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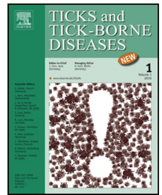

Short communication

Occurrence of *Francisella* spp. in *Dermacentor reticulatus* and *Ixodes ricinus* ticks collected in eastern Poland

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ABSTRACT

A total of 530 questing *Dermacentor reticulatus* ticks and 861 questing *Ixodes ricinus* ticks were collected from Lublin province (eastern Poland) and examined for the presence of *Francisella* by PCR for 16S rRNA (*rrs*) and *tul4* genes. Only one female *D. reticulatus* tick out of 530 examined (0.2%) was infected with *Francisella tularensis* subspecies *holarctica*, as determined by PCR of the *rrs* gene. None of 861 *I. ricinus* ticks were infected with *F. tularensis*. In contrast, the presence of *Francisella*-like endosymbionts (FLEs) was detected in more than half of the *D. reticulatus* ticks (50.4%) and 0.8% of the *I. ricinus* ticks. The nucleotide sequences of the FLEs detected in *D. reticulatus* exhibited 100% homology with the nucleotide sequence of the FLE strain FDrH detected in Hungary in *D. reticulatus*. In conclusion, our results suggest a low contribution of *D. reticulatus* and *I. ricinus* ticks to the circulation of *F. tularensis* in eastern Poland. This finding, however, needs to be confirmed by further studies in other areas. Our study confirmed the common infection of *D. reticulatus* with *Francisella*-like endosymbionts (FLEs) of unknown pathogenic potential and revealed, for the first time, a low grade of infection of *I. ricinus* with FLEs.

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Introduction

Francisella tularensis (*F. tularensis*) is a fastidious, aerobic, gram-negative coccobacillus that affects vertebrates, especially lagomorphs and rodents. In humans, this coccobacillus causes tularemia, a potentially fatal multi-systemic zoonotic disease that occurs in the northern hemisphere, including North America, Europe, and Asia. The species *F. tularensis* is divided into three subspecies: subsp. *Tularensis*, which has high virulence and occurs predominantly in North America, subsp. *Holarctica*, which has mild virulence and occurs throughout the northern hemisphere, and subsp. *Mediasiatica*, which has mild virulence and occurs in Central Asia (Carvalho et al., 2014). Humans can become infected by *F. tularensis* by being bitten by hematophagous arthropods (ticks, mosquitos, and deer flies), by direct contact with infected animals (mostly hares and rabbits), by ingestion of contaminated food or water, and by inhalation of infected aerosols. The bacterium is highly infectious and is considered as a potential bioweapon (Carvalho et al., 2014; CDC, 2005; Foley and Nieto, 2010; Zhang et al., 2008; Franke et al., 2010; Kaysser et al., 2008; Kugeler et al., 2005; Kreizinger et al., 2013).

The transovarial transmission of *F. tularensis* in ticks is controversial and may depend on what subpopulations of *F. tularensis* and what tick species are present (Petersen et al., 2009). The principal tick vectors include species of the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes* and *Ornithodoros* (Gordon et al., 1983). Many tick species are also hosts of bacteria that are closely related to *F. tularensis*, called *Francisella*-like endosymbionts (FLEs) (Dergousoff and Chilton, 2012). The pathogenic potential of FLEs remains unknown, although sequences homologous to the *iglC* and *mglA* genes of *F. tularensis*, which have been implicated in bacterial pathogenicity, have been detected in FLEs (Machado-Ferreira et al., 2009). FLEs appear to replicate intracellularly, and they are transmitted transovarially. To date, there is no evidence of horizontal transmission through tick bites (Ivanov et al., 2011). FLEs are widely distributed, and a number of diverse FLEs have been reported in various tick genera on at least four continents (2011; Scoles, 2004). In Europe, thus far, FLEs have been isolated from *Dermacentor reticulatus* (*D. reticulatus*) in Hungary (Kreizinger et al., 2013; Sréter-Lancz et al., 2009), Portugal (De Carvalho et al., 2011), France (Michelet et al., 2013) and Germany (Gehringer et al., 2013) and from *Hyalomma marginatum marginatum*,

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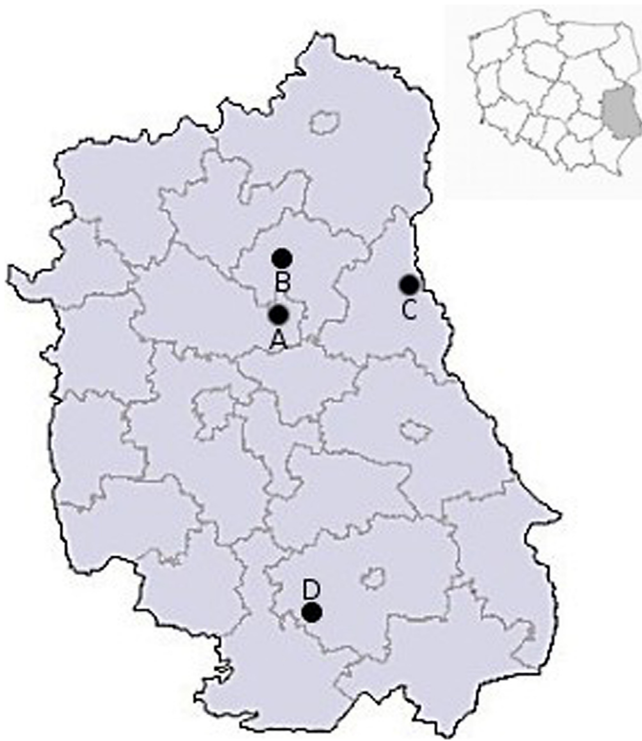

Fig. 1. Map of collection of ticks located in the Lublin province, eastern Poland (source of map http://www.polskainfo.pl). Locality A: Ostrów Lubelski; B: Parczew; C: Włodawa; D: Zwierzyniec.

