

Hare-to-Human Transmission of *Francisella tularensis* subsp. *holarctica*, Germany

Peter Otto, Rebekka Kohlmann, Wolfgang Müller, Sandra Julich, Gabriele Geis, Sören G. Gatermann, Martin Peters, Peter Johannes Wolf, Edvin Karlsson, Mats Forsman, Kerstin Myrtenäs, and Herbert Tomaso

In November 2012, a group of 7 persons who participated in a hare hunt in North Rhine-Westphalia, Germany, acquired tularemia. Two *F. tularensis* subsp. *holarctica* isolates were cultivated from human and hare biopsy material. Both isolates belonged to the FTN002–00 genetic subclade (derived for single nucleotide polymorphisms B.10 and B.18), thus indicating likely hare-to-human transmission.

Tularemia is a zoonotic disease caused by the gram-negative bacterium *Francisella tularensis* (1). Currently, there are 4 validly published subspecies. *F. tularensis* subsp. *tularensis* is the most virulent subspecies and occurs only in North America. *F. tularensis* subsp. *holarctica* is less virulent and occurs throughout the Northern hemisphere. *F. tularensis* subsp. *mediasiatica* was isolated in central Asia, and *F. tularensis* subsp. *novicida*, which has low virulence in humans, seems to be distributed globally (2).

Various PCR-based assays have been established for the detection of *F. tularensis* or for the diagnosis of tularemia. An accurate population structure has been defined by using single nucleotide polymorphisms (SNPs) and insertion/deletion mutations (INDELs) with potential canonical properties. Currently, this population is divided into 4 major genetic clades: B.4, B.6, B.12, and B.16 (3–6). The taxonomic nomenclature of major clades in *F. tularensis* subsp. *holarctica* is based on clade-specific canonical SNP markers (3,4). In Europe, the strains of clades B.12 and B.6 dominate (6). The latter is found particularly in large areas in northern, western, and central Europe, including Germany (5–9).

Author affiliations: Friedrich-Loeffler-Institut Federal Research Institute for Animal Health, Jena, Germany (P. Otto, W. Müller, S. Julich, H. Tomaso); Institute of Medical Laboratory Diagnostics Bochum, Bochum, Germany (R. Kohlmann, G. Geis); Ruhr-University, Bochum (S.G. Gatermann); National Veterinary Laboratory Arnsberg, Arnsberg, Germany (M. Peters); Evangelisches Krankenhaus, Lippstadt, Germany (P.J. Wolf); and Swedish Defence Research Agency, Umeå, Sweden (E. Karlsson, M. Forsman, K. Myrtenäs)

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The Study

On November 2, 2012, 15 European brown hares (*Lepus europaeus*) were shot during a hunt in Rütthen-Meiste, district Soest in the federal state of North Rhine-Westphalia, Germany (Figure). The animals seemed healthy and showed normal escape behavior. Upon inspection, the animals that had been shot showed no signs of disease. Consequently, all animals were skinned, eviscerated, and dissected. Portioning of the hares was done 2 days later. Within a few days, 7 healthy persons who had contact with the hare carcasses showed varied symptoms of illness. Tularemia was suspected because of the signs and symptoms in combination with exposure in a tularemia-endemic area. Exposure, clinical symptoms, and time of onset of symptoms of all patients (A to G) are described in the Table. All patients were treated successfully with doxycycline.

Human serum samples were collected about 5 weeks after infection from patients C and E and pleural fluid was obtained from patient E. On day 2 of incubation, the human isolate (12T0062) showed small pale-white to gray colonies on Columbia blood agar and chocolate agar, whereas no growth occurred on MacConkey agar plates. Ten organ specimens (from aorta, back and thigh muscles, lymph nodes, spinal cord) from 4 of the hares handled by the patients were tested for *Francisella* spp. in the National Reference Laboratory for Tularemia at the Friedrich-Loeffler-Institut in Jena, Germany. From all hare organs, only a single *Francisella* sp. was isolated from a spinal cord sample (13T0009) on cysteine heart agar Becton Dickinson GmbH, Heidelberg, Germany), which contains antibiotics. The strains were susceptible to erythromycin with inhibition zones between 22 and 24 mm corresponding to biovar I. Details of further methods that have been applied in the study are shown in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/1/13-1837-Techapp1.pdf>).

Both samples were identified as *F. tularensis* subsp. *holarctica* of clade B.6. For B.19, the SNP was C; results of Ftind33 and Ftind38 assays were IN, and the result of the Ftind49 assay was DEL. Both samples had T for SNP B.7, G for B.10 and T for B.18. Therefore, the strains were considered derived from SNPs B.10 and B.18. Blood serum samples of patients C and E were positive for *F. tularensis* with very high values of the optical density in the ELISA, 2.886 and 3.121, respectively.



Figure. Area of Germany where hares were hunted on November 2, 2012: Rügen-Meiste (black star; latitude 51.512890, longitude 8.487493, altitude 380 m), Soest district (white) of the federal state of North Rhine-Westphalia (dark gray).

