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Recherche de nouvelles stratégies thérapeutiques pour le traitement de la tularémie : résistances bactériennes chez *Francisella tularensis* et développement de nouveaux antibiotiques bis-indoliques de synthèse

Yvan Caspar

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THÈSE

Pour obtenir le grade de

**DOCTEUR DE LA COMMUNAUTE UNIVERSITE
GRENOBLE ALPES**

Spécialité : **Virologie – Microbiologie - Immunologie**

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Hospitalier, Université Grenoble Alpes

préparée au sein du **Laboratoire TIMC-IMAG**
dans l'**École Doctorale Chimie et Sciences du Vivant**

Recherche de nouvelles stratégies thérapeutiques pour le traitement de la tularémie : résistances bactériennes chez *Francisella tularensis* et développement de nouveaux antibiotiques bis-indoliques de synthèse

Thèse soutenue publiquement le **22 Mai 2017**,
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LISTE DES ABREVIATIONS

ADN : acide désoxyribonucléique

ARN : acide ribonucléique

BCYE : *buffered charcoal yeast extract*

canINDELs : insertions/délétions canoniques

CDC : *Centers for Disease Control*

CLSI : *Clinical and Laboratory Standards Institute*

CMI : concentration minimale inhibitrice

CNR : Centre National de Référence

DCM : Département de Chimie Moléculaire

DI50 : dose infectieuse 50

DL50 : dose létale 50

ECDC : *European Center for Disease Control and Prevention*

EFL : endosymbiontes *Francisella*-like

ELISA : *enzyme-linked immunosorbent assay*

INDELs : insertions/délétions

InVS : Institut de Veille Sanitaire

LNSB3 : laboratoire de niveau 3 de sécurité biologique

LPS : lipopolysaccharide

LVS : *live vaccine strain*

MALDI-TOF : *matrix assisted laser desorption ionisation- time of flight*

MLVA : *multi-locus variable number tandem repeat analysis*

MRSA : *methicillin-resistant Staphylococcus aureus*

OMS : Organisation Mondiale de la Santé

PCR : *polymerase chain reaction*

PSM : poste de sécurité microbiologique

QRDR : *quinolone resistance determining region*

RD : région de différence

SELDI-TOF : *surface enhanced laser desorption ionisation- time of flight*

SNP : *single nucleotide polymorphism*

TLR : *Toll-like receptor*

UE : Union Européenne

UFC : unité formant colonie

VISA : *vancomycin-intermediate Staphylococcus aureus*

WHO : *World Health Organization*

1. INTRODUCTION GENERALE :

la tularémie

La tularémie est une zoonose dont l'agent responsable est *Francisella tularensis*. Il s'agit d'une bactérie hautement infectieuse pour l'homme, notamment en cas de contamination par voie respiratoire, ce qui a conduit à sa classification dans la catégorie A des agents potentiels de bioterrorisme par le CDC aux Etats-Unis (Centers for Disease Control, Atlanta, USA). Avant l'apparition des traitements antibiotiques, la tularémie était fatale dans la moitié des formes septicémiques ainsi que dans 5% des formes ulcéroganglionnaires (1). Ce n'est qu'à partir de 1947 que le pronostic de la maladie s'est fortement amélioré grâce à un aminoside, la streptomycine. Puis la tétracycline a permis un traitement par voie orale des formes peu sévères de la maladie. Les modifications structurales de ces deux familles d'antibiotiques, permettant d'améliorer leurs propriétés pharmacodynamiques et de réduire leurs effets indésirables, ont depuis conduit à la commercialisation et à l'utilisation préférentielle de la gentamicine et de la doxycycline pour le traitement de la tularémie. La première utilisation des fluoroquinolones dans le traitement de la tularémie est bien plus tardive car elle ne date que de 1989, lors de la description du succès obtenu chez 5 patients finlandais avec la ciprofloxacine ou la norfloxacine. (2) La faible prévalence de la maladie ne permet pas de conduire des études cliniques randomisées, en double aveugle afin de comparer l'efficacité de ces différents antibiotiques. En France, les formes cliniques majoritaires déclarées à Santé Publique France (ex. InVS) sont les formes ganglionnaires et ulcéro-ganglionnaires (> 50%). On observe environ 20% de formes systémiques (pulmonaires et typhoïdiques principalement) et 30% de cas nécessitant une hospitalisation. (3) Actuellement, bien que très rarement fatale, la tularémie conduit à des situations d'échecs thérapeutiques et de récurrences dans 5 à 40% des cas malgré un traitement antibiotique adapté. La recherche de nouvelles stratégies thérapeutiques pour le traitement de la tularémie demeure donc indispensable.

1.1. Historique de la maladie

La tularémie est décrite depuis plus d'un siècle, à la fois comme la « maladie des chasseurs » ainsi que comme une fièvre associée aux lapins, aux lièvres, aux taons ou encore aux lemmings, autant de reflets des principaux vecteurs classiquement associés à cette pathologie. Elle est également connue sous le nom de maladie d'Ohara ou Yato-byo au Japon. La maladie a été vraisemblablement décrite il y a plusieurs siècles. Des symptômes ressemblant à la tularémie ont été décrits en 1653 chez des lemmings en Norvège et dans des rapports médicaux européens anciens, puis au Japon dans la première moitié du 19^{ème} siècle par Homma Soken. (1,4–6)) Néanmoins la première description détaillée de la pathologie est le fait de Mc Coy. Alors Directeur du laboratoire de recherche sur la peste du service public de santé américain, il fut envoyé en Californie en 1909. Des cas suspects de peste étaient observés chez des rongeurs suite à l'important tremblement de terre de San Francisco de 1906. Il décrivit ainsi en 1911 une épidémie affectant les écureuils terrestres (*Citellus beecheyi*) du comté de Tulare en Californie mais dont les caractéristiques ne ressemblaient pas au bacille de la peste lors de l'observation microscopique de tissus animaux infectés. (7–9) La bactérie ne put être isolée par Mc Coy et Chapin qu'à partir de 1912 grâce à l'utilisation d'un milieu de culture supplémenté avec du jaune d'œuf coagulé. Après 3 à 5 jours d'incubation, des colonies de 0,3 à 0,7 μ M apparaissaient transparentes et coalescentes entourées d'un halo capsulaire clair. Mc Coy et Chapin nommèrent la bactérie *Bacterium tularense* en référence au site de découverte de ce pathogène. (10) Ils prouvèrent que cette bactérie était responsable de la pathologie observée et étudièrent les lésions engendrées chez les écureuils terrestres. Elles se caractérisaient le plus fréquemment par la présence de bubons. Ils mirent également en évidence la transmission de cette bactérie à plusieurs autres espèces de mammifères parmi lesquels les cochons d'Inde, les souris, les lapins, les singes et les marmottes, alors que les rats (*Mus norvegicus*) n'étaient que peu sensibles à l'infection. Les morsures de puce furent identifiées comme possible vecteur de la maladie. (10)

Le premier cas humain authentifié ne fut publié que 2 ans plus tard (en 1914) par Wherry et Lamb, qui isolèrent la bactérie chez un commis de cuisine dans un restaurant de l'Ohio (USA). (11) Ce dernier souffrait d'une conjonctivite de l'œil gauche avec des

adénopathies pré-auriculaires et cervicales, associées à de la fièvre et à une prostration. Wherry et Lamb reproduisirent les symptômes chez le lapin et le cochon d'Inde. (11) Le second cas identifié fut décrit chez une fermière qui présentait des symptômes analogues. (12) Les lapins furent identifiés comme potentielle source de la contamination. Wherry observa que cette bactérie apparaissait sous la forme de bacilles au sein des tissus frais de lapins infectés après coloration par l'aniline et le violet de gentiane, mais que la bactérie prenait un aspect coccoïde quelques heures après la mort de l'animal. (12)

En 1919, l'officier de santé publique Edward Francis fut envoyé en Utah pour investiguer des cas de fièvre au long cours consécutifs à des piqûres de taons, associés à des adénopathies régionales satellites de la zone cutanée de la piqûre, certains patients présentant également une prostration. Plus de vingt cas, dont un fatal, avaient été rapportés entre 1917 et 1919. (13) Francis réussit à reproduire la maladie en inoculant des échantillons humains chez des lapins et des cochons d'Inde. Il réussit à cultiver la bactérie et la décrivit comme similaire à celle identifiée par Mc Coy. (13) L'isolement de la bactérie à partir de sang humain, ainsi que la transmission de cette bactérie via des arthropodes hématophages se nourrissant chez un patient aux décours d'une bactériémie conduisit au nom de tularémie. (1,6)

Après plusieurs années de recherche, E. Francis publia un important manuscrit qui apporta les preuves expérimentales de la transmission de la tularémie via les morsures de taons, les puces portées par les lapins et les souris, ainsi que par les punaises de lit. (Figure 1) Il proposa dans cet ouvrage un nouveau milieu de culture glucosé supplémenté avec du sang ou du sérum, et de la cystéine, permettant l'isolement de cette bactérie. Il décrivit un test d'agglutination pour le diagnostic sérologique rétrospectif de la tularémie. Ce manuscrit contenait également les premiers avertissements de sécurité concernant la virulence de cette bactérie pour l'homme car E. Francis y rapportait six cas de tularémie acquis par du personnel de laboratoire. (8,9,14) E. Francis résuma ses connaissances sur la tularémie dans une deuxième publication en 1928, fort de son expérience de plus de 800 cas humains. (15) La transmission de la maladie par morsure de tique fut documentée entre 1924 et 1934 pour les espèces de tiques *Dermacentor andersoni*, *D. variabilis* et *Amblyomma americanum*. (6,9,16–18)



Figure 1 : Sir Edward Francis (d'après les archives de l'Institut Smithsonian disponibles sur www.siarchives.si.edu)

Au Japon, une pathologie humaine appelée Yato-byo (maladie du lièvre) transmise lors d'un contact cutané avec un lièvre contaminé et comparable à la tularémie fut mise en évidence par Hachiro Ohara qui collabora avec E. Francis pour prouver que les deux affections étaient causées par le même agent pathogène. (5,8,19) Ohara mit au point un test d'intradermoréaction utile pour le diagnostic précoce de la maladie, ce qui n'était pas possible avec le test d'agglutination sérologique de Francis, et utilisable pour le diagnostic à grande échelle en cas d'exposition de masse à cette bactérie. (20)

Après sa dénomination initiale de *Bacterium tularensis*, puis sa classification transitoire en *Pasteurella tularensis* dans les années 1920, l'agent de la tularémie fut dénommé *Francisella tularensis* en 1959, en reconnaissance de la contribution de Francis à la compréhension de la tularémie. (21,22) Les analyses plus récentes basées sur l'hybridation ADN/ADN et le séquençage de l'ADNr16S confirmèrent la validité du genre *Francisella*, au sein

de la classe gamma des protéobactéries. (4,8,23) La première séparation au sein de l'espèce *F. tularensis* fut le fait de N. Olsufiev qui distingua en 1959 deux biovars : *F. tularensis* biovar *tularensis* responsable d'une forme sévère de la tularémie chez l'homme et identifié uniquement dans le « Nouveau Monde », c'est à dire en Amérique ; et *F. tularensis* biovar *palearctica* qui ne donnait lieu qu'à une forme clinique modérée de la tularémie dans « l'Ancien Monde », c'est à dire en Europe. (24) Cette taxonomie fut modifiée deux ans plus tard lorsque Jellison démontra que deux variants étaient en réalité présents en Amérique du Nord. Ce dernier distingua les souches de « type A » présentes uniquement en Amérique du Nord, des souches de « type B » présentes dans tout l'hémisphère Nord. (25,26) Cette classification est toujours utilisée actuellement. En 1970, Olsufiev proposa la dénomination des deux types de *F. tularensis* sous la forme de deux sous-espèces, choisissant les dénominations de *F. tularensis* subsp. *tularensis* pour les souches de type A et de *F. tularensis* subsp. *holarctica* pour les souches de type B correspondant au précédent biovar *palearctica*. (27) La troisième sous-espèce *F. tularensis* subsp. *mediasiatica* fut ajoutée par la suite par Aikimbaev pour désigner des souches présentes dans la zone d'Asie Centrale de l'URSS. (28)

L'analyse de la résistance aux antibiotiques des souches de *F. tularensis* subsp. *holarctica* permit à Kudelina et Olsufiev à la fin de années 1970 de définir deux biovars au sein de cette sous-espèce. Lors de l'analyse de la sensibilité de 330 souches à l'érythromycine, ils remarquèrent qu'un tiers des souches étaient sensibles à 25 mg/L d'érythromycine alors que les deux-tiers restant étaient capables de pousser sur des milieux contenant 6400 mg/L d'érythromycine, sans qu'aucune souche ne soit dans la zone intermédiaire. Aucune différence de virulence, de propriétés biochimiques ou de résistance à d'autres antibiotiques n'étaient corrélées à cette dichotomie des souches au sein de la sous-espèce *F. tularensis* subsp. *holarctica*. Cependant ils observèrent que la répartition des souches était singulière, avec une prédominance des souches sensibles à l'érythromycine en Europe de l'Ouest, dans l'Est de la Sibérie et dans l'Est de l'Asie, alors que les souches résistantes à l'érythromycine étaient regroupées en Europe Centrale, dans la partie européenne de l'URSS et la partie Ouest de la Sibérie. Ils proposèrent donc en 1980 de distinguer les souches de *F. tularensis* subsp. *holarctica* sensibles à l'érythromycine en tant que *F. tularensis* subsp. *holarctica* biovar I et les souches résistantes en tant que biovar II. (29,30) Toutes les souches de *F. tularensis* subsp.

holarctica présentes en Amérique du Nord appartenait en revanche au biovar I. (29,30) Ces deux biovars furent complétés deux ans plus tard par un troisième biovar appelé *japonica* pour désigner les souches japonaises qui sont biochimiquement et phylogénétiquement différenciables des deux autres biovars (28,30,31) Des souches de biovar *japonica* ont également été détectées plus récemment en Chine et en Turquie. (32,33)

La recherche d'un vaccin efficace contre la tularémie débuta dès les années 1930 mais les premiers vaccins préparés à partir de bactéries inactivées ne permirent pas d'induire une protection suffisante contre les souches les plus virulentes de *F. tularensis*. La production d'une souche vivante mais de virulence atténuée fut alors entreprise afin d'obtenir un vaccin efficace. Une telle souche fut obtenue par l'URSS qui procéda à des campagnes de vaccination de masse dès 1946. Une ampoule de cette bactérie atténuée fut transférée en 1956 de l'Institut Gamaleia en URSS à l'Institut de recherche médicale de l'armée américaine (Fort Detrick, Maryland) où fut sélectionnée la souche atténuée LVS (Live Vaccine Strain) couramment utilisée aujourd'hui comme modèle pour l'étude de *F. tularensis*. Cette souche atténuée permet de réduire drastiquement l'incidence des formes pulmonaires graves de la tularémie au sein du personnel travaillant sur cette bactérie à Fort Detrick (de 5,7 à 0,27 cas/1000 employés à risque). (8) Ce vaccin n'est aujourd'hui plus utilisé en vaccination humaine (bien qu'il n'ait pas été remplacé par un vaccin plus efficace) car son efficacité est moindre sur les autres formes de tularémie et car il existe un risque théorique de réversion de cette souche vaccinale vers la virulence.

F. tularensis a également été inclus dans des programmes de création d'armes biologiques dès la Deuxième Guerre Mondiale, de par sa contagiosité et son fort pouvoir infectieux. De larges quantités d'armes biologiques contenant *F. tularensis* sous forme lyophilisée ont été stockées jusqu'à la fin de ces programmes dans les années 1960 par l'armée américaine et jusque dans les années 1990 par l'URSS. (4,34,35) En 1970, l'OMS estimait que la dispersion sous forme d'aérosol d'une arme biologique contenant 50 Kg de *F. tularensis* sur une zone urbaine de 5 millions d'habitants d'un pays développé conduirait au décès de 19000 personnes et à plus de 250000 cas sévères. De plus, sur les deux-tiers de la population qui n'auraient pas fui la zone en 48h, le traitement d'un million de personnes par 10 jours

d'antibiothérapie serait nécessaire. (36) Ces chiffres expliquent la classification de cette bactérie dans la classe A des agents potentiels de bioterrorisme par le CDC.

1.2. Taxonomie

Le genre *Francisella* appartient à la sous-classe gamma des protéobactéries. Il s'agit du seul genre présent dans la famille des *Francisellaceae*.

1.2.1. F. tularensis

L'espèce *F. tularensis* est actuellement scindée en trois sous-espèces, différant par leurs propriétés biochimiques et leur virulence :

- la sous-espèce *F. tularensis* subsp. *tularensis* (souche de type A) qui est la sous-espèce la plus virulente pour l'homme, localisée en Amérique du Nord. Quelques souches environnementales ou isolées de tiques ou d'acariens en Slovaquie et en Autriche ont été identifiées, mais l'analyse de leur génome complet a démontré qu'elles étaient très proches de la souche de laboratoire Schu S4 et donc probablement liées à la dispersion de cette souche de type A d'un laboratoire vers l'environnement. (37)
- la sous-espèce *F. tularensis* subsp. *holarctica* (souche de type B) qui est responsable d'une forme plus modérée de la tularémie et présente dans tout l'hémisphère Nord, avec de très rares cas décrits dans l'hémisphère Sud, en Australie et au Soudan. (38,39)
- la sous-espèce *F. tularensis* subsp. *mediasiatica* qui regroupe quelques souches isolées en Asie Centrale ; cette souche n'a jamais été isolée chez l'homme.

La sous-espèce *F. tularensis* subsp. *holarctica* est elle-même divisée en trois biovars : le biovar I correspondant aux souches sensibles à l'érythromycine, le biovar II correspondant aux souches résistantes à l'érythromycine, et le biovar *japonica* désignant les souches japonaises capables de fermenter le glycérol et dont la séparation phylogénétique a depuis été confirmée par séquençage de génomes complets. (31,33,40) Il a été démontré très récemment que la résistance aux macrolides des souches de biovar II est liée à la présence

d'une mutation ponctuelle A2059C dans les trois copies du domaine V du gène *rrl*, codant pour l'ARNr 23S de *F. tularensis* subsp. *holarctica* (41). Cette mutation strictement clonale et acquise par transmission verticale est retrouvée uniquement dans le groupe phylogénétique B.12 présent dont nous reparleront dans un prochain paragraphe et qui se superpose exactement aux souches de biovar II. (41)

1.2.2. Autres espèces de *Francisella* exceptionnellement pathogène pour l'homme

Deux clades génétiques distincts existent au sein du genre *Francisella*, différant notamment par le contenu en séquences d'insertions de leur génome et leur taux de recombinaison. (Sjödin 2012) Le premier clade est constitué des espèces *F. tularensis*, *F. novicida*, *F. hispaniensis* et d'endosymbiontes d'arthropodes nommés *Francisellalike* (EFL). Le deuxième clade contient les espèces *F. philomiragia* et *F. noatunensis*. (Sjödin 2012) L'adaptation progressive des espèces pathogènes à leurs hôtes semble associée à une perte de gènes plutôt qu'à l'acquisition de gènes de virulence. (42) Cette adaptation a conduit de façon indépendante à l'évolution des souches environnementales de *Francisella* vers des espèces pathogènes pour les mammifères (*F. tularensis*) dans le clade 1 ou pathogène pour les poissons (*F. noatunensis*) dans le clade 2. (Figure 2)

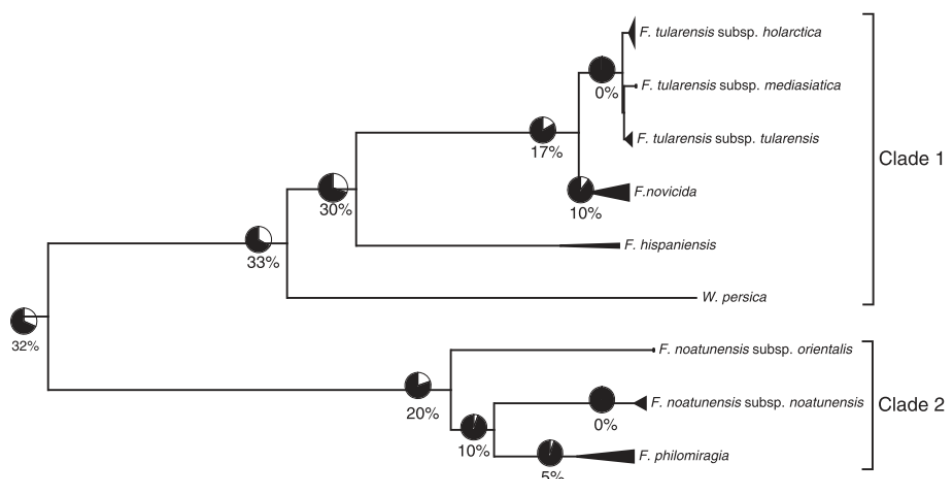


Figure 2 : Phylogénie du genre *Francisella* avec représentation de la proportion de gènes de chaque espèce présentant des évènements de recombinaison. (D'après Sjödin et al., 2012 (42))

1.2.2.1. *F. novicida*

F. novicida est la bactérie environnementale la plus proche de *F. tularensis*. Il s'agit d'une bactérie généralement avirulente pour l'homme. Seuls 12 cas humains ont été décrits avec un tableau clinique proche de celui de la tularémie. La plupart de ces cas d'infection ont été observés chez des patients immunodéprimés ou présentant d'autres pathologies sous-jacentes, après une contamination par voie hydrique. (43–45). La place de *F. novicida* au sein de la taxonomie du genre *Francisella* fait encore l'objet de débats controversés quant à son positionnement, soit en tant que quatrième sous-espèce au sein de l'espèce *F. tularensis*, soit en tant qu'espèce distincte. (46,46–48) Les deux dénominations de *F. novicida* et *F. tularensis* subsp. *novicida* coexistent actuellement dans la littérature, ce qui peut prêter à confusion. Au vu notamment de la forte différence de pathogénicité pour l'homme, il semble préférable de séparer *F. novicida* et *F. tularensis* en deux espèces distinctes. Les aspects réglementaires liées à l'isolement, la possession et l'utilisation de souches de *F. tularensis* sont très contraignants du fait de la virulence de cette espèce pour l'homme et ne doivent pas s'appliquer à *F. novicida*, qui est utilisé comme modèle avirulent pour étudier la physiopathologie des infections à *Francisella*.

1.2.2.2. *F. philomiragia*

A l'instar de *F. novicida*, il s'agit d'une bactérie pathogène opportuniste pour l'homme en cas d'immunodépression. Des cas d'infections par transmission hydrique, notamment après noyade ont été décrits, en adéquation avec le réservoir aquatique de cette bactérie. (49–52)

1.2.2.3. Endosymbiontes *Francisella*-like (EFL)

De découverte récente, les EFL sont des bactéries endosymbiontes d'arthropodes (notamment de tiques), cultivables uniquement sur milieux cellulaires et non pathogènes pour l'homme. (53) Ces EFL présentent de fortes similarités de séquence d'ADNr 16S avec le genre

Francisella, tout en étant plus proches de *F. tularensis* et *F. novicida* que de *F. philomiragia*. (42) En cas de recherche d'ADN de *F. tularensis* au sein de tiques, il est important d'utiliser des techniques moléculaires capables de différencier les EFL de *F. tularensis*. (212)

1.2.2.4. *F. hispaniensis*

Une seule souche de *Francisella hispaniensis* a été isolée chez un patient septicémique en Espagne. (47)

1.2.2.5. *F. noatunensis*

F. noatunensis est un pathogène de poisson, adapté à son hôte par rapport à son équivalent environnemental non pathogène *F. philomiragia*. Décrit pour la première fois chez la morue *F. noatunensis* est divisé en deux sous-espèces : *noatunensis* et *orientalis* qui sont en réalité des groupes paraphylétiques. (42,54,55)

1.2.2.6. Autres espèces

F. cantonensis et *F. gangzhouensis* ont été retrouvées dans des systèmes d'air conditionnés. Les autres espèces décrites au sein du genre *Francisella* sont *F. haliotida*, *F. piscida* et *F. asiatica*. (56)

1.3. Génomique moderne des souches de *F. tularensis*, phylogénie et phylogéographie

Les avancées technologiques en biologie moléculaire au cours des deux dernières décennies ont apporté de nouveaux outils permettant le typage et l'analyse de la phylogénie et de la phylogéographie des souches de *F. tularensis*, bien que l'évolution constante de ces techniques et la multiplicité des types de classification aient pu apporter de la confusion. Les techniques de typage classiques basées sur le séquençage de l'ADNr 16S ne permettent que la différenciation des espèces *F. tularensis*, *F. philomiragia* et *F. novicida*. La distinction des sous-espèces de *F. tularensis* n'est pas possible par cette technique. (57) Les techniques d'électrophorèse en champ pulsé (58,59), d'analyse multi-locus de séquences répétées en tandem en nombre variable (MLVA : multi-locus variable number tandem repeat analysis) (60–62,62,63), d'analyse de régions de différence (RD) (64,65), et de séquençage des génomes complets avec recherche de Single Nucleotide Polymorphism (SNPs) ou d'insertions/délétions (INDELs) canoniques (31,66–68) ont permis successivement de distinguer de nombreux clades et sous-clades au sein des sous-espèces *tularensis* et *holarctica*. (69)

Actuellement le génotypage des souches de *F. tularensis* repose sur le séquençage de génomes complets avec la recherche de SNP canoniques (canSNPs) et de mutations (insertions ou délétions) canoniques (canINDELs) (66). Avec cette technique de génotypage, de nouvelles souches peuvent être facilement implémentées dans l'arbre phylogénétique construit *in silico* à partir des données disponibles des séquences des génomes complets. Il est possible d'individualiser l'ensemble des souches typées tout en détaillant précisément les relations phylogénétiques entre les souches.

En résumé, la phylogénie de *F. tularensis* divise actuellement les souches de type A en deux clades A.1 et A.2 (Figure 3), elles-mêmes scindées respectivement en trois (Figure 4) et quatre sous clades. (31,70)

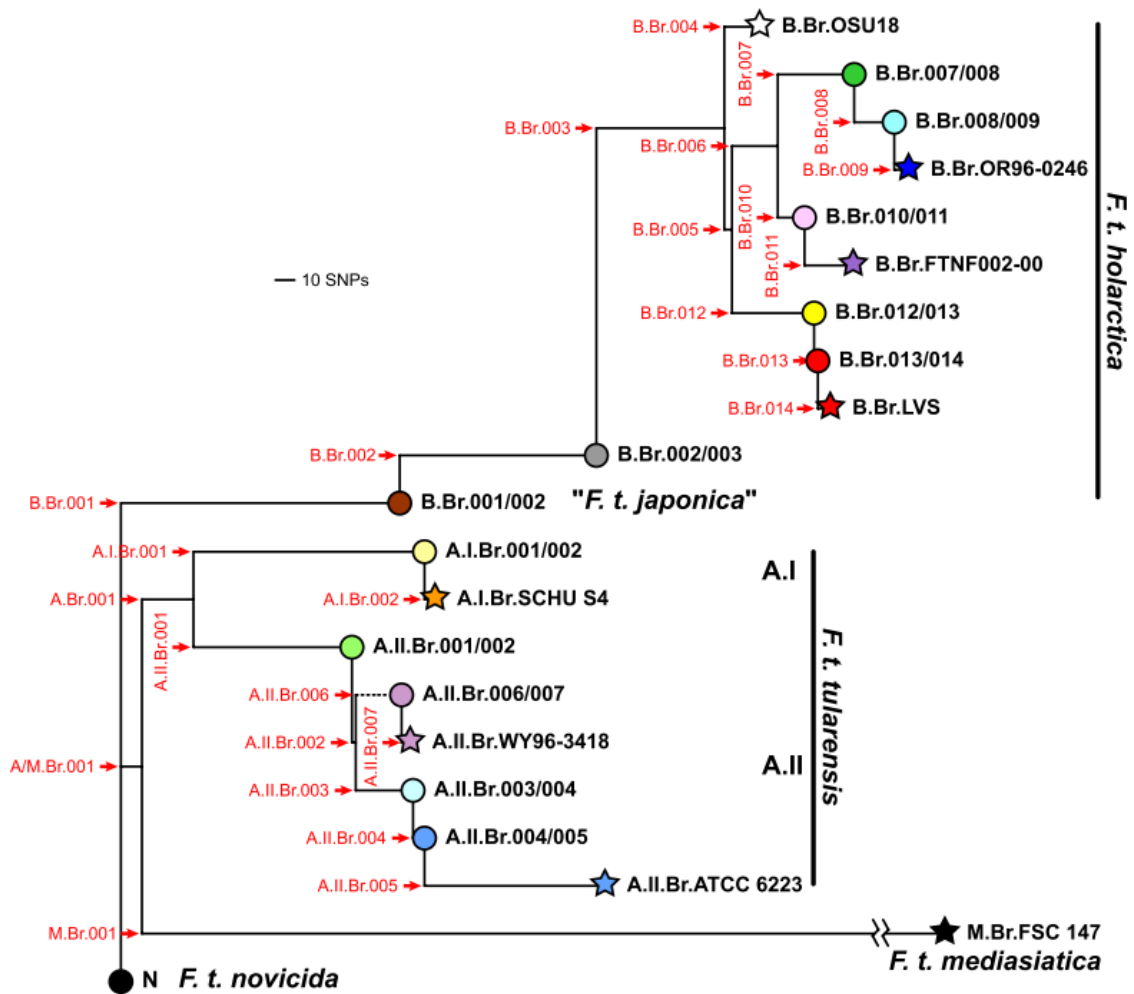


Figure 3 : Phylogénie de *F. tularensis*, divisant les souches de type A et B en plusieurs clades et sous-clades à partir de SNP canoniques. Les sous clades sont nommés à partir des 2 SNP canoniques permettant leur individualisation. Les étoiles indiquent les sous-clades terminaux définis par un des 8 génomes de *F. tularensis* qui ont permis la construction de cet arbre phylogénétique. Les cercles regroupent toutes les souches du sous-clade concerné. (D'après Vogler et al., 2009 (31)).

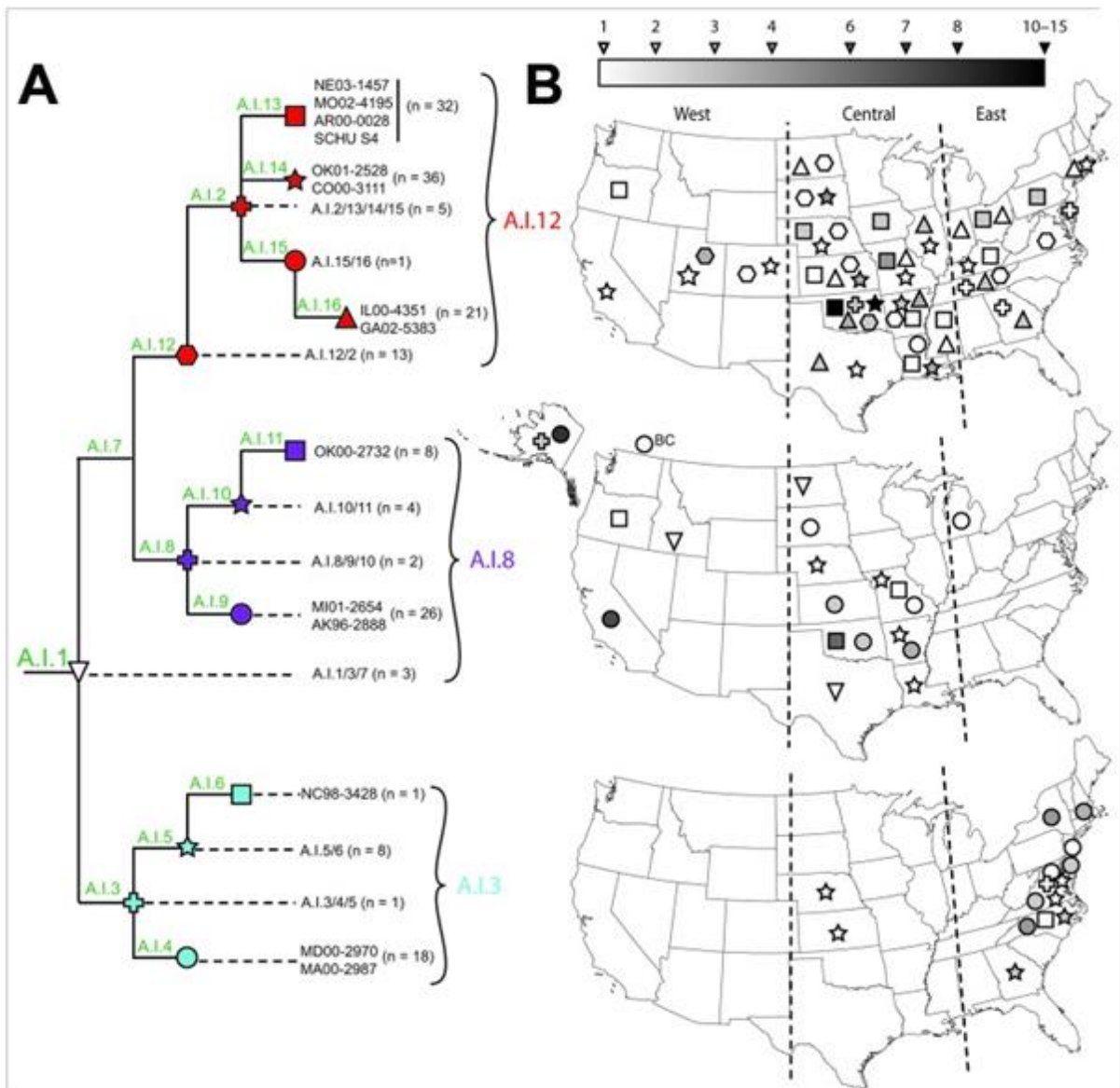


Figure 4 : Distribution géographique et analyse de la phylogénie des souches de type A1 par séquençage de génomes complets et recherche de SNPs canoniques. Le gradient d'opacité des symboles sur les cartes est fonction du nombre de souches (foncé = nombre de souches élevé). (D'après Birdsell et al., 2014 (70))

Les souches de type B sont également divisées en de nombreuses branches phylogénétiques (Figure 3, 5, 6). Les principales sont :

- le clade B.4 correspondant à la majorité des souches Nord-américaines

- le clade B.6 regroupant principalement les souches de biovar I d'Europe de l'Ouest (France, Espagne, Pays-Bas, Italie, Suède, Norvège, Finlande) sensibles à l'érythromycine
- le clade B.12 contenant les souches de biovar II d'Europe de l'Est et Centrale (Autriche, République Tchèque, Hongrie, Ukraine, Roumanie, Russie, Géorgie, et Slovaquie) résistantes à l'érythromycine
- le clade B.16 englobant les souches japonaises (biovar *japonica*) mais également des souches retrouvées plus récemment en Turquie, en Chine et au Tibet (Figure 5). (31,33,40,67,71-73)

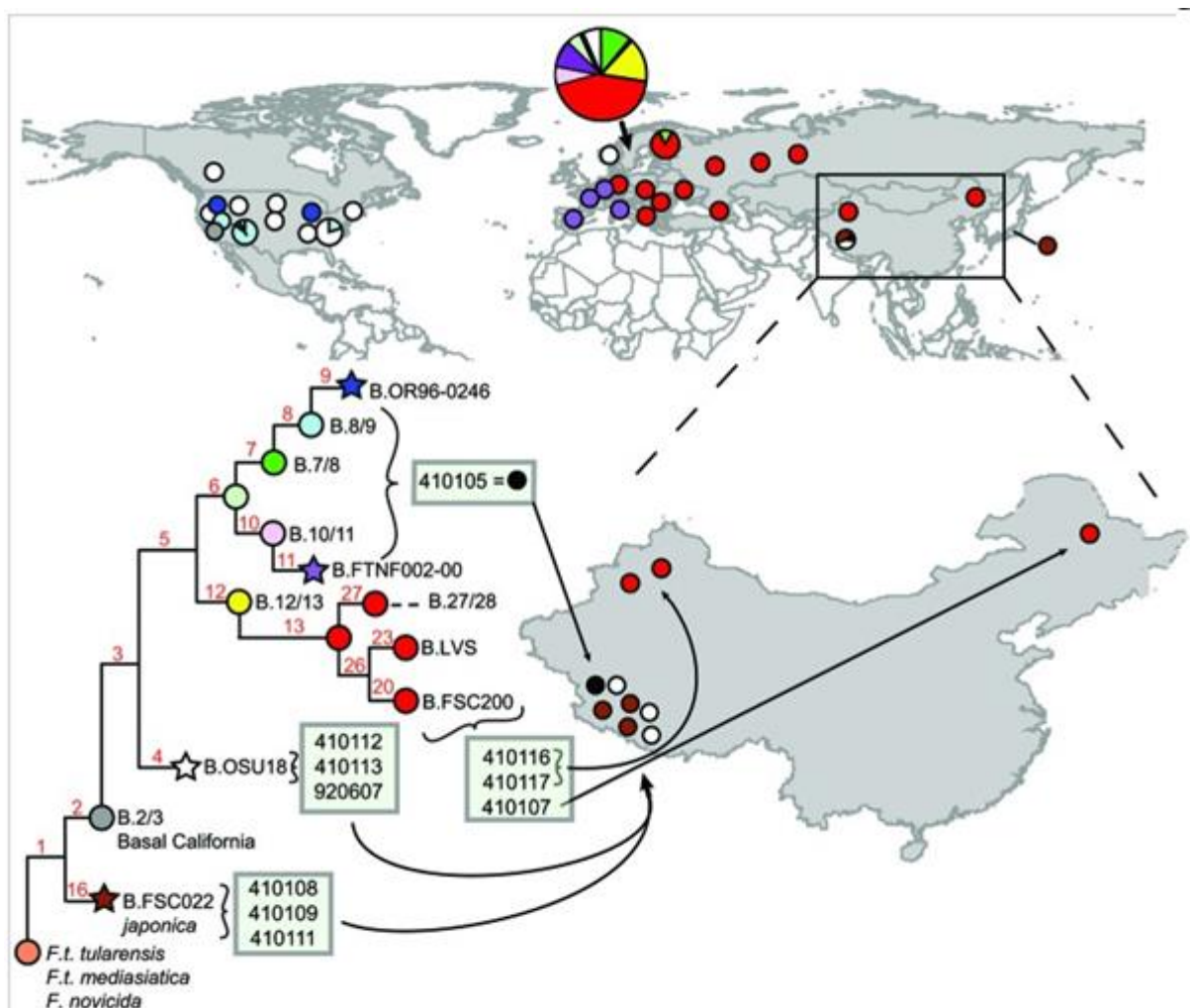


Figure 5 : Phylogéographie des souches de type B par analyse des SNP canoniques. Les numéros d'isolats encadrés indiquent la position des souches chinoises dans cet arbre phylogénétique. (D'après Wang et al., 2014 (33))

En Europe la plus grande diversité génétique des souches de type B est observée dans les pays scandinaves où l'on trouve des souches appartenant à la fois aux clades B.4, B.6 et B.12. (74) L'Allemagne et la Suisse forment l'interface géographique entre les souches appartenant aux clades B.6 et B.12. (75,76) Des souches de biovar I et II sont donc retrouvées dans ces deux pays. Enfin, toutes les souches françaises, espagnoles, italiennes et néerlandaises responsables de cas humains font parties du clade B.6 et sont regroupées dans le sous-clade particulier B.FNTF002-00. Ce dernier est issu de l'expansion clonale d'une souche caractérisée par une délétion caractéristique de 1,59 kb dans la région de différence RD23 qui permet de confirmer rapidement l'appartenance d'une souche à cette sous-clade (à l'exception d'une souche identifiée en Suède possédant également cette délétion) (31,77,78).

