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ORIGINAL ARTICLE

The first evidence for vertical transmission of *Babesia canis* in a litter of Central Asian Shepherd dogs

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Abstract

Introduction and objective. Tick-borne infections constitute an increasing health problem in dogs and may lead to death, especially in young or elderly individuals. Canine babesiosis constitutes a serious health problem in dogs worldwide. The aim of the study was to verify the probability of vertical transmission of *Babesia canis* between the bitch and the pups.

Materials and methods. In Autumn 2011, cases of babesiosis were diagnosed in a litter of 6-week-old puppies of a Central Asian Shepherd dog. Immediately following the first case of infection, blood samples were collected from all the pups in the litter (n=10) and from the female. Detection of *Babesia* infection was performed by molecular and microscopical techniques.

Results. The presence of *B. canis* DNA was detected using PCR in three pups, presenting at the time or 24–48 hours later with babesiosis symptoms, and in their asymptomatic mother. The isolates derived from the pups and the female – 520 bp 18S rRNA gene fragment – were compared and analyzed. All isolates from the pups and their mother were identical and showed 100% homology with *B. canis* group B (EU622793), supporting the same source of infection. Additionally, the USG of the peritoneal cavity was performed in the female, presenting evidence for splenomegaly.

Conclusions. On the basis of (1) the same timing of three pup cases; (2) the identical *B. canis* sequences derived from all positive dogs; (3) evident splenomegaly in the asymptomatic female, this provides the first evidence of the vertical transmission of this piroplasm in dogs.

Key words

Babesia canis, congenital, transplacental, vertical transmission, dog, asymptomatic infections

INTRIODUCTION

Vector-borne infections constitute increasing health problems in dogs worldwide, [1, 2, 3] and also in Poland [4, 5, 6] a country where babesiosis remains an emerging disease in dogs, and is expanding from the Eastern to the Western part of the country. Infections begin with fever, lethargy, anorexia, progressive anaemia and haemoglobinuria. Acute haemolytic anaemia, and kidney and liver dysfunctions often lead to death despite applied treatment [5]. Babesiosis may be especially severe in pups and in elderly dogs, and young or advanced age was proved to be the important risk factor for the acute manifestation [5]. However, the proportion of *B. canis* infections may be mild or even asymptomatic, developing into a chronic form [5, 7], thus possessing a risk for different modes of transmission, including vertical/ transplacental infections. To-date, only limited evidence for the vertical transmission of *B. gibsoni* and *B. microti*- like (*T. annae*) in dogs have been reported [8, 9]. According to official website of the Center for Disease Control and Prevention (CDC) in Atlanta, USA, congenital babesiosis is the third identified route of infection also for humans – additional to tick- or transfusion-borne cases (www.cdc.gov/parasites/features/babesia).

In Poland, there are noted cases of babesiosis in litters of puppies younger than 10 weeks; however, they are usually explained by a simultaneous tick infestation, which is rather doubtful, as pups of this age are usually kept indoors or in pup kennels, until the last vaccination at the age of 12 weeks. *Dermacentor reticulatus* tick is the main vector of canine babesiosis in Central Europe [10], and as this species feeds only once in each stage of life, horizontal transmission of *B. canis* among pups in the litter by one tick is unlikely, as the 'transfer' of feeding tick from a female to her offspring. Additionally, the rate of *B. canis* infection in adult *D. reticulatus* ticks in Central Poland ranges from 1–10% [11, 12], therefore the probability of simultaneous infections in four dogs can be calculated as $0.1 \times 0.1 \times 0.1 \times 0.1 = 0.0001$ (1: 10000) – extremely low. Simultaneous babesiosis cases in such the litters are usually explained by the transmammary route of infection, which had been previously excluded [8]. Taking all these suggestions together, it was planned to perform a detailed investigation on the possibility of *B. canis* vertical transmission, when the first case of babesiosis was diagnosed in a litter of 6-week-old, indoor kept pups.

OBJECTIVES

The aims of this work were:

1) to detect *B. canis* infection in pups and their mother using polymerase chain reaction (PCR);
2) to compare the parasite isolates derived from the pups and the bitch to confirm their similarity;
3) to identify other features of asymptomatic/ chronic infection in the female, thus finally to test the hypothesis on the vertical transmission of *B. canis* in dogs.