Conclusions

The re-emergence of tularemia in Germany has been described in previous studies (10). Infected hares are believed to be the sources of most cases of tularemia in Germany. However, to our knowledge, route of transmission has not been demonstrated by isolation and genotyping of the pathogen from the suspected source and the patient (11–13). In this study, we therefore described not only the clinical and epidemiologic data and the laboratory diagnostic findings for determining tularemia, but also the results of genotyping the *Francisella* spp. isolated from epidemiologically linked hares and humans.

All 7 infected persons (A to G) in this outbreak showed influenza-like symptoms of varying intensity (Table), but symptoms were also related to the route of infection. The 5 patients (A to E) who had fever as well as respiratory and topical symptoms were exposed to aerosols and had direct skin contact when skinning and processing hare carcasses. The 2 patients (F and G) who portioned the meat had lesions on their hands, enlarged lymph nodes, and fever.

The isolated *F. tularensis* subsp. *holarctica* strains were susceptible to erythromycin and thus belong to the *F. tularensis* subsp. *holarctica* biovar I group. Because of the inability of the duplex PCR assay to distinguish between *F. tularensis* subsp. *holarctica* strains (8), we performed a combined SNP and INDEL analysis using real-time PCR. Here, we were able to isolate *F. tularensis* subsp. *holarctica* biovar I strains from a hare and a human; both isolates could be assigned to the genetic clade B.6 in the first order of discrimination [B.19(C), Ftind33(IN), Ftind38(IN), and Ftind49(DEL)]. The isolates also showed an identical genotyping profile for B.7(T), B.10(G), B.18(T) in the second order of discrimination, which corresponds with a previously described subclade represented by the strain FTNF002–00 that was isolated from a patient from France who had bacteremia (3,4). Thus, the genetic subtyping results are consistent with the proposed transmission route of the epidemiologically linked (hare–human transmission) *F. tularensis* subsp., since both belonged to the same genetic subclade.

The current phylogeography of *F. tularensis* subsp. *holarctica* revealed that 2 major groups of virulent strains exist in Europe (5). In the western European countries of Spain, France, Switzerland, and Italy, strains of the FTNF002–00 group dominate, whereas strains of clade B.12 seem to predominate in eastern and northern Europe as reported from Austria, Czech Republic, Finland, Georgia, Hungary, Romania, Russia, Slovakia, Sweden, and Ukraine (4,5,8,13). Vogler et al. (4) suggest that it is likely that the spread of strains in subclade FTNF002–00 throughout France and the Iberian Peninsula was a very recent event. In Germany, isolates of both groups have been identified and a sharp dividing line in terms of occurrence of the clades B.12 and B.6 from the northwest to the southeast of the country has been shown (8). The reasons for this are not known; possible causes could be environmental and epidemiologic differences. Alternately, a mixture of both genetic clades and biovars have been reported in Bulgaria, Kazakhstan, Norway, Russia, and Sweden (7,14,15).

The genome of *F. tularensis* subsp. *holarctica* is highly conserved and strains can hardly be discriminated. Therefore, the discriminatory power of the applied assays is limited and other field isolates from this area may show identical characteristics (H. Tomaso, unpub. data). For epidemiologic and forensic purposes, whole-genome sequencing of a multitude of strains from well-documented outbreaks and the surrounding areas should be performed to clarify and possibly quantify the genetic changes that can finally confirm or rule out the route of transmission.

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Table. Exposure, clinical presentation, and time of onset of clinical symptoms of tularemia in patients, Germany, 2012

Patient	Exposure type	Clinical symptoms	Symptom onset
A	Skinning	Fever, cold, cough	Nov 12
B	Skinning	Chills, fever, joint pain	Nov
C	Dissection	Fever, nodes and skin ulcers	Nov
D	Cut up carcasses	Fever, cold, cough, joint pain, convulsions	Nov
E	Cut up carcasses	Cough, pleural effusion, weight loss	Unknown
F	Portioning of hares	Fever, skin ulcers, lymphadenopathy	Unknown
G	Portioning of hares	Fever, skin ulcers, weight loss	Unknown

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Dr. Otto is the head of the working group, Noro- and Rotaviruses, and an employee of the National Reference Laboratory of Tularemia at the Federal Institute of Bacterial Infections and Zoonoses of the Federal Research Institute of Animal Health, Friedrich Loeffler Institut, Jena, Germany. His interests are focused on the development of diagnostic methods, the incidence and epidemiology of *Francisella* spp., and other bacterial zoonotic agents in wild animals.

