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Original article

Five cases of vector-borne *Francisella tularensis holarctica* infections in south-western Germany and genetic diversity

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ABSTRACT

Tularemia is a rare zoonotic disease in Germany. *Francisella tularensis* has been isolated previously from ticks in southern Germany underscoring the importance of ticks (*Ixodes ricinus*) in tularemia transmission, but there have been only few reports from this region with single cases or small case series of tick-borne transmissions of tularemia. We report five cases of non-game animal associated tularemia diagnosed from 2010 to 2016 in southwestern Germany – Baden-Wuerttemberg. Our case series and molecular typing (MLVA) results add published clinical experience to this underdiagnosed disease and consolidate previous findings regarding tick-borne transmission of tularemia and phylogenetic diversity in Germany.

1. Introduction

Tularemia is a rare zoonotic disease in Germany and is increasingly reported from southwest Germany (Boone et al., 2015; Grunow, 2007). The causative agent *Francisella tularensis* is a Gram-negative, coccoid, facultative intracellular, fastidious bacterium. National surveillance data (SurvStat RKI 2.0, data as of 2016-12-01) report 243 human tularemia cases between 2001 and 2016, with ~16 cases per year on average. In total, 72 cases were diagnosed in this period in the south-western federal State of Baden-Wuerttemberg (see Fig. 1). In the northern hemisphere only *F. tularensis* subspecies *holarctica* (type B strains) is present, including Europe. It is associated with milder clinical symptoms than *F. tularensis* subspecies *tularensis* (type A strains), which is mainly isolated in North America and is related to severe clinical patterns (Kohlmann et al., 2014; Maurin and Gyuranecz, 2016). *F. tularensis* subspecies *holarctica* are categorized into three different biovars. Biovar I (erythromycin sensitive) has been isolated in western Europe, whereas biovar II (erythromycin resistant) has been found in eastern European regions. Biovar japonica (ferment glycerol) has been mainly reported from far east Asia (Maurin and Gyuranecz, 2016). Hierarchical genotyping, identification and differentiation could be done using a variety of molecular techniques such as canSNP typing, which is based on single nucleotide polymorphisms (SNPs) and insertion-deletion mutations (INDELS) (Svensson et al., 2009), Multi Locus Sequence Typing (MLST) and Multiple-Locus Variable tandem repeat Analysis (MLVA) (Vogler et al., 2009).

The clinical symptoms are variable and depend on the portal of entry (Maurin and Gyuranecz, 2016). The most frequent manifestation is ulcero-glandular tularemia with prominent lymphadenopathy, which is caused by direct skin contact or percutaneous inoculation of the pathogen via vectors. *F. tularensis* associated pneumonia is the result of inhalating contaminated aerosols (Kohlmann et al., 2014) and is linked to a high mortality rate of about 30–60% without anti-infective therapy. Oropharyngeal tularemia or gastrointestinal tularemia, presenting with pharyngitis or a typhus-like clinical picture, is caused ingesting of infected meat or contaminated water. Many animal species, including arthropods, birds, rodents, lagomorphs, carnivores and ruminants, can carry *Francisella*, but a definitive reservoir has not been identified yet (Maurin and Gyuranecz, 2016). The typical route of transmission is direct skin or mucosal contact with infected meat from game animals – especially meat and skin from hares, roe deer and boars. In this context, there were several outbreaks linked to hunting and processing of game animal meat (Otto et al., 2015). Systematic surveillance data from France (2002–2012, n = 380) indicate the importance of arthropod transmission – in particular tick-borne tularemia (Mailles and Vaillant, 2014). Approximately half of the reported human cases (179/380) were directly associated to hare handling, whereas ~18% (70/380) were linked to confirmed tick bites. In southern Germany there are anecdotal reports and small case series on tick-borne transmissions of tularemia (Boone et al., 2015). However, this possibly

