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Molecular detection of vector-borne bacteria and protozoa in healthy hunting dogs from Central Italy

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PEER REVIEW

Peer reviewer

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Comments

This is an interesting and well-written paper. The authors investigated the occurrence of some vector-borne pathogens in a category of dogs (hunting dogs) which were few investigated in Italy, even though they were frequently exposed to ticks as a result of the environment.
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ABSTRACT

Objective: To determine the prevalence of vector-borne bacteria and protozoa in hunting dogs living in Central Italy.

Methods: Molecular testing was executed on DNA which was extracted from blood specimens collected from 117 asymptomatic dogs to detect *Anaplasma phagocytophilum, Babesia canis* (*B. canis*), *Bartonella* spp., *Coxiella burnetii* (*C. burnetii*), *Ehrlichia canis, Hepatozoon canis,* and *Leishmania infantum*.

Results: A total of 48 dogs (41.0%) were infested by *Ixodes ricinus* and *Rhipicephalus sanguineus* ticks. Tick-borne infections were observed in 64 (54.7%) animals. More in detail, 38 dogs (32.5%) screened positive for *Hepatozoon canis*, 24 (20.5%) for *Bartonella vinsonii* subsp. *berkhoffii*, 20 (17.1%) for *Leishmania infantum*, 6 (5.1%) for *C. burnetii*, 5 (4.3%) for *B. canis* (3 *B. canis vogeli* and 2 *B. canis canis*), 3 (2.5%) for *Anaplasma phagocytophilum*, and 2 (1.7%) for *Ehrlichia canis*. Mixed infection by 2 agents occurred in 17 (14.5%) subjects, by 3 agents in 7 (6.0%) dogs, and by 4 agents in 1 (0.9%) animal.

Conclusions: The results demonstrated that several vector-borne pathogens were circulating in this region and dogs infected by these agents were usually asymptomatic. A relevant finding was the presence of DNA of *C. burnetii*, a severe zoonotic agent, in the 5.1% of tested dogs, which can be source of infection for their owners not only through tick bites, but also directly with urine, feces and birth products.

KEYWORDS
Arthropod-borne infection, Bacteria, Dog, PCR, Protozoa

1. Introduction

Vector-borne diseases are caused by parasites, bacteria or viruses which are transmitted by hematophagous arthropods. The past few years have seen the emergence of new diseases, or re-emergence of existing ones. These epidemiological changes are supposed to be due to human factors and climatic changes that can influence arthropod distribution and activity[1].

Dogs, in particular hunting ones, are frequently exposed to ticks. Evidence of current or past infections in these animals can be used to determine whether there is a risk of infection by tick-borne pathogens in a given geographic area.

Humans are susceptible to many tick-borne bacteria and protozoa which usually infect animals. Among them, *Coxiella burnetii* (*C.*

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burnetii) may be transmitted by different hematophagous arthropods, even if infection is usually acquired by humans and animals through inhalation of contaminated aerosol or ingestion of contaminated food, which are mainly raw milk and dairy products[2].

Anaplasma phagocytophilum (*A. phagocytophilum*) is a tick-borne bacterium mainly transmitted by *Ixodes ricinus* and a variety of wild animals, including rodents and deer, which act as reservoir hosts. Dogs are accidental hosts, which may develop acute or sub-clinical infections[3].

Several *Bartonella* species are zoonotic with their main natural hosts that exist in felids, canids, rodents and lagomorphs. Canids have been reported as the main reservoirs for *Bartonella vinsonii* subsp. *berkhoffii* (*B. vinsonii* subsp. *berkhoffii*), but other *Bartonella* species have been detected in domestic dogs, including *Bartonella henselae*, which is traditionally associated to cats[4].

Ehrlichia canis (*E. canis*) causes the canine monocytic ehrlichiosis and it is mainly transmitted by the brown dog tick *Rhipicephalus sanguineus* (*R. sanguineus*). Dogs and other canids are the natural hosts of *E. canis*, which has a worldwide distribution. *E. canis* is generally not considered as a zoonotic agent, but some cases of human infection have been reported in Venezuela[5].

Among protozoan parasites, *Babesia canis* (*B. canis*) and *Babesia gibsoni* cause significant disease in dogs. *B. canis* includes three subspecies: *Babesia canis rossi*, usually transmitted by *Haemaphysalis* spp., *Babesia canis canis* (*B. canis canis*) by *Dermacentor* spp., and *Babesia canis vogeli* (*B. canis vogeli*) by *R. sanguineus* ticks[6].

Hepatozoon canis (*H. canis*) which is transmitted by *R. sanguineus* is distributed throughout the old world. Disease associated with the infection is usually asymptomatic, while disease, when present, may range from subclinical and chronic, especially in the absence of concurrent infections, to severe and life-threatening[7].

