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In vitro and in vivo evaluation of fluoroquinolone resistance associated with DNA gyrase mutations in *Francisella tularensis*, including in tularaemia patients with treatment failure

V. Sutera [a,b,c,1], G. Hoarau [a,b,c,1], P. Renesto [b,c], Y. Caspar [a,b,c], M. Maurin [a,b,c,*]

[a] *Centre National de Référence des Francisella, Laboratoire de Bactériologie-Hygiène Hospitalière, Institut de Biologie et de Pathologie, Centre Hospitalier Universitaire Grenoble Alpes, CS 10217, 38043 Grenoble Cedex 9, France*
[b] *Laboratoire Techniques de l'Ingénierie Médicale et de la Complexité—Informatique, Mathématiques et Applications (TIMC-IMAG), Université Grenoble Alpes, CS 10170, 38042 Grenoble cedex 9, France*
[c] *Unité Mixte de Recherche 5525, Centre National de la Recherche Scientifique, 38042 Grenoble cedex 9, France*

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ABSTRACT

Fluoroquinolones (FQs) are highly effective for treating tularaemia, a zoonosis caused by *Francisella tularensis*, but failures and relapses remain common in patients with treatment delay or immunocompromised status. FQ-resistant strains of *F. tularensis* harboring mutations in the quinolone–resistance determining region (QRDR) of *gyrA* and *gyrB*, the genes encoding subunits A and B of *DNA gyrase*, have been selected in vitro. Such mutants have never been isolated from humans as this microorganism is *difficult* to culture. In this study, the presence of FQ-resistant mutants of *F. tularensis* was assessed in tularaemia patients using combined culture- and PCR-based approaches.

We analyzed 42 *F. tularensis* strains and 82 tissue samples collected from 104 tularaemia cases, including 32 (30.7%) with FQ treatment failure or relapse. Forty *F. tularensis* strains and 55 clinical samples were obtained before any FQ treatment, while 2 strains and 15 tissue samples were collected after treatment. FQ resistance was evaluated by the minimum inhibitory concentration (MIC) for the bacterial strains, and by newly developed PCR-based methods targeting the *gyrA* and *gyrB* QRDRs for both the bacterial strains and the clinical samples. None of the *F. tularensis* strains displayed an increased MIC compared with FQ–susceptible controls. Neither *gyrA* nor *gyrB* QRDR mutation was found in bacterial strains and tissue samples tested, including those from patients with FQ treatment failure or relapse. Further phenotypic and genetic resistance traits should be explored to explain the poor clinical response to FQ treatment in such tularaemia patients.

1. Introduction

Francisella tularensis is a Gram-negative, facultative intracellular bacterium responsible for the zoonosis tularaemia [1] and is classified as a category A bioterrorism agent by the CDC [2]. Human infections are caused by *F. tularensis* subsp. *tularensis* (type A) in North America and subsp. *holarctica* (type B) throughout the northern hemisphere [3]. The clinical symptoms of tularaemia are classically grouped into six clinical forms [3–5]: the ulceroglandular and glandular forms, a regional lymphadenopathy with a skin inoculation lesion only detectable in the former presentation; the oculoglandular and oropharyngeal forms, respectively a conjunctivitis or a pharyngitis with a regional lymphadenopathy; the pneumonic form, a pneumonia occurring after inhalation of contaminated aerosols or hematogenous spread of bacteria from other infection sites; and the typhoidal form, a systemic disease mimicking typhoid. Complications include lymph node suppuration in approximately 30% of patients with lymphadenopathy [3,6,7] and *F. tularensis* bacteremia with possible secondary infection sites and severe sepsis [3–5]. Mortality rates vary from less than 1% for type B infections in Europe [7], to up to 30% for type A pneumonia cases reported in the USA [8].

Fluoroquinolones (FQs) and tetracyclines are considered first-line drugs for treatment of cases of mild–to–moderate severity, whereas aminoglycosides (streptomycin or gentamicin) are advocated for severe infections [1,4,5]. FQs are bactericidal against *F. tularensis* in axenic media [9–12], infected cells [13,14], and animal

Abbreviations: FQ: fluoroquinolone; QRDR: quinolone–resistance determining region.

* Corresponding author. Laboratoire de Bactériologie-Hygiène Hospitalière, Département des agents infectieux, Institut de Biologie et de Pathologie, Centre Hospitalier Universitaire Grenoble Alpes, CS 10217, 38043 Grenoble Cedex 9, France.
E-mail address: mmaurin@chu-grenoble.fr (M. Maurin).
[1] These authors contributed equally to this work.

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models [15–17]. They are highly effective when administrated early in the course of disease [18–20]. In contrast, treatment failures and relapses are still observed in 5–30% of patients with treatment delay, complications (e.g., suppurated lymphadenopathy) and/or altered immune status [21,22]. We hypothesized that poor clinical response to FQ treatment might be caused by acquisition of FQ-resistance in *F. tularensis*. Bacterial resistance to these antibiotics is mainly related to mutations in genes encoding type II topoisomerases (DNA gyrase and topoisomerase IV) and overexpression of efflux pumps [23]. DNA gyrase, which is described as the main FQ target in Gram-negative bacteria, is encoded by *gyrA* and *gyrB,* whereas topoisomerase IV is encoded by *parC* and *parE*. FQ resistance may occur through single mutations in a specific region of these genes, referred to as the quinolone-resistance determining region (QRDR). In vitro *F. tularensis* strains resistant to FQ were thus selected because of mutations in type II topoisomerases encoding genes [24–26], the first mutations occurring in *gyrA* and *gyrB* [24]. No FQ resistance has been observed so far in natural strains of *F. tularensis* [9–12,22,27–29], but this fastidious bacterium is rarely isolated from tularaemia patients. In the present study, we assessed the possibility to detect FQ-resistant *F. tularensis* strains in humans using combined culture- and PCR-based approaches.