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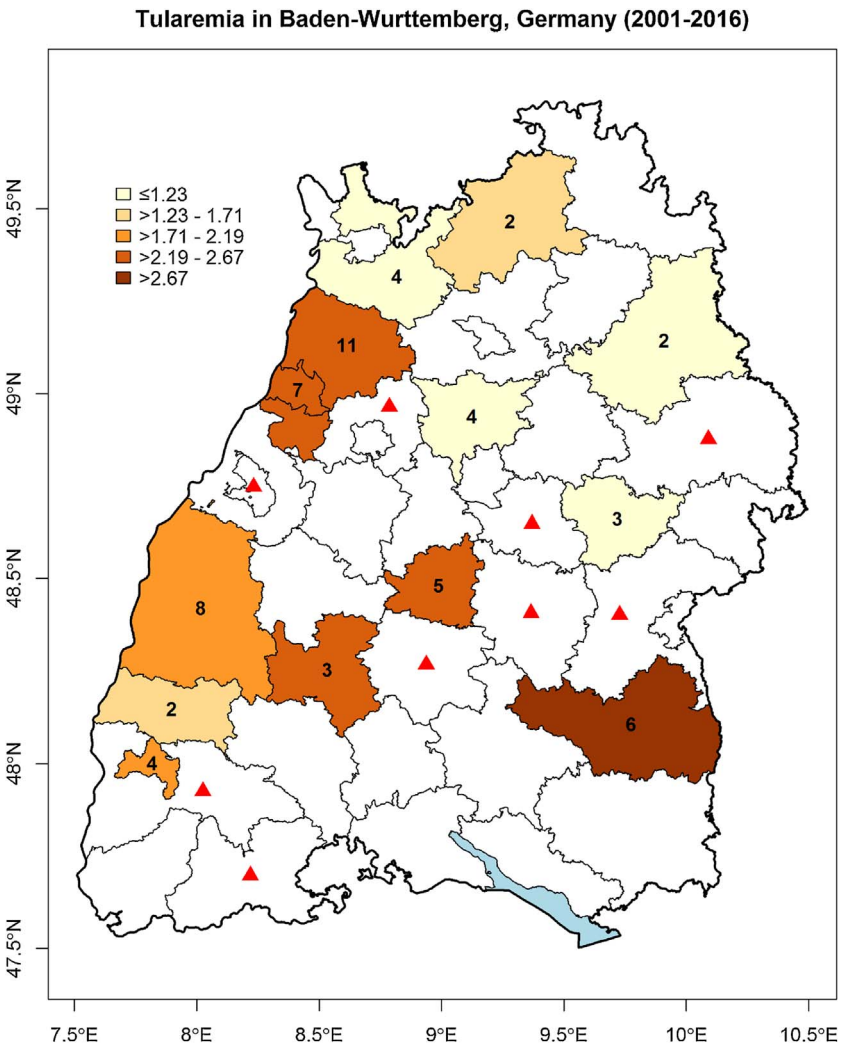

Fig 1. Reported cases of tularemia in Baden-Wuerttemberg, Germany. Cumulative case numbers from 2001 to 2016 per county are displayed. Counties with only one single case during the whole period are marked with a red triangle; otherwise cumulative incidences per 100,000 inhabitants are calculated and color-coded. In total, 70 cases occurred in 22 out of 44 counties. Source of data: Federal Statistical Office, GENESIS-Online database (data as of 2016-02-07). Robert Koch-Institute: SurvStat@RKI 2.0, https://survstat.rki.de (data as of 2016-12-01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

emerging mode of infection seems to be underreported. *F. tularensis* has been isolated from ticks in southwestern Germany underscoring the importance of ticks (*Ixodes ricinus*) in tularemia transmission (Gehringer et al., 2013). Overall, there are little data available regarding the presence of *F. tularensis* in questing ticks. However, one single location study from Thuringia (Germany) indicated, that 1,6% of the collected questing ticks were infected with *F. tularensis* (Franke et al., 2010). We report five cases of tularemia diagnosed from 2010 to 2016 in southwestern Germany – Baden-Wuerttemberg. Our case series and molecular typing results add published clinical experience to this underdiagnosed disease and consolidate previous findings regarding tick-borne transmission of tularemia and phylogenetic diversity in Germany.

2. Material and methods

2.1. Patients

Patients were diagnosed at a tertiary medical care center and in primary care facilities in the south-western regions of the federal state of Baden-Wuerttemberg. The medical history was obtained from medical records. These patients had their residence and place of employment in the corresponding counties and reported no journeys in the context to the diagnosis (except patient D), so that the infection in the federal state of Baden-Wuerttemberg could be plausible (see Supplemental Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.ttbdis.2017.06.009).

2.2. MLVA12

Multiple-Loci Variable number tandem repeat Analysis (MLVA) was applied to samples using 12 markers according to Vogler (Vogler et al., 2009), amended by one additional marker Ft-M26 introduced by Svensson (Svensson et al., 2009) which is a suitable marker in order to increase the resolution especially for European isolates of *F. tularensis* ssp. *holartica*. For fragment length determination, a 4-capillary Applied Biosystems 3130 Genetic Analyzer (Life Technologies) with GeneScan™ 1200 LIZ® Size Standard was used. The obtained fragment lengths were calibrated to *F. tularensis* ssp. *holarctica* LVS and *F. tularensis* ssp. *tularensis* SchuS4 in silico data.

2.3. Francisella tularensis diagnostics

Serological testing was performed for all patients except patient D at the Bundeswehr Institute of Microbiology using a polyvalent screening test (Serazym® Anti-Francisella tularensis, Seramun Diagnostica GmbH, Germany) and an IgG-specific *F. tularensis* LPS Immunoblot (Serablot® Anti-Francisella tularensis, Seramun Diagnostica GmbH, Germany) combined with IgM and IgG specific *F. tularensis* ELISA (SERION ELISA classic *F. tularensis* IgG/IgM, Virion\Serion GmbH, Germany). Molecular diagnostics were performed for patient A, patient B, patient C and patient E at the Bundeswehr Institute of Microbiology. DNA from clinical specimens was extracted using QIAamp DNA mini Kit (Qiagen, Germany) and for paraffin embedded specimens using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany), respectively. Real-time-PCR for