



Short communication

Detection of *Theileria* and *Babesia* infections amongst asymptomatic cattle in PortugalJacinto Gomes^{a,*}, Ricardo Soares^a, Marcos Santos^a, Gabriela Santos-Gomes^b, Ana Botelho^a, Ana Amaro^a, João Inácio^a^a Instituto Nacional de Recursos Biológicos, I.P., Laboratório Nacional de Investigação Veterinária (INRB, I.P. – LNIV), Estrada de Benfica 701, 1549-011 Lisboa, Portugal^b Unidade de ensino e Investigação de Parasitologia Médica, Centro de Malária e outras Doenças Tropicais, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (IHMT/UNL), Rua da Junqueira 100, 1349-008 Lisboa, Portugal

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ABSTRACT

Piroplasmosis caused by tick-borne hemoprotezoans of several *Theileria* and *Babesia* species has a major impact on livestock production worldwide. A reverse line blotting assay that includes genus- and species-specific probes for *Theileria* and *Babesia* species was used to assess the occurrence of these parasites in blood samples collected from 1407 healthy bovines throughout mainland Portugal. The global prevalence of piroplasm-infected animals was 36.8%, although significant differences were found between various regions. Higher prevalence was found in the southern regions (42.4% in Lisbon and Tagus Valley, 51.6% in Alentejo, and 40.0% in Algarve) compared to central (23.1%) and northern (12.8%) Portugal. *Theileria annulata* was the most frequently found species with a prevalence of 21.3%. The prevalence values of this pathogenic species were higher in the southern regions. The second most prevalent species was *T. buffeli* that infected 10.1% of the bovines. A low prevalence was found for *Babesia* infections (7.9%) with *B. bigemina* as the most frequent species. Some animals were infected with *T. ovis* and *B. occultans*. Veterinary practitioners and stakeholders should be made aware of the existence of a relatively high prevalence of carrier animals infected with *T. annulata* in Portugal, given the potential threat this pathogenic parasite represents to the cattle industry.

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Introduction

Piroplasmosis caused by tick-borne hemoprotezoans of several *Theileria* and *Babesia* species has a major impact on livestock production worldwide. Animals that survive the acute disease can become chronic piroplasm carriers and play an important role as a reservoir for the maintenance of the parasite life cycle. Tropical theileriosis, caused by *Theileria annulata*, is considered an endemic infection of cattle in some Portuguese regions. However, several cases of fatal acute disease in calves were reported recently in southern Portugal (Branco et al., 2010). Given the potential threat of tropical theileriosis and of other piroplasmoses to the Portuguese livestock industry, an enhanced awareness of the epidemiological traits of *Theileria* and *Babesia* parasites in this country is essential for assessing exposure risks and a better planning of prophylactic and control measures. Although previous studies confirmed the occurrence of these parasites in some geographically confined Portuguese regions (Brígido et al., 2004; Silva et al., 2010),

a comprehensive picture of their prevalence and distribution covering all regions of Portugal is still lacking. An epidemiological survey was therefore conducted in order to assess the *Theileria* and *Babesia* species infecting bovines (*Bos taurus*) from all regions of mainland Portugal.

Materials and methods

Cattle blood samples

This study included 1407 *Bos taurus* from the 5 mainland administrative regions of the Portuguese Ministry of Agriculture: DSVN (here named North), DSVC (Center), DSVLVT (Lisbon and Tagus Valley), DSA (Alentejo), and DSVAl (Algarve). The cattle population in these regions was approximately 1.3 million in 2009, according to the Census 2009 of the Portuguese Veterinary Authorities (Table 1). A stratified sampling was performed to ensure a similar proportion between the number of sampled animals and the cattle population from different regions. Blood samples were collected into sterile tubes with EDTA from apparently healthy animals, during 2008, and stored at 4 °C until further processing for DNA extraction.

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Table 1
Size of cattle populations in each Portuguese region (according to Census 2009) and respective number of bovines from which blood samples were collected.

Region	Total number of cattle	% of the national herd	Average number of animals per farm	Samples collected	
				Number of blood samples	Number of sampled farms
North	346,674	26.15	11	337	106
Center	174,345	13.15	12	169	77
Lisbon and Tagus Valley	171,506	12.94	46	257	89
Alentejo	623,024	47.00	120	599	176
Algarve	9991	0.75	24	45	22

