



HAL
open science

Hepatozoon canis and Babesia vogeli infections of dogs in Tunisia

Ali Bouattour, Ahmed Chabchoub, Iheb Hajjaji, Youmna M'Ghirbi

► **To cite this version:**

Ali Bouattour, Ahmed Chabchoub, Iheb Hajjaji, Youmna M'Ghirbi. Hepatozoon canis and Babesia vogeli infections of dogs in Tunisia. *Veterinary Parasitology: Regional Studies and Reports*, 2021, 23, pp.100512. 10.1016/j.vprsr.2020.100512 . hal-03493214

HAL Id: hal-03493214

<https://hal.science/hal-03493214>

Submitted on 15 Dec 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

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

2

3 Ali Bouattour ^{a*}, Ahmed Chabchoub ^{ab}, Iheb Hajjaji ^b, Youmna M'ghirbi ^a

4

5 ^a Laboratoire de Virus, Vecteurs et Hôtes, Institut Pasteur de Tunis, Université Tunis El
6 Manar, 13, Place Pasteur, 1002, Tunis-Belvédère. Tunisia.

7 ^b Ecole Nationale de Médecine Vétérinaire, Sidi Thabet, Université Manouba- Tunisia

8 Ahmed Chabchoub : chabchoubahmed2009@yahoo.fr

9 Iheb Hajjaji : ihebhajjaji@hotmail.fr

10 Youmna M'ghirbi : youmna.mghirbi@pasteur.tn

11 Corresponding author.

12 E-mail: ali.bouattour@pasteur.tn

13 Postal address: Institut Pasteur de Tunis, 13 Place Pasteur, 1002 Tunis

14 Tele: +2016 71893340, Fax: +21671791833

15

16 **Abstract**

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

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

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

29

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

32

33 **1. Introduction**

34

35 Species of *Babesia* and *Hepatozoon* infect a wide variety of mammals, including dogs
36 (Smith, 1996; Solano-Gallego and Baneth, 2011). Some of the pathogens transmitted to
37 dogs by ticks, such as hemoprotozoa, cause severe disease and serious illness. Several
38 *Babesia* species, including *B. canis*, *B. rossi*, *B. vogeli* and *B. gibsoni*, which are the
39 primary causes of babesiosis, have been reported in dogs (Carret et al., 1999; Irwin,
40 2009; Solano-Gallego et al., 2016; Baneth et al., 2019) and can cause infections whose
41 clinical and pathological signs range from subclinical to severe (fever, haemolytic
42 anemia, splenomegaly, thrombocytopenia...) and even death (Solano-Gallego and
43 Baneth, 2011).

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

50 2011), in North and South America (Birkenheuer et al., 2005; Passos et al., 2005), Asia
51 (Abd Rani et al., 2011) and in Australia (Jefferies et al., 2003).

52 Active during the warm season in Tunisia, *R. sanguineus* heavily infests dogs and can
53 be a vector for several pathogens. In addition to *B. vogeli*, this tick species was reported
54 to be infected with *Anapalsma platys*, *Rickettsia massiliae* Bar 29 and *Rickettsia conorii*
55 (Bouattour et al., 1999; Sarih et al., 2005; M'ghirbi and Bouattour 2008; Khrouf et al.,
56 2014). However, haemoparasites of the genera *Hepatozoon* have never been reported in
57 dogs in Tunisia. Canine infections with *H. canis* have been reported in many parts of
58 Africa (Ibrahim et al., 1989; Oyamada et al., 2005, Medkour et al., 2020), Europe,
59 America, Asia, and Australia (Eiras et al., 2007; Li et al., 2008; Abd Rani et al.; 2011;
60 Barker et al., 2012). The cosmopolite brown dog tick *R. sanguineus* was ascertained to
61 be the primary invertebrate host for this Protozoa (Christophers, 1907).

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

67
68

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

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

87

88 **2. Materials and methods**

89 2.1. Dogs and blood samples

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

98 2.2. DNA extraction

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

105

106 2.3. PCR

107 PCR was performed using a set of primers that amplified a 460 to 520 bp fragment in
108 the V4 variable region of the 18S rRNA of *Babesia* and *Theileria* species (Georges et
109 al., 2001). Sequences of genus-specific primers were used to amplify the V4 region of
110 the 18S rDNA were RLB-F2 5'-GACACAGGGAGGTAGTGACAAG-3' and RLB-R2
111 -5'-CTAAGAATTTACCTCTGACAGT-3'. In our experiments, sequencing showed
112 that these primers, previously reported as being able to amplify the *Babesia* and
113 *Theileria* genus, also amplified *Hepatozoon*.

