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The presence of *Ehrlichia canis* in *Rhipicephalus bursa* ticks collected from ungulates in continental Eastern Europe

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Abstract

Introduction: *Rhipicephalus bursa* is a common tick parasite of small-to-medium size ungulates, principally in warm, temperate, and subtropical areas. Although common in livestock and showing a wide geographic distribution, its epidemiological role in tick-borne bacterial disease is barely known. This study addressed the knowledge gap and aimed to screen for the presence of *Anaplasmataceae* and spotted fever group (SFG) *Rickettsia* species in *R. bursa* ticks collected from domestic animals in Romania, Eastern Europe. **Material and Methods:** A total of 64 pools of *R. bursa* ticks collected from small ungulates were tested by PCR for *Anaplasmataceae* DNA presence using group-specific primers. Specific testing was performed for *Anaplasma marginale/A. centrale/A. ovis*, *A. platys*, *A. phagocytophilum*, *Ehrlichia canis*, and SFG *Rickettsia*. The positive samples were purified and sequenced, and sequences analysis was used to identify the species and to confirm the PCR results. **Results:** The only pathogen identified in this study was *E. canis.* The obtained sequences confirmed the PCR results. The presence of *E. canis* in *R. bursa* in Romania and in ticks from sheep was shown for the first time in this study. **Conclusion:** Based on these findings, it may be presumed that the *E. canis* DNA originated from ticks; however, the vectorial role of *R. bursa* (and other arthropod species) in the transmission of *E. canis* should be proved experimentally.

Keywords: *Anaplasmataceae*, spotted fever group *Rickettsia*, Ixodidae, livestock, tick-borne diseases.

Introduction

The genus *Rhipicephalus* is the second most speciose in the Ixodidae family, with 74 species recognised worldwide (31). Most species have a tropical or subtropical distribution, with colder regions hosting only a few representatives. *Rhipicephalus bursa* is one of the few species occurring in temperate regions, being the only exophilic *Rhipicephalus* species reaching middle latitudes in Europe (30). It is largely distributed in the Mediterranean, occurring in the Palearctic region between the latitudes of 31° and 45° N (31). *Rhipicephalus bursa* prefers the warmer, grassy slopes of low-altitude mountains, drier lowlands or semi-desert environments (20). However, recently it has also been recorded in colder regions, implying a potential tendency to spread to the north (1). In Romania, *R. bursa* is distributed in the southern lowland region, but there are also sporadic reports from central and north-western parts, suggesting a possible human-mediated colonisation through livestock transfers (15). It is a two-host tick of which the immature stages commonly infest the same host and the adults a different one. The usual hosts are livestock (goats, sheep, cattle and horses), but rarely they may be other mammals, birds, lizards and snakes (31). It was also recorded in free-living carnivores and wild

ungulates (30), but its presence is sporadic in non-domesticated species, at least in Eastern Europe. Human infestations are rare but possible, and were also reported locally (15).

The vectorial role of *R. bursa* is not as well known as that of other *Rhipicephalus* species (*e.g. R. sanguineus* (6)). It was recognised as a vector of *Babesia ovis* (8) and *Coxiella burnetii* (20). However, several other pathogens such as *Theileria* spp., *Anaplasma marginale*, *A. ovis* and *Ehrlichia canis* were reported in *R. bursa* (10, 13, 23, 31).

Ehrlichia is one genus of obligate intracellular parasitic bacteria transmitted mainly by ticks from one host to another, and *Anaplasma* and *Rickettsia* are two further significant genera of with tick vectors. They have a wide geographical distribution and are important disease-causing agents in livestock, pets and humans (24). These bacteria may infect different haematopoietic cells, causing persistent infections in many vertebrate hosts and thus having many zoonotic reservoirs in nature (21). The most common bacterial species causing infections in domestic animals are *A. phagocytophilum*, *A. marginale*, *A. centrale*, and *A. ovis*, and less involved species are several of the *Ehrlichia* and spotted fever group (SFG) *Rickettsia* (5, 29). The management of these infections and generally of vector-borne diseases requires an appropriate understanding of their epidemiology in possible endemic areas. Knowledge of the presence of infectious agents in *R. bursa* ticks and the possible epidemiological role of this species in tick-borne diseases is scant in Eastern Europe, although this species is a common parasite of domestic ungulates (15). The aim of this study was to screen for the presence and the prevalence of rickettsial agents in *R. bursa* ticks collected from domestic livestock in south-eastern Romania, an area where this tick is commonly found.

Material and Methods

The tick sampling took place in June 2016 in south-eastern Romania; Fig. 1. presents the distribution of collection locations. Ticks were collected from sheep (*Ovis aries*) and goats (*Capra hircus*) kept in mixed flocks, foraging on extensive pastures. The ixodids were morphologically identified based on common features of dichotomous keys (31) and stored in 70% ethanol at −20°C until examination.

