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Evidence of *Anaplasma* infections in European roe deer (*Capreolus capreolus*) from southern Spain

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Abstract

Anaplasma spp. (Rickettsiales: Anaplasmataceae) are tick-borne pathogens of veterinary and human importance. The wildlife hosts for these pathogens are not well characterized and may play an important role in the epidemiology of the disease. The objective of this research was to study the infection with *A. marginale*, *A. ovis* and *A. phagocytophilum* in free-ranging European roe deer (*Capreolus capreolus*) from Cádiz, Andalucía, Spain. Of 17 roe deer tested, 14 (82%) and 5 (29%) had antibodies reactive to *Anaplasma* spp. and *A. phagocytophilum* by competitive ELISA and indirect immunofluorescent antibody testing, respectively. Polymerase chain reaction and sequence analysis of *Anaplasma* major surface protein 4 (*msp4*) gene was conducted on blood samples from all roe deer examined. Nine (53%) animals had evidence of infection with *A. ovis* and 3 (18%) were positive for *A. phagocytophilum*. Concurrent infections were not detected. Despite the presence of *A. marginale* infections in cattle in the study site (36% *msp4* PCR-positive animals), none of the *msp4* amplicons from roe deer corresponded to *A. marginale* sequences. *A. ovis msp4* sequences were identical to a genotype previously identified in sheep in Sicily, Italy. Two different *A. phagocytophilum* genotypes were identified in infected roe deer. This is the first report of roe deer naturally infected with *A. ovis*. These results demonstrate that roe deer are infected with *A. ovis* and *A. phagocytophilum* in Spain and suggest that this species may be involved in the natural cycle of these pathogens in this region, thus acting as potential reservoir for transmission to domestic and wild animals.
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1. Introduction

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) contains obligate intracellular organisms found exclusively within membrane-bound vacuoles in the cytoplasm of both vertebrate and invertebrate host cells (Dumler et al., 2001). The genus *Anaplasma* includes pathogens of ruminants, *A. marginale*, *A. centrale*, *A. bovis* and *A. ovis*. Also included in this genus is *A. phagocytophilum* which infects a wide range of hosts including humans, horses, rodents, birds, dogs and ruminants, and *A. platys* which infects dogs. These pathogens are biologically transmitted by ticks and vertebrate or tick hosts with persistent infection serve as reservoirs of the pathogen in nature (Kocan et al., 2004).

Concern about transmission of infectious agents between wildlife and domestic livestock is increasing, especially in areas where free-ranging wildlife and cattle share common grazing grounds (Chomel et al., 1994). *Anaplasma* infections have been well characterized in domestic animals and ticks in Spain (García Fernández and Huelli, 1984;

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Sevilla et al., 2002; de la Fuente et al., 2005a,b; Naranjo et al., 2006). However, information about potential wildlife reservoirs of infection is limited to Iberian red deer (*Cervus elaphus hispanicus*) and European wild boar (*Sus scrofa*) but other wildlife species may play an important role in the epidemiology of the disease in this region (de la Fuente et al., 2004a,b, 2005a,b; Naranjo et al., 2006). The objective of this research was to explore other potential wildlife reservoir hosts for *Anaplasma* infection in southern Spain. Herein, we studied the infection with *A. marginale*, *A. ovis* and *A. phagocytophilum* in free-ranging European roe deer (*Capreolus capreolus*) from Cádiz, Andalucía, Spain. Cattle were sampled to demonstrate the presence of *A. marginale* infections in the study site.

