

ORIGINAL ARTICLE

## In vitro susceptibility of isolates of *Francisella tularensis* from Turkey

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### Abstract

**Background:** Tularemia is an infection caused by *Francisella tularensis*, which has a wide distribution in the northern hemisphere and diverse clinical manifestations. For decades, the drug of choice for the treatment of tularemia has been streptomycin, with tetracycline and chloramphenicol being used as alternatives. The purpose of the present study was to determine the in vitro antimicrobial susceptibility of a large panel of geographically diverse *F. tularensis* isolates from Turkey against traditional and newer antimicrobial agents. **Methods:** The antibiotic susceptibilities of 250 *F. tularensis* strains were examined using the Epsilon test for 9 antimicrobial agents. Each isolate was identified by conventional and molecular techniques. **Results:** All the strains were confirmed biochemically and using a combination of species- and subspecies-specific polymerase chain reaction (PCR) assays to be *F. tularensis* subsp. *holarctica*. One isolate was assigned to *F. tularensis* subsp. *holarctica* biovar *japonica* based on erythromycin susceptibility, an ability to ferment glycerol, and the nucleotide sequence of the region of difference 1 (RD1). All strains were susceptible to aminoglycosides (streptomycin and gentamicin), tetracyclines (tetracycline and doxycycline), chloramphenicol, 2 fluoroquinolones (ciprofloxacin and levofloxacin), and rifampicin. In addition, all isolates except 1 had a minimal inhibitory concentration (MIC) for erythromycin of > 256 µg/ml. **Conclusions:** Since the fluoroquinolones showed the lowest MIC values and have advantages such as excellent bioavailability and activity, availability of oral formulations, and lower toxicities, they represent candidate therapeutic options in the first-line treatment of tularemia. To the best of our knowledge, this is the first report of the presence of *F. tularensis* subsp. *holarctica* biovar *japonica* outside Japan.

**Keywords:** *Francisella tularensis*, tularemia, antimicrobial susceptibility, Epsilon test, Turkey

### Introduction

Tularemia, caused by *Francisella tularensis*, is a potentially fatal multisystem disease in humans and some animals. Tularemia occurs widely across the northern hemisphere, with great variations in the geographic and temporal occurrence, but has rarely been found in the southern hemisphere [1,2].

Currently there are 4 recognized subspecies of *F. tularensis*: *tularensis* (type A), *holarctica* (type B), *novicida*, and *mediasiatica*, which differ in their pathogenicity and geographical distributions. While the highly virulent type A is usually confined to North America, the less virulent type B occurs in Europe and Asia and to a lesser extent in North America. Other subspecies, *mediasiatica*, *novicida*,

and a Japanese variant of *holarctica*, show a restricted geographical range and play little or no role in human disease [1–4]. Biochemical characterization (utilization of carbohydrates) and susceptibility to erythromycin separates subspecies *holarctica* into 3 distinct biovars: biovar I (erythromycin-sensitive), biovar II (erythromycin-resistant), and biovar *japonica* fermenting glycerol [5,6].

Tularemia generally presents as an acute febrile disease, with the major clinical presentations including the 6 classic forms of tularemia: ulceroglandular, glandular, oculoglandular, oropharyngeal, respiratory, and typhoidal [1,2]. Since infections with either subspecies *tularensis* or subspecies *holarctica* take a protracted course,

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antibiotic treatment is necessary [4]. For decades, streptomycin has been considered the drug of choice for the treatment of all forms of tularemia. Gentamicin, tetracycline, chloramphenicol, and quinolones have been recommended as alternatives [7–11]. The purpose of the present study was to determine the *in vitro* antibiotic susceptibility of a large panel of geographically diverse *F. tularensis* isolates from Turkey against traditional and newer antimicrobial agents.

## Materials and methods

### Bacterial strains

Two-hundred and fifty *F. tularensis* strains isolated from humans ( $n = 210$ ), water ( $n = 39$ ), and a rodent ( $n = 1$ ) were examined in this study (see Supplementary, Table I to be found online at <http://informahealthcare.com/doi/abs/10.3109/00365548.2012.751125>). The isolates were selected to include those obtained from diverse geographical areas of Turkey during the period October 2009 to July 2012 (Figure 1).

