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Short note

Canine babesiosis in Slovenia: Molecular evidence of *Babesia canis canis* and *Babesia canis vogeli*

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Abstract – Canine babesiosis, caused by intraerythrocytic *Babesia* spp., is a tick-borne disease of worldwide importance. No information on canine babesiosis has been documented in Slovenia. Therefore, 238 dogs admitted to the Small animal clinic in Ljubljana from the years 2000 to 2002 were tested for the presence of babesial parasites in the blood. Based on clinical, microscopic and molecular investigations, 14 dogs (5.9%) were determined as being infected with babesiae. Clinical signs relating to acute haemolysis, fever, anorexia, depression and haematological abnormalities such as anaemia and thrombocytopenia were noticed in most of the 14 infected dogs. The morphology of the parasites was indicative of *Babesia canis* infection. Two subspecies were detected, namely *B. canis canis* (11 dogs, 4.6%) and *B. canis vogeli* (3 dogs, 1.3%) using PCR and subsequent sequence analysis of portions of nns rRNA gene. In addition, based on nucleotide sequence analysis, the 11 isolates of *B. c. canis* could be subdivided into three groups, whereas the three *B. c. vogeli* isolates were genetically identical. The results of this study demonstrate the presence of canine babesiosis due to *B. c. canis* and *B. c. vogeli* in Slovenia.

***B. canis canis* / *B. canis vogeli* / molecular analysis / Slovenia**

1. INTRODUCTION

Canine babesiosis, one of the most important tick-transmitted infectious diseases of dogs, is an emerging veterinary problem worldwide. *Babesia canis* and *B. gibsoni* are recognised as the main causative agents of the disease [6]. The two species represent the large and small group of babesial parasites of dogs, respectively. They are easily distinguished on the basis of phenotypic and genotypic characteristics [5, 11]. Furthermore, significant genetic differences within *B. canis* and within the small group of canine babesiae have been reported. Recently, Kjemtrup et al. [5] suggested that the small group of canine babesia does not consist exclusively of *B. gibsoni*. Instead they have shown the existence of at least three genetically distinct small piroplasms isolated from dogs. In contrast to *B. gibsoni*, marked differences in vector specificity, geographical distribution and pathogenicity have been long described among *B. canis* isolates. Therefore, three subspecies of *B. canis* have been proposed,

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namely *B. canis canis*, *B. canis vogeli* and *B. canis rossi* [10]. These differences among the subspecies at the expression level are also reflected genetically as proven by the characterisation of the nss rRNA gene and internally transcribed spacers [2, 3, 11].

In Europe, cases of canine babesiosis caused by *B. gibsoni* and other genetic variants of small piroplasms are rarely reported [4, 8, 12]. The majority of cases throughout Europe can be undoubtedly ascribed to infection caused by the *B. c. canis* parasite [2, 11, 13]. *B. c. vogeli* has been primarily found in Northern Africa. Nonetheless, a few cases have been recently detected in France and Spain by means of molecular identification [2, 11].

To date, no information on babesiae in dogs from Slovenia has been documented. In this study the occurrence of babesiosis in Slovenia was studied. Dogs admitted to the Small animal clinic in Ljubljana, showing clinical signs compatible with babesiosis (e.g. thrombocytopenia, anaemia) were tested for the presence of babesial parasites. Molecular analysis of portions of the nss rRNA gene was performed to characterise detected babesiae.

2. MATERIALS AND METHODS

2.1. Clinical examination

From the years 2000 to 2002, 238 dogs from different parts of Slovenia were admitted to the Small animal clinic in Ljubljana on the basis of different clinical manifestations. Dogs showing clinical signs compatible with babesiosis were tested for the presence of babesial parasites. They were subjected to routine physical examination. Venous blood samples were collected for haematological analysis. For a few dogs, additional serum biochemical parameters and the analysis of urine were performed. Basic information on the breed, age, travel history and tick infestation of the dogs was provided by the owners.

