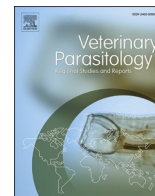




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Regional report

Detection of tick-borne pathogens in *Rhipicephalus bursa* ticks collected from the autochthonous Garrano breed of horses in Portugal

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ABSTRACT

The Garrano is a semi-feral horse breed native to several mountains in the northern Iberian Peninsula. Despite being endangered, this unique breed of pony has managed to survive in the wild and continues to be selectively bred, highlighting their remarkable resilience and adaptability to harsh environments.

Wildlife plays a critical role in the survival of tick vectors in their natural habitats and the transfer of tick-borne pathogens, as they can serve as reservoir hosts for many agents and amplifiers for these vectors. The semi-feral lifestyle of the Garrano horses makes them particularly vulnerable to exposure to numerous tick species throughout the year.

Therefore, the aim of this study was to investigate the occurrence of *Anaplasma*, *Ehrlichia*, *Babesia*, *Theileria*, and spotted fever rickettsiae in the Garrano horse ticks to obtain a knowledge of circulating agents in this host population.

The collected ticks ($n = 455$) were identified as *Rhipicephalus bursa*. DNA specimens were organized in pools of 5 ticks, for molecular screening. Pools PCR results confirmed the presence of *Candidatus Rickettsia barbariae* ($n = 12$ for the *ompB* gene, $n = 11$ for the *ompA* gene and $n = 6$ for the *gltA* gene), *Babesia bigemina* ($n = 1$), *Babesia caballi* ($n = 3$), *Theileria equi* ($n = 15$) and *Theileria haneyi* ($n = 1$). These results confirm the circulation of an emerging rickettsial spotted fever group member, *Candidatus R. barbariae*, in *R. bursa* ticks. Our findings demonstrated that *Candidatus R. barbariae* co-circulates with *B. bigemina* and *T. equi*, which are vectored by *R. bursa*. We are reporting for the first time, the detection of *T. haneyi* among *R. bursa* ticks feeding in the Garrano horses in Portugal.

Surveillance studies for tick-borne infections are essential to provide information that can facilitate the implementation of preventive and control strategies.

1. Introduction

Over the centuries, human and horses have held a long-term relationship, with a significant position in the growth and progress of Portugal. Four Portuguese autochthonous horse breeds have been officially recognized, Lusitano, Sorraia, Terceira and Garrano (Lopes et al., 2015). The Garrano is an endangered Portuguese semiferous horse breed, that still inhabits wild conditions in several mountains north of the

Iberian Peninsula (Brito & Silva, 2019). This distinct breed of pony continues to be selectively bred in a wild manner, which demonstrates the breed's exceptional durability and ability to adapt to challenging conditions (Brito & Silva, 2019).

Due to their semiferous lifestyle, these horses are highly exposed to several tick species throughout the year, obligate parasites that can transmit medically significant pathogens, such as the causative agents of equine piroplasmiasis, spotted fever rickettsiae, and equine granulocytic

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anaplasmosis (Parola et al., 2013).

Ixodid ticks play a critical role in the perpetuation and dissemination of pathogenic agents, through effective transmission mechanisms, such as transstadial perpetuation seen in *Theileria* spp. (Bishop et al., 2004; Olds et al., 2018) or a combination of transstadial and/or transovarian transmission as observed in *Rickettsia*, *Babesia*, *Anaplasma*, and *Ehrlichia* (Chauvin et al., 2009; Socolovschi et al., 2009).

The dissemination of tick-borne pathogens is determined by the transmission dynamics of different vertebrate species that coexist in the same geographic region and interact frequently, providing opportunities for these agents to propagate beyond their traditional host range, abundance, and geographic distribution (Krawczyk et al., 2020). This has led to the emergence of various arthropod-borne pathogens that originate from wildlife. Pathogens belonging to the family Anaplasmataceae (Order Rickettsiales) are of particular concern due to their worldwide distribution and zoonotic potential (Cossu et al., 2023). These include *Anaplasma*, *Ehrlichia* and *Rickettsia* spp. (Rickettsiaceae family), obligate intracellular Gram-negative bacteria that infect a wide range of arthropods and vertebrates (Cossu et al., 2023; Parola et al., 2013).

Elements from the *Anaplasma* and *Ehrlichia* genera can induce persistent infection in vertebrates, allowing them to be reservoirs (Rikihisa, 2010). The nature and virulence of the infection cycle of different strains of these bacteria depend on the susceptibility of the vertebrate host and the availability and frequency of ticks (Dumler et al., 2001). Both *Anaplasma* and *Ehrlichia*, can infect domestic and wild animals, but also humans (Doudier et al., 2010).

Anaplasma phagocytophilum has been described as the agent responsible for causing equine, canine and human granulocytic anaplasmosis (El Hamiani Khatat et al., 2021). Previous studies have described that *Ixodes* spp. are the main vectors for *A. phagocytophilum*, such as *Ixodes ricinus* (Santos et al., 2004) and *Ixodes ventralis* found in Portugal (Santos et al., 2018). However, it has been reported that *Rhipicephalus sanguineus* and *Hyalomma marginatum* ticks may also play a role in this issue (Hamidinejat et al., 2021).

Rickettsia is transmitted by arthropods that bite to animals and humans, being Ixodid ticks as the primary vectors of this bacterium. However, fleas, lice and mites also contribute to rickettsiae transmission (Blanton, 2019). The genus *Rickettsia* is classified into more than 31 species (List of Prokaryotic names with Standing in Nomenclature) and is divided into four major groups: spotted fever group (as *Rickettsia conorii*, *Rickettsia massiliae* and many others), typhus group (*Rickettsia prowazekii* and *Rickettsia typhi*), transitional group (*Rickettsia felis* and *Rickettsia akari*), and ancestral group (*Rickettsia bellii* and *Rickettsia canadensis*) (Blanton, 2019).