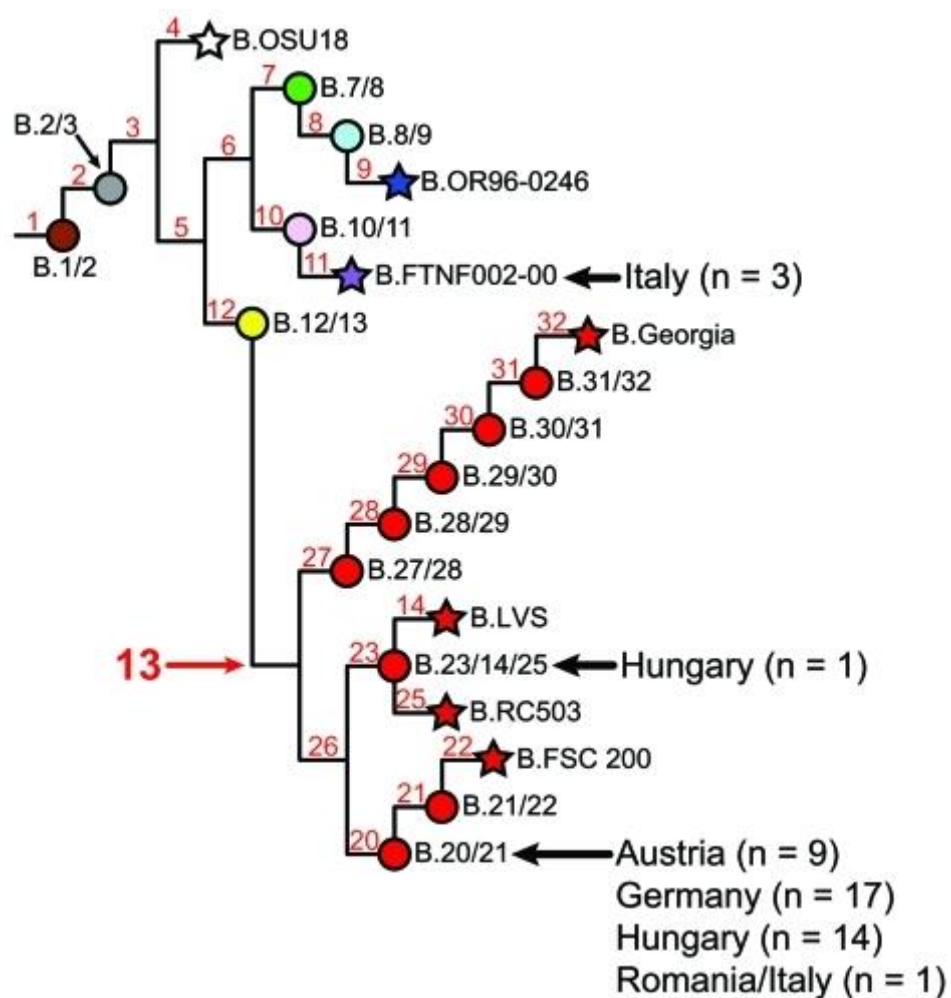


Figure 6 : Phylogénie des souches de type B en Europe par analyse des SNP canoniques. (D'après Gyuranecz et al., 2012 (67))

1.4. Morphologie et caractéristiques biochimiques de *Francisella tularensis*

F. tularensis est un coccobacille à Gram négatif, intracellulaire facultatif, de 0,2-0,7 μm de longueur sur 0,2 μm de largeur, dont la coloration de Gram peut être variable. Son aspect peut se révéler pléiomorphe lors de l'observation de tissus infectés ou lorsque la croissance de la bactérie a atteint la phase stationnaire. *F. tularensis* est une bactérie immobile, aérobie stricte qui ne produit ni toxines, ni spore, ni gaz. (79) Du point de vue des tests biochimiques rapides d'orientation, la catalase est faiblement positive alors que l'oxydase et l'uréase sont négatives.



Figure 7 : Colonies de *F. tularensis* subsp. *holarctica* LVS sur gélose chocolat + Polyvitex™. (CNR des *Francisella*, CHU de Grenoble)

F. tularensis est auxotrophe pour la cystéine ce qui rend nécessaire l'utilisation de milieux enrichis (ex : gélose chocolat + PolyViteX™) pour cultiver cette bactérie (Annexe 1). Sa croissance est accrue en atmosphère enrichie avec 5% de CO₂. Après 2 à 4 jours d'incubation, les colonies apparaissent de forme convexe, d'aspect opalescent blanchâtre ou grisâtre pâle, sans élasticité ni résistance lorsqu'on les prélève. Un fin halo clair entoure la colonie correspondant à la présence d'une capsule. (Figure 7) Cette capsule de 100 à 250 kDa, ainsi que la présence d'une activité citrulline uréidase ou citrullinase sont corrélés à la virulence des souches et peuvent être présentes chez les sous-espèces *tularensis*, *holarctica* et *mediasiatica*. (79–82) La richesse en acides gras saturés (acide caprique : C_{10:0}, acide laurique : C_{12:0}, acide myristique : C_{14:0}) et en acides gras saturés hydroxylés (3-OH C_{16:0}, 3-OH C_{18:0}) de la capsule et de la paroi bactérienne de *F. tularensis* est également singulière et peut être identifiée par chromatographie en phase gazeuse. (83) La structure particulière du lipide A du LPS de *F. tularensis* est à l'origine d'un faible effet endotoxinique du fait de l'absence d'activation de la voie des récepteurs TLR4 (Toll-Like Receptors) (84–86) *F. tularensis* est capable d'assimiler un nombre limité de glucides. (8) Les sous espèces *tularensis* et *holarctica* sont capables de fermenter le D-glucose alors que les sous espèces *tularensis*, *mediasiatica* et *holarctica* biovar *japonica* sont capables d'utiliser le glycérol comme source carbonée, ce qui permettait de différencier les sous-espèces avant l'avènement de la biologie moléculaire. (79,80)

1.5. Virulence des souches de *F. tularensis*

La virulence des souches de *F. tularensis* diffère entre les sous espèces *tularensis* et *holarctica*. Des différences de virulence ont également été observées au sein de la sous-espèce *tularensis*. La virulence des souches a été principalement étudiée chez le lapin, la souris et le cochon d'Inde. La différence de virulence des souches de type A et B peut être observée avec ces trois modèles si elle est exprimée en médiane du temps de survie mais elle ne peut être objectivée sous forme de dose létale 50 (DL50) que chez le lapin. (87–89) Les résultats obtenus chez le lapin montrent ainsi que la DL50 par voie sous-cutanée est inférieure à 10 UFC (Unité formant colonie) chez cet animal. L'injection sous-cutanée de 10 bactéries de type A

conduisent à une mortalité proche de 100%, alors que l'infection sous-cutanée ou par voie respiratoire par 1 million d'UFC d'une souche de type B ne parvient pas à tuer les lapins (DL100 estimée à 10^9 UFC).(24) De même *F. tularensis* subsp. *mediasiatica* et *F. tularensis* subsp. *holarctica* biovar *japonica* ont des DL50 supérieures à 10^6 UFC. (80) La virulence des souches de type B semble comparable chez le lapin quel que soit le biovar concerné.

Chez l'homme, la dose infectieuse 50 (DI50) est proche d'une bactérie par voie aérienne. La contamination par 50 UFC d'une souche de type A peut engendrer une infection très sévère, surtout en cas de transmission aérienne, alors que seules des infections modérées surviennent avec 12000 UFC d'une souche de type B. (Hornick, 2001; Jones et al.,)

Il existe également des différences de virulence au sein des souches de type A. L'analyse des souches par électrophorèse en champ pulsé (typage exprimé en chiffres romains) a dans un premier temps identifié des souches de clade A.I entraînant une mortalité humaine de 14%, bien supérieure à la mortalité des souches de type B (7%). Par contre, les souches de type A.II ne semblaient pas mortelles (0%) et donc de virulence inférieure aux souches de type B. (58) Cette différence de virulence a été confirmée par la suite chez la souris. (89) La comparaison des génomes des souches de type A.I et A.II dans le but d'expliquer leur différence de virulence n'a identifié des différences génétiques que dans un seul gène complet codant pour une protéine de fonction inconnue, ainsi que de multiples SNPs, petites insertions/délétions, variations de séquences d'insertion et grands réarrangements génomiques. (92) Des analyses plus précises ont permis d'affiner les distinctions au sein des souches de type A.I en terme de virulence. Les souches catégorisées en A.Ib ont été associées à une mortalité de 24% chez l'homme, alors que les souches A.Ia n'étaient fatales que dans 4% des cas. Ces différences ont également été confirmées en modèle murin. (59,89) Seules ces différences de génotypes permettaient d'expliquer cette variation du taux de mortalité chez l'homme. Aucun facteur lié à l'hôte tel que l'âge ou la pathologie sous-jacente n'ont pu être relié à la différence de mortalité observée. (59)

L'avènement du séquençage complet de génome (typage exprimé en chiffre arabes) a depuis divisé les souches de type A.1 en A.1.3, A.1.8 et A.1.12. Toutes les souches préalablement identifiées dans la classe A.Ia sont regroupées dans la classe A.1.12 alors que les souches hypervirulentes A.Ib sont réparties dans les trois classes. Des tests de confirmation

chez la souris ont révélés que les souches A.1.12 sont moins virulentes que les souches A.1.3 mais aucune étude n'a étudié ou réévalué la mortalité chez l'homme au sein de chacune de ces sous-clades. (70,89) On peut supposer que seule une des deux sous clades A.1.3 ou A.1.8 est responsable d'une très forte mortalité pour l'homme mais ceci doit être confirmé. A l'opposé, aucune différence de virulence n'a été démontrée au sein des souches de type A.2 ou de type B. (59,93)

Au vu de ces données, il est donc important de porter une attention particulière au modèle animal et à la souche utilisée lors de l'analyse de la virulence de *F. tularensis*. En effet, la plupart des études sont conduites avec la souche *F. tularensis* subsp. *tularensis* SchuS4. Cette souche a été isolée d'un ulcère du doigt chez un patient originaire d'Ohio (USA) à la fin des années 1940. (94) Très virulente chez le lapin, cette souche originelle a été caractérisée par une DL100 de 1 à 10 UFC chez le lapin et avait démontré une virulence plus grande que les souches de type B sur des modèles de souris, lapins et cochons d'Inde. (24,95) Une hétérogénéité des colonies a permis d'identifier des colonies très virulentes présentant un aspect « lisse » (smooth). Un clone virulent de phénotype smooth n°4 a été sélectionné et renommé SchuS4. (95) Depuis ce jour, la souche SchuS4 est considérée comme la souche modèle pour l'étude de la pathogénicité des souches de type A. Or, premièrement cette souche a été manipulée et sub-cultivée dans de nombreux laboratoires, ce qui a pu conduire à des souches ayant évolué de façon indépendante les unes des autres, bien que toujours appelée SchuS4 et qui peuvent présenter des différences de pathogénicité entre les études. Deuxièmement, en 2006, Twine et al ont comparé leur clone de SchuS4 à une autre souche de type A (FSC033) et à d'autres clones de SchuS4 (FSC043 et FSC237). Leur étude a révélé que la souche la plus virulente était la souche FSC033 avec une DL inférieure à 10 UFC lors d'une administration intraveineuse de la bactérie chez la souris. (88) En revanche, ils ont observé que le clone de SchuS4 FSC043 était moins virulent que la souche de type B de virulence atténuée LVS, avec une DL50 supérieure à 10^8 CFU chez la souris. A l'opposé la souche FSC237 présentait la même DL100 que FSC033 mais une médiane de survie supérieure à FSC033. (88) La virulence des souches de type A utilisée lors d'expériences de pathogénicité doit donc être évaluée avec précaution avant de débiter des expérimentations animales. Cette différence de virulence entre les souches SchuS4 et FSC033 peuvent être expliquée par le fait que SchuS4

appartient aux souches de type A.Ia alors que la souche FSC033 est décrite comme appartenant au type A.I sans plus de précision. La pathogénicité accrue de FSC033 pourrait s'expliquer s'il s'agit d'une souche appartenant au clade hypervirulent A.Ib. Une deuxième étude plus récente de Molins et al., comparant la virulence de SchuS4 à des souches de type A.Ia, A.Ib, A.II et B a montré que SchuS4 présentait une virulence comparable à la souche de type B évaluée dans l'étude, et une virulence atténuée par rapport à tous les autres souches de type A testées. (96) Par conséquent, les résultats de pathogénicité obtenus avec la souche SchuS4 doivent être interprétés avec précaution. De plus il semble judicieux d'étudier plus particulièrement la pathogénicité des souches de type A.Ib, hypervirulentes pour l'homme, et de les inclure dans les études futures évaluant l'efficacité thérapeutique des antibiotiques actuels ou en développement pour le traitement de la tularémie.

1.6. Cycle de vie intracellulaire chez l'homme

F. tularensis est une bactérie intracellulaire facultative qui peut infecter et se multiplier dans une grande variété de tissus, mais qui peut également exister sous forme extracellulaire dans le sang des mammifères infectés. (97,98) Les principales cellules infectées par la bactérie sont les cellules phagocytaires (macrophages, cellules dendritiques, monocytes, polynucléaires neutrophiles). Les hépatocytes, les cellules épithéliales rénales ou alvéolaires, les cellules endothéliales et les fibroblastes sont également des cellules cibles. (99–102) L'invasion des hématies peut avoir lieu, sans réplication intracellulaire de la bactérie. A ce titre, les hématies représentent peut-être une niche permettant la persistance prolongée de la bactérie dans le sang et sa transmission par des arthropodes hématophages. (34,101) Les macrophages et les cellules dendritiques sont considérés comme étant le principal compartiment cellulaire de multiplication de *F. tularensis*. Ces cellules participent à la dissémination de la bactérie dans l'organisme. (98,100) La plupart des modèles intracellulaires sont de ce fait réalisés à l'aide de cellules phagocytaires mononuclées. L'interprétation des résultats d'infection cellulaire doit se faire avec précaution et tenir compte du type de cellule utilisé (macrophages humains, monocytes humain THP-1, macrophages murins J774) et du

type de souche bactérienne utilisée (*F. tularensis* LVS, *F. tularensis* Schu S4, *F. novicida*, souches cliniques...).

Le cycle intracellulaire de *F. tularensis* est bien caractérisé, même si tous les mécanismes moléculaires sous-jacents ne sont pas encore élucidés. Les principales étapes du cycle sont l'endocytose de la bactérie par la cellule, l'échappement de la bactérie hors du phagosome, la multiplication et la survie de la bactérie à l'intérieur du cytosol de la cellule infectée puis enfin, la libération de la bactérie suite à lyse de la cellule infectée ou la transmission de la bactérie de cellule à cellule par le mécanisme récemment découvert de trogocytose (Figure 8).

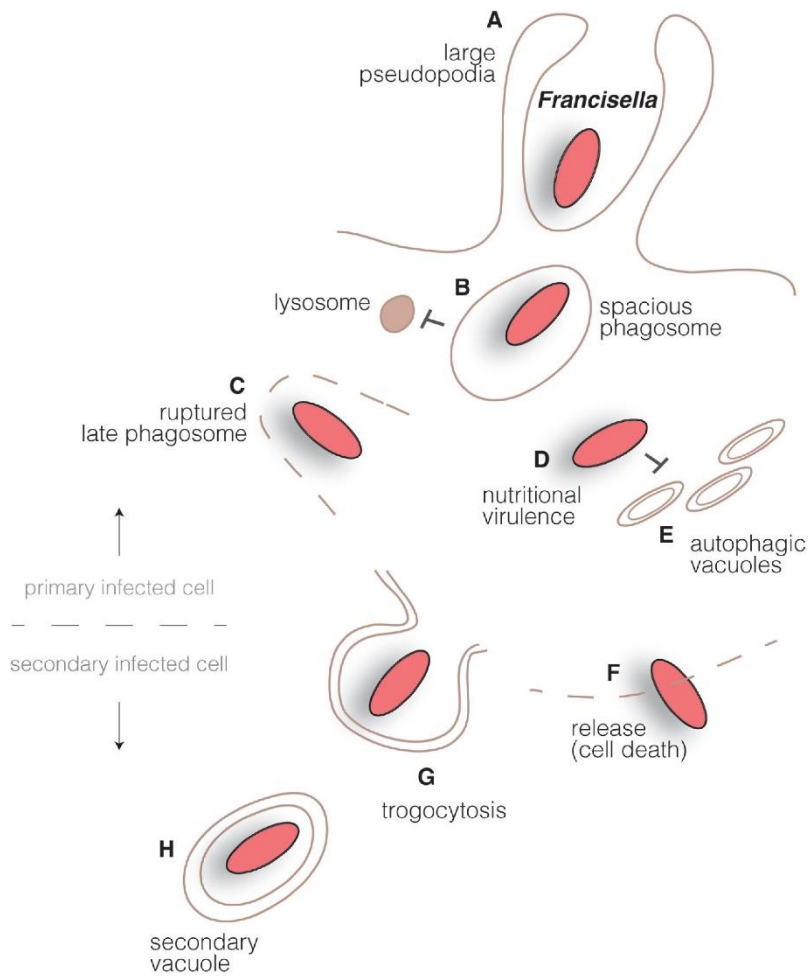


Figure 8 : Cycle intracellulaire de *Francisella*. L'entrée de la bactérie à l'intérieur des macrophages se fait par l'interaction avec des récepteurs à la surface des macrophages et la formation de pseudopodes (A). La bactérie est capable de survivre à l'intérieur de la cellule dans le phagosome (B), puis d'échapper à ce phagosome (C) pour atteindre le cytoplasme de la cellule et s'y multiplier (D). Le transfert de la bactérie d'une cellule à une autre se fait par lyse de la cellule infectée (F) ou directement de cellule à cellule par trogocytose (G). (D'après Pizarro-Cerda et al., 2016 (103))

1.6.1. Adhésion et Phagocytose

Les mécanismes de phagocytose et d'échappement hors de la vacuole d'endocytose de *F. tularensis* sont différents de ceux d'autres pathogènes bactériens intracellulaires tels que *Legionella*, *Listeria* ou *Salmonella*. La phagocytose de la bactérie se fait grâce à :

- l'adhésion de la bactérie à divers récepteurs présents sur la membrane des macrophages (ex : le récepteur du complément CR3 reconnaissant le facteur du complément C3 qui se fixe le lipopolysaccharide et la capsule de la bactérie, le récepteur du mannose (MR), le scavenger receptor A (SRA), les récepteurs du fragment Fc des IgG (FcγRs) et la nucleolin)(86,102,104)
- la formation d'un pseudopode asymétrique sous l'action de la polymérisation de l'actine, permettant l'internalisation de la bactérie à l'intérieur d'une vacuole d'endocytose qui rétrécit progressivement pour devenir un phagosome. (104,105)
- des mécanismes d'internalisation de la bactérie par macropinocytose qui ont été observés dans les cellules épithéliales. (106)

Grâce à la structure particulière du LPS de *F. tularensis*, cette bactérie n'active pas la voie de défense cellulaire via les TLR-4, mais les TLR-2 reconnaissent néanmoins les lipoprotéines à la surface de la bactérie et permettent d'initier la réponse inflammatoire. (103)

1.6.2. Echappement hors du phagosome et survie intracellulaire

Le phagosome contenant la bactérie interagit rapidement avec les endosomes précoces (acquisition de l'antigène endosomal précoce 1 (EEA-1) à la surface du phagosome) puis avec les endosomes tardifs (acquisition des marqueurs protéiques membranaire associés aux lysosomes LAMP-1 et 2), (86,99,104) mais la fusion avec les lysosomes n'a pas lieu (absence de cathepsine D à la surface du phagosome). (103) *F. tularensis* est capable de survivre à l'intérieur du phagosome en bloquant la formation de dérivés réactifs de l'oxygène via l'inhibition de la formation de la NADPH oxydase et via la réduction de l'acidification du phagosome. (98) La sortie de la bactérie hors du phagosome a lieu dans les premières heures après internalisation de la bactérie (50% des bactéries dans le cytosol 6h après l'infection).

(104). Certains des gènes impliqués dans cet échappement du phagosome sont regroupés au sein d'un îlot de pathogénicité des *Francisella* (FPI). Deux copies de cet îlot de pathogénicité sont présentes chez *F. tularensis* alors qu'une seule copie a été identifiée chez *F. novicida* et *F. philomiragia*. Cet îlot génomique regroupe entre 16 et 19 gènes participant pour certains à la formation d'un système de sécrétion de type VI (Figure 9), alors que d'autres sont toujours de fonction inconnue. (99,107,108)

A l'intérieur du cytosol, la bactérie doit proliférer et survivre aux mécanismes de défenses intracellulaires. En plus des gènes présents dans l'îlot de pathogénicité, des gènes (*mgIA*, *fevR* et *migR*) codant des régulateurs transcriptionnels semblent impliqués dans ces mécanismes et être sous la dépendance de la concentration en ppGpp (alarmone inhibant la synthèse des ARN) dans la bactérie. (86,99). D'autres gènes essentiels pour le métabolisme ou le catabolisme sont essentiels pour la croissance de *F. tularensis*. On peut citer la γ -glutamyl transferase qui permet à la bactérie de subvenir à ces besoins en cystéine à partir du glutathion intracellulaire, certains transporteurs d'acides aminés ou certaines protéines impliquées dans la biosynthèse des purines ou de l'uracile. (86,98,109)

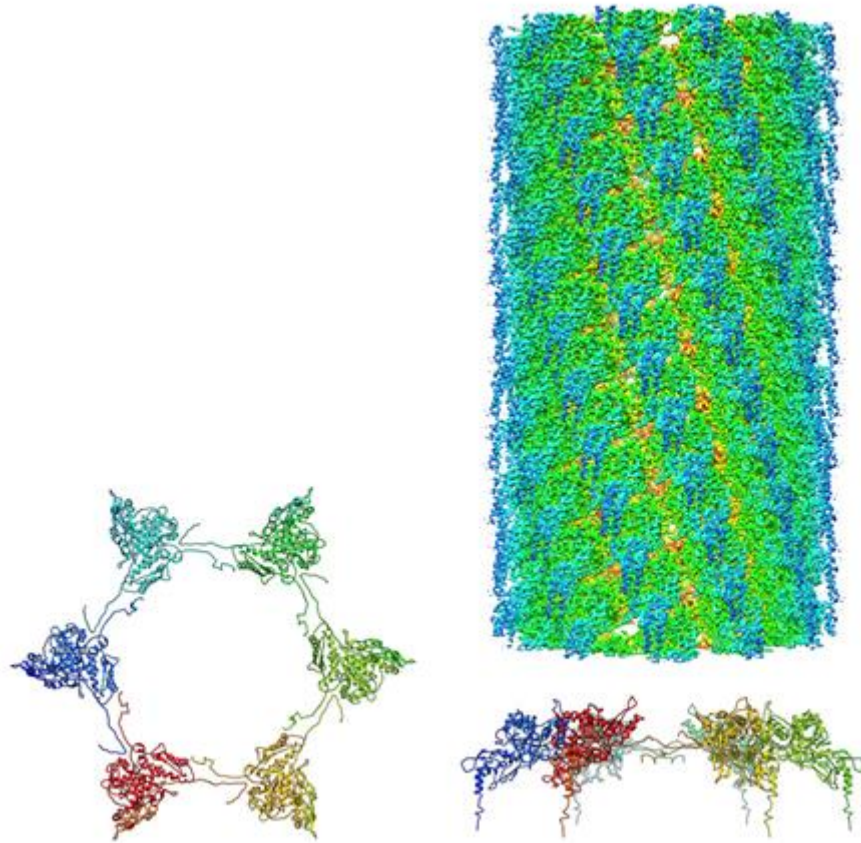


Figure 9 : Structure tridimensionnelle par cryo-électron-microscopie du système de sécrétion de type VI de *Francisella*, formé par assemblage des protéines IglA et IglB. Ce système de sécrétion de type VI est essentiel pour les fonctions de sécrétions, d'échappement au phagosome et de répllication intracellulaire de *Francisella*. (D'après Clemens et al., (107))

1.6.3. Diffusion de l'infection

La diffusion de cellule à cellule de l'infection par *F. tularensis* a lieu via deux mécanismes distincts. Le premier mécanisme identifié correspond à la dissémination de la bactérie d'une cellule à une autre lors de la lyse de la cellule infectée, suite à des mécanismes d'apoptose ou de pyroptose. (103) En 2016, un nouveau mécanisme de diffusion appelé trogocytose, n'impliquant pas la destruction de la cellule hôte, a été mis en évidence. Ce mécanisme permet l'échange de contenu intracellulaire entre deux cellules par contact et

bourgeonnement d'une partie de la membrane de la cellule « donneuse » piégeant une fraction de cytoplasme et potentiellement des bactéries au travers de la membrane de la cellule « receveuse » pour former une vacuole secondaire dans la cellule « receveuse ». Ce mécanisme permet ainsi le passage de bactéries d'une cellule infectée directement vers une cellule naïve sans lyse de la cellule infectée. (110)

1.7. Epidémiologie

Les cas de tularémie causés par la sous-espèce *Francisella tularensis* subsp. *tularensis* sont observés uniquement en Amérique du Nord, à l'exception de souches isolées d'arthropodes en Slovaquie et en Autriche et liées à la dispersion dans l'environnement de la souche de laboratoire SchuS4, comme mentionné plus haut. (37) A l'opposé, la sous-espèce *Francisella tularensis* subsp. *holarctica* est à l'origine de cas de tularémie dans tout l'hémisphère Nord. De rares cas liés à des souches de type B ont néanmoins été décrits dans l'hémisphère Sud :

- en Tasmanie en 2011, suite à la morsure d'une femme australienne par un possum à queue en anneau (*Pseudocheirus peregrinus*). (38) La présence de cette bactérie chez les possums à queue en anneau a depuis été confirmée en Australie. (111)
- au Soudan en 2012 au décours d'une bactériémie chez un patient présentant des adénopathies sous-mandibulaires. Mais l'identification bactérienne de la souche n'est pas formelle en ce qui concerne ce cas, car elle repose uniquement sur un diagnostic d'exclusion après isolement d'un coccobacille à Gram négatif n'appartenant pas au groupe des HACEK. (39)

L'épidémiologie de la tularémie est caractérisée par un niveau basal de cas sporadiques plus ou moins nombreux suivants le niveau d'endémie du pays concerné, avec la possibilité d'observer des épidémies (Tableau 1 et 2). Une surveillance nationale avec la notification obligatoire des cas confirmés de tularémie existe dans la plupart des pays de l'Union

Européenne (UE). Le Centre Européen de Contrôle et Prévention des Maladies (European Center for Disease Control and Prevention : ECDC) rapporte annuellement les cas de tularémie au sein de l'UE depuis 2005. D'après ces rapports, le nombre de cas de tularémie semble en diminution sur la période 2006-2014. Le nombre de cas confirmés par an passe de 1034 en 2006 à 526 en 2014 (range : 309 en 2013 – 1281 en 2007). En 2014 l'incidence des cas confirmés est donc de 0,11 cas/100 000 habitants (Tableau 2). Le pays ayant le plus fort taux de notification en 2014 est la Suède avec 1,56 cas/100 000 habitants.

	Total number of cases	Median per year (range)	Incidence per 100 000 inhabitants	Years of peak incidences
Austria	86	3 (0-19)	0.00-0.20	1998
Belgium	1	0 (0-1)	0.00-0.01	..
Bulgaria	308	7 (0-96)	0.00-1.20	1998, 2003
Croatia	97	4 (0-29)	0.00-0.60	1999
Czech Republic	1490	64 (36-255)	0.34-2.20	1999, 2008
Denmark	6	0 (0-4)	0.00-0.10	..
Estonia	9	0 (0-2)	0.00-0.15	..
Finland	4223	116 (15-926)	0.28-17.90	2000, 2003
France	325	16 (0-104)	0.00-0.16	2008
Germany	172	5 (0-31)	0.00-0.04	..
Hungary	1173	69 (15-148)	0.15-1.40	1997, 2006, 2010
Italy	125	4 (0-43)	0.00-0.07	2008
Latvia	6	0 (0-6)	0.00-0.29	..
Lithuania	15	0 (0-4)	0.00-0.14	..
Netherlands	1	0 (0-1)	0.00-0.00	..
Norway	529	18 (0-180)	0.00-3.66	2011
Poland	46	2 (0-8)	0.00-0.02	..
Romania	5	0 (0-4)	0.00-0.02	..
Slovakia	520	23 (5-133)	0.09-2.50	2002
Slovenia	21	1 (0-4)	0.00-0.20	..
Spain	1181	1 (0-585)	0.00-1.50	1997, 2007
Sweden	4622	241 (14-698)	0.20-7.80	2000, 2003, 2010
Switzerland	105	0 (0-40)	0.00-0.50	..
UK	2	0 (0-1)	0.00-0.00	..

Tableau 1 : Nombre de cas de tularémie chez l'homme et taux d'incidence en Europe entre 1997 et 2013. (D'après Maurin et Gyuranecz, 2016 (74))

Pays	2010		2011		2012		2013		2014			
	Cas	Taux	Cas	Taux	Cas	Taux	Cas	Taux	Cas rapportés	Cas confirmés	Taux	
Autriche	3	0	0	0	2	0	2	0	0	0	0	0
Belgique	0	0	0	0	0	0	1	0	0	0	0	0
Bulgarie	3	0	0	0	0	0	1	0	1	1	1	0
Croatie	1	0	2	0	2	2	2	0,1
Chypre	0	0	0	0	0	0	0	0	0	0	0	0
République Tchèque	50	0,5	57	0,5	42	0,4	36	0,3	48	48	48	0,5
Danemark
Estonie	0	0	2	0,2	0	0	1	0,1	1	1	1	0,1
Finlande	91	1,7	75	1,4	233	4,3	15	0,3	9	9	9	0,2
France	22	0	16	0	5	0	21	0	57	19	19	0
Allemagne	31	0	17	0	21	0	20	0	21	21	21	0
Grèce	0	0	0	0	0	0	0	0	0	0	0	0
Hongrie	126	1,3	15	0,2	18	0,2	48	0,5	140	140	140	1,4
Islande	0	0	0	0	0	0	0	0	0	0	0	0
Irlande	0	0	0	0	0	0	0	0	0	0	0	0
Italie	1	0	1	0	4	0	1	0	0	0	0	0
Lettonie	0	0	0	0	6	0,3	0	0	0	0	0	0
Liechtenstein
Lituanie	1	0	0	0	3	0,1	4	0,1	4	4	4	0,1
Luxembourg	0	0	0	0	0	0	0	0	0	0	0	0
Malte	0	0	0	0	0	0	0	0	0	0	0	0
Pays Bas	1	0	5	5	5	0
Norvège	33	0,7	180	3,7	50	1	28	0,6	46	46	46	0,9
Pologne	4	0	6	0	6	0	8	0	11	11	11	0
Portugal
Roumanie	4	0	0	0	0	0	1	0	0	0	0	0
Slovaquie	17	0,3	5	0,1	8	0,1	9	0,2	6	6	6	0,1
Slovénie	0	0	0	0	4	0,2	2	0,1	1	1	1	0
Espagne	1	0	1	0	1	0	0	0	90	62	62	0,1
Suède	484	5,2	350	3,7	590	6,2	108	1,1	150	150	150	1,5
Royaume-Uni	1	0	0	0	0	0	0	0	0	0	0	0
UE/EEE	872	0,2	725	0,2	994	0,2	309	0,1	592	526	526	0,1

Tableau 2 : nombre de cas de tularémie et taux d'incidence pour 100000 habitants dans l'Union Européenne (UE) et l'Esace Economique Européen (EEE) entre 2010 et 2014. (Données de l'ECDC, 2014 (112))

La répartition des cas en Europe fait donc apparaître des pays de forte endémie comme la Suède, la Norvège et la Finlande avec un taux moyen de cas $> 1.0/100000$ habitants sur la période 2006-2014 mais en forte diminution depuis 2013. A l'opposé, la Grèce, l'Irlande, Chypre, Le Luxembourg, Malte, la Macédoine, la Lettonie, l'Islande et la grande Bretagne semblent exempt de cas de tularémie mais il se peut qu'il y ait eu des biais de notification, de surveillance ou de transmission des données à l'ECDC. Entre ces deux extrêmes on retrouve des pays qui ont un taux faible ou intermédiaire de cas de tularémie :

- entre 0 et 0,1 cas/100000 habitants en moyenne par an en Albanie, Belgique, Bosnie Herzégovine, Danemark, Allemagne, Italie, Suisse, Pays-Bas, Pologne, Roumanie et Slovénie
- entre 0,1 et 1 cas/100000 habitants en moyenne par an en Autriche, Croatie, Estonie, France, Espagne, Hongrie, Lituanie, Ukraine, Slovaquie, République Tchèque et Serbie

(112–114)

Aux USA, la tularémie était plus commune dans les années 1950 avec un nombre moyen de cas de 500 à 700 par an, réduit à 100-200 cas par an depuis 1990. Le taux d'incidence de la tularémie était de $0,06/100000$ habitants en 2014. (<https://www.cdc.gov/tularemia/statistics/index.html>) Au Japon, 1300 cas ont été diagnostiqués entre 1924 et 1985 alors que sur les dernières années, moins de 10 cas par an seulement ont été notifiés. (4,115,116) La tularémie est par contre devenue un réel enjeu de santé publique en Turquie où la maladie est à déclaration obligatoire depuis 2005. Le nombre moyen de cas notifiés par an est de 679 sur la période 2005-2012, avec des pics en 2010 et 2011 (respectivement 1531 et 2151 cas) supérieurs au nombre de cas observés dans toute l'Europe à cette période. (117)

Des taux d'infection plus élevés sont observés chez les hommes après 45 ans, probablement lié à la chasse et aux activités en extérieur, à l'exception de la Turquie où les femmes développent plus fréquemment la maladie. (112,117) Les cas sporadiques liés à la chasse sont principalement observés en automne et en hiver. La majorité des cas en relation avec les piqûres de moustiques, de taons ou les morsures de tiques sont en revanche observés entre mai et septembre. (58,112) En Turquie et en Norvège, la plupart des cas surviennent en

hiver, via l'ingestion d'eau contaminée. (117,118) Le nombre de cas fluctue également selon les réservoirs et les modes de transmission présents dans chaque pays.

