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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.

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

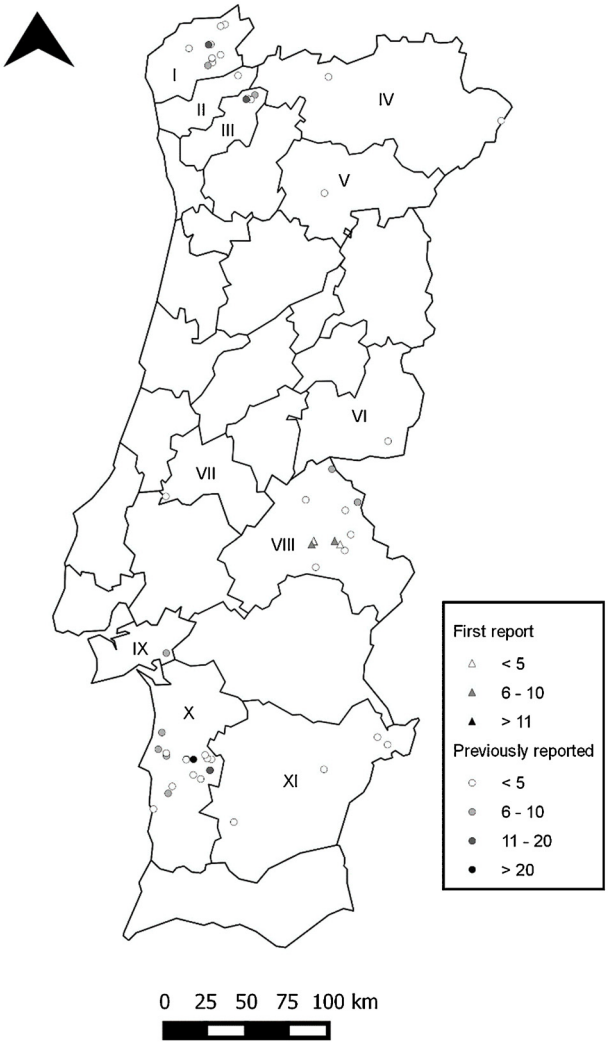

Fig. 1. Geographical locations and number of *Rhipicephalus bursa* found according to NUTS III Subregions. Map was performed using the QGIS 2.4.0. Chugiak program. Circles – Corresponds to the exact coordinates of collection sites in previously reported municipalities; Triangles – Corresponds to the 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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Table 1
Results of PCR screening for tick-borne pathogens in *Rhipicephalus bursa* from Mainland Portugal.

Sub regions/no municipalities[a]		No of positives/tested ticks[b]								Sequencing results
		Overall	*B. taurus*	*C. familiaris*	*C. hircus*	*E. caballus*	*O. aries*	*S. scrofa*	Vegetation	
IMC Minho-Lima	4	33	–	–	–	–	–	–	15m; 18f	
IMC Cávado	1	3	3 m	–	–	–	–	–	–	
IMC Ave	1	**2**/23	–	–	–	**2**/9 m; 14f	–	–	–	*2 T. equi*
Alto Trás-os-Montes	2	6	3 m; 2f	1 m	–	–	–	–	–	
IMC Douro	1	3	–	3f	–	–	–	–	–	
Beira Interior Sul	1	**1**/4	–	–	–	–	**1**/4f	–	–	1 *A. marginale*
IMC Médio Tejo	1	1	–	–	–	–	–	–	1 m	
Peninsula de Setúbal	1	**2**/6	–	–	–	–	–	–	3 m; **2**/3f	1 *A. marginale*
Alto Alentejo	7	**1**/61	1f; 43n; 3l	–	3 m; **1**/7f	1 m; 1f; 1n	–	1n	–	1 *A. marginale*
IMC Alentejo Litoral	2	**4**/120	**2**/65 m; **2**/49f	2 m; 3f	–	–	1 m	–	–	3[c] *A. marginale*; 1 *T. annulata*
IMC Baixo Alentejo	3	6	3f	–	–	–	3f	–	–	
Total	24	**10**/266	**2**/71 m; **2**/55f; 43n; 3l	3 m; 6f	3 m; **1**/7f	10 males, 15 females, 1 nymph	1 m; **1**/7f	1n	19 m; **2**/21f	

[a] Portuguese Mainland according to Nomenclature of Territorial Units for Statistics regions – NUTS III and local administrative units or municipalities (LAU I).
[b] According to tick instars: m-male; f-female; n-nymph; l-larva.
[c] One male and two females with *Anaplasma marginale*.

