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1 **Research paper**

2 Distribution and risk factors associated with *Babesia* spp. infection in hunting dogs from 3 Southern Italy

4 Vincenzo Veneziano[a], Diego Piantedosi[a,*], Nicola Ferrari[b], Benedetto Neola[a], Mario Santoro[c],
5 Laura Pacifico[a], Giovanni Sgroi[a], Nicola D'Alessio[c], Tullio Panico[d], Christian Leutenegger[e], Phyllis
6 Tyrrell[e], Jesse Buch[e], Edward B. Breitschwerdt[f], Ramaswamy Chandrashekar[e]

7 [a]*Department of Veterinary Medicine and Animal Productions, Università degli Studi di Napoli*
8 *"Federico II", Via F. Delpino 1, 80137 Naples, Italy*

9 [b]*Department of Veterinary Medicine, Università degli Studi di Milano, Via Celoria 10, 20133*
10 *Milan, Italy*

11 [c]*Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute 2, 80055 Portici, Italy*

12 [d]*Centro Interdipartimentale di Ricerca "Ambiente", Università degli Studi di Napoli "Federico II",*
13 *Via Mezzocannone 16, 80134 Naples, Italy*

14 [e]*IDEXX Laboratories, Inc., Westbrook, Maine 04092, USA*

15 [f]*College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607, USA.*
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18 *Corresponding author: Tel.: +39 081 2536004
19 *E-mail address*: dapiante@unina.it (D. Piantedosi)
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Abstract

Babesiosis is a hard tick, vector-borne or bite-transmitted disease of dogs caused by haemoprotozoan organisms of the genus *Babesia*. The aim of the present survey was to determine *Babesia* species prevalence in hunting dogs from Southern Italy and assess related risk factors. Blood samples were collected from 1,311 healthy dogs in the Napoli, Avellino and Salerno provinces of Campania region of Southern Italy. Serological testing was performed using two enzyme-linked immunosorbant assays (ELISA), with one designed to detect *B. canis* and *B. vogeli* antibodies and the other designed to detect *B. gibsoni* antibodies. Blood samples were also tested by real-time polymerase chain reaction (qPCR) assays for amplification of *B. canis*, *B. vogeli* and *B. gibsoni* DNA.

The overall seroprevelance for *B. canis/B. vogeli* was 14.0%, compared to 0.2% for *B. gibsoni*. *B. canis* and *B. vogeli* PCR prevalences were 0.15% and 1.1%, respectively. *B. gibsoni* DNA was not amplified by RT-PCR. Male gender (OR 1.85), adult age (OR 1.01), long hair coat (OR 1.61) and living in Salerno province (OR 1.71) represented risk factors for *B. canis/B. vogeli* seroreactivity. Hunting dogs in Southern Italy are often exposed to *B. canis/B. vogeli;* however, *Babesia* spp. infection was infrequently detected using qPCR. Further studies are needed to determine the extent to which *Babesia* spp. cause clinical disease in hunting dogs, and to evaluate the potential epidemiological relationships between hunting dogs and wild animal populations sharing the same area.

Key words: *Babesia canis*; *Babesia vogeli*; *Babesia gibsoni*; Hunting dogs; Italy.

59 **Introduction**

60 Canine babesiosis is vector-borne disease caused by haemoprotozoan organisms of the
61 genus *Babesia* (Apicomplexa: Piroplasmorida) and transmitted by hard ticks (Ixodidae) throughout
62 much of the world. In Europe, four *Babesia* species have been identified by molecular methods: *B.*
63 *canis*, *B. vogeli*, *B. gibsoni* and *B. microti*-like (reported in the literature as *B.* "Spanish dog isolate",
64 *B. annae*, *Theileria annae* and more recently as *B. vulpes*). Futhermore, these parasites are divided
65 into large (as *B. canis, B. rossi, B. vogeli*) and small (as *B. gibsoni* and *B. microti*-like) morphotypes
66 on the basis of their size in erythrocytes (Lempereur et al., 2017).