En dehors des cas sporadiques, on peut observer des épidémies de faible ou grande ampleur, principalement dans les pays de forte endémie. Des épidémies ont notamment été décrites en République Tchèque (119), en Suède (120–122), en Norvège (123), en Bulgarie (124,125), au Kosovo (126), en Allemagne (127), en Espagne (128), en Turquie (129–133), en France (134,135), aux USA (136,137) ou en Chine (138). Ces épidémies peuvent être très importantes comme le prouve l'épidémie Suédoise de 2003 impliquant 698 patients ou l'épidémie Norvégienne de 2011 avec 180 cas identifiés, incluant toutes les formes cliniques de tularémie. (120,123) Dans cette dernière épidémie, les sources de contaminations furent multiples. Les formes oro-pharyngées étaient principalement liées à la consommation d'eau contaminée à partir de puits privés, les formes ulcéro-ganglionnaires provoquées par des piqûres d'insectes ou la manipulation d'animaux contaminés et les autres formes cliniques probablement la conséquence d'activités en extérieurs telles que la chasse, le trekking ou le travail en forêt et à la ferme.(118) Ainsi certaines épidémies, principalement celles observées sur toute une année, semblent davantage correspondre à une augmentation globale de la transmission de la tularémie correspondant à une situation hyperendémique, c'est à dire une forte augmentation du nombre de cas pendant une période donnée dans une zone d'endémie. Ces situations hyperendémiques semblent favorisées par des conditions environnementales propices à la transmission de la bactérie durant une période plutôt qu'à l'émergence et à l'expansion géographiquement limitée d'une souche virulente. En effet il a été démontré que certaines épidémies même de faible ampleur, peuvent ne pas être liées à des infections par un clone bactérien particulièrement virulent, mais plutôt correspondre à de multiples clones co-existants dans des zones géographiques proches. (93,122) Cela a notamment été observé dans une petite épidémie en Utah (USA) qui a concerné 5 patients contaminés par piqûres de taons en 2007, chez lesquels ont été retrouvées à la fois des souches de type A des clades A.1 et A.2. (137) De même deux épidémies dues à des souches de type B en Suède, ont impliquées différentes souches appartenant aux clades B.13/14, B.7/8 et B.OSU18 dans la première épidémie et aux clades B7., B.10 et B.12 dans la seconde épidémie. (121,122)

Des épidémies clonales sont malgré tout possibles, correspondant à l'introduction de la maladie (parfois d'une souche hypervirulente) dans une population non immune. L'épidémie espagnole observée entre juin 1997 et avril 1998 fait état de 529 cas de tularémie dont tous étaient liés à la souche clonale FTNF002-00 portant la délétion RD23 typique des souches de *F. tularensis* subsp. *holarctica* strains retrouvées en Europe de l'Ouest.(139) Les périodes de guerre semblent également favoriser les épidémies de tularémie du fait des conditions sanitaires précaires. Des épidémies engendrant des dizaines voire des centaines de milliers de cas par an furent observées pendant la seconde guerre mondiale dans les pays d'Europe de l'Est. Le conflit au Kosovo a provoqué deux épidémies en 1999-2000 et 2001-2002, avec respectivement 327 et 247 cas confirmés par sérologie, probablement liées à l'augmentation de la présence de rongeurs contaminés, ainsi qu'à la consommation d'aliments et d'eau souillés. Depuis cette période le Kosovo est considéré comme une région endémique pour la tularémie avec environ 4 cas pour 100000 habitants entre 2003 et 2010. (Grunow et al., 2012; Reintjes et al., 2002)

1.8. Réservoir/Hôtes/Vecteurs /Modes de transmission

1.8.1. Réservoirs potentiels et vecteurs : mammifères, autres vertébrés, arthropodes, environnement

1.8.1.1. Espèces animales

Plus de 250 espèces animales dont au minimum 190 mammifères (lagomorphes, rongeurs, ours, primates, opossums, félins, ...) (141–143), 88 espèces d'invertébrés, 23 espèces d'oiseaux, (144), 3 amphibiens et quelques reptiles ont été identifiés comme hôtes potentiels de *F. tularensis*. (57) La plupart d'entre eux sont probablement des hôtes accidentels et ne représentent pas un vrai réservoir bien qu'ils participent au cycle de vie de ce pathogène. (9) Les lièvres sont un réservoir potentiel de *F. tularensis* ou tout au moins une source importante de contaminations humaines. Leur sensibilité à l'infection par *F. tularensis*

est variable selon les espèces de lièvre, ce qui permet la persistance de cette bactérie chez les lièvres en dehors des périodes d'épizootie. (67,142). A l'opposé, les campagnols sont des hôtes très sensibles. La contamination des mammifères peut avoir lieu de 3 façons différentes : 1/ d'animal à animal en cas d'épizootie ou d'ingestion d'un animal contaminé par les espèces carnivores ; 2/ par ingestion ou contact avec des eaux contaminées ; ou 3/ via la transmission par un arthropode hématophage.

1.8.1.2. Arthropodes hématophages

Les arthropodes hématophages (tiques, moustiques, taons, puces, punaises, acariens, ...) sont un réservoir potentiel de la maladie. Les espèces de tiques hébergeant la bactérie appartiennent aux genres *Dermacentor*, *Amblyomma*, *Haemaphysalis*, *Hyalomma*, *Rhipicephalicus* et *Ixodes* avec une prévalence allant jusqu'à 3% dans certaines régions. (57,145–148) La transmission entre les différents stades de vie de la tique a été démontré pour le genre *Ixodidae* et les souches de type A1, A2 et B. (149,150) Mais la transmission transovarienne demeure contestée, ce qui laisse planer un doute sur le rôle des tiques ou des moustiques en tant que réservoir de la maladie. La survie des tiques pendant plusieurs décennies pourrait cependant jouer un rôle dans la persistance à long terme de la bactérie dans la nature. (145,151)

Les taons ne sont quant à eux que des vecteurs mécaniques car la bactérie ne survit pas dans ces arthropodes. Ils sont cependant capables de se nourrir rapidement sur plusieurs hôtes consécutifs. (146)

Les moustiques des genres *Aedes*, *Culex*, *Anopheles* ou *Ochlerotatus* ont été identifiés comme vecteurs et réservoirs potentiels. (57,146,152,153) Les moustiques ont été longtemps considérés uniquement comme des vecteurs mécaniques mais il a été démontré que la transmission de *F. tularensis* entre les différents stades de développement du moustique était possible. (152) La persistance de *F. tularensis* dans les formes larvaires jusqu'aux formes adultes de moustiques a lieu dans un quart des larves infectées. (153) Il est possible que les moustiques soient contaminés dans leur environnement aquatique initial, soit directement à partir de l'eau contaminée, soit par prédation de protozoaires infectés comme les amibes.

(9,146,152,154) Néanmoins, la transmission transovarienne de la bactérie n'a pas été démontrée à l'heure actuelle. Plus qu'un réservoir, les moustiques sont donc considérés principalement comme des vecteurs permettant la transmission de la bactérie à partir de son réservoir animal ou aquatique vers l'homme ou d'autres animaux par pique hématophage.(153)

1.8.1.3. Environnement aquatique et amibes

La présence dans l'eau de formes viables et infectieuses de *F. tularensis* est connue depuis le début des années 1950. (155) Plus récemment, de l'eau renfermant ce pathogène et conservée à 8°C a permis de maintenir la viabilité de la bactérie et de la ré-isoler en culture pendant au moins 21 jours. (156) En revanche, la persistance dans l'eau pendant des périodes plus longues (140 jours) semble se faire sous un état viable mais non cultivable, avirulent pour la souris. (157) L'amélioration de la survie de la bactérie a été observée dans des eaux riches en soufre, possiblement du fait de l'auxotrophie pour la cystéine de cette bactérie. (158) Il est donc supposé que *F. tularensis* persiste dans les environnements aqueux sous forme non-répliquative, ce qui rend très difficile la détection de cette bactérie par culture à partir dans d'échantillons hydriques. (153) La biologie moléculaire peut alors s'avérer utile pour détecter la présence de l'ADN du pathogène. Lors d'une étude menée en Turquie analysant 154 échantillons d'eaux prélevés dans des rivières, des sources et des fontaines localisées dans des régions dans lesquelles des épidémies de tularémie avaient eu lieu, seuls 4 prélèvements permirent d'isoler la bactérie en culture alors que 17 prélèvements étaient positifs en PCR en temps réel. (159) Des analyses moléculaires en Suède ont également permis de confirmer la persistance de souches de type B dans des échantillons d'eau récoltés au cours de trois années consécutives au niveau d'un même site géographique. (154) Il est possible que la survie de *F. tularensis* dans l'eau soit médiée par des protozoaires. En effet, il a été démontré que cette bactérie est capable d'infecter, de survivre et de se multiplier dans des amibes telles qu'*Acanthamoeba castellanii*. (160) La bactérie a été observée non seulement dans les trophozoïtes mais également dans les formes kystiques. A l'intérieur de ces kystes la bactérie est capable de survivre 3 semaines après l'infection des amibes, protégée de la dessiccation. (161) La co-culture de *F. tularensis* avec *A. castellanii* stimule la multiplication de *F. tularensis*

dans les formes trophozoïtes alors que la réplication de la bactérie dans les kystes est faible. (160,161) Les amibes représentent donc peut-être un réservoir important mais méconnu à ce jour dans le cycle aquatique de la tularémie. L'ingestion d'amibes contaminées par les moustiques permettant alors de boucler le cycle aquatique de la tularémie (cf chapitre : cycle de vie de *Francisella*).

1.8.2. Modes de transmission à l'homme

F. tularensis est capable d'infecter l'homme à partir d'une très large variété d'hôtes, de vecteurs et d'environnements allant des mammifères aux taons, des oiseaux aux tiques, des moustiques aux eaux et sols contaminés. (57)

1.8.2.1. Transmission par contact ou morsure d'animaux infectés

Les lagomorphes tels que les lièvres, les rongeurs tels que les campagnols, les souris, les ragondins, les ratons laveurs, les rats musqués, les castors, les écureuils et les rats jouent un rôle important dans la transmission de la tularémie notamment :

- envers les chasseurs, lors de la manipulation ou du dépeçage du gibier
- en cas de morsure de personnes lors d'activités en extérieur (74,162–165)

En Europe, la tularémie est principalement transmise par le lièvre d'Europe (*Lepus europaeus*), très sensible à l'infection par cette bactérie. La proportion de cette voie de transmission semble néanmoins en diminution, inférieure à 50%, alors qu'elle représentait 90% des cas de transmission depuis des décennies. (3,142,145) La chasse du lièvre japonais (*Lepus brachyurus*) est responsable de plus de 90% des cas de tularémie au Japon. (116) Les épidémies animales sont souvent à l'origine d'épidémies humaines comme cela a été observé après une enzootie chez les lièvres en France, après la prolifération de lemmings en Norvège ou de campagnols en Hongrie. (67,118,166) Par conséquent, ces animaux infectés pourraient être utilisés comme sentinelles pour la surveillance de la tularémie. Enfin, des cas de tularémie ont été observés par contact ou morsure d'animaux de compagnie tels que des chats ou des rongeurs. (141,167–169)

1.8.2.2. Transmission par ingestion d'aliments contaminés

Des cas de tularémie oropharyngés, parfois groupés ou familiaux, ont lieu lors de la consommation de gibier contaminé sous la forme de viande insuffisamment cuite ou de terrines. Des cas indirects peuvent être consécutifs à la contamination d'autres aliments par des excréments (selles ou urine) d'animaux infectés ou par leurs cadavres. Cela a notamment conduit à une épidémie Tchèque en 1978 suite à la consommation de jus de pomme contaminé dans une maison de retraite. (118,119,131)

1.8.2.3. Transmission par ingestion d'eau contaminée

La contamination de l'eau, notamment de certains puits, est à l'origine de nombreux cas de tularémie, principalement en Turquie. (126,130,131,170) La contamination de l'eau semble dans ces cas, avoir lieu principalement par le biais de déjections animales ou de carcasses d'animaux infectés plutôt que par la persistance de la bactérie sur de longues périodes dans des points d'eau. L'insuffisance de protection de ces points d'eau vis-à-vis des rongeurs ainsi que l'insuffisance de traitement de ces eaux par chloration conduisent à de nombreux cas de forme oropharyngée de tularémie. (154,159) Les épidémies liées à des contaminations de sources hydriques sont préoccupantes en Turquie où la consommation d'eau non traitée est encore fréquente.(130,132,171).

1.8.2.4. Transmission par morsure d'arthropodes hématophages

Les arthropodes hématophages ont été identifiés comme vecteurs de la maladie très précocement. Francis en 1922 avait déjà démontré la transmission de *F. tularensis* à des animaux sains via des mouches (*Chrysops discalis*), des poux du lapin (*Haemodipsus ventricosus*), des punaises de lit (*Cimex lectularius*), des mites (*Liponyssus isabellinus*) et des puces de souris (*Polyplex serratus*). (14) Depuis, de nombreuses variétés d'arthropodes hématophages dont plusieurs espèces de tiques, de taons, de moucheron, de puces, d'acariens et de moustiques sont reconnus comme vecteurs de la maladie pour l'homme,

principalement pendant les mois d'été lorsque les activités en extérieur augmentent. (146) Les morsures de tiques représentent la première source de transmission aux Etats-Unis et une part importante en Europe (17-26% des cas en France entre 2007 et 2013) (145,172) Dans les forêts de Scandinavie, Russie et Alaska, les moustiques sont le vecteur principal de *Francisella tularensis* subsp. *holarctica* à l'homme, pouvant être à l'origine d'épidémies. Cette transmission par pique de moustique a également été rapportée sporadiquement dans d'autres pays d'Europe. (74,173) L'abondance des moustiques selon les années ou les saisons influence directement la variabilité du nombre de cas dans les pays concernés. (174) La distribution des espèces de moustiques capable de transmettre la tularémie pourrait expliquer le regroupement géographique des cas liés aux moustiques dans les régions proches du cercle polaire.

1.8.2.5. Transmission par voie aérienne

Les cas d'infection par *F. tularensis* par voie pulmonaire sont dus à l'inhalation de gouttelettes ou de poussières porteuses de la bactérie (manipulation de foin contaminé, tonte de pelouse, suspension liquide en laboratoire aérosolisée, ...). En théorie, l'introduction d'une bactérie dans les voies respiratoires humaines peut être à l'origine de formes pulmonaires de tularémie dans 50% des cas. (91). Les cas d'infection pulmonaire sont en général sporadiques. Les épidémies de tularémie transmises par voie aérienne sont heureusement rares mais ont été rapportées notamment en Suède (600 cas en 1966-1967) (175) en Finlande en 1982 avec l'infection de 52 fermiers (176) et plus récemment, en Allemagne en 2005 (127) et en Suède en 2010. (122) Des formes pulmonaires de tularémie ont également été notifiées sur l'île de Martha's Vineyard pendant deux décennies (Massachusetts, USA ; résidence d'été des présidents américains) (136,177).

Ces cas d'infection respiratoire représentent environ 5 à 15% des cas de tularémie sporadiques. A titre d'exemple 12% de formes pulmonaires ont été observés dans le Missouri (USA) entre 2000 et 2007, (178), 10% en France entre 2006 et 2010 (3), 7.7% dans le comté d'Örebro en Suède entre 2000 et 2004 (179) et 13.3% en Norvège en 2011. (118)

La sévérité des infections respiratoires par des souches de type A est corrélée à la charge bactérienne inhalée. Ces formes présentent une mortalité de 40 à 60% en l'absence de traitement. (91) L'aérosolisation de gouttelettes ou poussières contenant la bactérie peut avoir lieu lors de la tonte de pelouse ou de la manipulation de foin contenant des carcasses ou des excréments d'animaux contaminés, (136,180), lorsqu'un animal secoue sa fourrure pour la sécher alors qu'elle est contaminée par de l'eau ou des poussières contenant la bactérie, (134,177,181), lors du dépeçage d'animaux contaminés, (127) ainsi que lors de manipulation de cultures liquides de *F. tularensis* en laboratoire.

La transmission interhumaine par voie aérienne reste très exceptionnelle, mais est théoriquement possible. (35) La notification d'un cas de contamination secondaire, d'un patient souffrant de pleuropneumopathie vers une infirmière, a été rapportée en 1951. (91) Des précautions doivent donc être mises en place pour éviter la transmission via des aérosols de fluides corporels notamment en cas de chirurgie de résection d'adénopathies chez un patient atteint de tularémie.

1.8.2.6. Transmission par l'environnement (aquatique et tellurique)

Des cas de tularémie ont été observés suite à des activités de jardinage, de sport en extérieur (canyoning), de blessures par des végétaux (ronces, ...), de ramassage de champignons ou de manipulation de bois, ce qui témoigne d'une transmission environnementale possible de la bactérie. (3,182)

1.8.2.7. Contamination par des souches de laboratoire

Des cas secondaires de tularémie ont eu lieu suite à l'isolement de souches de *F. tularensis* en laboratoire (laboratoire d'analyses médicales, laboratoire vétérinaire, laboratoire de recherche) ce qui justifie la manipulation des souches de *F. tularensis* sous un poste de sécurité microbiologique (PSM), en laboratoire de niveau 3 de sécurité biologique (LNSB3).

1.8.3. Le ou les cycles de vie de *F. tularensis*: entre animaux et environnement

Malgré quelques avancées, l'écologie et le cycle complet de *F. tularensis* n'est toujours pas entièrement compris et sa caractérisation complète reste très difficile. La très large diversité d'hôtes et de vecteurs potentiels de ce pathogène, la variabilité des réservoirs entre les pays ainsi que les larges différences climatiques et environnementales entre les pays endémiques fait qu'il est impossible de définir précisément un schéma général de la transmission de ce pathogène. Actuellement deux cycles de vie et de transmission de cette bactérie sont décrits, l'un terrestre, l'autre aquatique (Figure 10). (9,57,74)

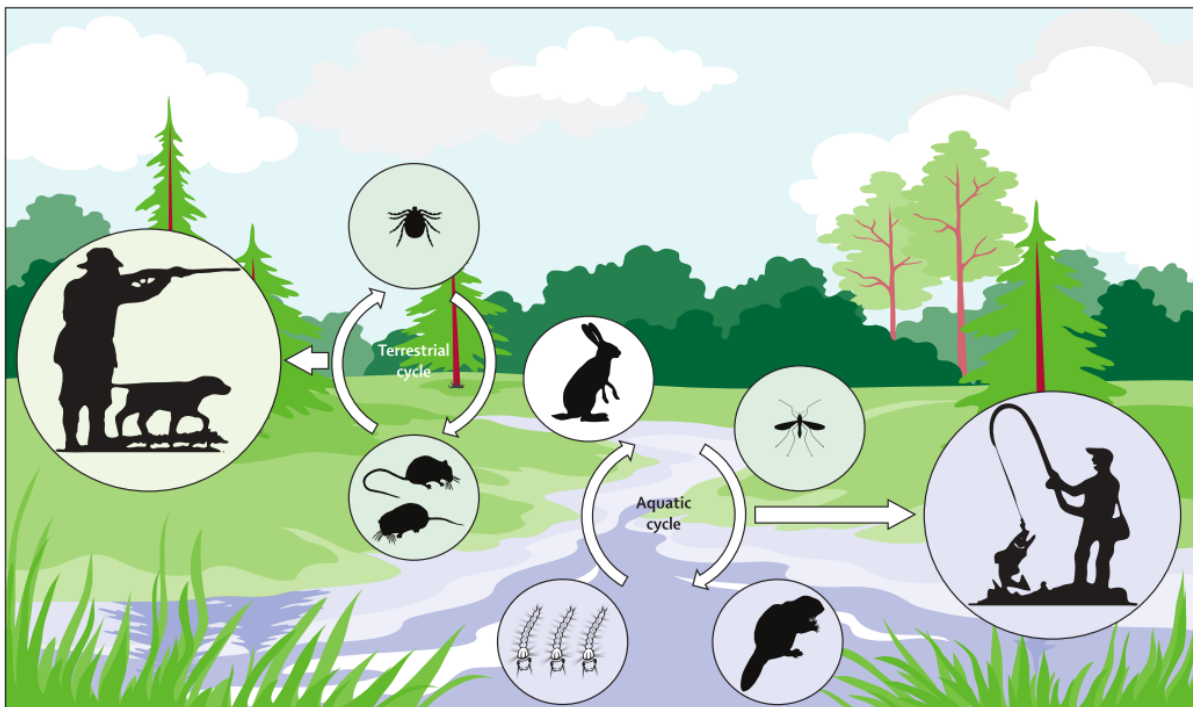


Figure 10 : Cycles terrestres et aquatiques de *F. tularensis* en Europe. (D'après Maurin et Gyuranecz, 2016 (74))

La sous-espèce *Francisella tularensis* subsp. *tularensis* semble plus associée au cycle terrestre, impliquant à la fois les petits mammifères comme les lièvres ou les rongeurs et les arthropodes hématophages parmi lesquels on retrouve en premier lieu les tiques et les taons.

La transmission de ces souches de type A à l'homme semble plus associée aux taons et aux tiques dans l'Ouest des Etats-Unis mais uniquement aux tiques à l'Est. (146)

Concernant la sous-espèce *Francisella tularensis* subsp. *holarctica*, un cycle aquatique est rapporté en sus du cycle terrestre, notamment dans certains pays d'Europe comme la Bulgarie, le Kosovo, la Turquie et la Scandinavie. (74,146) Ce cycle aquatique décrit comme possible réservoir des sources aquatiques comme les lacs, les rivières, les étangs ou les puits et impliquerait également les moustiques. (74,183,184) Les eaux seraient contaminées via la décomposition de carcasses de mammifères infectés, notamment de rongeurs semi-aquatiques. (74) Mais on ne sait toujours pas formellement si ce sont les animaux ou les eaux contaminés qui sont le principal réservoir et engendrent l'écologie observée dans ce cycle aquatique. (9) D'autant plus que le réservoir de certaines espèces proches comme *F. philomiragia* ou *F. noatunensis* est aquatique et qu'il a été démontré expérimentalement que cette bactérie est capable de survivre à l'intérieur d'amibe dont le rôle dans le cycle de vie de *Francisella* reste inconnu. (160,161)

1.9. Formes cliniques

La tularémie se caractérise par six formes cliniques principales, dépendant du mode de transmission et du site primaire d'inoculation de la bactérie : les formes ulcéroganglionnaire, ganglionnaire, oculoganglionnaire, oropharyngée, pulmonaire et typhoïdique. La période d'incubation de la maladie est habituellement de 3 à 5 jours mais peut aller jusqu'à deux semaines. La phase initiale est aiguë et caractérisée par la présence d'un syndrome pseudo-grippal, pouvant associer de la fièvre, des frissons, des céphalées, des myalgies et des arthralgies. Des adénopathies satellites élargies et souples doivent être recherchées en regard du point d'inoculation suspecté, dans la zone de drainage lymphatique du site d'inoculation. Parmi les populations les plus exposées on retrouve notamment les chasseurs, les gardes forestiers, les vétérinaires, le personnel des abattoirs et le personnel des laboratoires d'analyses biomédicales, vétérinaire ou de recherche travaillant sur *F. tularensis*.

1.9.1. Formes ulcéroganglionnaire et ganglionnaire

Ce sont les deux formes cliniques les plus fréquentes. Elles sont liées à l'inoculation de la bactérie via contact cutané avec des animaux infectés (manipulation, dépeçage,...), par morsure d'arthropodes hématophages, par morsure d'animaux infectés ou en cas de blessures avec de la terre, des végétaux ou des eaux contaminées. L'infection d'une peau saine n'est pas formellement démontrée à ce jour mais reste possible. En revanche, la présence de lésions cutanées augmente la sensibilité à l'infection. (83) La forme ulcéroganglionnaire associe un ulcère cutané bénin au site d'inoculation de la bactérie (pouvant persister plusieurs semaines) ainsi qu'une adénopathie satellite. La lésion primaire débute sous la forme d'une petite papule devenant une pustule inflammatoire et peut persister sous la forme d'une petite cicatrice. L'ulcère d'inoculation est absent de la forme ganglionnaire. L'adénopathie devient palpable, puis de plus en plus volumineuse et représente le principal motif de consultation des patients atteints de tularémie. Si le traitement antibiotique n'est pas débuté de façon précoce (dans les deux semaines après inoculation), l'adénopathie peut suppurer dans 30 à 40% des cas, voire nécessiter une résection chirurgicale. (83)

1.9.2. Forme oculo-ganglionnaire

Les formes oculoganglionnaires restent rares (environ 3-4% des cas en France, 1% au niveau mondial) et sont liées à des cas de contamination manu-portée de la bactérie vers la conjonctive oculaire suite à la manipulation d'animaux ou d'environnements contaminés ou suite à des projections oculaires de gouttelettes contaminées. (3,83,172) On observe des signes locaux : une conjonctivite unilatérale douloureuse, associée à des larmoiements intenses, un gonflement des paupières, un œdème périorbitaire et parfois une photophobie (Figure 11). (185) Une adénopathie satellite pré-auriculaire ou cervicale se développe dans les jours qui suivent l'atteinte conjonctivale. (83) Des complications sont possibles mais restent exceptionnelles : syndrome de Parinaud (atteinte neurologique avec paralysie oculaire verticale), uvéite. (186,187)



Figure 11 : Forme oculoganglionnaire de tularémie. (D'après Eren Gok et al., 2014 (185))

1.9.3. Forme oropharyngée

Le nombre de cas de tularémie oropharyngée varie selon les pays et est liée à l'ingestion d'aliments ou d'eaux contaminés. Des cas groupés, notamment intrafamiliaux sont possible lors de la consommation de viande contaminée insuffisamment cuite au cours d'un même repas. Les formes oropharyngées sont majoritaires dans certaines parties de la Turquie car la source principale de contamination dans ces régions se fait par l'ingestion d'eaux souillées par des déjections ou des carcasses d'animaux infectés. (188,189) En France cette forme clinique ne représente que 5 à 18% des cas observés. (3,172) L'examen clinique retrouve une stomatite ulcéreuse, une inflammation des muqueuses buccales ainsi qu'une pharyngite, avec ou sans inflammation des amygdales. Les adénopathies cervicales associées sont le plus souvent unilatérales et peuvent suppurer, notamment lorsque la tularémie est évoquée et traitée tardivement (Figure 12). Cette étiologie peut être évoquée en cas d'angine avec inflammation des amygdales, réfractaire au traitement par β -lactamine. (190,191)



Figure 12 : Adénopathie cervicale observée lors d'un cas de tularémie oropharyngée. (D'après Kaya et al., 2011 (192))

1.9.4. Forme pulmonaire

L'inhalation de la bactérie sous forme d'aérosols est à l'origine de la majorité des cas de tularémie pulmonaires ou pleuraux-pulmonaires. Certains cas peuvent néanmoins être secondaires à d'autres formes cliniques de tularémie en cas de bactériémie. Les symptômes observés lors des formes pulmonaires sont caractéristiques d'une pneumonie : toux, douleur thoracique, fièvre, polypnée pouvant être accompagnés de vomissement et de nausées. Les formes pulmonaires liées aux souches de type A peuvent être fulminantes en l'absence de traitement antibiotique et simuler une fièvre typhoïde notamment en cas de bactériémie concomitante. Les atteintes respiratoires liées aux souches de type B ne sont pas aussi

sévères. Les radiographies pulmonaires peuvent objectiver des adénopathies hilaires et/ou médiastinales ainsi que des infiltrats dans un ou plusieurs lobes pulmonaires.

1.9.5. Forme thyphoïdique

Les formes aiguës de tularémie associant une fièvre intense, des céphalées, une asthénie, des arthralgies et myalgies ainsi qu'une atteinte neurologique (confusion, stupeur, troubles du comportement), ressemblant à la fièvre typhoïde, et dans lesquelles on n'observe ni ulcération, ni adénopathie, sont regroupées sous le terme de forme « typhoïdique ». Ces formes sont associées à la présence d'une bactériémie et diagnostiquées généralement par l'isolement de la bactérie lors de la réalisation d'hémocultures.

1.9.6. Complications

Les complications potentielles de la tularémie sont nombreuses. (74,83) Elles peuvent être :

- Cutanées et se manifester sous la forme d'éruptions papulaires ou papulovésiculaires, d'érythème noueux, d'érythème polymorphe, d'urticaire ou de cellulite (83,193–195)
- Ganglionnaires avec suppuration et possible fistulisation à la peau jusque dans 30% des cas d'adénopathies chroniques, pouvant alors nécessiter la résection chirurgicale de l'adénopathie (3)
- ORL avec des abcès péri-amygdaliens, parotidiens et des otites
- Neurologiques : méningites avec hypoglycorachie mais prédominance de lymphocytes, encéphalites, abcès cérébraux (196–200)
- Plus rarement vasculaires : anévrisme aortique (201)

1.10. Diagnostic biologique

En France, les cas confirmés de tularémie associent la présence de signes cliniques compatibles avec la tularémie, avec l'un des critères suivants :

- un isolement de la bactérie à partir de prélèvement cliniques
- la séroconversion ou la multiplication par 4 du titre d'anticorps entre deux sérums prélevés à 15 jours d'intervalle
- une PCR positive sur un prélèvement clinique

Un cas probable de tularémie associe un tableau clinique évocateur à un seul sérum positif. (Fiche de déclaration obligatoire de la tularémie, Santé Publique France, Annexe 2).

Le diagnostic de la tularémie est en général tardif (> 3 semaines par rapport à l'apparition des signes cliniques) hormis dans les pays de forte endémie tels que les pays Scandinaves, dans lesquels les médecins généralistes sont plus au fait de la maladie.

1.10.1. Sérologie

Le diagnostic biologique de la tularémie repose principalement sur la sérologie. La sérologie ne se positive qu'au minimum deux semaines après le contage, c'est à dire 10 jours en moyenne après l'apparition des symptômes, et atteint son pic après environ six semaines. (202) Puis les titres en anticorps décroissent, tout en pouvant persister à des taux résiduels pendant plusieurs mois. En cas d'adénopathie chronique des taux significatifs d'IgG persistent néanmoins en général plusieurs mois. (3) Des taux significatifs d'IgG peuvent être détectés plusieurs années après la séroconversion, y compris en cas de traitement. (203). L'utilisation de méthodes permettant la distinction des IgM et des IgG est importante pour le diagnostic de primo-infection récente. (203)

Des tests sérologiques de type Micro-agglutination, Immunofluorescence, ELISA et Western Blot sont disponibles pour le diagnostic sérologique de la tularémie. (184) La sensibilité des kits commerciaux de type ELISA est en général supérieure à 95% mais leur spécificité inférieure aux techniques d'immunofluorescence. (3,204) Malheureusement

l'absence de standardisation entre les techniques et entre les laboratoires fait que des seuils différents sont appliqués selon la technique de sérologie utilisée, ce qui justifie la réalisation de sérologies successives pour un même patient dans un même laboratoire. La confirmation d'une sérologie positive en ELISA par une technique plus spécifique (Immunoblot ou Immunofluorescence) est recommandée. Une sérologie positive peut également être confirmée par l'envoi du sérum à un Centre National de Référence. Dans de rares cas confirmés de tularémie, les sérologies réalisées peuvent néanmoins rester négative. Enfin, des cas de faux positifs liés à des réactions croisées peuvent être observés notamment avec *Brucella* ou *Yersinia* et sont plus fréquents en cas d'utilisation d'une technique de type ELISA. (204)

1.10.2. Culture

F. tularensis est une bactérie fastidieuse de culture très difficile, qui doit être manipulée en LNSB3. Les souches sont cultivables à partir de divers prélèvements telles que des lésions cutanées, muqueuses ou conjonctivales, des biopsies ganglionnaires ou des prélèvements sanguins. La culture doit se faire sur milieu riche, enrichi en cystéine du fait de l'auxotrophie de la bactérie pour ce composé. Les milieux classiquement employés sont :

- les géloses au sang cuit enrichi en vitamines de type Polyvitex[®], supplémentées ou non en antibiotiques
- les géloses glucosées au sang enrichies en cystéine et en Polyvitex[®]
- les géloses coeur cystéine au sang cuit, additionnées ou non d'hémoglobine
- les géloses de type BCYE (buffered charcoal yeast extract) avec cystéine

La bactérie est également capable de pousser dans les flacons d'hémocultures aérobies. Malheureusement ces cultures ne sont positives que dans environ 10% des cas, principalement au décours de bactériémies, grâce à l'utilisation de flacons d'hémoculture. (74)

La conservation du prélèvement ainsi que le délai avant mise en culture influencent fortement l'efficacité de la culture. En cas de délai avant mise en culture du prélèvement, la congélation du prélèvement semble recommandée pour permettre sa conservation optimale. L'utilisation de milieu de culture additionné d'antibiotiques semble également améliorer la

culture de la bactérie en cas de prélèvement polymicrobien. (205) Les géloses doivent être incubées à 37°C. Une atmosphère enrichie en 5% de CO₂ semble requise pour la croissance de certaines souches. La croissance de la bactérie est suffisante en 48h en général mais peut prendre plusieurs jours à partir d'un prélèvement clinique. Les colonies font 1 à 2 mm de diamètre et sont de couleur blanches à légèrement grisâtres, brillantes et opalescentes, avec un halo transparent sur le pourtour de la colonie, correspondant à la capsule. Des tests biochimiques simples comme la fermentation du glucose ou du glycérol peuvent être utiles pour la distinction des différentes espèces ou sous-espèce des *Francisella*, bien que ces tests soient actuellement remplacés par le typage des souches grâce à des techniques de biologie moléculaire ou de spectrométrie de masse MALDI-TOF.

