}$  for 30 s, attachment of primers at  $50^{\circ}\text{C}$  (SC1 and SC2 and 132f and 905r) or at  $54^{\circ}\text{C}$  (220f and 824r) for 45 s, and elongation of DNA chain at  $72^{\circ}\text{C}$  for 1 min, and also final elongation for 7 min. The reaction products were separated on 2% ethidium bromide-stained gel (Sigma-Aldrich, Germany) in TBE buffer (0.089 M Tris, 0.089 M of boric acid, 0.002 M EDTA) and saved in a computer using BioCapt software (Vilber Lourmat, France).

**Identification of genospecies of *B. burgdorferi* s.l. using PCR-RFLP.** DNA amplified using primers 220f and 824r was digested with a restrictive enzyme *DdeI* (Sigma-Aldrich), recognizing the sequence CTNAG. The digestion products were separated on 2% agarose gel, and their digitized images were saved in a computer.

**Sequencing *fla* gene.** To confirm the genospecies identity of the acquired products of nested PCR and PCR-RFLP, the fragments of *fla* gene, obtained with primers 220f and 824r, were sequenced at the Institute of Biochemistry and Biophysics of the Polish Academy of Science (Warsaw). The sequences obtained were compared with the sequences of the reference strains at the GenBank, using DNAMAN software.

**DNA of *Anaplasma phagocytophilum*.** DNA of *Anaplasma phagocytophilum* was detected with a PCR technique, using two pairs of primers: *msp3f/msp3r* complementary to the *msp2* gene fragment [15] (product of 334 bp) and primers 16S EHR.D/16S EHR.R complementary to the 16S rRNA gene fragment (product of 345 bp) [3].

**Table 1.** Sex, breed, age and some symptoms of borreliosis in the examined dogs; date of examination, and results of first and second PCR study showing the presence of *B. burgdorferi* s.l. DNA. \*PCR+ for DNA of *A. phagocytophilum*.

No. of dog/ No. of study	Breed	Age	Sex	Body temp.	Arthritic pains/ swelling	Lymph nodes enlargement	Loss of weight/ appetite	Apathy	Infestation with <i>I. ricinus</i>	Date of 1 <sup>st</sup> examination	PCR I	PCR II
1/I	Golden retriever	4.5 years	male	39.8	-/+	Left submandibular	+/+	+	26.05	2.06.	+	+
1/II				38.3	-/-	-	-/-	-	-	23.06.	-	-
2/I	Golden retriever	3 years	male	39	+/-	L & R submandibular	+/+	+	26.05	2.06.	+*	-
2/II				38.6	+/-	-	+/+	+	-	23.06.	+	+
2/III				38	+/-	-	+/+	-	-	12.07.	-	-
3/I	mastif	3.5 years	female	38.9	+/+	R underarm	+/+	+	+	23.06.	+	-
3/II				37.8	-/-	-	+/+	-	-	12.07.	-	-
4/I	Shar-pei	8 months	male	40.2	-/+	-	-/+	+	+	23.06.	-	-
5/I	Collie	11 years	male	38.5	+/-	-	+/+	-	24.05.	28.06.	-	-
6/I	German shepherd	6 years	female	38.5	-/-	-	-/-	+	+	30.06.	-	-
7/I	Crossbreed	4 years	female	39	+/+	-	-/-	+	30.06	12.07.	-	-
8/I	rottweiler	6.5 years	male	38.5	-/-	-	+/+	+	+	12.07.	-	-
9/I	German shepherd	7.5 years	female	39	+/+	-	+/+	+	+	16.07.	+	+
9/II				38	-/-	-	-/+	-	-	6.08.	-	-
10/I	retriever	4 years	male	39.2	+/+	R underarm	+/+	+	10.07	16.07.	+	-
10/II				38.7	+/-	-	-/-	+	-	4.08.	+	-
10/III				37.8	-/-	-	-/+	-	-	30.08.	-	-
11/I	Dachshund	3 years	female	38.8	+/-	-	-/+	-	+	4.08.	-	-

The temperature-time profiles, the composition of reaction mixtures, and the electrophoresis conditions were similar to those described by afore-mentioned authors.

**DNA of *Babesia* spp.** DNA of *Babesia* spp. was detected with a PCR technique using primers PIRO-A and PIRO-B complementary to 18S rRNA gene fragment of the genus *Babesia* [2]. The temperature-time profile used was as follows: initial denaturation at 94°C for 2 min, 40 cycles involving denaturation at 94°C for 45 s, attachment of primers at 58°C for 45 s, and elongation of DNA chain at 72°C for 1 min, and final elongation at 72°C for 3 min. The reaction products were separated at 2% agarose gel stained with ethidium bromide.