The Mediterranean Spotted Fever, caused by two strains of *Rickettsia conorii* complex, is endemic in Portugal, in which Ixodid ticks are the main vectors and reservoirs (Sousa et al., 2003). Spotted fever rickettsiosis is an important vector-borne disease, closely related not only to economic losses in animal production, including horses, but also to risks posed to humans (Li et al., 2020).

Another tick-borne disease is Equine piroplasmiasis (EP), caused by the eukaryotic hemoparasites *Theileria equi*, *Theileria haneyi*, and *Babesia caballi*, with a considerable veterinary and economic impact on the horse industry worldwide (Tirosch-Levy et al., 2020). Furthermore, affected horses remain persistently infected or at least infected for an extended period, which increases the potential for protozoa transmission when suitable vectors are present, despite the absence of transovarian transmission (Ueti et al., 2008; Uilenberg, 2006). Equine piroplasmiasis is considered a reportable disease by the World Organization for Animal Health. Various tick species that may act as vectors for EP are present in mainland Portugal, namely *Rhipicephalus sanguineus*, *Rhipicephalus annulatus*, *Rhipicephalus bursa*, *Dermacentor marginatus*, *Hyalomma lusitanicum* and *Hyalomma marginatum* (Santos-Silva et al., 2011). The high prevalence of *T. equi* and *B. caballi* in horses from Portugal has been demonstrated by previous studies (Fuehrer et al., 2020; Ribeiro et al.,

2013).

Wild animals have a crucial role in the natural life cycles of numerous tick-borne pathogens, as exophilic Ixodid ticks can feed on not only sylvatic and domestic animals, but also humans. As such wildlife can serve as reservoirs host for many pathogenic agents and/or as amplificatory hosts for the tick vectors. Consequently, the presence of wild animals is important for the continued existence of tick vectors in their natural habitats and the spillover of tick-borne pathogens (Otranto et al., 2015; Tomassone et al., 2018).

The aim of our study was to investigate the occurrence of several tick-borne pathogens namely *Anaplasma*, *Ehrlichia*, *Babesia*, *Theileria* and spotted fever rickettsiae in Garrano horse ticks to obtain a knowledge of circulating agents in this host population.

2. Materials and methods

2.1. Study area and tick sampling

Peneda-Gerês National Park (PGNP) in northern Portugal (longitude 8°25'W and latitude 41°41'N), is the only protected area with national park status in the country. With 69,594.48 ha, the PGNP is a mountainous region, essentially granitic, with deep, steep-sided valleys that support a dense hydrographic network that allows for a wide variety of life forms.

The Garrano population is essential for the PGNP ecosystem, in its landscape and as an important food chain element, contributing to the different species ecological balance, of the wolf, after the game disappearance. The horses are kept free-grazing, during all the year, usually in the wasteland, moving in herds in variable numbers.

Removing tick samples from wild animals is difficult because monitoring (or catching) of them is typically arduous. To address this issue, the researchers in this study gathered tick samples from Garrano during the marking process, for herd control, identification, and deworming purposes.

In July 2022, a total of 455 ticks were manually collected from 35 Garrano horses during the marking process, when the animals were guided through a management sleeve for herd control and sanitary control measures in a rural zone of PGNP. Specimens were preserved in 96% ethanol solution at room temperature until further processing. All collected ticks were taxonomically classified according to their morphology using standard taxonomic keys (Estrada-Peña et al., 2017).

2.2. DNA extraction methods

DNA of partially and engorged ticks was extracted and processed individually. Each tick was washed in 200- μ l of a 10% bleach solution for five minutes and then rinsed three times in deionized water to remove residual bleach. Arthropods were dried on filter paper and transferred to 1.5 ml tubes. A modification of the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) was used to extract DNA from the ticks, following previously described methods for nucleic extraction in ticks (Crowder et al., 2010).

In brief, each frozen tick was disrupted mechanically with a mortar and a pestle. The tubes were filled with 420- μ l of lysis buffer and 25- μ l proteinase K solution. The tubes were briefly mixed by vortexing for 30 s and then centrifuged for 2 min at 6000 \times g. A 350- μ l aliquot of the recoverable supernatant was transferred to a fresh microcentrifuge tube and 350- μ l of RTL buffer was added. The tubes were briefly mixed by vortexing for 30 s, pulse centrifuged, and incubated at 37 °C for 10 min. The next steps followed the QIAamp® DNAMini Kit (Qiagen, Valencia, CA, USA) using an automated QIAcube (Qiagen GmbH, Germany).

A negative extraction control was processed along with each batch of arthropods (12 samples).

To confirm tick morphological identification, conventional PCR reactions targeting a partial region of the mitochondrial 12S rDNA (Black and Piesman, 1994) were performed on a randomly selected sample of

ticks, representative of the identified species (two individual ticks and 22 pools of ticks).

2.3. Molecular detection of *Rickettsia*, *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria*

After taxonomic classification, ticks were organized in pools. Tick pools (5 ticks in each pool) were composed exclusively of adult ticks.