Caractère	<i>F. tularensis</i>			<i>F. novicida</i>	<i>F. philomiragia</i>
	<i>ssp. tularensis</i>	<i>ssp. holarctica</i>	<i>ssp. mediasiatica</i>		
Besoin en cystéine/cystine	+	+	+	-	-
Fermentation du maltose	+	+	-	faible	+
Fermentation du sucrose	-	-	-	+	+
Fermentation du D-glucose	+	+	-	+	faible
Fermentation du glycérol	+	-	+	faible	-
Production de citrulline uréidase	+	+/-	+	+	NT
Oxydase	-	-	-	-	+

Tableau 3 : Caractères biochimiques permettant la distinction entre les espèces et sous-espèces de *Francisella*. (Adapté de Tarnvik, 2007 (83))

Actuellement, l'identification des souches à l'espèce est possible en routine par spectrométrie de masse MALDI-TOF mais peut nécessiter l'utilisation d'une base de donnée additionnelle. L'identification à la sous-espèce semble également possible après construction d'une base de données intégrant des spectres de plusieurs souches appartenant aux différentes espèces et sous-espèces de *Francisella*. La construction d'une grande base de données de spectre de *Francisella* reste encore nécessaire pour obtenir des scores d'identification suffisants. (206,207) Par ailleurs l'analyse combinée de 3 pics spécifiques au

sein des spectres du genre *Francisella* obtenus par MALDI-TOF, correspondant à trois protéines (la forme B de la protéine histone-like HU, la chaperonine de 10 kDa Cpn10, et la protéine ribosomale L24 de la sous-unité 50S), semble en mesure de permettre la distinction des espèces et sous-espèces au sein du genre *Francisella*. (208) L'identification des souches par spectrométrie de masse MALDI-TOF constitue donc une nouvelle méthode prometteuse d'identification très rapide, peu coûteuse et efficace des bactéries au sein du genre *Francisella*. De même, la distinction des sous-espèces semble possible avec la spectrométrie de masse SELDI-TOF. (209,210)

1.10.3. PCR

De nombreux tests de biologie moléculaire (PCR, PCR en temps réel, PCR multiplexe) pour le diagnostic de la tularémie sur des prélèvements humains, animaux ou environnementaux ont été développés depuis l'apparition de la PCR. La PCR 16S pan bactérienne ne permet pas de faire la distinction entre les sous-espèces au sein du genre *Francisella* car les séquences sont trop homologues. (211,212) Pour la pratique clinique, il est important de pouvoir distinguer rapidement et efficacement *F. tularensis* des autres espèces du genre *Francisella*, de même qu'il est important de distinguer les endosymbiontes *Francisella*-like (EFL) lors de l'analyse de la prévalence de *F. tularensis* au sein des arthropodes hématophages vecteurs de la tularémie. (212) Actuellement les principaux gènes cibles pour le diagnostic de la tularémie sont les gènes :

- *ISFtu2* codant une séquence d'insertion spécifique du genre *Francisella*, présent en multi-copies dans le génome (12 à 17 copies chez *F. tularensis* subsp. *tularensis*, 26 à 30 copies chez *F. tularensis* subsp. *holarctica*, 6 à 18 copies chez *F. tularensis* subsp. *novicida*, et 1 à 2 copies chez *F. philomiragia*), ce qui en fait actuellement la PCR la plus sensible pour la détection des *Francisella* (< 20 CFU/mL). (213) Cependant ce gène n'est pas spécifique de l'espèce *F. tularensis*. Des réactions positives sont possibles avec *F. novicida*, *F. philomiragia* et les endosymbiontes *Francisella*-like. (213,214) Cette PCR est donc une PCR de screening très sensible mais qui doit être confirmée par une technique plus spécifique pour *F. tularensis*.

- *tul4* (aussi appelé *LpnA*) codant pour une lipoprotéine de 17 KDa associée à la membrane externe de la bactérie. (97) La sensibilité de la PCR ciblant ce gène est proche de 20 CFU/mL mais sa spécificité est accrue par rapport à *ISFtu2* car cette PCR ne se positive pas en présence de *F. philomiragia* ou d'endosymbiontes *Francisella*-like. Elle détecte néanmoins *F. novicida*. (213)
- *23 kDa* codant une protéine exprimée par la bactérie importante pour l'infection des macrophages. Sa sensibilité est proche de celle de *ISFtu2* mais cette PCR amplifie également *F. novicida* et certaines souches de *F. philomiragia*. (213)
- *fopA* codant une protéine de la membrane externe de la bactérie. Plusieurs amorces différentes sont décrites. Les PCR ciblant ce gène sont généralement de sensibilité moindre que celles ciblant les 3 précédents gènes et leur spécificité est variable vis-à-vis du genre *Francisella*. (213–215)

La détection de *ISFtu2*, *23 kDa* et *tul4* a pu être combinées dans un outil de PCR multi-cibles afin d'améliorer certains défauts de sensibilité ou spécificité de ces PCR individuelles. (213).

Plusieurs techniques permettant la distinction entre les souches de type A et de type B ou entre les espèces et sous-espèces au sein du genre *Francisella* ont été développées au cours du temps, parmi lesquelles :

- l'amplification par PCR couplé au séquençage de la séquence intergénique 16S-23S (3)
- l'amplification et l'analyse génomique au hasard utilisant de longues amorces arbitraires (216)
- l'amplification et l'analyse de séquences répétées palindromiques extragéniques à l'aide d'amorces spécifiques (REP PCR) (216)
- l'amplification et l'analyse de séquences répétées consensus intragéniques (ERIC PCR) (216)
- l'analyse multi-locus de séquences répétées en tandem de nombre variable (MLVA) (60–62)
- la recherche de SNP et de délétions/insertions canoniques (66,78,217)

Néanmoins ces techniques de typage sont longues et ne permettent pas un diagnostic de routine rapide. Ainsi d'autres techniques ont été mises au point afin de différencier simplement et rapidement les souches de type A et B :

- PCR en temps réel ciblant le gène *pdpD* pour l'identification des souches de type A et une séquence spécifique chevauchant la jonction 3' du gène *ISFtu2* pour l'identification des souches de type B (83,218), à réaliser en cas de première PCR de screening positive.
- PCR classique amplifiant un marqueur appelé FtM19 couplée au séquençage de l'amplicon obtenu, permettant d'identifier les souches de type B par la présence d'une délétion caractéristique de 30 nucléotides par rapport aux autres souches (souches de type A, souches de *F. tularensis* subspecies *mediasiatica* et souches de *F. novicida*). (216)
- PCR classique amplifiant la région de différence RD1 couplé à l'analyse de la taille des fragments en gel d'agarose, permettant de différencier les sous espèce de *F. tularensis* ainsi que *F. novicida* et de distinguer les souches japonaises au sein de la sous espèce *F. tularensis* subsp. *holartica*. (65)
- PCR en temps réel TaqMan recherchant 11 SNP capables d'identifier *Francisella tularensis* et de différencier les sous espèces et les sous populations. Ce test identifie en 3 heures les espèces *F. tularensis*, *F. novicida* et *F. hispaniensis* (*F. TNH* SNP) puis distingue spécifiquement *F. tularensis* (*F.t.*-specific SNP) et les sous espèces *F. tularensis* subsp. *tularensis* et *mediasiatica* (*F.t.* A et M SNP), *F. tularensis* subsp. *mediasiatica* (*F.t.* M SNP), *F. tularensis* subsp. *tularensis* (*F.t.* A SNP) et *F. tularensis* subsp. *holarctica* (*F.t.* B SNP). Ce test permet également la classification des souches de type A en *F. tularensis* subsp. *tularensis* clade A.I (*F.t.* A.I SNP), *F. tularensis* subsp. *tularensis* sous-clade A.Ia (*F.t.* A.Ia SNP) et *F. tularensis* subsp. *tularensis* clade A.II (*F.t.* A.II SNP), et les souches de type B en *F. tularensis* subsp. *holarctica* souches japonaises (*F.t.* JB SNP) ou souches non-japonaise (*F.t.* nonJB SNP). Mais cette technique nécessite de réaliser de multiples PCR en temps réel en parallèle. (219)

Enfin pour les souches françaises, la recherche de la région de différence RD23 permet de confirmer l'appartenance de ces souches au clone d'Europe de l'Ouest *F. tularensis* subsp. *holarctica* B.FTNF-002. (77)

1.11. Traitement

Le dernier consensus pour le traitement de la tularémie a été publiée en 2001 (35) Les recommandations de l'OMS qui datent de 2007 sont basées sur ce consensus. (83) Des recommandations européennes ont également été publiées en 2004. (220) Les choix thérapeutiques pour le traitement de la tularémie reposent principalement sur trois classes d'antibiotiques : les aminosides (gentamicine et streptomycine), les fluoroquinolones et les tétracyclines (Tableau 3). Le choix de l'antibiothérapie dépend principalement de la sévérité de la maladie, de l'âge du patient et de la présence d'une immunodépression.

		Sévérité de la maladie	
		Sévère (hospitalisation)	Modérée
Adultes	Gentamicine IV: 5 mg/kg/j en 2 doses (≥10 jours selon la réponse clinique) + monitoring des concentrations sériques	Ciprofloxacin e IV ou per os: 800-1000mg/j en 2 doses (10-14 jours).	
	<u>Alternative</u> : Streptomycine IM: 2g/j en 2 doses (10 jours)	<u>Alternative</u> : Doxycycline per os: 200mg/j en 2 doses (≥ 15 jours)	
Enfants	Gentamicine IV: 5 mg/kg/j en 2 doses (≥10 jours selon la réponse clinique) + monitoring des concentrations sériques	Ciprofloxacin e IV ou per os: 15mg/Kg/j en doses (≥10 jours).	
	<u>Alternative</u> : Streptomycine IM: 15mg/Kg/j en 2 doses (≥10 jours)		

Tableau 4 : Traitement de la tularémie chez l'adulte et l'enfant en fonction de la sévérité de la maladie. (Adapté des recommandations 2007 de l'OMS ; Tarnvik, 2007 (83))

Dans les formes sévères de l'adulte et de l'enfant (forme pulmonaire, bactériémie, cas nécessitant une hospitalisation), une antibiothérapie par voie parentérale par de la gentamicine (5 mg/Kg/jour) pendant 10 jours est recommandée. Un traitement plus long peut s'avérer nécessaire selon la sévérité du cas ou le délai avant instauration du traitement. Cependant les recommandations de l'OMS font encore état d'une administration en deux fois par jour alors que les aminosides sont actuellement administrés en monodose journalière afin de limiter leur toxicité. Un monitoring des concentrations plasmatiques résiduelles de gentamicine est recommandé afin de limiter ses effets toxiques. La streptomycine, administrable par voie intramusculaire, est une alternative dans les pays dans lesquels cette molécule est encore disponible. La streptomycine a notamment permis de traiter avec succès 11 cas d'enfants atteints de tularémie oropharyngée en échec thérapeutique sous gentamicine. (192)

La ciprofloxacine en monothérapie est le traitement recommandé pour les formes modérées de la maladie avec une période de traitement de 10 à 14 jours. La doxycycline est une alternative pour le traitement des formes modérées mais la durée de traitement doit dans ce cas être au minimum de 15 jours car il s'agit d'un antibiotique uniquement bactériostatique. Il semblerait néanmoins que la doxycycline soit associée à davantage d'échecs thérapeutiques. (74) En ce qui concerne le traitement des formes modérées chez l'enfant, la doxycycline est contre-indiquée avant l'âge de 8 ans car elle entraîne une coloration irréversible de la dentine.

Le traitement des patients immunodéprimés nécessite une antibiothérapie bactéricide de 14 jours minimum. Les molécules de choix dans ce cas sont à nouveau la gentamicine et la ciprofloxacine.

Les options thérapeutiques pour le traitement des femmes enceintes sont limitées. La doxycycline est contre-indiquée pendant les deux derniers trimestres de la grossesse ainsi que pendant l'allaitement. Les aminosides et les fluoroquinolones ne sont en principe pas non plus recommandés en cas de grossesse mais le consensus de 2001 recommande malgré tout ces molécules devant l'absence d'alternatives thérapeutiques. Certains macrolides comme l'azithromycine pourraient être une option envisageable pour le traitement des souches de

biovar I de la sous-espèce *F. tularensis* subsp. *holarctica*, qui sont sensibles aux macrolides *in vitro* mais ces données demandent à être confirmées. (221)

Enfin, les mesures prophylactiques en cas d'exposition à cette bactérie sont variables selon la situation :

- L'exposition avérée et directe à la bactérie par le personnel de laboratoire par contact cutané doit conduire à l'instauration d'un traitement sans délai pendant une période de 14 jours. La ciprofloxacine (2 x 500 mg/j) ou la doxycycline (2 x 100 mg/j) sont les molécules recommandées. La réalisation immédiate d'une sérologie tularémie (pour déterminer le statut sérologique initial) ainsi que 14 jours plus tard est préconisée.
- Dans le cas d'une exposition peu probable ou sans contact rapproché avec la bactérie, une surveillance clinique semble suffisante avec réalisation d'une sérologie immédiate et à 14 jours post-exposition.
- L'aérosolisation accidentelle de la bactérie doit également conduire à une surveillance clinique forte et à l'instauration d'un traitement de 14 jours en cas de fièvre. (83)

1.12. Sensibilité de *F. tularensis* aux antibiotiques

Actuellement, aucune souche de *F. tularensis* résistante à l'un des antibiotiques indiqués pour le traitement de la tularémie (gentamicine, streptomycine, doxycycline et ciprofloxacine) n'a été isolée chez l'homme. De ce fait les antibiogrammes ne sont pas réalisés en routine sur les souches humaines dans le cadre du diagnostic de la tularémie. Cependant, des souches résistantes soit aux tétracyclines, soit aux fluoroquinolones, soit aux macrolides, soit à la streptomycine, ont été facilement obtenues *in vitro*. (83,222–224) L'apparition de mutation de résistance *in vivo* semble donc possible, notamment en cas de mauvaise diffusion d'un antibiotique au site de l'infection ou en cas de mauvaise observance thérapeutique conduisant à des concentrations plasmatiques sub-inhibitrices au site de l'infection, favorable au développement de résistances. D'autres antibiotiques ont été évalués vis-à-vis de cette bactérie mais la standardisation des conditions de réalisation des antibiogrammes reste faible

malgré la publication de recommandations par le Clinical and Laboratory Standard Institute (CLSI) en 2005. Selon ce référentiel, les antibiogrammes de *F. tularensis* doivent être réalisés en microdilution en milieu liquide en utilisant un bouillon de Mueller-Hinton ajusté en cation et supplémenté avec 2% de facteurs de croissance type Polyvitex® (Annexe 1). Tous ces aspects seront développés ci-dessous dans une revue de la littérature qui fait l'objet du premier article de cette thèse, intitulé « *Francisella tularensis* susceptibility to antibiotics: a comprehensive review of the data obtained *in vitro* and in animal models ».

1.13. Echec thérapeutique sous aminoside, fluoroquinolone ou doxycycline

Très peu de données ont été regroupées concernant les échecs thérapeutiques observés lors du traitement de la tularémie. La rareté de la maladie fait qu'aucune étude randomisée de grande échelle comparant l'efficacité d'un traitement par aminoside, fluoroquinolone ou doxycycline n'a pu être conduite à ce jour. La dernière revue colligeant des données d'efficacité thérapeutique par molécules ou par classe d'antibiotique date de 1994. (225) Selon cette revue, le taux d'efficacité de la gentamicine est de 86% (6% de rechutes et 8% d'échec thérapeutiques), alors que celui de la streptomycine est de 97%. Cette revue n'intègre que 6 cas traités avec succès par de la ciprofloxacine ou de la norfloxacine car le traitement de la tularémie par cette classe d'antibiotique n'est rapporté que depuis 1989. (2) D'après cette même revue l'efficacité de la tétracycline semble être de 88% avec 12% de rechutes sans qu'il n'y ait d'échec thérapeutique.

Une nouvelle analyse des données cliniques d'efficacité du traitement de la tularémie a été réalisée en se basant sur les données publiées en langue anglaise et française à l'aide des mots clés « *Francisella* » et/ou « tularaemia » dans la base de données Pubmed, depuis l'utilisation des fluoroquinolones pour le traitement de la tularémie (1989 à 2016). Seuls les articles traitant de plus de 5 cas de tularémie ont été inclus dans l'analyse. Seuls les articles offrant suffisamment d'informations sur l'évolution des patients (succès, rechute, échec thérapeutique) détaillée au minimum par classes d'antibiotiques ont été sélectionnés pour être analysés.

Au total, vingt-huit études traitant plus de 5 cas de tularémie ont été identifiées depuis 1989, regroupant 2740 cas de tularémie issus des Etats-Unis, d'Espagne, de Turquie, de France, de Finlande, de Suède, de Bulgarie et de Georgie. (3,128–130,133,170,171,179,226–245) La majorité des cas provenait néanmoins de Turquie (1977/2740 soit 72%) démontrant qu'il s'agit d'un pays de forte endémie pour cette pathologie. Huit études furent exclues du fait de l'absence de détail des succès et échecs thérapeutiques selon la classe d'antibiotique utilisée ou à cause de définition des échecs thérapeutiques non conforme aux autres études. (133,170,171,179,231,235,237,239,243,244) Enfin, une étude a été éliminée car elle ne traitait que de cas d'échecs après traitement par gentamicine, introduisant ainsi un biais dans l'analyse. (232) Sur les dix-sept études restantes, seules deux sont multicentriques et seules trois prospectives, ce qui témoigne de la difficulté à réaliser des études à haut niveau de preuve en ce qui concerne le traitement de la tularémie. Toutes les formes cliniques sont représentées : 559 formes oro-pharyngées, 206 ulcéro-ganglionnaires, 116 ganglionnaires, 50 typhoïdiques, 43 oculo-ganglionnaires et 31 formes pulmonaires. Néanmoins, seuls 785 patients présentaient des données suffisantes pour évaluer l'efficacité des traitements par classe d'antibiotique : 483 patients en Turquie, 130 patients en Espagne, 85 patients aux USA, 51 patients en France, 24 patients en Bulgarie et 12 patients en Suède (Tableau 5).

Référence	Délai avant mise en place d'une antibiothérapie efficace (jours)	Traitement				Echec thérapeutique			
		Aminoside	DOXY	FQ	Aminoside + DOXY	Aminosides	DOXY	FQ	Aminoside +DOXY
(226)	< 21 jours : 50 /138 patients	48		82	6	18		24	
(227)	13 jours (dispersion: 0–82 jours)	17	18	13		1	2	3	
(228)	19,1 +/- 7,3 jours	20		7		5		1	
(128)	47,5 +/- 31 jours	94	14	22		22	6	1	

(229)	21 +/- 13,7 jours	44	53	48		14	27	14	
(230)	21,6 +/- 6,9 jours		2	2	11		0	0	5
(3)	ND	1	25	25		0	8	12	
(245)	> 5 jours : 2/12 patients			12				2	
(233)	17.7 jours (dispersion : 8 - 40 jours) en 2003 et 8 jours (dispersion : 3 - 12 jours) en 2004.			8	8			0	7
(234)	40,7 +/- 22,8 jours	1		1	15	1		0	7
(130)	ND		1	5	12		0	2	8
(236)	≤ 3 semaines : 9 patients ; >3 semaines : 7 patients				16				≤ 3 semaines : 0/9 ; >3 semaines : 7/7
(238)	ND	2	8			0	0		
(129)	ND	4	2		1	0	1		0
(240)	10-14 jours (dispersion 3-30 jours)	28	1	1		3	5	1	
(241)	30 jours				12				3
(242)	12 jours	8				3			
Total		267	124	226	81	66	45	59	37

ND : non déterminé ; FQ : fluoroquinolone ; DOXY : Doxycycline

Tableau 5 : Analyse des échecs thérapeutiques du traitement de la tularémie par classe d'antibiotique entre 1989 et 2016

Les traitements identifiés étaient très variables selon les cas. Les traitements de première intention par des aminosides en monothérapie représentent 34% des cas avec 172 patients traités avec de la streptomycine, 93 avec de la gentamicine et 2 avec de l'amikacine. Les fluoroquinolones comptent pour 29% des traitements de première ligne avec 199 patients traités par de la ciprofloxacine, 12 par de la moxifloxacine, 9 par de l'ofloxacine et 5 par de la lévofloxacine. Les tétracyclines n'arrivent qu'en troisième position (18%) avec le traitement de 124 patients par de la doxycycline et 17 par de la tétracycline. Plusieurs combinaisons d'antibiotiques sont également utilisées en première intention. Les bithérapies associent principalement un aminoside et une tétracycline (17% des traitements initiaux : streptomycine + doxycycline : 78 patients ; streptomycine + tétracycline : 51 patients ; gentamicine + tétracycline : 1 patient ; gentamicine + doxycycline : 3 patients). Le traitement initial par une bithérapie combinant gentamicine et ciprofloxacine n'est retrouvé que dans deux cas et la combinaison ciprofloxacine et doxycycline que dans un cas.

Au total, des échecs thérapeutiques sont rapportés dans 25% (66/267) des cas de traitement en monothérapie par un aminoside, 26% (59/225) des cas de traitement en monothérapie par une fluoroquinolone et 36% (45/124) des cas de traitement en monothérapie par de la doxycycline. Il faut néanmoins garder à l'esprit que les aminosides sont utilisés pour traiter des formes plus sévères que celles traitées classiquement par de la ciprofloxacine ou de la doxycycline. En revanche, l'absence de définition consensuelle d'un échec thérapeutique entre les études rend l'analyse statistique très difficile et l'interprétation de ces chiffres doit être faite avec précaution. Au vu des définitions proposées dans les différentes études, il semble opportun de définir :

- l'échec thérapeutique par : l'incapacité d'un traitement oral ou parentéral, avec un antibiotique actif sur *F. tularensis*, à guérir la maladie. L'absence de guérison pouvant se traduire par un des signes suivants : fièvre persistante (> à 7 jours) ou récurrente, persistance d'un syndrome inflammatoire, élargissement des adénopathies présentes ou apparitions de nouvelles adénopathies, persistance d'une hyperleucocytose ou d'une CRP élevée à la fin du traitement, apparitions de complications telles qu'une suppuration ganglionnaire avec drainage spontané ou nécessitant un drainage chirurgical ou une résection de l'adénopathie.

- la rechute qui se manifeste par la réapparition des signes cliniques alors que les symptômes avaient disparus suite à un traitement efficace bien conduit.

Ces estimations de l'efficacité des différentes classes d'antibiotiques actives sur la tularémie semblent toutefois indiquer la supériorité d'un traitement par aminoside ou par fluoroquinolone par rapport à l'utilisation de la doxycycline en première intention. Malheureusement les taux de succès thérapeutiques n'étaient pas détaillés en fonction du délai d'instauration de l'antibiothérapie efficace pour le traitement de la tularémie. Plusieurs études font part d'un taux de succès thérapeutique supérieur en cas d'instauration du traitement dans les trois semaines suivant les premiers signes cliniques.(130,133,226,233,235,236,243)

Au vu de ces taux d'échec thérapeutique importants, y compris avec des antibiotiques bactéricides comme les fluoroquinolones ou la gentamicine, il semble nécessaire d'investiguer les causes potentielles de ces échecs (notamment la présence de résistance bactérienne) et de rechercher des stratégies thérapeutiques alternatives. L'objet de ce travail de thèse a été de répondre à une partie de ces interrogations.

La première partie de cette thèse a consisté à faire le point sur la sensibilité de *F. tularensis* aux antibiotiques et à évaluer la sensibilité des souches françaises isolées au Centre National de Référence des *Francisella* (CNR des *Francisella*, Laboratoire de bactériologie, CHU de Grenoble Alpes). Pour cela, une revue de la littérature a été réalisée en complément de cette introduction pour colliger les données de sensibilité *in vitro* et *in vivo* de *Francisella tularensis* aux différents antibiotiques actuellement évalués sur cette bactérie. L'objectif était de déterminer s'il existe d'autres possibilités thérapeutiques que celles utilisées aujourd'hui, ou s'il existe des données sur la supériorité ou l'infériorité d'une classe d'antibiotiques par rapport aux autres parmi celles utilisées actuellement pour le traitement de la tularémie. Puis la sensibilité à 18 antibiotiques actuellement sur le marché, dont certains encore peu évalués sur *F. tularensis*, a été déterminée sur des souches françaises de *F. tularensis* dans le but d'identifier de potentielles nouvelles pistes thérapeutiques pour le traitement de ces souches ou/et d'identifier des souches résistantes aux traitements recommandés, pouvant expliquer certaines situations d'échec thérapeutique en France.

La deuxième partie de cette thèse s'est attelé à caractériser et rechercher des mutations de résistance de *F. tularensis* aux fluoroquinolones à partir de souches résistantes obtenues lors d'expérimentations *in vitro* avec des espèces de *Francisella* de virulence atténuée pour l'homme. Tout d'abord, la caractérisation fonctionnelle de l'impact de mutations de résistance aux fluoroquinolones générées *in vitro* sur une souche de *F. novicida* a été réalisée. Puis la présence des principales mutations de résistance aux fluoroquinolones retrouvées *in vitro* a été recherchée sur des prélèvements cliniques de patients atteints de tularémie, en situation d'échec thérapeutique sous fluoroquinolone.

Enfin, la troisième partie de cette thèse a consisté à évaluer de nouveaux agents antibactériens de structure bis-indolique, actifs vis-à-vis de *F. tularensis* et ayant un mécanisme d'action différent des antibiotiques actuellement disponibles sur le marché. Le

spectre d'activité de ces composés de structure originale a été défini. La détermination des motifs chimiques structuraux importants pour l'activité antibactérienne de ces composés originaux a été réalisée sur un modèle de *Staphylococcus aureus*, bactérie également sensible à l'activité antibactérienne de ces composés bis-indoliques, mais de manipulation plus aisée que les bactéries du genre *Francisella*.

2. SENSIBILITE AUX ANTIBIOTIQUES DE *F. tularensis*

2.1. Publication 1 : *Francisella tularensis*
susceptibility to antibiotics: a comprehensive review
of the data obtained *in vitro* and in animal models

(Soumis à *Frontiers in Cellular Infection and Microbiology*)

Dans ce premier article nous avons cherché à faire un état des lieux des données de sensibilité de *F. tularensis* aux antibiotiques, obtenus *in vitro* et en modèle animal, car peu de données ont été colligées aux cours des deux dernières décennies, notamment en ce qui concerne la sensibilité des souches aux fluoroquinolones. Le but de ce travail a également été d'identifier les antibiotiques encore peu évalués vis-à-vis de *F. tularensis* afin de pouvoir les inclure dans l'étude de la sensibilité des souches françaises aux antibiotiques que nous souhaitons réaliser (Article 2). Nous avons ainsi pu retrouver des données de sensibilité aux antibiotiques pour 900 souches de *F. tularensis* (107 souches de type A, 789 souches de type B et 4 souches appartenant à la sous-espèce *mediasiatica*). Des données de sensibilité concernant 33 antibiotiques ont été colligées, incluant quatre aminosides (gentamicine, streptomycine, amikacine, et tobramycine), neuf fluoroquinolones (ciprofloxacine, lévofloxacine, moxifloxacine, ofloxacine, norfloxacine, grépafloracine, sparfloxacine, trovafloxacine, et gatifloxacine), trois tétracyclines (tétracycline, doxycycline et tigécycline), quatre macrolides (érythromycine, azithromycine, roxithromycine et clarithromycine), un kétolide (télithromycine), neuf β -lactamines (ampicilline, amoxicilline/acide clavulanique, ceftazidime, pipéracilline/tazobactam, ceftriaxone, céfépime, imipénème, méropénème et aztréonam) ainsi que le chloramphénicol, la rifampicine et le linézolide. Nous avons également cherché à analyser les diverses techniques utilisées pour déterminer la sensibilité de *F. tularensis* aux antibiotiques afin de faire un état des lieux des pratiques et d'identifier des techniques potentiellement discordantes par rapport aux recommandations et aux concentrations critiques du Clinical and Laboratory Standard Institute, utilisées pour la classification des souches en sensible/intermédiaire/résistante aux antibiotiques. Enfin nous avons voulu rassembler les données disponibles en modèle animal concernant l'efficacité des différents antibiotiques utilisés dans le traitement de la tularémie, afin de voir si les mêmes conclusions que celles observées lors de l'analyse des cas humains dans l'introduction de cette thèse (Tableau 4) étaient obtenues en modèle animal, notamment en ce qui concerne la comparaison de l'efficacité des aminosides, des fluoroquinolones et de la doxycycline.

1 ***Francisella tularensis* susceptibility to antibiotics: a comprehensive**
2 **review of the data obtained *in vitro* and in animal models.**

3
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5
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16
17 **Keywords:** *Francisella tularensis*, antimicrobial susceptibility, MIC, MBC, intracellular, animal
18 model, tularaemia, antibiotic therapy

19

20 **Abstract (311/350 words)**

21 The antibiotic classes that are recommended for tularaemia treatment are the aminoglycosides, the
22 fluoroquinolones and the tetracyclines. However, cure rates vary between 60 and 100% depending
23 on the antibiotic used, the time to appropriate antibiotic therapy setup and its duration, and the
24 presence of complications such as lymph node suppuration. Thus, antibiotic susceptibility testing
25 (AST) of *F. tularensis* strains remains of primary importance for detection of the emergence of
26 antibiotic resistances to first-line drugs, and to test new therapeutic alternatives. However, the AST
27 methods reported in the literature were poorly standardized between studies and AST data have not
28 been previously evaluated in a comprehensive and comparative way. The aim of the present review
29 was to summarise experimental data on antibiotic susceptibilities of *Francisella tularensis* obtained
30 in acellular media, cell models and animal models since the introduction of fluoroquinolones in the
31 treatment of tularaemia in 1989. We compiled MIC data of 33 antibiotics (including
32 aminoglycosides, fluoroquinolones, tetracyclines, macrolides, β -lactams, chloramphenicol,
33 rifampicin and linezolid) against 900 *F. tularensis* strains (504 human strains), including 107 subsp.
34 *tularensis* (type A), 789 subsp. *holarctica* (type B) and four subsp. *mediasiatica* strains, using
35 various AST methods. Specific culture media were identified or confirmed as unsuitable for
36 antibiotic susceptibility testing of *F. tularensis*. Overall, MICs were the lowest for ciprofloxacin (\leq
37 0.002–0.125 mg/L) and levofloxacin, and ranged from \leq 0.016 to 2 mg/L for gentamicin, and 0.064
38 to 4 mg/L for doxycycline. No resistant strain to any of these antibiotics was reported.
39 Fluoroquinolones also exhibited a bactericidal activity against intracellular *F. tularensis* and lower
40 relapse rates in animal models when compared with the bacteriostatic compound doxycycline. As
41 expected, lower MIC values were found for macrolides against type A and biovar I type B strains,
42 compared to biovar II type B strains. The macrolides were more effective against *F. tularensis* grown
43 in phagocytic cells than in acellular media.

44 **Search strategy and selection criteria**

45 Data on antibiotic susceptibility testing (AST) of *Francisella tularensis*, both *in vitro* and in animal
46 models, were collected from the English and French literature in the PubMed database, since the
47 introduction of fluoroquinolones in the treatment of tularaemia in 1989 until December 2016. They
48 were extracted using the key words “tularemia” and “*Francisella*” in order to achieve a broad
49 screening of the entire body of literature on the subject since 1989. Only studies evaluating more
50 than five *F. tularensis* strains were selected for analysis.

51 **1. *Francisella tularensis* and tularemia**

52 *F. tularensis*, the etiological agent of tularaemia, is a Tier 1 biological threat agent according
53 to the classification of the Centers for Disease Control and Prevention (Dennis DT et al., 2001). It
54 was first isolated from ground squirrels in 1911 in Tulare County, California (USA), and from a
55 human tularaemia case in 1914 in Ohio (USA) (Mccoy and Chapin, 1912; Wherry and Lamb, 1914).
56 The name “*Francisella tularensis*” was coined in 1959 to honor Dr. Edward Francis, who greatly
57 contributed to improve the knowledge on human tularaemia (Francis et al., 1922; Francis, 1928;
58 Rockwood, 1983). *F. tularensis* is currently divided into three subspecies: subsp. *tularensis* (type A
59 strains), mainly found in North America; subsp. *holarctica* (type B strains) found throughout the
60 northern hemisphere; and subsp. *mediasiatica* found in Central Asia (Jellison, 1974; Jellison and
61 Owen, 1961; Olsufiev et al., 1959; Olsufjev, 1970; Olsufjev and Meshcheryakova, 1983). Debate
62 continues on whether *Francisella novicida* is a fourth subspecies of *F. tularensis* or a separate
63 species, but we agree with Johansson et al. in keeping *F. novicida* in a separate species because of its
64 aquatic reservoir and very low virulence in humans (Busse et al., 2010; Huber et al., 2010; Johansson
65 et al., 2010). Type B strains are also classically differentiated into three biovars (Kudelina and
66 Olsufiev, 1980; Olsufjev and Meshcheryakova, 1982, 1983): biovar I (naturally susceptible to
67 erythromycin) is found in Western Europe and North America; biovar II (naturally resistant to
68 erythromycin) is found in Eastern Europe and Asia; and biovar *japonica* (susceptible to erythromycin
69 but fermenting glycerol) is mainly found in Japan, although it has recently been described in China

70 and Turkey (Kiliç et al., 2013; Wang et al., 2014). Biovar II strains are found in the Eastern part of
71 Europe (Czech Republic, Finland, Georgia, Russia, Slovakia, Ukraine, Austria, Hungary and
72 Romania) (Chanturia et al., 2011; Gyuranecz et al., 2012; Svensson et al., 2009b; Vogler et al., 2009)
73 and in Asia and both biovar I and II strains coexist in Germany, Switzerland and Scandinavia
74 (Maurin and Gyuranecz, 2016; Muller et al., 2013; Origgi et al., 2014)

75 Although the *F. tularensis* genome displays very low variability, four distinct clades have
76 been identified by pulse-field gel electrophoresis (PFGE) within type A strains in the United States
77 (A.Ia, A.Ib, A.IIa and A.IIb) with the A.Ib clade being associated with a 24% mortality rate in
78 humans (Kugeler et al., 2009). Type B strains have also been divided into several clades by whole
79 genome sequencing. The four main clades include clade B.4 corresponding to North American
80 strains, clade B.6 to biovar I Western European strains, clade B.12 to biovar II Eastern European
81 strains, and clade B16 to strains belonging to biovar japonica (Fujita et al., 2008; Karlsson et al.,
82 2016; Kiliç et al., 2015; Vogler et al., 2009).

83 *F. tularensis* is a Gram-negative, facultative intracellular coccobacillus (Broman et al., 2011).
84 It is strictly aerobic, nonmotile, nontoxigenic and non-spore-forming. It is a fastidious bacterium that
85 may be grown in cysteine-enriched media, under 5% CO₂ atmosphere. The main virulence factor of
86 *F. tularensis* corresponds to its ability to multiply within eukaryotic cells, especially in the cytosol of
87 macrophages (Clemens and Horwitz, 2007). Virulence is associated with the presence of a duplicated
88 pathogenicity island in the bacterial genome, encoding a type VI-like secretion system (Nano and
89 Schmerk, 2007). However, the high variations in virulence observed among *F. tularensis* genotypes
90 remain currently unexplained.

