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Anaplasma phagocytophilum infection in a fallow deer (*Dama dama*) population in a preserve of central Italy

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SUMMARY

From autumn 2004 to spring 2005, 70 fallow deer (*Dama dama*), 27 female and 43 male, living in a natural preserve of central Italy were examined by indirect immunofluorescence assay (IFA) to detect specific antibodies to *Anaplasma phagocytophilum*. Thirty-one (44.28%) sera scored positive: in particular 10 fallow deer (8 male and 2 female) scored positive at 40 antibody titer, 21 deer (8 male and 13 female) at ≥80 titer. EDTA anticoagulated blood samples collected from 29 of the 70 deer examined were tested by a nested-PCR assay to disclose a 546 bp fragment, specific of *A. phagocytophilum* 16S rRNA gene. Twenty (72.41%) blood samples (8 male and 13 female deer) resulted positive. Fifteen PCR-positive deer also resulted positive to IFA, whereas the remaining six did not show specific antibodies. Three serologically positive animals gave negative results at the nested PCR. Five deer scored negative both to serological tests and PCR.

KEY WORDS: Anaplasma phagocytophilum, fallow deer, indirect immunofluorescence assay, nested polymerase chain reaction

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Anaplasma phagocytophilum is an obligate intracellular bacterium belonging to the order *Rickettsiales*, family *Anaplasmataceae*. It is the causative agent of an emerging infectious disease mainly of humans, but also of horses and dogs, called granulocytic ehrlichiosis or anaplasmosis (Dumler *et al*., 2001).

Ticks of *Ixodes* species have been implicated in the transmission of this bacterium. *Ixodes ricinus* is usually present in northern Europe. It can be recovered in the wooded areas of north and central Italy.

Ixodes ricinus feed on a large number of vertebrate hosts; the immature stages are found mainly on small-size vertebrates and can feed also on humans. Sheep and cattle are hosts for adult *Ixodes* ticks, but wild animals represent the primary natural reservoir.

The role of white-tailed (*Odocoileus virginianus*), red (*Cervus elaphus*) and roe (*Capreolus capreolus*) deer as reservoirs of Ehrlichiosis (by *Ehrlichia chaffeensis*) and Anaplasmosis agents has been well documented by several studies carried out in the United States and Europe (Dawson *et al*., 1996; Liz *et al*., 2002; Petrovec *et al*., 2002; Stuen *et al*., 2002; De la Fuente *et al*., 2004; Massung *et al*., 2005).

Data on the granulocytic anaplasmosis of fallow deer (*Dama dama*), the typical deer present in Europe, are not available in literature and for this reason it is not clear if the granulocytic anaplasmosis has the same epidemiological and pathological characters also in this animal species.

Our objective was to use serology and PCR to investigate the presence of *A. phagocytophilum*

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infection among a fallow deer population living in a natural preserve of central Italy.
In the period from autumn 2004 to spring 2005, 70 fallow deer (*Dama dama*), 27 female and 43 male, living in a natural preserve of central Italy were tested for *A. phagocytophilum* infection. Whole-blood samples were collected from the jugular vein of each animal. Sera were separated by centrifugation and stored at -20°C until tested. EDTA- anticoagulated blood, collected from 29 among these animals, was centrifuged at 2,500 g for 10 min to obtain the buffy coat cells for the molecular analysis, and stored at -20°C.
Sera were tested by indirect immunofluorescence assay (IFA) using substrate slides with *Anaplasma phagocytophilum* antigen (Fuller Laboratories Fullerton, CA, USA) and a rabbit fluorescein isothiocyanate-conjugated anti-deer IgG (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD, USA) diluted 1:10 in Evans Blue solution. Sera were screened at dilutions of 1:40 and 1:80.
DNA was extracted from buffy coat cells using the DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and stored at 4°C until used as template for a nested PCR assay.
Primary PCR amplification allowed 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') previously described by Massung *et al*., 1998. The PCR amplification was performed in 50 µl of reaction mixtures containing 200 µM of deoxynucleoside triphosphates, 0.5 µM of each primer, 1.25 U of Taq polymerase (Qiagen GmbH, Hilden, Germany), 5 µl of 10x Qiagen PCR buffer and 4 µl of extracted DNA. PCR amplifications were performed in an automated thermal cycler (Gene-Amp PCR System 2700, Perkin-Elmer, Norwalk, Connecticut, USA). An initial 5 min denaturation at 95°C was followed by 40 cycles, each consisting of a 30 s denaturation at 94°C, a 30 s annealing at 55°C, and a 1 min extension at 72°C. A single 5 min extension at 72°C followed the last cycle.
The nested PCR employed the primers GE 9f (5' AACGGATTATTCTTTATAGCTTGCT 3') and GE 2 (5' GGCAGTATTAAAAGCAGCTCCAGG 3') and amplified a 546 bp fragment of the same gene (Massung *et al*., 1998). The PCR mixture contained 200 µM of deoxynucleoside triphosphates, 0.5 µM of each primer, 1.25 U of Taq polymerase (Qiagen GmbH, Hilden, Germany), 5 µl of 10x Qiagen PCR buffer and 2 µl of the primary PCR product as template. Nested-cycling conditions were identical to those for the primary reactions. A negative control was added in each PCR amplification, but no positive control was included to avoid cross-contaminations.
All the amplification products were visualized on ethidium bromide-stained 1% agarose gel.
Antibodies against *A. phagocytophilum* were found in 31 of 70 serum samples (44.28%). In particular, 10 deer (8 male and 2 female) scored positive at titers of 40 and 21 deer (8 male and 13 female) at a titer of ≥80.
The nested PCR disclosed the 546 bp fragment, specific of *A. phagocytophilum* 16S rRNA gene, in the DNA samples in 21 of 29 (72.41%) buffy coats examined (Figure 1). Eight animals that scored positive were male and 13 female deer. Fifteen PCR-positive deer also resulted positive to IFA, whereas the remaining six did not show specific antibodies. Only three animals positive to serology gave negative results with the nested PCR. Five deer scored negative in both serology and PCR.
The serological and molecular investigations carried out on the fallow showed the presence of *A. phagocytophilum* among these wild ruminants.

