Screening for bacterial DNA in the hard tick *Hyalomma marginatum* (Ixodidae) from Socotra Island (Yemen): detection of *Francisella*-like endosymbiont

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Abstract

Thirty-four adult ticks collected from livestock on Socotra Island (Yemen) were identified as *Hyalomma marginatum* using traditional morphological characteristics. Morphological identification was confirmed for all the collected specimens using a molecular approach targeting a fragment of the mitochondrial gene 12S rRNA. All the specimens were examined for the presence of tick-borne pathogens and the tick endosymbiont *Candidatus* Midichloria mitochondrii using polymerase chain reaction. Three specimens out of the 34 analyzed tested positive to the presence of *Francisella* spp. leading to the first detection of these bacteria in *H. marginatum* on Socotra Island. The phylogenetic analyses conducted on a 660 bp fragment of the ribosomal gene 16S rRNA of *Francisella* spp. (including *F. philomiragia* as outgroup, the four subspecies of *F. tularensis* and the *Francisella*-like endosymbiont of ticks) confirm that the newly detected *Francisella* strains cluster into the *Francisella*-like endosymbionts of ticks. Interestingly, the detected *Francisella*-like endosymbiont, shows a different genotype to that previously isolated from *H. marginatum* collected in Bulgaria. No specimen was positive for the presence of *Rickettsia* spp., *Coxiella burnetii*, *Borrelia burgdorferi* or *M. mitochondrii*.

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Introduction

Ticks are blood-sucking ectoparasites able to parasite a multitude of terrestrial vertebrates such as mammals, birds, reptiles and amphibians (Sonenshine, 1991, 1993). Nowadays, ticks are considered to be the group of arthropods that can transmit the widest variety of pathogenic agents to humans and animals (Jongejan & Uilenberg, 2004). Microorganisms such as bacteria (*e.g., Rickettsia* spp., *Borrelia burgdorferi*, *Erlichia* spp. and *Francisella* spp.), protozoa and viruses (like Crimean-Congo hemorragic fever, Tick Borne Encephalitis) can be transmitted to the host as a result of a tick bite (Sonenshine, 1991; Jongejan & Uilenberg, 2004). Ticks also play an important role as reservoirs for populations of these bacteria in nature (Parola & Raoult, 2001). Recently, the intra mitochondrial bacterium *Candidatus* Midichloria mitochondrii (hereafter *M. mitochondrii*; Sassera *et al.,* 2011), originally discovered in the tick *Ixodes ricinus,* was found to be widespread in many tick genera (Lo *et al.*, 2006; Epis *et al.*, 2008).

Approximately 870 species of ticks are described (Nava *et al.*, 2009), subdivided into three families: Argasidae, Ixodidae and Nuttalliellidae (Horak *et al.*, 2003; Nava *et al.*, 2009). The 26 species belonging to the genus *Hyalomma* Koch, 1844 are widespread in Palearctic and Afrotropical biogeographic regions (Horak *et al*., 2003; Apanaskevich & Horak, 2008; Estrada-Peña *et al.*, 2012). *Hyalomma marginatum* Koch, 1844 is widespread in Central and Southern Europe, North Africa and in Asia, eastwards to Iran (Manilla, 1998; Apanaskevich & Horak, 2008). *H. marginatum* is a 2-host species showing a low host specificity. In fact, the adults feed on different species of large mammals (ungulates and livestock) while the immature individuals feed on birds or small mammals (Manilla, 1998) increasing their ability to spread. This species can transmit a variety of pathogens to humans and animals (Hoogstraal, 1956) and is considered one of the most important tick species involved in the transmission of the virus of the Crimean-Congo hemorrhagic fever (Hoogstraal, 1979; Estrada-Peña *et al*., 2012). Furthermore, it is known to transmit bacteria of the genus *Rickettsia* (*e.g., R. conori* the causative agent of the Mediterranean spotted fever) and *Coxiella burnetii*, the causative agent of Q-fever (Hoogstraal, 1956). Recently, Ivanov and colleagues (2011) isolated *Francisella*-like endosymbionts (FLEs) in *H. marginatum* collected from Bulgaria.