91 Human tularaemia is a zoonotic disease usually occurring as sporadic cases or small familial
92 outbreaks (Bicakci and Parlak, 2008; Tärnvik and Berglund, 2003). However, a number of
93 tularaemia outbreaks have been reported, including in the last two decades (Akalın et al., 2009; Barut

94 and Cetin, 2009; Celebi et al., 2006; Cerný, 2001; Christova et al., 2004; Feldman et al., 2001; Hauri
95 et al., 2010; Helvacı et al., 2000; Johansson et al., 2014; Kantardjiev et al., 2006; Karlsson et al.,
96 2013; Larssen et al., 2011, 2014; Mailles et al., 2010; Mengelöglu et al., 2014; Payne et al., 2005;
97 Pérez-Castrillón et al., 2001; Petersen et al., 2008; Reintjes et al., 2002; Siret et al., 2006; Svensson
98 et al., 2009; Wang et al., 2011). Humans may be infected with *F. tularensis* through direct contact
99 with infected animals (manipulation of live or dead infected animals, animal bites or scratches),
100 consumption of contaminated food or water, exposure to contaminated environments or through
101 arthropod bites (Keim et al., 2007). *F. tularensis* can infect a large number of animal species, but
102 lagomorphs and small rodents are considered key hosts for this pathogen (Gyuranecz et al., 2011;
103 Maurin and Gyuranecz, 2016). This bacterium may also persist in water and soil environments for
104 several months, which might be related to an ability to multiply in protozoa such as amoebae (Abd et
105 al., 2003; Broman et al., 2011; El-Etr et al., 2009; Keim et al., 2007). A terrestrial and an aquatic
106 lifecycle have been described for this bacterium (Maurin and Gyuranecz, 2016). Arthropods such as
107 ticks and mosquitoes may be contaminated by *F. tularensis* from the animal or environmental
108 reservoirs (Maurin and Gyuranecz, 2016; Sjøstedt, 2007). Large tularaemia outbreaks have occurred,
109 for which multiple sources of contamination and several *F. tularensis* clones were identified (Akalin
110 et al., 2009; Barut and Cetin, 2009; Celebi et al., 2006; Cerný, 2001; Christova et al., 2004; Feldman
111 et al., 2001; Hauri et al., 2010; Helvacı et al., 2000; Johansson et al., 2014; Kantardjiev et al., 2006;
112 Karlsson et al., 2013; Larssen et al., 2011, 2014; Mailles et al., 2010; Mengelöglu et al., 2014; Payne
113 et al., 2005; Pérez-Castrillón et al., 2001; Petersen et al., 2008; Reintjes et al., 2002; Siret et al.,
114 2006; Svensson et al., 2009a; Wang et al., 2011). Symptoms vary according to the infection route
115 and classically correspond to six different clinical forms: ulceroglandular, glandular, oropharyngeal,
116 oculoglandular, pneumonic and typhoidal (Tarnvik A., 2007). Ulceroglandular and glandular forms
117 are consecutive to skin inoculation of bacteria (e.g. through an arthropod bite or contact with infected
118 animals). A regional lymphadenopathy develops with (ulceroglandular form) or without (glandular

119 form) a skin ulcer at the inoculation site. When contamination occurs through the ocular conjunctiva,
120 painful conjunctivitis with regional lymphadenopathy develops, which corresponds to oculoglandular
121 tularaemia. The oropharyngeal form is characterised by pharyngitis with regional lymphadenopathy,
122 usually occurring after consumption of contaminated meat or water. Pneumonic tularaemia results
123 from airborne contamination or hematogenous spread of bacteria to the lungs and is characterised by
124 unspecific symptoms such as cough, fever, dyspnea and occasionally a mediastinal or hilar
125 lymphadenopathy. The typhoidal form corresponds to systemic disease with neurological symptoms
126 mimicking typhoid, often with *F. tularensis* bacteraemia, but without detection of any portal of entry
127 of bacteria (especially no skin ulcer) and without symptoms of localised infection (especially no
128 regional lymphadenopathy) (Tärnvik and Chu, 2007). Complications may occur such as skin
129 eruptions, abscess formation, lymph node suppuration with occasionally skin fistulisation, and
130 secondary infectious locations due to hematogenous spread of bacteria (Erdem et al., 2014; Maurin et
131 al., 2011; Meric et al., 2008; Tärnvik and Chu, 2007). Diagnosis is usually based on compatible
132 clinical and epidemiological features, and positive serological tests. PCR is useful to detect *F.*
133 *tularensis* DNA in various clinical samples, especially before specific antibodies can be detected
134 (Tärnvik A., 2007). Isolation of a *F. tularensis* strain from blood or tissue samples is obtained in less
135 than 20% of patients, which makes antibiotic susceptibility testing (AST) of *F. tularensis* strains rare
136 (Maurin et al., 2011).

137 The 2007 WHO guidelines for treatment of tularaemia are based on the 2001 consensus
138 recommendations from Dennis et al (Dennis DT et al., 2001). According to these publications,
139 tularaemia treatment is mainly based on three antibiotic classes: the aminoglycosides, the
140 fluoroquinolones and the tetracyclines, although many other antibiotic classes have been tested
141 against *F. tularensis in vitro*. Here we have compiled all available experimental data on the
142 evaluation of the activity of antibiotics against *F. tularensis in vitro*, in acellular and cell models, and
143 *in vivo* in animal models to provide useful information for clinical microbiologists and physicians.

144 **2. Antibiotic susceptibility testing of *F. tularensis***

145 AST of *F. tularensis* strains is usually not performed on a routine basis because: 1) isolation
146 of the strain involved is only obtained in a few tularaemic patients (Maurin et al., 2011); 2) this
147 procedure is at risk for the laboratory personnel handling cultures of this highly infectious pathogen
148 and requires biosafety level 3 (BSL3) facilities (Tärnvik and Chu, 2007); 3) acquired resistance to
149 antibiotics has never been reported so far in clinical strains of *F. tularensis* (Tarnvik A., 2007) and 4)
150 *in vitro* data may not be predictive of treatment failure in tularaemia patients. However, reference
151 laboratories have reported AST surveys for collections of human and animal strains of *F. tularensis*
152 (García del Blanco et al., 2004; Georgi et al., 2012; Hotta et al., 2013; Ikäheimo et al., 2000;
153 Johansson et al., 2000, 2002a; Kiliç et al., 2013; Kreizinger et al., 2013; Origgi et al., 2014; Scheel et
154 al., 1993; Tomaso et al., 2005; Urich and Petersen, 2008; Valade et al., 2008a; Velinov, 2011;
155 Yeşilyurt et al., 2011).

156 For this review, we collected *F. tularensis* AST results from all studies of more than five
157 tularaemia cases, published in the literature since 1989. We obtained data from 898 *F. tularensis*
158 strains isolated from humans (510 strains), animals (200 strains), arthropods (four strains), natural
159 water samples (39 strains) and unknown sources (147). They included 107 type A, 789 type B and
160 four *F. tularensis* subsp. *mediasiatica* strains (Tables 1 and S1) (García del Blanco et al., 2004;
161 Georgi et al., 2012; Hotta et al., 2013; Ikäheimo et al., 2000; Johansson et al., 2000, 2002a; Kiliç et
162 al., 2013; Kreizinger et al., 2013; Origgi et al., 2014; Scheel et al., 1993; Tomaso et al., 2005; Urich
163 and Petersen, 2008; Valade et al., 2008a; Velinov, 2011; Yeşilyurt et al., 2011). AST results were
164 available for 33 antibiotics, although only some of them were tested in all studies (Tables 1–5 and
165 S1). These antibiotics included four aminoglycoside compounds (gentamicin, streptomycin,
166 amikacin and tobramycin), nine fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin,
167 ofloxacin, norfloxacin, grepafloxacin, sparfloxacin, trovafloxacin and gatifloxacin), three
168 tetracyclines (tetracycline, doxycycline and tigecycline), four macrolides (erythromycin,

169 azithromycin, roxithromycin and clarithromycin), one ketolide (telithromycin), nine β -lactams
170 (ampicillin, amoxicillin + clavulanic acid, ceftazidime, piperacillin + tazobactam, ceftriaxone,
171 cefepime, imipenem, meropenem and aztreonam), chloramphenicol, rifampicin and linezolid.

172 **2.1. MICs: Methods and results**

173 According to the Clinical and Laboratory Standards Institute (CLSI) 2005 and WHO
174 guidelines, AST of *F. tularensis* strains should be performed using cation-adjusted Mueller-Hinton
175 broth enriched with 2% defined growth supplement such as Polyvitex® (referred to here as enriched
176 caMHB), in order to fulfill the cystein growth requirement of this bacterium (CLSI 2005; Tarnvik A.,
177 2007). Adjustment of pH to 7.1 ± 0.1 is mandatory after addition of 2% growth supplement because
178 it significantly reduces the pH of caMHB medium. The bacterial inoculum must be calibrated at 5
179 10^5 CFU/mL of final concentration. Culture media should be incubated for 48 h in ambient air, but
180 incubation in 5% CO₂-enriched atmosphere may be needed for some strains, although it can lead to
181 acidification of the culture medium and therefore overestimation of aminoglycoside and macrolide
182 MICs, or underestimation of tetracyclines MICs (CLSI. M45-A2. 2010).

183 In the past three decades, three different methods for *F. tularensis* AST were most frequently
184 reported in the literature: antibiotic agar dilution, broth microdilution and E-test strips. These
185 techniques were poorly standardised between studies, including after CLSI guidelines were available
186 (CLSI. M45-A2. 2010). Most particularly, growth media, incubation conditions (atmosphere and
187 duration) and bacterial inocula used for *F. tularensis* AST greatly varied between studies. Therefore,
188 the results obtained in these different studies should be compared with caution.

189 As for the broth microdilution method, a number of authors used Mueller-Hinton II broth
190 supplemented with glucose, Ca²⁺ and Mg²⁺ ions, and ferric pyrophosphate (a medium referred to as
191 modified MHII) in spite of the recommended enriched caMHB medium (Baker et al., 1985; García
192 del Blanco et al., 2004; Origgi et al., 2014). The use of MHII resulted in overestimation of the MICs

193 of aminoglycosides and tetracyclines compared to caMHB (Table S1). The use of MHII for *F.*
194 *tularensis* AST should be discouraged to avoid erroneous classification of some strains as resistant to
195 aminoglycosides and/or tetracyclines using the CLSI breakpoints for susceptibility (concentration
196 value threshold used for the categorization of a bacterial strain as susceptible, intermediately
197 susceptible or resistant to an antibiotic). To date, antibiotic resistance reported in the literature for
198 natural strains of *F. tularensis* have never been formally characterized by a reference laboratory. In
199 the following paragraphs, the term resistant is applied to strain whose MICs are not classified as
200 susceptible according to CLSI breakpoints for susceptibility, even though the mechanism of
201 resistance has not been characterized. The MHII medium may also affect the activity of
202 fluoroquinolones since ciprofloxacin MICs up to 0.25mg/L were reported with this medium (García
203 del Blanco et al., 2004; Maurin et al., 2000), while MICs for this antibiotic range from ≤ 0.002 to
204 0.125 mg/L (MIC₉₀, ≤ 0.016 to 0.047 mg/L) for all other studies reported (Tables 1 and S1).

205 Several agar media were used for MIC determination using the E-test strip technique (Tables
206 1 and S1). MIC ranges observed with these different solid media were similar to each other and to
207 those obtained with the broth microdilution method using enriched caMHB. No strain was classified
208 as resistant to any of the drugs used for tularaemia treatment and for which CLSI breakpoints have
209 been defined (i.e. gentamicin, streptomycin, ciprofloxacin, levofloxacin, tetracycline, doxycycline
210 and chloramphenicol). Thus, the E-test strip methodology appears to be a convenient alternative for
211 antibiotic susceptibility categorisation of *F. tularensis* strains compared to the more time-consuming
212 and fastidious broth microdilution method. Moreover, this method is less risky for laboratory
213 personnel because it does not require handling large volumes of liquid suspensions of *F. tularensis*.
214 However, no study has compared AST results using MIC strips to those of the microdilution
215 reference method using enriched caMHB. Standardisation of agar media for *F. tularensis* AST would
216 be beneficial for comparison of studies conducted in different laboratories.

217 A few studies have compared *F. tularensis* antibiotic susceptibilities when using the agar
218 dilution technique with different solid media (Table 1 and S1). Blood cysteine agar should be
219 avoided since it identified resistant strains of *F. tularensis* for gentamicin or streptomycin, which was
220 never confirmed by characterising the resistance mechanisms involved. For the Thayer Martin agar
221 and enriched Mueller-Hinton agar, results were concordant with those of the broth microdilution and
222 MIC strip assays. One study compared MIC results using the agar dilution method, the MIC strip
223 method or the broth microdilution reference method. Unfortunately, results obtained with the
224 reference method were not reproducible. The correlation of results obtained with either the agar
225 dilution or MIC strip tests, using enriched Mueller Hinton agar, were 87% for doxycycline, 94% for
226 ciprofloxacin, but only 42% for gentamicin. The agar dilution method poorly correlated with the
227 broth microdilution method, with only 72% correlation for doxycycline, 68% for ciprofloxacin and
228 51% for gentamicin.

229

230 According to our previous comments on MIC methods, we excluded MIC values obtained
231 with the broth microdilution method using modified MHII broth and those determined by the agar
232 dilution method using blood cystein agar plates. (Table S1) MICs obtained with all other methods
233 were recorded, although analysed cautiously because of poor methodological standardisation, as
234 previously mentioned. The remaining 812 *F. tularensis* strains were categorised as susceptible to
235 first-line antibiotics for tularaemia, including the aminoglycosides, the fluoroquinolones and the
236 tetracyclines. MIC ranges and CLSI breakpoints for these antibiotics are shown in Table 2.

237 **2.1.1. Aminoglycosides, fluoroquinolones and doxycycline**

238 Altogether, among antibiotic classes recommended for first-line treatment of tularaemia,
239 ciprofloxacin displayed the lowest ranges for MICs (≤ 0.002 – 0.125 mg/L) and MIC_{S90} (≤ 0.016 –
240 0.064 mg/L) between studies. Gentamicin MICs ranged from ≤ 0.016 to 2 mg/L, and MIC_{S90} from

241 0.064 to 1 mg/L. The MIC and MIC₉₀ ranges for doxycycline were 0.064–4 mg/L and 0.25–2 mg/L,
242 respectively (Table 1). Only slight differences in susceptibility to these antibiotics were observed
243 between type A and type B strains of *F. tularensis*, or between various biovars or clades within type
244 A and type B strains.

245 Among fluoroquinolones, ciprofloxacin and levofloxacin were the most active compounds *in*
246 *vitro* (Table 1). All strains tested displayed MIC levels to these antibiotics at least fourfold less than
247 the CLSI breakpoint for susceptibility (Table 2). Moxifloxacin, norfloxacin, gatifloxacin,
248 grepafloxacin, trovafloxacin and sparfloxacin were also effective against *F. tularensis in vitro* (Table
249 3), but the three latter antibiotics have been withdrawn from the market because of potential severe
250 side effects.

251 As for the aminoglycosides, tobramycin and amikacin were evaluated in three studies, only
252 against type B strains. (Ikäheimo et al., 2000; Tomaso et al., 2005; Yeşilyurt et al., 2011) Tobramycin
253 displayed MIC values (0.125–3 mg/L) close to those of gentamicin and streptomycin. Interestingly,
254 Enderlin et al., reported in 1994 a cure rate with this antibiotic of only 50% but for a limited number
255 of patients (3/6 patients) (Enderlin et al., 1994). Conversely, amikacin was less active *in vitro*, with
256 MICs up to 16 mg/L (Table 3) (Tomaso et al., 2005).

257 Thus all strains were confirmed as susceptible to the first-line antibiotics recommended for
258 tularemia treatment, with ciprofloxacin and levofloxacin showing the lowest MICs *in vitro*.

259

260

2.1.2. Macrolides

261 Erythromycin MICs are much higher for biovar II *F. tularensis* subsp. *holarctica* strains, than
262 for biovar I strains of the same subspecies and for *F. tularensis* subsp. *tularensis* strains. Kudelina et
263 al first described erythromycin-resistant strains of *F. tularensis* as those able to grow on media

264 containing up to 6400 mg/L of this antibiotic, while susceptible strains could be killed by 25 mg/L of
265 this antibiotic (Kudelina and Olsufiev, 1980). In the literature, type B biovar II strains, which
266 currently correspond to East European strains belonging to the B12 subclade, were all resistant to
267 erythromycin with MIC > 256 mg/L using the E-test strip method (Table 4) (Karlsson et al., 2016).
268 Only Tomaso et al. reported one biovar II strain with an erythromycin MIC of only 4 mg/L, but this
269 result can be questioned according to current literature (Tomaso et al., 2005). Using the broth
270 microdilution method, all type B biovar II strains had erythromycin MICs of ≥ 32 mg/L (Table 1). In
271 contrast, all type B biovar I strains displayed erythromycin MICs ≤ 8 mg/L using the broth
272 microdilution method and ≤ 1 mg/L using MIC strips. Among erythromycin-susceptible *F. tularensis*
273 strains, subtle differences in erythromycin MICs could be found in the literature between the US
274 strains belonging either to type B biovar I (MIC range, 0.125–2 mg/L; MIC₉₀ range, 0.5–1 mg/L) or
275 to type A (MIC range, 0.125–1 mg/L), and the West European type B biovar I strains (MIC range, 1–
276 8 mg/L; MIC₉₀, 4 mg/L). This difference might be correlated with the B6 clade displaying higher
277 MIC levels, or to methodological differences such as incubation of media in ambient air versus under
278 5% CO₂ atmosphere, the latter reducing the activity of the macrolides. The Japanese type B strains,
279 including those formally identified as belonging to biovar japonica, displayed erythromycin MICs
280 ranging from 0.094 to 1.5 mg/L.

281 According to the above data, a breakpoint for the biovar II definition might be set at
282 erythromycin MIC ≥ 32 mg/L, in agreement with Kudelina's above-mentioned study (Kudelina and
283 Olsufiev, 1980). Moreover, erythromycin resistance in type B biovar II strains has recently been
284 correlated with the presence of a single mutation A2059C in the three copies of the *rrl* gene,
285 encoding the 23S rRNA (Karlsson et al., 2016). Thus, determination of the *rrl* gene sequence
286 provides a confirmatory identification of biovar II subtype.

287 Azithromycin MICs against *F. tularensis* were also determined in three studies, with the same
288 dichotomy between biovar I and biovar II of type B strains. MIC ranges were similar to those

289 observed for erythromycin (Table 4). Biovar II strains displayed azithromycin MICs > 256 mg/L,
290 whereas MIC ranges were 0.064–2 mg/L for 16 type B and 0.125–2 mg/L for eight type A strains
291 from the USA (Ikäheimo et al., 2000; Johansson et al., 2002b; Yeşilyurt et al., 2011). Telithromycin,
292 a ketolide compound, displayed MICs ranging from 0.125 to 0.25 mg/L with the agar dilution
293 method against type B biovar I strains (Valade et al., 2008a).

294 Thus, low MIC values are observed for macrolides and one ketolide against biovar I strains
295 although. No susceptibility breakpoints are currently defined by the CLSI for these antibiotics
296 against *F. tularensis*, although they may represent useful therapeutic alternatives for infection related
297 to biovar I strains, especially if first-line antibiotics are contraindicated. Further experimental data in
298 animal models are needed for the *in vivo* evaluation of the activity of these compounds against *F.*
299 *tularensis*. In contrast, the macrolides and ketolides are ineffective against biovar II strains.

300 **2.1.3. Beta-lactams**

301 Many β -lactams have been tested *in vitro* against *F. tularensis*, although they are considered
302 unreliable for treatment of tularaemia (Cross et al., 1995; Cross and Jacobs, 1993; Maurin et al.,
303 2011; Tarnvik A., 2007). In the literature, all the *F. tularensis* strains tested were resistant to
304 penicillin A, ticarcillin and piperacillin, whether or not associated with a β -lactamase inhibitor, with
305 MICs \geq 64 mg/L (Table 5). In contrast, the cephalosporins, monobactams and carbapenems
306 displayed larger MIC ranges. Although most *F. tularensis* strains displayed MICs higher than 32
307 mg/L to these β -lactams, a few strains displayed MICs lower than 1 mg/L. (Table 5). In a study from
308 Hotta et al., 30–60% of 36 Japanese strains displayed MICs lower than 1 mg/L for cefotaxime,
309 ceftriaxone, cefoxitin, aztreonam, imipenem and meropenem (Hotta et al., 2013). Velinov et al.
310 described 21 Bulgarian strains with a MIC range for ceftriaxone between 2 and 4 mg/L (Velinov,
311 2011). To date, two β -lactamase genes (*bla1* and *bla2*) have been identified in the LVS strain (with
312 corresponding homologues in the Schu S4 strain), but the expression of recombinant LVS proteins in

313 *Escherichia coli* only revealed the *bla2_{LVS}* gene to encode a functional β -lactamase.(Bina et al., 2006)
314 In 2012, Antunes et al. reported a class A β -lactamase (FTU-1) present in 14 strains belonging to all
315 *F. tularensis* subspecies, including various type B strains from the USA, France, Japan, Russia and
316 Sweden (Antunes et al., 2012). Actually, the FTU-1 corresponds to the *bla2_{LVS}* gene, as evidenced by
317 comparison of gene sequences (YP_513599.1 and FTT_0611c, respectively). This class A β -
318 lactamase, which was partially inactivated by β -lactamase inhibitors, induced resistance to penicillin
319 and a fourfold increase in ceftazidime MIC when cloned and expressed in *E. coli*. This β -lactamase
320 had no effect on other cephalosporins, monobactams and carbapenems (Antunes et al., 2012; Bina et
321 al., 2006). The presence of the FTU-1/*bla2_{LVS}* gene is thus compatible with the cephalosporin-
322 susceptibility phenotype observed in Japanese strains, whereas additional resistance mechanisms are
323 probably involved in strains displaying resistance to cephalosporins, monobactams and carbapenems
324 (Table 5). In 2008, Bina et al. characterised an AcrAB RND efflux system in the LVS strain, which
325 conferred increased resistance to all beta-lactams tested in the study (i.e. ampicillin, carbenicillin and
326 cefoperazone) but not to carbapenems (Bina et al., 2008). Thus, β -lactams are not recommended for
327 the treatment of tularemia.

328 **2.1.4. Chloramphenicol**

329 The reported chloramphenicol MICs range from ≤ 0.023 mg/L to 4 mg/L, with an MIC₉₀
330 range of 0.25–2 mg/L (Table 4). Chloramphenicol has been used occasionally (alone or in
331 combination with other antibiotics) to treat patients with tularaemia meningitis, owing to its high
332 distribution in brain tissue and cerebrospinal fluid (Hofinger DM et al., 2009; Tarnvik A., 2007).
333 Considering the MIC breakpoint of 8 mg/L, *F. tularensis* can be considered susceptible to
334 chloramphenicol, but the clinical use of this antibiotic is currently restricted to meningitis because of
335 potential severe bone marrow toxicity.

336 **2.1.5. Rifampicin**

337 Rifampicin is active *in vitro* against *F. tularensis* but with MICs ranging from ≤ 0.094 to 3
338 mg/L, and a MIC₉₀ range of 0.25–1.5 mg/L (Table 4). However, this antibiotic is not recommended
339 for tularaemia treatment because of potential rapid selection of resistant mutants, as described by
340 Johansson et al (Johansson et al., 2000; Tarnvik A., 2007). It has been used in combination with
341 ciprofloxacin for treatment of an infected total knee arthroplasty, with resolution of symptoms only
342 after addition of rifampicin and a successful outcome with this antibiotic combination (Cooper et al.,
343 1999).

344 **2.1.6. New antibiotics**

345 Among more recently developed antibiotics, tigecycline, a new glycylicycline, has been
346 evaluated in two studies using the E-test method, with reported MICs (0.094–0.38 mg/L) and MIC₉₀
347 (0.19–0.25 mg/L) ranges lower than those determined in the same studies for tetracycline and
348 doxycycline (Tables 1 and 4) (Kreizinger et al., 2013; Yeşilyurt et al., 2011). No breakpoints are
349 currently defined for *F. tularensis* susceptibility to tigecycline, but all tested strains displayed MICs
350 ≤ 0.5 mg/L. Further studies are needed to evaluate tigecycline MICs using the microdilution
351 reference method. Importantly, fresh medium (< 24 h) must be used to prevent overestimation of
352 tigecycline MICs (Bradford et al., 2005). Tigecycline might be a suitable alternative to doxycycline
353 for treatment of tularaemia, but its much broader antibacterial spectrum is a disadvantage due to a
354 greater deleterious effect on the gut commensal flora.

355 Conflicting results were observed for linezolid, an oxazolidinone compound. The activity of
356 this antibiotic is considered mainly restricted to Gram-positive bacteria, with a CLSI susceptibility
357 breakpoint for the corresponding species set at ≤ 2 mg/L. However, MICs of 4–8 mg/L have been
358 found for Gram-negative bacteria of the genera *Bacteroides*, *Moraxella* and *Pasteurella*, owing to
359 the absence or low efficacy of efflux systems, or a high affinity of their ribosomes for linezolid
360 (Livermore, 2003). As for *F. tularensis*, linezolid displayed MICs of 0.5–2 mg/L for 39 biovar II

361 type B strains from Turkey, 2–4 mg/L for eight type A strains from the USA, and 4–16 mg/L for 16
362 type B strains from the USA, when using the E-test strip procedure. In contrast, MICs ranged from
363 12 to 48 mg/L for 29 Hungarian biovar II type B strains using the same technique, although such
364 differences could be related to the use of different solid media for AST (Tables 1 and 4). The relative
365 susceptibility of *F. tularensis* to linezolid may be related to its small genome with a limited number
366 of efflux systems, while MIC variations between strains may reflect variable expression of such
367 efflux systems. This hypothesis needs to be further assessed on a larger panel of *F. tularensis* strains.

368 **2.1.7. Conclusion**

369 In conclusion, MIC data confirm that among the antibiotics recommended as first-line
370 treatment of tularaemia, ciprofloxacin and levofloxacin display the lowest MIC ranges against *F.*
371 *tularensis*. Gentamicin ranks second, while doxycycline has the highest MIC range with some strains
372 displaying an MIC at 4 mg/L, which is the breakpoint for susceptibility (4 mg/L). However, no
373 resistant strains to any of these antibiotics have been characterised so far. Moreover,
374 chloramphenicol is active and may be used in combination for rare meningitis due to *F. tularensis*.
375 Azithromycin and telithromycin may be useful alternatives for infection related to biovar I strains of
376 *F. tularensis* subsp. *holarctica*, when all first line antibiotics are contraindicated, but still require
377 confirmation of their efficacy in animal models. Tigecycline and rifampicin are active *in vitro* and
378 should be also further evaluated in animal models. Rifampicin may be an interesting antibiotic in
379 association to fluoroquinolones for rare bone and joint infections.

380

381 **2. 2. MBCs and other bactericidal assays**

382 A few *in vitro* studies have reported either MBC or time-kill kinetic experiments for *F.*
383 *tularensis*. MBC testing is performed similarly to MIC testing using a broth microdilution method,

384 but an aliquot of each well with no visible growth after 48 h incubation is plated on enriched
385 chocolate agar media for CFU count determination. After 48 h incubation of the chocolate agar
386 plates at 37°C, under 5% CO₂ atmosphere, CFU counting can determine the MBC, which is the
387 lowest antibiotic concentration allowing 3-log or greater reduction of the primary bacterial inoculum.

388 Time-kill kinetic experiments determine CFU counts over time in antibiotics containing
389 cultures, incubated for 48 h at 37°C, under 5% CO₂ atmosphere. Experiments are conducted using
390 enriched caMHB medium, containing two, four or eight times the MIC of the tested antibiotic
391 compound (Caspar et al., 2014; Maurin et al., 2000).

392 The MBCs of several antibiotics (ceftriaxone, streptomycin, amikacin, gentamicin,
393 thiamphenicol, telithromycin, erythromycin, clarithromycin, rifampicin, ofloxacin, ciprofloxacin,
394 pefloxacin, doxycycline and cotrimoxazole) were determined against a single human strain of *F.*
395 *tularensis* subsp. *holarctica* biovar I, using the modified MHII as the experimental medium (Maurin
396 et al., 2000). MBCs were equal to MICs for ofloxacin and ciprofloxacin; twofold higher for the
397 aminoglycosides (streptomycin, amikacin, and gentamicin), thiamphenicol, telithromycin,
398 rifampicin, pefloxacin and doxycycline; fourfold higher for erythromycin; 16 times higher for
399 cotrimoxazole; and 32 times higher for clarithromycin. Despite a MIC of 2 mg/L, a bactericidal
400 effect was never observed with ceftriaxone against *F. tularensis*, although β -lactams are classically
401 considered bactericidal drugs against most other bacteria susceptible to these compounds. The latter
402 results reinforce prior statements that β -lactams and cotrimoxazole are not reliable for tularaemia
403 treatment (Tarnvik A., 2007). According to this work, most antibiotics tested could be considered as
404 bactericidal against *F. tularensis*. However, it should be mentioned that the use of modified MH2 in
405 these experiments may have influenced both the bacteriostatic and the bactericidal activity of
406 antibiotics compared to caMHB, as previously discussed. Indeed, in that study, the MIC value for
407 doxycycline was 8 mg/L, which is higher than usually found in other studies.

408 We recently evaluated MBCs for gentamicin and doxycycline against the LVS strain and two
409 human strains of *F. tularensis* subsp. *holarctica* biovar I from France, using caMHB medium
410 (Caspar et al., 2014). The MBC was eightfold higher than the MIC for gentamicin (respectively, 2
411 and 0.25 mg/L) and could not be determined for doxycycline, which was mainly bacteriostatic (MIC
412 = 0.25 mg/L) (Caspar et al., 2014). These data are more in agreement with the known bactericidal
413 and bacteriostatic nature of these two antibiotics, respectively. Such a difference may participate to
414 the lower relapse rates reported with gentamicin (Enderlin et al., 1994).

415 Finally, a third study measured the MBCs of ciprofloxacin, gentamicin and doxycycline
416 against the LVS strain, using enriched caMHB medium (Aloni-Grinstein et al., 2015). The
417 MBC/MIC ratios were measured at 1–2 for gentamicin and ≤ 4 for ciprofloxacin, confirming the
418 bactericidal nature of these antibiotics against *F. tularensis*. The MBC/MIC ratio was equal to 4 for
419 chloramphenicol. In contrast, the MBC/MIC ratio was ≥ 64 for doxycycline, demonstrating a
420 bacteriostatic activity (Aloni-Grinstein et al., 2015).

421 In conclusion, MBC data investigated with suitable media for *F. tularensis* AST revealed a
422 bactericidal activity for the aminoglycosides and the fluoroquinolones, but a bacteriostatic activity
423 for doxycycline. These data should be further confirmed using a larger sample of *F. tularensis*
424 strains.

425

426 **3. Cell systems**

427 The activity of antibiotics against *F. tularensis* has rarely been evaluated in cell models.
428 These models used either of the following cell systems: murine macrophage-like cell lines J774.1 or
429 P388D1; kidney epithelial Vero cells from African green monkey; human cell lines, including lung
430 epithelial A549 cells, pulmonary fibroblastic MRC5 cells, non-phagocytic kidney epithelial HEK

431 293 and THP-1 human monocytes; or human monocytes purified from buffy coat (Golovliov et al.,
432 2003; Madrid et al., 2013; Maurin et al., 2000; Schmitt et al., 2013; Sutera et al., 2014). In these
433 models, the multiplicity of infection (MOI, bacteria/eukaryotic cell ratios used for cell infection)
434 varied between 10 and 3000 according to the *F. tularensis* strain used (i.e. *F. tularensis* subsp.
435 *tularensis* SchuS4 strain, *F. tularensis* subsp. *holarctica* FSC200 strain, or LVS) and the nature of
436 the eukaryotic cells. Cell infection usually occurs after 2–3 h of bacteria–cell contact. The
437 penetration of bacteria within eukaryotic cells may be enhanced by centrifugation of infected cell
438 monolayers (Aloni-Grinstein et al., 2015; Madrid et al., 2013; Maurin et al., 2000; Schmitt et al.,
439 2013; Sutera et al., 2014). Then non-phagocytised bacteria are removed by adding gentamicin to the
440 extracellular medium for 1–4 h, which is referred to as the gentamicin protection assay. The cell
441 monolayers are then washed and incubated at 37°C in 5% CO₂ atmosphere, either in drug-free
442 medium (growth control) or in the presence of the tested antibiotics. At various incubation times, cell
443 monolayers are washed and lysed with detergents, and CFU counts in cell lysates are determined.
444 Antibiotic activity is deduced from the ratio of CFU counts in antibiotic-containing cultures
445 compared to the drug-free growth control.

446 Alternative methods for the evaluation of antibiotic activity against intracellular *F. tularensis*
447 have been proposed. A dye-uptake assay based on the capacity of eukaryotic cells to internalise a
448 vital dye has recently been reported. In this model, activity of antibiotics is deduced from their ability
449 to inhibit bacterial growth within eukaryotic cells, preventing lysis of the cell monolayers. The term
450 “minimal inhibitory extracellular concentration” (MIEC) was coined to define the minimal
451 extracellular antibiotic concentration capable of preventing cytotoxic effects of *F. tularensis*
452 multiplication (Sutera et al., 2014). The turnaround time of the dye uptake assay was 2 days
453 compared to 4–5 days for the CFU count-based model. Cell mortality could also be evaluated by
454 measuring lactate dehydrogenase activity in cell supernatants (Madrid et al., 2013). More recently, a
455 qPCR assay was used to determine *F. tularensis* growth in Vero cells (Aloni-Grinstein et al., 2015).

456 MIECs were determined after 24 h infection, with results comparable to those obtained with the CFU
457 method (Aloni-Grinstein et al., 2015). However, qPCR may overestimate viable bacterial loads since
458 it quantifies DNA from both viable and non-viable bacteria.

459 **3.1. Fluoroquinolones**

460 The intracellular activity of antibiotics against a French type B biovar I strain of *F. tularensis*
461 (erythromycin MIC of 4 mg/L) was evaluated in a P388D1 murine macrophage-like cell model,
462 using the CFU count methodology (Maurin et al., 2000). The results showed that ciprofloxacin and
463 ofloxacin at 1 mg/L were the most potent compounds, with more than a 4-log and 3-log reduction of
464 bacterial inocula after only 48 h incubation of cultures, respectively (Maurin et al., 2000). In a
465 systematic screening of FDA-approved drugs to identify compounds that may inhibit multiplication
466 of biological threat agents, including *F. tularensis*, norfloxacin (50 μ M, i.e. 16 mg/L) exhibited 83%
467 protection of cells against cytotoxicity of the SchuS4 strain in J774.1 murine macrophages, as
468 determined by the supernatant's lactate dehydrogenase activity (Madrid et al., 2013). In a dye-uptake
469 assay using MRC5 cells, MIECs and MICs were equal for ciprofloxacin, levofloxacin and
470 moxifloxacin, suggesting good penetration of these antibiotics within *F. tularensis*-infected cells
471 (Sutera et al., 2014).

472 **3.2. Tetracyclines**

473 In the P388D1 murine macrophage-like model, doxycycline at 4 mg/L only induced a 2-log
474 reduction of bacterial inocula after 72 h incubation (Maurin et al., 2000). In the systematic screening
475 assay of FDA-approved drugs, tetracycline completely protected infected cells from cytotoxicity of
476 the SchuS4 strain at 50 μ M (22 mg/L), and minocycline induced 92% survival of infected cells at 23
477 mg/L, (Madrid et al., 2013). In the dye uptake assay, MIECs were also equal to MIC for
478 doxycycline, also demonstrating good penetration within infected cells. However, MIEC values were
479 eight times higher for doxycycline (0.5 mg/L) compared to ciprofloxacin (0.064 mg/L) against the

480 two biovar I type B strains evaluated in the study. In another study, the same ciprofloxacin MIEC
481 was found for the LVS strain in Vero cells, as determined by both qPCR and CFU count methods,
482 but a lower MIEC (0.125–0.5 mg/L) was found for doxycycline (Aloni-Grinstein et al., 2015).

