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***Hepatozoon canis* and *Babesia vogeli* infections of dogs in Tunisia**

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Abstract

A paucity of studies is available on haemoparasites in dogs in Tunisia. In this study, we used molecular techniques (PCR/sequencing) to detect and characterize haemoprotozoa in sick dogs from Tunisia. A total of 99 dogs displaying such clinical symptoms as fever, anorexia, and depression were presented for treatment to the hospital of the Veterinary School of Sidi Thabet (Tunisia). Among dogs screened by PCR, five (5%) proved to be infected with a hemoprotozoa species.

An analysis of all the sequences that were obtained enabled us to identify two species of Protozoa: *Babesia vogeli* (in three dogs) and *Hepatozoon canis* (in two other dogs). This is the first time that an infection of dogs by *Hepatozoon canis* in Tunisia has been

reported. Veterinary practitioners should be aware that these two hemoparasites can infect dogs and should include them in any differential diagnosis of clinical illnesses with manifestations compatible with tick-borne diseases.

Keywords: Canine Babesiosis, *Babesia*, *Hepatozoon*, Dogs, Tunisia, *Rhipicephalus sanguineus*.

1. Introduction

Species of *Babesia* and *Hepatozoon* infect a wide variety of mammals, including dogs (Smith, 1996; Solano-Gallego and Baneth, 2011). Some of the pathogens transmitted to dogs by ticks, such as hemoprotozoa, cause severe disease and serious illness. Several *Babesia* species, including *B. canis*, *B. rossi*, *B. vogeli* and *B. gibsoni*, which are the primary causes of babesiosis, have been reported in dogs (Carret et al., 1999; Irwin, 2009; Solano-Gallego et al., 2016; Baneth et al., 2019) and can cause infections whose clinical and pathological signs range from subclinical to severe (fever, haemolytic anemia, splenomegaly, thrombocytopenia...) and even death (Solano-Gallego and Baneth, 2011).

Small animal practitioners in Tunisia have frequently reported cases of canine babesiosis, which is most commonly caused by *Babesia vogeli* and transmitted by the dog brown tick *Rhipicephalus sanguineus* (M'ghirbi and Bouattour, 2008). Considered to be the least pathogenic species (Köster et al., 2015), *Babesia vogeli* has also been reported in other African countries (Collett, 2000; Oyamada et al., 2005), in southern Europe (Caccio et al., 2002; Solano-Gallego et al., 2016; Solano-Gallego and Baneth,

2011), in North and South America (Birkenheuer et al., 2005; Passos et al., 2005), Asia (Abd Rani et al., 2011) and in Australia (Jefferies et al., 2003).

Active during the warm season in Tunisia, *R. sanguineus* heavily infests dogs and can be a vector for several pathogens. In addition to *B. vogeli*, this tick species was reported to be infected with *Anapalsma platys*, *Rickettsia massiliae* Bar 29 and *Rickettsia conorii* (Bouattour et al., 1999; Sarih et al., 2005; M'ghirbi and Bouattour 2008; Khrouf et al., 2014). However, haemoparasites of the genera *Hepatozoon* have never been reported in dogs in Tunisia. Canine infections with *H. canis* have been reported in many parts of Africa (Ibrahim et al., 1989; Oyamada et al., 2005, Medkour et al., 2020), Europe, America, Asia, and Australia (Eiras et al., 2007; Li et al., 2008; Abd Rani et al.; 2011; Barker et al., 2012). The cosmopolite brown dog tick *R. sanguineus* was ascertained to be the primary invertebrate host for this Protozoa (Christophers, 1907).

Babesia and *Hepatozoon* are known to occur in endemic areas, which could partially explain the variations in clinical presentation and pathogenicity in infected dogs (Shaw et al., 2001). When examining tick-infested dogs that show clinical signs suggestive of hemoprotozoosis, veterinary practitioners often presume the cause to be babesiosis and omit hepatozoonosis as a possible cause without seeking laboratory confirmation.