The reference strain, *F. tularensis* National Collection of Type Cultures (NCTC) 10857 (*F. tularensis* subsp. *holarctica* LVS; live vaccine strain) was used as the control for the identification and antimicrobial susceptibility testing. Quality control strains were *Escherichia coli* American Type Culture Collection (ATCC) 25922, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853. All strains were stored in skim milk at  $-80^{\circ}\text{C}$  and twice subcultured before starting the study.

### Confirmation of identity

*F. tularensis* strains were identified using standard criteria as described previously [12,13]. DNA was extracted from pure cultures of *F. tularensis* strains using a commercial kit based on silica gel-membrane technology (QIAamp DNA Extraction Mini Kit; Qiagen GmbH, Germany). Affiliation to the genus *Francisella* was confirmed by amplification of the 17-kDa outer membrane lipoprotein gene fragment (species-specific *tul4* gene), as described previously by Sjöstedt et al. [14]. After confirmation of isolates as *F. tularensis* by PCR with *tul4* primers, 2 different conventional PCRs targeting the region of difference 1 (RD1) were used to determine subspecies identity [15,16].

### Antimicrobial susceptibility

Minimal inhibitory concentrations (MICs) of 9 antimicrobial agents (streptomycin, gentamicin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, chloramphenicol, rifampicin, and erythromycin) were determined by Epsilometer test (MIC test strip, **Liofilchem s.r.l., Italy**) on cysteine heart agar plates supplemented with 10% sheep blood (CHAB). Prior to susceptibility testing, *F. tularensis* isolates were subcultured from frozen stocks onto CHAB, followed by 2 additional subcultures on chocolate agar plates for 48 h at  $35^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. Clinical and Laboratory Standards Institute (CLSI) guidelines have established breakpoints for *F. tularensis* susceptibility testing with tetracycline, doxycycline, streptomycin, gentamicin, chloramphenicol, ciprofloxacin, and levofloxacin. In the

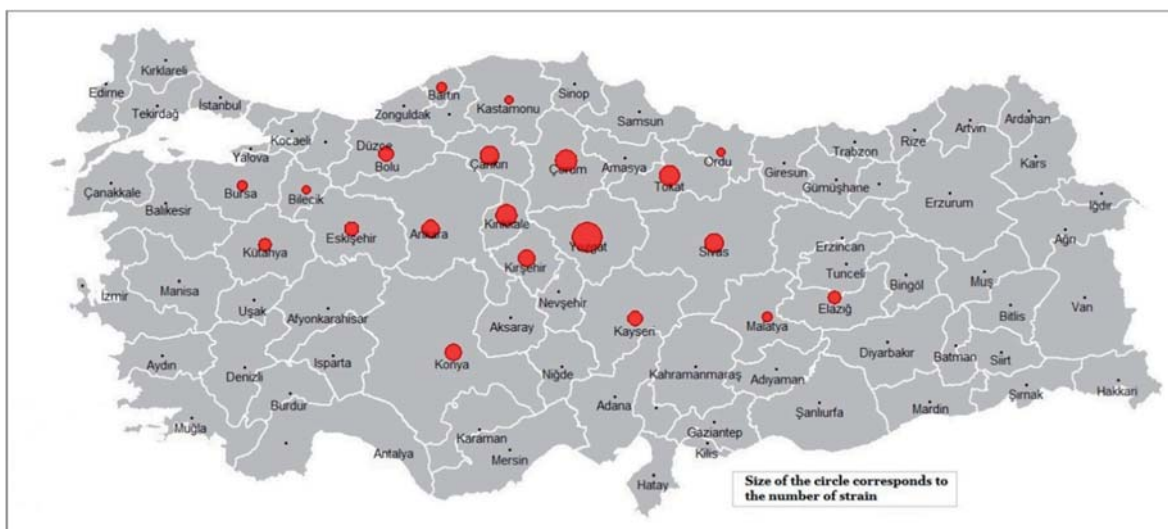


Figure 1. Geographic distribution of *Francisella tularensis* strains in Turkey.

absence of specific breakpoint data for *F. tularensis*, MIC values were interpreted according to CLSI criteria for Enterobacteriaceae [17].

