Ticks and Tick-borne Diseases 6 (2015) 31–37

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Risks of suffering tick-borne diseases in sheep translocated to a tick infested area: A laboratory approach for the investigation of an outbreak

Ana Hurtado, Jesús F. Barandika, Beatriz Oporto, Esmeralda Minguijón, Inés Povedano, Ana L. García-Pérez*

Department of Animal Health, NEIKER – Instituto Vasco de Investigación y Desarrollo Agrario, Berreaga 1, 48160 Derio, Bizkaia, Spain

ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form 8 August 2014
Accepted 7 September 2014
Available online 23 September 2014

Keywords:
Babesia
Anaplasma
Ticks
Outbreak
Real-time PCR
Sheep

ABSTRACT

This study was designed to investigate an outbreak of high mortality that occurred in naïve Assaf sheep introduced into a Latxa sheep flock in the Basque Country, a region where piroplasmosis is endemic. To identify the causes of this outbreak, a panel of different methods, including traditional pathological, biopathological and parasitological analyses combined with recently developed molecular methods, was used. These novel molecular methods included a multiplex real-time PCR assay to screen for the presence of the most important tick-borne pathogens (piroplasms and anaplasmas), followed by a second species-specific multiplex real-time PCR assay for the identification of *Anaplasma*-positive samples. The identification of piroplasm-positive samples was carried out by a multiplexed microsphere-based suspension array using a Luminex® xMAP technology-based procedure. Anaplasmas and/or piroplasms were detected in 7/10 lambs and 11/13 ewes, with *Babesia ovis* being detected in 12 of the 23 animals, *Theileria ovis* in 6 and *Anaplasma ovis* in 4, both as single and mixed infections. Most of the animals infected with *B. ovis* had a marked decrease in the values of the red blood cell parameters. Ticks collected from the animals were identified as *Riphicephalus bursa*, recognised vector of *B. ovis*. Other haemolytic pathologies (clostridial disease, copper poisoning and leptospirosis) were ruled out and, considering all clinical, laboratory and epidemiological data, babesiosis by *B. ovis* was diagnosed. A detailed description of the clinical outcome, with *ca.* 60% of mortality, laboratory results and epidemiological findings are provided. The implications of the introduction of naïve animals into a piroplasmosis endemic area are discussed.

Introduction

Tick-borne diseases have a major impact on extensive management systems. Sheep that spend long periods grazing in mountain pastures are exposed to tick bites that can transmit a variety of tick-borne pathogens, the most important being piroplasmosis and anaplasmosis (Uilenberg, 1995). Piroplasmoses are worldwide distributed tick-borne diseases caused by intracellular apicomplexan parasites of the genera *Theileria* and *Babesia* (Preston, 2001; Uilenberg, 2006). Previous studies carried out in the region (Nagore et al., 2004; Ros-García et al., 2013) showed a relatively high prevalence of sub-clinical infections in the sheep population and identified five different piroplasms: *Babesia ovis*, *Babesia motasi*, *Theileria ovis*, *Theileria luwenshuni*/OT1 and *Theileria* sp. OT3. The most virulent species in small ruminants is *B. ovis* (Uilenberg, 2006), which following infection, directly invades red cells causing a metabolic disease more complex than a simple syndrome of intravascular haemolysis, where animals develop haemolytic anaemia, haemoglobinuria, and jaundice (Valli, 2007). Parasitaemia is typically low (Habela et al., 1990), and body temperature rises to 42 °C before the onset of overt parasitaemia. *B. motasi* is considered a moderately virulent species, and the *Theileria* species that infect sheep in Europe have been generally described as low pathogenic and are rarely associated with overt clinical signs (Preston, 2001; Uilenberg, 2006). Animals that survive acute piroplasm infection generally become low-level carriers of the parasites, and could remain persistently infected for years without apparent clinical signs (Preston, 2001; Uilenberg, 2006). Asymptomatic carrier animals can act as source of infection for vector ticks. Ticks of the genera *Rhipicephalus* and *Haemaphysalis* are considered the vectors for ovine piroplasms.

* Corresponding author. Tel.: +34 944034300; fax: +34 944034310.
E-mail address: agarcia@neiker.net (A.L. García-Pérez).

http://dx.doi.org/10.1016/j.ttbdis.2014.09.001
1877-959X/

Anaplasmosis are common tick-borne zoonotic diseases of livestock and wildlife caused by intracellular gram-negative bacteria of the family *Anaplasmataceae* (Dumler et al., 2001), *Anaplasma phagocytophilum* and *Anaplasma ovis* being the main species found in sheep in Europe. *A. phagocytophilum* is the most frequently occurring and most important pathogen of this family (Woldehiwet, 2006). It is transmitted by Ixodid ticks, mostly *Ixodes ricinus* (Ogden et al., 2003). It invades granulocytes and in ruminants causes tick-borne fever (Woldehiwet, 2010) associated with hyperthermia, abortion, immunosupression, and other clinical signs including reduced appetite, apathy, decreased milk production, and respiratory abnormalities. Besides, the temporary state of immunodeficiency caused by *A. phagocytophilum* has been postulated as relevant in the onset of other tick-borne diseases (Brodie et al., 1986; Woldehiwet, 2006). *A. ovis* is an intraerythrocytic pathogen of sheep, goats, and wild ruminants. Infection is usually asymptomatic but it can cause haemolytic anaemia and haemoglobinuria (Hornok et al., 2007). *A. ovis* is transmitted by ticks of the genera *Dermacentor*, *Rhipicephalus* and *Hyalomma* (Friedhoff, 1997).