2.2. Microscopy

Thin blood smears were prepared from all dogs included in the study. They were air-dried and stained with Dade Diff-Quik Staining Set (Dade AG, Dudingen, Switzerland) which is a modification of the Pappenheim technique (Giemsa-May-Grünwald). Blood smears were examined by light microscopy at × 1000.

2.3. Analysis of DNA sequences

All 238 dogs were tested for the presence of babesial parasites using PCR and sequence analysis. DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Kit (Qiagen, GmbH, Hilden, Germany), according to the manufacturers' instructions.

To detect babesial parasites, each sample was tested with primers PIRO-A and PIRO-B, which were designed to amplify the 407-, 408- and 435-basepair (bp) fragments of the nss rRNA gene of *B. odocoilei, B. divergens* and *B. microti*, respectively [1, 7]. The alignment of complete sequences of nss rRNA from different species of babesiae deposited in the GenBank revealed that with this primer set, the 407-bp fragment of *B. canis* nss rRNA could be amplified as well. When using the primers PIRO-A and -B, the amplification protocol was slightly changed from the original description; the annealing temperature was raised to 60 °C.

All samples that demonstrated a positive reaction with the PIRO-A and -B primer set were sequenced on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI Prism, PE Applied Biosystems, California, USA). The sequences obtained were analysed with the modules SeqMan and EditSeq of the Lasergene 5.0 software package (Dnastar, Madison, Winsconsin, USA). Sequence alignments were performed with the MegAlign module of the same software package using the Clustal W algorithm.

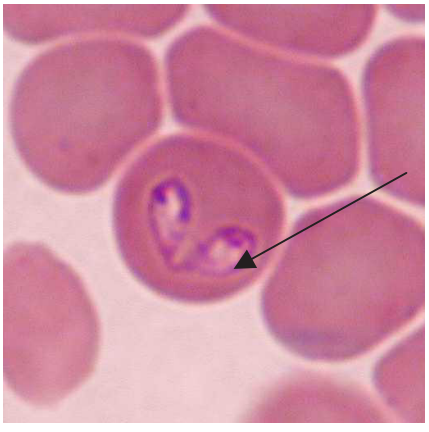

Figure 1. Thin blood smear of a dog from Slovenia infected with *B. canis.* Pear-shaped paired babesial parasites are present (magnification × 1000).

3. RESULTS

By examination of thin blood smears of the 238 dogs, intraerythrocytic parasites were observed in 14 dogs (5.9%). Large pear-shaped microorganisms mostly present in pairs were characteristic of *B. canis* (Fig. 1). The same dogs were found positive by *Babesia*-specific PCR. Infected dogs displayed clinical signs indicative of canine babesiosis including fever, anorexia, depression and pale mucous membranes with the exception of one dog for which clinical data were not available. The haematological abnormalities such as anaemia and thrombocytopenia were frequent as well. The results of the clinical examination and PCR for the infected dogs are presented in Table I in which the data on age and breed are provided as well. It is also very important to note, that all of the tested dogs originated from Slovenia and they had no travel history as stated by the owners.

Sequencing of the nss rRNA gene was performed on all 14 amplicons sized 407 bp. The gene sequences obtained confirmed that these dogs are infected with *B. canis*. Furthermore, two subspecies of *B. canis* were identified, namely *B. c. canis* and *B. c. vogeli.* The unique sequences determined in this study were deposited in the GenBank and may be accessed under the Accession No.: AY259123, AY259124. The prevalence rate of infection differed between the subspecies: 4.6% (11/238) for *B. c. canis* and 1.3% (3/238) for *B. c. vogeli*. The gene sequences from the dogs infected with *B. c. vogeli* were indistinguishable from one another and identical to the *B. c. vogeli* isolate from France (Acc. No.: AY072925). By contrast, genetic variation was noticed within the *B. c. canis* isolates. In comparison to the *B. c. canis* isolate from Croatia (Acc. No.: AY072926), three groups could be distinguished. Three isolates showed 100% identity to *B. c. canis* of Croatia. Two isolates displayed one transition mutation (G→A) at the 184 nucleotide position and were thus 99.7% identical to *B. c. canis* of Croatia. Six isolates displayed two transition mutations (G→A, A→G) at the 184 and 185 nucleotide positions, respectively (99.5% identity to *B. c. canis* of Croatia; Fig. 2).