## Results

### Confirmation of species, subspecies, and biovars

All isolates were confirmed to be *F. tularensis* subsp. *holarctica* based on a *F. tularensis*-specific PCR targeting the *tul4* gene, followed by a subspecies-specific PCR targeting the genetic region named RD1. Based on erythromycin susceptibility, 249 of the 250 isolates were assigned to *F. tularensis* subsp. *holarctica* biovar II (erythromycin-resistant). One isolate (TUR-F083) was assigned to biovar *japonica* based on erythromycin susceptibility, an ability to ferment glycerol, and a nucleotide sequence of RD1 that is specific to biovar *japonica*. The RD1 amplicon was sequenced and the 1136-bp amplicon (GenBank accession number JX436321.1) demonstrated 100% homology with the RD1 complete sequence (RD1) from *F. tularensis* subsp. *holarctica* strain FSC075 stored in GenBank (AF469618.1).

### Antimicrobial susceptibility

The MIC distributions for subspecies *holarctica* strains investigated are presented in Table I. Briefly, all isolates were susceptible to aminoglycosides (gentamicin and streptomycin), quinolones (ciprofloxacin and levofloxacin), tetracyclines (tetracycline and doxycycline), chloramphenicol, and rifampicin. The

MICs that inhibited the growth of 50% and 90% of the isolates (MIC<sub>50</sub> and MIC<sub>90</sub>, respectively) are shown in Table I. According to the MIC<sub>90</sub> values, levofloxacin (0.012 mg/l) was found to be the most active agent, followed by ciprofloxacin (0.016 mg/l), gentamicin (0.25 mg/l), doxycycline (0.25 mg/l), tetracycline (0.38 mg/l), chloramphenicol (0.5 mg/l), rifampicin (0.75 mg/l), and streptomycin (1.5 mg/l). All strains except 1 (TUR-F083) were resistant to erythromycin with minimal inhibitory concentrations > 256 mg/l (Table I). Erythromycin resistance was observed in 249 isolates, which were therefore assigned to biovar II. The remaining isolate was identified as biovar III (biovar *japonica*) based on being erythromycin-susceptible, biochemical test, and molecular methods.

## Discussion

We assayed the in vitro susceptibilities to 9 antimicrobials, some of which are in common use in the treatment of tularemia, using the Epsilometer test. The MIC values of streptomycin, gentamicin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, and chloramphenicol interpreted according to the CLSI criteria for potential bioterrorism agents, showed ranges below the breakpoints for sensitivity determination (Table I). Since the breakpoint MICs for susceptibility to rifampicin have not yet been established, the CLSI interpretive criteria for Enterobacteriaceae were taken into consideration in order to evaluate the results of the MIC determinations in the literature [12,18]. According to the CLSI interpretive criteria

Table I. In vitro activities of 9 antibiotics against 250 *Francisella tularensis* subsp. *holarctica* isolates and a reference strain.

Antimicrobial	MIC (mg/l) for clinical strains			MIC (mg/l) for reference strain NCTC 10857	CLSI breakpoints for susceptibility (mg/l)
	MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>		
<b>Aminoglycosides</b>					
Streptomycin	0.5–2.0	1.0	1.5	0.75	≤ 8 mg/l <sup>a</sup>
Gentamicin	0.094–0.38	0.19	0.25	0.125	≤ 4 mg/l <sup>a</sup>
<b>Tetracyclines</b>					
Tetracycline	0.094–0.5	0.25	0.38	0.19	≤ 4 mg/l <sup>a</sup>
Doxycycline	0.064–0.38	0.19	0.25	0.125	≤ 4 mg/l <sup>a</sup>
<b>Fluoroquinolones</b>					
Ciprofloxacin	0.004–0.023	0.012	0.016	0.008	≤ 0.5 mg/l <sup>a</sup>
Levofloxacin	0.003–0.016	0.008	0.012	0.006	≤ 0.5 mg/l <sup>a</sup>
<b>Others</b>					
Chloramphenicol	0.094–0.75	0.38	0.5	0.19	≤ 8 mg/l <sup>a</sup>
Rifampicin	0.125–1.0	0.5	0.75	0.19	≤ 4 mg/l <sup>b</sup>
Erythromycin	1.0–256	> 256	> 256	> 256	± <sup>c</sup>

MIC, minimal inhibitory concentration; NCTC, National Collection of Type Cultures; CLSI, Clinical and Laboratory Standards Institute.

<sup>a</sup>CLSI breakpoints for *F. tularensis*.

<sup>b</sup>CLSI criteria for Enterobacteriaceae.

