SHORT COMMUNICATION

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Serological and molecular survey of *Anaplasma phagocytophilum* in Italian hunting dogs

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Abstract

The presence of *Anaplasma phagocytophilum,* a tick-transmitted zoonotic pathogen, has been reported in Italy in humans and several animal species, including dogs, but data concerning its prevalence in the Italian canine population are lacking. The aim of this study was to determine the prevalence of *A. phagocytophilum* infection in hunting dogs which live in geographic areas of central Italy where the infection had been previously detected in wild animals. Sera from 215 hunting dogs were tested by indirect immunofluorescence antibody test (IFAT) to detect antibodies to *A. phagocytophilum*. Buffy coat samples from the same 215 animals were submitted to a polymerase chain reaction (PCR) assay specific for the *A. phagocytophilum* 16S rRNA gene. Thirty-two (14.8%) dogs proved to be seropositive with antibody titres from 1:40–1:2,560. The highest seroprevalence was observed in dogs 6–10-year-old. Two seropositive (0.9%) animals were also PCR positive. Sequencing of PCR products revealed gene sequences of *A. phagocytophilum* in both cases. These results confirm the presence of *A. phagocytophilum* in the Italian areas studied; thus, the zoonotic potential of this agent should be considered particularly for people, as hunters, at risk of exposure to tick bites.

Key words

Anaplasma phagocytophilum, hunting dogs, indirect immunofluorescence antibody test, polymerase chain reaction

INTRODUCTION

Anaplasma phagocytophilum is an obligate intracytoplasmic bacterium belonging to the family *Anaplasmataceae*, order *Rickettsiales* [1]. It resides within granulocytes, particularly neutrophils, but also the eosinophils of its host. It is transmitted by ticks of *Ixodes persulcatus* complex: *Ixodes scapularis* in the upper midwestern and northeastern USA, *Ixodes pacificus* in the western United States, *Ixodes persulcatus* in Asia and eastern Europe, and *Ixodes ricinus* in western Europe [2, 3]. Reservoir hosts for *A. phagocytophilum* are represented by a variety of wild animals, depending on the geographical area. In Europe in particular, *A. phagocytophilum* infection has been documented in roe deer (*Capreolus capreolus*) [4, 5, 6], red deer (*Cervus elaphus*) [6, 7], fallow deer (*Dama dama*) [8, 9] and red foxes (*Vulpes vulpes*) [10].

Dogs, horses and humans are accidental hosts. The first report of canine granulocytic anaplasmosis came from California in 1982 [11]. Successively, other cases have been reported in America, Asia, Africa and Europe [3].

Many canine *A. phagocytophilum* infections are subclinical and self-limiting, but acute infection has been associated with a febrile illness and a wide range of clinical signs. Fever, lethargy, anorexia, lameness, reluctance to move, and musculoskeletal pain are the most common clinical signs. Vomiting, diarrhoea, polyuria, polydipsia, cough, uveitis, spontaneous haemorrhage, and nervous system dysfunction have sometimes been observed. Splenomegaly and mild lymphadenopathy may also be present [12, 13, 14, 15, 16, 17, 18]. The laboratory abnormalities most frequently observed are thrombocytopenia, lymphopenia, anaemia, hypoalbuminemia, hyperglobulinemia, and elevated serum alkaline phosphatase activity [12, 17, 19].

The seroprevalence values observed in different studies carried out worldwide were in relationship to the state of health of the sampled dog population, the clinical suspicion for the presence of a vector-borne infectious disease, and the geographical variation in exposure to tick vectors and reservoirs hosts. In addition, because antibodies to *A. phagocytophilum* cross-react with other *Anaplasma* species, such as *A. platys*, seropositivity may not necessarily reflect previous exposure to *A. phagocytophilum* [3]. Molecular investigations are more suitable for detecting cases of infection in the canine population.

In Europe, a few data are available about the molecular prevalence of *A. phagocytophilum* in dogs [20, 21, 22, 23, 24, 25].

In Italy, cases of *A. phagocytophilum* infection in humans, domestic and wild animals have been observed and confirmed by molecular methods [8, 9, 10, 26, 27, 28, 29]. Serological and molecular investigations have also been performed in the canine population; however, data concerning the prevalence of canine *A. phagocytophilum* infection are lacking, and those available are not recent [21, 22, 30, 31].

The aim of the presented research was to evaluate, by serological and molecular methods, the prevalence of *A. phagocytophilum* infection in hunting dogs which live in geographic areas where the infection had been previously detected in wild animals.

Specimen collection. From autumn 2008 to spring 2011, peripheral whole blood and ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood samples were collected from 215 hunting dogs. Blood was drawn from the left or right cephalic vein. Whole blood samples (2–5 ml) were centrifuged at 1,500 x g for 15 min. and sera were collected. EDTA-blood samples (2–4 ml) were centrifuged at 2,500 × g

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for 5 min. and the buffy coat was separated. Sera and buffy coat samples were stored at -20 °C until examinations.