4. DISCUSSION

Intraerythrocytic protozoan parasites of the genus *Babesia* represent a health threat to dogs all over the world. A systematic review of the distribution of the major vector-borne parasitic infections in dogs in Europe including canine babesiosis was recently published [9]. Since only a few prevalence and incidence studies on *B. canis* and reports of autochthonous cases in Europe could be retrieved, the resulting map is at the moment probably only a partial presentation of the endemicity of this parasite [9].

Based on the results of our study, we can add a small piece to the existing knowledge of canine babesiosis distribution in Europe. In the light of the clinical, microscopic and molecular investigation of 238 dogs from Slovenia admitted to the Small animal clinic

Table I. Clinical features and results of haematological testing for 14 dogs from Slovenia infected with babesiae as determined by PCR and subsequent sequence analysis.

Breed	Sex	Age	Clinical signs	Hct % (35–50)	PLT × 10^9/L (200–500)	PCR	Subspecies
Great dane	M	7	Anorexia, vomiting	54	37	+	*B. canis canis*
Brandel brake	M	3	Depression, anorexia, fever	35	39	+	*B. canis canis*
Golden retriever	F	6	Pale mucous membranes	35	14	+	*B. canis canis*
Golden retriever	M	4	Anorexia, polydipsia	32	23	+	*B. canis canis*
German shepherd	F	7	Depression, anorexia	24	86	+	*B. canis canis*
Poodle	F	4	Depression, anorexia	44	14	+	*B. canis canis*
Mixed breed	F	1	Depression, anorexia	ND	ND	+	*B. canis canis*
E. cocker spaniel	F	2	Depression, anorexia, fever	25	8	+	*B. canis canis*
E. cocker spaniel	M	10	Depression, anorexia, fever	20	9	+	*B. canis canis*
Mixed breed	M	2	Pruridermatitis	ND	ND	+	*B. canis canis*
Mixed breed	M	6	Depression	ND	ND	+	*B. canis canis*
Labrador retriever	F	13	Chronic cough	ND	ND	+	*B. canis vogeli*
Golden retriever	F	2	Depression, anorexia, fever	44	19	+	*B. canis vogeli*
Airedale terrier	F	NI	NI	ND	ND	+	*B. canis vogeli*

Hct: haematocrit, normal values are stated in the brackets; Age in years; PLT: platelets, normal values are stated in the brackets; M: male; F: female; ND: not done; NI: no information provided.

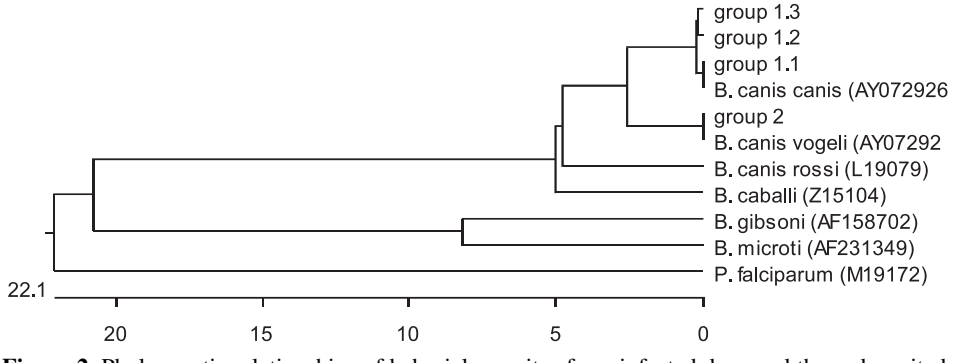

Figure 2. Phylogenetic relationships of babesial parasites from infected dogs and those deposited in the Genbank inferred from multiple sequence alignment of 364-bp (primer sequences were removed) of the nss-rRNA gene. Groups 1.1, 1.2, 1.3 and 2 consists of 3, 2, 6 and 3 infected dogs, respectively. Numbers at the bottom of the phylogenetic tree indicate the percentage of nucleotide substitutions.

