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W. Mueller, H. Bocklisch, G. Schueler, H. Hotzel, H. Neubauer, et al.. Detection of subsp. in a European brown hare () in Thuringia, Germany. *Veterinary Microbiology*, 2007, 123 (1-3), pp.225. 10.1016/j.vetmic.2007.03.025 . hal-00532230

**HAL Id: hal-00532230**

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Submitted on 4 Nov 2010

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## Accepted Manuscript

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

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PII: S0378-1135(07)00141-1  
DOI: doi:10.1016/j.vetmic.2007.03.025  
Reference: VETMIC 3618

To appear in: *VETMIC*

Received date: 19-7-2006  
Revised date: 13-3-2007  
Accepted date: 22-3-2007

Please cite this article as: Mueller, W., Bocklisch, H., Schueler, G., Hotzel, H., Neubauer, H., Otto, P., Detection of *Francisella tularensis* subsp. *holarctica* in a European brown hare (*Lepus europaeus*) in Thuringia, Germany, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.03.025

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1 Short communication

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61 Short communication

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63 **Detection of *Francisella tularensis* subsp. *holarctica* in a European brown hare**  
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76

77 **Abstract**

78

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

85

86 **Keywords:** tularemia, *Francisella tularensis*, PCR, *Lepus europaeus*

87 **1. Introduction**

88

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

93 The main hosts are believed to be hares and rabbits (*Leporidae*), hamsters, water  
94 and field voles (*Cricetidae*), water and wood rats, lemmings and field mice (*Muridae*),  
95 squirrels (*Sciuridae*), and also aquatic rodents like beaver (*Castoridae*) and muskrats  
96 (*Ondatrae*). Other mammalian species susceptible for *F. tularensis* are monkeys  
97 (*Anthropoidae*), dogs and coyotes (*Canidae*), cats (*Felidae*), sheep (*Ovidae*) and  
98 cattle (*Bovidae*) but also predators like bears (*Ursidae*), and foxes (*Vulpes*). Infection  
99 of lagomorphs and rodents with *F. tularensis* often causes fatal disease. Usually,  
100 infected animals are found moribund or dead. Also several bird species as well as  
101 fish and amphibians are considered to be incidental hosts (Spletstoeser et al.,  
102 2005, Anda et al. 2001). Further vectors for transmission of *F. tularensis* are ticks,  
103 biting flies and mosquitoes (Blaškovič and Barak, 2005, Hubalek et al., 1996).

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

111 In Germany in 1983, 1990, 1991 and 1992 four cases of tularemia in hares or rabbits  
112 were notified from Lower-Saxony, Rhineland-Palatinate, North-Westfalia and

113 Baden-Württemberg, respectively (BMELV, 2006).  
114 In November 2004 an outbreak of tularemia was reported among marmosets  
115 (*Callithrix jacchus*) in Central Lower Saxony, Germany (Splettstoesser et al., 2007).  
116 The detection of *F. tularensis* subsp. *holarctica* was a re-emergence of tularemia in  
117 Germany (BMELV, 2006).  
118 In 2005 15 cases of tularemia were serologically confirmed in persons in Hesse who  
119 had contact with hares (Hofstetter et al., 2006, Splettstoesser et al., 2006). *F.*  
120 *tularensis* subsp. *holarctica* was also detected in several organ samples taken from  
121 these hares.  
122 A variety of PCR methods has been established for the detection of *F. tularensis*  
123 DNA in both clinical and environmental specimens (Splettstoesser et al., 2005). For  
124 species identification PCR assays were used targeting the *tul4* gene (accession  
125 number M32059) which encodes a 17 kDa outer membrane protein (Sjöstedt et al.,  
126 1997). By Johansson et al. (2000) a PCR assay was evaluated to identify *F.*  
127 *tularensis* subsp. *holarctica* and distinguish it from all other subspecies (accession  
128 number AF240631).  
129 Here we report a case of tularemia in a European brown hare. The causative agent  
130 *Francisella tularensis* was identified by phenotypic characterisation, Gram staining,  
131 agglutination test, DNA sequencing, PCR, and susceptibility to erythromycin.