This paper is a first study on ticks (Acarina, Ixodida) collected from Socotra Islan, an archipelago in the Indian Ocean, that has a particular fauna as it was isolated 35-41 million years ago (Girdler & Styles, 1974). The paper also reports the bacterial community associated with these ticks in this area.

Materials and methods

Sample collection, morphological identification and image acquisition

A total of 34 adult tick specimens were collected on Socotra Island, Yemen, directly from livestock (10 specimens from 3 sheep and 24 from 5 goats, respectively) during field research in December 2010. All the collected specimens were immediately stored in absolute ethanol for further DNA extraction. Genomic DNA was extracted from all specimens individually following a procedure that allows the morphology to be preserved for further analyses. Specimen manipulation was completed using the Leica MS5 stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). All ticks were identified using standard taxonomic keys (Starkoff, 1958; Manilla, 1998; Apanaskevich & Horak, 2008). Male and female images were acquired by a machinery made and C-optimized in order to scan the sample at different focus layers that were mounted with a 1.0 64 bit Zerene Stacker (Student Edition; Zerene Systems LLC, Richland, WA, USA).

DNA extraction and polymerase chain reaction

Total genomic DNA was extracted and purified individually using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). All the ticks preserved in ethanol were washed with distilled water and dried before DNA extraction. Ticks were then cut with a scalpel along the idiosome and left for 12 h at 56°C in 180 μL of ATL lysis buffer (Qiagen) with 200 ng/mL proteinase K (Sigma Aldrich, St. Louis, MO, USA). Extraction was performed according to the manufacturer's instructions. In detail, extracted DNAs were quantified with Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA). In order to confirm the morphological identification of the ticks, a fragment of the mitochondrial ribosomal small subunit 12S rRNA gene was amplified (Beati & Keirans, 2001) and sequenced for all the samples. The extracted DNAs were examined, for the presence of *Francisella* spp., *Rickettsia* spp., *C. burnetii, B. burgdorferi* and *M. mitochondrii*, using specific polymerase chain reaction (PCR) protocols. The primers used for the screening of bacterial species are reported in Table 1. PCR amplification was performed in 25 μL reaction mix containing: 1 X GoTaq reaction Buffer (10 mM Tris-HC at pH 8.3, 50 mM KCl and 1.5 mM MgCl2), 0.2 mM of each deoxynucleoside triphosphate, 0.5 pmol of each primer, 0.625 U of GoTaq DNA Polymerase and 50 ng of template DNA. Successful amplification was determined by gel electrophoresis. Positive and unambiguous PCR products were directly sequenced in both strands by ABI technology (Applied Biosystems, Foster City, CA, USA). The obtained sequences were manually corrected using Geneious Pro 5.3 (Biomatters Ltd., Auckland, New Zealand) and deposited in the European Molecular Biology Laboratory data library (Accession numbers: *H. marginatum* partial 12S rRNA gene, HE819515; *Francisella*-like endosymbiont partial 16S rRNA gene, HE819516).

Bioinformatic and phylogenetic analyses

The tick mitochondrial 12S rRNA and the bacterial 16S rRNA consensus sequences obtained were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/blast) and compared to the sequences available in GeneBank (http://www.ncbi.nlm.nih.gov/genbank/). A 16S rRNA sequence of *Francisella* spp. was retrieved from GeneBank in order to perform phylogenetic analyses. Sequences belonging to the four subspecies of *Francisella tularensis,* FLEs of tick and other *Francisella* spp. were included in the dataset (Accession numbers are reported in Figure 1). The 18 sequences obtained were aligned using MUSCLE (Edgar, 2004) then trimmed with Gblocks (Castresana, 2000) and analyzed with jModelTest 0.1.1 (Posada, 2008) to choose the most suitable model of nucleotide evolution. Phylogenetic reconstructions were performed with Bayesian inferences using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Bayesian analyses were performed using general time reversible (Lanave *et al.*, 1984) as model of evolution +I +G; two parallel analyses, each composed of one cold and three incrementally heated chains were run for 2.5 million generations. Trees were sampled every 100 generations and burn-in fraction was calculated as 25% of total sampled trees, according to the likelihood scores (LnL) stationary analyses. The majority rule consensus tree was rooted with the branch leading to *F. philomiragia* and *F. noatunensis*, node with values of Bayesian posterior probability (BPP) less than 0.5 were collapsed (Figure 1). Pairwise p-distance between the FLEs sequence obtained in this study and the two closely related FLEs of *Rhipicephalus sanguineus* (HQ705171) and of *H. m. marginatum* (HQ705170) was calculated using MEGA 5 (Tamura *et al.,* 2011).

