Research paper

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

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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 seroprevalence 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.
Introduction

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

Based upon *Babesia* spp. epidemiological studies in dogs conducted across Europe (Solano-Gallego et al., 2016), prevalence varies due to the species of *Babesia* investigated, geographical area, canine population analyzed, number of samples tested, differences in sensitivity of the diagnostic methods, season of sampling, acaricide use and other tick management practices. In Italy, *B. canis* is referred more diffusely distributed in the northern region (Cassini et al., 2009; Vascellari et al., 2016), coinciding with the distribution of its relevant vector *Dermacentor reticulatus*, while *B. vogeli* is mainly reported in Central and Southern Italy, where *Rhipicephalus sanguineus* sensu lato is the predominant tick species (Solano-Gallego et al., 2008; Olivieri et al., 2016). In contrast to the large *Babesia* spp., the epidemiology and geographic distribution of *B. gibsoni* infection in dogs residing in the Italian Peninsula remains unclear. Trotta et al. (2009) described babesiosis by *B. gibsoni* infection confirmed with PCR in a Pitt Bull Terrier dog living in Rome, without history of tick infestation. The principal vector of *B. gibsoni* is actually unknown, although epidemiological evidence suggests a possible role of *R. sanguineus* sensu lato (Solano-Gallego et al., 2016). In Italy, other modes of transmission, such as dog fighting, are considered unlikely (Yeagley et al., 2009). *B. microti*-like infection has been detected in canine species in different European countries, especially in the Iberian Peninsula (Solano-Gallego et al., 2016). Actually, the red foxes are considered the natural reservoirs of this pathogen in Europe and a source
for domestic dog infection (Baneth et al., 2015). It has been suggested that *Ixodes hexagonus* is a potential vector of this parasite in dogs, but *B. microti*-like DNA has been detected in several tick species collected on foxes in Germany (Najm et al., 2014). In Italy, Cassini et al. (2009) detected *B. microti*-like DNA in one *R. sanguineus* sensu lato and in two *Ixodes ricinus* ticks collected in central and northern regions of Italian Peninsula, but this *Babesia* species has not yet been identified in dogs living in Italy.

Clinical manifestations, virulence, prognosis and treatment vary among *Babesia* spp., in conjunction with dog’s age, nutrition, immune status and concurrent infections (Schnittger et al., 2012). Fever, splenomegaly, anaemia, jaundice and hemoglobinuria are the most common clinico-pathological disorders reported in sick dogs, regardless of the causative *Babesia* species. In general, *B. canis* is more virulent, while *B. vogeli* causes a relatively mild or non-clinical disease (Köster et al., 2015). The pathogenicity of *B. gibsoni* varies from moderate to severe, but subclinical infections are possible and are common among Pitt Bull Terrier dogs in USA (Köster et al., 2015).

Although there are few published reports, hunting dogs may be at greater risk for *Babesia* spp. exposures compared to other dogs (e.g. household dogs), due to increased risk of tick infestations and closer contact with wooded and rural areas. In Romania, *B. canis* seroprevalence in hunting dogs was significantly higher compared to other dogs (Imre et al., 2013). A case-control study of *B. microti*-like infection reported hunting lifestyle as a major risk factor for dogs living in Northwestern Spain (Guitián et al., 2003).

The purpose of this study was to determine exposure (serology) and infection (PCR) prevalences, and the distribution of *B. canis*, *B. vogeli* and *B. gibsoni* in hunting dogs from Southern Italy. We also investigated potential risk factors associated with their presence.
Materials and Methods

Study area

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

Study animals and sample size

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

Ten milliliters of blood collected by jugular venepuncture after 12 hours of fasting was divided into two fractions. The first fraction was placed in tubes containing potassium ethylene diamine tetra-acetic acid (EDTA) and the second was placed in tubes without anticoagulant, allowed to clot and centrifuged at 908 g for 15 min at 4 °C. Whole blood and serum samples were stored at -80 °C and defrosted immediately before batch analysis.
The sample size to estimate prevalence was calculated using the formula proposed by Thrusfield (1995) for a theoretically “infinite” population considering the following epidemiological data: expected seroprevalence of 2% for *B. canis* based on the results of a similar study in the general canine population from Northeast Italy (Vascellari et al., 2016); confidence interval (99%) and desired absolute precision (1%).

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

**Serological assay**

Sera were tested for *B. gibsoni* antibodies by a previously described recombinant protein-based ELISA (Cannon et al., 2016). *B. canis* and *B. vogeli*-specific antibodies were detected with a second recombinant antigen ELISA, also described previously (Yang et al., 2012). *B. canis*-derived recombinant protein (IDEXX Laboratories, Inc.) was coated on microtiter plates at 1 µg/mL in 0.05M sodium carbonate buffer (pH 9.6). *B. gibsoni*-derived recombinant protein (IDEXX Laboratories, Inc.) was coated on microtiter plates at 0.5 µg/mL in 0.05M sodium carbonate buffer, pH 9.6. Both antigen-coated plates were blocked with 2% Tween-20 (Sigma-Aldrich) in 0.1M Tris buffer, pH 7.4. The plates were incubated with serum samples diluted 1:200 in pH 7.4 sample diluent (IDEXX Laboratories, Inc.), followed by color development with horseradish peroxidase-
conjugated rabbit anti-dog IgG (Jackson Immuno Research 304-035-003) diluted 1:2000 in enzyme
diluent, pH 7.4 (IDEXX Laboratories, Inc.) and TMB substrate (SeraCare). Optical
density of the resulting color development was measured at 650 nm. Samples were considered
positive if the optical density (OD) was greater than OD cut-offs pre-established by receiver-
operator curve analysis based on an independent set of known positive and negative canine samples
obtained from globally distributed populations characterized by PCR and immunofluorescence
assays (data not shown).