Tick DNA specimens were initially screened for the presence of SFG rickettsiae using a conventional PCR, targeting a broad spectrum 511 bp fragment of the outer membrane protein B (*ompB*) gene, as previously described (Choi et al., 2005). To confirm positive results and genetically characterize *Rickettsia* spp., ticks were further tested for a 532 bp fragment of the outer membrane protein A (*ompA*) gene (Regnery et al., 1991) and the near-complete (806 bp) of the citrate synthase (*gltA*) gene (de Sousa et al., 2005). For the detection of *Ehrlichia* and *Anaplasma*, DNA specimens were tested targeting a 345 bp fragment of the 16S rRNA gene (Gal et al., 2008). For the detection of *Babesia* and *Theileria*, DNA specimens were tested by a conventional PCR targeting a fragment (408 bp) of the 18S rRNA gene (Olmeda et al., 1997). All end-point PCR reactions were run on a T100 Thermal Cycler (Bio-Rad, Germany). The reaction mixtures contained Xpert Fast Hotstart Mastermix 2× with dye (GriSP, Portugal). The amplified DNA fragments were detected by subjecting the PCR amplification products to electrophoresis on 1% agarose gels stained with Xpert Green Safe DNA gel dye (GriSP, Portugal) at a voltage of 120 V for 30 min. UV light was used to validate and verify the obtained outcomes.

All positive amplicons were purified with Exo/SAP Go – PCR purification kit (GriSP, Portugal) and bidirectional sequencing was performed by Sanger method, using the respective primers of different target genes.

2.4. Blast search and phylogenetic data analysis

Sequences were manually corrected using the BioEdit Sequence Alignment Editor v 7.1.9 software, version 2.1 and further analysis was performed by comparison with the sequences available in the NCBI (GenBank) nucleotide database (<http://blast.ncbi.nlm.nih.gov/Blast>). For the phylogenetic analysis, the software MEGA version XI (Tamura et al., 2021) was applied. The analysis incorporated sequences obtained in this study, as well as representative sequences obtained from GenBank. A maximum-likelihood (ML) method was applied (Kumar et al., 2018; Tamura, 1992). The ML bootstrap values were estimated using 1000 replicates with Tamura 3-parameter (Tamura, 1992) as the correction model for 12S rDNA gene and *Rickettsia ompA* gene, Tamura-Nei (Tamura and Nei, 1993; Tamura et al., 2021) as the correction model for 18S rRNA gene and Hasegawa-Kishino-Yano (Hasegawa et al., 1985) as the correction model for *Rickettsia ompB* gene. MEGA version XI determined these models to be the most suitable replacement models.

The sequences obtained in this study were deposited in GenBank with accession numbers, OR544390 to OR544392 (*Babesia caballi*), OR544375 to OR544389 (*Theileria equi*), OR544393 (*Babesia bigemina*), OR544374 (*Theileria haneyi*), OR539295 to OR539306 (*Candidatus R. barbariae ompB* gene), OR539307 to OR539317 (*Candidatus R. barbariae ompA* gene), OR539318 to OR539323 (*Candidatus R. barbariae gltA* gene), OR552411 to OR552414 (*Rhipicephalus bursa*).

3. Results

3.1. Morphological and molecular identification of ticks

From the total of 455 adult ticks collected ($n = 165$ males and $n = 290$ females), all of them were morphologically identified at genus level as *Rhipicephalus* sp. Analysis of the 12S rDNA mitochondrial gene for molecular identification of the tick species was performed. BLAST

analysis of the 12S segments obtained from *Rhipicephalus bursa* demonstrated that these tick species are identical, sharing between 99.42 and 100% nucleotide identity with *Rhipicephalus bursa* (KC243833) from Italy.

3.2. Identification of rickettsiae in the examined ticks

From the total of 91 pools of ticks screened for *Rickettsia*, 12 were found positive for *ompB* gene. Further characterization of the BLAST analyses of the partial *ompB* gene showed an identity of 100% with *Candidatus Rickettsia barbariae* obtained from *Rhipicephalus bursa* ticks from Algeria (MK028339). To confirm positive results, the 12 tick pools were further studied for the *ompA* and *gltA* partial gene regions. When screening ticks for the *ompA* partial gene, 11 pools were amplified for this region. Out of these 11 pools, nine were also positive for *ompB* gene. Further characterization of the *ompA* sequences showed an identity between 99.8% and 100% with *Candidatus Rickettsia barbariae* obtained from *Rhipicephalus turanicus* from Turkey (MN482129). Furthermore, out of the tick pools tested for the *gltA* region, six pools that were *ompB* and *ompA* positive were found to have amplified this partial gene region.

BLAST analyses of the partial *gltA* gene indicated an identity of 100% with *Candidatus Rickettsia barbariae* (MK652441) from France and *Candidatus Rickettsia barbariae* (OQ076762) from Uzbekistan.

3.3. Identification of *Babesia* and *Theileria* spp. in the examined ticks

Out of the total of 91 pool ticks tested for *Babesia* and *Theileria* using the 18S rDNA mitochondrial gene, 20 tick pools amplified a product of the expected size for both genera. Based on sequence comparisons, two *Babesia* species (*B. bigemina* and *B. caballi*) and two *Theileria* species (*T. equi* and *T. haneyi*) were identified.

The BLAST analysis of the mitochondrial gene 18S rDNA of three pools of ticks showed that all were identical, being 99.80% identical to *B. caballi* (MK288107) from Israel and one pool of ticks presented a 100% identity with *B. bigemina* (MH257721) from South Africa. Fifteen tick pools showed an identity between 99.8% and 100% with *T. equi* detected in India (OQ992767), Brazil (MG052902) and Chile (MT463613) and one tick pool showed a 100% with *T. haneyi* detected in China (MT093500).

3.4. Identification of *Anaplasma* and *Ehrlichia* spp in the examined ticks

DNA samples from tick pools were also analyzed for the presence of *Anaplasma* and *Ehrlichia* targeting the 16S rRNA gene however all the samples were negative for both genera.

Phylogenetic analysis was performed for *Rhipicephalus bursa* mitochondrial 12S rDNA gene (Fig. 1), partial *Rickettsia ompB* gene (Fig. 2), partial *Rickettsia ompA* gene (Fig. 3) and, *Babesia* and *Theileria* 18S rDNA mitochondrial gene (Fig. 4) sequences to obtain information about their genetic relatedness with other species reference sequences.