Hyalomma aegyptium, *Rhipicephalus sanguineus* and *D. reticulatus* in Bulgaria (Ivanov et al., 2011).

No recent reports are available on the occurrence of *F. tularensis* in potential arthropod vectors in Poland. To fill this gap, we examined samples of *D. reticulatus* and *I. ricinus* ticks collected from eastern Poland for the presence of *F. tularensis*; we also considered the potential for FLEs in the sampled ticks.

Materials and methods

Collection of ticks

A total of 530 questing *D. reticulatus* ticks (273 females and 257 males) and 861 questing *I. ricinus* ticks (200 females, 179 males and 482 nymphs) were collected during the spring/summer season in 2011–2012 in 4 localities situated in the Lublin province (eastern Poland). *D. reticulatus* ticks were collected in 3 localities situated on the Łęczyńsko-Włodawskie Lakeland: Ostrów Lubelski (locality 'A'), Parczew (locality 'B'), and Włodawa (locality 'C'). *I. ricinus* ticks were collected in a forest inspectorate, Zwierzyniec (locality 'D'), situated on the Roztocze Highland (Fig. 1). Ticks were collected by dragging a woolen flag over the lower vegetation and litter along the paths and edges of deciduous and mixed forests.

DNA isolation from ticks

Total DNA was isolated from the adult ticks separately and from nymphs in pools of 5 specimens (Rijpkema et al., 1996) by boiling in 0.7 M ammonium hydroxide. The concentration of DNA in the isolates was determined with the NanoDrop ND1000 Spectrophotometer (USA). For *D. reticulatus*, the determined DNA concentrations ranged from 520 to 672 for males and from 670 to 878 ng/μl for females. For *I. ricinus*, the DNA concentrations ranged from 309 to 506 ng/μl for females and from 175 to 328 ng/μl for males, and for nymphs the DNA concentrations ranged from 18 to 80 ng/μl.

Detection of Francisella *spp. DNA in ticks by PCR*

For the 16S rRNA (*rrs*) and *tul4* (encoding 17 kDa lipoprotein) genes, PCR assays were performed according to the methods of Michelet et al. (2013) with some modifications. A 50 μl reaction volume was used and contained the following: 1 U *Taq* DNA polymerase (Qiagen, USA), 1× PCR buffer containing 15 mM $MgCl_2$, 2 mM dNTP (final concentration 0.2 mM) (Fermentas, Lithuania), 2.5 μl 10 μM each of primer (Eurogentec, Seraing, Belgium), 2 μl of DNA and nuclease-free water (Applied Biosystems, USA). For the *rrs* and *tul4* genes, the primers F11 and F5 (Forsman et al., 1994) and FT393 and FT642 (Long et al., 1993) were applied, respectively. The amplification was carried out in C1000 Thermal Cycler (BioRad, USA). After electrophoresis in a 1.5% agarose gel under standard conditions and staining with ethidium bromide solution (2 μg/ml), the products of the 16S rRNA amplification were identified as a 1140 bp band. For the amplification products of *tul4*, electrophoresis was performed in 2% agarose gels, and a 248 bp-long electrophoresis band was considered positive. The *F. tularensis* strain FSC043 was used as the positive control and was kindly provided by Prof. M. Kondrusik (Department of Infectious Diseases and Neuroinfection in Białystok, Poland). For the negative control, instead of using matrix DNA, we used nuclease-free water. In addition, positive specimens were examined using the *lpnA* gene, which encodes a region of a 17 kDA lipoprotein that is different from *tul4*. A 233-bp fragment of *lpnA* has been recommended as an appropriate marker for differentiating between *F. tularensis* and FLEs (Forestal et al., 2008; Escudero et al., 2008).

The PCR was performed according to the method of Gehringer et al. (2013). After PCR, the samples that contained *rrs, tul4* and *lpnA* genes were classified as positive.

DNA sequencing

DNA sequencing was performed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA, USA) using Abi Prism Big Dye Terminator v. 3.1. Cycle Sequencing Kits and Big Dye XTerminator Purification Kits (Applied Biosystems). The same primers as those used for the PCR of the *rrs*, *tul4*, *lpnA* genes were also used for sequencing. The resulting sequences were compared with sequences in the GenBank database using the BLAST server on the National Center for Biotechnology Information website (Bethesda, MD, USA).

Statistical analysis

The obtained results were analyzed by χ^2 test and Student's *t*-test using the STATISTICA v. 6.0 package (Statsoft, Tulsa, OK, USA). A p value <0.05 was considered statistically significant.

Results

Only one female *D. reticulatus* tick out of 530 examined (0.2%) was infected with the *F. tularensis* subspecies *holarctica* according to the PCR results for the *rrs* gene (accession number CP007148). Unfortunately, the sequence analyses of *tul4* and *lpnA* genes were not successful because of the poor quality of the PCR products (the bands on the agarose gel were too weak).

In contrast, *Francisella*-like endosymbionts (FLEs) were detected in more than half of the *D. reticulatus* ticks examined (50.4%) (Table 1). FLE infection of ticks was significantly dependent on locality ($p = 0.0083$) and sex: females were infected significantly more often than males ($p = 0.0034$). None of the 861 *I. ricinus* ticks

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