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Serological survey of *Borrelia burgdorferi sensu lato*, *Anaplasma phagocytophilum*, and *Ehrlichia canis* infections in rural and urban dogs in Central Italy

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Abstract

Introduction. *Borrelia burgdorferi sensu lato* (*s.l.*) and *Anaplasma phagocytophilum* are well known zoonotic pathogens, whereas *Ehrlichia canis* is usually considered to be of veterinary concern, although on the basis of recent reports it also seems to be able to infect humans.

Objective. The aim of the study was to determine the seroprevalence of *B. burgdorferi s.l.*, *A. phagocytophilum* and *E. canis* in an Italian canine population, and to verify if there are differences between dogs living in urban areas and those from a rural environment.

Materials and method. Blood sera of 1,965 dogs, 1,235 from cities and 730 from rural areas, were tested by indirect immunofluorescent assay (IFAT).

Results. The overall seroprevalence was highest for *E. canis* (7.07%), followed by *A. phagocytophilum* (4.68%), and *B. burgdorferi s.l.* (1.47%). Rural dogs showed the highest seroprevalence to *B. burgdorferi s.l.* and *A. phagocytophilum*. No significant differences were observed between rural and urban *E. canis*-positive dogs. A low percentage (1.32%) of dogs with dual seropositivity was detected, and no triple positive reactions were observed. No significant differences were detected in the seroprevalence of the three agents in relationship to the age and gender of the dogs. Seroprevalence in the five years considered were not statistically different, except for the lowest rate for *E. canis* observed in 2012.

Conclusions. The results confirm the presence of *B. burgdorferi s.l.*, *A. phagocytophilum* and *E. canis* in Italian dogs in both urban and rural areas. Monitoring pet dogs, which share the same environment with their owners, is useful for identifying the presence of tick-borne disease agents of both veterinary and public health significance.

Key words

Borrelia burgdorferi sensu lato, *Anaplasma phagocytophilum*, *Ehrlichia canis*, dog, indirect immunofluorescent assay, zoonosis

INTRODUCTION

Borrelia burgdorferi sensu lato (*s.l.*), *Anaplasma phagocytophilum* and *Ehrlichia canis* are tick-borne pathogens which can elicit serious illness in dogs.

B. burgdorferi s.l. is a spirochete which causes Lyme disease, mainly in humans and dogs. Lyme borreliosis is characterized in humans by an early set of skin-related and flu-like symptoms and, in the absence of treatment, may be followed by arthritic or neurologic complications [1].

The canine disease is often mild with non-specific clinical manifestations, commonly characterized by lameness, fever, anorexia, lethargy, and lymphadenopathy. In some cases, the disease can be severe with arthritis and neurologic dysfunction, whereas glomerulonephritis has been associated to borreliosis in dogs with antibodies against *B. burgdorferi*, even if a causative role of this bacterium in the development of renal disease has not been confirmed [2, 3, 4, 5].

B. burgdorferi is usually transmitted by *Ixodes* sp. ticks, in particular in Europe the main vector is *Ixodes ricinus*. These arthropods are three-host ticks that acquire spirochetes when feeding on rodents, the main reservoirs, as larvae or nymphs, and can then transmit infection as nymphs or adults [6].

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A. phagocytophilum is an obligate intracellular Gram-negative coccus of the family *Anaplasmataceae,* which infects granulocytes, mainly neutrophils. Infection has been reported in dogs, horses, cattle, small ruminants [7]. It also causes the human granulocytic ehrlichiosis (HGE) or anaplasmosis (HGA), characterized by fever, chills, headache, myalgia, anaemia, thrombocytopenia and leukopenia [8].

Dogs naturally infected with *A. phagocytophilum* may remain healthy or manifest clinical signs including fever, lethargy, lameness, reluctance to move, vomiting, diarrhoea, polyuria, polydipsia, nervous system dysfunction [9].

A. phagocytophilum is mainly transmitted by *I. ricinus*, and a variety of wild animals, including rodents and deer, acts as reservoir hosts [10].