132

133

## 134 **2. Materials and Methods**

135

### 136 **Material**

137

138 In February 2006, an European brown hare (*Lepus europaeus*) was shot and sent  
139 for microbiological investigation with the report of abnormal flight behaviour. It was an  
140 adult male weighing 3,600 g. No externally visible distinctive pathological-anatomical  
141 changes were observed. After opening of the carcass and examination of the organs  
142 the following significant morphological changes were found: swelling of body lymph  
143 nodes, moderate hyperplastic spleen tumour, swelling of the kidneys, diffuse  
144 reddening and petechiae of the tracheal mucosa and mucoid inflammation of the  
145 colon.

146

147 Isolation and cultivation of *Francisella tularensis*

148

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

158

159 Differential diagnostic investigations

160

161 Other differential diagnostic assays applied were hemadsorption test for detection of  
162 European brown hare syndrome virus (EBHS-V), ELISA antigen assay for  
163 *Chlamydia/Chlamydomphila* detection, direct immunofluorescence test for rabies

164 detection, general bacteriological investigation, and parasitological investigation of  
165 lung, small intestine and colon sections by means of flotation, sedimentation and  
166 migration techniques.

167

168 PCR detection and partial sequencing of 16S rRNA gene

169

170 A bacterial colony was resuspended in 100  $\mu$ l phosphate-buffered saline and boiled  
171 for 10 minutes. The DNA was extracted using the High Pure PCR Template  
172 Preparation Kit<sup>TM</sup> (Roche Diagnostics, Mannheim, Germany) according to the  
173 recommendations of the manufacturer. The DNA was eluted in 200  $\mu$ l elution buffer  
174 and 5  $\mu$ l were applied in each PCR assay.

175 The PCR was carried out as described by Johansson et al. (2000). Briefly, the  
176 reaction mixture consisted of 5  $\mu$ l 10x PCR buffer with 1.5 mmol MgCl<sub>2</sub> (Genaxxon,  
177 Stafflangen, Germany), 2  $\mu$ l of dNTP mix (2,5 mM of each dNTP, Carl Roth GmbH,  
178 Karlsruhe, Germany), 1  $\mu$ l of each of forward and reverse primers (10 pmol/ $\mu$ l,  
179 JenaBioscience, Jena, Germany), 0.2  $\mu$ l of *Taq* DNA polymerase (5 U/ $\mu$ l, Genaxxon),  
180 5  $\mu$ l of DNA extract and deionised water to a final volume of 50  $\mu$ l. The primers used  
181 are shown in table 1. After denaturation at 94 °C for 60 s, 35 cycles of amplification  
182 were performed according to the following protocol: denaturation at 94 °C for 30 s,  
183 primer annealing at 60 °C for 60 s, and primer extension at 72 °C for 30 s. After the  
184 final extension step at 72 °C for 60 s, each reaction mixture was subjected to  
185 electrophoresis in a 2% agarose gel (Eurogentech, Cologne, Germany). After  
186 ethidium bromide staining, the PCR products were visualized by UV light and  
187 documented using BioImage system GeneGenius (Syngene, Synoptics Ltd., UK).  
188 Amplicons obtained by using primer pair 16S UNI-L/R (Kuhnert et al., 1996) and DNA  
189 of isolate 06T0001 were cut out of agarose gel plug. DNA was purified using



190 QIAquick Gel Extraction Kit™ (Qiagen, Hilden, Germany) according to the  
191 manufacturer's instructions.  
192 The extract was subjected to cycle sequencing with BigDye™ Terminator Cycle  
193 Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany).  
194 Amplification primers 16S UNI-L and 16S UNI-R were used as sequencing primers,  
195 too. Nucleotide sequences were determined on an ABI PRISM 310 Genetic Analyzer  
196 (Applied Biosystems). Resulting sequences were compared with those from public  
197 database entries (<http://www.ncbi.nlm.nih.gov/blast/>).

198

199

### 200 **3. Results**

201

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

210 Using the primer pair 16S UNI-L/16S UNI-R a 1,200 bp fragment of the 16S rRNA  
211 gene was amplified. Partial sequencing of this fragment and BLAST search resulted  
212 in the identification of the bacterium as a member of the species *F. tularensis*. This  
213 result was in concordance with species-specific PCR results using primers TUL4-  
214 435/TUL4-863 (Sjöstedt et al., 1997).