4 were rejected. Two phylogenetic trees were built, one for members of Anaplasmataceae and the other for Apicomplexan parasites. Phylogenetic trees were reconstructed using neighbor joining (NJ) based on Kimura 2-parameters genetic distances. Phylogenetic inference and model selection were performed using MEGA 6 (Tamura et al., 2013). The reliability for the internal branches of NJ was assessed using the bootstrapping method (1000 bootstrap replicates). Graphical representation and editing of the phylogenetic trees were performed with EvolView (Zhang et al., 2012). The accession numbers and geographic origins of the isolates used in the phylogenetic analysis are shown in the phylogenetic trees. The sequences obtained in this study were submitted to GenBank and identified with the respective accession number.

3. Results

From the 266 *R. bursa* studied, 226 were found feeding on animals (88 males, 90 females, 45 nymphs and 3 larvae) and 40 were questing ticks (19 males and 21 females). As previously mentioned, ticks were obtained from 24 municipalities that correspond to 11 Portuguese Mainland subregions (NUTS III). Fig. 1 shows the number and geographical location of *R. bursa* according to those subregions. Most of the ticks were obtained from Alentejo Litoral (45.1%), followed by Alto Alentejo (22.9%), Minho-Lima (12.4%) and Ave (8.6%). A smaller number of ticks were found in the remaining subregions, ranging from 2.3 to 0.4% (Table 1). Of note is the fact that two of the seven municipalities from Alto Alentejo represent new data records for current *R. bursa* distribution (Alter do Chão and Crato) as the occurrence of this tick species was not previously reported in those places (Fig. 1). Regarding seasonality, adults were mostly found during spring and summer months, contrasting with immatures that were obtained from fall to winter. Considering tick-host associations, *R. bursa* were removed from cattle (*Bos taurus*; $N = 172$), followed by horses (*Equus caballus*; $N = 26$), goats (*Capra hircus*; $N = 10$), sheep (*Ovis aries*; $N = 8$), dogs (*Canis familiaris*; $N = 9$) and pigs (*Sus scrofa*; $N = 1$) (Table 1). Ticks were observed in body locations as varied as the head, namely in the inner surface of the ears; abdominal area, including scrotum and udder; perineum, tail and limbs.

PCR screening for the presence of tick-borne pathogens revealed an overall prevalence of infection in *R. bursa* of approximately 3.8% (10/266). Seven ticks (2.6%) were positive for *16S rDNA* of *Anaplasma*/*Ehrlichia* spp. and three were positive for *18S rDNA* subunit of *Babesia*/*Theileria* spp. (1.1%). Amplicon sequencing confirmed the presence of *A. marginale*, *T. annulata* and *Theileria equi* in *R. bursa* ticks. *A. marginale* infected ticks were obtained from cattle ($N = 3$, 1 male and 2 females), sheep ($N = 1$, female), goat ($N = 1$, female), and vegetation ($N = 2$, females), in the four subregions IMC Alentejo Litoral, Beira Interior Sul, Alto Alentejo and Peninsula de Setúbal, respectively. *T. annulata* was found in one male feeding in cattle from IMC Alentejo Litoral; whereas *T. equi* in two males feeding in horses from IMC Ave. No *C. burnetii* DNA was detected nor the presence of co-infections.

Phylogenetic analysis using *16S rDNA* from different worldwide isolates of *A. marginale* and other pathogens closely related as *A. platys*, *A. phagocytophilum*, *E. canis*, *Ehrlichia ruminantium*, *E. ewingii* and *E. chaffeensis* confirmed infection with *A. marginale* (Fig. 2). Blast analysis showed that the four sequences obtained both from questing and cattle parasitizing ticks (with the accession no. *A. marginale* Portugal KT004409, KT004411 and KT004412 in Fig. 2) presented between 98 and 100% identify to the bovine strain KNP/M8/a (labeled as *A. marginale* South Africa accession no. KC189852 in Fig. 2). The remaining sequences from ticks collected from small ruminants and vegetation (with the accession no. *A. marginale* Portugal KT004414, KT004410 and labeled as *A. marginale* Portugal.2 in Fig. 2) presented between 97 and 99% identify to the clone Luzon (labeled as *A. marginale* Philippines accession no. LC007100 in Fig. 2). The two sequences from *T. equi* detected in an infected horse (with the accession no. *T. equi* Portugal KT004407 and KT004408 in Fig. 3) clustered together with *T. equi* isolates from Brazil and South Africa. Finally, an isolated labeled as *Theileria* sp. Portugal KT004406 (Fig. 3) did not show clear phylogenetic relationship with any of the *Theileria* spp. used in the phylogenetic analysis. However, this isolate showed 96% similarity to a sequence detected in *Hyalomma anatolicum* from Tajikistan (labeled as *T. annulata* Tajikistan accession no. KM288519 in Fig. 3).