Canine leishmaniosis due to *Leishmania infantum* (*L. infantum*) is enzootic in Mediterranean countries[1] and it can be responsible for asymptomatic to patient clinical forms. The parasite is injected into the skin of the host by biting of female sandflies, and it is an emerging zoonosis in canine endemic foci.

Wild boar hunting in Central Italy is by largely practiced dogs. These animals spend most of their life outdoor and are frequently exposed to tick infestations. Although the arthropod-borne infections in dogs are well known, data about the prevalence of bacteria and protozoa which were transmitted by haematophagous vectors in canine population living in this region are scant.

The aim of the present study was to determine, by molecular testing, the prevalence of tick-borne bacteria and protozoa, such as *A. phagocytophilum*, *Bartonella* spp., *B. canis*, *E. canis* and *Hepatozoon* spp., in dogs living in Central Italy that were employed for wild boar hunting in the Maremma Region, an area traditionally endemic for canine leishmaniosis due to *L. infantum*.

2. Material and methods

The Maremma Region is a very extensive area, which comprises part of Southwestern Tuscany and part of Northern Lazio. It is characterized by a rich vegetation which varies base on the territory, in particular where sandy coast, palus and forest vegetation are present. Several wild animal species live in the forest area such as wild boars (*Sus scrofa*), foxes (*Vulpes vulpes*), roe deers (*Capreolus capreolus*), fallow deers (*Dama dama*), hares (*Lepus europaeus*), hedgehogs (*Erinaceus europaeus*), badgers (*Meles meles*), porcupines (*Hystrix cristata*), and a wide range of birds.

A PCR survey was conducted in the hunting season (November 2012 to January 2013) to investigate the prevalence of vector-borne agents which infected hunting dogs with no history of recent tick treatments and no overt clinical manifestations. A total of 117 animals, of both genders (69 males and 48 females), aged from 8 to 132 months [(54.0±31.5) months], were randomly selected among dogs which were brought to the veterinary physician because of wild boar attacks during hunting activity.

Ticks occurring on the canine hosts were removed into tubes with 75% ethanol and stored at 20 ˚C . Later, the ticks were morphologically identified[8]. A blood specimen was drawn from the cephalic vein of each animal and DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Milano, Italy) and used for PCR purposes.

2.1. A. phagocytophilum

A primary amplification was carried out to amplify a 932 bp fragment of the 16S rRNA gene of *A. phagocytophilum*, using the primers GE 3a and GE 10r. A nested PCR, with the primers GE 9F and GE 2, amplified a 546 bp fragment of the same gene. Primary and secondary amplifications were performed with the same cycling conditions[9].

2.2. B. canis

B. canis was detected by using primer PIRO-A and antisense oligonucleotide primer PIRO-B that amplify an approximatively 400 bp portion of the small subunit ribosomal DNA of most *Babesia* species. A PCR-restriction fragment length polymorphism analysis of amplification products was carried out using HinfI and TaqI restriction enzymes to discriminate among *B. canis* subspecies[10].

2.3. Bartonella spp.

DNA samples were employed in a PCR assay to identify *Bartonella* genus. The primers p24E and p12B were used to amplify a 296 bp fragment of the *Bartonella* 16S rRNA gene[11].

2.4. C. burnetii

C. burnetii was identified by amplifying a 687 bp fragment of the IS1111a gene using primers Trans-1 and Trans-2[2].

2.5. E. canis

An initial reaction amplified a 478 bp fragment common among all known *Ehrlichia* species employing the primers ECB and ECC[12]. In the nested reaction, a 389 bp of 16S rRNA gene was amplified

only from the first PCR product of *E. canis* with the primers HE3 and ECA[13].

2.6. Hepatozoon spp.

A fragment of the 18S rRNA gene was amplified by PCR, using the primers HepF and HepR to detect the presence of *Hepatozoon* sp., as previously described[14], with slight modifications. The amplification was performed as follows: 95 ℃ for 12 min (for polymerase activation), followed by 34 cycles of 95 ℃ for 30 seconds (denaturation); 57 ℃ for 30 seconds (annealing); 72 ℃ for 1 min and 30 seconds (extension), followed by 72 ℃ for 7 min (final extension).

2.7. L. infantum

DNA was characterized using the primers R221 and R332, and thereafter the primary PCR products were added as a template for the second amplification step, with specific primers R223 and R333, to amplify a *Leishmania* specific gene small subunit ribosomal DNA[15].

