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Short Communication

Francisella-Like Endosymbiont in *Dermacentor reticulatus* Collected in Portugal

Isabel Lopes de Carvalho,[1] Nuno Santos,[2] Teresa Soares,[3] Líbia Zé-Zé,[1] and Maria Sofia Núncio[1]

Abstract

In Portugal, recent studies have confirmed the presence of *Francisella tularensis* in *Dermacentor reticulatus*. Bacterial endosymbionts with significant homology to *F. tularensis* have been described in several species of ticks. In this work we identified *Francisella*-like endosymbionts in *D. reticulatus* ticks (39%), confirming the presence of these bacteria in Portugal. This finding should be considered in future studies using molecular approaches to detect *Francisella* prevalence in ticks and environmental samples.

Key Words: *Dermacentor reticulatus*— *Francisella*-like endosymbionts—Portugal.

Introduction

Francisella tularensis is an important etiologic agent transmitted during tick feeding that causes tularemia in humans. These bacteria can be acquired through consumption of contaminated food or water, inhalation of aerosols, contact with infected animals, or by the bite of an infected vector. Increased concern over the potential use of agents such as *F. tularensis* in bioterrorism and the frequent occurrence of outbreaks of tularemia in several European countries has highlighted the importance of implementing prevention measures, through precise characterization of the epizootiology and epidemiology of the infection. Currently, there are four subspecies recognized within *F. tularensis* species: *F. tularensis* subsp. *tularensis*, *holarctica*, *mediasiatica*, and *novicida* (Huber et al. 2009). On the basis of a high degree of similarity in 16S rDNA gene sequences, several other organisms have been classified as probable members of the Francisellaceae family, including *Wolbachia persica* (proposed remove from the genus *Wolbachia*) and *Francisella*-like endosymbionts (FLEs) (Sjöstedt 2005). Currently, *Francisella* spp. is detected using molecular methods, and the homology between *Francisella* spp. and FLEs may cause some difficulties in interpretation of tick surveys. The implications of this fact in public health are important due to the different pathogenicity of the two organisms. *F. tularensis* can cause a wide variety of clinical syndromes, including severe, sometimes fatal, disease, compared to FLEs, which seem to be nonpathogenic for humans (Niebylski et al. 1997). To overcome the difficulties in diagnostics due to the homology between *Francisella* spp. and FLEs, recent studies have been performed to differentiate both pathogens (Kugeler et al. 2005, Escudero et al. 2008).

FLEs have a worldwide distribution in both hard and soft ticks (Vun et al. 2000, Machado-Ferreira et al. 2009), namely, in the genera *Ixodes*, *Amblyomma*, *Dermacentor*, and *Ornithodoros* (Scoles 2004, Machado-Ferreira et al. 2009). The effect of FLEs, if any, on vector competency and transmission of *F. tularensis* by ticks is still unknown, as are the factors responsible for the maintenance of both *F. tularensis* and FLEs in nature (Petersen et al. 2009). A phylogenetic study shows that some FLEs form a monophyletic clade most closely related to pathogenic *Francisella* species transmitted by ticks (Scoles 2004). Two other studies support the hypothesis that distinct FLE populations emerged from an infective ancestral organism (Scoles 2004, Machado-Ferreira et al. 2009).

In Portugal, recent studies have confirmed the presence of *F. tularensis* subsp. *holarctica* (1.1%) in *Dermacentor reticulatus* ticks (Lopes de Carvalho et al. 2007). Thus, to contribute to the evaluation of the ecological and epidemiological situation of tularemia in Portugal, adding to the reasons above mentioned, the presence of FLEs in the same tick species was investigated in this study.

Materials and Methods

A total of 75 ticks—62 *D. reticulatus* (51 females and 11 males), 4 *Ixodes hexagonus* (males), 2 *Ixodes ricinus* (females), 2 *Ixodes frontalis* (nymphs), and 5 *Dermacentor marginatus*

[1]Centro de Estudos de Vectores e Doenças Infecciosas Dr. Francisco Cambournac, Instituto Nacional de Saúde Dr. Ricardo Jorge, Águas de Moura, Portugal.
[2]Parque Nacional da Peneda-Gerês, Bragança, Portugal.
[3]Direcção Geral de Veterinária–DIVR, Santarém, Portugal.

