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Short communication

Detection of *Francisella tularensis* subsp. *holarctica* in a European brown hare (*Lepus europaeus*) in Thuringia, Germany

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Abstract

The isolation of *Francisella tularensis* subsp. *holarctica* biovar II (strain 06T0001) from a European brown hare (*Lepus europaeus*) from Thuringia, Germany, is described for the first time. Identification of the microorganism was carried out by phenotypic characterisation, partial sequencing of the 16S rRNA gene and specific PCR using the primers TUL4-435/TUL4-863 and FtC1/FtC4. The epidemiology of tularemia in Germany is discussed and a risk assessment for humans is made.
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1. Introduction

Tularemia is an infectious disease caused by the small, pleomorphic, heat-labile, Gram-negative, rod-shaped bacterium *Francisella* (*F.*) *tularensis*. The microorganism is a facultative intracellular pathogen affecting a wide range of animal species with more hosts than any other known zoonotic pathogen (Johansson et al., 2004).

The main hosts are believed to be hares and rabbits (*Leporidae*), hamsters, water and field voles (*Cricetidae*), water and wood rats, lemmings and field mice (*Muridae*), squirrels (*Sciuridae*), and also aquatic rodents like beaver (*Castoridae*) and muskrats (*Ondatrae*). Other mammalian species susceptible for *F. tularensis* are monkeys (*Anthropoidae*), dogs and coyotes (*Canidae*), cats (*Felidae*), sheep (*Ovidae*) and cattle (*Bovidae*) but also predators like bears (*Ursidae*), and foxes (*Vulpes*). Infection of lagomorphs

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and rodents with *F. tularensis* often causes fatal disease. Usually, infected animals are found moribund or dead. Also several bird species as well as fish and amphibians are considered to be incidental hosts (Splettstoesser et al., 2005; Anda et al., 2001). Further vectors for transmission of *F. tularensis* are ticks, biting flies and mosquitoes (Blaškovič and Barák, 2005; Hubalek et al., 1996).

Tularemia is endemic in many regions of the Northern hemisphere worldwide. The species *F. tularensis* includes three subspecies (2 types). Type A strains, *F. tularensis* subsp. *tularensis*, have so far been found predominantly in North America, although type A strains have been isolated in the Danube region close to Bratislava (Guryčova, 1998). Type B strains (*F. tularensis* subsp. *holarctica*), *F. tularensis* subsp. *mediasiatica* and *F. novicida* are found in Europe, Northern Asia and Northern America (Ellis et al., 2002).

In Germany in 1983, 1990–1992 four cases of tularemia in hares or rabbits were notified from Lower-Saxony, Rhineland-Palatinate, North Rhine-Westfalia and Baden-Wuerttemberg, respectively (BMELV, 2006).

In November 2004, an outbreak of tularemia was reported among marmosets (*Callithrix jaccus*) in Central Lower Saxony, Germany (Splettstoesser et al., 2007). The detection of *F. tularensis* subsp. *holarctica* was a re-emergence of tularemia in Germany (BMELV, 2006).

In 2005, 15 cases of tularemia were serologically confirmed in persons in Hesse who had contact with hares (Hofstetter et al., 2006; Splettstoesser et al., 2006). *F. tularensis* subsp. *holarctica* was also detected in several organ samples taken from these hares.

A variety of PCR methods have been established for the detection of *F. tularensis* DNA in both clinical and environmental specimens (Splettstoesser et al., 2005). For species identification PCR assays were used targeting the *tul4* gene (accession number M32059) which encodes a 17 kDa outer membrane protein (Sjöstedt et al., 1997). By Johansson et al. (2000) a PCR assay was evaluated to identify *F. tularensis* subsp. *holarctica* and distinguish it from all other subspecies (accession number AF240631).

Here, we report a case of tularemia in a European brown hare. The causative agent *F. tularensis* was identified by phenotypic characterisation, Gram staining, agglutination test, DNA sequencing, PCR, and susceptibility to erythromycin.

2. Materials and methods

2.1. Material

In February 2006, a European brown hare (*Lepus europaeus*) was shot and sent for microbiological investigation with the report of abnormal flight behaviour. It was an adult male weighing 3600 g. No externally visible distinctive pathological–anatomical changes were observed. After opening of the carcass and examination of the organs the following significant morphological changes were found: swelling of body lymph nodes, moderate hyperplastic spleen tumour, swelling of the kidneys, diffuse reddening and petechiae of the tracheal mucosa and mucoid inflammation of the colon.