483 **3.3. Aminoglycosides**

484 In the P388D1 murine macrophage-like cell model, gentamicin at 3 mg/L was not bactericidal
485 after 48 h incubation of cultures and only induced a 1-log reduction of the bacterial inoculum after
486 72 h incubation. When tested at 10 mg/L, a 2-log reduction of bacterial counts was observed after 48
487 h incubation and more than 3-log reduction after 72 h (Maurin et al., 2000). In another study using
488 the attenuated LVS strain and the Vero cell line, gentamicin at 20 mg/L did not show any
489 intracellular activity after 32 h incubation (Aloni-Grinstein et al., 2015). In the dye-uptake assay
490 using MRC5 cells, the gentamicin MIEC after 48 h incubation of cultures was 2 mg/L against two
491 French type B biovar I strains of *F. tularensis*, while the MICs of this antibiotic were, respectively,
492 0.25 and 0.5 mg/L for the same strains (Sutera et al., 2014). Delayed activity of gentamicin
493 correlated with the slow penetration of this antibiotic within eukaryotic cells, usually detectable only
494 after 48–72 h of antibiotic–cell contact.

495 The other aminoglycosides also penetrate slowly within eukaryotic cells. Amikacin and
496 streptomycin, at 10 mg/L, only decreased *F. tularensis* inoculum by 1 log after 48 h, and 2 logs after
497 72 h (Maurin et al., 2000). In contrast, netilmicin did not prevent cell lysis in J774.1 cells infected
498 with the SchuS4 strain (Madrid et al., 2013).

499 **3.4. Macrolides/lincosamides/ketolides**

500 Conflicting results have been reported between studies testing the intracellular form of *F.*
501 *tularensis*, which may be due to the use of different cell types and bacterial strains. In P388D1
502 murine macrophage-like cells, erythromycin and clarithromycin at 4 mg/L exhibited no intracellular

503 activity against a type B biovar I strain of *F. tularensis* displaying MICs of 4 mg/L and 8 mg/L in
504 acellular media for these antibiotics, respectively. In contrast, telithromycin at 4 mg/L (MIC at 0.5
505 mg/L in acellular medium) was able to reduce the bacterial inoculum between two to three log₁₀ after
506 72 h (Maurin et al., 2000). Unlike telithromycin, the intracellular concentration of erythromycin and
507 clarithromycin was probably not sufficient in this experiment to kill bacteria, as the extracellular
508 concentration used was equal or superior to their MIC. In another study, a high concentration of
509 erythromycin (50 μM, 37 mg/L) conferred full protection of J774.1 cell viability after infection with
510 the SchuS4 type A strain (Madrid et al., 2013). In the same assay, clindamycin (50 μM, 21 mg/L)
511 exhibited 83% protection of cells against the cytotoxic effect of *F. tularensis* proliferation (Madrid et
512 al., 2013). In the dye uptake assay reported by Sutera et al., erythromycin MIECs were four times
513 lower than MICs (respectively, 1–2 mg/L and 4–16 mg/L) for two French biovar I type B strains in
514 MRC5 cells (Sutera et al., 2014). Altogether, these experiments show that erythromycin may be
515 effective in preventing intracellular replication of type A and biovar I type B strains.

516 In another experiment reported by Ahmad et al., azithromycin (a C15 macrolide) showed
517 complete killing of the LVS strain at 5 mg/L in murine macrophage J774.1 cells, although this
518 antibiotic displays an MIC of 25 mg/L against this biovar II type B strain (Ahmad et al., 2010). This
519 result suggests a high bactericidal concentration of azithromycin within *F. tularensis*-infected J774.1
520 macrophages. This may not be true for all cell types since a previous experiment from Ahmad et al.,
521 demonstrated that azithromycin was fully bactericidal at only 25 mg/L against the LVS strain grown
522 in human lung epithelial A549 cells (Ahmad et al., 2010).

523 **3.5. Beta-lactams**

524 In the P388D1 murine macrophage-like model, β-lactams at 10 mg/L (penicillin G,
525 amoxicillin, ceftriaxone) displayed no activity against intracellular *F. tularensis*.(Maurin et al., 2000)

526 Meropenem was ineffective against both intracellular and extracellular *F. tularensis* in the dye
527 uptake assay (Sutera et al., 2014).

528 **3.6. Other antibiotics**

529 In the P388D1 murine macrophage-like model, thiamphenicol at 4 mg/L displayed no
530 intracellular activity. In contrast, rifampicin at 4 mg/L induced a 2-log reduction of bacterial inocula
531 after 72 h incubation (Maurin et al., 2000). In the dye uptake assay using MRC5 cells, MIECs were
532 close to MICs for rifampicin, also suggesting good penetration of this compound within *F.*
533 *tularensis*-infected cells. However, MIEC values were 16 times higher than those of ciprofloxacin (1
534 mg/L versus 0.064 mg/L, respectively) against the two biovar I type B strains tested for *F. tularensis*
535 (Sutera et al., 2014). Interestingly, linezolid exhibited higher activity against intracellular than
536 extracellular *F. tularensis*, suggesting its accumulation within MRC5 cells, with a MIEC of 1 mg/L
537 and an MIC of 8 mg/L (Sutera et al., 2014). Finally, daptomycin was ineffective both intracellularly
538 and extracellularly (Sutera et al., 2014).

539 **3.7. Conclusion of intracellular activity of antibiotics against tularaemia**

540 To summarise the data from the intracellular models, the fluoroquinolones ciprofloxacin,
541 levofloxacin, moxifloxacin and ofloxacin displayed the lowest MIECs and the fastest bactericidal
542 activity against intracellular *F. tularensis*, suggesting rapid and efficient concentration of these
543 compounds within infected eukaryotic cells. The tetracyclines were less effective and mainly
544 bacteriostatic against intracellular *F. tularensis*. The aminoglycosides displayed a bactericidal
545 activity, but only after 48–72 h of antibiotic exposure of infected cells, which correlated with the
546 slow intracellular accumulation of these basic compounds (Maurin and Raoult, 2001). Thus, the
547 aminoglycosides are probably much more effective against the extracellular form of *F. tularensis* at
548 the early stage of antibiotic treatment, while these antibiotics may also be active against intracellular
549 *F. tularensis* after several days of treatment. Streptomycin and amikacin were slightly less effective

550 than gentamicin at the same concentration, and netilmicin was ineffective. The beta-lactams and
551 daptomycin were not effective in cell systems. In contrast, the activity of linezolid against
552 intracellular *F. tularensis* should be further investigated both *in vitro* and *in vivo*.

553 Interestingly, in two studies, the macrolides erythromycin and azithromycin were found to be
554 active against the intracellular form of type A and biovar I type B *F. tularensis* strains, which are
555 naturally susceptible to these antibiotics. Azithromycin was also effective against the intracellular
556 form of biovar II type B LVS strain, although naturally more resistant to macrolides, but only in
557 phagocytic and fibroblastic cells. Indeed, the intracellular accumulation of the macrolides varies
558 according to the eukaryotic cell type considered, with a lower accumulation in epithelial cells
559 compared to phagocytic and fibroblast cells (Ahmad et al., 2010; Sutera et al., 2014). PK/PD studies
560 have demonstrated the accumulation of azithromycin within human phagocytic and fibroblast cells,
561 and in many human tissues such as lungs, soft tissues, prostate and tonsils (Matzneller et al., 2013;
562 McDonald and Pruul, 1991). The intracellular/extracellular azithromycin ratio in tissues is generally
563 between 10 and 100, and can be greater than 200 in polymorphonuclear (PMN) leukocytes, with
564 probable large amounts of this antibiotic at the site of infection (Hall et al., 2002; Hand and Hand,
565 2001; Matzneller et al., 2013). The intracellular accumulation of erythromycin is lower, with
566 intracellular/extracellular ratios generally between 1 and 10 in tissues and PMNs.(Hand and Hand,
567 2001; McDonald and Pruul, 1991) The activity of azithromycin against intracellular *F. tularensis*
568 warrants further evaluation of its activity in animal models. These experiments should be conducted
569 using type A and type B strains, especially the type B biovar II strains.

570

571 **4. Animal models**

572 Although several animal models have been developed to study the pathogenesis of *F.*
573 *tularensis* infection and vaccine efficacy, few of these models have evaluated *in vivo* antibiotic

574 efficacy (Rick Lyons and Wu, 2007). This may be related to the absence of an optimal animal model
575 mimicking human infection. In these models, antibiotic activity is usually deduced from the death
576 rate and survival time of infected animals and the bacterial load in their organs (especially in the
577 spleen) at the time of death or sacrifice, when treated with various antibiotic regimens compared to
578 infected and untreated controls. BALB/c and C57BL/6 mice and guinea pigs are highly susceptible
579 hosts to *F. tularensis* infection, but also to the LVS strain of *F. tularensis* subsp. *holarctica* and *F.*
580 *novicida* strains, which have often been used as surrogates of *F. tularensis*, although they are much
581 less virulent in humans (Kingry and Petersen, 2014; Stundick et al., 2013). Rabbits and Fisher 344
582 rats are less susceptible to *F. tularensis* infection and may better mimic human infection. However,
583 variable immune responses to *F. tularensis* infection also exist between rat strains, with Sprague-
584 Dawley being much more resistant than Fisher 344 rats (Raymond and Conlan, 2009). Results may
585 also vary according to the route of infection, i.e. the intraperitoneal, intradermal, subcutaneous or
586 intranasal routes for Fischer 344 rats (Stundick et al., 2013). Moreover, the “Animal Rule” states that
587 experimental animal models should be developed using the true etiologic agent causing human
588 disease. Thus, evaluation of antibiotic activity in Fisher 344 rats infected with virulent type A or type
589 B strains would be more predictive of the clinical situation.

590 Few experiments have evaluated the activity of antibiotics in *F. tularensis*-infected animal
591 models. However, interesting data have been obtained regarding the *in vivo* efficacy of antibiotics,
592 and relapse rates according to delay in antibiotic therapy and treatment duration. Unfortunately, these
593 models are highly heterogeneous with respect to the route of infection, the route of antibiotic
594 administration, the antibiotic dose administered and the *F. tularensis* strain tested.

595 **4.1. Fluoroquinolones and doxycycline**

596 In 1998, Russel et al evaluated the effect of subcutaneous injection of doxycycline or
597 ciprofloxacin on the median lethal dose (MLD) of the Schu S4 strain injected intraperitoneally to

598 Porton outbred mice (Russell et al., 1998). The animals either received a 2-day antibiotic prophylaxis
599 before the bacterial challenge, or were treated 48 h post-infection with 20 or 40 mg/kg twice a day,
600 of doxycycline or ciprofloxacin for 10 days, and then were monitored for 24 days. Although the
601 MLD was close to 1 CFU of the Schu S4 strain in untreated mice, full protection at the highest
602 bacterial inoculum tested (8.8×10^6 CFU) was achieved by a 2-day prophylaxis with ciprofloxacin or
603 doxycycline, and by 10 days of post-infection treatment with either of these antibiotics at 40 mg/kg
604 twice a day. At this dosage, the serum concentrations were higher than $4 \times$ MIC for at least 12 h for
605 ciprofloxacin, and $3 \times$ MIC for 9 h for doxycycline (Russell et al., 1998).

606 The *in vivo* efficacy of fluoroquinolones was confirmed in 2005, by Piercy et al. (Piercy et al.,
607 2005). BALB/c mice were infected subcutaneously with 10^6 CFU of the Schu S4 strain and treated
608 orally with 100 mg/kg of ciprofloxacin, gatifloxacin or moxifloxacin, from 6 h to 14 days post-
609 infection. Survival rates at day 42 post-infection were 94%, 100% and 100%, respectively, for these
610 three antibiotics (Piercy et al., 2005).

611 In 2008, Klimpel et al. showed that a 13-day course of levofloxacin (5 mg/kg/day,
612 intraperitoneally), starting 24 h following an intranasal challenge with 3 LD₅₀ of *F. tularensis* Schu
613 S4 strain, prevented death of all Balb/c mice (Klimpel et al., 2008). No bacteria were detectable in
614 the spleen of the animals after 10 days of treatment, while very few were detected in the lungs at this
615 time (Klimpel et al., 2008).

616 In 2012, Rotem et al. compared the efficacy of ciprofloxacin and doxycycline in Balb/c mice
617 infected by inhalation of a 100-LD₅₀ dose of *F. tularensis* LVS (10^5 CFU) or Schu S4 (10^2 CFU)
618 strains. (Rotem et al., 2012) Untreated controls died 5–7 days post-infection, whereas all LVS-
619 infected mice were cured by intraperitoneal injection of ciprofloxacin (50 mg/kg bid) for 7 days or
620 doxycycline (40 mg/kg bid) for 14 days, whether the antibiotic treatment was started at 24 h, 48 h or
621 72 h post-infection (Rotem et al., 2012). *F. tularensis* LVS strain was undetectable by culture in the

622 lungs, liver and spleen after completion of any of these treatments. In contrast, for animals infected
623 with the Schu S4 strain, no death occurred when ciprofloxacin was administered 24 h or 48 h post
624 infection, while doxycycline only cured 90% of the animals even when administered 24 h post-
625 infection (Rotem et al., 2012).

626 **4.2. Treatment delay**

627 In the study from Russel et al., a 48-h prophylaxis with ciprofloxacin or doxycycline
628 protected mice from a 3.7×10^6 CFU and 6.0×10^6 CFU challenge with the Schu S4 strain,
629 respectively (Russell et al., 1998). In contrast, when antibiotic treatment was started 24 h post-
630 infection, complete protection was only obtained against an intraperitoneal challenge with 880 CFU
631 for ciprofloxacin and 100 CFU for doxycycline. Thus, antibiotic efficacy was dramatically reduced
632 after only 24 h infection (Russell et al., 1998). In the study by Rotem et al., in which mice were
633 infected via Schu S4 strain aerosols, no death occurred when ciprofloxacin was administered 24–48
634 h post infection, while a 30% mortality rate was observed when this antibiotic was administered 3
635 days post-infection (Rotem et al., 2012). Doxycycline only cured 90%, 30% and 0% of the mice
636 when started at 24 h, 48 h and 72 h post-infection, respectively (Rotem et al., 2012). In the study
637 reported by Piercy et al., the survival rates decreased dramatically (94%, 67% and 0%) when
638 ciprofloxacin was administered 6 h, 24 h or 48 h post-infection, respectively (Piercy et al., 2005). In
639 contrast, the survival rates were 100%, 96% and 84% for gatifloxacin, and 100%, 100% and 62% for
640 moxifloxacin, respectively, showing the better therapeutic efficacy of these drugs compared to
641 ciprofloxacin. In this study, the infectious dose was much higher than in that from Rotem et al (10^6
642 CFU intraperitoneally versus 10^2 CFU intranasally). Finally, in the Klimpel et al. study, full
643 protection against the SchuS4 strain (99 CFU intraperitoneally) was observed for levofloxacin at 40
644 mg/kg/day, as long as the treatment delay did not exceed 72 h (Klimpel et al., 2008). If started at 72
645 h, bacterial load decreased approximately from 10^6 to 10 CFU per organ after 1 week of treatment.

646 Delaying the treatment of more than 120 h resulted in the death of all infected mice (Klimpel et al.,
647 2008).

648 **4.3. Bactericidal activity and treatment duration**

649 In the Russel et al. study, when treatment was administrated for 5 days rather than 10 days
650 post-infection, the MLD was reduced from $> 8.8 \times 10^6$ CFU to 3.7×10^6 CFU for ciprofloxacin and
651 6.0×10^6 CFU for doxycycline (Russell et al., 1998). The protective effect of the antibiotic therapy
652 also decreased when animals were treated with a lower dose (20 mg/kg bd rather than 40 mg/kg bd)
653 of ciprofloxacin or doxycycline. Moreover, the authors reported that death occurred in 90% of
654 animals after antibiotic treatment withdrawal, demonstrating that a 5-day course of antibiotic
655 treatment was not sufficient to eradicate *F. tularensis* (Russell et al., 1998). Thus, in this study, the
656 dose and length of the antibiotic therapy administered highly influenced the outcome of *F. tularensis*
657 infection in animals (Russell et al., 1998).

658 Significant insight into relapses came from the study reported by Piercy et al. (Piercy et al.,
659 2005). Among the nine groups tested (ciprofloxacin, moxifloxacin or gatifloxacin, initiated 6 h, 24 h
660 or 48 h post-infection), these authors showed that the administration of 7 days of dexamethasone to
661 surviving mice in order to abolish their immune system resulted in 17–73% relapse rates depending
662 on the treatment group. These findings suggested that quiescent bacteria controlled by the immune
663 system persisted in mice despite 14 days of fluoroquinolone therapy. Fluoroquinolones were thus not
664 fully bactericidal when a high inoculum of *F. tularensis* was injected to the mice (e.g. subcutaneous
665 injection of 10^6 CFU of the Schu S4 strain). Suppression of the immune system enabled latent
666 bacteria to multiply and kill mice, even in the 6-h post-exposure treatment group (respectively, 36%
667 and 42% mortality rates in the ciprofloxacin- and moxifloxacin-treated groups) (Piercy et al., 2005).

668 Data from the study by Rotem et al. corroborated this hypothesis (Rotem et al., 2012). In their
669 experiments with the virulent Schu S4 strain, no death occurred when ciprofloxacin was

670 administered 24 h or 48 h post-infection, while a 30% mortality rate was observed when this
671 antibiotic was administered 3 days post-infection. In the latter case, bacteria were undetectable in
672 organs at the time of antibiotic treatment withdrawal, while cultures were positive 3 days later for
673 three out of ten mice that died after antibiotic withdrawal. The extension of ciprofloxacin treatment
674 duration from 7 to 10 days cured all animals, even when the antibiotic was administered 72 h post-
675 infection. In contrast, when doxycycline was started 72 h post-infection, all animals had
676 approximately 100 CFU/g of tissue (lungs, liver or spleen) at the end of treatment, after which the
677 bacterial loads in organs increased in all mice until death. Extension of treatment from 14 to 21 days
678 resulted in no cultivable bacteria in lungs, liver or spleen at the end of treatment, but all mice
679 relapsed after 2 days of doxycycline withdrawal (Rotem et al., 2012). A different picture was
680 observed with the LVS strain, which was below detectable level by culture in the lungs, liver and
681 spleen after completion of any of the treatment regimens (ciprofloxacin for 7 days or doxycycline for
682 14 days, started 24 h, 48 h, and 72 h post-infection) in the mice sacrificed at the end of the treatment.
683 However, 2 days after doxycycline treatment withdrawal, the LVS strain was detected in all organs,
684 and bacterial loads gradually decreased to an undetectable level in the following 7 days and resulted
685 in no death. Thus, although undetectable after doxycycline treatment, the attenuated LVS strain was
686 not fully eradicated and regrowth of the bacteria was observed as soon as doxycycline was stopped,
687 although it did not kill the mice. In contrast, re-emergence of bacteria did not occur for the
688 ciprofloxacin group of LVS-infected mice after ciprofloxacin withdrawal.

689 **4.4. Conclusions drawn from animal model data**

690 Altogether, these data indicate that both ciprofloxacin and doxycycline are able to prevent
691 tularaemia in mice when treatment is started 48 h before a challenge with *F. tularensis* SchuS4
692 strain. These antibiotics are also able to cure the disease and eradicate *F. tularensis* when
693 administered post-infection at a concentration of 40 mg/kg or higher, for 5–10 days. Efficacy of the
694 antibiotic treatment is highly correlated with the antibiotic dose administered, which is probably

695 related to the duration over which serum concentrations of antibiotics are above the MIC values of
696 the infecting strain.

697 However, when treatment was started 24–72 h post-infection, ciprofloxacin was superior to
698 doxycycline: 1) in the study conducted by Rotem et al., doxycycline was not able to cure all mice
699 when infected with 10^2 CFU of the SchuS4 strain via the aerosol route, even when started at 24 h
700 post-infection, while ciprofloxacin cured all mice when started either 24 h or 48 h post-infection
701 (Rotem et al., 2012); 2) relapse rates were higher for doxycycline as it was unable to fully eradicate
702 the Schu S4 or LVS strains from the lungs, liver and spleen, even when administered for 14–21
703 days; 3) the time until antibiotic treatment initiation reduced doxycycline efficacy much more than
704 for ciprofloxacin.

705 *F. tularensis* multiplies within the slightly acidic cytosol of eukaryotic cells, and a low pH
706 may alter the activity of the basophilic antibiotics ciprofloxacin and doxycycline (Russell et al.,
707 1998). Data obtained in animal models indicate that persistent bacteria may develop in hosts infected
708 with *F. tularensis* when the antibiotic therapy administered has poor bactericidal activity or is too
709 short in duration. These data suggest that long antibiotic treatment duration should be used in
710 immunocompromised patients infected with *F. tularensis*. This could also be true for patients
711 suffering from suppurated lymphadenopathy, who often experience relapses despite administration
712 of ciprofloxacin for 14 days or doxycycline for 21 days.

713 Treatment must be initiated as soon as possible since antibiotic efficacy decreased
714 significantly when treatment was delayed by 24–48 h post-infection. It should be noted that, because
715 of diagnosis delays, antibiotics are often given several days to weeks after infection in patients
716 suffering from tularaemia. Importantly, moxifloxacin efficacy was less impacted by treatment delay
717 than ciprofloxacin and thus may represent an advantageous therapeutic option in case of late
718 diagnosis. MIC values are close for moxifloxacin and ciprofloxacin.

719 **5. General conclusion and perspectives**

720 Since the introduction of streptomycin in the mid-1950s, antibiotic treatment of human
721 tularaemia has remained challenging. In most tularaemia endemic countries, human infections are
722 now rare, and *F. tularensis* isolation is even rarer. Thus, AST data for isolated strains of *F. tularensis*
723 may not reflect the true situation of current antibiotic resistance in this pathogen. Importantly, the
724 analysis of AST methodological variations between studies shows an urgent need for international
725 standardisation. Following the CLSI guidelines, including using the appropriate experimental
726 medium, bacterial inoculum and incubation time are mandatory, although some *F. tularensis* strains
727 grow better or exclusively under 5% CO₂ atmosphere. The broth microdilution technique using
728 enriched caMHB medium should be considered the reference method. As recommended by the
729 CLSI, a final inoculum calibrated at 5 10⁵ CFU/mL should be used, as a tenfold higher or lower
730 inoculum is associated to interpretation errors (Georgi et al., 2012). However, the modified Mueller-
731 Hinton II liquid medium should not be used for *F. tularensis* AST to avoid reporting MICs that could
732 categorize *F. tularensis* strains as resistant to aminoglycosides or doxycycline whereas such
733 resistances have never been characterized so far in this pathogen. From the data currently available,
734 use of the CLSI broth microdilution method can be recommended to test large collections of *F.*
735 *tularensis* strains in reference laboratories, while the E-test method would be more convenient for
736 testing one or a few strains. However, the E-test MIC strip method, although more convenient to
737 perform, still requires comparative studies with the reference method.

738 Altogether, available AST data indicate that the fluoroquinolones display the lowest MICs,
739 are strongly and rapidly bactericidal in cell models and are highly effective in curing *F. tularensis*
740 infection in infected mice when administered for at least 10 days. Among the fluoroquinolones,
741 ciprofloxacin and levofloxacin have the lowest MICs. In contrast, doxycycline MICs are closer to the
742 CLSI susceptibility breakpoint and this antibiotic is only bacteriostatic in cell models. In the mouse
743 model, a 14-day course of doxycycline did not eradicate *F. tularensis* when treatment was started 72

744 h post-infection (Rotem et al., 2012). A 21-day course of treatment was still not fully effective to
745 eradicate bacteria, whereas 10 days of ciprofloxacin treatment was effective. Therefore, doxycycline
746 should be considered less effective than fluoroquinolones for treatment of tularaemia, which is
747 consistent with higher therapeutic failure rates in humans observed with this antibiotic. Because
748 antibiotic treatment is often administered several days after symptom onset in tularaemia patients,
749 ciprofloxacin may be a better choice than doxycycline. Moreover, treatment duration with
750 ciprofloxacin of at least 14 days should be recommended, while 3 weeks would be a minimum for
751 doxycycline (Rotem et al., 2012). A longer duration of antibiotic treatment should probably be
752 considered in case treatment is delayed longer than 2 weeks after symptom onset, especially if lymph
753 node suppuration or other local or systemic complications have occurred. The aminoglycosides
754 (especially gentamicin) also have low MICs. They are rapidly bactericidal in acellular media, but
755 their intracellular bactericidal activity only takes place after 72 h of cell exposure owing to their slow
756 penetration through the eukaryotic cell membrane. In the 1994 review by Enderlin et al.,
757 streptomycin was considered the most reliable treatment for human tularaemia, with almost no
758 relapse after treatment removal (Enderlin et al., 1994). The aminoglycosides are still considered the
759 most reliable treatment of severe tularaemia cases (e.g. the pneumonic and typhoidal forms of
760 tularaemia, and all other systemic infections). Experimental data suggest that the combination of an
761 aminoglycoside with a fluoroquinolone may currently be the most effective alternative in patients
762 with severe tularaemia, especially to obtain a rapid bactericidal activity against extracellular and
763 intracellular *F. tularensis*. However, one has always to remember that antimicrobial potency deduced
764 from MIC values, MBC values, *in vitro* and *in vivo* bactericidal activity in animal models is only one
765 of many factors (including pharmacokinetic and pharmacodynamic parameters, tolerability, plasma
766 protein binding, tissue penetration, bactericidal activity and contraindication) that may influence the
767 decision of what drug to use in a clinical setting.

768 The macrolides (especially azithromycin) could be an alternative in patients infected with
769 erythromycin-susceptible strains of *F. tularensis* (type A and type B biovar I) and in those for whom
770 the above first-line treatments are contraindicated, including young children and pregnant women.
771 However, the macrolide-resistant type B biovar II strains are currently found in Eastern Europe and
772 in Asia, and co-exist with biovar I strains in Germany, Switzerland and Scandinavia. The empirical
773 use of a macrolide in tularaemia patients cannot be considered reliable in these areas. The use of a
774 macrolide in patients with mild to moderate severity tularaemia is safer in Western Europe, where
775 only type B biovar I strains cause human infections. In this context, quick discrimination between
776 type B biovar I and II strains either by determination of erythromycin susceptibility (using MIC
777 strips or sequencing of *rml* gene) or by detection of particular genetic markers (detection of RD23
778 deletion only found in West European strains) would be of particular interest (Dempsey et al., 2007;
779 Karlsson et al., 2016; Pilo et al., 2009). Since these antibiotics mainly display a bacteriostatic activity
780 against *F. tularensis*, they cannot be considered reliable to treat severe forms of tularaemia.
781 Azithromycin combined with lymph node resection proved to be effective to cure a pregnant women
782 suffering from oropharyngeal tularaemia with no complications for the fetus (Dentan et al., 2013).
783 Further *in vivo* data in animal models and humans are needed before azithromycin can be
784 recommended as a reliable alternative for treatment of tularaemia

785 Finally, the causes of the high relapse rates observed in tularaemia patients after administration of a
786 fluoroquinolone or a tetracycline remain undetermined. Several hypotheses can be proposed,
787 including low penetration of antibiotics in tissues and eukaryotic cells *in vivo*; low susceptibility of
788 bacteria *in vivo* especially because of low replication and persistence; inactivation of antibiotic
789 activity *in vivo* owing to local conditions (e.g. acidic pH, enzymatic inactivation, etc.) and acquired
790 resistance to antibiotics *in vivo* in patients under fluoroquinolone or tetracycline treatment. Further
791 evaluation of such hypotheses in *in vitro* and *in vivo* experimental models is warranted.

792

793 **Transparency declarations**

794 The authors have no conflicts of interest to declare.

795

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797

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1106 **TABLES**

1107 **Table 1: Characteristics of *F. tularensis* strains included in this study and MICs for aminoglycosides (gentamicin and streptomycin), the tetracycline**
 1108 **compound doxycycline and fluoroquinolones (ciprofloxacin and levofloxacin) according to the culture medium used and to the AST assay used.**

Reference	Year(s)	Origin	Nb of strains Type/biovar	Culture medium	MIC (mg/L)									
					Gentamicin		Streptomycin		Doxycycline		Ciprofloxacin		Levofloxacin	
					Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀
<u>Broth microdilution</u>														
Origgi et al	1996–2013	Switzerland	19 B6 5 B12	caMHB + 2% IsoVitaleX	≤0.12–0.25	0.25	4	4			≤0.06	≤0.06		
Georgi et al	UNK	Europe North America Central Asia	69 B 7 A 4 ssp. <i>mediasiatica</i>	caMHB + 2% IsoVitaleX caMHB + 2% IsoVitaleX caMHB + 2% IsoVitaleX	≤0.25–0.5 ≤0.25–0.5 ≤0.25	0.5	≤0.5–2	2			≤0.031–0.125 0.063 ≤0.031–0.125	0.063	≤0.031–0.125	0.063
Urich et al	1974–2005	North America	92 A 77 B	caMHB + 2% IsoVitaleX caMHB + 2% IsoVitaleX	0.03–0.5 0.03–0.5	0.25 0.12	0.25–4 0.25–4	2 2	0.25–4 0.25–2	2 2	0.004–0.06 0.008–0.06	0.06 0.03	0.015–0.12 0.015–0.12	0.06 0.06
<u>E-test</u>														
Johansson et al, 2000	1998	Sweden	7 B	Modified Thayer-Martin	0.5–1				0.25–0.5		0.008–0.015			
Hotta et al	1926–1989	Japan	36 B	Chocolate II agar	0.023–0.5	0.125			0.094–1.5	1	0.003–0.023	0.016		
Kreizinger et al	2003–2010	Hungary	29 B12	Modified Francis Agar	0.38–1	0.75	3–8	6	0.12–1.5	1	0.012–0.047	0.047	0.004–0.023	0.023
Yeşilyurt et al	2009–2010	Turkey	39 B biovar II	GBCA	0.094–0.25	0.25	0.75–1.5	1.5			0.008–0.016	0.016	0.006–0.016	0.012
Tomaso et al	1992–1998	Austrian	50 B biovar II	CHAB	0.094–2	0.75	0.75–8	3	0.38–3	2	0.004–0.125	0.032	0.008–0.047	0.032
Ikäheimo et al	UNK	Finland	38 B	CHA + 2% Haemoglobin	0.38–1.5	1	0.25–4.0	4			0.008–0.023	0.016	0.008–0.023	0.016
Kiliç et al, 2013	2009–2012	Turkey	249 B biovar II + 1 biovar <i>japonica</i>	CHAB	0.094–0.38	0.25	0.5–2	1.5	0.064–0.38	0.25	0.004–0.023	0.016	0.003–0.016	0.012
Johansson et al, 2002	1996–2001	USA	8 A 16 B	MHII + 1% IsoVitaleX or CHAB	0.032–0.25 0.016–0.125		0.064–2 0.064–1		0.125–2 0.125–2		0.016–0.064 0.016–0.064		0.016–0.064 0.064	0.125
Velinov et al	UNK	Bulgaria	21 B biovar II	caMHB + 2% IsoVitaleX	0.064–0.5	0.125	0.25–2	1	0.25–4	2	0.002–0.06	0.047	0.016–0.125	0.094
<u>Agar dilution</u>														
Valade et al	1996–2005	France	71 B	MHII + 2% IsoVitaleX	0.03–0.5		<0.5–1		0.125–1		0.015–0.03			
Johansson et al, 2000	1998	Sweden	7B	Modified Thayer-Martin	1				0.5		0.03			

1109 caMHB: cation-adjusted Mueller Hinton Broth; GBCA: glucose blood cystein agar; MHII: Mueller Hinton II; CHA: cystein heart agar; CHAB: cystein heart agar enriched with blood; MIC₉₀: minimal inhibitory
 1110 concentration that inhibits 90% of the strains; MIC range: range between the lowest and the highest MIC observed

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1115 **Table 2: MIC and MIC₉₀ ranges of aminoglycosides, tetracyclines and fluoroquinolones against *F. tularensis*. Data are summarised from all studies**
1116 **selected for this review**

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Antibiotics	MIC range (mg/L)	MIC ₉₀ range (mg/L)	CLSI breakpoint for susceptibility (mg/L)
Gentamicin	≤ 0.016–2	0.064–1	≤ 4
Streptomycin	≤ 0.064–8	0.25–6	≤ 8
Tetracycline	≤ 0.094–2	≤ 0.25- 1	≤ 4
Doxycycline	0.064–4	0.25–2	≤ 4
Ciprofloxacin	≤ 0.002–0.125	≤ 0.016–0.064	≤ 0.5
Levofloxacin	≤ 0.004–0.125	0.012–0.125	≤ 0.5
Chloramphenicol	≤ 0.023–4	≤ 0.25–2	≤ 8

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1136 **Table 3: MICs for the other aminoglycosides and fluoroquinolones evaluated against *F. tularensis***

Reference	Origin	Nb of strains Type/biovar	Tobramycin		Amikacin		Moxifloxacin		Norfloxacin		Grepafloxacin		Trovafoxacin		Gatifloxacin		Sparfloxacin	
			Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀
<u>Broth microdilution</u>																		
Georgi et al	Europe	69 B			≤0.5–2	2												
<u>E-test</u>																		
Yeşilyurt et al	Turkey	39 B biovar II	0.125–0.38	0.25	0.75–1	1	0.012–0.032	0.032										
Tomaso et al	Austria	50 B biovar II	0.19–3	0.75	1–16	4	0.016–0.19	0.125	0.023–0.19	0.125					0.008–0.047	0.032	0.003–0.047	0.023
Ikäheimo et al	Finland	38 B	0.5–2	1.5							0.016–0.047	0.047	0.012–0.047	0.032				
Johansson et al, 2002	USA	8 A					0.032–0.125				0.008–0.125				0.016–0.125		0.008–0.032	
		16 B					0.032–0.125	0.125			0.016–0.125	0.064			0.008–0.064	0.064	0.008–0.064	0.064

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1147 **Table 4: MICs of tetracycline, tigecycline, macrolides (erythromycin, azithromycin and telithromycin), chloramphenicol, rifampicin and linezolid against**
 1148 ***F. tularensis*.**

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Reference	Origin	Nb of strains Type/biovar	Tetracycline		Tigecycline		Erythromycin		Azithromycin		Telithromycin	Chloramphenicol		Rifampicin		Linezolid	
			Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range
Broth microdilution																	
Origi et al	Switzerland	19 B6 5 B12	≤0.25	≤0.25			2-8	4				≤2	≤2				
Georgi et al	Europe	69 B	≤0.125–2	1			1->16	>16				1–4	2	≤0.5–2			
	North America	7 A	≤0.125–0.5									≤0.5					
	Central Asia	4 ssp. <i>mediasiatica</i>	0.25–2									1–4					
Urich et al	North America	92 A 77 B	0.25–2	1			0.5–4	2				0.5–4	2				
			0.12–2	1			0.5–2	0.5				0.5–4	2				
E-test MIC strips																	
Johansson et al, 2000	Sweden	7 B					>256					0.25		0.5			
Hotta et al	Japan	36 B					0.094–1.5	1.5									
Kreizinger et al	Hungary	29 B12	0.19–0.72	0.5	0.094–0.19	0.19	>256	>256				0.5–1.5	1.5	0.5–2	1	12–48	32
Yeşilyurt et al	Turkey	39 B biovar II	0.125–0.5	0.38	0.094–0.38	0.25	>256	>256	>256	>256		0.094–0.25	0.25	0.25–1	0.75	0.5–2	1.5
Tomaso et al	Austria	50 B biovar II	0.125–0.75	0.75			4->256	>256				0.023–2	0.75	0.25–3	1.5		
Ikäheimo et al	Finland	38 B	0.094–0.5	0.38					>256	>256		0.125–0.5	0.38	0.094–0.38	0.25		
Kiliç et al, 2013	Turkey	249 B biovar II + 1 biovar <i>japonica</i>	0.094–0.5	0.38			1->256	>256				0.094–0.75	0.5	0.125–1	0.75		
Johansson et al, 2002	USA	8 A 16 B					0.125–1		0.125–2			0.5–1		0.25–2		2–4	
Velinov et al	Bulgaria	21 B biovar II					0.125–1	1	0.064–2	1		0.25–1	1	0.125–1	1	4–16	8
							>256	>256				1–4	2				
Agar dilution																	
Valade et al	France	71 B									0.125–0.25		0.25–2		0.015–0.5		
Johansson et al, 2000	Sweden	7B											0.5–1		1		