Infection with *Hepatozoon canis* (Family Hepatozoidae) is also part of the differential diagnosis of hemoprotozoosis affecting dogs as intermediate hosts; the brown dog tick *R. sanguineus* is the definitive host and vector (Baneth et al., 2001). Dogs with canine hepatozoonosis are often asymptomatic with low levels of parasitaemia whereas dogs with high levels of parasitaemia can present severe disease and show fever, lethargy, anaemia, and cachexia (Ivanov and Tsachev, 2008). A diagnosis of *Hepatozoon* cannot

rely only on clinical or biological observations. The detection of other haemoprotozoa such as small *Babesia* is not obvious even after an examination of blood smears stained with Giemsa. The diagnosis of these parasites is even more problematic in chronic cases of infection because of less virulent species. A blood smear examination by light microscopy remains the easiest, most accessible and least expensive diagnostic test for most veterinarians, but this technique detects less commonly small hemoprotozoa such as *B. gibsoni* and *H. canis*. The development of more sensitive molecular tools such as PCR and sequencing has made it possible to identify and differentiate the various species of *Babesia* (Baneth et al., 2000; Caccio et al., 2002; Inokuma et al., 2004) and active *Hepatozoon* (Oyamada et al., 2005; Medkour et al., 2020) infections in dogs. This study therefore aimed to diagnose the causal agents of infections in dogs presenting suspected haemoparasitosis using PCR and sequencing methodologies.

2. Materials and methods

2.1. Dogs and blood samples

Our study was carried out during the warm season (April-July 2018) during which ticks are active. A total of 99 dogs of various breeds with clinical manifestations compatible with tick-borne diseases including apathy, dehydration, fever, anorexia, weight loss, and pale mucous membranes, were presented to Veterinary School of Sidi Thabet Hospital (Tunisia) for treatment. The dogs underwent clinical examinations and blood samples were collected from the radial vein in 5 mL tubes containing ethylenediaminetetraacetic acid (EDTA)-anticoagulated. Whole blood was collected for DNA extraction and hematological analysis.

2.2. DNA extraction

We used the QIAmp®DNA Mini kit (Qiagen GmbH, Hilden, Germany) for DNA extraction as per the manufacturer's instructions, adjusted to 100 µL with the elution buffer (AE) provided with the kit, and stored at –20°C for further use. To verify contamination, a control tube (distilled water) for each of the ten samples was included in the extraction protocol. The DNA yield was determined with a NanoDrop_ND-1000 Spectrophotometer (NanoDrop Technologies, DE, USA).

2.3. PCR

PCR was performed using a set of primers that amplified a 460 to 520 bp fragment in the V4 variable region of the 18S rRNA of *Babesia* and *Theileria* species (Georges et al., 2001). Sequences of genus-specific primers were used to amplify the V4 region of the 18S rDNA were RLB-F2 5'-GACACAGGGAGGTAGTGACAAG-3' and RLB-R2 -5'-CTAAGAATTTACCTCTGACAGT-3'. In our experiments, sequencing showed that these primers, previously reported as being able to amplify the *Babesia* and *Theileria* genus, also amplified *Hepatozoon*.

The PCR reactions were performed in a final volume of 25 µL containing 75 ng of genomic DNA, 200 nM of each primer, 1× PCR buffer, 1.5 mM MgCl₂, 200 µM of each dNTPs and 1 U of Taq Platinum Polymerase (Invitrogen, CA, USA).

The reactions were performed on an automated DNA thermal cycler (Perkin-Elmer 2720, CA) as follows: starting with 15 min at 95° C and followed by 45 cycles of 94° C for 35 sec; 51° C for 35 sec and 72° C for 35 sec and a final elongation, 72° C for 10 min. Extraction controls and PCR negative (water) controls were included in each PCR reaction. Plasmid including the amplicon of the V4 region of the 18S rRNA gene of *Babesia canis* species was used as a positive control.

