Characterization and genotyping of strains of *Francisella tularensis* isolated in Bulgaria

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A tularaemia focus was detected in 1998 in Bulgaria, in an area where tularaemia had never been reported. The properties of *Francisella tularensis* subsp. *holarctica* strains isolated from 1998 to 2005 were studied. The strains showed heterogeneity, based on acid production from glycerol and erythromycin susceptibility. Genotyping by analysis of seven loci containing variable-number tandem repeats showed four genotypes among eight strains.

INTRODUCTION

Tularaemia is a zoonotic infection. In humans, the disease is usually severe and occasionally fatal. The causative agent, *Francisella tularensis*, is a Gram-negative, aerobic bacterium. Currently, there are four recognized subspecies (*tularensis*, *holarctica*, *mediasiatica* and '*novicida*') (Sjöstedt, 2005). Most cases of tularaemia are caused by *F. tularensis* subsp. *tularensis* or subsp. *holarctica*. *F. tularensis* subsp. *tularensis* is distinctly more virulent than the other subspecies. In Europe, only *F. tularensis* subsp. *holarctica* has been isolated. There is a single report of isolation of *F. tularensis* subsp. *tularensis* from rodents in Slovakia (Gurycova, 1998). Tularaemia in Europe does not generally cause human mortality.

Genetic and phenotypic heterogeneity among the species is limited. *F. tularensis* subsp. *holarctica* can be distinguished from *F. tularensis* subsp. *tularensis* on the basis of its inability to produce acid from glycerol, the absence of citrulline ureidase activity and its moderate virulence in rabbits (Olsufjev & Meshcheryakova, 1982; Sjöstedt, 2005). In contrast, *F. tularensis* subsp. *tularensis* strains produce acid from glycerol, show citrulline ureidase activity and are highly virulent for rabbits. *F. tularensis* is susceptible to quinolones, aminoglycosides and tetracyclines. Differentiation of erythromycin-susceptible and erythromycin-resistant *F. tularensis* subsp. *holarctica* strains has been described and is associated with geographical distribution (Olsufjev & Meshcheryakova, 1982).

Several tularaemia epidemics have been reported in Europe over the last 10 years (Anda *et al.*, 2001; Eliasson *et al.*, 2002; Reintjes *et al.*, 2002; Celebi *et al.*, 2006). There was a tularaemia focus in northern Bulgaria during the 1960s (Dinev & Zlatanov, 1972). No more cases were reported

until 1997, when an outbreak occurred in 1997–1998 in western Bulgaria and a new focus was detected. From October 1997 to April 2007, 298 cases of tularaemia were registered. More than 95% of the patients had the oropharyngeal form of the disease (Kantardjiev *et al.*, 2006).

The purpose of this study was to characterize *F. tularensis* strains isolated in a new focus of tularaemia, in an area where this infection has not been reported previously.

METHODS

Strains and growth conditions. Eight Bulgarian *F. tularensis* strains isolated from 1998 to 2005 were used in this study (Table 1). With the exception of L2, all of the strains were isolated from three villages (Slivnitsa, Breznik and Meshtitsa) or their vicinity. The distance between each was no more than 25 km. More than 100 tularaemia cases were registered in this area. Strain L2 was isolated in northern Bulgaria, in an area about 250 km away. The isolates were investigated in comparison with nine *F. tularensis* strains of different geographical origins (Table 2). The control strains were supplied by the Bulgarian Type Culture Collection. All strains investigated were identified by immunofluorescence assay and by PCR using primers TUL4-435 and TUL4-863 (Sjöstedt *et al.*, 1997). Bacteria were grown on glucose–cysteine blood agar (GCBA), containing 40 g blood agar base (Merck) l⁻¹, 500 mg L-cysteine.HCl l⁻¹, 5% human blood and 1% glucose at 37 °C.

Cultural and biochemical characterization. The requirement for cysteine for growth was tested on GCBA and on the same medium without cysteine.

The test for oxidase activity was performed using Kovacs' reagent. The production of H₂S was estimated on GCBA with 1.8 g cysteine l⁻¹ using lead acetate strips and on triple-sugar iron agar.

The production of acid from carbohydrates was estimated on solid bloodless medium, containing 20 g blood agar base l⁻¹, 1.8 g L-cysteine.HCl l⁻¹, 0.9 g DL-histidine l⁻¹, 0.2 g haemin l⁻¹, 0.01 g thiamine l⁻¹, 0.45 g FeSO₄·7H₂O l⁻¹, 0.18 g KCl l⁻¹, 0.12 g bromothymol blue l⁻¹ and 2% glucose, 2% glycerol or 2% sucrose.

Abbreviations: MLVA, multilocus variable-number tandem repeat analysis; VNTR, variable-number tandem repeat.