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

117 The reactions were performed on an automated DNA thermal cycler (Perkin-Elmer
118 2720, CA) as follows: starting with 15 min at 95° C and followed by 45 cycles of 94° C
119 for 35 sec; 51° C for 35 sec and 72° C for 35 sec and a final elongation, 72° C for 10
120 min. Extraction controls and PCR negative (water) controls were included in each PCR
121 reaction. Plasmid including the amplicon of the V4 region of the 18S rRNA gene of
122 *Babesia canis* species was used as a positive control.

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

125

126

127 2.4. DNA sequencing and data analysis

128 To confirm the PCR results, positive *Babesia/Theileria* amplified DNA (n=5) was
129 analyzed by sequencing. The PCR products were purified using the ExoSAP clean-up
130 procedure (Amersham Biosciences, Germany). All nucleotide sequences were obtained
131 using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) and the
132 3130 automated sequencer (Applied Biosystems).

133 The sequences generated in this study were edited and aligned using BioEdit (Hall,
134 1999) and ClustalW software programs (Thompson et al., 1994). The BLAST program
135 (<http://www.ncbi.nlm.nih.gov/BLAST>) was used to compare and analyze the data
136 sequences.

137 2.5. Phylogenetic analysis

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

143 2.6. Nucleotide sequences data

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

146 2.7. Hematological analysis

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

149

150

151

152

153 **3. Results**

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

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

169

170 **4. Discussion**

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

182 In our study, the DNA of 99 dogs presenting various clinical manifestations to the
183 hospital of the Veterinary School of Sidi Thabet (Tunisia) was screened by PCR using a
184 set of a genus-specific primers amplifying *Babesia* and *Theileria* species (Georges et al.,
185 2001). After sequencing the positive amplicons, we discovered that these same primers
186 can also amplify *Hepatozoon*. Oyamada et al. (2005) have reported similar results using
187 *Babesia* spp. primers. Sequencing analysis of the positive amplicons that were obtained
188 revealed that *Babesia vogeli* was present in three dogs (15, 18 and 51) and *Hepatozoon*
189 *canis* was present in two dogs (32 and 93).

190 Our results support and confirm the presence of *B. vogeli* in dogs in Tunisia as we have
191 previously reported. However, in the investigated sick dogs, the prevalence of the
192 infection was only 3%, which is lower than that reported in our previous investigation
193 (6.6%) on randomly studied dogs (M'ghirbi and Bouattour, 2008). In fact, infections in
194 dogs with this large *Babesia* have been reported with prevalence range values that vary
195 widely by locality: *B. vogeli* prevalence was 13.2% among 227 dogs screened by PCR

196 in Algeria a neighboring country (Medkour et al., 2020); 2% in 78 randomly chosen
197 dogs from eastern Sudan (Oyamada et al., 2005); 0.6% in dogs with clinical
198 manifestations in Nigeria, (Kamani et al., 2013); in southern France, the prevalence was
199 13.6 % in 140 dogs tested by nested PCR (René-Martellet et al., 2015); in southeastern
200 Rio de Janeiro (Brazil), the prevalence was 15.66 % in 390 dogs tested by qPCR
201 (Paulino et al., 2018). The variation in prevalence might be attributable to several
202 factors including the DNA extraction methodology, the primers that were used and the
203 targeted gene, which can have different sensitivities (Valkiunas et al., 2008). In
204 addition, host-related variables such as age or breed and mainly tick infestations should
205 also be considered when comparing varying levels of regional prevalence.