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1153 **Table 5: MICs of beta-lactams against *F. tularensis***

Reference	Origin	Nb of strains Type/biovar	MIC range (mg/L)								1154
			AMP	AMC	PIP/TAZ	CAZ	COX	FEP	IMI	MER	AZT
<u>Broth Microdilution</u>											
Goergi et al	Europe	69 B		64->64		>32				16->16	1156
<u>E-test</u>											
Hotta et al.	Japan	34 B					0.047->256		0.047->32	0.094->32	0.75->256
Yeşilyurt et al	Turkey	39 B biovar II	>256	>256	>256	>256	>256	>256	>32		
Tomaso et al	Austria	50 B biovar II	>256		>256	>256	>32	>32	0.5->32	1.5->32	>256
Ikäheimo et al	Finland	38 B			>256	>256	>32		>32	>32	
Velinov et al	Bulgaria	21 B biovar II			>256		2-4				
<u>Agar dilution</u>											
Scheel et al	Scandinavia	20 B				>32			>32	>32	>32

*if available, MIC₉₀ value corresponded every time to the highest value of MIC range. AMP:

Ampicillin; AMC: Amoxicillin/Clavulanate; PIP/TAZ: Piperacillin/tazobactam; CAZ:

Ceftazidime; COX: Ceftriaxone; FE: Cefepime; IMI: Imipenem; MER: Meropenem; AZT:

Aztreonam

Supplementary Material

***Francisella tularensis* susceptibility to antibiotics: a comprehensive review of data obtained *in vitro* and in animal models.**

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1 Supplementary Figures and Tables

Reference	Year(s)	Origin	Nb of strains Type/biovar	Culture medium	MIC (mg/L)															
					Gentamicin		Streptomycin		Doxycycline		Ciprofloxacin		Levofloxacin		Tetracycline		Erythromycin		Chloramphenicol	
					Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀
<u>Broth microdilution</u>																				
Origgi et al	1996–2013	Switzerland	19 B FTNF002 5 B13	Modified MHII	1–4	4	2–4	4			≤0.06	≤0.06			2–8	8	1–8	4	≤2	≤2
					2		2–4				≤0.06				4		>32		≤2	
García del Blanco et al	1997–1999	Spain	42 B	Modified MHII	1–8	8	4–32	32	2->32	>32	0.06–0.25	0.25	≤0.25	≤0.25	4–64	64	2->4	>4	≤8	≤8
<u>Agar dilution</u>																				
Scheel et al	UNK	Scandinavia	20 B	Blood cystein agar	2–8		2–16		0.25–2		0.031–0.125								Range: 0.5–8 (7 strains) or >32 (13 strains)	1–8

Table S1: Characteristics and MICs of strains from studies excluded from the analysis

2.2. Publication 2 : Antibiotic susceptibilities of
Francisella tularensis subsp. *holarctica* strains
isolated from tularaemia patients in France between
2006 and 2016

(A soumettre à *Journal of Antimicrobial Chemotherapy*)

Suite à cette revue des données de sensibilité de *F. tularensis* aux antibiotiques, il nous a semblé opportun et nécessaire de déterminer la sensibilité des souches isolées en France et identifiées au Centre National de Référence des *Francisella*, selon la technique de référence en microdilution en milieu liquide, devant la quasi absence de telles données pour les souches françaises. (246) Une étude réalisée sur des souches française isolées entre 1996 et 2005 avait été conduite quelques années auparavant mais en utilisant les techniques de diffusion en agar ou des bandelettes à gradients de concentration, qui ne sont pas la technique de référence pour l'évaluation de la sensibilité des *Francisella*. Les souches françaises appartiennent au biovar I de la sous espèce *F. tularensis* subsp. *holarctica* et plus précisément à la lignée clonale B. FTNF002-00 présente en Europe de l'Ouest, (77,78,78) pour laquelle peu de données sont disponibles avec la technique de référence. (76,246) En plus des antibiotiques recommandés pour le traitement de la tularémie (gentamicine, ciprofloxacine, doxycycline), nous avons inclus dans notre analyse :

- des macrolides (érythromycine, azithromycine) pour confirmer l'appartenance des souches françaises au biovar I de la sous espèce *F. tularensis* subsp. *holarctica* et pouvoir établir des recommandations d'utilisation ou de non utilisation thérapeutique pour ces antibiotiques vis-à-vis des souches appartenant au biovar I
- des antibiotiques rarement évalués tels que le méropénème, l'amikacine, la tobramycine, l'ofloxacine, la moxifloxacine, la tigécycline, la télithromycine, la clindamycine et la daptomycine afin de fournir des données pour ces antibiotiques
- des antibiotiques pour lesquels des résultats contradictoires ont pu être rapportés tels que le linézolide
- des antibiotiques dont l'utilisation est limitée à certains contextes particuliers : chloramphénicol pour les méningites, rifampicine pour les infections ostéo-articulaires
- la sensibilité des souches à l'éthanol pour :1/ évaluer le pouvoir antibactérien de ce composé sur *F. tularensis* et 2/ car l'éthanol est employé comme solvant pour l'évaluation de la sensibilité de certains antibiotiques, lors de la réalisation des solutions stocks d'antibiotiques à forte concentration.

Nous avons ainsi analysé la sensibilité de 59 souches de *F. tularensis* subsp. *holarctica* isolées entre 2006 et 2016 de cas humains de tularémie. Toutes les formes cliniques étaient représentées avec des souches isolées de formes ulcéroganglionnaires (n = 19), ganglionnaires

(n = 1), oropharyngées (n = 6), oculoganglionnaire (n = 2), pulmonaires (n = 16) ou typhoïdiques (n = 11) ainsi que de formes cliniques plus rares telles que les formes méningées (n = 1), vasculaires (n = 1) ou ostéo-articulaires (n = 1). Les objectifs secondaires de cette étude étaient également de :

- pouvoir émettre des recommandations thérapeutiques pour le traitement des cas de tularémie en France
- identifier des souches résistantes aux antibiotiques utilisés pour le traitement de la tularémie, permettant d'expliquer certaines situations d'échec thérapeutique.

1 **Antibiotic susceptibilities of *Francisella tularensis* subsp. *holarctica* strains isolated from**
2 **tularaemia patients in France between 2006 and 2016.**

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15 **Running Title:** Antibiotic susceptibilities of *Francisella tularensis* strains isolated in France

16 **Keywords:** *Francisella tularensis* subsp. *holarctica*, antibiotic susceptibility, MIC,
17 tularaemia, France

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20 **SYNOPSIS (250/250)**

21 **Objectives:** We determined the *in vitro* susceptibility to 18 antibiotics of *Francisella*
22 *tularensis* subsp. *holarctica* strains isolated in France between 2006 and 2016.

23 **Methods:** MIC data were determined for fifty-nine strains belonging to the phylogenetic
24 subclade B.FTNF002-00, using the recommended broth microdilution method in cation-
25 adjusted Mueller-Hinton medium supplemented with 2% Polyvitex[®].

26 **Results:** All strains were susceptible to fluoroquinolones (ofloxacin, ciprofloxacin,
27 levofloxacin and moxifloxacin; MIC range: 0.016-0.25 mg/L), aminoglycosides (gentamicin
28 and tobramycin; MIC range: $\leq 0,03$ -0,25 mg/L), doxycycline and chloramphenicol (MIC
29 range 0.5-2 mg/L). Erythromycin MIC range (0,5-2 mg/L) confirmed that all isolates
30 belonged to biovar I of *F. tularensis* subsp. *holarctica*. Telithromycin displayed lower MIC
31 range (0.03-0.5 mg/L). Tigecycline MIC range was slightly higher than doxycycline (0,25-1
32 mg/L and 0,125-0,25 mg/L, respectively). All strains were resistant to beta-lactams,
33 daptomycin, clindamycin and linezolid.

34 **Conclusions:** *F. tularensis* subsp. *holarctica* strains isolated in France are susceptible to
35 antibiotic classes recommended for tularemia treatment. However, fluoroquinolones display
36 the lowest MICs compared to aminoglycosides and doxycycline. Moreover, fluoroquinolones
37 are bactericidal against *F. tularensis* and associated with lower rates of therapeutic failures
38 and relapses than doxycycline which is only bacteriostatic. Thus, fluoroquinolones should
39 probably be advocated as first-line treatment of mild cases of tularemia, which are
40 predominant in Europe, whereas doxycycline should be restricted to patients with
41 contraindications to fluoroquinolones. In contrast, a combined therapy using a single daily
42 dose of gentamicin with ciprofloxacin may be useful in more severe cases, both to limit the
43 nephrotoxicity and improve the intracellular bactericidal activity of the antibiotic treatment.

44 INTRODUCTION (3456 words)

45 Tularemia is a zoonotic disease caused by two subspecies of the bacterium *Francisella*
46 *tularensis*. *F. tularensis* subsp. *tularensis* (type A), the most virulent one, is only present in
47 North America. *F. tularensis* subsp. *holarctica* (type B) is present in the whole northern
48 hemisphere.¹ Biovar I strains (erythromycin susceptible) are found in Western Europe
49 (France, Spain, Italy) and in North America.²⁻⁶ Biovar II strains (erythromycin resistant) are
50 found in the Eastern part of Europe and Asia.^{2,6-9} Both biovars exist in Germany and
51 Switzerland.^{10,11} High-level resistance to erythromycin in biovar II strains is related to the
52 presence of an A2059C mutation in the three copies of the domain V of the 23S rRNA (*rrl*)
53 gene.⁸ Biovar Japonica corresponds to erythromycin-susceptible strains found in Japan, China
54 and Turkey.¹

55 According to the mode of transmission of *F. tularensis*, six clinical forms are
56 classically recognized in humans. The ulceroglandular form associates a skin ulcer with a
57 regional lymphadenopathy in the corresponding lymphatic draining area. A regional
58 lymphadenopathy with no detectable skin ulcer corresponds to the glandular form. Pharyngitis
59 with mouth ulcerations and cervical lymph nodes is observed in the oropharyngeal form. The
60 oculoglandular form is characterized by a painful conjunctivitis and lymphadenopathy in the
61 pretragal, submandibular or cervical areas. A severe sepsis often associated with neurological
62 symptoms, but without lymphadenopathy or cutaneous ulcer is designated as typhoidal
63 tularemia.¹ Pneumonic tularemia corresponds to *F. tularensis* lung involvement with
64 unspecific symptoms such as cough, dyspnea or chest pain, often with mediastinal
65 lymphadenopathy on the chest radiograph.

66 Current treatment guidelines from the World Health Organization (WHO) are derived
67 from the 2001 consensus statement from Dennis et al.^{12,13} Gentamicin and streptomycin are
68 recommended in case of severe tularemia, requiring hospitalization. Ciprofloxacin and

69 doxycycline can be used for milder forms of the disease.¹³ For children, doxycycline is
70 contraindicated under the age of eight.¹³ In France, although endemic, tularemia is responsible
71 for less than 100 cases per year. All cases are caused by the subspecies *F. tularensis* subsp.
72 *holarctica*.^{14,15} Moreover, strains isolated in France are clonal and belong to specific
73 B.FTNTF002-00 subclade, which is characterized by a genetic deletion in a specific region of
74 difference (RD23).²⁻⁴ Despite appropriate treatment with gentamicin, ciprofloxacin or
75 doxycycline, many therapeutic failures and relapses are still observed in tularemia patients in
76 France. Approximately 30% of patients suffering from lymphadenopathy require surgical
77 lymph node removal because of suppuration.¹⁵ The aim of this study was to evaluate the *in*
78 *vitro* antibiotic susceptibilities of *F. tularensis* subsp. *holarctica* strains isolated from French
79 tularemia patients between 2006 and 2016.

80

81 **MATERIAL AND METHODS**

82 **Bacterial strains**

83 All experiments were conducted in a biosafety level 3 laboratory. We evaluated the
84 antibiotic susceptibilities of 59 *F. tularensis* subsp. *holarctica* strains identified at the French
85 National Reference Center for *Francisella* using PCR amplification and sequencing of the
86 intergenic 16S-23S rRNA region, as previously described.¹⁵ We also tested reference strains
87 as controls: *F. tularensis* subsp. *holarctica* LVS NCTC10857, *F. tularensis* subsp. *novicida*
88 CIP56.12, *F. philomiragia* ATCC25015, and *Staphylococcus aureus* ATCC29213 (ATCC,
89 Mannasas, VA, USA). All strains were kept frozen in cryotubes (MastDiagnostic, Amiens,
90 France) at -80°C and cultured on chocolate agar medium supplemented with Polyvitex[®]
91 (CHA-PVX medium, bioMérieux, Marcy l'Etoile, France).

92 **Antibiotic compounds**

93 Gentamicin, tobramycin, doxycycline, azithromycin, erythromycin, clindamycin,
94 ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin, ampicillin, meropenem,
95 chloramphenicol, rifampicin, tigecycline, ethanol and acetic acid were purchased from Sigma-
96 Aldrich (Saint-Quentin Fallavier, France), and telithromycin from TOKU-E (Zwijnaarde,
97 Belgium). For daptomycin (Cubicin, Cubist Pharmaceuticals) and linezolid (Zyvoxid, Pfizer)
98 we used commercialized powders for intravenous infusion. Some antibiotic powders were
99 first dissolved in ethanol (chloramphenicol and azithromycin) or glacial acetic acid
100 (telithromycin) as recommended by the Clinical and Laboratory Standards Institute.¹⁶

101 **Antibiotic susceptibility testing (AST)**

102 MICs were determined in 96-well microplates using the broth microdilution method,
103 which is the reference method for AST of *Francisella* strains according to the CLSI.¹⁶ The
104 assay medium was cation-adjusted Mueller-Hinton2 (MH2) broth supplemented with 2%
105 PolyViteX[®] (MH2-PVX, bioMérieux, Marcy L'Etoile, France) and adjusted to pH 7.1 +/- 0,1
106 as recommended. We determined MICs for eighteen antibiotics including compounds rarely
107 evaluated against tularemia (gentamicin, tobramycin, doxycycline, ofloxacin, ciprofloxacin,
108 levofloxacin, moxifloxacin, erythromycin, azithromycin, telithromycin, clindamycin,
109 ampicillin, meropenem, chloramphenicol, rifampicin, linezolid, tigecycline, daptomycin).
110 We also determined the percentage of ethanol and acetic acid able to inhibit visible bacterial
111 growth. In brief, one row of a 96-well microplate was filled with 75 µL of twofold serial
112 dilutions of the tested antibiotic compound in the assay medium (See Table 2 for antibiotic
113 dilution range tested). For tigecycline, fresh medium was prepared the day of testing as
114 recommended.¹⁷ The final bacterial inoculum was standardized at 5×10^5 CFU/mL by adding
115 75 µL of a calibrated bacterial suspension to each well. Antibiotic-free cultures were used as
116 positive controls and bacteria-free cultures as negative controls. Microplates were incubated
117 at 37°C in a 5% CO₂ atmosphere. The MICs were read after 48 h for *Francisella* strains. As

118 for *S. aureus* ATCC 29213, MICs were read in MH2-PVX medium after 48h incubation or in
119 MH2 medium after 24h, according to CLSI recommendations. MICs were determined using a
120 microplate reader (TECAN M200 Pro, Männedorf, Switzerland) at 600nm. Experiments were
121 performed at least in duplicate for each strain-antibiotic combination to confirm results. The
122 MICs were interpreted using the CLSI susceptibility breakpoints, which are specific for *F.*
123 *tularensis* only for gentamicin, doxycycline, ciprofloxacin, levofloxacin, and
124 chloramphenicol. We used the *Haemophilus influenzae* EUCAST breakpoints for
125 erythromycin, azithromycin, telithromycin, and rifampicin. Finally, we used the PK/PD
126 breakpoints from the European Committee on Antimicrobial Susceptibility Testing
127 (EUCAST), which are not species specific, for ampicillin, meropenem, ofloxacin,
128 moxifloxacin, linezolid, and tigecycline. Bacterial growth inhibition by ethanol and acetic
129 acid (used as solvents for some antibiotic for the preparation of stock solutions), after pH
130 adjustment to 7.1, was evaluated

131

132 **RESULTS**

133 The 59 *F. tularensis* subsp. *holarctica* strains evaluated in this study were isolated
134 between 2006 and 2016 from various human samples (Table 1). Most patients corresponded
135 to the classical tularaemia clinical forms: ulceroglandular (19 strains), glandular (1),
136 oropharyngeal (6), oculoglandular (2), pneumonic (16) and typhoidal (11). Three strains were
137 isolated from patients with unusual clinical presentations, including meningitis (1), bone and
138 joint infection (1), and aortitis (1) (Table 1). Most strains were isolated from blood samples
139 collected from bacteremic patients, while the others were isolated from other clinical samples
140 directly inoculated to chocolate agar Polyvitex® media (Biomérieux).

141 The most effective antibiotics were the fluoroquinolones (Table 2), with ciprofloxacin
142 displaying the lowest MIC₉₀ (0,03 mg/L). We observed a narrow distribution of MIC values
143 for these compounds (Table 3). The aminoglycosides were also highly active, gentamicin
144 being the most effective compound tested (MIC₉₀ = 0,125 mg/L). The MIC₉₀ of doxycycline
145 was only slightly higher at 0,25 mg/L. All *F. tularensis* strains tested were considered
146 susceptible to these three classes of antibiotics according to CLSI susceptibility breakpoints.
147 Chloramphenicol displayed a ten times higher MIC₉₀ (2 mg/L) compared to doxycycline, but
148 all strains were still considered susceptible to this antibiotic. In contrast, resistance was
149 observed for all strains tested, for the beta-lactams ampicillin (MIC₉₀ > 128 mg/L) and
150 meropenem (MIC₉₀ > 32 mg/L), and for daptomycin (MIC₉₀ > 8 mg/L), linezolid (MIC₉₀ =
151 16 mg/L) and clindamycin (MIC₉₀ = 16 mg/L). Other antibiotics displayed intermediate-level
152 MIC₉₀, including telithromycin (0,25 mg/L), erythromycin (1 mg/L), azithromycin (1 mg/L),
153 rifampicin (1 mg/L) and tigecyclin (1 mg/L). Ethanol alone was able to inhibit growth of all
154 *F. tularensis* strains when diluted at 2% (vol/vol), but the residual concentration of this
155 compound in working solutions of chloramphenicol and azithromycin was 0,016% or lower.
156 Acetic acid also had a bacteriostatic activity against *F. tularensis* strains at 0,5%, but its
157 concentration in working solutions of telithromycin was 0,004% or lower.

158

159 **DISCUSSION**

160 In this study, we tested the antibiotic susceptibilities of 59 human isolates of *F.*
161 *tularensis* subsp. *holarctica* collected throughout France over an 11-year period. Our goal was
162 to check for the presence of susceptibility heterogeneity among this clonal population of
163 strains belonging to the B.FTNF002-00 subclade.²⁻⁴ These data are needed to detect the
164 emergence of acquired resistances to antibiotics in *F. tularensis* and determine if antibiotic

165 treatments currently recommended for tularemia in Western Europe are still adequate. The
166 intermediate-level erythromycin MICs of the strains confirmed their affiliation to *F.*
167 *tularensis* subsp. *holarctica* biovar I, while biovar II strains display MICs higher than 32
168 mg/L. (Caspar and Maurin, *Frontiers in cellular infection and microbiology*, submitted)
169 Importantly, the MICs distribution for all antibiotics tested were very narrow, almost all of
170 them being distributed within three consecutive two-fold dilutions. Only tigecyclin,
171 rifampicin and telithromycin displayed MIC ranges of 4 to 5 dilutions (Table 3). This finding
172 is consistent with the clonality of the strains isolated in France and the absence of acquired
173 resistance mechanism.

174 According to the CLSI susceptibility breakpoints for *F. tularensis*, all the strains tested
175 were susceptible to gentamicin, doxycycline, fluoroquinolones and chloramphenicol (Table
176 2). Thus, no resistant strains to any clinically useful antibiotics had emerged in our strains
177 collection, which is concordant with the literature data. The lowest MIC values were observed
178 for fluoroquinolones, especially ciprofloxacin and levofloxacin, with MICs at least eightfold
179 lower than the susceptibility breakpoint. MICs were slightly higher for ofloxacin and
180 moxifloxacin, with rare strains reaching the EUCAST PK/PD breakpoint defined for
181 susceptibility to the later drug. Thus ciprofloxacin and levofloxacin should probably be
182 preferred to ofloxacin and moxifloxacin for tularemia treatment. Indeed, the WHO guidelines
183 for tularemia treatment only recommend ciprofloxacin (500 mg bid) treatment for 10 to 14
184 days, but levofloxacin could be a possible alternative in the light of its similar *in vitro* activity.
185 No clinical trial has compared the efficacy of both molecules in the context of tularemia. It is
186 to be noted that the bioavailability of levofloxacin is better than that of ciprofloxacin (99-
187 100% versus 70-80%).^{18,19} Levofloxacin (500mg or 750mg) can be administrated once a day,
188 which could allow better compliance of patients compared to the twice a day administration
189 of ciprofloxacin.

190 Gentamicin is currently considered the drug of choice for treatment of severe
191 tularemia usually requiring hospitalization. The 2007 WHO guidelines recommend 10 days of
192 treatment with gentamicin at 5 mg/Kg/day in two doses, a duration that can be prolonged if
193 necessary.¹³ In contrast, the consensus statement from Dennis et al., recommend using
194 gentamicin once daily for adults.¹² The bactericidal activity of the aminoglycosides is
195 concentration-dependent and these antibiotics are currently most often administered once
196 daily to limit their toxicity. These two regimens have not been compared in tularaemia
197 patients. It should be emphasized that *F. tularensis* is an intracellular bacterium and the
198 aminoglycosides slowly penetrate within eukaryotic cells (48 to 72 hours *in vitro*) (Caspar and
199 Maurin, *Frontiers in cellular infection and microbiology*, submitted). The combination of an
200 aminoglycoside with a fluoroquinolone, which penetrates very rapidly into the intracellular
201 compartment, may represent the current best alternative to obtain a rapid clinical
202 improvement in patients with severe infection. Also, such a combination may allow reducing
203 the duration of the administration of gentamicin for 5-7 days to limit its nephrotoxicity while
204 obtaining a rapid bactericidal activity, with the possibility of an early switch from intravenous
205 to oral treatment after improvement of the patient's condition. Tobramycin is not
206 recommended as first line drug for tularaemia treatment. However, in the present study,
207 tobramycin displayed similar MIC₅₀ and MIC₉₀ compared to gentamicin. Using the E-test
208 method, three studies evaluating AST of type B strains from Turkey, Austria and Finland
209 reported tobramycin MIC ranges of 0,125-0,38mg/L; 0,19-3mg/L and 0,5-2 mg/L,
210 respectively.²⁰⁻²² We found an even lower MIC range for this compound using the broth
211 microdilution method. However, Enderlin et al., reported in their review from 1994, a cure
212 rate of only 50% (3/6 patients) with tobramycin.²³ Although this patients series was too small
213 to draw any conclusion, the use of tobramycin in tularaemia patients should be discouraged
214 unless more clinical data will be available.²³

215 Doxycycline (100 mg bid) is an alternative drug for the treatment of mild cases of
216 tularemia, but this antibiotic must be administrated for a minimum of 14 or preferably 21 days
217 owing to its bacteriostatic nature.¹³ All the strains we tested were susceptible to doxycycline
218 with a very narrow MIC range (0,125-0,25 mg/L, Table 3). A larger MIC range (0,25-4 mg/L)
219 was reported for type A and type B strains from North America using the same AST
220 method.²⁴ Treatment of tularaemia patients with doxycycline is associated with a higher rate
221 of failures and relapses compared to fluoroquinolones and aminoglycosides, both in humans
222 and in animal models.²⁵⁻²⁷ As an example, a tularaemia outbreak involving 142 patients in
223 Spain was characterized by a dramatic superiority of ciprofloxacin treatment compared to
224 doxycycline one. Treatment failures occurred in 4,5% of the 22 patients treated with
225 ciprofloxacin, but in 42,8% of the 14 patients treated with doxycycline ($p < 0,018$).²⁶ Because
226 the outbreak was caused by a clonal type B biovar I strain of *F. tularensis* (FTNT002-00
227 subclade), such a difference was likely related to the antibiotic administrated rather than to the
228 involved strain. As French isolates all belong to this particular subclade, we suggest that
229 ciprofloxacin should probably be advocated as a first-line therapeutic option for mild cases of
230 tularemia in Western Europe. Doxycycline treatment may be considered an acceptable
231 alternative only in patients with a contraindication to fluoroquinolone treatment, and thus
232 should be considered as a second-line therapeutic option. Tigecycline did not show an
233 increased activity compared to doxycycline against type B biovar I strains of *F. tularensis*
234 (MIC range: 0,25-1 mg/L for tigecycline versus 0,125-0-25 mg/L for doxycycline). Previous
235 evaluation of type B biovar II strains from Hungary and Turkey, using the E-test method,
236 reported MIC₉₀ of 0,19 mg/L and 0,25 mg/L, respectively, slightly inferior to MIC₉₀ of
237 doxycycline and tetracycline evaluated in the same studies.^{22,28} Using EUCAST PK/PD (non-
238 species related) breakpoints for tigecycline, 9/59 (15%) strains from our study were

239 categorized as resistant and 11/59 (20%) intermediately susceptible to tigecycline. Thus
240 tigecycline might be less effective than doxycycline for the treatment of tularemia in France.

241 Among antibiotics for which CLSI breakpoints are available for *F. tularensis*,
242 chloramphenicol was also active against all strains tested. It may be considered in combined
243 therapy for tularemia despite its bacteriostatic activity and important side-effects, only in case
244 of tularaemia meningitis or meningo-encephalitis because of its high penetration into the brain
245 tissue and cerebrospinal fluid.¹³

246 In this study, we confirmed that the aminopenicillins and carbapenems are ineffective
247 against *F. tularensis* subsp. *holarctica* belonging to B.FTNTF002-00 subclade. This was
248 previously reported by Georgi et al., using the same AST medium, and by Origgi et al., using
249 a modified Mueller Hinton 2 medium, although this later medium should be considered less
250 appropriate for AST of *Francisella* strains.^{11,29} (Caspar and Maurin, *Frontiers in cellular*
251 *infection and microbiology*, submitted) In addition, daptomycin and clindamycin, two
252 antibiotics that have been rarely evaluated against *Francisella*, were also ineffective. Similar
253 results were previously obtained in a cell model of infection for daptomycin.³⁰

254 As for the macrolides, there are currently no consensus breakpoints for susceptibility
255 or resistance of *F. tularensis* strains, or for differentiation between the biovars I and II of type
256 B strains. The introduction of a A2059C mutation in at least one of the three copies of the *rrl*
257 gene encoding the 23S rRNA, conferred erythromycin resistance to the type B biovar I strain
258 FSC274, with an MIC increase from 1-2 mg/L to ≥ 256 mg/L using the E-test method.⁸ We
259 previously mentioned that for type B biovar I strains, erythromycin MICs reported in the
260 literature were ≤ 8 mg/L using the broth microdilution method, and ≤ 1 mg/L using the E-test
261 method. (Caspar and Maurin, *Frontiers in cellular infection and microbiology*, submitted).
262 Thus erythromycin MIC cut-off for the distinction between the biovars I and II of *F.*
263 *tularensis* subsp. *holarctica* could be set at 8 mg/L using the microdilution method. Particular

264 attention should be paid to adjustment of the pH for erythromycin and azithromycin, as
265 acidification of the medium rapidly results in a significant four- to eightfold MIC
266 increase.(Caspar and Maurin, unpublished data). It is to be noted that, when using
267 susceptibility breakpoints available for macrolides against *Haemophilus influenzae* (Table 2),
268 another small Gram-negative fastidious bacterium, all tested *F. tularensis* strains would be
269 classified as intermediately susceptible to erythromycin and azithromycin. In contrast, 33/59
270 (56 %) strains tested would be classified as susceptible to telithromycin and only 26/59 (45%)
271 as intermediately susceptible. Telithromycin was previously evaluated against 71 *F. tularensis*
272 subsp. *holarctica* strains from France using an agar dilution method.³¹ The reported MIC
273 range (0,125-0,25 mg/L) would categorize all those strains as intermediately susceptible
274 when using the *H. influenzae* breakpoints.³¹ Moreover, telithromycin at 4 mg/L was effective
275 against a type B biovar I strain in a cellular model of infection.³² Although the activity of
276 telithromycin against *F. tularensis* should be further evaluated *in vivo*, especially in animal
277 models, this drug could represent an interesting second- or third-line therapeutic option for
278 infections caused by type B biovar I strains, especially when first-line antibiotics are
279 contraindicated. Azithromycin (500 mg/day for 6 weeks), although categorized as
280 intermediately susceptible, has already been used for the treatment of a pregnant women
281 suffering from oropharyngeal tularemia in France.³³

282 Among other antibiotic tested, linezolid MIC ranged from 4 to 16 mg/L using a broth
283 microdilution method. Variable studies using the E-test method have reported MICs ranging
284 from 0,5 to 48 mg/L between three published studies evaluating either type A and B strains
285 from North America or type B biovar II strains from Turkey and Hungary.^{19, 25, 32} According
286 to our *in vitro* results, biovar I strains belonging to the B.FTNTF002-00 subclade should be
287 considered resistant to linezolid. Moreover, using *H. influenzae* breakpoints, the *F. tularensis*
288 strains evaluated in this study would also be categorized as susceptible to rifampicin, although

289 the MIC range (0.125-1 mg/L) was close to the resistance cut-off ($\text{MIC} \leq 1 \text{ mg/L}$). Higher
290 MIC values have already been described for rifampicin (up to 3 mg/L) for *F. tularensis*
291 strains, but only when using the E-test method. (Caspar and Maurin, *Frontiers in cellular*
292 *infection and microbiology*, submitted). One study of 71 French isolates reported MIC range
293 of 0,015-0,5 mg/L when using the agar dilution method.³¹ Thus, although rifampicin cannot
294 be used alone because of rapid selection resistant mutants, it could be useful in combination
295 with a first-line drug to treat rare bone and joint infections owing to its excellent diffusion in
296 these tissues.¹³ One patient with prosthetic joint infection showed a dramatic clinical
297 improvement after the addition of rifampicin to ciprofloxacin antibiotic treatment.³⁵ Moreover
298 we observed that the growth of all *F. tularensis* subsp. *holarctica* strains tested was inhibited
299 by 2% ethanol and 0.5% acetic acid (after adjustment of the pH of the AST medium to 7.1).

300 Considering this and previous studies on *F. tularensis* MIC data obtained with the
301 standardized broth microdilution method using cation-adjusted MH2-PVX medium as
302 recommended by the CLSI,^{11,24,29} we propose to establish epidemiological cut-offs (E_{coff})
303 values for the first-line antibiotics currently used for tularemia treatment. These cut-offs
304 would help to better define wild type strains with no identified resistance mechanism and to
305 better detect the emergence of acquired resistances to these drugs. They are different from the
306 clinical breakpoints, which are used to predict the clinical efficacy of the corresponding
307 antibiotics. As no significant MIC differences have been observed between type A and type B
308 strains for fluoroquinolones and aminoglycosides with this technique, we proposed to set up
309 an E_{coff} at $\leq 0,125 \text{ mg/L}$ for ciprofloxacin and levofloxacin, and $\leq 0,5 \text{ mg/L}$ for gentamicin.
310 As a larger MIC range was observed by Urich et al. for doxycycline against *F. tularensis*
311 strains from North America, using the same experimental medium, a general E_{coff} for
312 doxycycline may not be suitable. Thus, according to the present study, we set up an E_{coff} at
313 $\leq 0,25 \text{ mg/L}$ for doxycycline, for strains belonging to B.FTNNF002-00 subclade.

314 In conclusion, we found that *F. tularensis* subsp. *holarctica* strains of the B.FTNNF002-
315 00 subclade isolated in France over the last decade remain fully susceptible to gentamicin,
316 ciprofloxacin and doxycycline, which are currently advocated as first-line drugs for tularemia
317 treatment.¹³ However, our results indicate that alternative therapeutic schemes should be
318 evaluated in Western Europe, where mild to moderate severity infections predominate.
319 Firstly, because of lower rates of failures and relapses, the fluoroquinolones should be
320 considered the best oral alternative, while the use of doxycycline should be restricted to
321 patients with contraindications to the former antibiotics. For rare patients suffering from
322 severe systemic diseases, the combination of gentamicin and ciprofloxacin parenterally should
323 be considered as a first alternative to obtain rapid clinical improvement. Finally, because
324 tularaemia cases in Western Europe are only caused by type B biovar I strains, azithromycin
325 and telithromycin may represent useful alternatives in pregnant women and young children.
326 All these proposals should be validated by further clinical evaluation.