The PCR products were separated by electrophoresis using 2% agarose gel containing ethidium bromide (10 mg/mL) before it was analyzed by sequencing.

2.4. DNA sequencing and data analysis

To confirm the PCR results, positive *Babesia/Theileria* amplified DNA (n=5) was analyzed by sequencing. The PCR products were purified using the ExoSAP clean-up procedure (Amersham Biosciences, Germany). All nucleotide sequences were obtained using the Big Dye Terminatorv.3.1 Cycle Sequencing Kit (Applied Biosystems) and the 3130 automated sequencer (Applied Biosystems).

The sequences generated in this study were edited and aligned using BioEdit (Hall, 1999) and ClustalW software programs (Thompson et al., 1994). The BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) was used to compare and analyze the data sequences.

2.5. Phylogenetic analysis

A phylogenetic tree was inferred using neighbor-joining analysis with MEGA5.2 software with a bootstrap of 1000 replications and evolutionary distances adjusted through Kimura-2 parameter nucleotide substitution (Tamura et al., 2007). The sequence of *Plasmodium falciparum* (M19172.1) was used as distant phylogenetic organisms to root the tree.

2.6. Nucleotide sequences data

Nucleotide sequences data reported in this paper is available in the GenBank™, EMBL and DDBJ databases under the accession numbers MT588813-MT588817.

2.7. Hematological analysis

White blood cell counts, Hematocrit, Hemoglobin, and platelet counts were performed by a fully automatic hematology analyzer -- the BC-2800 Vet (Mindray, China).

3. Results

Of the 99 dogs examined by PCR, five (5%) showed a specific band of 460 bp for Protozoa. The sequences obtained were analyzed using Bioedit® software and then compared with the reference sequences in the GenBank (www.ncbi.nlm.nih.gov/BLAST). This enabled us to identify two species of Protozoa: *Babesia vogeli* and *Hepatozoon canis*. Three sequences (354 sequenced nucleotides) were found to be 100% identical to the partial sequence of *B. vogeli* (MK910150) reported in a dog from Colombia. In addition, two sequences (339 sequenced nucleotides) were 100% identical to the partial sequence of *H. canis* (MN628318) isolated in India (Fig. 1).

All positive dogs had a fever (39.4-41.3 °C) and were anorexic (Table1). Of these, only dogs 51 and 93, which were infected by *B. vogeli* and *H. canis* respectively, developed a mild non-regenerative anaemia. The hematocrit showed low values in dogs 32, 93 and 51. Dog 51 also presented a leukopenia ($WBC = 5.2 \times 10^3/\mu L$). Dogs 15, 18 and 51 suffered from thrombocytopenia (Table1). All dogs had at least two specimens of *Rhipicephalus sanguineus* ticks on their ears.

4. Discussion

Infections with *Babesia* or *Hepatozoon* provoked a wide range of clinical manifestations in dogs from asymptomatic to severe disease. Acute clinical and hematological signs include fever, lethargy, hemolytic anemia, and thrombocytopenia, (Criado-Fornelio et al., 2007; Irwin, 2009; Solano-Gallego and Baneth, 2011; Baneth et al., 2003, Kamani et al., 2019). However, clinical manifestations caused by haemoprotozoa are not so clearly defined that veterinary practitioners can make a diagnosis and adapt their treatments. Molecular methods must be used to identify the causative species. Several techniques have been described, and conventional PCR used on blood proved more sensitive and specific than classical methods for diagnosing haematozoa (Oyamada et al., 2005; Wahlang et al., 2019). In addition, sequencing facilitated the identification of hemoparasite species (Solano-Gallego and Baneth, 2011).