206 In addition to *B. vogeli*, we detected *H. canis* in the group of investigated dogs. This
207 study is the first molecular report of *H. canis* in Tunisia. This parasite has been reported
208 recently in dogs in Algeria with a prevalence of 41% (Medkour et al., 2020). In
209 addition, this *Hepatozoon* species was recorded in several countries of Africa: Sudan,
210 Nigeria and Zambia with prevalence rates of 42.3%, 41.4%, and 13% respectively
211 (Oyamada et al., 2005; Kamani et al., 2013, 2019; Williams et al., 2014). Studies in
212 several European countries have reported different prevalence rates from low (1% and
213 3% in France and Spain respectively; Criado-Fornelio et al., 2009; Tabar et al., 2009) to
214 high (50.6% in southern Italy; Otranto et al., 2011). The prevalence of *H. canis*
215 similarly varies by country in America: 53.3% in Brazil (Rubini et al., 2008) to 2.5% in
216 the USA, where *H. canis* occurs together with *H. americanum* (Allen et al., 2008). Dogs
217 in several counties in Asia were also reported to show prevalence: in India where the
218 hemoparasite was identified for the first time, the rate of prevalence was 30% (Abd
219 Rani et al., 2011).

220 *Babesia vogeli* and *Hepatozoon canis* are canine hematic Protozoa that are widespread
221 due to the cosmopolitan nature of their main vector, *Rhipicephalus sanguineus* (Shaw et
222 al., 2001; M'ghirbi and Bouattour, 2008; Irwin, 2009; Solano-Gallego and Baneth,
223 2011), the most prevalent tick species infesting dogs worldwide. However, while
224 *Babesia* is transmitted to a dog by the tick's salivary gland during the blood meal
225 (Solano-Gallego et al., 2016), *H. canis* is transmitted by the ingestion of ticks
226 containing mature oocysts (Baneth et al., 2001, 2003).

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

244

245 **5. Conclusion**

246 In Tunisia, warm weather from March to October prolongs the active season of
247 *Rhipicephalus sanguineus* ticks that heavily infest dogs. This tick is a vector of several
248 pathogens such as species of *Ehrlichia* and *Babesia*, previously reported (Sarih et al.,
249 2005; M'ghirbi and Bouattour, 2008), and *Hepatozoon canis* that is described in this
250 work. It should be noted that co-infections can occur in dogs, which might partially
251 explain the variations in often unspecific clinical presentations (Shaw et al., 2001;
252 Sasanelli et al., 2009; Kamani et al., 2013). It is therefore clinically important that these
253 diseases be diagnosed with a sensitive, specific, simple method and that the veterinary
254 practitioner be aware of the existence of these pathogens in Tunisia and select a
255 treatment adequate to each one.

256

257 **Ethical statement**

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

262 **Conflict of interest**

263 The authors declare that there is no conflict of interest.

264 **Acknowledgments**

265 We thank Ministry of High Education and Research of Tunisia (Direction Generale de
266 la Recherche) for financial support. We thank also Dr. Deborah Glassman, Washington
267 D.C., for English corrections.

268 **Authors' contribution:** A.B (MDV, PhD) conceived, designed and wrote the
269 manuscript. A. Ch (MDV, Professor) and I. H. (MDV) were the veterinary clinicians
270 who examined the dogs and performed the hematological analysis.
271 Y.M (PhD) performed the molecular analysis and contributed to the manuscript.
272 All authors read and approved the final manuscript.

273 **References**

- 274 Abd Rani, P.A.M., Irwin, P.I., Coleman G.T., Gatne, M., Traub R.J., 2011. A survey of
275 canine tick-borne diseases in India. *Parasit. Vectors*, 4:141-148.
- 276 Allen K.E, Li, Y., Kaltenboeck, B., Johnson, E.M., Reichard, M.V., Panciera, R.J.,
277 Little, S.E., 2008. Diversity of *Hepatozoon* species in naturally infected dogs in the
278 southern United States. *Vet. Parasitol.* 154:220–225.
- 279 Baneth, G., Barta, J.R., Shkap, V., Martin, D.S., Macintire, D.K., Vincent-Johnson N.,
280 2000. Genetic and antigenic evidence supports the separation of *Hepatozoon canis* and
281 *Hepatozoon americanum* at the species level. *J. Clin. Microbiol.* 38:1298–1301.
- 282 Baneth, G., Cardoso, L., Brilhante-Simoes, P., Schnittger, L., 2019. Establishment of
283 *Babesia vulpes* n. sp. (Apicomplexa: Babesiidae), a piroplasmid species pathogenic for
284 domestic dogs. *Parasite. Vector*, 12:129-137.
- 285 Baneth, G., Mathew, J.S., Shkap, V., Macintire, D.K., Barta, J.R., Ewing, S.A., 2003.
286 Canine hepatozoonosis: two disease syndromes caused by separate *Hepatozoon* spp.
287 *Trends. Parasitol.* 19:27–31.
- 288 Baneth, G., Samish, M., Alekseev, E., Aroch, I., Shkap, V., 2001. Transmission of
289 *Hepatozoon canis* to dogs by naturally-fed or percutaneously injected *Rhipicephalus*
290 *sanguineus* ticks. *J. Parasitol*, 87: 606-611.