2. Material and methods

2.1. Patients and clinical specimens

Bacterial strains and clinical samples were collected at the French National Reference Center for *Francisella tularensis*, during our routine expertise of human tularaemia cases in France. This sample collection was declared to the French Ministry of Education and Research (DC–2008-677), and we obtained the authorization to use it for research purposes from our ethics committee (Comité de Protection des Personnes Sud-Est V). In accordance with French law, patient information for this type of research was through a hospital medical booklet and only a non-opposition of the patients was needed.

We studied 42 *F. tularensis* strains and 82 tissue samples collected from 104 confirmed tularaemia cases, according to the WHO classification [1], including 32 (30.7%) who experienced failure or relapse after FQ treatment (Table 1). All patients were infected with a *F. tularensis* subsp. *holarctica* strain, as determined by PCR amplification and sequencing of the 16S-23SrRNA region from isolated strains or tissue samples [7].

Group A included 42 cases (9 females, 33 males), which mainly occurred as sporadic infections (39/42 cases) between 2004 and 2014, for which a *F. tularensis* strain was isolated before any antibiotic treatment (except 2 patients already under FQ treatment) (Table 1). The clinical forms were ulceroglandular (12 cases), glandular (3), oropharyngeal (7, including 3 family cases), oculoglandular (2), pneumonic (8) and typhoidal (10).

Group B included 70 cases for which 82 clinical samples tested positive for *F. tularensis* DNA using a qPCR-*ISFtu2* assay targeting the insertion sequence Ftu2 of this species (Table 1) [7]. The clinical forms were ulceroglandular (22 cases), glandular (24), oropharyngeal (16), oculoglandular (4), pneumonic (3) and typhoidal (1). Group B patients were split into subgroups B1 and B2 for patients who received or did not receive FQs (mostly ciprofloxacin) before clinical sample collection, respectively (Table 1). One group B1 patient and seven group B2 patients also belong to group A.

2.2. Bacterial strains

Five *Francisella* sp. strains with low virulence in humans were used (Table 2): *F. tularensis* subsp. *holarctica* Live Vaccine strain (FthLVS, NCTC 10857, provided by the Institut de recherche biomédicale des armées, Grenoble, France); *F. novicida* strain CIP56.12 (Centre de Ressources Biologiques de l'Institut Pasteur, Paris, France); and three FQ–resistant mutants, either derived from FthLVS (Fth1P14 and Fth2P14) or from *F. novicida* CIP56.12 (Fno1P14) [24]. The specificity of the qPCRFtgyrA assay was evaluated using 34 strains outside the *Francisella* genus (Table 3). All cultures were performed in a biosafety level 3 (BSL3) laboratory.

Table 1
Distribution of tularaemia patients from groups A, B1 and B2, according to sex, age, clinical forms and collected samples.

Patient groups		A	B1	B2
Number, sex ratio		42, 3.7	15, 1.1	55, 1.1
Mean age, SD (years)		60.6, 18.5	48.3, 17.8	52.7, 18.2
Clinical form	Ulceroglandular	12	6	16
	Glandular	3	5	19
	Oropharyngeal	7	1	15
	Oculoglandular	2	1	3
	Pneumonic	8	1	2
	Typhoidal	10	1	0
Clinical sample for isolation or DNA detection of *F. tularensis*	Lymph node tissue	6	9	39
	Lymph node suppuration	0	9	13
	Skin ulcer exudate	4	0	1
	Subcutaneous abscess	5	0	0
	Psoas abscess	0	1	0
	Pharyngeal exudate	3	0	5
	Ear exudate	2	0	0
	Conjunctival exudate	2	0	1
	Pleural fluid	2	0	1
	Cerebrospinal fluid	1	0	0
	Blood	17	0	0
	Serum	0	1	2
Fluoroquinolone treatment before sampling		2/42	15/15	0/55
Fluoroquinolone treatment failure or relapse		2/42	15/15	16/55

2.3. MIC determination

The MICs of several antibiotics for clinical strains of *F. tularensis* were determined using a previously described procedure [24], taking into account the recommendations of the Clinical and Laboratory Standards Institute [30]. The FQs tested were ciprofloxacin, levofloxacin (Fresenius Kabi, Sèvres, France) and moxifloxacin (Bayer, Puteaux, France). Erythromycin (Fluka, Lausanne, Switzerland) was also tested for biovar identification [31,32]. Each MIC experiment included a negative control (no bacteria), a growth control (no antibiotics), and three control strains (*E. coli* ATCC25922, *S. aureus* ATCC29213, and *P. aeruginosa* ATCC 27853).

Table 2
Fluoroquinolone MICs (mg/L) for *F. tularensis* strains tested in this study.

Strains	Ciprofloxacin	Levofloxacin	Moxifloxacin
F. tularensis subsp. *holarctica* live vaccine strain (FthLVS)	0.016	0.016	0.032
Francisella novicida strain CIP 56.12	0.064	0.064	0.125
Fth1P14 (*gyrA83* mutation, T83I amino acid change)	32	32	32
Fth2P14 (*gyrA83* and *gyrA87* mutations, T83K and D87Y amino acid changes)	32	32	64
Fno1P14 (*gyrA87* mutation, D87Y amino acid change)	32	32	64
5 LVS-derived lineages with *gyrA83* mutation (MICp)	0.25–1		
5 LVS-derived lineages with *gyrA87* mutation (MICp)	0.125–0.5		
42 clinical strains of *F. tularensis* subsp. *holarctica*	0.016–0.064	0.032–0.064	0.125–0.25