4. Discussion

The Garrano horse breed is known to forage in the mountains of PGNP throughout the year, often in groups of varying sizes. The diverse range of natural environment conditions, including Atlantic, Mediterranean, and Continental climates, contributes to the region's exceptional botanic variety, abundant wildlife, and the presence of a wide array of migratory bird species (Lopes et al., 2015), recognized as the most significant carriers of ticks to far-flung biogeographic areas in the world (Buczek et al., 2020).

These obligatory hematophagous ectoparasites play a crucial role as both vectors and reservoirs for a variety of pathogens, including viruses such as tick-borne encephalitis virus and Crimean-Congo hemorrhagic fever virus, and bacteria such as *Anaplasma*, *Ehrlichia*, and *Rickettsia*, protozoa such as *Babesia* and *Theileria*. As such, they contribute to the

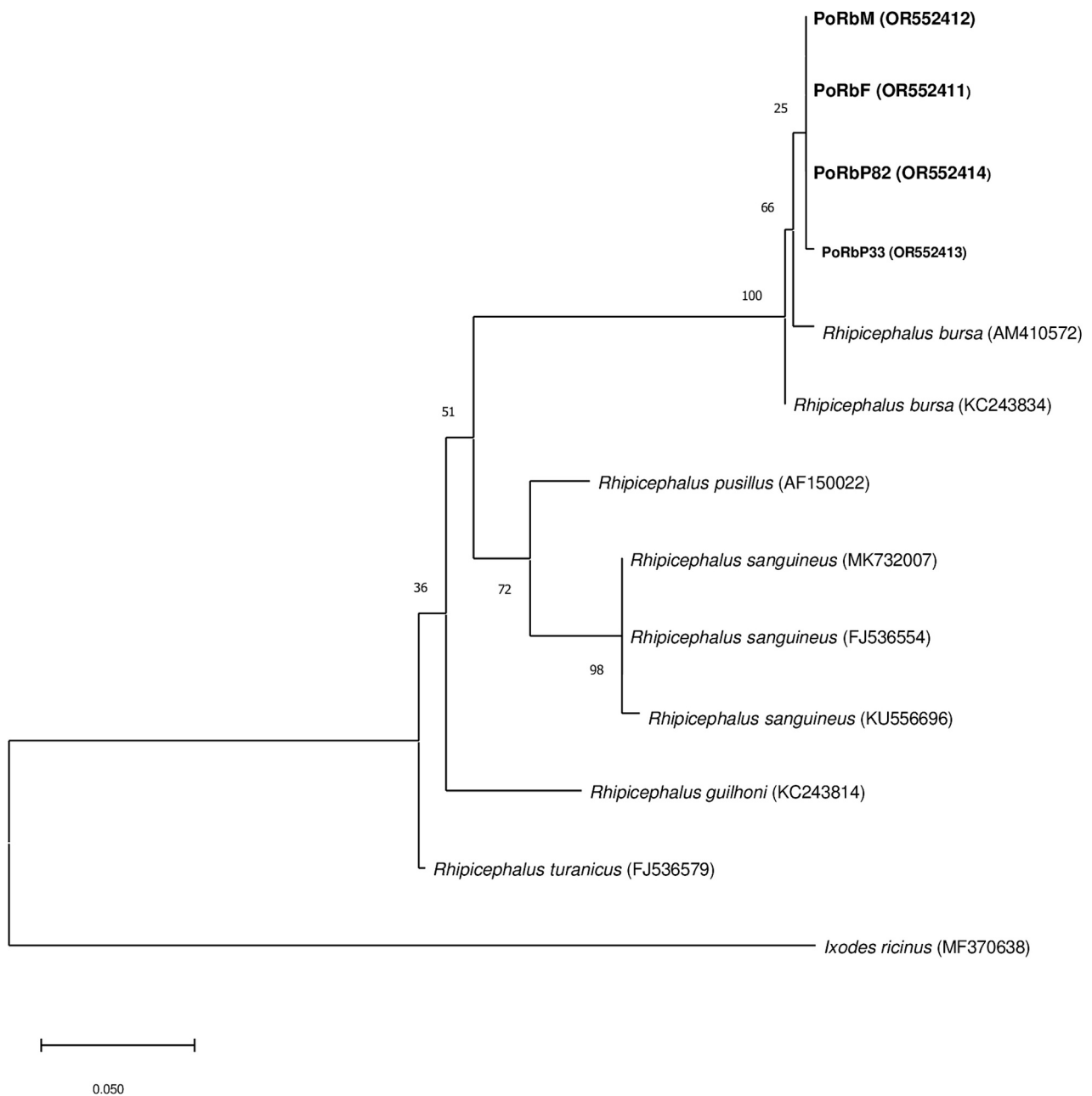


Fig. 1. Phylogenetic analysis based on partial 12S rRNA gene sequences of *Rhipicephalus* spp.

A maximum likelihood method based on the Tamura 3-parameter model phylogenetic tree was constructed based on *Rhipicephalus* 12S mitochondrial gene DNA sequences. Reliability of internal branches was assessed using the bootstrapping method (1000 replicates). *Rhipicephalus bursa* characterized in this study are shown as country/ tick species/ identification of tick (P, M or F).

Po: Portugal; Rb: *Rhipicephalus bursa*; P: pool number, M: male, F: female.

circulation of pathogenic agents in nature and the maintenance of foci for tick-borne diseases (Tomassone et al., 2018).

Like other wild animal species, Garrano horses are largely exposed to Ixodidae ticks, whose survival and dissemination depend on climatic and environmental conditions as well as the presence of potential hosts for all tick developmental stages.