in Ljubljana we demonstrated the presence of canine babesial parasites in our country.

Canine babesiosis presents clinical signs relating to anemia, fever, anorexia, depression and pale mucous membranes [6]. Most of the 14 infected dogs included in our study displayed the above mentioned clinical signs. The severity of the disease could, among other factors, depend on the age, breed and immune status. The infected dogs from our study were of different breeds, both sexes and of age from 1 to 13 years.

More important data were obtained on the basis of the haematological abnormalities since mild to severe anaemia and severe thrombocytopenia was detected in these dogs. The detected haematological profile is typical of canine babesiosis, although it may vary in different parts of the world [6]. The prevalence of infection as determined by PCR and examination of thin blood smears was 5.9% (14/238 dogs). The same 14 dogs were determined as being infected with babesiae by using both methods.

Although both methods showed a similar sensitivity, only PCR allowed discrimination between the morphologically similar *B. c. canis* and *B. c. vogeli* parasites. Eleven of 238 dogs (4.6%) were infected with *B. c. canis* and 3 of 238 dogs (1.3%) with *B. c. vogeli*. The existence of both *B. canis* subspecies is in agreement with the other studies in Europe and expands the data of distribution of *B. canis* [9]. In addition, the sequencing of PCR products revealed that the *B. canis* isolates could be subdivided into three genetically related groups of parasites. Similar intrasubspecies polymorphism has been previously detected and it is important as an additional evidence for clear genetic separation of *B. canis* subspecies [2, 11]. Moreover, data on different phenotypic and genotypic properties of large-type babesiae is likely to warrant classification on the species level in the future, i.e. *B. canis*, *B. vogeli* and *B. rossi*.

B. c. canis, transmitted by *Dermacentor reticulatus,* is the most common agent of canine babesiosis in Europe and it shows a variable pathogenicity [10]. Regarding clinical observations and the presence of *D. reticulatus* ticks in Slovenia (personal communication, T. Trilar, Natural History Museum, Ljubljana, Slovenia), we expected the majority if not all cases of canine babesiosis in Slovenia to be caused by *B. c. canis*. Although, the presence of *B. c. vogeli* in Slovenia was not expected, it is not entirely surprising. This subspecies is transmitted by *Rhipicephalus sanguineus* and it causes a mild, often clinically in apparent disease [10]. Moreover, it is morphologically indistinguishable from other *B. canis* subspecies. Therefore, even if *B. c. vogeli* is present in Slovenia and in Europe, it was probably not often determined. *B. c. vogeli* has only recently been detected in Europe with the use of molecular methods [2, 11]. Infection of Slovenian dogs with *B. c. vogeli* could not be acquired in other European countries since the owners claimed that the infected dogs had no travel history and that they noticed the presence of ticks on their dogs. Furthermore, *R. sanguineus* has been described in the Mediterranean region of Slovenia (thesis, D. Erjavec, Occurrence of ticks (Acarina: Ixodidae) in three test areas in Southwest Slovenia, Biotechnical Faculty, Department of Biology, University of Ljubljana, 2002). However, additional studies are needed to determine the respective vectors of canine babesiae in different parts of Slovenia.

In conclusion, the results of our study demonstrate the presence of *B. c. canis* and *B. c. vogeli* in dogs in Slovenia based on clinical, microscopic and molecular means of investigation.

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