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# Antibiotic susceptibility of *Francisella tularensis* subsp. *holarctica* strains isolated from tularaemia patients in France between 2006 and 2016

Yvan Caspar<sup>1,2\*</sup>, A. Hennebique<sup>1,2</sup> and Max Maurin<sup>1,2</sup>

<sup>1</sup>Centre National de Référence *Francisella tularensis*, Laboratoire de Bactériologie-Hygiène Hospitalière, Département des agents infectieux, Institut de Biologie et de Pathologie, Centre Hospitalier Universitaire Grenoble Alpes, CS 10217, F-38043 Grenoble cedex 9, France; <sup>2</sup>Université Grenoble Alpes, CNRS, Grenoble INP, TIMC-IMAG, F-38000 Grenoble, France

\*Corresponding author. Tel: +33-4-76-76-63-12; Fax: +33-4-76-76-62-28; E-mail: YCaspar@chu-grenoble.fr

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**Objectives:** To determine the *in vitro* susceptibility to 18 antibiotics of human strains of *Francisella tularensis* isolated in France between 2006 and 2016, to check the absence of acquired resistance and to evaluate potential therapeutic alternatives.

**Methods:** Fifty-nine clinically unrelated *F. tularensis* subsp. *holarctica* strains identified at the French National Reference Centre for *Francisella* as belonging to the phylogenetic subclade B.FTNF002-00 were used. MICs were determined in CAMHB medium supplemented with 2% PolyViteX<sup>®</sup>, using the CLSI broth microdilution method.

**Results:** All strains were susceptible to fluoroquinolones (ofloxacin, ciprofloxacin, levofloxacin and moxifloxacin; MIC range: 0.016–0.25 mg/L), aminoglycosides (gentamicin and tobramycin; MIC range: ≤0.03–0.25 mg/L), doxycycline (MIC range: 0.125–0.25 mg/L) and chloramphenicol (MIC range: 0.5–2 mg/L). The erythromycin MIC range (0.5–2 mg/L) confirmed that all isolates belonged to biovar I of *F. tularensis* subsp. *holarctica*. Azithromycin and telithromycin displayed lower MIC ranges (0.25–1 and 0.03–0.5 mg/L, respectively). The tigecycline MIC range (0.25–1 mg/L) was slightly higher than that of doxycycline. All strains were resistant to ampicillin, meropenem, daptomycin, clindamycin and linezolid.

**Conclusions:** *F. tularensis* strains isolated in France remain susceptible to antibiotic classes recommended for tularaemia treatment. Because fluoroquinolones display the lowest MIC<sub>90</sub>, have bactericidal activity and have lower therapeutic failure rates compared with doxycycline, they may be advocated as first-line treatment of mild cases of tularaemia, predominant in Europe. MIC data also indicate that azithromycin or telithromycin may be possible therapeutic options against biovar I strains from Western Europe in case of contraindication to first-line antibiotics.

## Introduction

Tularaemia is a zoonosis caused by the highly infectious Gram-negative bacterium *Francisella tularensis*.<sup>1</sup> In Europe, tularaemia cases are caused by *F. tularensis* subspecies *holarctica* (type B), including biovar I strains (erythromycin susceptible) in Western Europe (France, Spain, Italy)<sup>2–4</sup> and biovar II strains (erythromycin resistant) in Eastern Europe.<sup>4,5</sup> Both biovars exist in Germany and Switzerland.<sup>6,7</sup> In France, fewer than 100 human tularaemia cases are notified annually to Santé Publique France (the national public health system), and the ulceroglandular and glandular forms (a regional lymphadenopathy with or without a skin eschar, respectively) of tularaemia are predominant.<sup>8,9</sup> Type B strains isolated in France are clonal and belong to the subclade B.FTNF002-00, which is characterized by a deletion in region of difference 23 (RD23) of the genome.<sup>2,3,10</sup>