Table 1. Characteristics of *F. tularensis* subsp. *holarctica* strains isolated in a new focus of tularaemia in Bulgaria

Source, year of isolation	Strain	Acid production from glycerol	ES*
Hare, 1998	L1	No	R
Tick, 1998	Dm1	No	R
Tick, 1998	Dm2	No	R
Tick, 1999	B1	Yes	S
Tick, 1999	B2	Yes	S
Common vole, 2003	MMM	No	R
Human, 2003	MN	No	R
Hare, 2005	L2	Yes	R

*ES, Erythromycin susceptibility: S, MIC ≤ 2 mg l⁻¹; R, MIC > 256 mg l⁻¹.

The pH of the medium was adjusted to ~ 7.4 . This medium is a modification of a liquid medium described previously (Sandström *et al.*, 1992). Additionally, growth on cysteine blood agar was estimated in comparison with growth on cysteine blood agar supplemented with 2 % glycerol or 2 % glucose. All tests were repeated at least five times for all strains, in separate experiments.

Citrulline ureidase activity was estimated on the basis of conversion of citrulline to ornithine.

Bacterial suspensions ($\sim 10^{10}$ bacteria ml⁻¹) in PBS (pH 6.5) containing 21 mM Na₂HPO₄ and 46 mM KH₂PO₄ were used. A 500 μ l sample of the suspension was mixed with 500 μ l 50 mM L-citrulline and incubated for 22 h at 30 °C. The mixtures were centrifuged and the supernatant was removed and sterilized by heating for 20 min at 100 °C. L-Citrulline and ornithine in the supernatant were separated by paper electrophoresis. We used electrophoresis for 1 h at 15 V cm⁻¹ in phosphate buffer (pH 6.0) containing 8 mM Na₂HPO₄ and 59 mM KH₂PO₄. Whatman chromatography paper 4 was used and 5 μ l spots of samples were applied. Controls consisted of standard solutions of L-citrulline and L-ornithine, and supernatants of bacterial suspensions incubated without L-citrulline were included. Following drying, the amino acids bands were located by spraying with a ninhydrin solution (0.1 %, w/v, in 95 % ethanol) and heating at 80 °C for 10 min. A sample was considered to be positive when a clearly visible band of ornithine appeared.

Table 2. *F. tularensis* control strains used in this study

Strain	Origin
<i>F. tularensis</i> subsp. <i>holarctica</i>	
Sweden	Sweden
Norway	Norway, 1953, tick
Chateauroux	France, 1952, hare
Gaiski-15	Russia
597-73	China, 1957
KF-479	USA, 1958
<i>F. tularensis</i> subsp. <i>tularensis</i>	
Schu	USA, 1949, human
8859	USA, 1958, horse
O-284	USA, 1959

Virulence determination. The virulence of each of the Bulgarian isolates was tested in ten white mice. Five mice were subcutaneously inguinally inoculated with suspensions in saline containing 10–20 cells, and five mice were inoculated with 100–200 cells. To investigate virulence in rabbits, groups of three animals were inoculated with $\sim 10^5$ or $\sim 10^6$ bacteria. Three *F. tularensis* subsp. *tularensis* control strains were inoculated with $\sim 10^2$ bacteria. The cell number was determined by colony counts on GCBA. All animals were observed for 20 days.

Spleen tissue samples from animals that did not survive the infection were examined by an immunofluorescence assay. Infection in the surviving rabbits was confirmed by a serological assay.

Molecular subspecies identification. Subspecies identification by PCR was carried out by amplification of regions of differences as described previously (Broekhuijsen *et al.*, 2003). This method allows differentiation of the four subspecies of *F. tularensis*.

Antibiotic susceptibility testing. *In vitro* susceptibility to ciprofloxacin, gentamicin and erythromycin was determined using Etests (AB Biodisk) on GCBA plates. The inoculum was adjusted to the density of a McFarland 0.5 turbidity standard and resulted in confluent growth. MICs were estimated after incubation for 48 h at 37 °C.

Genotyping. Strain genotyping was performed by analysis of seven loci containing variable-number tandem repeats (VNTRs) (Johansson *et al.*, 2004). Previously, it has been shown that six of these VNTR markers are discriminative for *F. tularensis* subsp. *holarctica* (Byström *et al.*, 2005). Multilocus VNTR analysis (MLVA) was performed as described previously (Gürçan *et al.*, 2008).