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Address for correspondence: Herbert Tomaso, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Naumburger Str. 96A, 07743 Jena, Germany; email: herbert.tomaso@fli.bund.de



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Material and Methods

Cultivation

The organ samples were streaked on modified Martin-Lewis-agar (BD Biosciences, Heidelberg, Germany) and cysteine heart agar (CHA; Becton Dickinson GmbH, Heidelberg, Germany) that was supplemented with 10% chocolatised sheep blood and was prepared with and without antibiotics. The selective CHA contained 100 mg ampicillin (Sigma-Aldrich Chemie, Taufkirchen, Germany) and 600,000 U polymyxin B (Sigma-Aldrich Chemie) per one liter of culture medium. All plates were incubated at 37°C with 5% CO₂ for up to 8 days.

Erythromycin Susceptibility

The isolates were tested for their erythromycin susceptibility by using erythromycin discs (30 µg; Oxoid, Wesel, Germany) according to the manufacturer's instructions to discriminate the susceptible *F. tularensis* subsp. *holarctica* biovar I from the resistant biovar II.

DNA Extraction

50 mg of organ material were lysed and the DNA was extracted by using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Colonies were suspended in 200 µL phosphate-buffered saline, boiled at 95°C for 10 minutes and DNA was prepared as described above. Finally, DNA was eluted in 200 µL elution buffer. Five µL were applied in duplex PCR assay.

Duplex PCR Assay

A duplex PCR targeting the locus FtM-19 that distinguishes the two major subspecies *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* based on the 30 bp-deletion was carried out as described by Johansson et al. (1).

Single Nucleotide Polymorphism and Insertion Deletion Mutants Discrimination Assay

Four loci (B.19, Ftind33, Ftind38 and Ftind49) described by Svensson et al. (2) have been found to be useful for identification of the genetic clades B.4, B.6 and B.12 of *Francisella tularensis* subsp. *holarctica*. Further single nucleotide polymorphisms (SNPs), B.7, B.10, (3) and B.18 (2) were selected for discrimination of clade B.6 into subclades. SNP and INDEL determination was carried out in duplicates by using real-time PCR assays with SYBR Green as described in Svensson *et al.* (2), with a few modifications. The reaction mixture consisted of 10 μ L 2x LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany), 0.8 μ L of each 10 μ M primer (TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany), 2 μ L sample DNA adjusted to 500 pg/ μ L, and deionized water to a total volume of 20 μ L. Each sample was amplified by using a mastermix containing either the derived or the ancestral primer as forward primers and the common reverse primer. Temperature time profile: 2 min at 50°C, 10 min at 95°C, 41 cycles [15 s at 95°C, 1 min at 60°C], 1 min at 95°C, 1 min at 20°C, then continuous heating to 95°C for melting curve analysis (heating rate: 0.11°C/s). Primer sequences are listed in the Technical Appendix Table.

The SNP status was determined by inspecting the amplification curves. Amplification appeared earlier in reaction mixtures containing the forward primers with a matching base. A positive result was assigned for the ancestral or derived assay with the lower Cq value, when there was a minimum difference of $\Delta Cq \geq 2$.

In case of a deletion (DEL), the Cq values belonging to the OUT primer were lower than for the IN primer. In case of an insertion (IN), both Cq values were almost similar with a $\Delta Cq < 2$.

Enzyme-linked Immunosorbent Assay

The ELISA for detection of *Francisella* antibodies was performed as published by Porsch-Ozcürümez et al. (4), but with some modifications (5).

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Technical Appendix Table: Canonical SNP and insertion/deletion mutations were detected by using a common reverse primer and primers specific for the ancestral or derived state (2,3)

Marker	SNP state	Primer	Sequence 5'→3'
B.19	A	Derived	gcgggcTTGCTACTGATGGTTAACTa
	C	Ancestral Common	gcgggcagggcggcTTGCTACTGATGGTTAACTc CAATACGTCACTTATGCAGTGAT
Ftind33		IN	TCTAAATTTAAGCAATGTTTCTAACT
		OUT	ATCATCGTATAAGAAATCAACTT
		CP	TCAACCTTACAGAATAAGAATGT
Ftind38		IN	ACCCAATAAGCTCACCATCA
		OUT	ATCTTCTCAGGTACAGACTTTA
		CP	AGTACTATTTGCTTATCCAAGTGAA
Ftind49		IN	AAGATTAAGTGGCAATTTAC
		OUT	TTCAACCTGGACAACCACTA
		CP	AGGATCCCAGTTAGGTTTAGTA
B.7	C	Derived	cggggcggggcggggcggggGTTTTGCAGCTAATAATTTTCATTGgC
	T	Ancestral Common	ttttttttttttttttTGGTTTTGCAGCTAATAATTTTCATTGgT GTCCTTGTTAGTCAAAGCGCTATAA
B.10	G	Derived	cggggcggggcggggcggggGCCATCACTAGTAAATACCACATTAAaC
	A	Ancestral Common	ttttttttttttttttTGCCATCACTAGTAAATACCACATTAAgT TTGTAATATTAGCTMGAAAAGTAGATGAA
B.18	T	Derived	gcgggcAGCAGCAGGACAAATAGt
	C	Ancestral Common	gcgggcagggcggcAGCAGCAGGACAAATAGc TTGTGTCGATTCAAACCAGACTTA