At the time of testing all dogs lived in the hunting areas of central Italy, characterized by big woods and hilly sites in which several species of wild mammals and birds are present. For all dogs, breed, age and gender were recorded.

The dogs' owners reported past and/or recent ticks' exposure, but no signs of illness and recent antibiotic treatment. The collection of blood samples was carried out in private clinics by veterinarians who kindly cooperated in the study. In all cases, the dogs' owners, after they had been informed, gave their consent to carry out the analysis.

Serological analysis. The indirect immunofluorescence antibody test (IFAT) was carried out on *Anaplasma phagocytophilum* IFAT slides (Fuller Laboratories Fullerton, California, USA).

Blood sera were diluted 1:40 (cut-off) in phosphate-buffered saline (PBS, pH 7.2) and incubated in the wells of 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 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 under 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 defined as the major dilution with a ≥ 2 score.

Molecular methods. DNA was extracted from 50 µl of each buffy coat samples using a DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions, and stored at 4 °C until used as the template for PCR assays.

A nested PCR was performed. The primary assay was carried out on the DNA samples to amplify a 932 bp fragment of the 16S rRNA gene of *A. phagocytophilum,* using the primers GE 3a (5' CACATGCAAGTCGAACGGATTATTC 3') and GE 10r (5' TTCCGTTAAGAAGGATCTAATCTCC 3'). The secondary reaction employed the primers GE 9f (5' AACGGATTATTCTTTATAGCTTGCT 3') and GE 2 (5' GGCAGTATTAAAAGCAGCTCCAGG 3') and amplified a 546 bp fragment of the same gene [32]. Each PCR amplification was performed in 50 µl of reaction mixtures containing 200 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP) (Qiagen), 0.5 mM of each primer, 1.25 U of Taq polymerase (Qiagen), 5 µl of 10x Qiagen PCR buffer. For the primary reaction, 4 µl of extracted DNA were added to the PCR mixture. The secondary reaction was carried out using 2 µl of the primary PCR product as template. Primary and nested amplifications were performed in an automated thermal cycler (Gene-Amp PCR System 2700, Perkin-Elmer, Norwalk, Connecticut, USA), with the following cycling conditions: an initial 5 min. denaturation at 95 °C, followed by 40 cycles, each consisting of a 30 sec. denaturation at 94 °C, a 30 sec. annealing at 55 °C, and a 1 min. extension at 72 °C. A single 5 min. extension at 72 °C followed the last cycle.

PCR products were analyzed by electrophoresis on 1.5% agarose gel at 100V for 45 min.; the gel was stained with ethidium bromide and observed. GelPilot 100 bp Plus Ladder (Qiagen) was used as the DNA marker.

Standard precautions were taken to avoid contamination of samples and reaction mixture, including strict separation of the areas for reagent preparation, DNA extraction and amplification. A negative control without template DNA was included to ensure the absence of contamination in the reaction mixture. The *A. phagocytophilum* genomic DNA, obtained from immunofluorescent slides, was used as positive control.

The 546 bp amplicons were sent to PRIMM (Milan, Italy) for DNA sequencing; the nucleotide sequences were compared with the sequences in GenBank database using the basic local alignment search tool (BLAST).

Statistical evaluation was carried out by the χ^2 test to analyze the results of serological tests in relationship to age, gender and breed of dogs. Values of $P<0.05$ were considered significant.

IFAT detected antibodies to *A. phagocytophilum* in 32 dogs with 14.8% total seroprevalence. The antibody titres ranged from 1:40–1:2,560 (Tab. 1).

Table 1. IFAT antibody titres to *Anaplasma phagocytophilum* compared to PCR results

IFAT antibody titre	No. IFAT positive dogs (%)	No. PCR positive dogs
1:40	9 (4.2)	0
1:80	4 (1.9)	0
1:160	7 (3.2)	1
1:320	7 (3.2)	0
1:640	0	0
1:1280	2 (0.9)	0
1:2560	3 (1.4)	1
Total	32 (14.8)	2

The seroprevalence was significantly higher in >6 – <10-year-old dogs compared to the other subjects (χ^2 test, $P<0.05$).

There were no statistically significant differences between male and female dogs. Irish red setter proved to be the breed with the highest seroprevalence. However, the number of dogs tested belonging to different breeds, in particular to Irish red setter, were low; a statistical evaluation was therefore considered not significant.

PCR detected *A. phagocytophilum* DNA in two (0.9%) seropositive dogs. Sequencing of these products revealed gene sequences of *A. phagocytophilum* in both cases. The two 16S rRNA gene sequences were identical. One sequence was submitted to the NCBI database with the following GenBank accession number: JX867096.

The first PCR positive animal was a mixed-breed, male dog of 3 – 6-years-old, with 1:160 antibody titre, whereas the second animal was a mixed-breed, female dog of 6 – 10-years-old with 1:2,560 antibody titre.