In our study, the DNA of 99 dogs presenting various clinical manifestations to the hospital of the Veterinary School of Sidi Thabet (Tunisia) was screened by PCR using a set of a genus-specific primers amplifying *Babesia* and *Theileria* species (Georges et al., 2001). After sequencing the positive amplicons, we discovered that these same primers can also amplify *Hepatozoon*. Oyamada et al. (2005) have reported similar results using *Babesia* spp. primers. Sequencing analysis of the positive amplicons that were obtained revealed that *Babesia vogeli* was present in three dogs (15, 18 and 51) and *Hepatozoon canis* was present in two dogs (32 and 93).

Our results support and confirm the presence of *B. vogeli* in dogs in Tunisia as we have previously reported. However, in the investigated sick dogs, the prevalence of the infection was only 3%, which is lower than that reported in our previous investigation (6.6%) on randomly studied dogs (M'ghirbi and Bouattour, 2008). In fact, infections in dogs with this large *Babesia* have been reported with prevalence range values that vary widely by locality: *B. vogeli* prevalence was 13.2% among 227 dogs screened by PCR

in Algeria a neighboring country (Medkour et al., 2020); 2% in 78 randomly chosen dogs from eastern Sudan (Oyamada et al., 2005); 0.6% in dogs with clinical manifestations in Nigeria, (Kamani et al., 2013); in southern France, the prevalence was 13.6 % in 140 dogs tested by nested PCR (René-Martellet et al., 2015); in southeastern Rio de Janeiro (Brazil), the prevalence was 15.66 % in 390 dogs tested by qPCR (Paulino et al., 2018). The variation in prevalence might be attributable to several factors including the DNA extraction methodology, the primers that were used and the targeted gene, which can have different sensitivities (Valkiunas et al., 2008). In addition, host-related variables such as age or breed and mainly tick infestations should also be considered when comparing varying levels of regional prevalence.

In addition to *B. vogeli*, we detected *H. canis* in the group of investigated dogs. This study is the first molecular report of *H. canis* in Tunisia. This parasite has been reported recently in dogs in Algeria with a prevalence of 41% (Medkour et al., 2020). In addition, this *Hepatozoon* species was recorded in several countries of Africa: Sudan, Nigeria and Zambia with prevalence rates of 42.3%, 41.4%, and 13% respectively (Oyamada et al., 2005; Kamani et al., 2013, 2019; Williams et al., 2014). Studies in several European countries have reported different prevalence rates from low (1% and 3% in France and Spain respectively; Criado-Fornelio et al., 2009; Tabar et al., 2009) to high (50.6% in southern Italy; Otranto et al., 2011). The prevalence of *H. canis* similarly varies by country in America: 53.3% in Brazil (Rubini et al., 2008) to 2.5% in the USA, where *H. canis* occurs together with *H. americanum* (Allen et al., 2008). Dogs in several counties in Asia were also reported to show prevalence: in India where the hemoparasite was identified for the first time, the rate of prevalence was 30% (Abd Rani et al., 2011).

Babesia vogeli and *Hepatozoon canis* are canine hematic Protozoa that are widespread due to the cosmopolitan nature of their main vector, *Rhipicephalus sanguineus* (Shaw et al., 2001; M'ghirbi and Bouattour, 2008; Irwin, 2009; Solano-Gallego and Baneth, 2011), the most prevalent tick species infesting dogs worldwide. However, while *Babesia* is transmitted to a dog by the tick's salivary gland during the blood meal (Solano-Gallego et al., 2016), *H. canis* is transmitted by the ingestion of ticks containing mature oocysts (Baneth et al., 2001, 2003).