DNA extraction and reverse line blotting

Total genomic DNA was extracted from bovine whole-blood samples by a BioSprint[®]96 automated workstation (Qiagen), using the BioSprint[®]96 Blood kit (Qiagen) according to the manufacturer's instructions, and stored at -20°C until further use. The average total DNA yield in these samples was around 35–40 ng/ μl (as assessed using a NanoDrop[™] 1000 Spectrophotometer from Thermo Scientific). The detection of *Theileria* and *Babesia* species in the DNA samples was performed by a reverse line blotting (RLB) assay (Gubbels et al., 1999; Georges et al., 2001). Briefly, primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (biotin-5'-CTA AGA ATT TCA CCT CTG ACA GT-3') (STAB Vida Lda, Portugal) were used to amplify and label a segment of the 18S rDNA spanning the hypervariable V4 region of all *Theileria* and *Babesia* species (Georges et al., 2001). The PCR products were then hybridized with universal and specific probes for *Theileria* and *Babesia* species linked to a commercial RLB membrane (TBD-RLB kit, ISOGEN Life Sciences; probe sequences are shown in Supplemental Table 1). It was reported that the *T. annulata*-specific probe used in this membrane cross-reacts with *T. lestoquardi*. However, this latter species is unable to infect cattle (Leemans et al., 1999). Additionally, when referring to *T. buffeli*, we are actually referring to the biologically related group of *T. buffeli*, *T. orientalis*, and *T. sergenti*. Preparation, hybridization, and stripping of the RLB membrane were performed according to manufacturer's instructions and as described previously (Gubbels et al., 1999). The nucleotide sequence of selected PCR products corresponding to samples that hybridized only with *Theileria/Babesia* catch-all probes was determined using the same above-mentioned RLB-F2 and RLB-R2 primers for the sequencing reactions (STAB Vida Lda, Portugal).

Statistical analysis

Pearson's chi-square test was used to determine differences between the prevalence of piroplasm from different regions (software SPSS, version 19.0). Each test was conducted at a 95% confidence interval ($P < 0.05$).

Results

The RLB assay detected 518 positive blood samples (out of 1407), corresponding to a prevalence of 36.8% (CI: 95%, 34.3–39.4) of piroplasm infected animals. The frequency and distribution of all *Theileria* and *Babesia* infections are summarized in Table 2. Positive samples included 399 single infections and 74 mixed infections. Additionally, 45 samples revealed to be positive by hybridizing with *Theileria/Babesia* catch-all probes (but not with any species-specific probe). When comparing different regions, the prevalence of piroplasm-infected animals ranged from 12.8% (CI: 95%, 9.6–16.7%) in the North and 51.6% (CI: 95%, 47.6–55.6%) in Alentejo, with significant differences observed among the

5 geographic regions ($P < 0.05$) (Table 2). The most abundant parasite was *T. annulata* with a total prevalence of 21.3% (CI: 95%, 19.3–23.5%). However, the prevalence of this species ranged from 3.3% (CI: 95%, 1.8–5.7%) in the North to 33.5% (CI: 95%, 28–39.4%) in Lisbon and Tagus Valley (Table 2), with significant differences observed among the 5 regions ($P < 0.05$). The total prevalence of *T. buffeli* was 10.1% (CI: 95%, 8.6–11.8%) (Table 2). Significant differences in prevalence were also observed for this species within the 5 regions ($P < 0.05$) (Table 2). *Babesia bigemina* was identified in 7.8% of samples (CI: 95%, 6.5–9.3%) and significant differences in prevalence were observed among the 5 regions ($P < 0.05$). One sample was positive for *B. divergens* (in Alentejo), and *B. bovis* was never detected in this study (Table 2). Alentejo is the main area of cattle production in Portugal, comprising 47% of the total bovine population. Therefore, we further studied the distribution of these parasites in the different veterinary administrative subregions of Alentejo (Portalegre, Elvas, Évora, Beja, and Alcácer do Sal). Statistical analysis showed significant differences only in the distribution of *T. annulata* and *T. buffeli*, but not of that of *B. bigemina*. The highest prevalence of *T. annulata* was in Elvas (44.7%) (35.0% in Portalegre and 39.0% in Évora) and of *T. buffeli* in Alcácer do Sal (51.0%).

The nucleotide sequences of the V4 region were obtained for 9 samples that hybridized only with *Theileria/Babesia* catch-all probes (deposited in NCBI-GenBank with accession numbers JX088587–JX088595). Sequences identical to the ones of *T. ovis*, *B. occultans*, *T. annulata*, and *B. bigemina* were found in 5, 2, 1, and 1 samples, respectively.

Discussion

The global prevalence of piroplasm-infected animals in mainland Portugal was 36.8%, although significant differences were found between the various regions of the country. A higher prevalence was found in the southern regions (42.4% in Lisbon and Tagus Valley and 51.6% in Alentejo) (Table 2). These differences are certainly related to the distinct vegetation and climatic characteristics of those regions, influencing vector tick distribution and abundance across the country and, as a result, the incidence of piroplasmosis. Several vectors of piroplasms such as *Hyalomma* tick species have been found concentrated in the southeastern regions, under hot and dry Mediterranean type climate conditions and where grazing ruminants and other animals are also abundant (Estrada-Peña and Santos-Silva, 2005). In contrast, the occurrence of ticks in central and northern Portuguese regions has a more or less patchy distribution as a consequence of lower temperatures and lower prevalence of grazing ruminants (Estrada-Peña and Santos-Silva, 2005). Different livestock management systems amongst regions can also influence the exposure of animals to vector ticks. The regions of Lisbon and Tagus Valley and Alentejo are the main areas of livestock industry, mainly for beef production. Animals are usually produced outdoors under extensive farming systems and are consequently more prone to exposure to ticks. In contrast, the northern regions harbor mainly small-scale dairy farms where animals are raised

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