PCR products obtained from positive samples for *Hepatozoon* spp., *Bartonella* spp. and *Leishmania* spp. were then sequenced and analyzed. All sequencing procedures were performed by a commercial laboratory (BMR-Genomics, Padova, Italy). Sequences were assembled and corrected by visual analysis of the electropherogram using Bioedit v.7.0.2, then compared with those available in GenBank using the basic local alignment search tool program (http://www.ncbi.nlm.nih.gov/BLAST) to assign the species.

3. Results

A total of 48 animals (41.0%) were infested with ticks, showing a number of parasites ranging from 1 to 11 (2.6±2.1). A total of 119 specimens were recovered and recognized to belong to *Ixodes ricinus* (*n*=83) and *R. sanguineus* (*n*=36). Mixed infestations were recorded in dogs.

Tick-borne infections were observed in 64 out of 117 (54.7%) animals. Among them, 33 dogs were infested with ticks at the sampling time. The remaining 15 tick-infested dogs resulted PCR negative. More in detail, 38 dogs out of 117 (32.5%) screened positive for *Hepatozoon* spp. Analysis of sequences allowed us to recognize *H. canis* species in all samples. A total of 24 (20.5%) dogs scored positive for *Bartonella* spp. Analysis of sequences revealed *B. vinsonii* susp. *berkhoffii* in all cases. *L. infantum* DNA was found in 20 animals, with a prevalence of 17.1%. *C. burnetii* was detected in 6 (5.1%) dogs. *B. canis* DNA was recovered from 5 (4.3%) blood specimens. PCR–restriction fragment length polymorphism revealed the presence of *B. canis vogeli* in 3 samples and *B. canis canis* in 2 dogs. *A. phagocytophilum* was detected in 3 dogs, *E. canis* in 2, with prevalence values of 2.5% and 1.7%, respectively.

Mixed infection by 2 agents occurred in 17 (14.5%) subjects, by 3 agents were detected in 7 (6.0%) dogs, and by 4 in 1 (0.9%) animal. More detailed results are showed in Table 1.

Table 1

Dogs resulted PCR positive to two or more pathogens.

Dog No.	*A. phagocytophilum*	*B. canis*	*B. vinsonii* susp *berkhoffii*	*C. burnetii*	*E. canis*	*H. canis*	*L. infantum*
4	-	-	+	-	+	+	-
7	-	-	+	-	-	-	+
15	-	-	+	-	-	+	+
17	-	-	+	-	-	-	+
18	+	-	+	-	-	-	-
20	+	-	-	-	-	+	-
22	-	-	+	-	-	+	-
25	-	-	+	+	-	-	-
27	-	-	+	+	-	+	+
30	-	-	+	-	-	-	+
31	-	-	+	+	-	+	-
37	-	+*	-	-	-	+	-
38	-	+*	-	-	-	+	-
41	-	+**	+	-	-	-	-
46	-	-	+	-	-	-	+
49	-	-	-	+	-	-	+
51	-	-	+	-	-	+	+
53	-	-	+	-	-	+	-
55	-	-	-	-	-	+	+
63	-	-	-	-	-	+	+
70	-	-	+	-	-	+	+
72	-	-	+	-	-	+	+
74	-	-	+	-	-	+	-
98	-	-	+	-	-	+	-
109	-	-	+	-	-	+	+

+: Positive; -: Negative; *: *B. canis vogeli*; **: *B. canis canis.*

4. Discussion

Ticks in the present study, belonging to most frequently reported species infecting dogs were in agreement with literature[16]. The animals being examined showed an overall prevalence (54.7%) of infection by investigated tick-borne agents. It indicated that such pathogens are very spread among the healthy animals in Central Italy. Mixed infections have been detected in 25 (21.4%) dogs. Infections due to more tick-borne pathogens were quite frequent, as previously reported by other authors[17,18]. They were related to the transmission of pathogens by more ticks and/or arthropod infected by more agents.

The survey was carried out in an endemic area for canine leishmaniosis. The record of 17.1% of *L. infantum* DNA in blood specimens of asymptomatic animals would confirm the presence of the parasite in these subjects.

A relevant prevalence value (32.5%) was observed for *H. canis*. This protozoan showed a higher prevalence, when compared to similar surveys carried out both in Italy and in Europe in healthy subjects. In particular, Cassini *et al.* reported a prevalence of 3.6% in Central and Northern Italy[16]. Furthermore, 11.8% of *H. canis* infected dogs were obtained in Croatia, while 3.3% and 7.0% were reported in Spain and Grenada, respectively[17-19]. Dogs result positive to *H. canis* from autumn, since they become infected during summer. In fact, in this season ticks are abundant and highly infected by the protozoan[20]. For this reason, in the present survey most infections contracted during the previous transmission season were probably detected.