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

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

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

229

230

#### 231 **4. Discussion**

232

233 Tularemia is widespread in Europe, its presence is reported from all countries except  
234 UK, Iceland, and Portugal (Tärnvik et al., 2004). In most countries, only sporadic  
235 human infections are reported every year, whereas in others, e. g. Sweden or  
236 Finland, outbreaks comprising hundreds of cases are recorded (Tärnvik et al., 2004).  
237 The first case of tularemia in Germany was reported 50 years ago (BMELV 2006). In  
238 October 2004 an outbreak of this disease in a semi-free living group of marmosets in  
239 Lower Saxony was detected (Splettstoesser et al., 2007), and in December 2005  
240 infections in humans were reported from Hesse (Hofstetter et al. 2006, Splettstoesser

241 et al., 2006 ). Here, a case of tularemia in a hare in Thuringia is described. All these  
242 cases were found within a region known as “Central Germany”. The confirmation of  
243 autochthonous infections in the geographical centre of Germany will stimulate the  
244 historical debate on the origin of tularemia in Central Europe and on its natural  
245 reservoirs, routes of transmission and the ecological niches of the bacteria. In this  
246 geographical area characteristic conditions are predominant for typical natural foci of  
247 tularemia (Pikula et al., 2003): alluvial forests, < 200 m above sea level, 8°-10°C  
248 mean annual temperature, 450-700 mm mean annual precipitation, and 2,000-2,200  
249 h mean annual sunshine duration.

250 The most important natural occurring reservoir of *F. tularensis* may be a moeba or  
251 protozoa in surface water and ticks (e. g. *Dermacentor reticulatus* and *Ixodes*  
252 species) as well as fleas (Guryčova et al., 2001). Therefore the tenacity of the  
253 infectious bacteria was found in dust, damp hay, carcasses, water etc. The bacterium  
254 can survive in the environment and in vectors for a relevant period of time. The  
255 assumption that rodent and tick populations of non-endemic areas are very  
256 susceptible to a newly introduced, highly contagious and virulent agent may explain  
257 the focal emergence of tularemia in hitherto unaffected areas like Thuringia.  
258 In the reported case a hare shot by a hunter was sent to a local authority for  
259 investigation because of its abnormal flight behaviour. It showed significant  
260 morphological changes of organs, like swelling of body lymph nodes, moderate  
261 spleen tumour, swelling of kidneys and diffuse reddening and petechiae of tracheal  
262 mucosa. These lesions are typical for tularemia. The investigation of the bacterial  
263 colonies revealed results, which were concordant with *Francisella* bacteria. Using  
264 DNA sequencing of 16S rRNA gene the occurrence of *F. tularensis* was confirmed.  
265 Consequently, we used the PCR assays as the latest diagnostic method for detection  
266 of *F. tularensis* (Spletstoeser et al. 2005). With PCR using primer pair FtC1/FtC4 for

267 subspecies identification of the investigated bacteria the agent could be identified as  
268 *F. tularensis* subsp. *holarctica*. Erythromycin resistance was observed in the isolate  
269 and was therefore assigned to biovar II (Tomaso et al., 2005). This finding indicates  
270 that the possible geographic origin of the isolate is Eastern Europe or that the  
271 Western border of this well-known region of endemicity is located in the German  
272 mountain range.

273 This case of tularemia in a hare is the first report on the presence of this zoonosis in  
274 Thuringia, Germany. The increased epizootic activity of *Francisella tularensis* in the  
275 endemic region of Central Europe shows that tularemia has to be considered an  
276 emerging disease for animals and humans too. The influence of Greenhouse effect  
277 and the obviously changing climate in Central Europe might have an crucial impact  
278 on the future spread of the disease and the stable establishing of permanent  
279 endemic areas. Thus a higher risk for wildlife, pet animals and humans has to be  
280 expected. Therefore, the aim of further investigations will be the characterisation of  
281 the epidemiology of tularemia in whole Europe.

282

### 283 **Acknowledgements**

284

285 We thank the staff of the collaborating laboratories for their support. We wish to thank  
286 Prof. Ian N. Clarke, Southampton, for reviewing the English language of the  
287 manuscript.