2.2. Isolation and cultivation of *F. tularensis*

Bacteria originating from brain, lung, liver, spleen, kidney, sections of small intestine, colon and bile were cultivated on blood agar plates by means of loop inoculation and incubated under aerobic conditions at 37 °C for 3–4 days. In parallel, growth of *Francisella* bacteria was tested under aerobic and microaerophilic conditions on chocolate agar with 2% cysteine and cystine heart agar (Oxoid, Wesel, Germany) with 9% sheep blood. Growth of *F. tularensis* was confirmed by the slide agglutination assay with a specific antiserum (BgVV, Berlin, Germany) and standard Gram-staining. The susceptibility of bacteria to erythromycin was examined (Etest™, erythromycin discs [15 μg], Oxoid).

2.3. Differential diagnostic investigations

Other differential diagnostic assays applied were hemadsorption test for detection of European brown hare syndrome virus (EBHS-V), ELISA antigen assay for *Chlamydia*/*Chlamydophila* detection, direct immunofluorescence test for rabies detection, general bacteriological investigation, and parasitological investigation of lung, small intestine and colon

sections by means of flotation, sedimentation and migration techniques.

2.4. PCR detection and partial sequencing of 16S rRNA gene

A bacterial colony was resuspended in 100 μl phosphate-buffered saline and boiled for 10 min. The DNA was extracted using the High Pure PCR Template Preparation Kit™ (Roche Diagnostics, Mannheim, Germany) according to the recommendations of the manufacturer. The DNA was eluted in 200 μl elution buffer and 5 μl were applied in each PCR assay.

The PCR was carried out as described by Johansson et al. (2000). Briefly, the reaction mixture consisted of 5 μl 10× PCR buffer with 1.5 mmol $MgCl_2$ (Genaxxon, Stafflangen, Germany), 2 μl of dNTP mix (2.5 mM of each dNTP, Carl Roth GmbH, Karlsruhe, Germany), 1 μl of each of forward and reverse primers (10 pmol/μl, JenaBioscience, Jena, Germany), 0.2 μl of *Taq* DNA polymerase (5 U/μl, Genaxxon), 5 μl of DNA extract and deionised water to a final volume of 50 μl. The primers used are shown in Table 1. After denaturation at 94 °C for 60 s, 35 cycles of amplification were performed according to the following protocol: denaturation at 94 °C for 30 s, primer annealing at 60 °C for 60 s, and primer extension at 72 °C for 30 s. After the final extension step at 72 °C for 60 s, each reaction mixture was subjected to electrophoresis in a 2% agarose gel (Eurogentech, Cologne, Germany). After ethidium bromide staining, the PCR products were visualized by UV light and documented using BioImage system GeneGenius (Syngene, Synoptics Ltd., UK).

Amplicons obtained by using primer pair 16S UNI-L/R (Kuhnert et al., 1996) and DNA of isolate 06T0001 were cut out of agarose gel plug. DNA was purified using QIAquick Gel Extraction Kit™ (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The extract was subjected to cycle sequencing with BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany). Amplification primers 16S UNI-L and 16S UNI-R were used as sequencing primers, too. Nucleotide sequences were determined on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Resulting sequences were compared with those from public database entries (http://www.ncbi.nlm.nih.gov/blast/).

3. Results

The pathological findings in the sick hare were concordant with the clinical picture of tularemia in this species. *F. tularensis* could be isolated from different organs (lung, liver, spleen, kidney, small intestine section and bile) of this animal. After incubation for 72–96 h at 37 °C, numerous glossy colonies were seen on chocolate agar plates. The characteristic glossy grey or dark green staining of the agar in the colony area was distinct on cysteine heart agar. The bacteria were Gram-negative, very small, had a coccoid rather than a bacillary shape, and showed resistance to erythromycin.

Using the primer pair 16S UNI-L/16S UNI-R a 1200 bp fragment of the 16S rRNA gene was amplified. Partial sequencing of this fragment and BLAST search resulted in the identification of the bacterium as a member of the species *F. tularensis.* This result was in concordance with species-specific PCR results using primers TUL4-435/TUL4-863 (Sjöstedt et al., 1997).

