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Detection of *Babesia* and *Theileria* species infection in cattle from Portugal using a reverse line blotting method

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ABSTRACT

Babesiosis and Theileriosis are tick-borne diseases widespread in tropical and sub-tropical regions with high economic impact worldwide. In Portugal there are at least 4 tick vectors known to be competent for the transmission of *Babesia* and *Theileria* sp. identified: *Rhipicephalus bursa*, *Rhipicephalus* (*Boophilus*) *annulatus*, *Ixodes ricinus* and *Haemaphysalis punctata*. All these potential *Babesia* and *Theileria* tick vectors are widely distributed in Portugal, although they are predominant in the Southern region. In this study, 1104 cattle blood samples were randomly collected from Central and Southern regions of Portugal and analyzed by PCR–reverse line blotting (RLB) for the detection of *Babesia* and *Theileria* sp. Testing indicated that 74.7% of the bovines tested were positive for either *Babesia* and/or *Theileria* sp. In addition, five different apicomplexan species, namely, *Theileria buffeli*, *Theileria annulata*, *Babesia divergens*, *Babesia bovis*, and *Babesia bigemina* were detected by RLB among the bovines tested. *T. buffeli* was the most frequently found species, being present in 69.9% of the positive samples either as single infections (52.4%), or as mixed infections (17.5%). The *Babesia* specie most frequently found was *B. divergens*, detected in 4.2% of the infected bovines. Overall, infected bovines were found in all regions tested; however the highest number of infected bovines was observed in Évora district (96.2%) and in cattle from Limousin breeds (81.7%). The results indicate widespread *Babesia* and *Theileria* infections in Portuguese bovines, suggesting the need for improved control of ticks and tick-borne diseases.

1. Introduction

Babesia and *Theileria* species are apicomplexan-hemoprotozoan parasites transmitted by Ixodidae ticks (Preston, 2001; Uilenberg, 2001). These infections are of worldwide importance and are characterized by anemia, icterus, hemoglobinuria and death (Wagner et al., 2002), having a high economic impact in several parts of the world, including tropical and temperate countries (Wagner et al., 2002).

In continental Portugal there are an estimated total of ~3.9 million food animals with economic importance. From them ~2.8 million are ovine and caprine, and 1.1 million are bovine, which are susceptible to tick infestation and consequently, to infection with tick-borne diseases (TBDs) including babesiosis and theileriosis. Twelve different species of ticks, which are grouped in two main families (Ixodiade and Argasinae) and six genera (*Ixodes*, *Rhipicephalus*, *Hyalomma*, *Dermacentor*, *Haemaphysalis* and *Ornithodoros*), were found infesting Portuguese cattle so far (Caeiro, 1999). Four of these type of ticks are competent

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vectors for both *Babesia* sp. and *Theileria* sp. transmission in cattle: *Rhipicephalus* (*Boophilus*) *annulatus*, *Rhipicephalus bursa*, *Ixodes ricinus* and *Haemaphysalis punctata* (Caeiro, 1999; Estrada-Peña et al., 2004), and are predominantly found in South Eastern regions of Portugal. Additionally, *R. bursa* can also be found in a small part of Central and Northern Portugal. All these ticks are located mainly in areas where natural pasture and *Quercus* sp. are the main vegetation (Estrada-Peña and Santos-Silva, 2005).

Microscopic detection, such as Giemsa staining of blood smears is mostly used as a confirmatory diagnosis of cattle suffering the acute phase of the *Babesia* and *Theileria* disease and, therefore, showing clinical symptomatology, but it cannot discriminate among parasites and is not appropriate for large scale epidemiology, prevalence and distribution studies. Detection of antibodies is used to test for previous exposure to the aetiological agent and, thus, widely used in epidemiological studies to estimate the seroprevalence of these diseases, usually in the absence of clinical disease, such as in subclinically infected carrier cattle. Other diagnostic techniques, based on the detection of DNA from the infective agent, such as PCR and reverse line blotting (RLB) are able to simultaneously detect and differentiate the infecting organisms in a given animal (Schnittger et al., 2004). In this study we provide data on the distribution of *Babesia* and *Theileria* species in predominant tick areas of Portugal, using the PCR–RLB technique.