The distribution of dogs according to their IFAT and PCR results and animal data (age, gender, breed) is shown in Table 2.

Previous investigations carried out in Europe found values of seroprevalence ranging from 5 to 50 % depending on the health state of dogs and the risk factors, such as exposure to ticks and environmental conditions [23, 24, 33, 34, 35, 36, 37].

Table 2. Serological and molecular results in relation to age, gender and breed of the canine study population

		Study population No. = 215	IFAT positive dogs No. = 32 (%)	PCR positive dogs No. = 2 (%)
Age (year)	< 1	38	2 (5.2)	0
	>1-<3	65	8 (12.3)	0
	>3-<6	43	6 (13.9)	1 (2.3)
	>6-<10	54	14 (25.9)	1 (1.8)
	>10	15	2 (13.3)	0
Gender	Male	98	14 (14.2)	1 (1.0)
	Female	117	18 (15.3)	1 (0.8)
Breed	Mixed-breed	118	22 (18.6)	2 (1.6)
	Segugio italiano	26	4 (15.3)	0
	Segugio maremmano	22	1 (4.5)	0
	Bracco italiano	12	0	0
	Pointer	11	0	0
	Irish Red Setter	9	2 (22.2)	0
	Epagnuel Breton	17	3 (17.6)	0

In the present study serology revealed a prevalence of 14.8% in the canine population. This value is higher than the seroprevalence (8.8%) previously detected in pet dogs living in central Italy in domestic conditions [31]. The higher value observed is probably due to the environment frequented by the dogs of this study; in fact the hunting areas are characterized by conditions favourable for the arthropods diffusion, as abundant vegetation and presence of animal species, in particular wild ruminants that serve as reservoir for *A. phagocytophilum*.

In central Italy, several species of ticks are present, mainly *Rhipicephalus* sp., *Dermacentor* sp., *Hyalomma* sp., *Haemaphysalis* sp., and *Ixodes ricinus*, which are well known as vectors of pathogens such as *Babesia* sp., Spotted Fever Group rickettsiae, and *Borrelia burgdorferi sensu lato* [38, 39, 40, 41]. *A. phagocytophilum* has been detected in *I. ricinus* ticks in this geographic area [9, 42] and in northern Italy [43, 44, 45].

The seroprevalence found in the presented study was significantly higher in 6 – 10-year-old dogs compared to younger and older subjects. These dogs had probably been exposed to an environment infested by *A. phagocytophilum*-contaminated ticks for a longer time compared to the younger animals. The low prevalence observed in the older dogs could be related to the low number of subjects tested, also considering that few >10-year-old dogs are usually employed for hunting.

The molecular survey detected only two positive animals with a 0.9% prevalence. This value is in agreement to the results obtained by other authors. In particular, previous molecular investigations carried out in Italy did not find positive dogs [21, 22], and the molecular surveys in other European countries detected prevalence values of 0% in Portugal [24], 0.8% in the United Kingdom [20], 0.4% in Warsaw, Poland [25], and 1% in northwestern Poland [46].

The molecular prevalence observed in the canine population in this study is considerably lower than the prevalence (16.6%) recently observed among foxes (*Vulpes vulpes*) living in the same geographic area [10].

This difference could be related to the different source of the tested samples. In fact, in previous research, PCR was carried out on DNA extracted from spleen specimens collected during the necroscopies of the foxes, whereas in this study DNA was extracted from buffy coat obtained from EDTA-anticoagulated blood samples. Positive PCR for *A. phagocytophilum* confirmed the infection, but a negative response does not always mean that the animal is not infected, because the bacteremia of *A. phagocytophilum* in dogs appears to be of short duration (<28 days) [3]. For this reason, diagnosis of *A. phagocytophilum* infection in dogs should be accomplished using PCR and serology, which allows the detection of IgG class antibodies approximately eight days after the initial exposure [3].

Gene sequencing is useful for confirming the PCR results. In the presented investigation, the two obtained sequences were identical, supposing that the two dogs were infected by the same *A. phagocytophilum* variant.

The two infected dogs appeared to be healthy, confirming that dogs with granulocytic anaplasmosis often do not have any signs of illness. On the basis of these considerations, dogs, particularly those living in endemic areas, should be periodically submitted to serological and molecular controls.

CONCLUSION

The serological and molecular prevalence of infection with *A. phagocytophilum* observed in the canine population studied is not very high considering that the hunting dogs are at risk for *A. phagocytophilum* infection. The low prevalence observed could be related to the behaviour of the owners who, being aware of the risks due to the tick bites, regularly treat the dogs with tick preventatives and, when possible, promptly remove the attached ticks.

These results confirm that *A. phagocytophilum* is present in the Italian areas studied; therefore the zoonotic potential of this agent should be considered particularly for people at risk as hunters, considering the close relationship between dogs and humans, as well as their ticks.

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