In our study, infections with *B. vogeli*, considered to be the least virulent *Babesia* species, caused clinical diseases in three infected dogs that displayed fever, anorexia, dehydration depression, lethargy and weakness. All three dogs also suffered from thrombocytopenia whereas only one dog (number 51) had mild regenerative anaemia since it was infected with *B. vogeli* (30% hematocrit; 9g/dl hemoglobin). Similar clinical signs (fever, anorexia and weakness) were observed in the two dogs infected with *H. canis*; hematological abnormalities (anemia and low hematocrit) were also noted. Similar clinical and hematological manifestations caused by these two parasites were reported (Sasanelli et al., 2009; Solano-Gallego and Baneth, 2011; Solano-Gallego et al., 2016). *H. canis* also causes subclinical infections in apparently healthy dogs. Indeed, infection with this protozoon can induce severe illness and can be fatal; dogs with high levels of *H. canis* parasitaemia that often approaches 100% of the peripheral blood neutrophils can display lethargy, cachexia and anaemia (Baneth et al., 2003). Two dogs presented to the clinic showed mild disease, which is the most common presentation of infection that is usually associated with low levels of *H. canis* parasitaemia (1–5% of neutrophils are infected) (Baneth et al., 2003; Ivanov and Tsachev, 2008).

5. Conclusion

In Tunisia, warm weather from March to October prolongs the active season of *Rhipicephalus sanguineus* ticks that heavily infest dogs. This tick is a vector of several pathogens such as species of *Ehrlichia* and *Babesia*, previously reported (Sarih et al., 2005; M'ghirbi and Bouattour, 2008), and *Hepatozoon canis* that is described in this work. It should be noted that co-infections can occur in dogs, which might partially explain the variations in often unspecific clinical presentations (Shaw et al., 2001; Sasanelli et al., 2009; Kamani et al., 2013). It is therefore clinically important that these diseases be diagnosed with a sensitive, specific, simple method and that the veterinary practitioner be aware of the existence of these pathogens in Tunisia and select a treatment adequate to each one.

Ethical statement

All dogs were examined and sampled by the veterinary clinicians according to the procedures and the ethics committee of the Veterinary School of Sidi Thabet, and after the consent of the owner. Owners presented their dogs to the Veterinary School Hospital for diagnosis and treatment.

Conflict of interest

The authors declare that there is no conflict of interest.

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Authors' contribution: A.B (MDV, PhD) conceived, designed and wrote the manuscript. A. Ch (MDV, Professor) and I. H. (MDV) were the veterinary clinicians who examined the dogs and performed the hematological analysis.

Y.M (PhD) performed the molecular analysis and contributed to the manuscript.

All authors read and approved the final manuscript.

