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Babesia canis canis in dogs from Hungary: detection by PCR and sequencing

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Abstract

Canine babesiosis in Hungary has always been a severe and frequent disease, attributed to infection with *Babesia canis* transmitted by *Dermacentor reticulatus*. Identification of the disease agent has been based merely on size and morphology of the intraerythrocytic parasites and no evidence has been found concerning the subspecies (genotype) of *B. canis*. Therefore, a molecular survey on natural *Babesia* infection of dogs in Hungary using PCR and sequence analysis was attempted to clarify the subspecies (genotype) and to obtain information on the occurrence of *B. canis*. A total of 44 blood samples from dogs showing clinical signs of babesiosis were collected. A piroplasm-specific PCR amplifying the partial 18S rRNA gene yielded an approximately 450 bp PCR product in 39 (88.6%) samples. Thirteen positive samples originated from Budapest and 26 from 21 other locations. Five PCR products were chosen randomly for sequencing. The partial 18S rDNA sequences were submitted to GenBank® (accession numbers AY611729; AY611730; AY611731; AY611732 and AY611733). The sequences showed 100% homology to one another or differed by one nucleotide. BLAST search against GenBank® revealed the highest similarity (99.8 or 100%) with *Babesia canis canis*. The implication of these data, for the further study and diagnosis of canine babesiosis is discussed.

Keywords: *Babesia canis canis*; Dog; PCR; 18S rDNA; Hungary

1. Introduction

Babesia species are tick-transmitted apicomplexan parasites infecting a wide range of wild and domestic vertebrate hosts (Kuttler, 1988). Traditionally, identification of species has been based on host specificity and morphology of the intraerythrocytic piroplasms. Based on these, canine piroplasms have been originally recognised to belong to two distinct species, the large (4–5 μm) *Babesia canis* and the small (1–2.5 μm) *Babesia gibsoni*. On the basis of differences in geographical distribution, vector specificity and antigenic properties (Uilenberg et al., 1989; Hauschild et al., 1995), the former species is subdivided into

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three subspecies, namely *Babesia canis canis* transmitted by *Dermacentor reticulatus* in Europe, *B. canis vogeli* transmitted by *Rhipicephalus sanguineus* in tropical and subtropical regions and *B. canis rossi* transmitted by *Haemaphysalis leachi* in South Africa. Genetic differences between these subspecies have also been proved by Zahler et al. (1998) and Carret et al. (1999). *B. gibsoni* occurs in Asia, North America, northern and eastern Africa, Australia and Europe (Birkenheuer et al., 1999; Muhlnickel et al., 2002; Criado-Fornelio et al., 2003a). Recent genetic characterisations demonstrated that small canine piroplasms also represent a greater diversity than previously thought (Zahler et al., 2000a, 2000b; Kjemtrup et al., 2000; Kocan et al., 2001). One of the recently identified small piroplasms, *Theileria annae* (Zahler et al., 2000a) is phylogenetically closer to *B. microti* than to *B. gibsoni* and can be found with a high frequency among Spanish dogs (Camacho et al., 2001). For both small and large canine piroplasms, the distinction between subspecies can only be made by genetic analysis.

Canine babesiosis in Hungary was first described by Wetzl (1905). Since then, it has been a severe and frequent disease in the country (Horváth and Papp, 1996; Csikós et al., 2001) attributed to infection with *Babesia canis,* transmitted by the tick, *Dermacentor reticulatus* (syn. *D. pictus*) (Janisch, 1986). Recent studies (Farkas and Földvári, 2001; Földvári and Farkas, 2004) showed that this vector species occurred in a greater geographical range than Horváth and Papp (1996) previously described. Identification of the pathogen has always been based merely on size and morphology of the intraerythrocytic parasites, and no evidence was found concerning the subspecies of this large canine piroplasm. Beside large *Babesia* infections, small canine *Babesia* infection has been detected by Farkas et al. (2004) in two Hungarian dogs. The genetic characters and vector species of this newly recognised small *Babesia* is not yet know.

Recent advances in molecular methodology (e.g. automated DNA sequencing) have made it possible to detect and identify piroplasms with greater sensitivity and specificity than traditional methods allowed (Birkenheuer et al., 2003; Jefferies et al., 2003). Beside PCR-RFLP (Zahler et al., 1998; Carret et al., 1999) and reverse line blot hybridization (Matjila et al., 2004), sequencing single PCR products remains a reliable and quick diagnostic method (Criado-Fornelio et al., 2003b). In this study, a molecular survey on natural *Babesia canis* infection of dogs in Hungary was carried out to clarify the subspecies and to obtain information on the occurrence of this piroplasm.

2. Materials and methods

2.1. Blood samples

Between 2002 and 2004, veterinary practitioners from all over the country were asked to collect blood samples from dogs showing clinical signs of babesiosis (e.g. fever, weakness, lethargy, loss of appetite, haemoglobinuria). The veterinarians were also asked to prepare a thin blood smear for each dog, to fix with methanol then to stain with Giemsa solution. Blood samples were taken into EDTA tubes and kept at −20 °C before transporting to our department in cold packs.