Results

All the collected ticks, 11 males and 23 semi-engorged females, were morphologically identified as *Hyalomma marginatum*. Male and female images in dorsal view are reported in Figure 2. The DNA extracted from the 34 specimens, quantified by Nanodrop 1000, result in concentrations ranging from 40 to 110 ng/μL. All tick samples were positive for

Table 1. Primers used for bacterial screening in the present study.

Organism	Target gene	Primer sets	References
Borrelia burgdorferi	16S rRNA	5'-ATGCACACTTGGTGTTAACTA-3' 5'-GACTTATCACCGGCAGTCTTA-3'	Marconi & Garon, 1992
Coxiella burnetii	Transposon-like repetitive region	5'-TATTGTATCCACCGTAGCCAGTC-3' 5'-CCCAACAACACCTCCTTATTC-3'	Willems *et al.*, 1994; Berri *et al.*, 2000
Francisella spp.	16S rRNA	5'-CAAGGTTAATAGCCTTGGGGGA-3' 5'-GCCTTGTCAGCGGCAGTCTTA-3'	Forsman *et al.*, 1994
Midichlori mitochondrii	16S rRNA	5'-GTACATGGGAATCTACCTTGC-3' 5'-CAGGTCGCCCTATTGCTTCTTT-3'	Epis *et al.*, 2008
Rickettsia spp.	Citrate synthase gltA	5'-GCAAGTATCGGTGAGGATGTAAT-3' 5'-GCTTCCTTAAAATTCAATAAATCAGGAT-3'	Labruna *et al.*, 2004

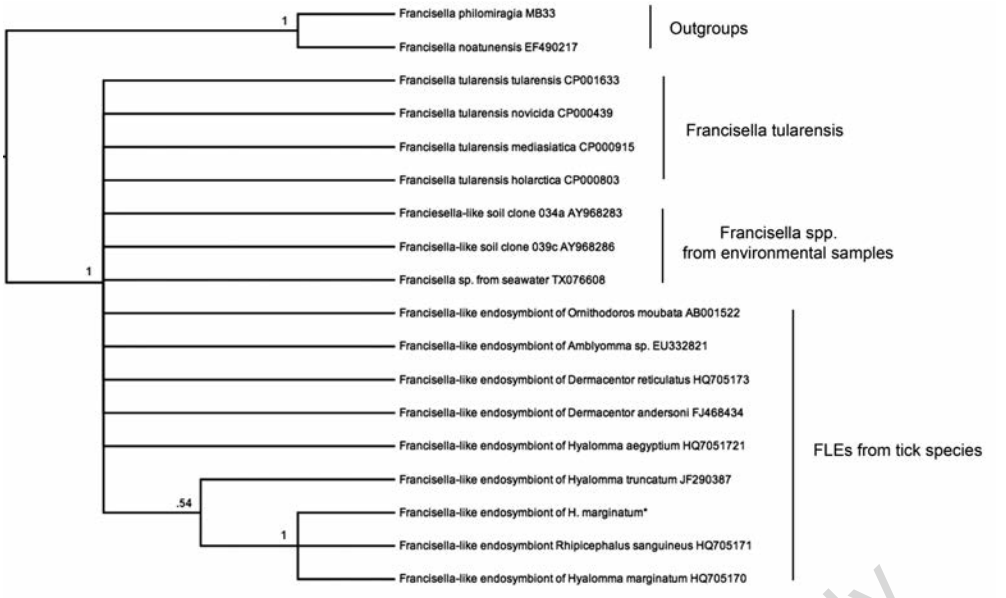

Figure 1. Bayesian consensus cladogram of *Francisella* spp. 16S rRNA gene, Bayesian posterior probability (BPP) values are reported above each node; branches with BPP values less than 0.5 were collapsed. *The sequence obtained in the present study.