Table 1
Sequences of primers

Primer	Sequence (5′–3′)	Fragment size	Reference
16S UNI-L 16S UNI-R	AGA GTT TGA TCA TGG CTC AG GTG TGA CGG GCG GTG TGT AC	1200 bp	Kuhnert et al. (1996)
TUL4-435 TUL4-863	GCT GTA TCA TCA TTT AAT AAA CTG CTG TTG GGA AGC TTG TAT CAT GGC ACT	428 bp	Sjöstedt et al. (1997)
FtC1 FtC4	TCC GGT TGG ATA GGT GTT GGA TT GCG CGG ATA ATT TAA ATT TCT CAT A	300 bp/330 bp	Johansson et al. (2000)

For distinguishing the *F. tularensis* subsp. *holarctica* from other *F. tularensis* subspecies, the primer set FtC1/FtC4 (Johansson et al., 2000) was applied. The amplicon obtained with DNA of isolate 06T0001 was only 300 bp in length suggesting that this isolate was a member of the subspecies *holarctica*. The amplicons for *F. tularensis* subsp. *tularensis,* subsp. *mediasiatica, F. novicida* and *F. philomiragia* were 330 bp in size.

Other differential diagnostic investigations were applied including the hemadsorption test for detection of European brown hare syndrome virus and, *Chlamydia*/*Chlamydophila* capture ELISA, direct immunofluorescence test for rabies detection were negative. Parasitological investigation of the lungs, small intestine and colon resulted in detection of *Trichuris* and other nematode species, *Dicrocoelium* flatworms and *Eimeria* spp.

In general bacterial investigations *Mannheimia haemolytica* was detected in lung tissue and *Clostridium perfringens* type A in the colon.

4. Discussion

Tularemia is widespread in Europe, its presence is reported from all countries except UK, Iceland, and Portugal (Tärnvik et al., 2004). In most countries, only sporadic human infections are reported every year, whereas in others, e.g. Sweden or Finland, outbreaks comprising hundreds of cases are recorded (Tärnvik et al., 2004). The first case of tularemia in Germany was reported 50 years ago (BMELV, 2006). In October 2004, an outbreak of this disease in a semi-free living group of marmosets in Lower Saxony was detected (Splettstoesser et al., 2007), and in December 2005, infections in humans were reported from Hesse (Hofstetter et al., 2006; Splettstoesser et al., 2006). Here, a case of tularemia in a hare in Thuringia is described. All these cases were found within a region known as ''Central Germany''. The confirmation of autochthonous infections in the geographical centre of Germany will stimulate the historical debate on the origin of tularemia in Central Europe and on its natural reservoirs, routes of transmission and the ecological niches of the bacteria. In this geographical area characteristic conditions are predominant for typical natural foci of tularemia (Pikula et al., 2003): alluvial forests, <200 m above sea level, 8–10 °C mean annual temperature, 450–700 mm mean annual precipitation, and 2000–2200 h mean annual sunshine duration.

The most important natural occurring reservoir of *F. tularensis* may be amoeba or protozoa in surface water and ticks (e.g. *Dermacentor reticulatus* and *Ixodes* species) as well as fleas (Guryčova et al., 2001). Therefore, the strongest tenacity of the infectious bacteria was found in dust, damp hay, carcasses, water, etc. The bacterium can survive in the environment and in vectors for a relevant period of time. The assumption that rodent and tick populations of non-endemic areas are very susceptible to a newly introduced, highly contagious and virulent agent may explain the focal emergence of tularemia in hitherto unaffected areas like Thuringia.

In the reported case, a hare shot by a hunter was sent to a local authority for investigation because of its abnormal flight behaviour. It showed significant morphological changes of organs, like swelling of body lymph nodes, moderate spleen tumour, swelling of kidneys and diffuse reddening and petechiae of tracheal mucosa. These lesions are typical for tularemia. The investigation of the bacterial colonies revealed results, which were concordant with *Francisella* bacteria. Using DNA sequencing of 16S rRNA gene the occurrence of *F. tularensis* was confirmed. Consequently, we used the PCR assays as the latest diagnostic method for detection of *F. tularensis* (Splettstoesser et al., 2005). With PCR using primer pair FtC1/FtC4 for subspecies identification of the investigated bacteria the agent could be identified as *F. tularensis* subsp. *holarctica.* Erythromycin resistance was observed in the isolate and was therefore assigned to biovar II (Tomaso et al., 2005). This finding indicates that the possible geographic origin of the isolate is Eastern Europe or that the Western border of this well-known region of endemicity is located in the German mountain range.

This case of tularemia in a hare is the first report on the presence of this zoonosis in Thuringia, Germany. The increased epizootic activity of *F. tularensis* in the endemic region of Central Europe shows that tularemia has to be considered an emerging disease for animals and humans too. The influence of Greenhouse effect and the obviously changing climate in Central Europe might have a crucial impact on the future spread of the disease and the stable establishing of permanent endemic areas. Thus, a higher risk for

wildlife, pet animals and humans has to be expected. Therefore, the aim of further investigations will be the characterisation of the epidemiology of tularemia in whole Europe.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2007.03.025.

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