67

68 Based upon *Babesia* spp. epidemiological studies in dogs conducted across Europe
69 (Solano-Gallego et al., 2016), prevalence varies due to the species of *Babesia* investigated,
70 geographical area, canine population analyzed, number of samples tested, differences in sensitivity
71 of the diagnostic methods, season of sampling, acaracide use and other tick management practices.
72 In Italy, *B. canis* is referred more diffusely distributed in the northern region (Cassini et al., 2009;
73 Vascellari et al., 2016), coinciding with the distribution of its relevant vector *Dermacentor*
74 *reticulatus*, while *B. vogeli* is mainly reported in Central and Southern Italy, where *Rhipicephalus*
75 *sanguineus* sensu lato is the predominant tick species (Solano-Gallego et al., 2008; Olivieri et al.,
76 2016). In contrast to the large *Babesia* spp., the epidemiology and geographic distribution of *B.*
77 *gibsoni* infection in dogs residing in the Italian Peninsula remains unclear. Trotta et al. (2009)
78 described babesiosis by *B. gibsoni* infection confirmed with PCR in a Pitt Bull Terrier dog living in
79 Rome, without history of tick infestation. The principal vector of *B. gibsoni* is actually unknown,
80 although epidemiological evidence suggests a possible role of *R. sanguineus* sensu lato (Solano-
81 Gallego et al., 2016). In Italy, other modes of transmission, such as dog fighting, are considered
82 unlikely (Yeagley et al., 2009). *B. microti*-like infection has been detected in canine species in
83 different European countries, especially in the Iberian Peninsula (Solano-Gallego et al., 2016).
84 Actually, the red foxes are considered the natural reservoirs of this pathogen in Europe and a source

85 for domestic dog infection (Baneth et al., 2015). It has been suggested that *Ixodes hexagonus* is a
86 potential vector of this parasite in dogs, but *B. microti*-like DNA has been detected in several tick
87 species collected on foxes in Germany (Najm et al., 2014). In Italy, Cassini et al. (2009) detected *B.*
88 *microti*-like DNA in one *R. sanguineus* sensu lato and in two *Ixodes ricinus* ticks collected in
89 central and northern regions of Italian Peninsula, but this *Babesia* species has not yet been identified
90 in dogs living in Italy.
91
92 Clinical manifestations, virulence, prognosis and treatment vary among *Babesia* spp., in
93 conjunction with dog's age, nutrition, immune status and concurrent infections (Schnittger et al.,
94 2012). Fever, splenomegaly, anaemia, jaundice and hemoglobinuria are the most common clinico-
95 pathological disorders reported in sick dogs, regardless of the causative *Babesia* species. In general,
96 *B. canis* is more virulent, while *B. vogeli* causes a relatively mild or non-clinical disease (Köster et
97 al., 2015). The pathogenicity of *B. gibsoni* varies from moderate to severe, but subclinical infections
98 are possible and are common among Pitt Bull Terrier dogs in USA (Köster et al., 2015).
99
100 Although there are few published reports, hunting dogs may be at greater risk for *Babesia*
101 spp. exposures compared to other dogs (e.g. household dogs), due to increased risk of tick
102 infestations and closer contact with wooded and rural areas. In Romania, *B. canis* seroprevalence in
103 hunting dogs was significantly higher compared to other dogs (Imre et al., 2013). A case-control
104 study of *B. microti*-like infection reported hunting lifestyle as a major risk factor for dogs living in
105 Northwestern Spain (Guitiàn et al., 2003).
106
107 The purpose of this study was to determine exposure (serology) and infection (PCR)
108 prevalences, and the distribution of *B. canis*, *B. vogeli* and *B. gibsoni* in hunting dogs from Southern
109 Italy. We also investigated potential risk factors associated with their presence.
110

111 **Materials and Methods**

112 *Study area*

113 The study area had surface of 5,698.81 square km, including the hunting district of Naples
114 (ATC NA), Avellino (ATC AV) and one of the two hunting districts of Salerno (ATC SA 1). These
115 are located in Southern Italy in the provinces of Naples (40° 50′ N - 14° 15′ E), Avellino (40° 54'
116 55" N - 14° 47' 22" E) and Salerno (40° 41' 00" N - 14° 47' 00" E). The territory of the three
117 provinces is contiguous and, those of Naples and Salerno overlook the Tyrrhenian Sea. It has a
118 typical Mediterranean temperate climate along the coast, which becomes progressively continental
119 in the inland and mountainous areas.

120

121 *Study animals and sample size*

122 The study included 1,311 healthy hunting dogs from 153 municipalities representative of
123 the three study provinces and, was conducted as a component of the hunting dog's health assistance
124 program of University of Naples, which was supported by the Italian management committees of
125 the respective hunting districts (ATCs). The study was approved by the Ethical Animal Care and
126 Use Committee of the University of Naples "Federico II" (number of approval 0039904; date of
127 approval 20 October 2014), and written consent was obtained from the owners of the hunting dogs.
128 Blood samples were collected in 36 private veterinary hospitals located in the study area between
129 March and October 2015. Sampling was performed by different veterinary operators during a
130 routine health check.