Genomic DNA was extracted from pools of *R. bursa* ticks (one pool per host) using commercial ISOLATE II Genomic DNA Kits (Bioline, London, UK), following the manufacturer's instructions. In order to assess cross-contamination between extracts, negative controls consisting of reaction mixes without DNA were used in each extraction procedure. The DNA quantity and purity were assessed using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), based on a representative number of randomly selected samples.

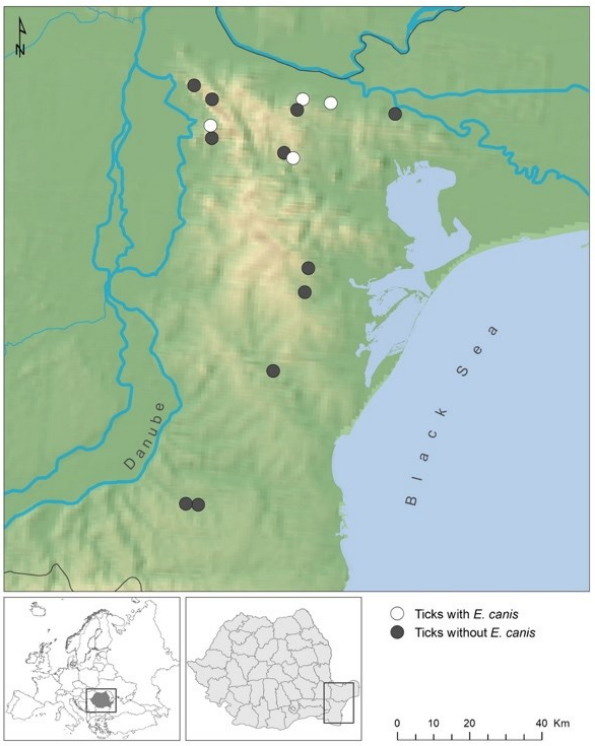

Fig. 1. Geographical origin of *Rhipicephalus bursa* (total collected and *Ehrlichia*-positive) in south-eastern Romania

The samples were assessed for the presence of Rickettsiales bacteria using general and specific primers. The first PCR was performed with EHR16SD and EHR16SR general primers (Macrogen Europe B.V., Amsterdam, the Netherlands) flanking a 345 bp fragment of 16S rRNA (*rrs*) of various species including *Ehrlichia canis*, *E. chaffeensis*, *E. muris*, *Cowdria ruminantium*, *A. phagocytophilum*, *A. platys*, *A. marginale*, *A. centrale*, *Wolbachia pipientis*, *Neorickettsia sennetsu*, *N. risticii*, and *N. helminthoeca* following the protocol developed by Parola *et al.* (19). Genus and species specific PCRs were performed using two nested PCRs: first using Ge3a and Ge10 primers (Macrogen Europe B.V., Amsterdam, the Netherlands) amplifying a 932 bp fragment of *rrs* of *Anaplasma* spp., and Ge2 and Ge9 primers (Macrogen Europe B.V., Amsterdam, the Netherlands) amplifying a 546 bp fragment of *rrs* of *A. phagocytophilum,* as described and used by Massung *et al*. (14) and the second using ECC and ECB primers (Macrogen Europe B.V., Amsterdam, the Netherlands) flanking a 500 bp *rrs* fragment of *Ehrlichia* spp., and respectively Canis and HE3 primers (Macrogen Europe B.V., Amsterdam, the Netherlands) flanking a 389 bp *rrs* fragments of *E. canis* as detailed in Siarkou *et al*. (27).

Conventional PCRs were employed for *A. platys* using specific EPLAT5 and EPLAT3 primers (Macrogen Europe B.V., Amsterdam, the Netherlands) targeting a 359 bp fragment of the *rrs* gene (18), and for *A. marginale/A. centrale/A. ovis* using Msp43 and Msp45 primers targeting an 842 bp fragment of the *msp4* gene (7). The samples were also assessed for the presence of SFG *Rickettsia* using group-specific

Rsfg877 and Rsfg1258 primers (Macrogen Europe B.V., Amsterdam, the Netherlands) targeting a 359 bp fragment of the *gltA* gene (22). Table 1 lists all the primers used.