2.2. DNA extraction, amplification and sequencing

DNA was isolated from 200 μl amounts of EDTA blood from each dog using QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The primers PIRO-A1 (5′-AGGGAGCCTGAGAGACGGCTACC-3′) and PIRO-B (5′-TTAAATACGAATGCCCCCAAC-3′) were used to amplify an approximately 450 bp region of the 18S rRNA gene. The forward primer, PIRO-A1 was developed by Muhlnickel et al. (2002) to amplify most *Babesia* species using sequence information from GenBank®. The reverse primer, PIRO-B, has been described previously by Olmeda et al. (1997). Two microliters of extracted DNA was added to a 48 μl reaction mixture comprised of 1.5 U of Taq DNA polymerase (Promega, Madison, WI, USA), 200 μM of each dNTP, 25 pmol of each primer and 5 μl 10X PCR buffer and 1.5 mM $MgCl_2$ (Promega, Madison, WI, USA). Amplification was performed using a Tpersonal 48 thermal cycler (Biometra GmbH, Göttingen, Germany). An initial denaturation step at 94 °C for 10 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Final extension was done at 72 °C for

5 min followed by a hold step at 4 °C. Amplified DNA was subjected to electrophoresis in a 1.5% agarose gel (120 V, 40 min), pre-stained with ethidium-bromide and viewed under ultra-violet light.

Five PCR products were chosen randomly for sequencing. After purification with Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA), ABI Prism® Big Dye Terminator v.3.1 Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA) was used for DNA sequencing reactions. Samples were then examined using an ABI Prism® 3100 Genetic Analyser at the Agricultural Biotechnology Centre Gödöllő, Hungary. Obtained sequences were checked with Chromas v.1.45 and compared to sequence data available from GenBank®, using the BLAST 2.2.9 program (http://www.ncbi.nlm.nih.gov/BLAST/). New sequences were submitted to GenBank® database.

3. Results

Blood samples from 44 dogs with clinical signs of babesiosis were collected. For 17 (38.6%) dogs, unusable (3) or no blood smears (14) were available. In the others (61.4%), 4–5 μm long, single or paired pyriform intraerythrocytic parasites, characteristic for *Babesia canis* were observed using light microscopy.

No PCR products were detected from 5 of 44 samples (11.4%) for which no blood smears were available. An approximately 450 bp PCR product was amplified from the blood of 39 dogs (88.6%). Thirteen positive samples originated from nine districts of Budapest and 26 from 21 other locations covering a considerable part of Hungary (Fig. 1). Five PCR products were sequenced and the partial 18S rDNA sequences were submitted to GenBank® (accession numbers AY611729; AY611730; AY611731; AY611732 and AY611733). The sequences showed 100% homology to one another or differed by 1 nucleotide which may represent sequencing error (being close to the primers) or minor variation. BLAST search against GenBank® revealed the highest similarity (99.8 or 100%) with *Babesia canis canis* 18S rDNA partial sequence published by Cacciò et al. (2002), accession number AY072926 (Table 1).

4. Discussion

Babesiosis is an endemic disease among dogs in Hungary. It has been demonstrated with traditional methods (e.g. size of the intraerythrocytic forms and experimental transmissions) that the causative agent is *Babesia canis* (Horváth and Papp, 1996) and its vector tick species is *Dermacentor reticulatus* (syn. *D. pictus*) (Janisch, 1986). This is the first molecular survey on canine babesiosis in the country attempting to identify and characterize the subspecies (genotype) of this large canine piroplasm.

Polymerase chain reactions with the piroplasm-specific primers, PIRO-A1/PIRO-B were positive in the case of 39 out of 44 samples sent to our department for *Babesia* analysis. The five PCR negative samples were from dogs diagnosed by clinical examination and not tested by blood smear examination. This points out, that diagnosis of canine babesiosis can not solely be based on the observed clinical symptoms. On the other hand, there can be cases, when samples found negative by microscopic examination of blood smears, still can turn out to be positive by the considerably more sensitive PCR methods (Jefferies et al., 2003; Birkenheuer et al., 2003). Notably, the sensitivity of the PCR used in this study needs further investigations. Because of the unspecificity of the primers used, positive products do not provide information on the subspecies or species level without sequencing.

Geographical origin of PCR positive samples proved the presence of piroplasms in many districts of Budapest and in several other parts of the country including north-eastern and south-eastern regions, from which no babesiosis has been reported (Horváth and Papp, 1996). The occurrence of canine babesiosis in these parts of Hungary is in accordance with the geographical distribution of the vector, *D. reticulatus* observed by Földvári and Farkas (2004), and suggests that large part of the country can be considered to be endemic for this disease.