In the present study, all the adult ticks removed from Garrano horses were identified as *R. bursa*, which confirms the occurrence of these tick species in the community of Minho-Lima, already described by other authors (Ferreiro et al., 2016). This level of host specificity (to the species level) is not surprising. In a previous study, the majority of tick species found in a diverse Neotropical tick community showed significant structural and/or phylogenetic specificity during the adult life

stage, with this specificity being found at the host species level (Esser et al., 2016).

We have found a higher number of females compared to males (male-to-female ratio of 0.57), which is consistent with a previous study (Lotfi and Karima, 2021). However, other reports have already described an equivalent ratio of male-to-female *R. bursa* collected in the summer season (Ferreiro et al., 2016). *Rhipicephalus bursa* is a two-host tick widely distributed in the Palearctic Mediterranean region. Preferentially lives in low-altitude mountains, grass slopes or semi-desert environments (Raele et al., 2015) however, *R. bursa* ticks were also reported in the North Atlantic part of Spain (Estrada-Peña and Santos-Silva, 2005) and the Northern Atlantic region of Portugal (Estrada-Peña and Santos-Silva, 2005; Ferreiro et al., 2016), both characterized by a warm

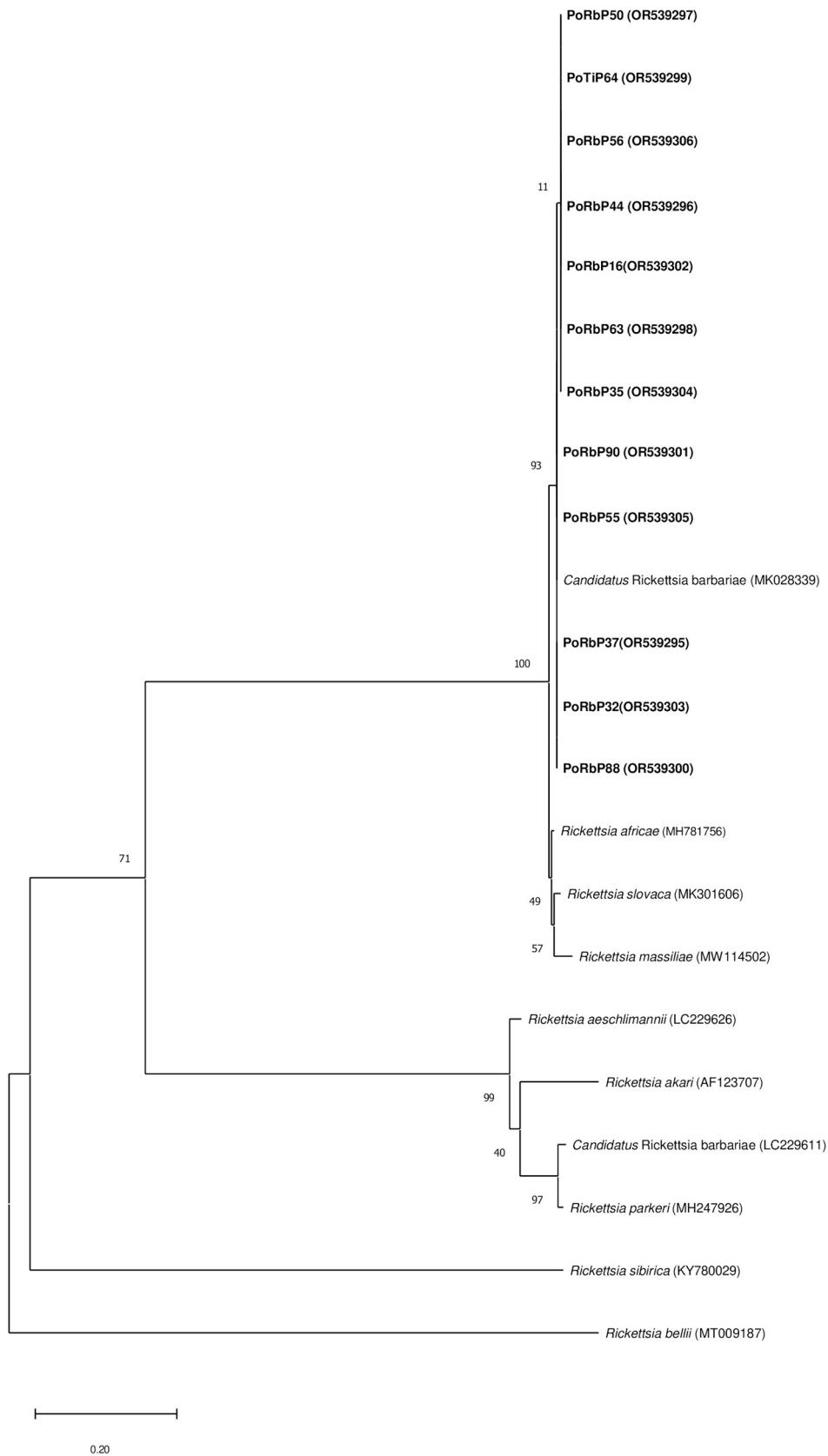


Fig. 2. Phylogenetic analysis based on *ompB* gene of *Candidatus Rickettsia barbariae* identified in *Rhipicephalus bursa* ticks. A maximum likelihood method based on the Hasegawa-Kishino-Yano model phylogenetic tree was constructed based on *Rickettsia ompB* DNA sequences. Reliability of internal branches was assessed using the bootstrapping method (1000 replicates). *Rickettsia* spp. characterized in this study are shown as country/ tick species/ pool. Po: Portugal; Rb: *Rhipicephalus bursa*; P: pool number.