**DNA of *Rickettsia helvetica*.** To detect the presence of *Rickettsia helvetica* DNA, the *gltA* gene-specific RpCS877 and RpCS1258 primers were used [26]. The amplification product should be the length for 382 bp. The temperature-

time profiles, the composition of reaction mixtures, and electrophoresis conditions were similar to those described by above-mentioned authors.

## RESULTS

The positive result of PCR with primers complementary to the *fla* gene, indicating the presence of DNA of *B. burgdorferi* s.l., was recorded in the blood of 7 dogs in study I, which constituted 38.8% of the 18 dogs examined. Each of these dogs were examined (study I) before the administration of antibiotic, and each of them was diagnosed (by a veterinarian) with elevated body temperature, symptoms of arthritic pain and/or arthritic swelling, loss of appetite, and apathy (Tab. 1). In 6 out of 7 PCR+, the dates of dogs' and ticks' contact were recorded and the first PCR study carried out during week 1. Study II revealed the presence of borrelia DNA only in three blood samples, collected from dogs proved to be PCR-positive in study I. In Study

III, all blood samples were negative for the presence of borrelia DNA. The same studies, carried out with primers complementary to the *rrs* gene, were positive only in study I and only for the same samples that were positive with the first pair of primers. All positive samples, subjected to PCR-RFLP turned out to represent a single genospecies i.e. *B. burgdorferi* sensu stricto.

The sequencing of the positive samples confirmed the genospecies identification performed with PCR-RFLP. The sequenced samples demonstrated an identical sequence and its similarity to the sequence of the reference strain GeHo (*B. burgdorferi* s.s. isolated from Germany) was 99.5%. One sequence was sent to the GenBank (accession number: DQ016625).

A positive PCR result for the presence of DNA of *Anaplasma phagocytophilum* was recorded only for the blood sample of borreliosis-suspected dog, and only in study I. The sample was also borrelia positive. Borreliosis in this dog was diagnosed based on such symptoms as elevated body temperature, enlargement of lymph nodes (left-and right submaxillary), loss of appetite, and apathy. All samples were negative for the presence of DNA of *Babesia* and *R. helvetica*.

## DISCUSSION

Chang *et al.* [4] used different genes as genetic markers for detection of DNA of *B. burgdorferi* s.l. and obtained different results from the same biological materials. Therefore, they suggested the usage of at least two pairs of primers for an objective assessment of the results in canine borreliosis. Our own studies on the efficacy of two different PCR protocols, applied to the blood of borreliosis-suspected patients [36], have also confirmed the need for two sets of primers, which we followed in this study. DNA amplification with primers complementary to the nucleotide sequence of the *fla* gene proved to be more sensitive, this was because it permitted detection of borrelia DNA as late as three weeks after the start of the therapy.

Molecular methods implemented for detection and identification of microorganisms are aimed at improvement of medical- and veterinary diagnostics, but they also enable monitoring of the treatment (recovery) process. The usefulness, however, of the PCR technique for monitoring of the borreliosis therapy, has not been conclusively confirmed. There have been reports on negative PCR results, despite persisting disease symptoms, as well as on positive results obtained after completion of an antibiotic treatment [12]. Our earlier blood studies of patients with confirmed borreliosis revealed that antibiotic therapy at early phase of borreliosis does not decrease the PCR sensitivity, because we detected the presence of DNA of *B. burgdorferi* in over 70% of patients prior to antibiotic therapy or in within the commenced therapy [13]. Also in the group of 14 patients, who started to use antibiotics 4–5 days before being examined, 12 (85.7%) had DNA of the spirochete in

their blood. After four-week antibiotic therapy, as many as 52% (45/86) patients demonstrated a positive PCR. These facts may raise questions about the dosage used or treatment period in terms of their efficacy [13].

On the other hand, the presented PCR results of 7 out of 11 dogs (38.8%) were positive before the treatment and 3 were positive after three weeks therapy. No PCR-positive results after the completion of the treatment indicated the efficacy of the treatment administered. In 6 out of 7 PCR+, the first study was carried out during week 1. Therefore, the PCR method is useful for monitoring an early infection of dogs with spirochete *B. burgdorferi* s.l. No PCR-positive results occurred after the completion of the treatment, thus indicating the efficacy of the treatment prescribed.

