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Evidence of co-infection with *Mycobacterium bovis* and tick-borne pathogens in a naturally infected sheep flock

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ABSTRACT

Ticks are responsible for the transmission of pathogens of veterinary importance, including those affecting sheep. The current study was designed to investigate co-infections with tick-borne and other pathogens in a naturally infected sheep flock with poor health condition using serology and PCR. Infection with *Anaplasma ovis* was detected by serology and PCR in 56% of the animals. The presence of *Rickettsia* spp. of the Spotted Fever Group (SFG) was detected by PCR and sequence analysis in 31% of the animals. All the animals were negative for *Anaplasma phagocytophilum* either by serology or PCR. Twelve sheep were randomly selected for anatomopathological studies. Five of these animals presented lesions consistent with *Mycobacterium tuberculosis* complex (MTBC) infection and spoligotyping confirmed infection with *Mycobacterium bovis* spoligotype SB0339. Co-infection with tick-borne pathogens and MTBC could contribute to the poor health condition observed in these animals but other uncontrolled factors may also be responsible. The differential expression of immune response genes supported previous findings in ruminants and suggested that infection with tick-borne pathogens and *M. bovis* may results in unique gene expression patterns in sheep. The results underline the need for further research into the possible role of sheep in the epidemiology of animal tuberculosis.

Introduction

Ticks are obligate blood-sucking arthropods that parasitize vertebrates and are distributed throughout the world. Ticks can transmit a broad range of infectious agents (viruses, bacteria, parasites) of veterinary importance and, after mosquitoes, are the most important vectors of diseases for humans (Parola and Raoult, 2001). In sheep, ticks transmit pathogens causing diseases such as tick-borne fever (*Anaplasma phagocytophilum*), anaplasmosis (*Anaplasma ovis*), theileriosis (*Theileria* spp.), babesiosis (*Babesia* spp.) and acute hemorrhagic gastroenteritis (Nairobi sheep disease virus) (Schnittger et al., 2004; de la Fuente et al., 2007; Torina et al., 2008; Gong et al., 2015). However, tick-borne pathogen co-infections and possible interactions with non vector-borne pathogens have not been well described in ruminants (Galindo et al., 2010).

The current study was designed to characterize co-infection with tick-borne pathogens in a sheep flock from Castilla-La Mancha (Spain). The possible interaction with non tick-borne pathogens of the *Mycobacterium tuberculosis* complex (MTBC) was also considered, as *Mycobacterium bovis* is endemic in the area (Vicente et al., 2013). The infection with *M. bovis* is infrequently diagnosed in sheep although clinical cases have been described previously (Houlihan et al., 2008; Muñoz-Mendoza et al., 2012). In specific regions, sheep are regarded as part of the MTBC maintenance host community (Muñoz-Mendoza et al., 2015). The results of the study showed co-infection with tick-borne pathogens and *M. bovis* and provided new information for designing effective control strategies for MTBC and tick-borne pathogens in sheep.

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Table 1
Oligonucleotide primers and PCR conditions used for the detection of tick-borne pathogens.

Pathogen	Gene	Primer sequences (5′–3′)	Annealing conditions	Amplicon size (bp)
A. phagocytophilum	Major surface protein 4 (*msp4*)	MSP4-L: CCTTGGCTGCAGCACCACCTG MSP4-R: TGCTGTGGGTCGTGACGCG	55 °C, 60 s	344
A. ovis	Major surface protein 4 (*msp4*)	MSP4-5: GGGAGCTCCTATGAATTACAGAGAATTGTTTAC MSP4-3: CCGGATCCTTAGCTGAACAGGAATCTTGC	60 °C, 30 s	849
Rickettsia spp.	*16S rRNA*	fD1: AGAGTTTGATCCTGGCTCAG Rc16S-452n: AACGTCATTATCTTCCTTGC	54 °C, 30 s	416
Rickettsia spp	Bar29 ATP synthase alpha subunit (*atpA*)	atpA-F: ACATATCGAGATGAAGGCTCC atpA-R: CCGAAATACCGACATTAACG	48 °C, 30 s	731
Rickettsia spp.	Outer membrane protein B (*ompB*)	ompB-F: GGGTGCTGCTACACAGCAGAA ompB-R: CCGTCACCGATATTAATTGCC	53 °C, 30 s	618
Rickettsia spp.	*recA*	recA-B: TGCTTTTATTGATGCCGAGC recA-R: CTTTAATGGAGCCGATTCTTC	52 °C, 30 s	428

Materials and methods

Ethics statement

Sampling was conducted complying with the current Spanish legislation, and designed and developed by scientists (B and C animal experimentation categories) in accordance with EC Directive 86/609/EEC and RD 1201/2005 for animal handling and experimentation.