Dogs tested during the present survey resulted positive to *B. vinsonii* subsp. *berkhoffii* with 20.5% prevalence, which confirmed the sensitivity of canine population to this zoonotic pathogen.

Currently, there was little information about *Bartonella* spp. infected dogs in Italy. A previous serological survey detected 6% of *Bartonella henselae* positive dogs[21], whereas DNA of *B. vinsonii* subsp. *berkhoffii* and a novel *Bartonella* sp. strain were found in clinically healthy dogs from South Italy[22].

A relevant result was the 5.1% of animals scored positive to *C. burnetii*. This is the agent of a severe zoonosis called Q fever, which is usually associated to farm animals. Male dogs infected by this pathogen are often asymptomatic, whereas females may develop abortion. Dogs, the same as other animal species, can contract the infection not only by bite of infected ticks, but also by inhalation or ingestion of contaminated materials. It couldn't be excluded that subjects of this investigation had become infected for the direct or indirect contact with infected animals. Considering the environments where they live, it is likely that their infection was due to arthropod bites.

The detection of *C. burnetii* DNA in the blood of examined dogs showed that exposure to this agent was existed to threaten humans, which was not only related to tick bites, but also because infected dogs may excrete the bacteria in feces, urine and birth products[23].

B. canis was recognized in 5 specimens, 3 of them belonged to the subspecies *B. canis vogeli* and the other belonged to *B. canis canis*. These prevalences were in agreement with Cassini *et al*.[16] and Tabar *et al*[18]. The epidemiology of these parasites in Italy would indicated that *B. canis canis* can be considered endemic in Northern Italy, while *B. canis vogeli* was predominantly found in Central and Southern Italy[24].

A. phagocytophilum DNA was found in 2.5% of the examined dogs. Central Italy was considered an endemic area, on the basis of previous serological and molecular investigations carried out in domestic and wild animals. In particular, *A. phagocytophilum* DNA was found in 72.0% of fallow deer[25] and 16.6% of red foxes[26], whereas a lower prevalence (0.9%) was detected in hunting dogs[27]. Results obtained in the present investigation could be not corresponding to the real prevalence value, because of false negative response. In fact, a negative PCR result did not always mean that the animal was not infected, because *A. phagocytophilum* bacteremia in dogs appeared to be of short duration (<28 d)[28].

The lowest detected prevalence was to *E. canis*. Although this canine pathogen is well known, updated data about its prevalence in Italian canine population are scanty. In particular, previous serological investigation showed seroprevalence values of 7.0%[29] and 46.0%[30] in Central and Southern Italy, respectively. A molecular study showed 2.9% of *E. canis* positive dogs in Northern Italy, 8.0% in Central Italy, and 9.7% in Southern Italy[31], which indicated that prevalence rates were influenced by the geographical region.

Obtained data could reflect a low spread of *E. canis* in the Maremma Region, probably because this pathogen had a reduced range of sensitive animal species (canids and rarely cats). Moreover, PCR was not always sensitive, in particular negative results may occur when organisms in circulation were below the level of detection[32].

In conclusion, the high PCR prevalence values which were recorded for some of these agents appeared to be striking, considering that investigated asymptomatic animals were involved in heavy work activities. The present results confirmed that dogs infected by these pathogens often develop asymptomatic or subclinical forms. Moreover, the prevalence values demonstrated the spread of several vector-borne pathogens in areas with ecological features, in particular vegetation and reservoir wild animals, which facilitate the circulation of hematophagous arthropods. Dogs should be periodically monitored for these pathogens, in particular for zoonotic agents, such as *C. burnetii*, in order to promptly identify positive subjects which could be source of infections for their owners.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Vector-borne diseases represent an re-emerging problem, mainly due to the climatic changes. Arthropod-borne dog infections are well known, but data about their occurrence in hunting dogs (especially exposed to ticks) are scarce in Italy.

Research frontiers

The present study evaluated the occurrence of some arthropod-borne infections in hunting dogs living in the Central Italy by molecular testing.

Related reports

The investigated dogs were asymptomatic, but 54.7% of them tested PCR positive for one or more infectious agents. These findings suggest the need to control hunting dogs, especially for zoonotic agents.

Innovations and breakthroughs

An interesting result which concerns the PCR positivity to *C. burnetii,* suggests that canine excreta, other than tick bites, may be a source of infection to humans.

Applications

The results of the present investigation showed a large spread of several vector-borne pathogens in hunting dogs, which are frequently exposed to ticks. As infections are usually subclinical, the risk for owners should be considered.

Peer review

This is an interesting and well-written paper. The authors investigated the occurrence of some vector-borne pathogens in a category of dogs (hunting dogs) which were few investigated in Italy, even though they were frequently exposed to ticks as a result of the environment.

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