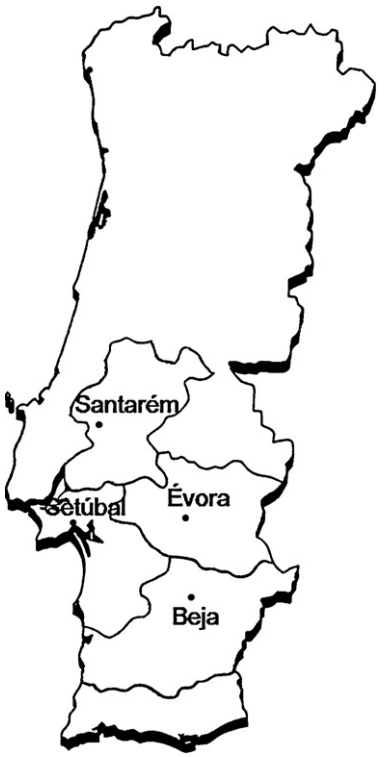

Fig. 1. Map representing the four regions where the cattle blood was randomly collected: Santarém (Central region), Setúbal (Southern West region of Portugal), Beja and Évora (Southern East region of Portugal).

2. Materials and methods

2.1. Overall design of the survey

Whole blood samples were randomly collected from 1104 clinically healthy bovines from randomly selected farms from Central and Southern regions of Portugal (Fig. 1). The samples were collected during 2006 in four different Portuguese districts: 98 samples from 2 farms in Santarém (Central), 348 samples from 47 farms in Setúbal (Southern West), 26 samples from 1 farm in Évora and 632 samples from 48 farms in Beja (Southern East). The number of cattle in farms range between 36 and 250. Blood samples from approximately 10% of the total number of cattle in the farm were used in this study. The presence of *Babesia* and *Theileria* sp. was studied among 5 distinct defined cattle breeds: Charolais crossbred, Limousin crossbred, Limousin, Friesian, Mertolenga and cattle from undefined, multiple-crossbreeds (designed as unknown breed). The ages of the cattle studied varies widely: <1 y: 18; >1–2 y: 33; >2–3 y: 163; >3 y: 890.

The 2006 annual average of maximum temperature in these districts was ~22 °C, and the average lowest temperature was ~10 °C. However, the accumulate precipitation changed widely through the districts. Santarém and Setúbal with annual average of 800 mm, Évora with 600 mm and Beja with lower than 600 mm.

2.2. Molecular detection of Babesia and/or Theileria sp. parasites

DNA was extracted from whole blood collected with EDTA as an anticoagulant using the DNA Purification System Blood Kit – Puregene™ (Gentra/Qiagen), according to manufacturer's instructions, and stored at −20 °C until used.

The first step was PCR amplification of DNA from both *Theileria* and *Babesia* species using a set of primers designed to amplify a variable region in the 18S ribosomal RNA (rRNA) gene. These primers were designed for specific amplification of a region within the rRNA genes that is conserved among *Theileria* and *Babesia* species. The primer's sequences are not complementary to the hosts or ticks rRNA genes, thus resulting in a high specificity for the PCR reaction. The sequence of the forward primer was 5′-GAC ACA GGG AGG TAG TGA CAA G-3′ and for the reverse primer was Biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′ (Nijhof et al., 2003). PCR amplifications were performed in a 50 μl volume using 35 repetitive cycles (94 °C for 60 s, 55 °C for 90 s, 72 °C for 90 s), and a final elongation step at 72 °C for 10 min. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

For the further detection of specific species of *Babesia* and *Theileria*, the amplicons obtained in the previously described PCR reaction were then hybridized onto a RLB membrane (from commercial TBD-RLB kit, Isogen Life Science, Ijsselstein, The Netherlands) designed to detect *Babesia*/*Theileria* sp. The RLB procedure was performed as described in detail in Gubbels et al. (1999). Briefly, a Biodyne C blotting membrane immobilized with specific oligonucleotide probes for *Theileria* and *Babesia* species