Animals and sampling

From February to April 2014, we started an investigation into pathogen infection of a flock of sheep (Manchego breed) from a local farm in the province of Ciudad Real, Spain. For the control of external and internal parasites, Ivermectin was regularly inoculated subcutaneously in spring and summer and the flock also received a cypermethrin dip-bath treatment twice during the summer. Fifty animals (Table S1) were randomly selected and blood was collected by the farmer's veterinary service into sterile tubes with EDTA and without anticoagulant and kept at 4 °C until arrival at the laboratory. Serum and Buffy coat were separated after centrifugation and stored at −20 °C and −80 °C, respectively until used for serological or DNA/RNA studies. Then, another 12 randomly selected adult female sheep from the same flock were submitted to the laboratory for necropsy. Serum and Buffy coat samples were also collected for DNA/RNA studies. Peripheral blood from these animals was used to prepare blood smears that were air-dried and stained by Diff-Quik (QCA, Tarragona, Spain) following manufacturers recommendations.

Analysis of serum antibodies

Antibodies for *Anaplasma* spp. were detected using a competitive enzyme-linked immunoassay (cELISA, VMRD, Pullman, WA, USA). Samples having <30% inhibition were considered negative (Torioni De Echaide et al., 1998).

Immunofluorescence antibody assay for *A. phagocytophilum*, based on *A. phagocytophilum* HGE-1 isolate antigens derived from HL-60 cells, was performed using Fuller Laboratories kits (Fullerton, CA, USA) according to the manufacturer's instructions but adapted to sheep using FITC-conjugated anti-sheep antibodies (Sigma, St. Louis, MO, USA). An antibody titre of 1:80 or greater was considered positive for *A. phagocytophilum* antigen.

PCR detection of pathogens

Total DNA was extracted from blood samples using a REALPURE Spin Kit (Durviz, Madrid, Spain) according to manufacturer's instructions. For the detection of *Anaplasma* and *Rickettsia* spp., 2 μl of DNA was used with 20 pmol of each primer (Table 1), in a 50 μl reaction PCR Master Mix (Promega, Madison, WI, USA) using a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Carlsbad, CA, USA). PCR products were electrophoresed on 1% agarose gels to check the size of the amplified fragments by comparison to DNA molecular weight marker GeneRuler 1 kb DNA ladder (Thermo Scientific, Waltham, MA, USA).

Sequence analysis of selected amplicons

For additional characterization, *A. ovis msp4*, and *Rickettsia* spp. *16S rDNA, atpA, ompB and recA* amplicons were purified with a MinElute Gel Extraction Kit (Qiagen, Valencia, CA, USA), cloned into TOPO TA (Invitrogen, Grand Island, NY, USA) and transformed in TOP10 chemically competent *Escherichia coli* cells. For each gene, three individual clones were selected and purified using the GeneJET Plasmid Miniprep Kit (Life Technologies, Grand Island, NY, USA) and sent for sequencing to the Centro Nacional de Investigaciones Oncológicas (CNIO, Spain). The sequences were searched for homology against sequences in the NCBI database using the BLAST search program (Altschul et al., 1990). Sequences were aligned using ClustalW software (Larkin et al., 2007) for multiple sequence alignment. Sequences were submitted to the GenBank database under accession numbers KR401150, KR401151, KT733035–KT733040 (*Rickettsia* spp. sequences), KR608305 (*Anaplasma* sequence).

Gene expression analysis by real-time RT-PCR

To analyze gene expression profiles in response to pathogen infection in peripheral blood mononuclear cells (PMBC), total RNA was extracted using a QIAamp RNA Blood Mini Kit (Qiagen Inc. Valencia, CA, USA) according to manufacturer's instructions using blood collected from uninfected ($n = 3$, adult female sheep from the same flock and the same breed, negative to *A. ovis*, *Rickettsia* spp. and *M. bovis*) and sheep selected for necropsy studies ($n = 12$). Quantitative RT-PCR was performed on the RNA samples with immune response gene specific primers and real-time RT-PCR conditions (Table 2) using a Quantitect SYBR Green RT-PCR Kit and a Rotor Gene Q thermocycler (Qiagen, Inc. Valencia, CA, USA) following manufacturer's recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample (Ririe et al., 1997). The mRNA values were normalized against *Ovis aries aldolase B* (ALDOB) using the genNormddCT method (Livak and Schmittgen, 2001). The mean of the duplicate values was used and data from infected