Current treatment guidelines recommend gentamicin and streptomycin as first-line drugs for severe tularaemia cases, while ciprofloxacin and doxycycline are recommended for milder forms of the disease.<sup>11</sup> However, the treatment of tularaemia still has many limitations. Doxycycline is contraindicated in children under 8 years of age.<sup>11</sup> Both doxycycline and the fluoroquinolones are contraindicated in pregnant women. Moreover, despite appropriate treatment, many patients experience therapeutic failures or relapses, especially when tularaemia diagnosis has been delayed. In France, approximately one-third of patients suffering from regional lymphadenopathy progress to lymph node suppuration.<sup>9</sup> Most of these patients experience therapeutic failures and relapses, and cure is usually only obtained after surgical removal of the suppurated lymph node tissues.<sup>9</sup> Because therapeutic options for tularaemia are limited, and no vaccine is currently licensed for human use, there is a concern that resistance to currently available treatments

**Table 1.** MIC results (mg/L) for 59 *F. tularensis* subsp. *holarctica* strains isolated from tularaemia patients in France between 2006 and 2016

Antibiotic	Dilution range tested (mg/L)	<i>F. tularensis</i> subsp. <i>holarctica</i> (59 strains)			<i>F. tularensis</i> subsp. <i>holarctica</i> LVS NCTC 10857	Breakpoints (mg/L)	
		MIC <sub>50</sub> (mg/L)	MIC <sub>90</sub> (mg/L)	range (mg/L)	MIC (mg/L)	S ≤	R >
Gentamicin	0.03–16	0.125	0.125	≤0.03 to 0.25	0.06	4 <sup>a</sup>	
Tobramycin	0.03–16	0.125	0.25	0.06–0.25	0.125		
Ofloxacin	0.008–4	0.06	0.125	0.06–0.125	0.06	0.25 <sup>b</sup>	0.5 <sup>b</sup>
Ciprofloxacin	0.001–0.5	0.03	0.03	0.016–0.06	0.016	0.5 <sup>a</sup>	
Levofloxacin	0.001–0.5	0.03	0.06	0.016–0.06	0.016	0.5 <sup>a</sup>	
Moxifloxacin	0.002–1	0.125	0.125	0.06–0.25	0.03	0.25 <sup>b</sup>	0.25 <sup>b</sup>
Doxycycline	0.03–16	0.25	0.25	0.125–0.25	0.125	4 <sup>a</sup>	
Tigecycline	0.03–16	0.25	1	0.125–1	0.06	0.25 <sup>b</sup>	0.5 <sup>b</sup>
Ampicillin	0.25–128	>128	>128	64 to ≥128	>128	2 <sup>b</sup>	8 <sup>b</sup>
Meropenem	0.06–32	>32	>32	>32	>32	2 <sup>b</sup>	8 <sup>b</sup>
Erythromycin	0.06–32	1	1	0.5–2	>32	0.5 <sup>c</sup>	16 <sup>c</sup>
Azithromycin	0.06–32	0.5	1	0.25–1	>32	0.125 <sup>c</sup>	4 <sup>c</sup>
Telithromycin	0.016–8	0.125	0.25	0.03–0.5	>8	0.125 <sup>c</sup>	8 <sup>c</sup>
Clindamycin	0.03–16	8	16	4–16	>16		
Linezolid	0.125–64	8	16	4–16	4	2 <sup>b</sup>	4 <sup>b</sup>
Chloramphenicol	0.06–32	1	2	0.5–2	1	8 <sup>a</sup>	
Rifampicin	0.004–2	0.5	1	0.125–1	0.03	1 <sup>c</sup>	1 <sup>c</sup>
Daptomycin	0.016–8	>8	>8	>8	>8		
Ethanol (%)	0.016–8	2	2	1–2	2		
Acetic acid (%), pH adjusted to 7.1	0.016–8	0.125	0.25	0.125–0.5	0.5		

S, susceptible; R, resistant.

The MICs of all antibiotics tested for the control strain *S. aureus* ATCC 29213 were within the acceptable range determined by CLSI.

<sup>a</sup>CLSI breakpoints.

<sup>b</sup>EUCAST PK/PD breakpoints.

