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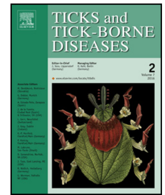

Short communication

Detection and phylogenetic characterization of *Theileria* spp. and *Anaplasma marginale* in *Rhipicephalus bursa* in Portugal

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ABSTRACT

Ticks are obligatory blood-sucking arthropod (*Acari*:*Ixodida*) ectoparasites of domestic and wild animals as well as humans. The incidence of tick-borne diseases is rising worldwide, challenging our approach toward diagnosis, treatment and control options. *Rhipicephalus bursa* Canestrini and Fanzago, 1877, a two-host tick widely distributed in the Palearctic Mediterranean region, is considered a multi-host tick that can be commonly found on sheep, goats and cattle, and occasionally on horses, dogs, deer and humans. *R. bursa* is a species involved in the transmission of several tick-borne pathogens with a known impact on animal health and production. The aim of this study was to estimate *R. bursa* prevalence in Portugal Mainland and circulating pathogens in order to contribute to a better knowledge of the impact of this tick species. *Anaplasma marginale* and *Theileria* spp. were detected and classified using phylogenetic analysis. This is the first report of *Theileria annulata* and *Theileria equi* detection in *R. bursa* ticks feeding on cattle and horses, respectively, in Portugal. This study contributes toward the identification of currently circulating pathogens in this tick species as a prerequisite for developing future effective anti-tick control measures.

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1. Introduction

During the last two decades, a growing interest in tick-borne diseases from veterinary, medical, and public health perspectives has emerged (Jongejan and Uilenberg, 2004). The worldwide prevalence of these diseases is steadily rising, challenging how we approach diagnosis, treatment and preventative control measures, and underlining the importance of the One Health concept (Dantas-Torres et al., 2012). Ticks are known to have a significant impact on host species through their feeding behavior, causing direct skin and sub-cutaneous tissue damage and blood depletion, whilst acting as vectors of different pathogens, such as viruses, bacteria, protozoa or fungi (Bell-Sakyi et al., 2007; Colebrook and Wall, 2004). It is estimated that approximately 10% of tick species exert an active role as biological vectors in the transmission of tick-borne pathogens, including several zoonotic agents (Heyman et al., 2010; Jongejan and Uilenberg, 2004; Labuda and Nuttall, 2004). Amongst these tick species is *Rhipicephalus bursa* Canestrini and Fanzago, 1877, classified in the Ixodidae family (Walker et al., 2000). Epidemiological studies have identified *R. bursa* as being widely distributed in the Mediterranean region where the climate is typically characterized by long dry summers and cold winters (Walker et al., 2000; Yeruham et al., 1985). Considered a multi-host tick, the primary hosts of this species include cattle, sheep, and goats (Santos-Silva et al., 2011; Walker et al., 2000). Though less common, this tick can also be found in other domestic animals, as well as in wild ungulates and small-medium sized mammals and sporadically, in humans (de la Fuente et al., 2004b; Mihalca et al., 2012; Psaroulaki et al., 2006; Santos-Silva et al., 2011; Satta et al., 2011; Walker et al., 2000). *R. bursa* has been described as being involved in the transmission of agents of the genus *Anaplasma* (de la Fuente et al., 2004a), *Babesia* (Altay et al., 2008; M'Ghirbi et al., 2010),

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Theileria (Garcia-Sanmartin et al., 2008), *Rickettsia* (Boudebouch et al., 2009; de Sousa et al., 2006; Ioannou et al., 2011; Toledo et al., 2009) among others, with a known impact on animal health. The main objective of this study was to provide up-dated information regarding the currently circulating pathogens in *R. bursa* and their phylogenetic characterization, in Portugal Mainland, for the future development and planning of effective tick control measures.

2. Materials and methods

2.1. Ticks samples

A total of 266 *R. bursa* ticks were included in this study. Ticks were collected from 2007 to 2014 in 24 local administrative units – municipalities (LAU I) belonging to 11 out of the 28 Mainland Portuguese subregions (Nomenclature of Territorial Units for Statistics regions – NUTS III), including the intermunicipal community (IMC) of Minho-Lima, IMC Cávado, IMC Ave, Alto Trás-os-Montes, IMC Douro, Beira Interior Sul, IMC Médio Tejo, Alto Alentejo, Peninsula de Setúbal, IMC Alentejo Litoral and IMC Baixo Alentejo. Fig. 1 shows the number of *R. bursa* specimens collected and the locations, according to geographical coordinates and subregions (QGIS 2.4.0. Chugiak). Ticks were either removed from domestic animals by local veterinarians or collected by flagging/dragging the vegetation and further identified to species level using morphological keys, as previously described (Santos-Silva et al., 2011). After identification, ticks were preserved in 70% alcohol, separated according to instars, origin and site of collection, until further manipulation.

2.2. DNA extraction, PCR screening and amplicon sequencing

Each tick was recovered from ethanol, rinsed in pH 7.4 phosphate-buffered saline (PBS), homogenized and used for DNA extraction using TriReagent (Sigma–Aldrich, Lisbon, Portugal), as previously described (Antunes et al., 2015). DNA concentration and purity was accessed by spectrophotometry (Thermo Scientific NanoDrop 2000, Lisbon, Portugal). DNA was stored at −20 °C for downstream application.