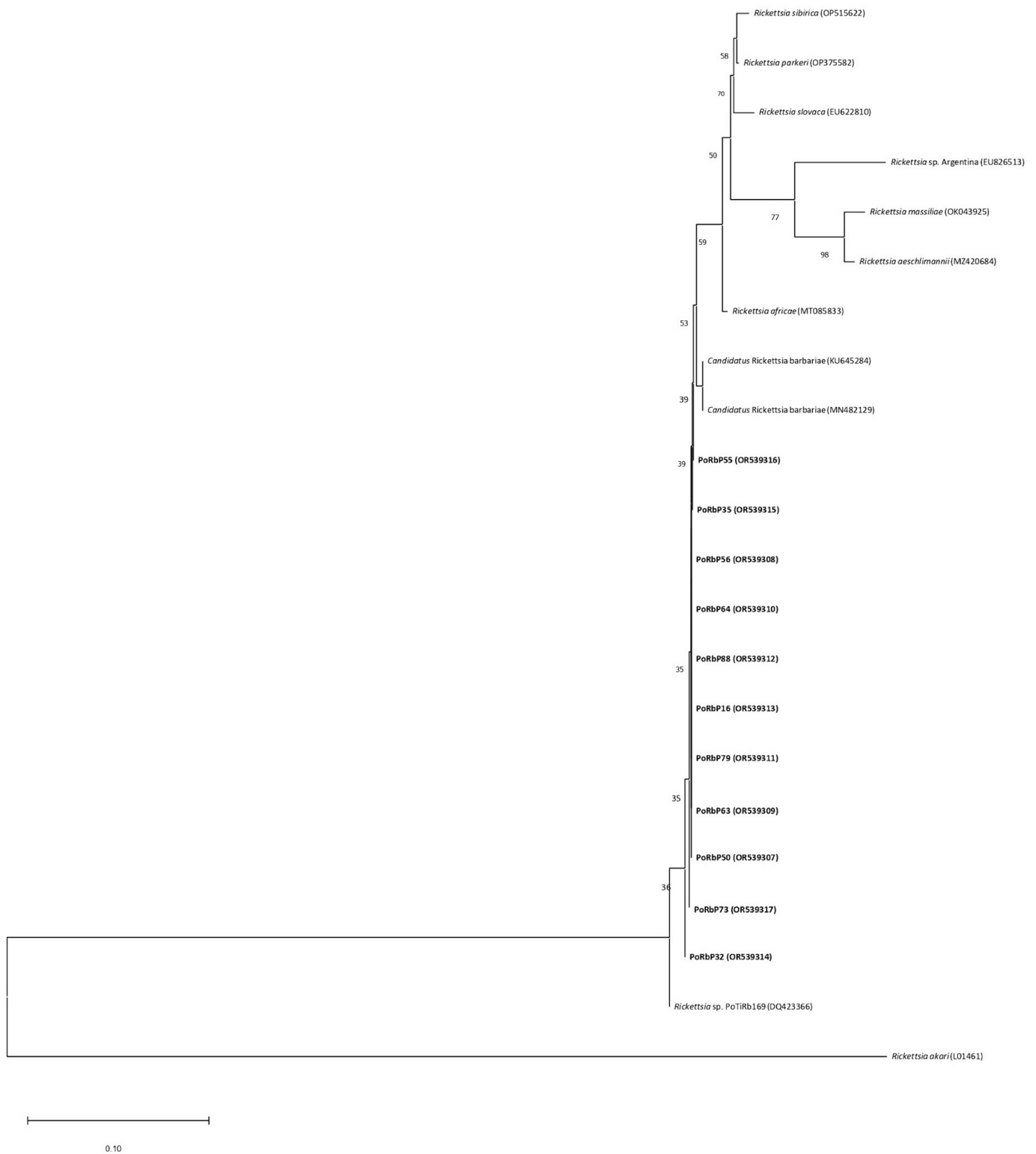
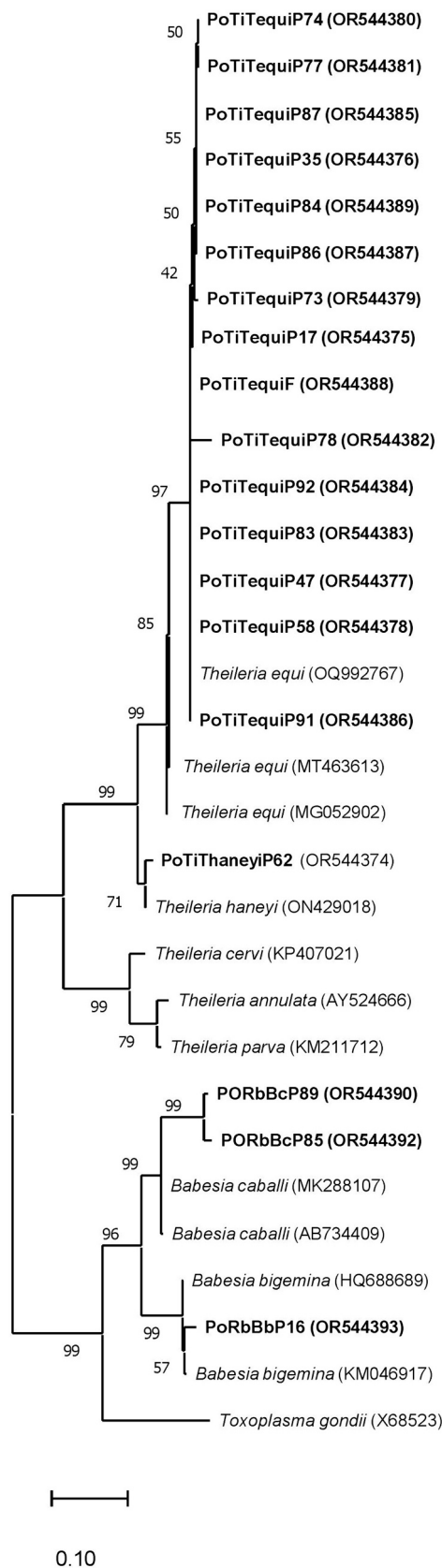