131

132 Ten milliliters of blood collected by jugular venepuncture after 12 hours of fasting was
133 divided into two fractions. The first fraction was placed in tubes containing potassium ethylene
134 diamine tetra-acetic acid (EDTA) and the second was placed in tubes without anticoagulant,
135 allowed to clot and centrifuged at 908 *g* for 15 min at 4 °C. Whole blood and serum samples were
136 stored at -80 °C and defrosted immediately before batch analysis.

137 The sample size to estimate prevalence was calculated using the formula proposed by Thrusfield
138 (1995) for a theoretically “infinite” population considering the following epidemiological data:
139 expected seroprevalence of 2% for *B. canis* based on the results of a similar study in the general
140 canine population from Northeast Italy (Vascellari et al., 2016); confidence interval (99%) and
141 desired absolute precision (1%).

142

143 A questionnaire was submitted to each owner to obtain information about the dog's
144 locality, breed category, type of coat (short and long hair), body size (small, medium, large), age
145 (registered as continuous variable), gender, pack size when cohabiting with other dogs (registered
146 as continuous variable), contact with other pet or farm animals (dogs, cats, horses and ruminants),
147 living environment (rural or urban), hunting months, hunting environment (grassland or
148 bush/woodland), travel abroad, history of tick infestation (estimated number of tick bites) and
149 ectoparasite control practices (frequency of ectoparasiticide treatment, ectoparasiticide drug used,
150 drug administrator, assessment of drug dosage). The distribution of these factors into the sample is
151 summarized in Table 1.

152

153 *Serological assay*

154 Sera were tested for *B. gibsoni* antibodies by a previously described recombinant protein-
155 based ELISA (Cannon et al., 2016). *B. canis* and *B. vogeli*-specific antibodies were detected with a
156 second recombinant antigen ELISA, also described previously (Yang et al., 2012). *B. canis*-derived
157 recombinant protein (IDEXX Laboratories, Inc.) was coated on microtiter plates at 1 μg/mL in
158 0.05M sodium carbonate buffer (pH 9.6). *B. gibsoni*-derived recombinant protein (IDEXX
159 Laboratories, Inc.) was coated on microtiter plates at 0.5 μg/mL in 0.05M sodium carbonate buffer,
160 pH 9.6. Both antigen-coated plates were blocked with 2% Tween-20 (Sigma-Aldrich) in 0.1M Tris
161 buffer, pH 7.4. The plates were incubated with serum samples diluted 1:200 in pH 7.4 sample
162 diluent (IDEXX Laboratories, Inc.), followed by color development with horseradish peroxidase-

163 conjugated rabbit anti-dog IgG (Jackson Immuno Research 304-035-003) diluted 1:2000 in enzyme
164 conjugate diluent, pH 7.4 (IDEXX Laboratories, Inc.) and TMB substrate (SeraCare). Optical
165 density of the resulting color development was measured at 650 nm. Samples were considered
166 positive if the optical density (OD) was greater than OD cut-offs pre-established by receiver-
167 operator curve analysis based on an independent set of known positive and negative canine samples
168 obtained from globally distributed populations characterized by PCR and immunofluorescence
169 assays (data not shown).

170

171 *Molecular assay*

172 *Babesia* spp. real-time PCR was performed after DNA extraction from EDTA-anti-
173 coagulated blood samples at a commercial laboratory as part of a broad screening panel for vector-
174 borne pathogens (Tick/Vector Comprehensive RealPCR Panel Canine, IDEXX Laboratories).
175 Real-time PCR was performed in conjunction with six quality controls, including quantitative PCR-
176 positive control, PCR-negative control, negative extraction control, quantitative DNA internal
177 sample quality control targeting the host 18S rRNA gene complex, an internal positive control
178 spiked into the lysis solution and an environmental contamination monitoring control. Blood
179 samples positive by *Babesia* genus PCR (ssrRNA, AF271082) were subsequently tested using
180 species-specific real-time PCR, including *B. canis* (heat shock protein 70, AB248735), *B. vogeli*
181 (heat shock protein 70, EF527401) and *B. gibsoni* (heat shock protein 70, AB248731). All assays
182 were designed and validated according to industry standards (Applied Biosystems, User Bulletin
183 #3).