4. Discussion

R. bursa is widely distributed across the Mediterranean region, including Portugal. In Portugal the presence of this tick species was first recognized in 1943 by Aboim-Inglês (Tendeiro, 1963), and subsequently reported in several areas and on different vertebrate hosts (de Sousa et al., 2011; Santos-Silva et al., 2011, 2012, 2014). In the present study, *R. bursa* were collected from six species of domestic animals (*B. taurus*, *E. caballus*, *C. hircus*, *O. aries*, *C. familiaris* and

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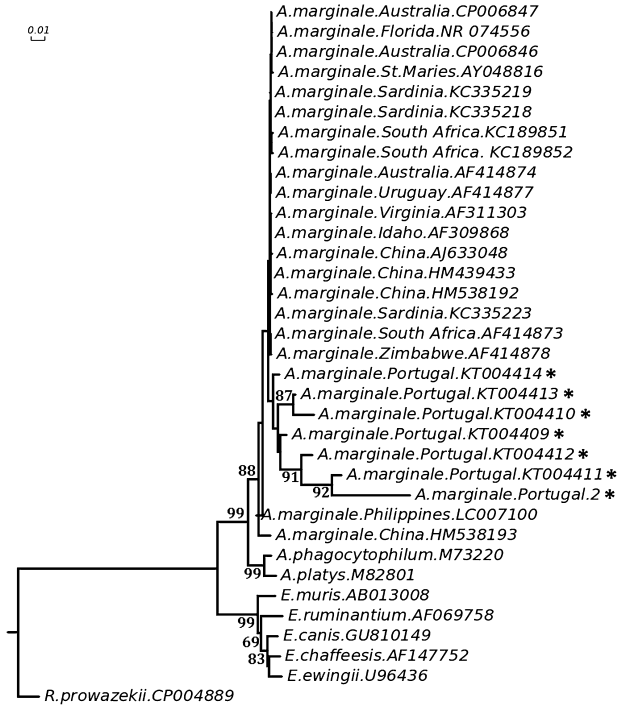

Fig. 2. Phylogenetic tree of Anaplasmataceae using *16S ribosomal RNA* gene sequences. The evolutionary history was inferred by using the NJ method based on the Kimura 2-parameter model. *16S rDNA* gene nucleotide sequences from *A. marginale*, *A. phagocytophilum*, *A. platys*, *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. canis* (family Anaplasmataceae) were used. Accession numbers of sequences are shown and isolates from Portugal reported in this study are marked with "*". Numerals on branches are bootstrap values (1000 replicates). *R. prowazekii* was used as outgroup to Anaplasmataceae. An accession no. was not attributed to the isolate *A. marginale*. Portugal. 2 due to the short size of the sequence.

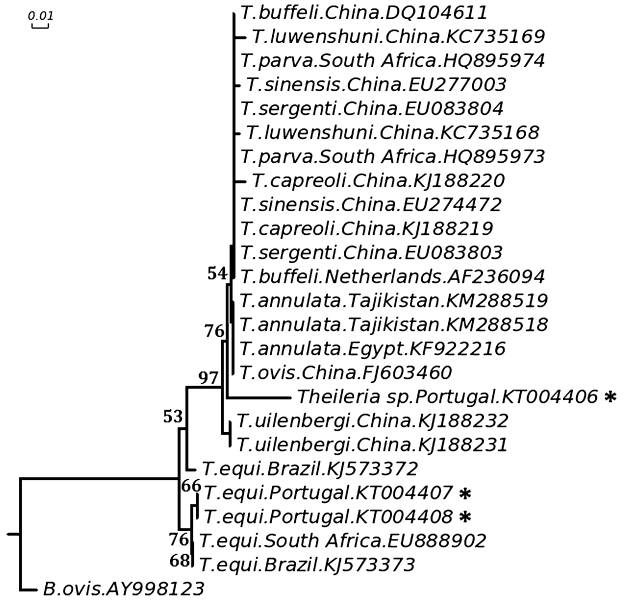

Fig. 3. Phylogenetic tree of *Theileria* using *18S ribosomal RNA* gene sequences. The evolutionary history was inferred by using the NJ method based on the Kimura 2-parameter model. *18S rDNA* gene nucleotide sequences from *T. buffeli*, *T. equi*, *T. annulata*, *T. parva*, *T. ovis*, *T. capreoli*, *T. luwenshuni*, *T. sinensis*, *T. sergenti*, *T. uilenbergi* (Order Piroplasmida) were used. Accession numbers of sequences are shown and isolates from Portugal reported in this study are marked with "*". Numerals on branches are bootstrap values (1000 replicates). *Babesia ovis* was used as outgroup to *Theileria*.