291 Barker, E.N., Langton D.A., Helps C.R., Brown, G., Malik, R., Shaw, S.E.,
292 Tasker, S., 2012. Haemoparasites of Free-Roaming Dogs Associated With Several
293 Remote Aboriginal Communities in Australia. *BMC Vet. Res.*, 14; 8:55.

294 Birkenheuer, A.J., Correa, M.T., Levy, M.G., Breitschwerdt, E.B., 2005. Geographic
295 distribution of babesiosis among dogs in the United States and association with dog
296 bites: 150 cases (2000–2003). *J. Am. Vet. Med. Assoc.* 227, 942–947.

297 Bouattour, A., Darghouth, M.A., Daoud, A., 1999. Distribution and ecology of ticks
298 (Acari: Ixodidae) infesting livestock in Tunisia: an overview of eight years of field
299 collection. *Parassitologia* 41:5–10.

300 Caccio, S.M., Antunovic, B., Moretti, A., Mangili, V., Marinculic, A., Baric, R.R.,
301 Slemenda, S.B., Pieniazek, N.J., 2002. Molecular characterisation of *Babesia canis*
302 *canis* and *Babesia canis vogeli* from naturally infected European dogs. *Vet. Parasitol.*
303 106, 285–292.

304 Carret, C., Walas, F., Carcy, B., Grande, N., Precigout, E., Moubri, K., Schetters, T.P.,
305 Gorenflot, A., 1999. *Babesia canis canis*, *Babesia canis vogeli*, *Babesia canis rossi*:
306 differentiation of the three subspecies by a restriction fragment length polymorphism
307 analysis on amplified small subunit ribosomal RNA genes. *J. Eukaryot. Microbiol.* 46,
308 298–303.

309 Christophers, S.R., 1907. The sexual life cycle of *Leucocytozoon canis* in the tick.
310 Scientific Memoirs by the Officers of the Medical and Sanitary Departments of the
311 Government of India 28: 1–11.

312 Collett, M.G., 2000. Survey of canine babesiosis in South Africa. *J. S. Afr. Vet. Assoc.*
313 71:180–186.

314 Criado-Fornelio, A., Buling, A., Pingret J.L., Etievant, M., Boucraut-Baralon, C.,
315 Alongi, A., Agnone, A., Torina, A., 2009. Hemoprotozoa of domestic animals in
316 France: prevalence and molecular characterization. *Vet Parasitol.* 159.1:73-76.

317 Criado-Fornelio, A., Rey-Valeiron, C., Buling, A., Barba-Carretro, J.C., Jefferies, R.,
318 Irwin, P., 2007. New advances in molecular epizootiology of canine hematic Protozoa
319 from Venezuela, Thailand and Spain. *Vet. Parasitol.* 144, 261–269.

320 Eiras, D.F., Basabe, J., Scodellaro, C.F., Banach, D.B., Matos, M.L., Krimer, A.,
321 Baneth, G., 2007. First molecular characterization of canine hepatozoonosis in
322 Argentina: evaluation of asymptomatic *Hepatozoon canis* infection in dogs from
323 Buenos Aires. *Vet. Parasitol.* 149, 275–279.

324 Georges, K., Loria, G.R., Riili, S., Greco, A., Caracappa, S., Jongejan, F., Sparagano,
325 O., 2001. Detection of haemoparasites in cattle y reverse line blot hybridisation with a
326 note on the distribution of ticks in Sicily. *Vet. Parasitol.* 99, 273-86.

327 Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and
328 analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.*, 41:95–98.

329 Ibrahim, N.D., Rahamathulla, P.M., Njoku, C.O., 1989. Neutrophilmyeloperoxidase
330 deficiency associated with canine hepatozoonosis. *Int. J. Parasitol.* 19:915–918.

