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Original paper

Natural Anaplasmataceae infection in *Rhipicephalus bursa* ticks collected from sheep in the French Basque Country

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ABSTRACT

Rhipicephalus bursa is one of 79 species of the genus *Rhipicephalus* in the family of Ixodidae. In this study, we investigated Anaplasmataceae bacteria associated with *R. bursa* collected after an epizootic outbreak of ovine anaplasmosis. 76 adult ticks, (60 male and 16 female ticks), were removed from sheep in two farms and all identified as *R. bursa,* all female**s** were partially engorged. We found that 50% of the ticks were positive in the initial Anaplasmataceae qPCR screening. Bacterial species was identified by analyzing the sequences of amplicons of 23S rRNA, *groEL* and rpoB *genes*. 22.4% of ticks contained DNA of *Anaplasma phagocytophilum* and 7.9% the DNA of *Anaplasma ovis*. Based on 23S rRNA and *groEL* genes analysis, we found that 19.7% of ticks contained a potentially new species of *Ehrlichia*. We propose the status of Candidatus for this uncultured species and we provisionally name it *Candidatus* Ehrlichia urmitei. No *Wolbachia* were identified. These results show that *R. bursa* can be a carrier of Anaplasmataceae bacteria.

1. Introduction

Rhipicephalus bursa is one of 79 species of the genus *Rhipicephalus* in the family of Ixodidae (Barker and Murrell, 2008). The meaning of the species' name originates from Middle Latin 'bursa' for 'pouch' or a 'purse made of skin', referring to the bloated pouch-like appearance of an engorged female of this species (Walker et al., 2000). Under laboratory conditions, this species has a life cycle that lasts from 99 to 214 days. Increased longevity may be also associated with the capacity of the unfed larvae to survive hot and dry climatic conditions (Yeruham et al., 2000).

Walker et al. (2000) state that the records of this species from outside the Palearctic region are linked to its misidentification or accidental importation. *R. bursa* is mainly diffused in Mediterranean areas and preferentially lives in low altitude mountain, grassy slopes or semi-desert environments (Raele et al., 2015). However, *R. bursa* was also recorded in the colder region in the Peninsula and mountain area of Crimea, Ukraine, with a tendency to move northwards to the places of its native habitat (Akimov and Nebogatkin, 2013). *R. bursa* is a two-host tick. The immature stages (larvae and nymphs) commonly infest the same host, whereas adults infest a second host. The usual hosts are mammals (Bovidae), birds, lizards and snakes (Walker et al., 2000; Guglielmone et al., 2014). *R. bursa* has also been removed from other mammals (Ovidae, Suidae, Camelidae, Perissodactyla, Carnivora, Rodentia, Insectivora and Lagomorpha) (Estrada-Peña et al., 2013). Human infestation is considered possible (Kar et al., 2013).

R. bursa is recognized as the vector of many important pathogens in livestock including *Babesia ovis* causing ovine babesiosis (Enayati et al., 2009) and *Coxiella burnetii*, the Q fever agent, (Raele et al., 2015). Furthermore, *Theileria* spp., *Anaplasma marginale* and *Anaplasma ovis* were also reported from *R. bursa* (Walker et al., 2000; Ferrolho et al., 2016; Renneker et al., 2013). Recently *A. ovis* and *Ehrlichia canis* DNA were amplified from *R. bursa* ticks collected in Turkey (Aktas, 2014) and Italy (Masala et al., 2012) respectively.

The development and implementation of a control strategy against Anaplasmataceae in animals requires identification of the spectrum of tick species implicated in their transmission. *R. bursa* is an exophilic tick (Sobrino et al., 2012). The role of these ticks in the epidemiology of Anaplasmataceae in the south of France and in the French Basque country has not yet been studied. In this work, we collected ticks from sheep in two farms situated in the

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French Basque Country (the Atlantic area in the south of France), after an epizootic outbreak of ovine anaplasmosis. The aim was to identify bacterial species belonging to the Anaplasmataceae family associated with these ticks.

2. Materials and methods

2.1. Collection of ticks and morphological identification

In September 2013, ticks on six sheep were collected from two ovine farms located in the village of Lantabate in the French Basque area (south-western France) by a veterinarian (F.R.) who practices in this region and who surveyed the outbreak of ovine anaplasmosis that occurred in this area. All adult ticks removed from host animals were partially engorged. Morphological identification was performed with a binocular microscope and carried out by an entomologist with a broad experience of arthropods of medical and veterinary importance. Ticks were classified by family, genus and species using available taxonomic keys and morphometric tables (Estrada-Peña et al., 1990, 2004a; Walker et al., 2000).

2.2. DNA extraction

Ticks were rinsed with distilled water and dried on sterile filter paper in a laminar-flow hood. Each tick was cut in half lengthways (the blades were discarded after each tick was cut). DNA was extracted from one-half and the remaining halves of the ticks were frozen at −80 °C for subsequent studies as described previously (Parola et al., 2003). DNA extraction was performed on the BioRobot EZ1 (Qiagen, Courtaboeuf, France) using a commercial EZ1 DNA Tissue Kit (Qiagen) according to the manufacturer's instructions.