184

185 *Statistical analysis*

186 To test the effects of risk factors on the probability of being seropositive for *B. canis*/*B.*
187 *vogeli*, a multiple logistic regression was performed. The serological status (seroreactive *vs.* non-
188 seroreactive) was considered as response variable, while the risk factors collected on the

189 questionnaire were considered as explanatory variables. Odds ratios (OR) were estimated from the
190 coefficient of the logistic regression. All statistical analyses were performed using the software R
191 3.4.2 (R Development Core Team R, 2017) and considering $p < 0.05$ as the threshold for statistical
192 significance. For the estimation of the 95% confidence intervals of the prevalence the package
193 "binom" was used applying the exact method.

194 **Results**

195 The overall *B. canis*/*B. vogeli* seroprevelance was 14.0% (184/1311; 95% C.I. 12.2-16.0%)
196 and 0.2% (3/1311; 95% C.I. 0.05-0.66%) for *B. gibsoni*. PCR overall prevalences for *B. canis* and
197 *B. vogeli* were 0.15% (2/1311; 95% C.I. 0.02-0.54%) and 1.1% (15/1311; 95% C.I. 0.6-1.8%),
198 respectively. *Babesia gibsoni* DNA was not amplified using qPCR. Only one dog PCR-positive for
199 *B. canis* was also antibody-positive, while ten dogs were positive to both PCR for *B. vogeli* and
200 serology. The distribution of the *Babesia* ELISA seroractive and PCR-positive dogs in the study
201 area is shown in Fig. 1.

202 Analyses of *B. canis*/*B. vogeli* seroprevalence in relation to the potential risk factors
203 associated with exposure to *Babesia* parasites is summarized in Table 1. The probability of being
204 ELISA seroreactive was influenced by dog's gender, age, coat and province (living locality). Risk
205 was higher in male dogs (OR 1.85; 95% C.I. 1.29-2.67), increased with age (OR 1.01; 95% C.I.
206 1.01-1.02) and was higher in dogs with long hair coat (OR 1.61; 95% C.I. 1.08-2.41). Dogs living in
207 Avellino and Salerno provinces had the highest risk (OR 1.71: 95% C.I. 1.32-2.59%), while dogs
208 from Naples had the lowest risk (OR 0.94; 95% C.I. 0.49-1.77) for *Babesia* spp. exposure. Due to
209 the low *B. gibsoni* seroprevalence, and low *B. canis* and *B. vogeli* PCR positivity, risk factor
210 statistical analyses were not examined.

211

212 **Discussion**

213 Consistent with previous surveys from Southern Italy (Solano-Gallego et al., 2008; Dantas-
214 Torres et al., 2013), this study documents that hunting dogs in Campania region are most often

215 exposed to *B. vogeli*. This latter *Babesia* species was detected in sick dogs from Central and
216 Southern Italy (16.3 % PCR+; 10/61) (Solano-Gallego et al., 2008). In a longitudinal study
217 involving young dogs exposed to multiple vector-borne pathogens, de Caprariis et al. (2011)
218 reported the presence of *B. vogeli* as single infection, or as co-infection with *Anaplasma platys,* in a
219 kennel located in the Apulia region. In contrast, *B. canis* was mainly detected in dogs with clinical
220 signs referable to tick-borne diseases in Northern Italy (29.1 % PCR+; 30/103) (Solano-Gallego et
221 al., 2008).

222 It is interesting underline that, considering the likelihood of frequent environmental
223 exposure to ticks, *Babesia* spp. PCR prevalence was low in hunting dogs living in Southern Italy.
224 Comparative studies involving other dog populations from the same area of Southern Italy, have not
225 been published. In Northern and Central Italy, exposure to *R. sanguineus* sensu lato in a kennel
226 setting was the most important risk factor for *B. canis* infection (Cassini et al., 2009). In Romania
227 hunting lifestyle was the only factor (OR 4.57) positively associated with *B. canis* seroprevalence in
228 dogs (Imre et al., 2013). In a case-control study in Northwestern Spain, hunting dogs had a 24.2-
229 fold greater risk of contracting *Babesia microti*-like infection than control dogs (Guitiàn et al.,
230 2003).

