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

**INTRODUCTION**

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 × 0.1 × 0.1 × 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.
**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 leukenopa, thrombocytopenia and anaemia with poikilocytosis, anisocytosis, hypochromasia and hypochromasia (Tab. 1). The biochemical 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), amoxicyclinum with clavulonic acid (12.5 mg/kg, Synulox Phizer), dexamethasone (0.2 mg/kg intravenously, Dexaven®, Phizer) 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 poikilocytosis, anisocytosis, hypochromasia and hypochromasia (Tab. 1). Leukopenia was moderate in the second and increased in the third pup blood sample, but was severe in third (Tab. 1). Again, no significant changes in the biochemical profile were detected. Both pups received treatment with imidocarb, amoxicillinum 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 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 immediately. The DNA in blood samples of pups and the bitch was 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’ GAAATGATCCCTCCGAGGTTCACCT 3’) and CRYPTOFL (5’ AACCCTGTTGGATCCTGGCCATGATCAT 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].

**RESULTS**

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

<table>
<thead>
<tr>
<th>Morphology:</th>
<th>Units</th>
<th>First pup 43rd day of life</th>
<th>Second pup 45th day of life</th>
<th>Third pup 46th day of life</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>G/l</td>
<td>3.9↓ 4.4↓ 2.8↓</td>
<td>6.0–12.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td></td>
<td>19 40↑ 44↑</td>
<td>12–30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils %</td>
<td></td>
<td>8↑ 56↑ 56↑</td>
<td>60–77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils %</td>
<td></td>
<td>4 6 4</td>
<td>0.1–6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes T/l</td>
<td></td>
<td>2.3↓ 1.96↓ 2.43↓</td>
<td>5.5–8.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin mmol/l</td>
<td></td>
<td>3.23↓ 2.86↓ 3.35↓</td>
<td>7.45–11.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT</td>
<td>l/l</td>
<td>0.16↓ 0.13↑ 0.16↑</td>
<td>0.37–0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCV</td>
<td>f/l</td>
<td>69 68.9 67</td>
<td>60.0–77.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCHC</td>
<td>mmol/l</td>
<td>20.4 21.18 20.7</td>
<td>19.8–22.3</td>
<td></td>
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<tr>
<td>Trombocytes G/l</td>
<td></td>
<td>21↓ 33↑ 45↑</td>
<td>200–580</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Biochemistry profile:**

| ALP | U/l | 28 26 26 | 3.0–50.0 |
| AST | U/l | 25 55↑ 69↑ | 1.0–37.0 |
| MCV – mean corpuscular volume |
| MCHC – mean corpuscular haemoglobin concentration |
| AST – aspartate aminotransferase |
| ALT – alanine aminotransferase |
| ALP – alkaline phosphatase |
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>
<thead>
<tr>
<th></th>
<th>43&lt;sup&gt;rd&lt;/sup&gt; day of life</th>
<th>44&lt;sup&gt;th&lt;/sup&gt; day of life</th>
<th>45&lt;sup&gt;th&lt;/sup&gt; day of life</th>
<th>46&lt;sup&gt;th&lt;/sup&gt; day of life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pup 1</td>
<td>B. canis present&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pup 2</td>
<td>ND</td>
<td>5/200</td>
<td>37/200&lt;sup&gt;2&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Pup 3</td>
<td>ND</td>
<td>9/200</td>
<td>ND</td>
<td>244/200&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bitch</td>
<td>ND</td>
<td>&lt;1/200</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>1</sup> diagnosed in diagnostics laboratory  
<sup>2</sup> onset of babesiosis symptoms

A USG of the peritoneal cavity was performed in the female on the 70<sup>th</sup> 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 7<sup>th</sup> 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 6<sup>th</sup> – 8<sup>th</sup> 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 7<sup>th</sup> week of a pup’s life is recommended for the first vaccination against canine distemper and parvovirus. 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 6<sup>th</sup> week of life. All babesiosis cases reported in this paper manifested in the 7<sup>th</sup> 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 7<sup>th</sup> 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 39<sup>th</sup> day of life and the parasitaemia was lower in this pup in comparison to the others. The other three pups died between the 14<sup>th</sup> – 20<sup>th</sup> 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 the puppies were infected 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