E. canis is a gram negative, obligate intracellular bacteria with a tropism for leucocytes. It causes the canine monocytic ehrlichiosis and is transmitted by the brown dog tick *Rhipicephalus sanguineus*. Dogs and other canids are the natural hosts of *E. canis,* which has a worldwide distribution. Infected dogs may develop a febrile illness, and the infection may persist for years. After a period of remission, severe chronic disease may develop, with fever, malaise, inappetence, weight loss, lymphadenopathy, pale mucous membranes, joint pain, bleeding tendency, hyperglobulinemia and pancytopenia [10].

E. canis is generally not considered a zoonotic agent, but some cases of human infection have been recently reported in Venezuela [11].

The presence of *B. burgdorferi, A. phagocytophilum* and *E. canis* has been reported in Italy, but data concerning their prevalence in the canine population are limited. Although it is difficult to compare information from studies using different diagnostic tools, the prevalence of these infections are related to geographical region, density of tick populations, and presence of reservoirs.

The purpose of the presented study was to evaluate, during a 5-year period, the seroprevalence of *B. burgdorferi s.l.*, *A. phagocytophilum* and *E. canis* among dogs living in Central Italy, and to verify if the risk of exposure to tick-borne infections in dogs from rural areas is higher than in those living in an urban environment.

MATERIAL AND METHODS

Animals. From January 2008 – December 2012, peripheral whole blood samples were collected from 1,965 dogs. Animals were included in the study if they were being seen for routine care, and were excluded if being evaluated for suspected vector-borne diseases or if undergoing antibiotic treatment. The collections of blood samples were executed by collaborating veterinarians in their private clinics.

Once received, all samples were given an identification number and catalogued by animal age, gender, habitat (urban or rural). 730 animals were from rural areas and 1,235 were urban dogs. All dogs lived in Central Italy, particularly in the Tuscan province and other districts bordering with Tuscany.

Whole blood samples, drawn from the left or right cephalic vein, were centrifuged at 1,500 × g for 15 min, the sera obtained were collected and tested immediately or stored at -20 °C until examinations.

Indirect immunofluorescence antibody test. The indirect immunofluorescence antibody test (IFAT) was executed on IFAT slides specific for *Borrelia burgdorferi* s.l., *Anaplasma phagocytophilum*, *Ehrlichia canis* (Fuller Laboratories Fullerton, California, USA).

Blood sera were diluted 1:64 and 1:40, the cut-off dilutions for *B. burgdorferi s.l.* and *A. phagocytophilum/E. canis,* respectively, in phosphate-buffered saline (PBS, pH 7.2) and incubated on wells of the slides in a humidified chamber at 37 °C for 30 min. The slides were rinsed three times in PBST (PBS + 0.4% Tween 80 – Sigma-Aldrich, St. Louis, Missouri, USA) and once in distilled water, and then air-dried. Each well of the slides was probed with fluorescein isothiocyanate-conjugated rabbit anti-Dog IgG (Sigma-Aldrich) diluted 1:30 in Evans Blue (Sigma-Aldrich) solution and incubated at 37 °C in a humid chamber for 30 min. The slides were washed and dried as described above and examined with a fluorescence microscope.

Positive samples were two-fold serially diluted to determine the endpoint titre. Scores from 1 – 4 were assigned to the intensity of specific fluorescence, and the antibody titre was defined as the major dilution with a ≥ 2 score.

Statistical analysis. Statistical evaluation was carried out by the χ^2 test to analyze the results of serological tests in relationship to age, gender, and urban or rural habitat of the examined dogs, and to the years in which samples were collected. Values of $P<0.05$ were considered significant.

RESULTS

The number of dogs serologically positive with any of the three pathogens surveyed in this study was 234 (11.9%), 129 (6.56%) from rural areas and 105 (5.34%) from cities.