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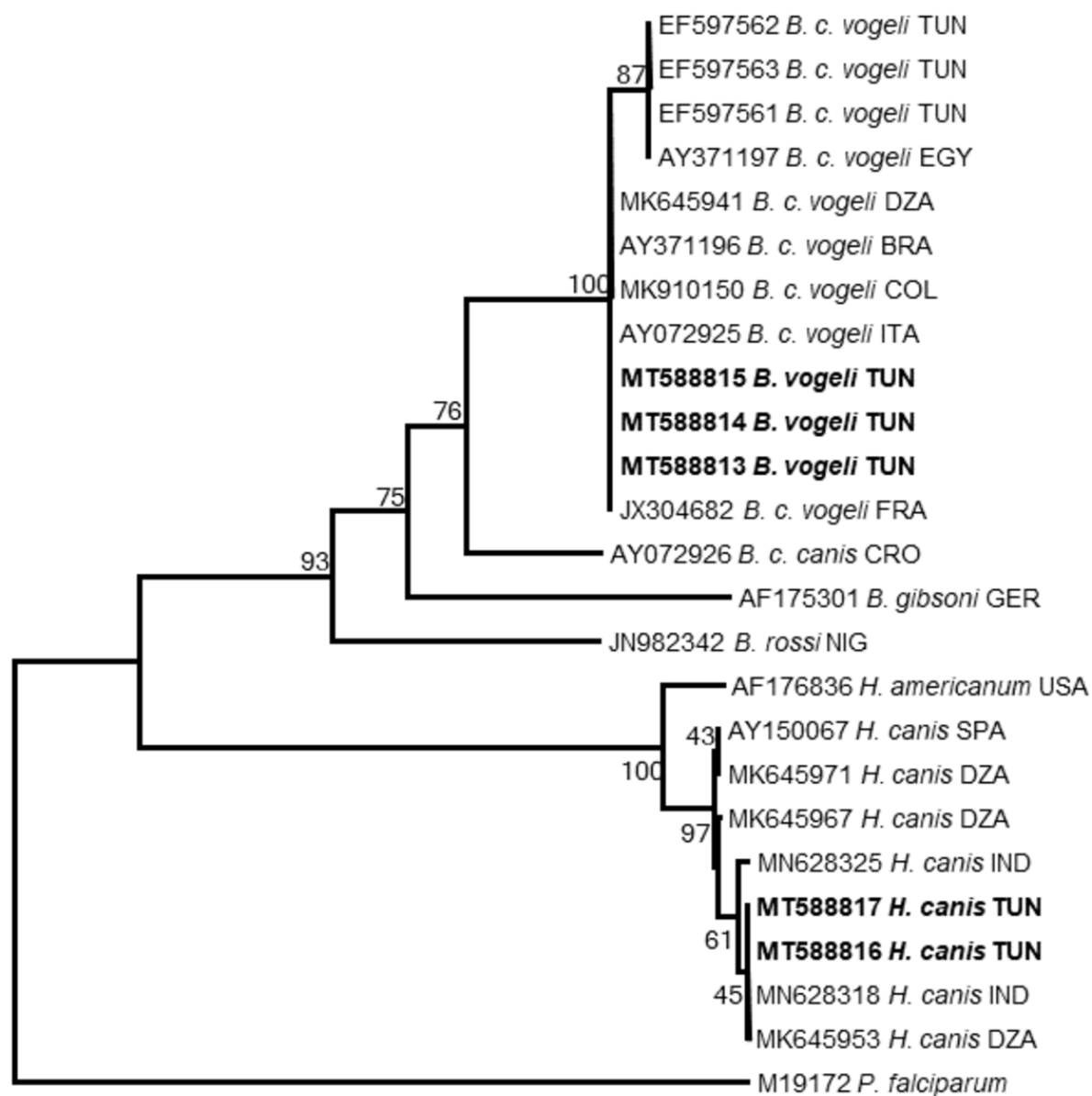
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Table 1. Results of sequencing PCR products and hematological values in dogs that showed positive for the first PCR

Fig. 1: A phylogenetic tree obtained using a small ribosomal subunit sequence *Theileria annulata*, 10 *Babesia* species/isolates and 6 *Hepatozoon canis* isolates. The Kimura-2 method was used to calculate distances, and tree topology was inferred by neighbour joining (MEGA 5.2 software). A total of 354 informative positions were used in the analysis. Numbers at the nodes represent the bootstrap support in 1000 replications. Scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. New sequences are marked in bold.



0.02

Table1 . Results of sequencing PCR products, clinical signs, and hematological values in dogs revealed positive by the PCR

Dog no.	Age / year	<i>Babesia vogeli</i>	<i>H. canis</i>	Accession number	Red blood cells (10 ⁶ /μl)	White blood cells (10 ³ /μl)	Hematocrit (%)	Hemoglobin (g/dl)	Platelet Count (10 ³ /μl)	T °C	Clinical signs
Reference range*					5.50 - 8.50	6.00 -17.0	37.0 - 55.0	12.0 - 18.0	200.0 - 500.0	38-39	
15	2	+		MT588815	6.40	11.1	44	13	66	40.3	Anorexia, dehydrated, depression
18	1	+		MT588814	7.09	7	54.4	15.8	120	41.3	Anorexia, dehydrated, depression
32	3		+	MT588816	5.11	15.4	28	11.4	569	39.5	Anorexia, lethargy
51	1.5	+		MT588813	5.20	5.2	30	9	140	39.4	Anorexia, lethargy, weakness
93	1		+	MT588817	5.54	9.9	25	8.63	292	39.7	Anorexia, Weakness

*From Sasanelli et al. (2009)

+ = sequencing PCR products result