Fig. 3. Phylogenetic analysis based on *ompA* gene of *Candidatus Rickettsia barbariae* identified in *Rhipicephalus bursa* ticks. A maximum likelihood method based on the Tamura 3-parameter model phylogenetic tree was constructed based on *Rickettsia ompA* DNA sequences. Reliability of internal branches was assessed using the bootstrapping method (1000 replicates). *Rickettsia* spp. characterized in this study are shown as country/ tick species/ pool. Po: Portugal; Rb: *Rhipicephalus bursa*; P: pool number.

summer Mediterranean climate (Csb) and mild winters. As a multi-host tick, it is commonly found on sheep, goats, cattle and horses, and occasionally other mammals, birds, lizards and snakes (Walker et al., 2000). Human infections, although rare, were already reported (Mihalca et al., 2012). *Rhipicephalus bursa* has been described as involved in the

circulation of several agents namely, *Anaplasma marginale* and *T. equi* (Ferrolho et al., 2016), *Anaplasma ovis* and *A. phagocytophylum* (Dahmani et al., 2017), *Babesia ovis* (Erster et al., 2016), *Coxiella burnetii* (Raele et al., 2015), and *Ehrlichia canis* (Matei et al., 2021). In the herein study, molecular results confirmed the presence of *Candidatus R.*



(caption on next column)

Fig. 4. Phylogenetic analysis based on 18S rRNA mitochondrial gene DNA sequences of *Babesia* and *Theileria* spp. identified in *Rhipicephalus bursa* ticks. A maximum likelihood method based on the Tamura-Nei model phylogenetic tree was constructed based on *Babesia* and *Theileria* 18S mitochondrial gene DNA sequences. Reliability of internal branches were assessed using the bootstrapping method (1000 replicates).

Babesia and *Theileria* spp. characterized in this study are shown as country/tick species/ *Babesia* and *Theileria* species/pool.

Po: Portugal; Rb: *Rhipicephalus bursa*; Teq: *Theileria equi*; Tha: *Theileria haneyi*, Bc: *Babesia caballi*, Bb: *Babesia bigemina*, P: pool number.

barbariae, *B. caballi*, *B. bigemina*, *T. equi* and *T. haneyi*. Of the total 91 *R. bursa* pools tested, 12 amplified the *Candidatus R. barbariae* for the *ompB* partial gene of SFG rickettsiae. Further characterization of the partial *ompA* and *gltA* genes showed the highest identity with *Candidatus R. barbariae* sequences from Turkey (MN482129), France (MK652441), and Uzbekistan (OQ076762).

Candidatus R. barbariae, was detected for the first time from *R. bursa* ticks in Portugal in 2006 and was identified as *Rickettsia* sp. PoTiRb169 (de Sousa et al., 2006). Further genetic characterization was performed from a strain obtained from *R. turanicus* removed from domestic animals in Italy (Mura et al., 2008).

Besides Portugal, *Candidatus R. barbariae* has been amplified in ticks from other European countries (Italy, Greece, Cyprus and France) (Chochlakis et al., 2012; Cicculli et al., 2019; de Sousa et al., 2006; Mura et al., 2008; Papa et al., 2016), from Asia (China, Lebanon, Israel, Turkey and Palestinian Territories) (Ereqat et al., 2016; Fernández de Mera et al., 2018; Guo et al., 2016; Orkun and Emir, 2020; Waner et al., 2014) and recently from Africa (Cameroon and Algeria) (Abdelkadir et al., 2019; Vanegas et al., 2018). This bacterium has been mainly detected in ticks, specifically from *Rhipicephalus* species (*Rhipicephalus turanicus* and *Rhipicephalus sanguineus*, and to a lesser extent from *R. bursa*, *Rhipicephalus annulatus*, *Rhipicephalus lunulatus* or *Rhipicephalus simus*) (de Sousa et al., 2006; Halajian et al., 2018; Mura et al., 2008). However, *Candidatus R. barbariae* was also detected in other tick genera such as *Amblyomma* or *Hyalomma* (Ereqat et al., 2016; Fernández de Mera et al., 2018; Vanegas et al., 2018). In addition to ticks, *Candidatus R. barbariae* was also amplified from the flea *Vermipsylla alakurt* (Zhao et al., 2016). In contrast to previous European studies, *Ehrlichia* and *Anaplasma* were not amplified from *R. bursa* pool ticks, known to be competent vectors for these agents (Ferrolho et al., 2016; Masala et al., 2012).

Equine piroplasmosis (EP) is a tick-borne disease caused by the protozoans *B. caballi*, *T. equi* and *T. haneyi*. The latter species was recently reported (Knowles et al., 2018; Sears et al., 2019).

Theileria equi, previously known as *Babesia equi*, can be transmitted by Ixodidae ticks, namely *R. bursa* already reported in Spain (García-Sanmartín et al., 2008), Iran (Abedi et al., 2014), France (Rocafort-Ferrer et al., 2022) and Portugal (Ferrolho et al., 2016). Similarly, we have amplified products of the expected size for *T. equi* from 15 *R. bursa* pool ticks collected from Garrano horses. *Theileria equi* infection is associated with long-life persisting parasitemia, allowing tick infection, and bridging tick transovarial transmission incapacity (Bělková et al., 2021).

Theileria haneyi was detected in a tick pool, a result that is consistent with the low molecular prevalence observed in Nigeria (Mshelia et al., 2020) but is much lower compared to the molecular prevalence of *T. haneyi* reported in horses and donkeys from Egypt (Elsawy et al., 2021). *Theileria haneyi* rarely causes clinical signs in field horses, even in splenectomized ones infected with this protozoan (Sears et al., 2022). Like *T. equi*, *T. haneyi* can cause a long-lasting infection in horses and provide a silent reservoir for their transmission. There are no studies on vector competence for *T. haneyi* (Sears et al., 2019). To the best of our knowledge, our results describe for the first time the occurrence of *T. haneyi* in Portugal, and thus extend the knowledge of the geographical distribution and dynamics of this pathogen infecting *R. bursa* ticks.