The number of dogs with singular and dual seropositivity was 208 (10.58%) and 26 (1.32%), respectively. Three rural dogs were coinfected by *B. burgdorferi s.l.* and *A. phagocytophilum*. 23 dogs – 5 from rural and 18 from urban areas – scored positive to *A. phagocytophilum* and *E. canis*. No triple positive reactions were detected.

Among the 1,965 dogs tested, 29 were seropositive to *B. burgdorferi s.l.*, 92 to *A. phagocytophilum* and 139 to *E. canis* with 1.47%, 4.68% and 7.07% total mean seroprevalence, respectively.

No significant differences in the seroprevalence to the three agents were observed in dogs of different ages and gender.

The seroprevalence for *B. burgdorferi s.l.* and *A. phagocytophilum* were statistically higher in rural than in urban dogs (χ^2 test, $p<0.05$), whereas no significant differences were detected in *E. canis* positive dogs living in different habitat.

Data relative to the seroprevalence in relationship to age, gender and habitat are summarized in Table 1.

Table 1. Serological results in relationship to age, gender and habitat of the canine study population

Category		Study population	*B. burgdorferi* s.l. positive (%)	*A. phagocytophilum* positive (%)	*E. canis* positive (%)
Age	< 1	157	3 (1.91)	8 (5.09)	11 (7.00)
	1–5	686	8 (1.16)	39 (5.68)	35 (5.10)
	6–10	892	14 (1.56)	33 (3.69)	72 (8.07)
	>10	230	4 (1.73)	12 (5.21)	21 (9.13)
Gender	Male	1078	17 (1.57)	59 (5.47)	65 (6.02)
	Female	887	12 (1.35)	33 (3.72)	74 (8.34)
Habitat	Urban	1235	7 (0.56)	29 (2.34)	87 (7.04)
	Rural	730	22 (3.01)	63 (8.63)	52 (7.12)
TOTAL		1965	29 (1.47)	92 (4.68)	139 (7.07)

Significant differences in the mean *E. canis* seroprevalence were detected in relationship to the years in which samples were collected (χ^2 test, $p>0.05$); in particular, the lowest value were observed in 2012 (3.39%). No significant differences in the mean seroprevalence to *B. burgdorferi s.l.* and *A. phagocytophilum* were observed in the respective years (Tab. 2).

Table 2. Seroprevalence of *B. burgdorferi s.l., A. phagocytophilum* and *E. canis* among tested dogs in relationship to the different years

Year	No. examined dogs	*B. burgdorferi* s.l. positive dogs	*A. phagocytophilum* positive dogs	*E. canis* positive dogs
2008	507 R 262 U 245	9 (1.77%) R 7 U 2	26 (5.12%) R 21 U 5	45 (8.87%) R 18 U 27
2009	418 R148 U 270	3 (0.71%) R 3 U 0	21 (5.02%) R 14 U 7	29 (6.93%) R 11 U 18
2010	413 R 102 U 311	6 (1.45%) R 3 U 3	14 (3.38%) R 10 U 4	32 (7.74%) R 9 U 23
2011	303 R115 U 188	6 (1.98%) R 4 U 2	14 (4.62%) R 9 U 5	22 (7.26%) R 12 U 10
2012	324 R103 U 221	5 (1.54%) R 5 U 0	17 (5.24%) R9 U 8	11 (3.39%) R 2 U 9

R – rural environment; U – urban environment

The antibody titres to *B. burgdorferi s.l.* varied between 1:64 – 1:512 (Tab. 3), those to *A. phagocytophilum* and *E. canis* between 1:40 – 1:1280 (Tab. 4).