<sup>c</sup>± Used for biovar designation.

for Enterobacteriaceae, all isolates were susceptible to rifampicin. For decades, streptomycin was considered the drug of choice for the treatment of tularemia, with defervescence usually within 48 h of treatment and the least number of relapses [7]. Although gentamicin may substitute for streptomycin, the main drawbacks such as toxicity, the necessity for parenteral administration, and the necessity for monitoring of serum levels, preclude its wider use, especially in the outpatient setting. Consistent with the results of previous studies [12,13,18–23], all isolates in this study were susceptible to streptomycin and gentamicin. Streptomycin had the higher MIC value ( $MIC_{90}$  1.5 mg/l) compared to gentamicin ( $MIC_{90}$  0.25 mg/l).

Tetracycline and doxycycline are among the effective drugs in the treatment of tularemia [4,7]. In our study, doxycycline ( $MIC_{90}$  0.25 mg/l) and tetracycline ( $MIC_{90}$  0.38 mg/l) showed good in vitro activity against all the isolates, and MICs showed ranges below the breakpoint for sensitivity determination (sensitive  $\leq$  1 mg/l) [17].

Although chloramphenicol has been shown to be efficacious against *F. tularensis*, it is only indicated for tularemia meningitis owing to the lack of experience using other treatment alternatives with good penetration into the cerebrospinal fluid [12,18]. All the isolates were susceptible to chloramphenicol ( $MIC$  0.094–0.75 mg/l). This result is in agreement with those previously reported [12,13,18–23].

There is growing evidence that the fluoroquinolones are effective for first-line therapy of tularemia because of their excellent bioavailability, intracellular penetration capability, the ability to achieve optimum tissue concentrations, and the fact that drug level monitoring is not necessary [8–11,24,25]. In the present study, quinolones had the lowest MIC values among the antibiotics tested. Levofloxacin ( $MIC_{90}$  0.012 mg/l) had a lower MIC value than ciprofloxacin ( $MIC_{90}$  0.016 mg/l) against subspecies holarctica. There are a few reports on the in vitro activities against *F. tularensis* and our results are in agreement with those studies [18,20,21,23]. Streptomycin, and to a lesser extent gentamicin, are today by far the most commonly used aminoglycosides in Turkish hospitals. However, aminoglycoside therapy usually requires hospitalization of the patient and has drawbacks such as vestibular toxicity and nephrotoxicity. On the other hand, their use is less feasible as the majority of our tularemia patients are not in need of hospitalization. Hence, fluoroquinolones appear suitable for ambulatory treatment and probably also for hospitalized cases in Turkey.

Although erythromycin has been used successfully in a few patients with pneumonia in the USA [26,27], macrolides are not recommended for

the treatment of tularemia due to resistance to erythromycin in some areas of Europe and Russia [5]. Our results with regard to erythromycin confirm previous data [12,18,20], indicating that European isolates are erythromycin-resistant, and almost all Turkish isolates were *F. tularensis* subsp. holarctica biovar II (erythromycin-resistant). Within the present collection, a single strain was assigned as biovar japonica due to its erythromycin susceptibility, biochemical characteristics, and sequencing of the RD1 region. This observation confirms previous data [22] indicating that biovar japonica is macrolide-sensitive. Since the prevailing *F. tularensis* subsp. holarctica biovar II is resistant, erythromycin and other macrolides should not be used for the treatment of tularemia in Turkey.

In this study, rifampicin exhibited good antimicrobial activity against all the strains tested ( $MIC$  0.125–1.0 mg/l). Even though drawbacks exist, such as some toxicities and the rapid emergence of resistance in monotherapy, our results as well as previous reports [18,19,21] emphasize that rifampicin might be useful in conjunction with aminoglycosides or quinolones in severe cases of tularemia owing to its advantage of oral administration.

In conclusion, the classically recommended therapeutics proved to be effective in vitro against *F. tularensis* subsp. holarctica strains from Turkey. On the basis of our results, erythromycin and other macrolides should not be used in the treatment of tularemia in Turkey. Additionally, fluoroquinolones are highly effective, suggesting that these antimicrobial agents might be useful for patients treated on an outpatient basis or for those who are intolerant to more standard treatment regimens. Moreover, we have described an important novel finding of *F. tularensis* subsp. holarctica: the existence of biovar japonica outside Japan. The discovery of the Japanese variant of subsp. holarctica in Turkey raises epidemiologic and epizootiologic questions and requires further study.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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### Supplementary material available online

Supplementary Table I showing characteristics of the *Francisella tularensis* isolates analyzed.