S. scrofa), all previously included in the list of hosts parasitized by this tick species. Moreover, this report also confirms the occurrence of *R. bursa* in 22 out of the 68 prior recorded municipalities, from a total of 278 Mainland LAU (de Sousa et al., 2011; Santos-Silva et al., 2011, 2012, 2014). New data on geographical distribution includes the identification of this species in two additional municipalities from the subregion of Alto Alentejo. Interestingly, this is also the first report of *R. bursa* larvae detection in Portugal. The three larvae were collected from cattle from Alto Alentejo; and together with a group of 45 nymphs, reinforces the association of the immature instars to ungulates, as discussed in previous studies (Santos-Silva et al., 2011). The presented data shows that *R. bursa* is active during all year, with a marked seasonal behavior according to the developmental stage, with adults being present mostly during the spring-summer months (May to September) and immature in autumn-winter (October to January–February). This is in agreement with the findings of other studies, although the majority of the available data refers to adult ticks (de Sousa et al., 2011; Santos-Silva et al., 2011, 2012, 2014). Although the highest numbers of ticks were collected in the summer (75.2%), this might reflect not only species seasonal tendency but also the tick active surveillance during cattle vaccination campaigns and other livestock management. Few surveys conducted in Portugal have found nymphs of *R. bursa*, and all specimens were obtained on cattle, goats, sheep and mice (Santos-Silva et al., 2011). Nevertheless, the reduced number of reports regarding immatures is likely to be due to a limited screening of ungulates during colder months. During our study we have found an equivalent ratio of male: female. Although a similar ratio of male: female has been reported (Garcia-Sanmartin et al., 2008), most studies report that the number of males generally outnumbers the number of females (Moshaverinia et al., 2012; Papadopoulos et al., 1996; Yeruham et al., 1996). As mentioned, the primary hosts of this tick species include cattle, sheep, and goats; and less frequently dogs and smaller mammals, such as rabbits (de la Fuente et al., 2004a; Masala et al., 2012; Mihalca et al., 2012; Psaroulaki et al., 2006; Santos-Silva et al., 2011; Satta et al., 2011; Walker et al., 2000). Here, *R. bursa* ticks were removed mainly from large mammals such as cattle and horses; and in lesser numbers from goats, sheep, and dogs. This result may be linked to the fact that the present survey was not designed to compare *R. bursa* host preference, but to primarily contribute to a broader insight on the tick *R. bursa* distribution. Nevertheless, our results are very similar to the ones described in a study conducted in Sardinia (Satta et al., 2011). Additionally, *R. bursa* is described to have preference for particular areas of the host body, according to tick instars and the host species (Estrada-Pena et al., 2004; Yeruham et al., 1989). Indeed, in this study distinct locations were recorded although the reduced number of collected ticks limits any statistical inference. In cattle, the highest number of adult ticks was collected from the abdominal and perineal areas, contrasting with the immatures that were mainly found on the head and dorsal regions. The same observations were recorded by other authors (Estrada-Pena et al., 2004; Feldman-Muhsam, 1953; Papadopoulos et al., 1996). More specifically, in horses and goats most of the adult *R. bursa* were collected from the posterior regions of the body, including perineum and tail and perineum and udder, respectively; in sheep and dogs, ticks were found mostly on the head, mainly from the inner surface of the ears, similarly to what have been reported elsewhere (Feldman-Muhsam, 1953; Papadopoulos et al., 1996). No specimens were found on other locations already described for these hosts, such as the tail, perianal and inguinal areas, interdigital spaces and axilla, for sheep (Moshaverinia et al., 2012; Papadopoulos et al., 1996; Yeruham et al., 1989); and neck, breast and the interdigital spaces, for dogs (Papadopoulos et al., 1996).