Table 3. Number of dogs positive to *B. burgdorferi s.l.* according to habitat and antibody titres

Agent	Habitat	No. positive dogs at the given antibody titre				Total
		1:64	1:128	1:256	1:512	
B. burgdorferi s.l.	Rural	7	11	3	1	22
	Urban	4	3	-	-	7

Table 4. Number of dogs positive to *A. phagocytophilum* and *E. canis* according to habitat and antibody titres

Agent	Habitat	No. of positive dogs at the given antibody titre						Total
		1:40	1:80	1:160	1:320	1:640	1:1280	
A. phago-cytophilum	Rural	15	22	5	8	11	2	63
	Urban	12	11	6	-	-	-	29
E. canis	Rural	13	21	9	4	4	1	52
	Urban	35	31	17	1	3	-	87

DISCUSSION

On the basis of the results obtained from the presented study, *E. canis* appears to be the most widespread tick-borne pathogen in canine population in Central Italy, with a mean seroprevalence of 7.07%, and no significant differences observed between urban and rural dogs.

E. canis is usually transmitted by *R. sanguineus* ticks which feed on dogs in all stages and can complete their entire life cycle indoors, houses and kennels. *R. sanguineus* ticks are active during the whole year in the Mediterranean area, specifically in Italy. Moreover, due to its high degree of adaptability to a different microenvironment, and its capability to occasionally feed on hosts other than dog, *R. sanguineus* represents one of the major threats not only to dogs, but also to cats and humans. *R. sanguineus* may also be found on wild mammals, thus it is present not only in the indoors environment, but also outside in peri-urban and rural areas. This adaptability to different habitat could explain the similar *E. canis* seroprevalence observed in urban and rural dogs in the presented study.

Since its discovery in 1935, *E. canis* has been considered as a pathogen for dogs, other canids, and rarely for cats. In 1996, Perez et al. [12] reported the first human infection with *E. canis* and culture isolation of an *E. canis* strain, called Venezuelan human *Ehrlichia* (VHE), from an apparently chronically-infected asymptomatic human in Venezuela. *E. canis* is closely related to *Ehrlichia chaffeensis* which causes human monocytic ehrlichiosis (HME), characterized by fever, chill, headache, myalgia, anorexia, nausea or vomiting, meningitis, encephalitis, thrombocytopenia and leukopenia [13].

The cases of human infection by *E. canis* recently observed in Venezuela in symptomatic patients [11], highlight the zoonotic potential of this pathogen, and further investigation should be carried out in the human population of the regions where *E. canis* is present.

The second pathogen affecting the canine population in the presented study appears to be *A. phagocytophilum* (4.68%), with a higher seroprevalence in animals living in a rural environment than in urban dogs.

Previous investigations carried out in dogs from the same geographic area revealed a higher seroprevalence: 8.8% in pet dogs during the years 2004–2007, 14% in hunting dogs in the period 2008–2011 [14, 15].

In the first case, the study was performed on a heterogeneous population, including healthy and symptomatic dogs, whereas in the current survey, the dogs were excluded if they were being evaluated for suspected vector-borne diseases.

The higher seroprevalence recognized in the second investigation was probably due to the environment frequented by the examined hunting dogs; in fact, the hunting areas are characterized by conditions favourable for the arthropods' diffusion, because of the abundant vegetation and presence of animal species, in particular wild animals, that serve as reservoir hosts for *A. phagocytophilum*.

Dogs examined in the presented survey showed the lowest seroprevalence to *B. burgdorferi* (1.47%). In particular, urban dogs had a lower rate than the rural ones.

The presence of Lyme borreliosis has been described in various regions of Italy, especially Trentin, Veneto, and Central-Southern areas of the Italian peninsula [16]. Although an official national surveillance system for reporting infectious diseases was started in Italy in 1990, Lyme borreliosis is under-reported, probably because many human infections are not recognized.

A previous survey on the prevalence and incidence of antibodies to *B. burgdorferi* in asymptomatic agricultural and forestry workers from Tuscany, found about the 7% of positive subjects [17].

Studies on the seroprevalence for *B. burgdorferi* infection within occupational groups at risk have been conducted also in Europe and in the USA, reporting values from 13 – 43% for exposed individuals and from 2.5 – 10% for healthy blood donors [18].