<sup>c</sup>*H. influenzae* EUCAST breakpoints.

may emerge. The aim of this study was to evaluate the *in vitro* antibiotic susceptibilities of *F. tularensis* subsp. *holarctica* strains isolated from French tularaemia patients over a period of 11 years (2006–16) to a large panel of antibiotics, to check the absence of acquired resistance to first-line drugs and to look for possible therapeutic alternatives.

## Materials and methods

### Bacterial strains

All experiments were conducted in a biosafety level 3 (BSL3) laboratory. We evaluated 59 *F. tularensis* subsp. *holarctica* strains corresponding to all *F. tularensis* strains isolated from human samples and investigated at the French National Reference Centre for *Francisella* during this period. The strains were identified using PCR amplification and sequencing of the intergenic 16S–23S rRNA region, as previously described.<sup>9</sup> RD23 genomic deletion was detected by PCR according to previously published protocols.<sup>3</sup> Reference strains used as controls for antibiotic susceptibility testing were *F. tularensis* subsp. *holarctica* LVS NCTC 10857 and *Staphylococcus aureus* ATCC 29213 (ATCC, Manassas, VA, USA). All strains were kept frozen in cryotubes (MastDiagnostic, Amiens, France) at –80 °C and cultured on chocolate agar medium supplemented with PolyViteX<sup>®</sup> (bioMérieux, Marcy-l'Étoile, France).

### Antibiotics

All antibiotics (Table 1), ethanol and acetic acid were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), except telithromycin

(TOKU-E, Zwijnaarde, Belgium), daptomycin (Cubicin, Cubist Pharmaceuticals, France) and linezolid (Zyvoxid, Pfizer, France) for which we used commercialized powders for intravenous infusion. Antibiotic powders were first dissolved in ethanol for chloramphenicol and azithromycin, or glacial acetic acid for telithromycin, as recommended.<sup>12</sup>

### Antibiotic susceptibility testing

MICs of 18 antibiotics (Table 1), ethanol and acetic acid (used as solvents for some antibiotics) were determined in 96-well microplates using the broth microdilution method recommended by CLSI for *Francisella* strains.<sup>12</sup> The assay medium was CAMHB supplemented with 2% PolyViteX<sup>®</sup> (CAMHB-PVX, bioMérieux) and adjusted to pH 7.1±0.1 as recommended. In brief, one row of a 96-well microplate was filled with 75 µL of 2-fold serial dilutions of the antibiotic compound tested in the assay medium (see Table 1 for the antibiotic dilution range tested). For tigecycline, fresh medium was prepared on the test day as recommended.<sup>13</sup> The final bacterial inoculum was standardized at 5×10<sup>5</sup> cfu/mL by adding 75 µL of a calibrated bacterial suspension to each well. Antibiotic-free cultures were used as positive controls and bacteria-free cultures were used as negative controls. Microplates were incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere. The MICs were read after 48 h for *Francisella* strains. As for *S. aureus* ATCC 29213, MICs were read in CAMHB-PVX medium after 48 h of incubation or in MH2 medium after 24 h, according to CLSI recommendations. MICs were determined using a microplate reader (TECAN M200 Pro, Männedorf, Switzerland) at 600 nm. Experiments were conducted in duplicate for each strain/antibiotic combination to confirm results. In case of discrepant