331 Inokuma, H., Yoshizaki, Y., Matsumoto, K., Okuda, M., Onishi, T., Nakagome, K.,
332 Kosugi, R., Hirakawa, M., 2004. Molecular survey of *Babesia* infection in dogs in
333 Okinawa, Japan. *Vet. Parasitol.* 121:341–346.

334 Irwin, P.J., 2009. Canine babesiosis: From molecular taxonomy to control. *Parasite.*
335 *Vector.* 2 (Suppl. 1): S4.

336 Ivanov, A., Tsachev, I., 2008. *Hepatozoon canis* and hepatozoonosis in the dog. *TJS.* 6.
337 2. 27-35

338 Jefferies, R., Ryan, U.M., Muhlneckel, C.J., Irwin, P.J., 2003. Two species of canine
339 *Babesia* in Australia: detection and characterization by PCR. J. Parasitol. 89, 409–412.

340 Kamani, J., Baneth, G., Mumcuoglu, K.Y., Waziri, N.E., Eyal, O., Guthmann, Y.,
341 Harrus, S., 2013. Molecular detection and characterization of tick-borne pathogens in
342 dogs and ticks from Nigeria. PLoS Negl. Trop. Dis. 7 (3), e2108.

343 Kamani J., Baneth G., Shimon Harrus S., 2019. An Annotated Checklist of Tick-Borne
344 Pathogens of Dogs in Nigeria. Vet Parasitol. Reg. Stud. Reports. 15,100255-1-9.

345 Khrouf, F., M'ghirbi, Y., Znazen, A., Ben Jemaa, M., Hammami, A., Bouattour, A.,
346 2014. Detection of *Rickettsia* in *Rhipicephalus sanguineus* ticks and *Ctenocephalides*
347 *felis* fleas from southeastern Tunisia by reverse line blot assay. J. Clin. Microbiol. 52(1).
348 268-274.

349 Köster, L., Lobetti, R., Kelly, P., 2015. Canine babesiosis: a perspective on clinical
350 complications, biomarkers, and treatment. Vet. Med.: Res. and Reports. 6. 119-128.

351 Li, Y., Wang, C., Allen, K.E., Little, S.E., Ahluwalia, S.K., Gao, D., Macintire, D.K.,
352 Blagburn, B.L., Kaltenboeck, B., 2008. Diagnosis of canine *Hepatozoon* spp. infection
353 by quantitative PCR. Vet Parasitol. 157. (1-2), 50–58.

354 M'ghirbi, Y., Bouattour, A., 2008. Detection and molecular characterization of *Babesia*
355 *canis vogeli* from naturally infected dogs and *Rhipicephalus sanguineus* ticks in
356 Tunisia. Vet. Parasitol. 152, 1–7.

357 Medkour, H., Laidoudi, Y., Lafri, I., Davoust, B., Mekroud, A., Bitam, I., Mediannikova,
358 O., 2020. Canine vector-borne Protozoa: Molecular and serological investigation for
359 *Leishmania* spp., *Trypanosoma* spp., *Babesia* spp., and *Hepatozoon* spp. In dogs from
360 Northern Algeria. Vet Parasitol. Reg. Stud. Reports. 19, 100353,

361 Otranto, D., Dantas-Torres, F., Weigl, S., Latrofa, M.S., Stanneck, D., Decapriaris, D.,
362 Capelli, G., Baneth, G., 2011. Diagnosis of *Hepatozoon canis* in young dogs by
363 cytology and PCR. *Parasit. Vectors*, 4:55.

364 Oyamada, M., Davoust, B., Boni, M., Dereure, J., Bucheton, B., Hammad, A., Itamoto,
365 K., Okuda, M., Inokuma, H., 2005. Detection of *Babesia canis rossi*, *B. canis vogeli*,
366 and *Hepatozoon canis* in dogs in a village of eastern Sudan by using a screening PCR
367 and sequencing methodologies. *Clin. Diagn. Lab. Immunol.* 12, 11, 1343–1346.

368 Passos, L.M., Geiger, S.M., Ribeiro, M.F., Pfister, K., Zahler-Rinder, M., 2005. First
369 molecular detection of *Babesia vogeli* in dogs from Brazil. *Vet. Parasitol.* 127, 81–85.