Serosurveys have been performed in dogs living in the USA the USA and overall seroprevalence of 1.2, 4.0, 6.7% have been detected in relationship to the different regions considered [6, 10]. The most recent investigations about the prevalence for *B. burgdorferi* infection in European canine population found different rates in relationship to the geographic area, healthy status of dogs and tests employed. In particular, the prevalences observed were as follows: 0.2–0.5% in Portugal [19], 0.6% [20] and 6.26–8.8% in Spain [21], 1.09% in France [22], 1.7% and 40.2% in Poland [23, 24], 4.5% in Germany [25], 6.52% in Romania [26], 10.3% in the Czech Republic [27] and 25.8% in Serbia [28].

In Italy, data concerning the prevalence of *B. burgdorferi* in dogs are lacking and where they exist are often outdated; it is therefore not possible to affirm if the results of the present investigation are in agreement to the prevalence in dogs living in other Italian regions, or in the same geographic area, but in different periods.

The presented survey detected a low number of co-infected dogs. Three animals from rural areas resulted positive both to *B. burgdorferi s.l.* and *A. phagocytophilum*. This result is not surprising given the shared tick vectors and mammalian reservoirs for the two pathogens.

23 dogs scored positive to *A. phagocytophilum* and *E. canis*. These results could be due to co-infections, but also to serologic cross-reactivity between the two bacteria [10].

On the basis of the results of the presented research, *B. burgdorferi s.l.* and *A. phagocytophilum* seem to be mainly widespread in rural areas. These data are related to the rural ecosystem, characterized by the presence of vegetation and domestic and wild mammals. *Ixodes* ticks, vectors of both pathogens, find optimal conditions in this habitat for surviving and spreading.

The positivities observed in urban dogs could be due mainly to the exposure to infected *Ixodes* ticks during recreational and leisure activities of their owners in urban public parks or peri-urban areas. In fact, in Central Italy, there are several areas, wooded or not, which are frequented by people and their pet dogs for open-air activities, such as jogging, trekking and mountain biking. These are the mountain wooded areas of the Mugello and Casentinese Forest in the east of Tuscany, and the Regional Parks of the Apuan Alps in the north, as well as the flat land covered by pinewoods, such as the natural San Rossore-Migliarino-Massaciuccoli reserve along the Tyrrhenian coast.

During the 5-year period considered, no statistically significative differences were observed in the seroprevalences to *B. burgdorferi s.l.* and *A. phagocytophilum*, whereas *E. canis* seroprevalence decreased in 2012. This result could be related to a lower circulation of *E. canis* and/or a reduced density of *R. sanguineus* population in the studied area, but data about these aspects are not available.

The overall seroprevalences of *B. burgdorferi*, *A. phagocytophilum* and *E. canis* found in the presented investigation are not very high, but these results are related to the fact that all dogs selected for the survey were clinically healthy, and excluded animals suspected of vector-borne diseases. However, the observed rates of seropositivity demonstrate the exposure of the animals to infected arthropods, and confirm the presence of these pathogens in the considered geographic area.

Pet dogs, such as those of this survey, shared the same environments with their owners. For this reason they can be used better than other animal species as sentinels to identify the presence of vector-borne disease agents of both veterinary and public health significance.

Monitoring of tick-borne diseases using molecular methods can be better than serological surveys, because the detection of antibodies could be related to prior exposure, whereas the molecular detection of pathogens reveals a current infection. However, serologic testing can be sufficient to provide preliminary valuable data regarding area-specific disease prevalence, which may contribute to an appropriate index of suspicion for disease in both human and animal patients.

CONCLUSION

1. Control of tick-borne infections is not only of veterinary concern, but also a public health priority.
2. Pet dogs give a good indication of the exposure of their human owners to infected ticks, since they largely share the same environment and visit the same outdoor areas.
3. *E. canis* is usually considered of veterinary concern, but on the basis of recent reports it seems to be able to infect humans as well. For this reason, investigations should be carried out in humans living in geographic area where *E. canis* is present.

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