Equine piroplasmosis is known to be endemic in several countries in

Africa (Onyiche et al., 2021; Selim and Khater, 2020), Asia (Ahmadi Afshar et al., 2020; Boldbaatar et al., 2005), and the Americas (Hébert et al., 2021; Souza et al., 2019) and mostly in the Mediterranean countries (Camino et al., 2021; Nadal et al., 2022; Zanet et al., 2017). The north of Portugal is endemic to equine piroplasmiasis (Ribeiro et al., 2013) as demonstrated by previous studies conducted in the detection of *T. equi* and *B. caballi* in blood (Baptista et al., 2013; Fuehrer et al., 2020; Ribeiro et al., 2013) and *R. bursa* collected from horses (Ferreiro et al., 2016).

Babesia caballi is transmitted in ticks both transstadially and transovarially, which results in the transition of the pathogen to the next tick generation, making the tick population a primary reservoir (Rothschild and Knowles, 2007). In this work, we have amplified *Babesia caballi* in three *R. bursa* pools, data that is in accordance with previous studies performed in southeastern France (Rocafort-Ferrer et al., 2022) and Italy (Romiti et al., 2020), although in a higher percentage in this last study. Previous research conducted in Portugal has documented the identification of this agent (Ribeiro et al., 2013). However, there have been no records thus far that establish a link between *R. bursa* and *B. caballi* in our country.

Bovine babesiosis is caused by *Babesia bovis*, *B. bigemina*, *Babesia divergens* and *Babesia major*. However, *B. bovis* and *B. bigemina* are the most common *Babesia* species worldwide and can significantly reduce bovine production (Uilenberg, 1995).

Rhipicephalus annulatus and *Rhipicephalus microplus* are considered the main vectors and reservoirs of *Babesia bigemina* (Bock et al., 2004). However, in this study, *B. bigemina* was detected in a *R. bursa* tick pool, data consistent with the low prevalence of *B. bigemina* observed in healthy bovines throughout mainland Portugal (Gomes et al., 2013). This suggests that *R. bursa*, a tick species that primarily parasitizes cattle, may have acquired the infection from a previous host's blood meal, most likely in free-grazing cattle in PGNP. The extensive diffusion of EP can be attributed to the migratory patterns of animals that are chronically infected, and this diffusion is perpetuated by international movements. Equine piroplasmiasis is of high concern for the entire equine sector (Onyiche et al., 2020), and measures must be taken to control the spread of this disease on a global scale. This is particularly important since there is currently no vaccine available for the prevention of these infections and only a limited number of therapeutic solutions exist (Onyiche et al., 2020).

Interestingly, *Anaplasma* and *Ehrlichia* spp. were not detected in this study. If this is due to *R. bursa* not being a competent vector for these agents, or the Garrano horse breed being genetically resistant to these agents, is yet to be known and deserves further studies. In fact, locally adapted breeds are known not only for being crucial to sustainability, but their genetic diversity is especially important in light of environmental challenges and also for offering greater resistance to various infectious diseases that circulate in the region (Fioravanti et al., 2020).

An eco-epidemiological approach delves into the interconnectedness of three separate components relevant to vector-borne diseases. These components are the pathogen, the vector, and the vertebrate host, as well as the connection between the different levels of organization (genetic, individual, population, socio-environmental) involved in the causal process of the disease. This comprehensive approach aids in the definition and clarification of the disease (Powell, 2019). In addition, the present study corroborates the circulation of an emerging member of the rickettsial spotted fever group, *Candidatus R. barbariae*, which is present in *R. bursa* ticks that were collected from Garrano horses. Our findings demonstrated that *Candidatus R. barbariae* co-circulates with *B. bigemina* and *T. equi*, vectored by *R. bursa*.

One limitation of our study stems from the relatively low number of Garrano horses included. However, it's crucial to note that the Garrano horse is a free-living animal, making it challenging to control sample size in field studies. The animals included in this study were gathered for a marking and a deworming purpose. To minimize the number of invasive procedures, blood collection was not feasible. Another

constraint lies in our pooling strategy, coupled with the inability to collect blood samples, which hindered our ability to determine if Garrano horses could be co-infected with the identified agents or if ticks might harbor more than one bacterial species. Nevertheless, this study provided an efficient approach to screen and confirm circulation of tick-borne agents in wildlife.

Here we report for the first time, the detection of *T. haneyi* among *R. bursa* ticks feeding on Garrano horses in Portugal. It is of utmost significance to confirm the presence of *Babesia* and *Theileria* in Portugal as both pathogens exert a great impact on equine health influencing animal production and trading. Therefore, surveillance studies for tick-borne infections are essential to provide information that can facilitate the implementation of preventive and control strategies.

Ethical statement for veterinary parasitology: regional studies and reports

I testify on behalf of all co-authors that our article submitted to Veterinary Parasitology: Regional Studies and Reports.

- 1) this material has not been published in whole or in part elsewhere;
- 2) the manuscript is not currently being considered for publication in another journal;
- 3) all authors have been personally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

Patrícia F. Barradas.

CRediT authorship contribution statement

Patrícia F. Barradas: Writing – review & editing, Writing – original draft, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Catarina Tavares:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Nuno Vieira Brito:** Writing – review & editing, Methodology, Formal analysis, Data curation. **João R. Mesquita:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Derived data supporting the findings of this study are available from the corresponding author [PFB] on request.

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