RESULTS AND DISCUSSION

Characteristics and subspecies identification of the isolates

All strains included in this study showed a requirement for cysteine for growth, the absence of oxidase activity, production of H₂S on GCBA and the absence of H₂S production on triple-sugar iron agar. Five of the isolates were negative for acid production from glycerol, as were all of the *F. tularensis* subsp. *holarctica* control strains used. Three isolates showed acid production from glycerol (Table 1) similar to the *F. tularensis* subsp. *tularensis* control strains used. All of the glycerol-positive strains grew on the bloodless medium with glycerol and acidified the medium, although growth was not abundant. On cysteine blood agar without glycerol, these strains formed whitish colonies, without a change in the medium. On cysteine blood agar with glycerol, the colonies were grey, distinctly larger and surrounded by a greenish discoloration of the medium. The more abundant growth and the change in the medium were probably a result of degradation of glycerol and acid production, respectively. The growth of the strains negative for acid production from glycerol was not affected by the addition of glycerol. The reproducibility of the tests was good. All strains investigated showed acid production from glucose, estimated by the same methods. No strain produced acid from sucrose. Citrulline ureidase activity

was found only in the *F. tularensis* subsp. *tularensis* control strains.

All Bulgarian isolates were highly virulent for white mice and moderately virulent for rabbits. The lethal dose for mice was ≤ 20 bacteria for seven strains and $< 10^2$ bacteria for one strain. The rabbits survived infection with $\sim 10^5$ bacteria, but infection with $\sim 10^6$ cells was lethal. In contrast, the virulence of the *F. tularensis* subsp. *tularensis* control strains for rabbits was $< 10^2$ cells. In all cases of lethal infection, the animals survived for no longer than 10 days.

Subspecies identification by PCR showed amplification of characteristic fragments of ~ 0.9 kb (Broekhuijsen *et al.*, 2003) in the Bulgarian strains and the *F. tularensis* subsp. *holarctica* control strains. Fragments of ~ 1.5 kb were amplified in the *F. tularensis* subsp. *tularensis* strains.

The characteristics of the strains isolated showed that they belong to *F. tularensis* subsp. *holarctica*. Three strains produced acid from glycerol. This is an unusual finding and, to our knowledge, is the first report of such a phenotype in European *F. tularensis* subsp. *holarctica* strains. The ability to produce acid from glycerol in some of the Bulgarian *F. tularensis* subsp. *holarctica* strains could be explained by the possible recovery of certain metabolic pathways due to reverse mutations. An alternative explanation might be that the Bulgarian *F. tularensis* subsp. *holarctica* subpopulation had undergone divergent evolution and the metabolic pathways remained intact. In this

case, the ability to produce acid from glycerol may represent the ancestral state, as in *F. tularensis* subsp. *tularensis*. Thus, at least in the Balkan Peninsula, there is the possibility that some *F. tularensis* subsp. *holarctica* strains may be misidentified as the highly virulent *F. tularensis* subsp. *tularensis* on the basis of acid production from glycerol.

Antibiotic susceptibility

For our isolates, the MICs of ciprofloxacin ranged from 0.023 to 0.064 mg l⁻¹. The MICs of gentamicin ranged from 0.023 to 0.250 mg l⁻¹. For the control strains, the MICs of ciprofloxacin and gentamicin ranged from 0.004 to 0.047 mg l⁻¹ and from 0.016 to 0.125 mg l⁻¹, respectively. The MICs found were similar to those reported previously in Europe (Ikäheimo *et al.*, 2000). The MICs of erythromycin for the *F. tularensis* subsp. *holarctica* control strains were > 256 or ≤ 6 mg l⁻¹. The MICs for six of the Bulgarian isolates were > 256 mg l⁻¹ and for two of the isolates were ≤ 2 mg l⁻¹ (Table 1).

MLVA

MLVA showed four genotypes represented among the eight strains (Fig. 1). Three genotypes were found among the five strains isolated in 1998 and 1999. Two strains isolated in 2003 did not show additional genotypes. However, a strain isolated in 2005 in an area 250 km away presented with a

Fig. 1. Dendrogram based on cluster analysis of seven VNTR (MLVA) loci (M3, M6, M10, M20, M21, M22 and M24). The size obtained for each locus is given in bp.

different genotype (L2; Fig. 1). Three alleles were found in the highly variable M3 VNTR locus, three alleles were found in the M6 locus and two alleles in the M20 locus (Fig. 1). Some strains isolated in a common area (L1, Dm1 and Dm2) in 1998 differed in their alleles for these three loci. The Bulgarian strains represented a heterogeneous group and some were more closely related to other European strains. The results showed high genetic diversity among these strains distributed in a new disease focus. This is comparable to the genetic diversity of strains isolated in tularaemia-endemic areas and was unexpected in a new focus, where a common parental strain is more likely (Farlow *et al.*, 2001).

The erythromycin-susceptible strains that were positive for acid production from glycerol were identical. These strains and erythromycin-resistant strains that were positive for acid production from glycerol appeared to be closely related, but showed distinct MLVA genotypes.

Investigation of the strains isolated in the outbreak focus showed high phenotypic and genotypic heterogeneity. We suggest that strains with different properties were distributed simultaneously in the area of the focus, probably because of ecological changes in Eastern Europe and the spread of *F. tularensis* vectors. Another possible explanation is a long-existing epizooty or preservation of the causative agent in other ecological niches, prior to the epidemic.

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