Table 1. Sequences Used for Phylogenetic Analysis

Sequence	*Strain/isolate*	*Country/origin*	*GenBank accession no.*
Francisella-like endosymbiont of *Dermacentor reticulatus*	PoTiEF1	Portugal	GU113085
Francisella-like endosymbiont of *D. reticulatus*	FDrH	Hungary	EU126640
Francisella endosymbiont of *D. albipictus*	02-045	United States	AY375409
Francisella endosymbiont of *D. andersoni*	01-151-1	Canada	AY375412
Francisella endosymbiont of *D. hunteri*	02-249	United States	AY375417
Francisella endosymbiont of *D. nitens*	DnT2-1	United States	AY375418
Francisella endosymbiont of *D. occidentalis*	02-241	United States	AY375419
Francisella endosymbiont of *D. variabilis*	01-109	United States	AY375420
Francisella endosymbiont of *D. variabilis*	01-175	United States	AY375421
Francisella endosymbiont of *Amblyomma maculatum*	02-240	United States	AY375422
Francisella endosymbiont of *Amblyomma dubitatum*	A45	Brazil	EU441945
Francisella endosymbiont of *Anocentor nitens*	C2	Brazil	EU441945
Francisella endosymbiont of *Ornitnodoros porcinus*	02-52/53	Southern Africa	AY375423
Francisella tularensis subsp. *holarctica*	PoHuF1	Portugal	DQ459299
Francisella tularensis subsp. *tularensis*	SCHU S4	North America	AJ749949
Francisella tularensis subsp. *mediasiatica*	FSC147	Kazakhstan	CP000915
Francisella tularensis subsp. *novicida*	U112	United States	CP000439
Francisella philomiragia subsp. *philomiragia*	ATCC 25017	—	CP000937

(2 females and 3 males) parasitizing vertebrate hosts, namely, wolf (*Canis lupus*) ($n = 57$) and dog (*Canis familiaris*) ($n = 8$), and questing ticks ($n = 7$)—were collected in the Bragança area, located in the north of Portugal; this is the same region where *F. tularensis* was described for the first time. Ticks were identified to the species level, washed in 70% ethanol and distilled water, and then processed individually for DNA extraction as described previously (Rijpkema et al. 1995).

DNA samples were tested with the specific primer set FT393 and FT642, amplifying a 250 bp fragment of the gene coding the 17 kDa lipoprotein (*Tul4)* of *Francisella* spp. as described previously (Karhukorpi and Karhukorpi 2001) and the *Francisella* 16S rRNA gene primer set F5 and F11 (Forsman et al. 1994). Polymerase chain reactions included negative controls. Resulting products were run in 1.5% low-melt agarose gels, purified using the Jetquick purification kit (Genomed), and sequenced with the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems) on an ABI 377 DNA sequencer. A partial sequence of the gene coding the 17 kDa lipoprotein (*Tul4*) was analyzed for all positive samples and a random set of four (17%) 16S rDNA amplicons were sequenced.

The sequences of the positive samples were assembled by combining the sequences generated by each primer, using BioEdit software (Table 1). For phylogenetic inference the alignments were made using amino acid sequences and converted to DNA sequences using BioEdit software. All alignments were made using ClustalX program (Thompson et al. 1997) and manually inspected for misalignments. Primers sequences were removed from the alignment before phylogenetic analyses. Neighbor-joining tree of DNA sequence alignment was conducted in PAUP* 4.0b10 software. Distance matrices were calculated using the Kimura two-parameter model to correct for multiple substitutions. Bootstrap analysis was obtained with 1000 replicates (Fig. 1).

Results and Discussion

Our aim was to evaluate the presence of FLEs in ticks collected in the same geographical area where the presence of *F. tularensis* subsp. *holarctica* was detected for the first time in Portugal. Concerning the 75 ticks tested, 24 *D. reticulatus* were positive for FLEs, with a prevalence rate of 32%, much higher than the prevalence of *Francisella* spp. previously detected (1.1%). Due to the small amount of ticks analyzed in this study, these results do not have statistical significance. The phylogenetic analyses based on the partial sequence of 17 kDa lipoprotein gene grouped the tick samples with other FLEs detected in *D. reticulatus* from Hungary and show a robust cluster (93 bootstrap). This study allowed the first detection of FLEs in Portuguese *D. reticulatus* ticks, confirming the presence of these bacteria and the need to take this into account in ticks and environmental specimen.

The distribution and prevalence of FLEs in tularemia-transmitting tick species is largely unknown (Kugeler et al. 2005). Experiments undertaken on *Dermacentor andersoni* ticks have shown the presence of FLEs in female reproductive tissues but not in salivary glands (Niebylski et al. 1997). The results obtained in this work are surprising comparing with a previous study in *D. reticulatus* ticks collected in northern Portugal where we detect only *F. tularensis* subsp. *holarctica* (Lopes de Carvalho et al. 2007). Because the methods of tick analysis in both studies were exactly the same, these results suggest that there is a discrepancy in distribution of FLEs in local tick population. This difference could be explained by tick origin, since in the first study the analyzed ticks were from other vertebrate hosts (as sheep's and cows) and were collected in a different site (about 100 km apart). Moreover, these findings can suggest that the presence of FLEs may interfere in the prevalence of pathogenic *Francisella* strains. However, the confirmation of this hypothesis remains to be tested using more ticks from other collection sites. The DNA sequence we detected presents 100% of similarity with the Hungarian sequence, using TUL4 partial sequence and 99.8% for 16S rDNA sequence (916 nt) (Sréter-Lancz et al. 2009).