It must be emphasized, however, that spirochetemia of the blood is a temporary phenomenon, and therefore a high level of detection is possible in a short period of primary infection only [6, 8], and this must be kept in mind while considering PCR methods in borreliosis diagnostics. Our earlier studies of the presence of DNA of borrelia in the blood of borreliosis-suspected dogs [38] demonstrated that the majority of dogs with confirmed DNA of the spirochete showed clinical symptoms, typical for an early infection. On the other hand, the presence of the detected sequences do not necessarily constitute a proof of the disease, because PCR method does not permit a discrimination between DNA of a live organism, a dead organism, or DNA from a disintegrated bacterium cell. In the synovial fluid in patients with untreated arthritic, the plasmid DNA was detectable, in contrast to rare cases of detection of chromosomal DNA. There is growing evidence that positive PCR results are most often related to an active form of the disease. After a well-conducted therapy, the PCR results are most often negative, and vice versa the therapy failure, expressed in the form of persisting disease symptoms, is usually associated with positive PCR results [28].

There has hitherto been little information on the pathogenic role of *B. burgdorferi* s.l. in dogs. In the USA, the role of the one genospecies (*B. burgdorferi* s.s.) dominant in North America [1] has been ascertained. In Europe, in naturally exposed dogs, the principal pathogens are *B. burgdorferi* s.s. and *B. garinii* with possible infections with the four most important genospecies of variable tropism [10, 11]. Our PCR-RFLP revealed a single genospecies, namely, *B. burgdorferi* s.s. In all PCR samples obtained through amplification of the *fla* gene with primers 220f and 824r we obtained only one band pattern, and this was consistent with the pattern obtained through sequence analysis of the reference *fla* gene of the isolate *B. burgdorferi* s.s. GeHo (X15660) taken from the GenBank. We obtained the same results in the course of earlier studies of DNA isolated from the blood of dogs with confirmed borreliosis [39].

Spirochetes *Borrelia burgdorferi* are the most common pathogen transmitted by ticks, *Ixodes ricinus*. Also, the common tick is a vector of, e.g. bacteria of the genera *Erlichia* and *Anaplasma* and protozoans of the genus

*Babesia*. In ticks collected from the recreational areas of the city of Szczecin and other areas of north-western Poland we detected DNA of the above-mentioned pathogens, and we also revealed cases of double or triple co-infections [34, 35]. Therefore, it would be reasonable to also expect mixed infections in vertebrate hosts of those pathogens. The most common *Ehrlichia* species, widely distributed is *E. canis* [25, 27]. Its vector is the tick, *Ripicephalus sanguineus*, not occurring in our part of Europe. Another, less frequently occurring in dogs, is the HE factor, presently identified as *Anaplasma phagocytophilum* [7, 20] transmitted by *I. ricinus*. In earlier studies, we detected DNA of this anaplasma species in a clinically healthy dog, and in a dog with clinically confirmed borreliosis [29, 30]. The dog with presently detected DNA of *A. phagocytophilum* did not show any clinical signs of active infection. The positive PCR for DNA of *Anaplasma phagocytophilum* was present in the blood of this dog only in study I, which was also positive for the presence of borrelia DNA. Therefore, the co-existence of the detected pathogens did not disturb the clinical picture of borreliosis, and the administered therapy was also effective in this mixed infection. In cases of infections inflicted by parasitic protozoans *Babesia* spp. the principal factor of canine babesiosis is *B. canis*. In Poland, this disease has been detected since the 1960s in eastern regions, particularly in the vicinity of Warsaw and the Lublin Heights. The only tick species naturally occurring in Poland and transmitting *B. canis* is *Dermacentor reticulatus*, which is known to occur solely east of the Vistula River. There have been no records of *I. ricinus* acting as an alternative vector for *B. canis*. Therefore, the lack of *D. reticulatus* in north-western Poland efficiently precludes any focal outbreaks of babesiosis there. In the course of this study, DNA of *B. canis* has not been found in any blood sample. Similarly, we did not detect any genetic material of other species of *Babesia*, known to be transmitted by *I. ricinus*, namely, *B. microti* and *B. divergens*, detected in other studies in Polish Western Pomerania [32, 35]. This finding confirms the assumption that dogs do not serve as reservoirs for the two afore-mentioned species.

### Acknowledgements

This work was financed by the State Committee for Scientific Research (KBN), Poland (Grant No. PCZ 014-26).

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