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MATERIALS AND METHOD

Dogs. The study concerned the litter of ten 6-week-old puppies, pure breed Central Asian Shepherd dogs, kept strictly indoors. The kennel is situated in the vicinity of Warsaw, which is the endemic region for canine babesiosis in Poland [5]. Only the bitch had free access to the garden, and the owner had found one semi-engorged tick attached to the skin on the same day as when the first symptoms of babesiosis occurred in the litter (October 2011). The tick was then identified as the *Dermacentor reticulatus* female. The bitch had been treated against ticks with spot-on fipronil-containing medicament, applied every 4 weeks from early spring – late autumn (as reported by the owner).

The first pup, a 6-week old female (on 43rd day of life), was presented to a veterinary clinic because of decreased appetite and activity. The pup was presented with a pale mucus membrane, slightly enlarged subpopliteal lymph nodes and an elevated body temperature (39.2˚C). Microscopical observation of blood smears revealed the *B. canis* trophozoites in the erythrocytes. Blood morphology examination revealed severe leukopenia, thrombocytopenia and anaemia with poikilocytosis, anisocytosis and hypochromasia (Tab. 1). The biochemistry profile was without significant changes. Due to fatal morphology results, progressive weakness and decreasing body temperature, a blood transfusion was performed immediately. The dog was given the standard treatment for babesiosis, including imidocarb (5mg/kg sc, Imizol, Intervet International BV, Boxmeer, Holland), amoxycyclinum with clavulonic acid (12.5 mg/kg, Synulox Phizer), dexamethasone (0.2 mg/kg intravenously, Dexaven®, Jelfa, Jelenia Góra, Poland) and vitamin B complex.

The other two cases, involving two male pups, were presented on the second day in the evening and third day in the morning (on the 45 and 46 day of life, respectively). In both cases, the symptoms were almost the same as in the first: a pale mucus membrane, elevated body temperature and an additionally enlarged spleen (by abdominal palpation). In both cases, severe thrombocytopenia, anaemia with anisocytosis, hypochromasia and poikilocytosis were observed (Tab. 1). Leukopenia was moderate in the second pup blood sample, but was severe in third (Tab. 1). Again, no significant changes in the biochemistry profile were detected. Both pups received treatment with imidocarb, amoxicilinum with clavulonic acid, and dexamethasone. Fluid therapy and a blood transfusion were completed.

Following the detection of *Babesia canis* in a blood sample, an abdominal ultrasound investigation (USG) was performed in the 6-year-old bitch, to visualize the spleen, because splenomegaly is the main symptom observed in asymptomatic or resolving cases of babesiosis in dogs.

Laboratory investigation. Blood smears were prepared and stained with Giemsa stain. Each smear was examined under oil immersion using a Nikon YS100 microscope. 200 fields of view were inspected for the presence of *B. canis*. Parasitaemia was calculated as the number of infected erythrocytes per 200 fields of view. Blood samples from 10 pups and the bitch were collected on the day 44 of the pups' life into 0.001M EDTA and frozen at -20˚C. DNA extractions were performed on 200 µl of whole blood using the AxyGen MiniPrep Blood kit (AxyGen, USA). Additionally, genomic DNA was isolated from 2 ml of bitch blood, precipitated with absolute ethanol (2 volumes) and re-suspended in 30 µl of 1 x TE buffer (Tris 10mM, EDTA 1 mM, pH= 8) for the condensation of target DNA. Amplification of the 18S rRNA *Babesia* gene fragment was performed using a previously described PCR protocol [13, 14]. Primers BAB GF2 (5' GYYTTGTAATT GGAATGATGG 3') and BABGR2 (5'CCAAAGACTTTGATTTCTCTC 3') were used to produce a ~550 bp fragment. Two µl of target DNA were used in 20 µl of the final PCR mixture volume. DNA of *Babesia microti* Kings Collage strain [15] was used as a positive control for each set of PCRs. Negative controls were set with deionised sterile water. Sequencing of the 1200 bp fragment of the *Babesia* 18S rRNA gene from three positive puppies was performed with the primers CRYPTOR (5' GAATGATCCTTCCGCAGGTTCACCTAC 3') and CRYPTOF (5' AACCTGGTTGATCCTGCCAGTAGTCAT 3') [13, 14]. Sequencing reactions were conducted with the ABI-PRISM 377 automatic DNA sequencer (Applied Biosystem). The resulting sequences were assembled using the program ABI™ BigDye™. BLAST comparisons were run against the GenBank database (www.ncbi.nlm.nih.gov/BLAST). DNA sequence alignments were conducted using MEGA version 5.0 [16].