Five samples were chosen randomly for sequencing, and showed 99.8–100% similarity with the *Babesia canis canis* sequence deposited in GenBank® (accession number AY072926) by Cacciò et al. (2002). This is in accordance with the prediction of the authors based on geographic, diagnostic (Horváth and Papp, 1996) and vector information (Janisch, 1986; Földvári and Farkas, 2004). However, this is the

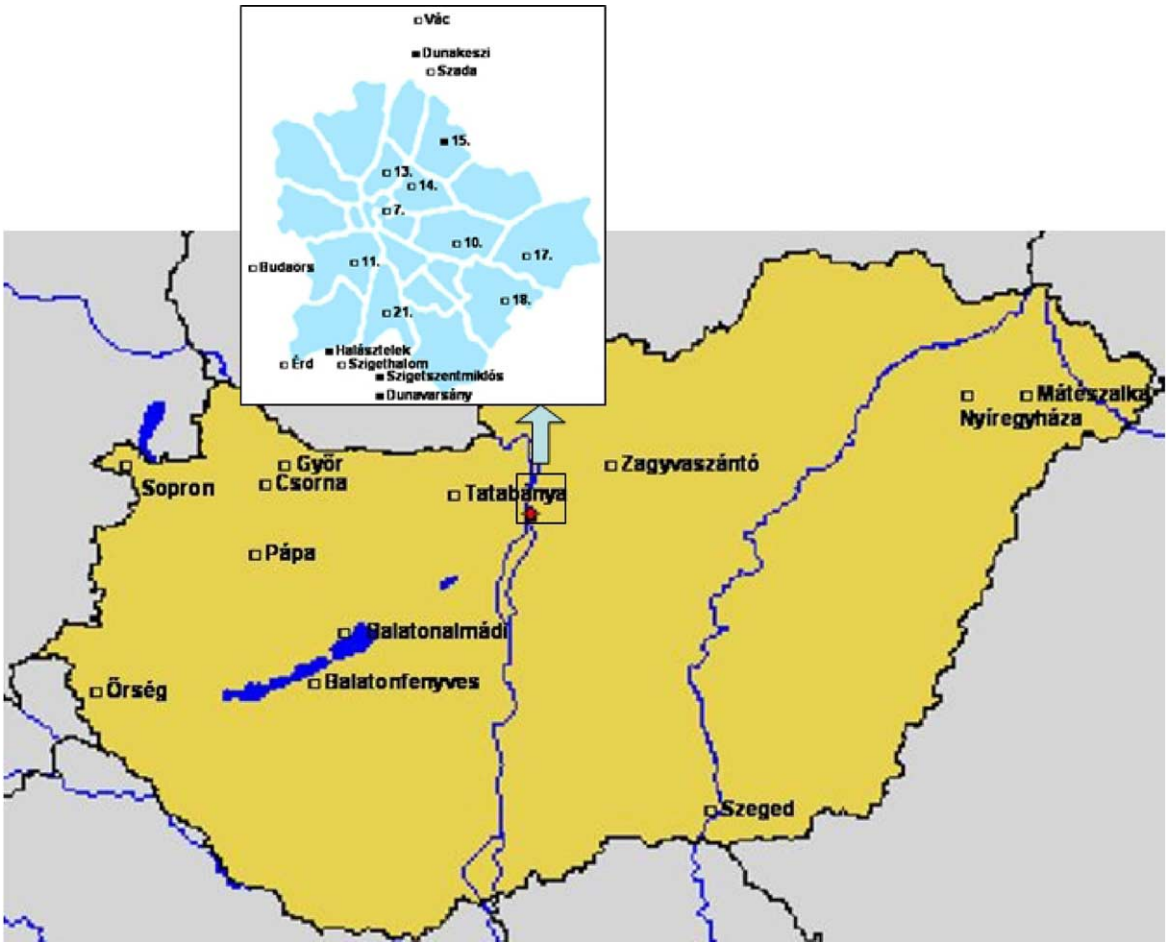

Fig. 1. Origin of PCR positive samples (Budapest region in higher magnification; (□) for PCR product, (■) for sequenced PCR product). District numbers within Budapest are indicated.

first study that provides evidence concerning the subspecies (genotype) of *B. canis* which has caused severe disease among dogs in Hungary.

The scarce information concerning immunological and clinical aspects of infections with different subspecies of *Babesia canis* indicates (Schetters et al., 1997), that the frequently used *diagnosis ex juvantibus* (i.e. diagnosis based on the recovery of dogs following antibabesial treatment) is not recommended. Furthermore, infections with other piroplasms, like

Table 1
New sequences submitted to the GenBank® database

Origin of sample	Accession No.	Sequence length (bp)	Similarity to *B. canis canis* (%)
Budapest	AY611729	405	100
Halásztelek	AY611730	411	100
Dunakeszi	AY611731	412	99.8
Szigetszentmiklós	AY611732	411	99.8
Dunavarsány	AY611733	412	99.8

Similarity values are in respect to AY072926.

Babesia canis vogeli (proved to be present in France by Cacciò et al. (2002)), *B. canis presentii* (recently observed as a new subspecies in cats from Portugal, Spain and Israel by Criado-Fornelio et al. (2003c) and Baneth et al. (2004)) or small canine *Babesia* (Farkas et al., 2004) cannot be excluded, especially in case of a previous visit in endemic areas. Molecular biology provides a powerful method not only in subspecies (genotype) identification, but also in cases when symptoms and/or blood smears do not provide definitive diagnostic information for the veterinarian. For these reasons, it can be also diagnostically important to determine the species, subspecies and genotype that causes canine babesiosis.

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