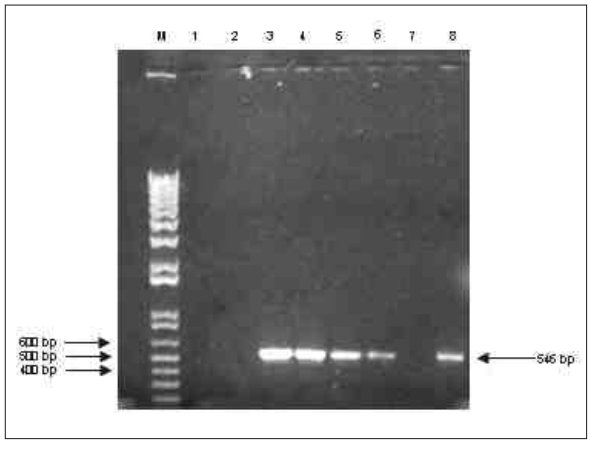

FIGURE 1 - *Agarose gel electrophoresis of nested PCR products. Lane M: molecular standard; lane 1: negative control; lanes 2 and 7: negative samples; lanes 3, 4, 5, 6 and 8: positive samples. The expected size (546 bp) is noted.*

In fact, about the half of the animals tested (44.28%) showed antibodies to *A. phagocytophilum*, and a high percentage (72.41%) resulted positive at the nested-PCR assay. A good correlation was observed between IFA and PCR results. Among the 21 deer that scored PCR positive, only 6 did not show antibodies to *A. phagocytophilum*; these animals could be in the early stages of infection when specific antibodies are absent. Three serological reactivities means only a contact with the pathogen but not a current infection.

The fallow deer population studied lived in a natural preserve of central Italy characterized by woodlands with grassy clearings and a typically mediterranean climate, factors which favour the presence of ticks. The European wild boar (*Sus scrofa*) is the other wild animal species widespread in the preserve. Also domestic animals, such as cattle and horses, sensitive to *A. phagocytophilum*, are present. Infected cattle rarely develop clinical signs, whereas this microorganism is able to cause a severe disease in horses (Pusterla *et al.*, 2002).

The preserve is frequently visited by humans for professional reasons (forest guards, veterinarians), but also for hobbies, particularly walking and riding increasing the possibility that people can be attacked by infected ticks. The human granulocytic ehrlichiosis (HGE) by *A. phagocytophilum* presents with nonspecific symptoms such as fever, headache and myalgia; leucopenia, thrombocytopenia and elevated liver enzymes are frequently observed (Massung *et al.*, 1998).

A. phagocytophilum has frequently been recovered in ixodid ticks in the United States and Europe (Pancholi *et al.*, 1995; Cinco *et al.*, 1998; Parola *et al.*, 1998; Schauber *et al.*, 1998; Kramer *et al.*, 1999; Petrovec *et al.*, 1999; Pusterla *et al.*, 1999). Results of serological and molecular investigations showed *A. phagocytophilum* infection in wild ruminants living in the USA (Belongia *et al.*, 1997; Little *et al.*, 1998; Foley *et al.*, 1998;Walls *et al.*, 1998; Magnarelli *et al.*, 1999) and European countries (Ogden *et al.*, 1998; Stuen *et al.*, 2001; Stuen *et al.*, 2002; Liz *et al.*, 2002; Groen *et al.*, 2002; Petrovec *et al.*, 2002). Human granulocytic ehrlichiosis has been described in Europe, even if some cases do not fulfill the diagnostic criteria for confirmed or probable HGE (De la Fuente *et al.*, 2005). In Italy, human seropositivity was detected mainly in workers at risk for tick bites (Santino *et al.*, 2004), but only three cases of HGE have been confirmed (Ruscio and Cinco, 2003; De la Fuente *et al.*, 2005). Molecular investigations have detected *A. phagocytophilum* in *Ixodes* ticks collected in northern Italy (Cinco *et al.*, 1998) and seropositivity was observed among the canine population in different Italian regions (Alberti *et al.*, 2005). Data on the diffusion of *A. phagocytophilum* among wild animals in Italy are not available in literature.

Deer, important hosts for *I. ricinus*, have often been used as sentinel animals to monitor tick-borne infections, such as the Lyme disease. The detection of *A. phagocytophilum* among fallow deer suggests that these animals, sensitive to granulocytic anaplasmosis, may serve in monitoring of this infection in a given area. However, the high *A. phagocytophilum* prevalence in deer compared to the low number of human anaplasmosis cases reported in Italy and in Europe may indicate the existence of strains less virulent or non pathogenic to humans (Skarphedinsson *et al.*, 2005).

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