A follow-up of this line of research with further phylogenetic studies using more FLE gene sequences would be crucial, since the knowledge of these bacteria is still scarce.

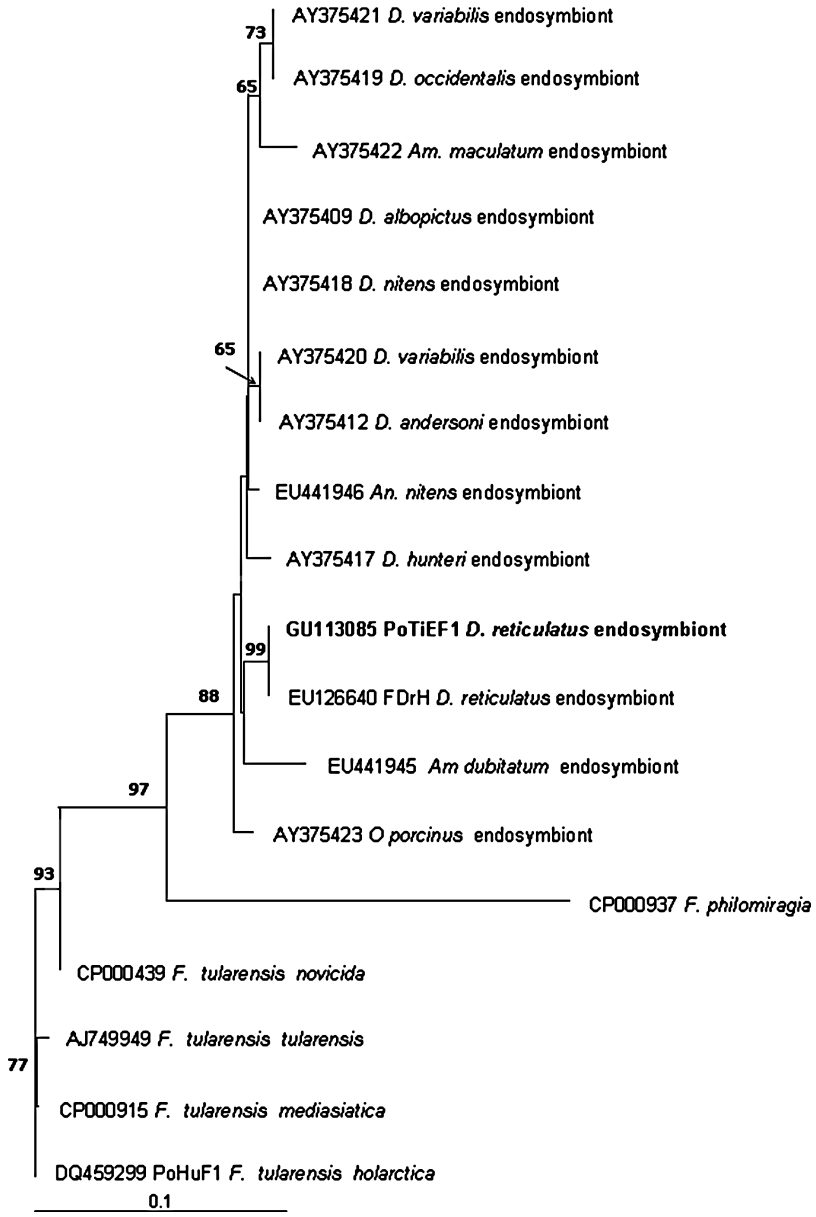

FIG. 1. Neighbor-joining tree inferred from partial TUL4 sequences from the tick detection obtained in this study (bold) compared to GenBank sequence data. Distance matrices were calculated using the Kimura two-parameter model to correct for multiple substitutions. Bootstrap values were obtained from 1000 replicate trees and are indicated at the nodes (>50%).

Additionally, the isolation and characterization of both *Francisella* and FLE strains would be necessary, for an assessment of the diversity in virulence action of these microorganisms circulating in Portugal and their public health relevance.

Nucleotide Sequence Accession Numbers

The GenBank nucleotide sequence accession number for partial sequences of 17 kDa lipoprotein gene generated in this study is GU113085 for PoTiEF1.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Isabel Lopes de Carvalho
Centro de Estudos de Vectores e Doenças Infecciosas
Dr. Francisco Cambournac
Instituto Nacional de Saúde Dr. Ricardo Jorge
Av. Liberdade n 5
Águas de Moura 1649-016
Portugal

E-mail: isabel.carvalho@insa.min-saude.pt