370 Paulino, P.G., Pires, M.S., da Silva, C.B., Peckle, M., da Costa R.L., Vitari, G.L.V., de
371 Abreu A.P.M., Massard, C.L., Santos, H.A., 2018. Molecular epidemiology of *Babesia*
372 *vogeli* in dogs from the southeastern region of Rio de Janeiro, Brazil. *Vet. Parasitol.*
373 *Reg. Stud. Reports*, 13, 160–165.

374 René-Martellet, M., Moro, C.V., Chêne, J., Bourdoiseau, G., Chabanne, L., Mavingui,
375 P., 2015. Update on epidemiology of canine babesiosis in Southern France. *BMC Vet.*
376 *Res.* 11:223–234.

377 Rubini, A.S., Paduan, K.S., Lopes, V.V.A., O’Dwyer, L.H., 2008. Molecular and
378 parasitological survey of *Hepatozoon canis* (Apicomplexa: Hepatozoidae) in dogs from
379 rural area of São Paulo state, Brazil. *Parasitol. Res.* 102, 895–899.

380 Sarih, M., M’ghirbi Y., Bouattour, A., Gern, L., Baranton, G., Postic, D., 2005.
381 Detection and identification of *Ehrlichia* spp. in Ticks collected in Tunisia and
382 Morocco. *J. Clin. Microbiol.* 43:1127–1132.

383 Sasanelli, M., Paradies, P., Lubas, G., Otranto, D., de Capriaris D., 2009. Atypical
384 clinical presentation of coinfection with *Ehrlichia*, *Babesia* and *Hepatozoon* species in a
385 dog. *Vet. Record.* 164, 22–23.

386 Shaw S.E., Day M.J., Birtles R.J. Breitschwerdt, E.B., 2001. Tick-borne infectious
387 diseases of dogs. Trends in Parasitology.17(2).74-80

388 Smith, T.G., 1996. The genus *Hepatozoon* (Apicomplexa: Adeleina). J. Parasitol. 82,
389 565-585.

390 Solano-Gallego, L., Baneth, G., 2011. Babesiosis in dogs and cats-Expanding
391 parasitological and clinical spectra. Vet. Parasitol.181. 48– 60,

392 Solano-Gallego, L., Sainz, A., Roura, X., Estrada-Peña, A., Miró, G., 2016. A review of
393 canine babesiosis: the European perspective. Parasit. Vectors. 9: 336.

394 Tabar, M.D., Francino, O., Altet, L., Sánchez, A., Ferrer, L., Roura, X., 2009. PCR
395 survey of vector borne pathogens in dogs living in and around Barcelona, an area
396 endemic for leishmaniasis. Vet. Rec. 24;164 (4):112-116.

397 Tamura, K., 2007. Mega4, Molecular Evolutionary Analysis (MEGA) software, version
398 4.0. Mol. Biol. Evol., 24, 1, 1596-1599.

399 Thompson, J.D., Higgins, D.G., Gibson, T.J., Clustal, W., 1994. improving the
400 sensitivity of progressive multiple sequence alignment through sequence weighting,
401 position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22:4673–
402 4680.

403 Valkiunas, G., Iezhova, T.A., Krizanauskiene, A., Palinauskas, V., Sehgal, R.N.M.
404 Bensch, S., 2008. A comparative analysis of microscopy and PCR-based detection
405 methods for blood parasites. J. Parasitol. 94: 1395–1401.

406 Wahlang, L., Lakshmanan, B., Thomas, N., Bosewell, A., Jain, J.K., Sunanda, C.,
407 Aravindakshan, T. V., 2019. Comparative analysis of conventional and real time PCR
408 for detection of haemoparasites in dogs. IJBT 18.1. 9-15.

409 Williams, B.M., Berentsen, A., Shock, B.C., Teixeira, M., Dunbar, M.R., Becker, M.S.,
410 Yabsley, M.J., 2014. Prevalence and diversity of *Babesia*, *Hepatozoon*, *Ehrlichia*, and

411 *Bartonella* in wild and domestic carnivores from Zambia, Africa. Parasitol. Res., 113,
412 911-918.