Table 1. Comparison of blood morphology and biochemical parameters in pups during acute phase of babesiosis

	Units	First pup 43rd day of life	Second pup 45th day of life	Third pup 46th day of life	Reference values
Morphology:					
Leukocytes	G/l	3.9↓	4.4↓	2.8↓	6.0–12.0
Lymphocytes	%	19	40↑	44↑	12–30
Neutrophils	%	81↑	56	-	60–77
Eosinophils	%	-	4	56↑	0.1–6.0
Erythrocytes	T/l	2.3↓	1.96↓	2.43↓	5.5–8.0
Hemoglobin	mmol/l	3.23↓	2.86↓	3.35↓	7.45–11.17
HCT	l/l	0.16↓	0.13↓	0.16↓	0.37–0.55
MCV	f/l	69	68.9	67	60.0–77.0
MCHC	mmol/l	20.4	21.18	20.7	19.8–22.3
Trombocytes	G/l	21↓	33↓	45↓	200–580
Biochemistry profile:					
ALT	U/l	28	26	26	3.0–50.0
AST	U/l	25	55↑	69↑	1.0–37.0
ALP	U/l	117	168↑	125	20.0–155.0
Blood glucose	mg/dl	169↑	132↑	131↑	70.0–120.0
Creatinine	mg/dl	0.9↓	0.7↓	0.8↓	1.0–1.7
Blood urea	mg/dl	32	41	49.3↑	20.0–45.0
Total serum protein	mg/dl	38↓	38↓	50↓	55.0–70.0

MCV – mean corpuscular volume
MCHC – mean corpuscular haemoglobin concentration
AST – aspartate aminotransferase
ALT – alanine aminotransferase
ALP – alkaline phosphatase

RESULTS

Detection of *B. canis* DNA in blood samples of pups and the bitch. Among 10 pup blood samples, *B. canis* infections

were confirmed in three pups by successful amplification of 18S rDNA (prevalence 30%). Positive results were obtained either for the blood sample collected from the first pup during the acute phase (43 day of life) or for the samples of the other two pups collected before (44 day of life) & after the first symptoms of disease appeared (45 and 46 days of life, respectively). The DNA of *B. canis* was also detected in the blood sample of the asymptomatic female, although this required the use of a double volume of target DNA solution in 50 µl of the final PCR mixture volume.

Genotyping of *Babesia* using 18S rRNA gene fragments.

a) *520 bp gene fragment*. The isolates derived from the pups and the female were compared and analyzed. All isolates from the pups and their mother were identical and showed 100% homology with *B. canis* group B (EU622793), suggesting the same source of infection. To confirm this finding, the amplification and analysis of the longer 18S rDNA fragment was conducted for all positive isolates.

b) *1200 bp gene fragment*. Sequencing of a 1200 bp fragment of 18S rDNA amplified from three infected pups revealed that all three isolates were identical (100% homology) to *B. canis* group B (EU622793), originally isolated from dogs in Poland. Amplification of this longer rDNA fragment was unsuccessful for the female.

Features of *Babesia* infection in dogs. Microscopical observation of the blood smears revealed *B. canis* trophozoites in the erythrocytes of three infected puppies, either before or during the clinical manifestation of the infection (Tab. 2). Parasitaemia was higher during the acute phase of the infection, and was the highest in the blood of the pup presenting the clinical symptoms at the latest point (day 46 of life). All other pups' blood smears tested negative. In the blood smear of the female, only one *B. canis* trophozoite was noted.

Table 2. Comparison of parasitaemia (no. of *B. canis* infected erythrocytes/ 200 fields of view) in dogs before and during the acute phase of babesiosis

	43rd day of life	44th day of life	45th day of life	46th day of life
Pup 1	*B.canis* present[1,2]	ND	ND	ND
Pup 2	ND	5/200	37/200[2]	ND
Pup 3	ND	9/200	ND	244/200[2]
Bitch	ND	<1/200	ND	ND

[1] diagnosed in diagnostics laboratory
[2] onset of babesiosis symptoms

A USG of the peritoneal cavity was performed in the female on the 70th day post the date of the pups delivery, presenting evidence of splenomegaly.

DISCUSSION

The main aim of the presented study was to investigate the source of parallel *B. canis* infections in three pups in a litter of Central Asian Shepherd dogs. To the best of our knowledge, this is the first study providing evidences for vertical transmission of *B. canis* between bitch and pups. Congenital *Babesia/ Theileria* infections have been previously suggested/reported in domestic animals [17, 18, 19]. Two reports on the vertical transmission of *Babesia/Theileria* in dogs have been published – experimental transplacental *B. gibsoni* infection in the litter of beagles [8] and possible vertical transmission of *B. microti*-like (syn. *Theileria annae*) between the bitch and pup of German shepherds [9].