231 The low *B. canis* PCR prevalence in our study may be explained by a lower abundance of
232 the tick vector, *D. reticulatus* that prefers cool and wet climates. In a recent study, *D. reticulatus*
233 was the only tick species collected on the ground and bushes in two parks located in the Northern
234 Italy (Lombardia region) (Olivieri et al., 2016). *B. canis* DNA has been detected in two *D.*
235 *marginatus* ticks removed from dogs with clinical babesiosis in Northern Italy (Trotta et al., 2012).
236 Cassini et al. (2009) PCR amplified *B. canis* DNA from *R. sanguineus* sensu lato and *B. vogeli*
237 DNA from *I. ricinus,* collected from asymptomatic dogs living in Northern and Central Italy, but
238 further field studies and experimental transmission trials are needed to verify if the vector
239 competence of *Babesia* spp. may differ among tick species geographically.

240 Only a few *B. gibsoni* serological or molecular prevalence studies have been reported for
241 dogs in Europe (Solano-Gallego et al., 2016). Clinical cases of *B. gibsoni* infection have been
242 described in dogs from Spain, Germany, Italy and Romania (Suarez et al., 2001; Hartelt et al., 2007;
243 Trotta et al., 2009; Imre et al., 2013). In our study, both the low *B. gibsoni* seroprevalence and the
244 absence of any PCR-positive animal among a large number of hunting dogs suggests that tick
245 transmission of this *Babesia* spp. may not occur in Southern Italy. The unique *B. gibsoni* clinical
246 case reported in Italy by Trotta et al. (2009) was in a 4-year-old American Pitt Bull Terrier, born in
247 Croatia from a bitch imported from the USA and transferred to Italy at 4 months of age, supporting
248 the possibility of transplacental transmission, which has been demonstrated in the experimental
249 setting (Fukumoto et al., 2005). In the last decade numerous cases of *B. gibsoni* infection have
250 reported outside Asia, where the vector tick is *Haemaphysalis longicornis*. Bite transmission by the
251 exchange of blood and/or saliva among fighting dog breeds, or from fighting breeds to pet dogs, has
252 been reported in the USA (Yeagley et al., 2009).

253 In these hunting dog population, adult age emerged as a risk factor for *B. canis*/*B. vogeli*
254 seroreactivity; this finding is probably due to a cumulative exposure to the vector ticks, as suggested
255 in other studies (Leschnik et al., 2013; Costa-Júnior et al., 2009), rather than a decline in adaptive
256 immunity, related to an impairment of T cell function, evidenced in an experimental mouse model
257 by Vannier et al. (2004). An increased risk of developing canine babesiosis in male dogs was
258 previously described in South Africa in association with *Babesia rossi* infection (Mellanby et al.,
259 2011). Male dogs may have a higher environmental exposure due to more roaming behavior, or
260 alternatively sex-related hormonal differences might influence disease susceptibility (Moore and
261 Wilson, 2002). The potential contribution of tick exposures, gender or genetic effects requires
262 further epidemiological studies. Our data indicates a significantly higher seroprevalence in long
263 hair dogs, because the hard ticks can cling and attach more easily and not be noticed, as described
264 for Komondor dogs in Hungary (Hornok et al., 2006). Finally, differences in *Babesia*
265 seroprevalence between the studied provinces highlight the geographical effects, including vector

266 distribution, density and temporal evolution of life cycles, all of which influence dog's exposure to
267 tick-borne diseases (Duscher et al., 2013).
268 All other observed characteristics were without statistical significance. In the interpretation
269 of the data of our study, it must be considered that the most of the dogs (99.1%) were treated with
270 ectoparasiticide drugs, probably as a result of the information campaigns toward the transmission
271 risks played by tick and other vector borne pathogens. However, the lack of any significant
272 difference among the anti-ectoparasite intervention strategies applied, suggests that they have a
273 similar efficacy, which does not depend on the ectoparasiticide drugs, the administrator, frequency
274 of treatment and criteria of dosage.
275

276 **Conclusions**

277 In conclusion, hunting dog population in Southern Italy shows low prevalence and
278 exposition toward *Babesia* spp. infection. The present study confirms an higher circulation of *B.*
279 *vogeli* within canine population of Southern Italy respect other *Babesia* species, adding useful data
280 to the scarce literature available about epidemiology of canine babesiosis in Italy. Further studies
281 should be addressed to determine the prevalence of clinical babesiosis in hunting dogs, and evaluate
282 the relationship between dogs and populations of wild animals sharing the same area in the
283 epidemiology of *Babesia* spp.
284

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288

289 **Conflict of interest statement**

290 None of the authors has any financial or personal relationships that could inappropriately
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393 **Figure captions**

394 Fig. 1. Distribution map of *Babesia* spp. ELISA seroreactive and PCR-positive hunting dogs in the

395 study area.