**Table 2.** Antibiotic MIC distribution for the 59 *F. tularensis* subsp. *holarctica* strains

Antibiotic	MIC (mg/L)																		
	0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Gentamicin						1	5	50	3	0	0	0	0	0	0				
Tobramycin						0	1	31	27	0	0	0	0	0	0				
Ofloxacin				0	0	0	42	17	0	0	0	0	0						
Ciprofloxacin	0	0	0	0	1	55	3	0	0	0									
Levofloxacin	0	0	0	0	1	29	29	0	0	0									
Moxifloxacin		0	0	0	0	0	12	45	2	0	0								
Doxycycline						0	0	11	48	0	0	0	0	0	0				
Tigecycline						0	0	5	34	11	9	0	0	0	0				
Ampicillin									0	0	0	0	0	0	0	0	1	0	58
Meropenem							0	0	0	0	0	0	0	0	0	0	59		
Erythromycin							0	0	0	16	42	1	0	0	0	0			
Azithromycin							0	0	9	38	12	0	0	0	0	0			
Telithromycin					0	2	19	12	23	3	0	0	0	0					
Clindamycin						0	0	0	0	0	0	0	1	37	21				
Linezolid								0	0	0	0	0	6	45	8	0	0		
Chloramphenicol								0	0	0	2	45	12	0	0	0	0		
Rifampicin			0	0	0	0	0	1	6	39	13	0							
Daptomycin					0	0	0	0	0	0	0	0	0	0	0	59			
Ethanol (%)					0	0	0	0	0	0	3	56	0	0					
Acetic acid (%), pH adjusted to 7.1					0	0	0	34	23	2	0	0	0	0					

results between the two replicates (an MIC difference equivalent to two or more doubling dilutions), one more replicate was performed and the two closest MICs were considered valid. The MICs were interpreted using the CLSI susceptibility breakpoints for *F. tularensis* when available (gentamicin, doxycycline, ciprofloxacin, levofloxacin and chloramphenicol). We used the *Haemophilus influenzae* EUCAST breakpoints for erythromycin, azithromycin, telithromycin and rifampicin. We used the pharmacokinetic/pharmacodynamic (PK/PD) EUCAST breakpoints for ampicillin, meropenem, ofloxacin, moxifloxacin, linezolid and tigecycline.

## Results

The 59 *F. tularensis* subsp. *holarctica* strains evaluated in this study were isolated mostly from blood samples (39%) collected from bacteraemic patients or from various other clinical samples (cutaneous ulcer/abscess: 22%; lymph node: 10%; see Table S1, available as [Supplementary data](#) at JAC Online). Fifty-five strains were isolated from patients suffering from classical forms of tularaemia: ulceroglandular (19 strains), glandular (1), oropharyngeal (6), oculoglandular (2), pneumonic (16) and typhoidal (11). Three strains were isolated from patients with unusual clinical presentations, including meningitis (1), bone and joint infection (1) and aortitis (1). For one strain, the clinical form was unknown. See Table S1. All strains belonged to the B.FTNF002-00 subclade as confirmed by detection of the RD23 deletion.

MIC results (Table 1) showed that all strains were susceptible to ciprofloxacin, levofloxacin, gentamicin, doxycycline and chloramphenicol according to CLSI susceptibility breakpoints for *F. tularensis*. MICs were slightly higher for ofloxacin and moxifloxacin compared with ciprofloxacin and levofloxacin, with rare strains reaching the EUCAST PK/PD breakpoint defining susceptibility for

moxifloxacin. As for aminoglycosides, tobramycin displayed similar MIC<sub>50</sub> and MIC<sub>90</sub> values compared with gentamicin. In contrast, all strains tested were resistant to the β-lactams ampicillin and meropenem, and to daptomycin, linezolid and clindamycin. The other antibiotics tested displayed intermediate-level MIC<sub>90</sub> values, including telithromycin (0.25 mg/L), erythromycin (1 mg/L), azithromycin (1 mg/L), rifampicin (1 mg/L) and tigecycline (1 mg/L). Importantly, the MIC distribution (Table 2) was very narrow for all antibiotics, almost all of them being distributed within three consecutive 2-fold dilutions. Only gentamicin, tigecycline, rifampicin and telithromycin displayed MIC ranges of four to five 2-fold dilutions (Table 2). Ethanol alone was able to inhibit growth of all *F. tularensis* strains when diluted at 2% (v/v), but the residual concentration of this compound in working solutions of chloramphenicol and azithromycin was ≤0.016%. Acetic acid also had a bacteriostatic activity against *F. tularensis* strains at 0.5%, but its concentration in working solutions of telithromycin was ≤0.004%.