Thus, a detailed investigation on the possibility of *B. canis* vertical transmission was performed when the first case of babesiosis was diagnosed in the 6-week-old litter, with the pups kept indoors, although the mother of the pups was asymptomatic. First of all, three pups from this litter suffered from babesiosis in a 4-day period in the 7th week of life, and microscopical and molecular diagnosis revealed that all pups were infected at the same time (day 44 of life) when the first case manifested. Additionally, the infection was confirmed in the asymptomatic bitch by means of sensitive molecular technique. No ticks had been ever noted on these pups, but there was some possibility of tick 'delivery' to the pups by the bitch from the garden. However, the probability of acquiring simultaneous infections from four different *B. canis*-infected *D. reticulatus* ticks (three pups + female) is very low (p=0.0001), as calculated on the basis of studies in ticks in the same region of Poland [11, 12].

In fact, the period between 6th – 8th week of life is believed to be crucial in the development of immunity in dogs. During this period, maternally derived immunity decreases rapidly, to enable the development of individual immunity [20]. This is the reason why the 7th week of a pup's life is recommended for the first vaccination against canine distemper and parvovirosis. Pups infected during the foetal period inherit the pathogen/parasite but are protected against development of the disease through the uptake of immunoglobulin-rich colostrum – maternal immunity against these infections. Thus, infection may remain asymptomatic until the time when maternally-derived immunity significantly decreases- starting from the 6th week of life. All babesiosis cases reported in this paper manifested in the 7th week of life, between day 43 and 46 of the pups' lives. The appearance of babesiosis in 30% of puppies in one litter in a 4-day period during the 7th week of life is the first strong suggestion for congenital infection. This conclusion is supported by the studies on neonatal asymptomatic *Theileria equi* infections in foals [19], and when analyzing the findings of two other reports in dogs. In the case of the bitch and the pup of German shepherds, the dogs were presented to veterinary clinics with symptoms of babesiosis when the pup was two months old. In the case of experimental *B. gibsoni* infection, the litter consisted of five pups, four alive and one stillborn [8]. All pups were infected and only one of the four living pups was left with its mother and was allowed to feed on colostrum. The other three were fed by an uninfected female. Babesiosis was fatal in all four pups; however, the pup with maternally- derived immunity was able to survive the longest, until the 39th day of life and the parasitaemia was lower in this pup in comparison to the others. The other three pups died between the 14th – 20th day of life [8]. Interestingly, the pups delivered by the uninfected female but fed by the infected one, did not become infected, thus acting against the transmammary route of infection.

Babesia canis infection in the bitch in the presented study was confirmed by microscopical observation of a blood smear, positive results from PCR, sequencing of the isolate and finally, by observed splenomegaly. Splenomegaly is a characteristic feature of babesiosis, either in the acute, asymptomatic or chronic phase of the infection [7, 15]. Genotyping of parasite isolates derived from puppies and the bitch revealed that the

obtained sequences were identical, belonging to *B. canis* group B, which is relatively rare when compared to group A in dogs in Poland [4, 5]. Although this comparison was performed on the 520 bp long, strongly conserved 18S rRNA gene fragment, it supported the identical source of the parasite for the bitch and the puppies. Molecular analysis of the longer 18S rDNA fragment (1200 bp) confirmed the identity of parasite isolates derived from the three ill puppies. However, the lack of a positive result for this PCR on the bitch sample suggested that *Babesia* infection in the bitch was probably resolved at this time, or was of a very low parasitaemia, characteristic for the chronic phase of the infection [15]. Interestingly, the vertical transmission of *B. gibsoni* was confirmed in the female which was experimentally infected 650 days before mating and resulted with the infection in all pups [8]. The fact that only three of 10 pups were infected in this study is not unusual, as even the success rate of transovarial or transstadial transmission of various pathogens in ticks is less than 100%.

Although the timing of babesiosis cases suggested congenital infection, it cannot be excluded that the transmission of the parasite via milk, given that the bitch was allowed to use the garden during the lactation period, and was found to be infested with the *D. reticulatus* tick just before the disease manifested in the puppies. However, the results of experimental infection of *B. gibsoni* mentioned above demonstrated the lack of such a mode of transmission [8].

Vertical/transplacental transmission of babesiosis in dogs may constitute a serious problem for dog breeders and new owners as the disease may easily become life-threatening in puppies under two months of age. Additionally, asymptomatic *Babesia/ Theileria* infections in dog or horse females may lead to abortion and unsuccessful breeding [9]. Thus, the results of the presented study suggest the advantage of molecular diagnosis of babesiosis in females in endemic regions prior to a decision on mating. The increasing number of evidences for vertical transmission of important blood parasites (i.e. *Babesia, Plasmodium*) should also be recognized as a new threat in pregnancy and medicine.