An initial screening to validate DNA extraction was performed in a group of samples randomly selected, representing 20% of all extracted ticks. Using the primer pair T1B/T2A that targets a 360 bp fragment of tick mitochondrial *12S rDNA*, a PCR was performed as previously described (Beati and Keirans, 2001).

To amplify *Anaplasma* spp. and *Ehrlichia* spp. a broad range PCR screen with the primers EHR16sD/EHR16sR was conducted as reported before (Inokuma et al., 2000). This primer set amplifies a 345 bp fragment of the *16S rRNA* gene of bacteria within the family Anaplasmataceae, including the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*. For piroplasms, a PCR targeting a 408 bp fragment from the small subunit of *18S rDNA* of *Babesia* spp. and *Theileria* spp. was conducted using the primer set Piro-A and Piro-B as described elsewhere (Harrus et al., 2011). To detect *Coxiella burnetii* DNA, a nested-touchdown PCR was done using the primer pairs Trans1/2 followed by Trans3/4 that amplify a 243 bp fragment of the repetitive insertion element *IS1111* (Lorenz et al., 1998). Primers were obtained from StabVida (Lisbon, Portugal). PCR were performed in 25 μl reactions with Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 1 μM primers and up to 5 μl of template DNA. Nuclease-free water was used as negative control. As positive controls, DNA extracted from reference strains was used: *Anaplasma marginale* Va-48 strain, *Babesia bigemina* Israel strain, *C. burnetii* Nine Mile strain (Vircell, Spain) and *Theileria annulata* (Uzbek strain). Amplifications were performed in a T100 thermal cycler (Biorad, Amadora, Portugal) according to references

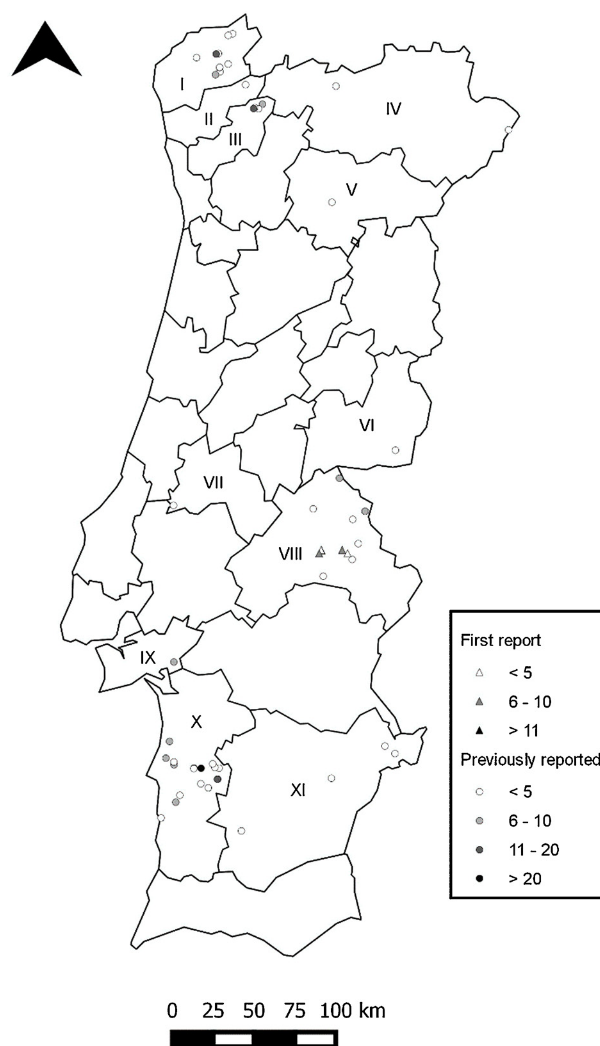

Fig. 1. Geographical locations and number of *Rhipicephalus bursa* found according to NUTS III Subregions. Map was performed using the QGIS 2.4.0. Chugiak program. Circles – Corresponds to the exact coordinates of collection sites in previously reported municipalities; Triangles – Corresponds to the exact coordinates of collection sites in new municipalities; I – Intermunicipal community (IMC) of Minho-Lima, II – IMC Cávado, III – IMC Ave, IV – Alto Trás-os-Montes, V – IMC Douro, VI – Beira Interior Sul, VII – IMC Médio Tejo, VIII – Alto Alentejo, IX – Peninsula de Setúbal, X – IMC Alentejo Litoral, XI – IMC Baixo Alentejo.

(Harrus et al., 2011; Inokuma et al., 2000; Lorenz et al., 1998). Positive amplicons were purified using the NZYGelpure kit (NZYtech, Lisbon, Portugal) and sent for Sanger sequencing at StabVida (Lisbon, Portugal). The obtained sequences were aligned, compared to those already deposited in the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast).

2.3. Phylogenetic analysis

The phylogenetic analyses were conducted with *A. marginale*, *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia canis 16S rDNA* (family Anaplasmataceae) and *Theileria* spp. *18S rDNA* (Apicomplexan) nucleotide sequences aligned with MAFFT (v7) configured for the highest accuracy (Katoh and Standley, 2013). After alignment, the sequences were cured using Gblocks (Castresana, 2000) with the following parameters: minimum length of a block after gap cleaning: 10, no gap positions were allowed in the final alignment and all segments with contiguous nonconserved positions bigger than

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