2.3. PCR amplification

Detection of Anaplasmataceae from the ticks' DNA was conducted using several pairs of genus-specific primers and probes (Table 1). Initially, all samples were screened by a qPCR targeting the 23S rRNA gene, this qPCR was reported amplifying most bacteria belonging to the Anaplasmataceae family (Dahmani et al., 2015a). Subsequently, all positive samples were subjected to a conventional PCR using the primers that amplify a 485 base pair (bps) long fragment of the 23S rRNA gene, as previously described (Dahmani et al., 2015a). In order to mine deeper into the identity of Anaplasmataceae species detected in ticks, all samples were also tested in 3 additional PCRs using a set of *Anaplasma* genus-specific primers targeting the 525 bps fragment of *rpoB* gene, *Ehrlichia* genus-specific set of primers targeting the 590 bps-long portion of the heat shock protein gene (*groEL*) and finally, a *Wolbachia* genus specific set of primers that amplify a 634 bps-long portion of *groEL* (Table 1).

The *rpoB* gene sequences of *A. bovis*, *A. ovis* and *A. platys* were not available in the Genbank. For these, we have amplified and deposited in Genbank the *rpoB* gene sequences of the *A. ovis* strain KMND Niayes 14 (Djiba et al., 2013), amplified from sheep blood sample in Senegal, and *A. platys* strain Gard1, France (Dahmani et al., 2015a). Unfortunately, we couldn't obtain the DNA of *A. bovis,* so, we did not include the sequences of *A. bovis* in the phylogenetic analysis based on the two genes 23S rRNA and/or *rpoB* genes.

PCR amplifications were performed as described previously (Dahmani et al., 2015a,b). Briefly, the real-time PCR assays were performed with the CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France) using Takyon Master Mix under the conditions specified by the manufacturer. The conventional PCRs were performed in automated DNA Thermal cyclers (GeneAmp PCR Systems Applied Biosystems, Courtaboeuf, France). The amplification reactions were performed under the following conditions: an initial denaturation step at 95 °C for 15′, followed by 40 cycles consisting of 1′ denaturation at 95 °C, 1′ annealing at a corresponding temperature (Table 1) and a 1′ extension at 72 °C. A final extension cycle at 72 °C for 7′ was performed and the reactions were cooled at 15 °C. Distilled water and DNA of *Anaplasma phagocytophilum, Ehrlichia canis and Wolbachia pipientis* were used in each test as negative and positive controls, respectively. After electrophoresis, the amplification products were visualized on 1.5% agarose gels stained with ethidium bromide and examined by UV transillumination. A DNA molecular weight marker (marker VI, Boehringer Mannheim, Mannheim, Germany) was used to estimate the sizes of the products.

2.4. Sequencing and phylogenetic analyses

Sequencing analyses were performed on Applied Biosystems Prism 3130xl Genetic Analyzer (Thermo Fisher Scientific, France) using the DNA sequencing BigDye Terminator Kit (Perkin-Elmer) as described by the manufacturer. The obtained sequences were assembled using ChromasPro 1.7 software (Technelysium Pty Ltd., Tewantin, Australia). Sequences obtained in this study were aligned with other species of the Anaplasmataceae family available on GenBank using CLUSTALW. Phylogenetic and molecular evolutionary analysis was conducted using MEGA 6 (http://www.megasoftware.net/). The genetic trees were constructed using 23S rRNA gene for the Anaplasmataceae species, the *rpoB* gene for the genus of *Anaplasma* spp., and the *groEl* gene for the genus of *Ehrlichia* spp. Phylogenetic analysis were inferred using the maximum likelihood method, with the complete deletion option, based on the Kimura 2-parameter model for nucleotide sequences. Uniform rates was used to model evolutionary rate differences among sites for the 23s rRNA and *groEl* gene, whereas, for the *rpoB* gene, we used a discrete Gamma distribution with the Gamma parameter 2. Initial trees for the heuristic search were obtained automatically by applying the Maximum Parsimony method. Statistical support for internal branches of the trees was evaluated by bootstrapping with 1000 iterations.

3. Results

In total, 76 adult ticks (60 male and 16 female ticks) were collected and identified morphologically as *R. bursa*, all females were partially engorged. In the first step, the 23S-based qPCR screening showed 38/76 (50%) ticks contained DNA of Anaplasmataceae bacteria. All positive samples were also amplified by the conventional PCR using primers targeting the same gene (Table 1). A nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov) of the sequenced amplicons showed the presence of *A. phagocytophylum* (100% identity with Dog strain, CP006618) in 17/38 ticks. 6/38 ticks contained DNA of *A. ovis* (100% identity with *A. ovis* strain KMND Niayes14 found in sheep in Senegal (KM021411)). Finally, in 15/38 ticks we found the DNA of a potentially new species of *Ehrlichia* that show 98.5% identity with *Candidatus* Ehrlichia urmitei (KT364334) identified in *R. microplus*, *Amblyomma variegatum* and *Hyalomma truncatum* in Côte d'Ivoire (Ehounoud et al., 2016). The closest recognized species are *Ehrlichia muris* (96.3% identity, NR 121968) (Fig. 1). The overall prevalence in collected ticks was as follows: *A. phagocytophilum* 22.4%, *A. ovis* 7.9% and *Ehrlichia* sp. 19.7%.

Results from the second amplification (using specific *Anaplasma* and *Ehrlichia* genus primers targeting respectively the RNA polymerase subunit beta (*rpoB*) gene, and the heat shock protein (*groEL*) gene) are the following. After the *rpoB* gene screening, 23/76 (47.37%) ticks were positive. 17/23 ticks contain DNA of *A. phagocytophilum* (100% identity with Dog strain, (CP006618)), 6/23 contain DNA of *A. ovis* (100% identity with *A. ovis* strain KMND Niayes14