On the basis of the following:

1) the same timing of the three pup cases;
2) the identical *B. canis* group B sequences derived from all positive dogs;
3) splenomegaly in the asymptomatic female, the presented study has provided the first evidence for the vertical transmission of *B. canis* in dogs.

REFERENCES

1. Baneth G, Breitschwerdt EB, Hegarty BC, Pappalardo B, Ryan J. A survey of tick-borne bacteria and protozoa in naturally exposed dogs from Israel. Vet Parasitol. 1998; 74: 133–142.
2. Matjila PT, Leisewitz AL, Jongejan F, Penzhorn BL. Molecular detection of tick-borne protozoan and ehrlichial infections in domestic dogs in South Africa. Vet Parasitol. 2008; 155: 152–157.
3. Solano-Gallego L, Trotta M, Razia L, Furlanello T, Caldin M. Molecular survey of *Ehrlichia canis* and *Anaplasma phagocytophilum* from blood of dogs in Italy. Ann N Y Acad Sci. 2006; 1078: 515–518.
4. Adaszek L, Winiarczyk S. Molecular characterization of *Babesia canis canis* isolates from naturally infected dogs in Poland. Vet Parasitol. 2008; 152: 235–241.
5. Welc-Falęciak R, Rodo A, Siński E, Bajer A. *Babesia canis* and other tick-borne infections in dogs in Central Poland. Vet Parasitol. 2009; 166: 191–198.
6. Zygner W, Gójska O, Rapacka G, Jaros D, Wedrychowicz H. Hematological changes during the course of canine babesiosis caused by large *Babesia* in domestic dogs in Warsaw (Poland). Vet Parasitol. 2007; 145: 146–151.
7. Bajer A, Rodo A, Welc-Falęciak R, Siński E. Asymptomatic babesiosis as a cause of splenomegaly and splenectomy in a dog. Med Wet. 2008; 64: 441–443.
8. Fukumoto S, Suzuki H, Igarashi I, Xuan X. Fatal experimental transplacental *Babesia gibsoni* infections in dogs. Int J Parasitol. 2005; 35: 1031–1035.
9. Simoes PB, Cardoso L, Araujo M, Yisaschar-Mekuzas Y, Baneth G. Babesiosis due to the canine *Babesia microti*-like small piroplasm in dogs – first report from Portugal and possible vertical transmission. Parasites and Vectors. 2011; 4: 50.
10. Zygner W, Wedrychowicz H. Occurrence of hard ticks in dogs from Warsaw area. Ann Agric Environ Med. 2006; 13: 355–359.
11. Zygner W, Jaros S, Wedrychowicz H. Prevalence of *Babesia canis, Borrelia afzelii,* and *Anaplasma phagocytophilum* infection in hard ticks removed from dogs in Warsaw (Central Poland). Vet Parasitol. 2008; 153: 139–142.
12. Bajer A, Welc-Falęciak R, Siński E. *Dermacentor reticulatus* tick as a vector of human and dog pathogens. Proceedings of the International Symposium 'Arthropods- Human and Animal Parasites', Lublin 2011. p.24.
13. Bonnet S, Jouglin M, Malandrin L, Becker C, Agoulon A, L'Hostis M, Chauvin A. Transstadial and transovarial persistence of *Babesia divergens* DNA in *Ixodes ricinus* ticks fed on infected blood in a new skin-feeding technique. Parasitology 2007; 134: 197–207.
14. Bonnet S, Jouglin M, L'Hostis M, Chauvin A. *Babesia* sp. EU1 from roe deer and transmission within *Ixodes ricinus*. Emerg Infect Dis. 2007; 13: 1208–1210.
15. Welc-Falęciak R, Bajer A, Bednarska M, Paziewska A, Siński E. Long term monitoring of *Babesia microti* infection in BALB/c mice, using nested PCR. Ann Agric Environ Med. 2007; 14: 287–290.
16. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol. 2011; 28: 2731–2739.
17. Neitz WO. Classification, transmission, and biology of piroplasms of domestic animals. Ann N Y Acad Sci. 1956; 64: 56–111.
18. Yeruham I, Avidar Y, Aroch I, Hadani A. Intra-uterine infection with *Babesia bovis* in 2-day-old calf. J Vet Med B Infect Dis Vet Public Health. 2003; 50: 60–62.
19. Allsopp MTEB, Lewis BD, Penzhorn BL. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. Vet Parasitol. 2007; 148: 130–136.
20. Day MJ. Immune system development in the dog and cat. J Comp Pathol. 2007; 137: 10–15.