413

414

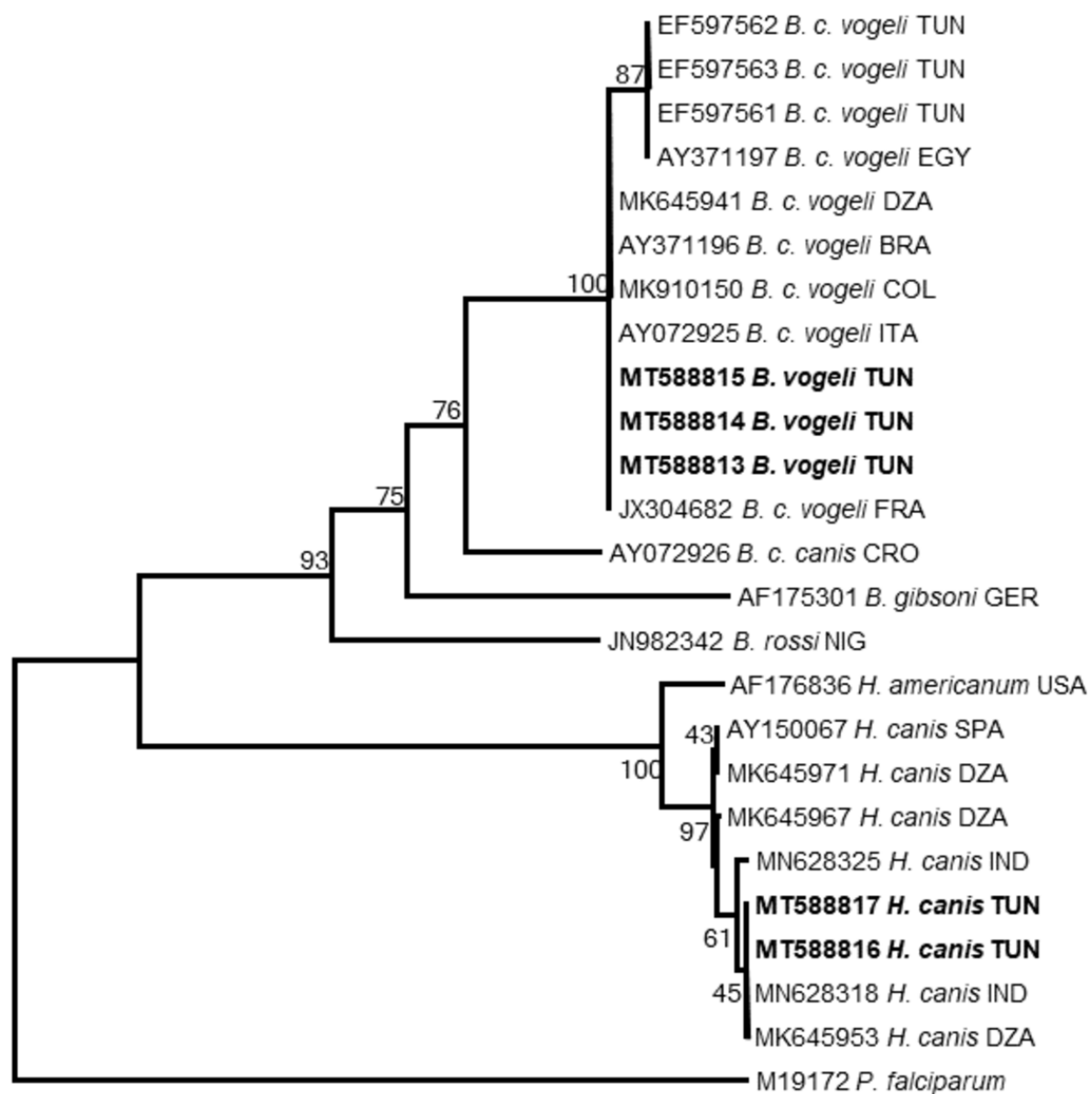
415

416

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

419

420 Fig. 1: A phylogenetic tree obtained using a small ribosomal subunit sequence *Theileria*
421 *annulata*, 10 *Babesia* species/isolates and 6 *Hepatozoon canis* isolates. The Kimura-2
422 method was used to calculate distances, and tree topology was inferred by neighbour
423 joining (MEGA 5.2 software). A total of 354 informative positions were used in the
424 analysis. Numbers at the nodes represent the bootstrap support in 1000 replications.
425 Scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the
426 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