## Discussion

Despite the availability of WHO and European guidelines,<sup>11,14</sup> tularaemia treatment remains challenging in France. Diagnosis is often delayed and therapeutic failures still occur despite appropriate antibiotic treatment.<sup>9</sup> Tularaemia diagnosis is still based on serological tests. *F. tularensis* cultures are not performed on a routine basis, and this pathogen is currently isolated in <10% of infected patients. Moreover, antibiotic susceptibility testing is not performed on a routine basis when *F. tularensis* strains are isolated because cultures of this pathogen must be handled under BSL3 conditions. In this study, our primary objective was to determine

whether first-line drugs currently recommended for tularaemia treatment are still effective in Western Europe, where only strains belonging to the B.FTNF002-00 subclade are present. We looked for the emergence of acquired resistance to these antibiotics in human strains of *F. tularensis* isolated in France over a period of 11 years. No strains resistant to any clinically recommended antibiotics were identified, which is consistent with previous data.<sup>7,15</sup> The narrow MIC distribution observed for each of the antibiotics tested is consistent with the low genetic diversity of the *F. tularensis* strains isolated in France.

In this study, the lowest MIC values were observed for ciprofloxacin and levofloxacin (MIC<sub>90</sub> 0.03 and 0.06 mg/L, respectively). These fluoroquinolone compounds should probably be preferred for tularaemia treatment over ofloxacin and moxifloxacin, which showed higher (2–4 times) MICs. The WHO guidelines only recommend ciprofloxacin (500 mg q12h) treatment for 10–14 days for tularaemia treatment, while European guidelines also recommend levofloxacin as first-line treatment,<sup>14</sup> which is supported by similar *in vitro* activity of both compounds. Doxycycline (100 mg q12h) is an alternative drug for treatment of mild cases of tularaemia, but this antibiotic must be administered for a minimum of 14 or preferably 21 days owing to its bacteriostatic nature.<sup>11</sup> However, doxycycline is associated with a higher rate of failures and relapses compared with fluoroquinolones and aminoglycosides, in both humans and animal models.<sup>15</sup> Interestingly, during a tularaemia outbreak involving 142 patients in Spain, treatment failures occurred in 4.5% of the 22 patients treated with ciprofloxacin, but in 42.8% of the 14 patients treated with doxycycline ( $P < 0.018$ ).<sup>16</sup> Because the outbreak was caused by a clonal strain of *F. tularensis*, the differences observed were not related to variations in the virulence or antibiotic susceptibilities of the strain involved, but rather the choice of the antibiotic treatment administered. Therefore, we suggest that ciprofloxacin should probably be advocated as a first-line therapeutic option for mild cases of tularaemia in Western Europe. Doxycycline treatment may be considered an acceptable alternative only in patients with a contraindication to fluoroquinolones. Indeed, in European guidelines, doxycycline is only recommended for adults as a third-line treatment option, whereas fluoroquinolones appear as a second-line option after aminoglycosides.<sup>14</sup> As regards severe tularaemia, gentamicin is currently considered the drug of choice. Although tobramycin is not recommended for treatment of tularaemia, this compound displayed similar MICs compared with gentamicin. Using the Etest method, three studies evaluating type B strains from Turkey, Austria and Finland also reported tobramycin-susceptible MIC ranges of 0.125–0.38, 0.19–3 and 0.5–2 mg/L, respectively.<sup>15</sup> Among other antibiotics for which CLSI breakpoints are available for *F. tularensis*, chloramphenicol was also active. It may be considered in combined therapy for tularaemia despite its bacteriostatic activity and significant side effects, though only for cases of tularaemia meningitis or meningoenzephalitis because of its high penetration into the brain tissue and CSF.<sup>11</sup>

Based on MIC data, a few antibiotics may be useful in cases where the previously mentioned antibiotic classes are contraindicated. Linezolid was tested because a wide range of MICs were previously reported with high variations between studies. Azithromycin, clindamycin, daptomycin, tigecycline and telithromycin were tested because few data were available for those antibiotics in the literature, especially for *F. tularensis* strains from