Molecular results confirmed the presence of *A. marginale, T. annulata* and *T. equi* in *R. bursa* ticks. *A. marginale* was found in

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ticks feeding in cattle, sheep, and goats, and among questing ticks. *T. annulata* was found in a tick feeding in cattle; and *T. equi* in ticks feeding in horses. Despite the detection of these three pathogens, it was not possible to conclude about the vector capacity of this tick species. To address this one would need more evidence, firstly because the biologic material was obtained from the complete tick and the pathogen could be present only in the midgut and not in the salivary glands from where it would be transmitted *via* saliva; secondly, a positive PCR does not confirm that these pathogens are capable of multiplication in this tick and thus transmitted. Additionally, once the blood of each animal was not tested the origin of each pathogen was not determined. Two possible sources of tick infection could be implicated: the blood meal on the host from where the tick was collected, or the blood meal from the previous host through transstadial transmission.

In Portugal and Spain, *R. bursa* is a proven vector of *A. marginale* and the infection of cattle, known as bovine anaplasmosis, caused by this pathogen has been already reported (Caeiro, 1999; de la Fuente et al., 2004a; Kocan et al., 2004). Bovine anaplasmosis is now known to be endemic in tropical and subtropical regions worldwide, causing substantial economic losses to cattle industries (Kocan et al., 2003). *T. annulata* is mainly transmitted by ticks of the genus *Hyalomma*; however, similarly to our study, *R. bursa* was also associated with the transmission of this pathogen in Tunisia (M'Ghirbi et al., 2010). In cattle, *T. annulata* is the causative agent of tropical theileriosis responsible for serious constraints on the animal health and productivity worldwide due to high morbidity and mortality (Branco et al., 2010; Doliveira et al., 1995). In Portugal, the presence of this tick-borne pathogen has already been reported in cattle during an acute lethal infection of calves highly infested by ticks of the genus *Hyalomma* (Branco et al., 2010), and during a survey conducted in asymptomatic cattle for detection of piroplasms (Gomes et al., 2013). Nevertheless, we were not able to identify any study conducted in Portugal that has isolated *T. annulata* from *R. bursa* ticks. *T. equi* (formerly known as *Babesia equi*) can be naturally transmitted by ticks of the family Ixodidae (Stiller et al., 2002) and *R. bursa* has been implicated as the vector of piroplasmosis caused by *T. equi* in horses in Spain and Iran (Abedi et al., 2014; Garcia-Sanmartin et al., 2008). Similarly, we have found *T. equi* in ticks of this species infesting horses. Previous studies conducted in Portugal report the detection of this pathogen in samples collected from horses (Baptista et al., 2013; Ribeiro et al., 2013) but, to date, no reports were found relating *R. bursa* with *T. equi* in our country. Equine piroplasmosis is endemic in the north of Portugal (Ribeiro et al., 2013) and it is estimated that 90% of the equine population worldwide lives in areas where equine piroplasmosis is present (Dewaal, 1992). Nowadays, equine piroplasmosis has serious implications for increasing international trade of horses (Ros-Garcia et al., 2013). To the best of our knowledge, our report is the first to identify *T. annulata* and *T. equi* in *R. bursa* ticks feeding in domestic cattle and horses, respectively, in Portugal.

5. Conclusions

Ticks and tick-borne diseases are a current and emerging global threat for human and animal species. The impact of global climate change, human activities, including land management, habitat destruction and control strategies that rely on the use of pesticides, might be responsible for a change in tick biology and possibly contribute to the rapid annual rise in the tick population (Heyman et al., 2010; Omeragic, 2011; Santos-Silva et al., 2011). The results of this study indicate that in Portugal, *R. bursa* can be found on hosts such as cattle, smaller ruminants, such as sheep and goats, horses, and occasionally dogs. Also, this tick is active during all year, with a marked seasonal behavior according to the developmental stage. Our study also demonstrates that several pathogens responsible for tick-borne diseases, particularly *A. marginale*, *T. annulata* and *T. equi*, do exist in *R. bursa* ticks and circulate in determined areas of Portugal. Moreover, all positive ticks only harbored a single infection. Here we report for the first time the detection of *T. annulata* and *T. equi* amongst *R. bursa* ticks feeding in domestic cattle and horses, respectively, in Portugal. The confirmation of the presence of *Anaplasma* and *Theileria* in Portugal is of extreme importance as both pathogens exert great impact in animal health, influencing animal production and trading. Finally, information about the prevalence of infection in ticks is essential to provide information in order to develop future effective preventive and control strategies.

Conflict of interest

The authors declare no competing personal or financial interests.

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