The amplification was performed with 25 µL of reaction mixture containing 12.5 µL of Green PCR Master Mix (Rovalab GmBH, Teltow, Germany), 6.5 µL of PCR water and 1 µL of each primer (0.01 mM) and a 4 µL aliquot of isolated DNA. One µL of the primary PCR products was used in the nPCR reaction. In each PCR reaction set, positive and negative controls were included in order to assess the specificity of the reaction and the possible presence of cross-contamination. Positive controls consisted of DNA extracted from the blood of two dogs naturally infected with *A. phagocytophilum* and *E. canis*, and from *Ixodes ricinus* infected with *Rickettsia helvetica*, all previously confirmed by sequencing. The negative control consisted of a reaction mix without DNA. The PCR was carried out using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using protocols described in the literature (7, 18, 19, 22, 27). PCR products were visualised by electrophoresis in a 1.5% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, part of Thermo-Fisher Scientific, Waltham, MA, USA). All positive PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing analysis was performed by a commercial provider (Macrogen Europe, Amsterdam, the Netherlands) and the obtained sequences were compared with those available in GenBank by basic local alignment search tool analysis. Statistical calculations were performed using Epi Info 7 software (CDC, Atlanta, GA, USA). A phylogenetic tree was constructed based on the maximum parsimony method, a bootstrap test with 1,000 replicates was carried out according to the Subtree–Pruning–Regrafting algorithm in MEGA6 software (28), and the percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test was calculated. The phylogenetic branches were >50% supported by bootstrap analysis. The *Anaplasma platys* sequence was used as the outgroup.

Results

Altogether, 88 domestic ruminants comprising 43 goats and 45 sheep belonging to 14 flocks were inspected for the presence of ticks. Ticks were found on animals from 13 of the flocks, with a mean tick prevalence of 92.04%, 100% prevalence in goats and 84.44% in sheep. Two tick species were found, *R. bursa* accounting for 98.3% of all ticks collected, and *Hyalomma marginatum* making up the remaining 1.7% and having 14.8% prevalence. All *R. bursa* individuals were adults and the male/female ratio was 1:4. Overall, 7 out of 64 pools of ticks (10.94%, CI 95%: 4.51–21.25) were positive for *Anaplasmataceae* DNA (Fig. 2). All these seven samples were positive for *Ehrlichia* spp. and for *E. canis*. The remaining tested pathogens were not amplified in any of the samples. The positive ticks originated from both sheep and goats, in which prevalences were 8.7% (2/23; CI 95%: 1.07–28.04) and 12.2% (5/41, CI 95%: 4.51–21.25), respectively. The animals carrying positive ticks originated from four different locations, and in one (Teliţa) both sheep and goats infested with positive ticks were found.

Among the obtained sequences, six were identical and revealed a 100% similarity to other *E. canis* isolates (Fig. 3), including those from dogs in Brazil (GenBank accession no. JX437966), Tunisia (accession no. EU781689) or India (accession no. KF888021) and a tick from Brazil (accession no. KF972452). The seventh sequence differed by one nucleotide and was 100% identical to three isolates from Brazil (accession nos KP642753, KF972452 and JQ260853). The obtained sequences were deposited in GenBank under accession nos MG241316 and MG241317.

Table 1. Primers used for the detection of Rickettsiales DNA in *Rhipicephalus bursa*

Pathogen	Primer sequence	Target gene	Target fragment length	Reference
Anaplasmataceae	EHR16SD: GGTACCYACAGAAGAAGTCC EHR16SR: TAGCACTCATCGTTTACAGC	16S rRNA	345bp	19
Anaplasma spp./ *A. phagocytophilum*	Ge3a: CACATGCAAGTCGAACGGATTATTC Ge10: TTCCGTTAAGAAGGATCTAATCTCC Ge2: AACGGATTATTCTTTATAGCTTGCT Ge9: GGCAGTATTAAAAGCAGCTCCAGG	16S rRNA	932bp 546bp	14
Ehrlichia spp./ *E. canis*	ECC: AGAACGAACGCTGGCGGCAAGCC ECB: CGTATTACCGCGGCTGCTGGCA Canis: CAATTATTTATAGCCTCTGGCTATAGGA HE3: TATAGGTACCGTCATTATCTTCCCTAT	16S rRNA	500bp 389bp	27
A. marginale/ *A. centrale*/*A. ovis*	Msp43: CCGGATCCTTAGCTGAACAGGAATCTTGC Msp45: GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	*msp4*	842bp	7
A. platys	EPLAT5: TTTGTCGTAGCTTGCTATGAT EPLAT3: CTTCTGTGGGTACCGTC	16S rRNA	359bp	18
SFG Rickettsia spp.	Rsfg877: GGGGGCCTGCT- CACGGCGG Rsfg1258: ATTGCAAAAAGTACAGTGAACA	*gltA*	359bp	22