The Basque Country is a region in northern Spain where climate and vegetation favour abundance of tick populations (Barandika et al., 2006, 2011). Latxa is the native dairy sheep breed of the Basque Country, a small, rustic animal semi-extensively managed in mountain pastures in contact with ticks (Ruiz et al., 1997). Piroplasm infection has been described to be present in ovine Latxa flocks, but incidence of clinical disease is very low (Nagore et al., 2004; Ros-García et al., 2013). The presence of *A. phagocytophilum* in Latxa sheep has been known for a long time and it has been associated with abortion in pregnant yearlings in their first contact with ticks (Garcia-Perez et al., 2003). Several studies indicated the risks of severe disease outbreaks when introducing naïve animals into a tick-borne pathogens endemic area (Corn and Nettles, 2001).

This study was designed to investigate the causes of an outbreak of disease occurred in Assaf sheep purchased from central Spain and introduced into a Latxa sheep flock in the Basque Country. A combination of already available laboratory methods and two newly developed multiplex real-time PCR assays for diagnosis of common tick-borne pathogens affecting livestock (piroplasms and anaplasmas) were used. The developed techniques and the clinical, laboratory and epidemiological findings of the case study are described, along with the implications of introducing naïve animals into an endemic region.

Materials and methods

Flock description and management characteristics

The studied flock was composed of Latxa dairy breed ewes ($N = 380$) and was located in the province of Alava (Basque Country, northern Spain). Latxa ewes and replacement lambs are managed under a traditional semi-extensive husbandry system. Ewes are kept indoors while lambing and at the beginning of milking (January–March). For the remainder of the milking season (April–July) ewes are raised at farmland pastures and housed at night. The rest of the year, animals graze on mountain pastures. In December 2012 the farmer purchased 120 Assaf ewes (some of them pregnant) and 40 newborn lambs from Castilla-León (central Spain) where animals had been managed under an intensive management system for milk production. Upon their entrance into the flock, Assaf sheep were maintained indoors until the first week of April, when animals started grazing on farmland pastures a few hours daily. A few days later, 4 Assaf ewes died and, since clostridial disease was suspected, all the animals were immediately vaccinated. The rest of the season was rainy and colder than usual, and Latxa and Assaf ewes followed the same management until the beginning of summer. Replacement lambs ($N = 61$) were weaned in February and raised indoors until the end of June. Then, ewes and lambs started grazing in a bushy pasture.

Case presentation and samples collection

First clinical signs appeared soon after animals started grazing at the beginning of summer 2013. On the 8th July, when ewes entered into the milking parlour, ticks infesting sheep were observed for the first time. One week later (15th July), animals started to die.

First samples were submitted to the laboratory on the 24th July and included 2 sheep (Ewes 1 and 2) and blood samples collected with EDTA from these and a third animal (Ewe 3) with clinical signs. Since tick-borne disease was suspected, additional blood and serum samples from affected animals was requested, and samples from another 20 affected animals (10 replacement lambs older than 6 months and 10 ewes) were later submitted (3rd August).

Pathological, biopathological and parasitological analyses and initial differential diagnosis

Before necropsy, the 2 animals were externally examined, and attached ticks were collected for species identification (Manilla, 1998). The sheep were then necropsied and the presence of lesions was assessed macroscopically. Selected tissues were fixed in 10% formalin and embedded in paraffin wax; 4 μm sections were cut and stained with hematoxylin-eosin for histological examination.

Haematological analyses (leukocyte and erythrocyte cell counts, packed cell volume (PCV), haemoglobin, leukocytic cell differentiation) were performed with an electronic counter (Hemavet 950, Drew, USA) in blood samples collected in EDTA-containing tubes. Thin blood smears were stained with Giemsa and analysed under an oil-immersion objective for the study of piroplasms (*Babesia* spp./*Theileria* spp.) or *Anaplasmataceae* bacteria (*A. phagocytophilum* in leukocytes or *A. ovis* in erythrocytes). Urine taken from dead animals during the necropsies was subjected to semi-quantitative standard commercial urine test (Combur^{10} test M, Roche Diagnostics, Germany) and the urine sediment collected after centrifugation was examined under the microscope.

Smears from intestine content of the 2 necropsied ewes were stained by Gram's method and examined under the microscope for the study of *Clostridium* spp. Tissues from udder (Ewe 2) were inoculated on Columbia agar, supplemented with 5% sheep blood, MacConkey agar, and PPLO enriched medium (agar and broth), for bacterial isolation. Presence of antibodies against *Leptospira interrogans* serovar Icterohaemorrhagiae in sera from affected and dead animals was analysed by Microscopic Agglutination Test (MAT), considering titres $\geq$1/100 as indicative of contact with the pathogen (OIE, 2008). In addition, the presence of *Leptospira* spp. DNA in urine samples from necropsied ewes was evaluated by a PCR assay targeting the *lipL32* gen (Levett et al., 2005). To rule out copper (Cu) poisoning, kidney and liver tissue samples from necropsied animals and sera from affected animals were analysed by atomic absorption spectrometry (Varian, SpectrAA 220 FS).

DNA extraction

DNA was extracted from 200 μl of blood using the BioSprint 96 DNA Blood Kit and the BioSprint 96 Workstation (Qiagen, Hilden, Germany). Negative controls were included to monitor for possible contamination. Extracted DNA was re-suspended in 100 μl of elution buffer.