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291

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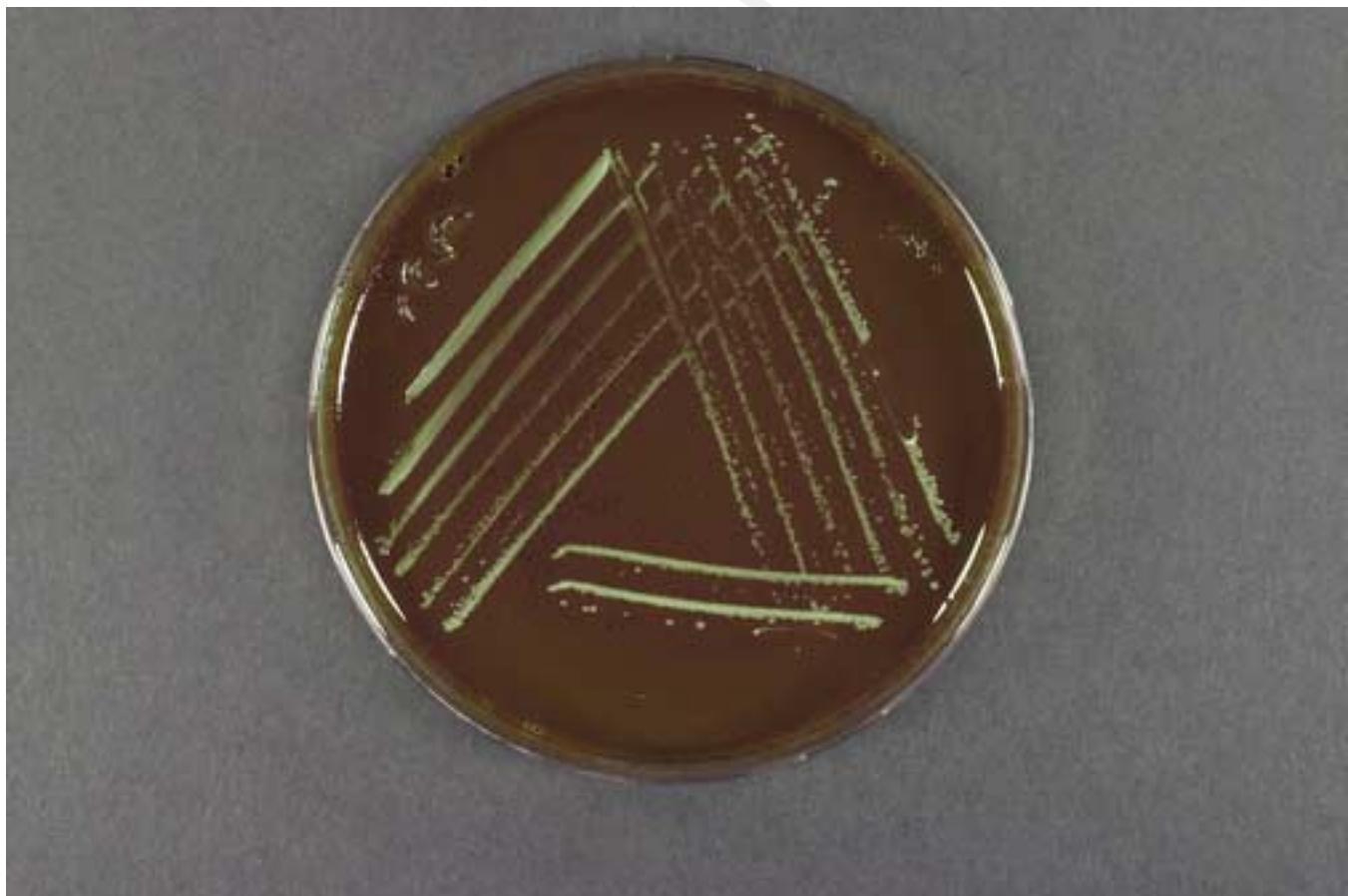
343 Table1: Sequences of primers

344

Primer	Sequence (5'-3')	Fragment size	Reference
16S UNI-L	AGA GTT TGA TCA TGG CTC AG	1,200 bp	Kuhnert 1996
16S UNI-R	GTG TGA CGG GCG GTG TGT AC		
TUL4-435	GCT GTA TCA TCA TTT AAT AAA CTG CTG	400 bp	Sjöstedt 1997
TUL4-863	TTG GGA AGC TTG TAT CAT GGC ACT		
FtC1	TCC GGT TGG ATA GGT GTT GGA TT	300 bp/330 bp	Johansson 2000
FtC4	GCG CGG ATA ATT TAA ATT TCT CAT A		

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