Western Europe.<sup>15</sup> The low erythromycin MICs and the RD23 deletion confirmed the affiliation of all human strains we tested to the biovar I of *F. tularensis*.<sup>5,15</sup> Importantly, we noted that particular attention should be paid to adjustment of the broth pH for erythromycin and azithromycin testing, as acidification of the growth medium resulted in a 4- to 8-fold MIC increase (Y. Caspar and M. Maurin, unpublished data). Using susceptibility breakpoints advocated for *H. influenzae* (Table 1), another small Gram-negative fastidious bacterium, all *F. tularensis* strains would have been classified as intermediately susceptible to erythromycin and azithromycin. Furthermore, 33 of 59 (56%) strains tested would have been classified as susceptible to telithromycin and only 26 of 59 (44%) as intermediately susceptible. Using the same breakpoints, 71 *F. tularensis* subsp. *holarctica* strains from France evaluated previously using an agar dilution method would have been categorized as intermediately susceptible to telithromycin (MIC range: 0.125–0.25 mg/L).<sup>17</sup> Moreover, telithromycin at 4 mg/L was effective against a type B biovar I strain in a cell model of infection.<sup>18</sup> Although the activity of azithromycin and telithromycin against *F. tularensis* should be further evaluated *in vivo*, especially in animal models, these drugs could be potential second-line therapeutic options for infections caused by type B biovar I strains when recommended antibiotics are contraindicated. Azithromycin (500 mg/day for 6 weeks) has already been used for the treatment of a pregnant women suffering from oropharyngeal tularaemia in France.<sup>19</sup> Moreover, using *H. influenzae* breakpoints, the *F. tularensis* strains evaluated in this study would also be categorized as susceptible to rifampicin, although the MIC range (0.125–1 mg/L) was close to the resistance cut-off set at  $\leq 1$  mg/L. Higher MIC values have already been described for rifampicin (up to 3 mg/L) for *F. tularensis* strains, but only when using the Etest method.<sup>15</sup> A previous study of 71 French isolates reported an MIC range of 0.015–0.5 mg/L when using the agar dilution method.<sup>17</sup> Thus, although rifampicin cannot be used alone because of the rapid selection of resistant mutants, it may be useful in combination with a first-line drug to treat rare bone and joint infections owing to its excellent diffusion in these tissues.<sup>11</sup> One patient with prosthetic joint infection showed a dramatic clinical improvement after the addition of rifampicin to ciprofloxacin antibiotic treatment.<sup>20</sup> Tigecycline was not more effective than doxycycline. Using EUCAST PK/PD breakpoints for tigecycline, 9 of 59 (15%) strains from our study were categorized as resistant and 11 of 59 (19%) intermediately susceptible to this antibiotic. Finally, according to our *in vitro* results, biovar I strains belonging to the B.FTNF002-00 subclade are confirmed to be resistant to linezolid, aminopenicillins, carbapenems, daptomycin and clindamycin.

In conclusion, we found that *F. tularensis* subsp. *holarctica* strains of the B.FTNF002-00 subclade isolated in France over the last decade remain fully susceptible to gentamicin, ciprofloxacin, levofloxacin and doxycycline, which are the currently advocated drugs for tularaemia treatment.<sup>11,14</sup> The therapeutic failure rate in France seems unrelated to antibiotic resistance and may be due to other parameters influencing antibiotic efficacy such as delayed diagnosis, delayed appropriate antibiotic therapy, insufficient patient compliance, penetration issues of the antibiotics to the site of infection or other resistance mechanisms (biofilm, bacterial persistence). In Western Europe, where mild to moderately severe infections predominate, fluoroquinolones may be considered the best oral alternative because of lower failure rates, while restricting

the use of doxycycline to patients with contraindications to the former antibiotics. Finally, because tularaemia cases in Western Europe are only caused by type B biovar I strains, azithromycin and telithromycin may be useful alternatives in pregnant women and young children. All these proposals should be validated by further clinical evaluation.

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## Transparency declarations

None to declare.

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## Supplementary data

Table S1 is available as [Supplementary data](#) at JAC Online.

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