12S rRNA PCR; the PCR products were sequenced and morphological identification was confirmed by BLAST analysis (100% identity with *H. marginatum marginatum*; Accession number AF150034).

All the specimens tested negative in PCR for the presence of *Rickettsia* spp., *Coxiella burnetii, Borrelia burgdorferi* and *M. mitochondrii*; while 3 specimens collected from 2 different sheep hosts (2 females and one male, 8.8% of prevalence) were positive for *Francisella* 16S rRNA amplicons. No nucleotide differences were recovered between the three consensus sequences after a pairwise comparison. BLAST analysis performed on the 3 sequences confirms their identity (99%) with *Francisella*-like endosymbiont. The best hit of the sequences were tthe FLEs of *Rhipicephalus sanguineus* and of *H. m. marginatum*, with a difference of one nucleotide (pairwise p-distance=0.15%).

Phylogenetic analyses were performed on a dataset of a 660 bp of the bacterial 16S rRNA composed of eighteen taxa belonging to *Francisella* spp. from different origin (*e.g.*, pure culture, soil samples, seawater, tick endosymbionts) in order to understand the relationships of the newly sequenced bacterial strains. Bayesian analysis (Figure 1) confirms that *Francisella* spp. harbored by *H. marginatum* collected from livestock on Socotra Island cluster within the group of tick FLEs. The new sequence clusters with a BPP of 1 within a well-supported group formed by two FLEs previously detected from *H. marginatum* and *Rhipicephalus sanguineus* collected from Bulgaria.

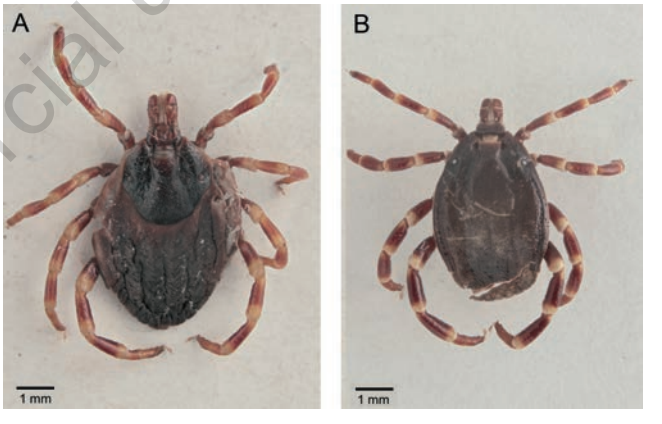

Figure 2. Dorsal view of *Hyalomma marginatum*: (A) female and (B) male.

Discussion

This is the first study on ticks collected from livestock on Socotra Island and on the harbored bacteria. Gram-negative bacteria belonging to the genus *Francisella* are known to be distributed mainly in the northern hemisphere (Foley & Nieto, 2010). Within this group there are bacteria of medical and veterinary importance, such as the etiological agent of Tularemia, *F. tularensis* and the FLEs of tick. At present, FLEs have been identified in both soft (genus *Ornithodoros*) and hard (*Amblyomma*, *Dermacentor*, *Rhipicephalus*, *Hyalomma*) ticks, furthermore their pathogenic role is unknown even if genes implicated in the pathogenicity of *F. tularensis* have been detected (Machado-Ferreira *et al.*, 2009). The results obtained disagree with the trees obtained in a previous study that have suggested the monophyly of the *Francisella*-like tick endosymbionts. These discrepancies are due to the fact that we used a fragment of the 16S rRNA that does not provide enough information for these relationships to be clarified.

Considering the importance to human health of bacteria of the genus *Francisella*, our results could help control new emerging diseases on Socotra Island.

In fact, this study provides the first evidence of *Francisella*-like endosymbionts harbored by ticks collected from livestock on Socotra Island, and should alert physicians and veterinarians working within the region to the possibility of infection from this organism.

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