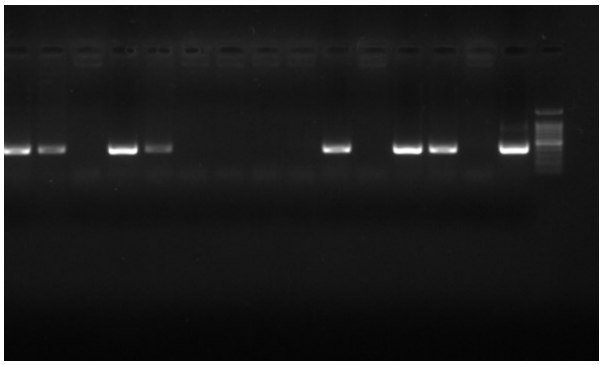

Fig. 2. Electrophoresis gel image showing positive results and including negative and positive controls and the 100-bp DNA ladder (Invitrogen, part of Thermo-Fisher Scientific, Waltham, MA, USA) as the last 3 lanes

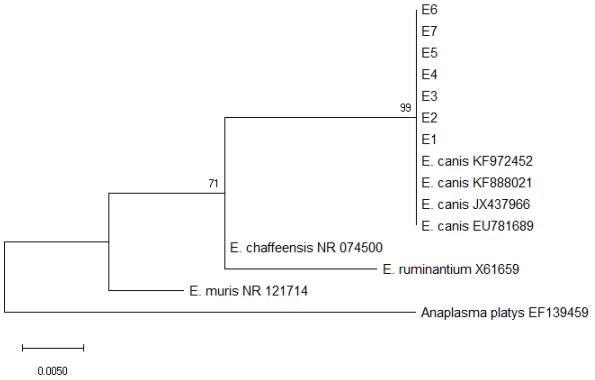

Fig. 3. Phylogenetic tree of *E. canis* detected in *R. bursa* collected from sheep and goats in south-eastern Romania

Discussion

The study results show the presence of *E. canis* in *R. bursa* in Romania for the first time. Moreover, to the best of our knowledge this is the first report of *E. canis* in *R. bursa* collected from sheep, this bacterium having been reported in *R. bursa* collected from goats in Sardinia, Italy (13) and in the same ixodids collected from a cow on Corsica, France (5).

In Europe, the main vector for *E. canis* is another representative of the *Rhipicephalus* genus, the brown dog tick, *Rhipicephalus sanguineus* sensu lato (s.l.). All European countries bordering the Mediterranean are endemic for *E. canis.* However, some surveys have indicated that the infectious agent is spreading to countries lying to the north (25). In Romania, *E. canis* was identified for the first time in ticks (*R. sanguineus* s.l.) from the south-east in 2013 (11) and later from a dog in the west (17). Before these reports, the only serological proofs of canine ehrlichiosis were published in south-eastern Romania, in an area partly overlapping with our study site (16). Infection with *E. canis* is reported commonly in dogs worldwide (25). Also, the infection was reported in other wild canids (foxes, wolves, raccoons, black-backed jackals and bush dogs) but experimental infection failed in other mammalian groups and positive infection cycles were only registered in domestic dogs (2, 25). Several studies reported the presence of *E. canis* DNA in cats and wild felids from Brazil, Portugal, Zimbabwe and Japan (2, 25). To the best of our knowledge, this DNA has not been reported up to now in ticks hosted by small ruminants in continental Europe, although it has already been isolated from one individual *R. bursa* tick collected from a cow, signifying prevalence of 0.8%, on the island of Corsica, France (5). In addition, Masala *et al*. (13) detected *E. canis* DNA in ticks collected from goats in one location on the neighbouring island of Sardinia, also in a highly localised manner. The presence of this bacteria was recorded in low prevalence (2.2%) in certain wild ungulates in China (12), but how the infection was acquired by these animals was not speculated upon. Furthermore, *R. bursa* ticks were found to commonly host other *Ehrlichia* spp. On Corsica, DNA of several *Ehrlichia* spp. (*E. minasensis*, *Candidatus* E. urmitei, and the potential new species *Candidatus* E. corsicanum) were recorded (3), although in low prevalence. In contrast, in this study a high prevalence of *E. canis* DNA was found in *R. bursa* ticks, and its geographical distribution in south-eastern Romania shows a more common and widespread presence of this pathogen (Fig. 1). While *R. sanguineus* s.l. (including *R. rossicus*), the proven vector of this pathogen, is widespread in dogs and wild carnivores in the region (4, 26), this study failed to document the presence of either of these two species in domestic herbivores. In this context it may be presumed that the *E. canis* DNA detected in ticks in the current study did not originate from another *Rhipicephalus* tick species but from *R. bursa*, suggesting a potential vectorial or reservoir role for this species for *E. canis*. However, the role of *R. bursa* and other arthropod species in the transmission of *E. canis* remains unknown, with only *R. sanguineus* s.l. being a proven vector. *Rhipicephalus bursa* is a common and important ectoparasite of sheep and goats in the Mediterranean basin (31), occasionally infesting carnivores and humans (15). Against the background of the high reported prevalence of *E. canis* DNA in livestock, the common occurrence of *R. bursa* in livestock warrants further investigations in order to elucidate its potential vectorial role for *E. canis.*

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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