2. Materials and methods

2.1. Study site

Roe deer ($n = 16$) and cattle ($n = 51$) were sampled in a 3000 hectares fenced hunting estate located in the Natural Park of "Los Alcornocales" in the province of Cádiz, Andalucía in southern Spain. Cork oak (*Quercus suber*), olive tree (*Olea europaea*, var. *sylvestris*) and evergreen oak (*Quercus ilex*) forests mixed with scrubland (mainly *Pistacia* spp., *Cystus* spp., *Erica* spp., *Rhamnus lyciodes*, and *Phillyrea angustifolia*) and pastures compose the main landscape in the hunting estate. Mediterranean climate is dominant in this area, and rains are mainly present during spring and autumn, with annual rainfall ranging 500–1000 mm. Wild ungulates (red deer and roe deer), wild rabbits (*Oryctolagus cuniculus*), red-legged partridges (*Alectoris rufa*), wild carnivores (*Genetta genetta, Herpestes ichneumon, Meles meles, Vulpes vulpes, Martes foina*) and a wide variety of birds are present in the hunting estate. Gibraltar Strait is located at less than 50 km from the hunting estate. Cattle ($n = 30$) and 1 roe deer were sampled in a neighbouring hunting estate, located at less than 10 km from the Gibraltar Strait. Cattle and roe deer are the only ungulates present in this estate where both species share pastures and water supply. Climate and vegetation in this estate is similar to that described above, except for higher annual rainfall (1000–1500 mm) and predominant cork oak and holm oak (*Quercus faginea*) forest.

2.2. Animals and sample preparation

Roe deer ($n = 17$; approx. 10% of the study site population) were captured by gamekeepers with fix capture boxes during June–July 2005 and bled by cervical puncture. Cattle ($n = 81$; approx. 10% of the study site population) were bled during the tuberculosis control campaign in 2004 and 2005. Blood was collected into sterile tubes with and without anticoagulant (EDTA) and maintained at 4 °C until arrival at the laboratory. Plasma and serum were then separated after centrifugation and stored at −20 °C. Two 1.5 ml blood aliquots were frozen at −20 °C. Ticks were collected from roe deer and cattle by inspection of the ears, head, axillae and ventral parts of each animal and classified as previously described (Ruiz-Fons et al., 2006).

2.3. Anaplasmosis serologic tests

Animals were tested for antibodies to *Anaplasma* spp. using the competitive ELISA (cELISA) anaplasmosis test (VMRD, Inc., Pullman, WA, USA), which detects antibodies to the MSP5 antigen conserved between *A. marginale*, *A. phagocytophilum* and *A. ovis* (Dreher et al., 2005). Percent inhibition values greater than 30% were considered positive as recommended by the manufacturer.

The immunofluorescence test for *A. phagocytophilum* was performed using the Indirect Immunofluorescence Antibody (IFA) Test Kit from Fuller Laboratories (Fullerton, CA, USA) following the manufacturer's instructions (Torina et al., 2007). The IFA test uses antigens derived from HL-60 cells infected with the HGE-1 isolate of *A. phagocytophilum*. For deer samples, an anti-protein G Alexa Fluor 488 conjugate (Invitrogen, Carlsbad, CA, USA) was used.

2.4. msp4 polymerase chain reaction (PCR) and sequence analysis

The *Anaplasma* spp. *msp4* gene was amplified by PCR and sequenced as reported previously (de la Fuente et al., 2002a, 2005c). Briefly, 1 μl (1–10 ng) DNA was used with 10 pmol of each primer (*A. marginale*/*A. ovis*: MSP45: 5′GGGAGCTCCTATGAATTACAGAGAATT GTTTAC3′ and MSP43: 5′CCGGATCCTTAGCTGAA CAGGAATCTTGC3′; *A. phagocytophilum*: MAP4A P5:5′-ATGAATTACAGAGAATTGCTTGTAGG-3′ and MSP4AP3:5′-TTAATTGAAAGCAAATCTTGCTCCTA TG-3′) in a 50 μl volume PCR (1.5 mM $MgSO_4$, 0.2 mM dNTP, 1X AMV/*Tfl* reaction buffer, 5 u *Tfl* DNA polymerase) employing the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler (Techne model TC-512, Cambridge, England, UK) for 35 cycles. After an initial denaturation step of 30 s at 94 °C, each cycle consisted of a denaturing step of 30 s at 94 °C, an annealing for 30 s at 60 °C and an extension step of 1 min at 68 °C for *A. marginale*/*A. ovis* and an annealing-extension step of 1 min at 68 °C for *A. phagocytophilum*. Negative control reactions were performed with the same procedures, but adding water instead of DNA to monitor contamination of the PCR. The program ended by storing the reactions at 10 °C. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 Kb DNA Ladder, Promega). Amplified fragments were resin purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) or used directly for sequencing both strands by double-stranded dye-termination cycle sequencing (Secugen SL, Madrid, Spain). When cloned, at least two independent clones were sequenced for each PCR.

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