Molecular assay

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

Statistical analysis

To test the effects of risk factors on the probability of being seropositive for *B. canis/B. vogeli*, a multiple logistic regression was performed. The serological status (seroreactive vs. non-
seroreactive) was considered as response variable, while the risk factors collected on the
questionnaire were considered as explanatory variables. Odds ratios (OR) were estimated from the coefficient of the logistic regression. All statistical analyses were performed using the software R 3.4.2 (R Development Core Team R, 2017) and considering p < 0.05 as the threshold for statistical significance. For the estimation of the 95% confidence intervals of the prevalence the package “binom” was used applying the exact method.

Results

The overall *B. canis/B. vogeli* seroprevalence was 14.0% (184/1311; 95% C.I. 12.2-16.0%) and 0.2% (3/1311; 95% C.I. 0.05-0.66%) for *B. gibsoni*. PCR overall prevalences for *B. canis* and *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%), respectively. *Babesia gibsoni* DNA was not amplified using qPCR. Only one dog PCR-positive for *B. canis* was also antibody-positive, while ten dogs were positive to both PCR for *B. vogeli* and serology. The distribution of the *Babesia* ELISA seroreactive and PCR-positive dogs in the study area is shown in Fig. 1.

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

Discussion

Consistent with previous surveys from Southern Italy (Solano-Gallego et al., 2008; Dantas-Torres et al., 2013), this study documents that hunting dogs in Campania region are most often
exposed to *B. vogeli*. This latter *Babesia* species was detected in sick dogs from Central and Southern Italy (16.3 % PCR+; 10/61) (Solano-Gallego et al., 2008). In a longitudinal study involving young dogs exposed to multiple vector-borne pathogens, de Caprariis et al. (2011) reported the presence of *B. vogeli* as single infection, or as co-infection with *Anaplasma platys*, in a kennel located in the Apulia region. In contrast, *B. canis* was mainly detected in dogs with clinical signs referable to tick-borne diseases in Northern Italy (29.1 % PCR+; 30/103) (Solano-Gallego et al., 2008).

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

The low *B. canis* PCR prevalence in our study may be explained by a lower abundance of the tick vector, *D. reticulatus* that prefers cool and wet climates. In a recent study, *D. reticulatus* was the only tick species collected on the ground and bushes in two parks located in the Northern Italy (Lombardia region) (Olivieri et al., 2016). *B. canis* DNA has been detected in two *D. marginatus* ticks removed from dogs with clinical babesiosis in Northern Italy (Trotta et al., 2012). Cassini et al. (2009) PCR amplified *B. canis* DNA from *R. sanguineus* sensu lato and *B. vogeli* DNA from *I. ricinus*, collected from asymptomatic dogs living in Northern and Central Italy, but further field studies and experimental transmission trials are needed to verify if the vector competence of *Babesia* spp. may differ among tick species geographically.
Only a few *B. gibsoni* serological or molecular prevalence studies have been reported for dogs in Europe (Solano-Gallego et al., 2016). Clinical cases of *B. gibsoni* infection have been described in dogs from Spain, Germany, Italy and Romania (Suarez et al., 2001; Hartelt et al., 2007; Trotta et al., 2009; Imre et al., 2013). In our study, both the low *B. gibsoni* seroprevalence and the absence of any PCR-positive animal among a large number of hunting dogs suggests that tick transmission of this *Babesia* spp. may not occur in Southern Italy. The unique *B. gibsoni* clinical case reported in Italy by Trotta et al. (2009) was in a 4-year-old American Pitt Bull Terrier, born in Croatia from a bitch imported from the USA and transferred to Italy at 4 months of age, supporting the possibility of transplacental transmission, which has been demonstrated in the experimental setting (Fukumoto et al., 2005). In the last decade numerous cases of *B. gibsoni* infection have reported outside Asia, where the vector tick is *Haemaphysalis longicornis*. Bite transmission by the exchange of blood and/or saliva among fighting dog breeds, or from fighting breeds to pet dogs, has been reported in the USA (Yeagley et al., 2009).

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

All other observed characteristics were without statistical significance. In the interpretation of the data of our study, it must be considered that the most of the dogs (99.1%) were treated with ectoparasiticide drugs, probably as a result of the information campaigns toward the transmission risks played by tick and other vector borne pathogens. However, the lack of any significant difference among the anti-ectoparasite intervention strategies applied, suggests that they have a similar efficacy, which does not depend on the ectoparasiticide drugs, the administrator, frequency of treatment and criteria of dosage.

Conclusions

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

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Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.
References


Seroprevalence of *Babesia canis* infection in clinically healthy dogs from western Romania. J. Parasitol. 99 (Suppl.1), 161-163.


**Figure captions**

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