327

328 **Transparency declarations**

329 The authors have no conflicts of interest to declare.

330

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332

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425 **TABLES**

426

427 **Table 1:** Characteristics of the 59 *F. tularensis* subsp. *holarctica* strains evaluated in this
 428 study and isolated from tularaemia patients in France between 2006 and 2016

Strain	Host	Year of isolation	Origin of the strain	Clinical form of tularaemia
CHUGA-Ft6	Human	2007	Blood culture	Typhoidal
CHUGA-Ft7	Human	2006	Conjunctivitis	Oculoglandular
CHUGA-Ft8	Human	2007	Skin ulcer	Ulceroglandular
CHUGA-Ft9	Human	2007	Blood culture	Typhoidal
CHUGA-Ft10	Human	2008	Lymph node	Pneumonic
CHUGA-Ft11	Human	2008	Pharynx	Oropharyngeal
CHUGA-Ft12	Human	2008	Pharynx	Oropharyngeal
CHUGA-Ft13	Human	2008	Pharynx	Oropharyngeal
CHUGA-Ft14	Human	2008	Blood culture	Typhoidal
CHUGA-Ft15	Human	2008	Cerebrospinal Fluid	Meningitis
CHUGA-Ft16	Human	2008	Blood culture	Typhoidal
CHUGA-Ft17	Human	2008	Blood culture	Typhoidal
CHUGA-Ft18	Human	2008	Blood culture	Typhoidal
CHUGA-Ft19	Human	2008	Blood culture	Typhoidal
CHUGA-Ft20	Human	2008	Skin ulcer	Ulceroglandular
CHUGA-Ft21	Human	2008	Conjunctivitis	Oculoglandular
CHUGA-Ft22	Human	2009	Finger abcess	Ulceroglandular
CHUGA-Ft23	Human	2009	Ear suppuration	Oropharyngeal
CHUGA-Ft24	Human	2009	Lymph node	Ulceroglandular
CHUGA-Ft25	Human	2010	Blood culture	Typhoidal
CHUGA-Ft26	Human	2010	Blood culture	Pneumonic
CHUGA-Ft27	Human	2010	Blood culture	Typhoidal
CHUGA-Ft28	Human	2010	Lymph node	Glandular
CHUGA-Ft29	Human	2010	Blood culture	Ulceroglandular
CHUGA-Ft30	Human	2011	Skin ulcer	Ulceroglandular
CHUGA-Ft31	Human	2011	Blood culture	Pneumonic
CHUGA-Ft32	Human	2012	Lymph node	Ulceroglandular
CHUGA-Ft33	Human	2011	Blood culture	Pneumonic
CHUGA-Ft34	Human	2012	Blood culture	Pneumonic
CHUGA-Ft35	Human	2012	Finger abcess	Ulceroglandular
CHUGA-Ft36	Human	2012	Unknown	Ulceroglandular
CHUGA-Ft37	Human	2010	Blood culture	Pneumonic
CHUGA-Ft38	Human	2012	Blood culture	Pneumonic
CHUGA-Ft39	Human	2012	Finger abcess	Ulceroglandular
CHUGA-Ft40	Human	2012	Pleural liquid	Pneumonic
CHUGA-Ft41	Human	2013	Pleural liquid	Pneumonic
CHUGA-Ft42	Human	2013	Skin ulcer	Ulceroglandular
CHUGA-Ft43	Human	2013	Lymph node	Ulceroglandular
CHUGA-Ft44	Human	2014	Finger abcess	Ulceroglandular
CHUGA-Ft45	Human	2014	Blood culture	Oropharyngeal
CHUGA-Ft46	Human	2014	Ear suppuration	Oropharyngeal
CHUGA-Ft48	Human	2014	Pleural liquid	Pneumonic
CHUGA-Ft50	Human	2014	Blood culture	Typhoidal
CHUGA-Ft51	Human	2014	Bronchoalveolar lavage	Pneumonic

CHUGA-Ft52	Human	2014	Elbow abcess	Ulceroglandular
CHUGA-Ft53	Human	2014	Skin ulcer	Ulceroglandular
CHUGA-Ft54	Human	2015	Blood culture	Unknown
CHUGA-Ft55	Human	2015	Finger abcess	Ulceroglandular
CHUGA-Ft56	Human	2015	Blood culture	Pneumonic
CHUGA-Ft57	Human	2016	Blood culture	Pneumonic
CHUGA-Ft58	Human	2015	Finger abcess	Ulceroglandular
CHUGA-Ft59	Human	2015	Blood culture	Pneumonic
CHUGA-Ft60	Human	2016	Blood culture	Typhoidal
CHUGA-Ft62	Human	2016	Lymph node	Ulceroglandular
CHUGA-Ft63	Human	2016	Finger abcess	Ulceroglandular
CHUGA-Ft64	Human	2016	Aortic anevrysm	Aortitis
CHUGA-Ft65	Human	2016	Articular hematoma (hip)	Bone and joint infection
CHUGA-Ft68	Human	2016	Pleural liquid	Pneumonic
CHUGA-Ft69	Human	2016	Pleural liquid	Pneumonic

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451 **Table 2:** MIC results (mg/L) for 59 *F. tularensis* subsp. *holarctica* strains isolated from tularaemia patients in France between 2006 and 2016.

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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 NCTC10857	Breakpoints (mg/L)	
		MIC50 (mg/L)	MIC90 (mg/L)	Range (mg/L)	MIC (mg/L)	S ≤	R >
Gentamicin	0,03-16	0,125	0,125	≤0,03 - 0,25	0,06	4	
Tobramycin	0,03-16	0,125	0,25	0,06 - 0,25	0,125	2	4
Ofloxacin	0,008-4	0,06	0,125	0,06 - 0,125	0,06	0,25	0,5
Ciprofloxacin	0,001-0,5	0,03	0,03	0,016 - 0,06	0,016	0,5	
Levofloxacin	0,001-0,5	0,03	0,06	0,016 - 0,06	0,016	0,5	
Moxifloxacin	0,002-1	0,125	0,125	0,06 - 0,25	0,03	0,25	0,25
Doxycycline	0,03-16	0,25	0,25	0,125 - 0,25	0,125	4	
Tigécycline	0,032-16	0,25	1	0,125 - 1	0,06	0,25	0,5
Ampicillin	0,25-128	>128	>128	64 - > 128	>128	2	8
Meropenem	0,06-32	>32	>32	>32	>32	2	8
Erythromycin	0,06-32	1	1	0,5 - 2	>32	0,5	16
Azitromycin	0,06-32	0,5	1	0,25 - 1	>32	0,125	4
Telithromycin	0,016-8	0,125	0,25	0,03 - 0,5	>8	0,125	8
Clindamycin	0,03-16	8	16	4 - 16	>16	0,25	0,5
Linezolid	0,125-64	8	16	4 - 16	4	2	4
Chloramphenicol	0,06-32	1	2	0,5 - 2	1	8	
Rifampicin	0,004-2	0,5	1	0,125 - 1	0,03	1	1
Daptomycin	0,016-8	>8	>8	>8	>8	1	1
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		

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455 **Table 3:** Antibiotic MIC distribution of the 59 *F. tularensis* subsp. *holarctica* strains isolated from tularaemia patients in France between 2006
 456 and 2016

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Antibiotic	MIC distribution (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	44	2	0	0								
Doxycycline						0	0	11	48	0	0	0	0	0	0				
Tigécycline						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			
Azitromycin							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					

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3. RESISTANCE AUX FLUOROQUINOLONES

3.1. Publication 3 : Functional Characterization of
the DNA gyrases in Fluoroquinolone-resistant
Mutants of *Francisella novicida*.

La deuxième partie de cette thèse a consisté à caractériser et rechercher des mutations de résistances aux fluoroquinolones chez les patients traités par cette classe d'antibiotique mais en situation d'échec thérapeutique. Bien que non retrouvées lors de la mesure de la sensibilité des souches de *F. tularensis* aux antibiotiques (Article 1 et 2), des résistances bactériennes aux fluoroquinolones peuvent malgré tout être à l'origine des échecs thérapeutiques rencontrés, car elles apparaissent rapidement lors d'expérimentations conduites *in vitro* et car ces mécanismes de résistance bactériens sont observés chez la plupart des espèces bactériennes pathogènes pour l'homme. A titre de comparaison, ce n'est que très récemment que la possibilité de sélection *in vivo* de mutation de résistance aux fluoroquinolones suite à un traitement par cette classe d'antibiotique, a été confirmée chez *Legionella pneumophila*. (247) Ces mutations ont été identifiées par le biais d'analyses moléculaires conduites sur des prélèvements de patients atteints de Légionellose, alors qu'aucune souche résistante aux fluoroquinolones n'avait jamais été isolée. (247) De plus, il est important de rappeler que *F. tularensis* est une bactérie de croissance très difficile, qui n'est isolée que dans environ 10% des cas de tularémie, principalement en cas de bactériémie et principalement avant l'instauration d'une antibiothérapie efficace. (74) Sur les 94 cas confirmés par le Centre National de Référence des *Francisella* entre 2006 et 2010 en France, seules 21 souches furent isolées alors que 39 prélèvements étaient positifs en PCR. Par ailleurs, l'acquisition de mutation de résistance touchant principalement les grandes fonctions cellulaires telles que la synthèse du peptidoglycane, la réplication de l'ADN, la synthèse protéique ou la transcription de l'ARN, celle-ci s'accompagne en général d'un « coût » répliatif pour la bactérie se traduisant par une diminution de son taux de croissance. (248) Ce phénomène laisse à penser que la culture de ces souches potentiellement résistantes à partir de prélèvements cliniques est encore plus difficile que celle des souches sensibles, ce qui peut contribuer au fait qu'aucune souche résistante n'ait été isolée à ce jour.

Une étude réalisée au sein de notre équipe a néanmoins permis de montrer que la sélection *in vitro* de souches résistantes aux fluoroquinolones en présence de concentrations sub-inhibitrices d'antibiotique était facile et rapide et pouvait conduire à des résistances de très haut niveau (jusqu'à une multiplication par 2048 de la CMI de la ciprofloxacine). (224) Dans cette étude, des lignées résistantes à la ciprofloxacine ont été obtenues à partir de souches de *F. tularensis* LVS, *F. novicida* et *F. philomiragia*, pris comme modèles avirulents ou

de virulence diminuée de *F. tularensis*. (224) Les principales mutations de résistance aux fluoroquinolones mises en évidence furent des mutations ponctuelles, identifiées dans les gènes *gyrA* et *gyrB* codant pour les sous-unités GyrA et GyrB des topoisomérases de type II de *F. tularensis* (Tableau 5), qui sont la cible des fluoroquinolones. Ces mutations furent principalement retrouvées dans des zones appelées Quinolone-Resistance-Determining-Region ou QRDR car ces zones concentrent les principales mutations de résistance aux fluoroquinolones identifiées à ce jour.

Bacterial lineage	CIP CMI (mg/L)	Mutations ^a			
		GyrA, <i>gyrA</i>	GyrB, <i>gyrB</i>	ParC, <i>parC</i>	ParE, <i>parE</i>
Fth wt	0.016				
Fth1P14	32	T83I, C248T	E467D, G1401T		L488I, C1462A
Fth2P14	32	T83K, C248A ; D87Y, G259T		S453X, +T1368	
Fth3P14	32	T83K, C248A	S465Y, C1394A	ΔC82, ΔGTT254-256	
Fno wt	0.064				
Fno1P14	32	D87Y, G259T	+P466, +CTC1397		P472S, C1414T
Fno2P14	64	D87G, A260G	D487E-ΔK488 ^b ΔTAA1460-1462 ^b		
Fno3P14	32	ΔE503-S504, ΔAGAGTC1509-1514 ^c			
Fno4P14	32	P43H, C128A			
Fph wt	0.032				
Fph1P14	32	D87G, A260G	E88K, G262A ; P746S, C2236T		
Fph2P14	64	T83M, C248T	P746S, C2236T		
Fph3P14	32	ΔT83, ΔACG247-249	P746S, C2236T		
Fph4P14	64	T83M, C248T ; ΔT570, ΔCAA1709-1711	P746S, C2236T		

CIP: ciprofloxacin; LVX: levofloxacin; MXF: moxifloxacin; GEN: gentamicin; ERY: erythromycin; DOX: doxycycline. Fth : *F. tularensis* subsp. *holarctica* ; Fno : *F. novicida* ; Fpo : *F. philomiragia* ; P14 : 14^{ème} repiquage en présence de concentrations sub-inhibitrices de ciprofloxacin. Wt : wild-type

^aLes modifications en acides aminés résultant des différentes mutations génétiques (en italique) sont numérotées selon la séquence des protéines chez *Francisella*. Les sigles Δ et + indiquent respectivement la délétion ou l'insertion d'un codon. X indique un codon stop. Les modifications au niveau des nucléotides sont numérotées selon la séquence des gènes chez *Francisella* sp. Les sigles Δ et + indiquent respectivement la délétion ou l'insertion d'un ou plusieurs nucléotide(s).

^bCette mutation consiste en un événement unique correspondant à une délétion de 3 paires de bases

^cCette mutation consiste en un événement unique correspondant à une délétion de 6 paires de bases

Tableau 6 : CMI à la ciprofloxacin et mutations de résistances aux fluoroquinolones observées chez 11 lignées de *Francisella* résistantes aux fluoroquinolones. Ces lignées ont été générées *in vitro* par culture successives (14 passages) de souches sauvages (wt) en présence de concentrations sub-inhibitrices d'antibiotiques. (Adapté de Sutera et al., 2013 (224))

Parmi les mutations observées sur les différentes lignées résistantes obtenues dans l'étude de Sutera et al, les plus fréquentes sont celles présentes sur le gène *gyrA* (100% des lignées générées présentant une mutation sur ce gène). Des sites particuliers sur la sous-unité GyrA sont considérés comme des points à fort taux de mutations. Il s'agit des positions 83 et 87. Les mutations observées en position 83 et 87 ont été retrouvées sur les différentes lignées résistantes de l'étude de Sutera et al. Ces mutations sont classiques. Elles ont déjà été observées dans d'autres études sur *Francisella* et bien caractérisées chez plusieurs autres bactéries dont *Escherichia coli*. (223,249–251) De la même façon, des mutations en position 426 et 447 du QRDR de la sous-unité GyrB ont déjà été décrites chez *E. coli* mais n'ont pas été retrouvées dans l'étude de Sutera et al. En revanche cette étude a mis en évidence de nouvelles mutations :

- P43H (C128A) en dehors du QRDR du gène *gyrA* chez une lignée résistante de *F. novicida*
- ΔE503-S504 (ΔAGAGTC1509-1514) en dehors du QRDR du gène *gyrA* chez une lignée résistante de *F. novicida*
- +P466 (+CTC1397) dans le QRDR du gène *gyrB* chez une lignée résistante de *F. novicida* possédant également une mutation D87Y (G259T) dans le QRDR du gène *gyrA*. Cette mutation est proche de deux mutations décrites sur deux lignées de *F. tularensis* LVS en position S465Y (C1394A) et E467D (G1401T) dans la même étude
- D487E-ΔK488 (ΔTAA1460-1462) dans le QRDR du gène *gyrB* chez une lignée résistante de *F. novicida* possédant également une mutation D87G (A260G) dans le QRDR du gène *gyrA*

La caractérisation fonctionnelle de toutes ces mutations n'a jamais été réalisée au sein du genre *Francisella*. Le but de cette troisième étude a donc été de déterminer les conséquences en terme fonctionnel des mutations de résistance aux fluoroquinolones identifiées sur les gènes *gyrA* et *gyrB* au sein des lignées de *F. novicida*, sur l'activité de superenroulement de l'ADN en présence de fluoroquinolones, afin de définir l'impact de chacune de ces mutations sur la résistance aux fluoroquinolones.

1 **Functional Characterization of the DNA gyrases in**
2 **Fluoroquinolone-resistant Mutants of *Francisella novicida***

3 Running title: *Francisella* DNA gyrase and fluoroquinolone resistance

4
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24 **Abstract**

25 Fluoroquinolone (FQ) resistance is a major health concern in the treatment of tularemia. With
26 DNA gyrase described as the main target of these compounds, our aim was to clarify the
27 contribution of both GyrA and GyrB mutations found in *Francisella novicida* clones highly
28 resistant to FQs. Wild-type and mutated GyrA and GyrB subunits were overexpressed so that
29 the *in vitro* FQ sensitivity of functional reconstituted complexes could be evaluated. Data
30 obtained were compared with the MICs of FQs against bacterial clones harboring the same
31 mutations and were further validated through complementation experiments and structural
32 modeling. Whole-genome sequencing of FQ-highly resistant lineages was also done.
33 Supercoiling and DNA cleavage assays demonstrated that GyrA-D87 is a hot-spot FQ
34 resistance target in *F. novicida* and pointed out the role of GyrA P43H substitution in
35 resistance acquisition. An unusual feature of FQ resistance acquisition in *F. novicida* is that
36 the first step mutation occurs in GyrB with direct or indirect consequences for FQ sensitivity.
37 Insertion of P466 in GyrB leads to an IC₅₀ value comparable to that observed for mutant
38 gyrase carrying GyrA-D87Y substitution, while the D487E-ΔK488 mutation not active on its
39 own, contributes to the high level of resistance that occurs following acquisition of GyrA-
40 D87G substitution in double GyrA/GyrB mutants. Involvement of other putative targets is
41 discussed, including the ParE mutation that was found to arise in the very late stage of
42 antibiotic exposure. This study provides the first characterization of the molecular
43 mechanisms responsible for FQ resistance in *Francisella*.

44 INTRODUCTION

45 *Francisella tularensis* is a Gram-negative, facultative intracellular bacterium responsible
46 for the zoonosis tularemia (1). Classically, two subspecies cause most human infections:
47 subsp. *tularensis* (type A) in North America and subsp. *holarctica* (type B) throughout the
48 northern hemisphere. Humans are contaminated through direct contact with infected animals
49 (especially hares), arthropod bites (mainly ticks), ingestion of contaminated food or water,
50 and contact with contaminated environments. Depending on the portal of entry of bacteria, the
51 disease may manifest in six clinical forms: the ulceroglandular and glandular forms, a
52 regional lymphadenopathy with or without a visible skin inoculation lesion, respectively; the
53 oropharyngeal form; a pharyngitis with cervical lymphadenopathy; the oculoglandular form, a
54 conjunctivitis with a pretragal or cervical lymphadenopathy; the pneumonic form after
55 inhalation of a contaminated aerosol or through hematogenous spread of bacteria; and the
56 typhoidal form combining high fever and neurological symptoms that may mimic typhoid.
57 Among these various clinical forms, respiratory tularemia has attracted the most attention
58 because it may cause up to 30% mortality in humans if left untreated (2, 3). This highly
59 infectious aerosolizable pathogen is considered as a class A bioterrorism agent by the Centers
60 for Disease Control and Prevention (USA). However, despite its potential use as a biological
61 weapon, no safe and potent vaccine is currently available (2, 3). A few antibiotic classes are
62 effective in treating tularemia including aminoglycosides, tetracyclines, and fluoroquinolones
63 (FQs) (4). Microbiological and clinical data show that ciprofloxacin, and possibly other FQs,
64 are the best alternative for oral therapy of tularemia in patients with clinical manifestations of
65 mild to moderate severity, but failures and relapses are frequent (5, 6).

66 Quinolones are one of the most commonly prescribed classes of antibacterial agents in the
67 world (7). These drugs inhibit DNA replication through interaction with complexes composed
68 of DNA and either of the two target enzymes, DNA gyrase or topoisomerase IV, that belong

69 to type IIA topoisomerases (8). As in most Gram-negative bacteria, the primary target for FQs
70 in *Francisella* strains is thought to be the DNA gyrase (9-11), which functions as an A₂B₂
71 heterotetrameric complex able to catalyze the negative supercoiling of the bacterial circular
72 chromosome (12). Resistance to FQs can result from single point mutations in GyrA and
73 GyrB, leading to conformational changes of the whole complex, in turn impairing antibiotic-
74 target interactions. Such mutations are described to be mainly restricted to discrete regions of
75 the so-called quinolone-resistance-determining regions (QRDRs). Another major mechanism
76 of resistance relies on mutations affecting the bacterial entry/efflux systems resulting in a
77 reduced quinolone accumulation in bacterial cells. Most of these efflux pumps are encoded on
78 the chromosome, but quinolone-specific (QepA) as well as multidrug (OqxAB) efflux pumps
79 were also found encoded by bacterial plasmids. Other plasmid-mediated quinolone resistance
80 include the *qnr* genes encoding pentapeptide repeat proteins that act as DNA mimics and
81 protect the type II topoisomerases, and an allele of the aminoglycoside acetyltransferase
82 (*aac*)-encoding gene able to decrease drug activity through acetylation (7, 13, 14).

83 Because of the widespread use (and overuse) of these drugs, the increased prevalence of
84 quinolone-resistant strains is a growing concern in several bacterial species (15). To date, no
85 resistant strain of *F. tularensis* has been isolated from patients (16), but successful culture of
86 this fastidious pathogen is achieved in only 10% of tularemia cases (5). *In vitro*, the c248t
87 (T83I) substitution has been found in the GyrA-QRDR of a *F. tularensis* subspecies
88 *holarctica* quinolone-resistant strain (URFtCIPR isolate) (9). This mutation was also observed
89 in a *F. tularensis* SchuS4 strain isolated after *in vitro* exposure to increasing amounts of
90 ciprofloxacin and was accompanied by the g259t (D87Y) GyrA-QRDR substitution (10).

91 An evolution experiment was conducted in our lab on several *Francisella* sp. strains with
92 attenuated virulence to humans exposed to increasing concentrations of FQs (11). This
93 approach, considered as a powerful tool to investigate the acquisition dynamics of drug

94 resistance (17, 18), led to the emergence of high-level resistant mutants on which phenotypic
95 and genotypic analyses were carried out (11). Besides mutations in the QRDR-A and the
96 QRDR-B hotspot regions, novel substitutions or deletions were identified both in *Francisella*
97 GyrA and GyrB subunits. An elegant study from Jaing *et al.* (19) recently reported genome-
98 wide mutations associated with ciprofloxacin-resistant *F. tularensis* LVS isolated *in vitro* and
99 confirmed GyrA and GyrB mutations described by Sutura *et al.* (11). However, while a
100 structural model of DNA gyrase was built to achieve a better understanding of how mutations
101 affect FQ resistance (19), their functional role was not investigated.

102 Here our aim was to clarify the functional consequences of GyrA and GyrB mutations on
103 FQ sensitivity observed in four independent lineages of *F. novicida* highly resistant to such
104 antibiotics. While not pathogenic to humans, this *Francisella* species is a widely used model
105 for studying *F. tularensis* infections (20). This is the first study describing the DNA
106 supercoiling activity and the FQ-induced DNA cleavage by *Francisella* proteins, these
107 functional tests being used as efficient ways to establish relationships between DNA gyrase
108 mutations and FQ resistance (21).

109 MATERIALS AND METHODS

110 **Sample collection.** The phenotypic and genotypic characteristics of the *F. novicida* lineages
111 selected for this study are reported Table 1. In contrast to the FQ-susceptible parental strain *F.*
112 *novicida* U112, the four lineages designated Fno1 to Fno4 and propagated for 14 passages in
113 the presence of ciprofloxacin are highly resistant to FQs (11). A common feature of these FQ-
114 resistant strains is the presence of mutations in the gene encoding GyrA. Concerning Fno1
115 and Fno2, the GyrA substitutions were localized within the QRDR and are accompanied with
116 additional GyrB mutations, whereas Fno3 and Fno4 were mutated in GyrA regions outside the
117 QRDR without an additional GyrB mutation. Bacterial cultures grown from glycerol stocks
118 stored at -80°C at each passage were used for sequencing, MIC determination and preparation

119 of competent cells. Whole-genome sequencing was performed for each lineage (Fno1 to
120 Fno4) on one clone at the end of the evolution experiment (passage 14) using the ABI SOLID
121 sequencing technology (AB 5500xl Genetic Analyzer), and mutations observed were further
122 confirmed by PCR amplification and sequencing of the corresponding DNA regions using a
123 dye terminator sequencing approach.

124 **Expression and purification of *F. novicida* GyrA and GyrB.** The genes were amplified
125 from genomic DNA of the selected isolates using gene-specific primers (Table S1) and cloned
126 into the pDEST-17 vector including a 6xHis-N-terminal tag (Invitrogen, Carlsbad, CA, USA).
127 The integrity of all constructs was confirmed by DNA sequencing (Eurofins, Ebersberg,
128 Germany). Protein expression was carried out in *Escherichia coli* BL21(DE3) or C41(DE3)
129 grown at 37°C to an OD₆₀₀ of 0.6 in Luria Broth (LB) supplemented with 100 µg/mL
130 ampicillin before addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). Optimized
131 conditions for each mutant are reported in Table S2 (see supplementary data). After
132 sonication the lysate was clarified by centrifugation, then passed over a Ni²⁺ affinity column
133 (Qiagen) extensively washed with Tris-HCl/NaCl buffer supplemented with 25–50 mM
134 imidazole before elution of the His-tagged proteins in the presence of 300 mM imidazole. The
135 purity of recombinant proteins was analyzed by sodium dodecyl sulfate polyacrylamide gel
136 electrophoresis (SDS-PAGE) and Coomassie blue staining before concentration using an
137 Amicon[®] Ultracentrifugal device. Purified proteins (0.5–1.0 mg/mL) were dialyzed overnight
138 at 4°C (50 mM Tris-HCl pH 7.9, 40% glycerol, 1 mM EDTA, 1 mM DTT) and stored at
139 –80°C after flash freezing in liquid nitrogen. For GyrA_Fno2, 500 mM NaCl was added to
140 the dialysis buffer to avoid protein precipitation.

141 **DNA supercoiling assays.** DNA supercoiling assays were carried out with reconstituted
142 combinations of the GyrA and GyrB subunits from the wild-type and/or mutated strains. The
143 protocol was similar to that applied for the analysis of *Mycobacterium tuberculosis* DNA

144 gyrase (22) with minor modifications. The experiments were conducted in a final volume of
145 10 μ L containing 35 mM Tris-HCl pH 7.5, 24 mM KCl, 4 mM MgCl₂, 2 mM DTT, 0.1
146 mg/ml BSA, 1.8 mM spermidine, 100 mM potassium glutamate, 1 mM ATP, 6.5% (w/v)
147 glycerol and 0.15 μ g relaxed pBR322 (Inspiralis, Norwich, UK) as substrate. After 30 min
148 incubation at 37°C, the reaction was stopped by adding gel loading dye (New England
149 Biolabs). Samples were analyzed by electrophoresis in 0.8% agarose gels run at 50 V for 6 h
150 in 1x TAE buffer, stained with GelRed™ and imaged on a UV transilluminator. For all
151 combinations of GyrA and GyrB tested, we determined the minimal amounts of wild-type
152 and/or mutated proteins required to convert 0.15 μ g of relaxed pBR322 DNA to the
153 supercoiled form (1 U). The inhibitory effect of ciprofloxacin (Sigma-Aldrich) or
154 moxifloxacin (Bayer) against the wild-type and mutated DNA gyrases was assessed by
155 determining the concentrations of drugs required to inhibit their supercoiling activity by 50%
156 (IC₅₀). Each experiment was conducted at least in triplicate using two different batches of
157 proteins. Statistical analysis was done using Prism v5.0 (GraphPad Software). An unpaired
158 Student *t*-test was used to determine the difference between the means of values obtained in
159 independent experiments with a *P*-value of <0.05 being considered significant.

160 **DNA cleavage assays.** DNA cleavage assays were performed in a 10 μ L reactional
161 volume using the same buffer as supercoiling assays but without ATP, 0.2 μ g supercoiled
162 pBR322 DNA (Inspiralis, Norwich, UK) as substrate and 125 nM of GyrA and GyrB
163 subunits. Following 1 h incubation at 25°C, SDS and proteinase K were added and the
164 incubation was continued for 30 min at 37°C as described (23). The extent of DNA cleavage
165 was quantified after electrophoresis as described above and the results expressed as the FQ
166 concentrations required to induce 25% of the maximum DNA cleavage (CC₂₅).

167 **Molecular modeling.** The breakage-reunion (GyrA N-terminal) and the Toprim (GyrB C-
168 terminal) domains were modeled using Phyre2 (24). The catalytic core model, composed of

169 the breakage-reunion and Toprim domains, DNA, and FQs, was generated by superposition
170 onto the crystal structure of the *M. tuberculosis* DNA gyrase (25) using SSM implemented in
171 Coot (26).

172 **Transformation of *F. novicida* with a GyrB-encoding plasmid.** The genes encoding the
173 *F. novicida* wild-type or GyrB_D487E-ΔK488 were amplified using a primer pair on which
174 NotI and AgeI restriction sites had been engineered (Table S1) and cloned into the pFNLTP6
175 shuttle plasmid downstream of the *gro* promoter (27) with the T4 DNA ligase (New England
176 Biolabs). Following restriction enzyme digestion and sequencing, the constructs were
177 introduced by electroporation in selected *F. novicida* isolates as described (28). Transformed
178 colonies appeared after 2 days of incubation and the presence of the plasmid was checked by
179 PCR using specific primers (Table S1).

180 **Accession numbers.** The sequences of the final evolved clones are available in the
181 Sequence Read Archive with the accession numbers SRX2188198, SRX2188197,
182 SRX2188196 and SRX2188195.

183 RESULTS

184 **Expression and purification of recombinant *F. novicida* DNA gyrases.** To assess the
185 relationships between resistance levels and mutations in GyrA and GyrB, we cloned and
186 expressed the wild-type and mutated proteins listed in Table 1. High yields of recombinant
187 His-tagged and soluble recombinant GyrB proteins were obtained with only a few changes of
188 the protocol described by Tari *et al.* (29). In contrast, several attempts were required to
189 optimize purification of the GyrA subunits, including variations in temperature and induction
190 times, salt concentrations and addition of detergents. As judged by SDS-PAGE analysis (Fig.
191 S1) the purity of all GyrA and GyrB constructs is greater than 90%.

192 **DNA supercoiling activity of *F. novicida* DNA gyrases.** The wild-type DNA gyrase of *F.*
193 *novicida* was reconstituted *in vitro* using equimolar amounts of GyrA_WT and GyrB_WT

194 proteins and examined for its capacity to convert relaxed pBR322 to its supercoiled form. As
195 shown Fig. 1, the assay carried out using 35 mM Tris-HCl pH 7.5, 24 mM KCl, 2 mM DTT,
196 0.1 mg/ml BSA, 1.8 mM spermidine and various concentrations of MgCl₂ and ATP
197 demonstrates that the reconstituted complex could catalyze a magnesium-dependent
198 supercoiling reaction, although the effect is not optimal. The catalytic activity of *F. novicida*
199 DNA gyrase is significantly improved by the addition of 100 mM potassium glutamate (K-
200 Glu) and requires MgCl₂ and ATP at optimum concentrations of 2 and 1 mM, respectively.

201 **Respective contributions of GyrA and GyrB mutations to FQ resistance.** To assess the
202 respective roles of the GyrA and GyrB mutations in ciprofloxacin resistance, the inhibitory
203 effect of FQs was first tested against the supercoiling activity of DNA gyrase complexes
204 corresponding to the wild-type sensitive strain or to complexes reconstituted using a
205 recombinant mutated target in combination with its complementary wild-type GyrA or GyrB
206 subunit. Representative inhibition profiles are shown Fig. 2A and 2B. For each condition we
207 verified that neither the GyrA nor the GyrB subunit alone induces DNA supercoiling that
208 could result from a putative contamination of the recombinant proteins with the *E. coli*
209 enzymes (wells 1 and 2). Given that similar but not strictly identical patterns were obtained
210 for experiments conducted with different batches of proteins, the data were expressed as
211 means \pm standard error (SEM) of the ciprofloxacin concentrations required to inhibit 50% of
212 DNA supercoiling activity (IC₅₀ value) in different assays. As illustrated in Fig. 2C, the
213 substitutions at position 87 in QRDR-A generate a high-level of FQ resistance with an IC₅₀
214 value approximately twice as high when the aspartic acid residue is exchanged for a tyrosine
215 than for a glycine (152.3 \pm 9.2 mg/L; *n*=4 for D87Y vs 83.82 \pm 15.2 mg/L; *n*=6 for D87G, *P* <
216 0.05). To a lesser extent but significantly, the P43H substitution in the GyrA N-terminus
217 domain exhibits a ciprofloxacin sensitivity that is four times lower (18.9 \pm 1.07 mg/L; *n*=6)
218 than the wild-type complex (4.11 \pm 0.91 mg/L; *n*=5). The 3D structure of the *F. novicida*

219 Quinolone-Binding Pocket (QBP) together with FQs and DNA that was generated through
220 homology modeling (Fig. 3) shows that this residue is located close to the catalytic Y122
221 tyrosines where the DNA is bound and close to the D87 residues that interact with FQ. From
222 this model, it can be assumed that the P>H substitution might potentially affect the inhibitory
223 effect of the drugs. Finally, the $\Delta E523, \Delta S524$ deletion found in the C-terminus of GyrA does
224 not affect drug activity (4.98 ± 0.65 mg/L; $n=3$).

225 Supercoiling data also evidenced that the QRDR-B insertion (+P466) leads to a high
226 resistance level (105.09 ± 18.86 ; $n=6$), in contrast with the D487E- $\Delta K488$ mutation found in
227 the C-terminal domain of the GyrB subunit, which fails to significantly affect FQ sensitivity
228 (9.6 ± 1.07 mg/L; $n=5$). The capacity of ciprofloxacin to inhibit the DNA supercoiling activity
229 was then evaluated against reconstituted DNA gyrase complexes corresponding to the high-
230 level resistant clones isolated after the final (14th) passage in the Fno1 and Fno2 lineages
231 sharing GyrA/GyrB double mutations (Fig. 2C, right panel). Thus, the IC_{50} value measured
232 with the reconstituted Fno1 complex, which possesses mutations both in QRDR-A (D87Y)
233 and QRDR-B (+P466), was 186.9 ± 40.69 mg/L ($n=6$). This value was not significantly
234 different from the value obtained with the reconstituted Fno2 complex (181.1 ± 43.09 mg/L;
235 $n=9$), which also has a QRDR-A substitution (D87G) but accompanied with a GyrB mutation
236 outside the QRDR (D487E- $\Delta K488$). A strictly similar pattern was obtained using
237 moxifloxacin (not shown). Overall these results are well correlated with data obtained from
238 the ciprofloxacin-mediated DNA cleavage assays (Fig. 2 D, E, F and Table S3).

239 **Stepwise appearance of GyrA and GyrB mutations.** The QRDR-A and the mutated
240 GyrB regions were sequenced from *F. novicida* Fno1 and Fno2 initial lineages as well as from
241 resistant isolates saved at the different passages during the course of antibiotic exposure (11).
242 For each passage of interest, six clones were analyzed with similar results. In both cases, and
243 in contrast to what was expected, the GyrB mutation occurred first. In Fno1 the ctc nucleotide

244 insertion leading to the appearance of an additional proline residue (+P466) in the GyrB
245 subunit was detected as early as the second passage. The g/t substitution responsible for the D
246 to Y change at the residue position 87 of the QRDR-A arose later (passage 4). Similarly, in
247 Fno2, the three-base deletion (taa 1460-1462), accounting for the D487E,ΔK488 mutation in
248 GyrB, was found at passage 4, while the a260g point mutation accounting for the GyrA-
249 QRDR D87G substitution occurred at passage 9. To evaluate the precise impact of the
250 mutations detected in ciprofloxacin resistance, MICs were then measured on the
251 corresponding bacterial clones both before and after each mutation occurred. The
252 chronological appearance of GyrA and GyrB mutations and the evolution of the ciprofloxacin
253 MICs are summarized in Fig. 4. The shifts in the MIC increase observed upon the acquisition
254 of QRDR-A substitution in bacterial isolates sharing a GyrB mutation (×4 in Fno1 and ×32 in
255 Fno2) were within the same range of magnitude as the IC₅₀ shifts obtained with the
256 recombinant DNA gyrase complexes carrying either single GyrB or double GyrA/GyrB
257 mutations (see above). This result reinforces the contribution of DNA gyrase mutations to the
258 FQ resistance of evolved bacterial clones. Moreover, the important shift in resistance
259 observed upon the acquisition of QRDR-A mutation in Fno2, together with the moderate
260 impact of FQ resistance-conferring D87G mutation, suggest that the D487E-ΔK488 mutation
261 found in GyrB, which fails to significantly affect the resistance phenotype on its own,
262 indirectly contributes to an increased FQ resistance level.

263 **Complementation study confirmed the contribution of the GyrB D487E-ΔK488**
264 **mutation to FQ resistance.** Complementation experiments were conducted on the *F.*
265 *novicida* Fno2 single mutant (passage 4; QRDR-B D487E-ΔK488) or double mutant (passage
266 11; QRDR-B D487E-ΔK488 plus QRDR-A D87G) using the pFNLTP6 plasmid carrying
267 either the gene encoding wild-type or mutated GyrB. Whatever plasmid was used, the MIC
268 values obtained upon complementation of the *F. novicida* Fno2 clone isolated at passage 4,

269 i.e., before acquisition of the GyrA mutation, remained unchanged. In contrast, we observed
270 that the ciprofloxacin susceptibility of the clone isolated at passage 11 was partially restored
271 upon complementation with the wild-type GyrB, this transformation conferring an
272 approximately two- to threefold decrease in the MIC relative to the same mutant transformed
273 with the mutated protein (64 mg/L).

274 **Genome sequencing of *F. novicida* clones highly resistant to FQs.** As shown above, the
275 GyrA deletion found in the Fno3 lineage is not associated with FQ resistance; concerning
276 Fno4, the inhibitory effect of ciprofloxacin on DNA supercoiling *in vitro* (Fig. 2C) did not
277 correlate with the high MIC measured on the final bacterial isolates (Table 1). In addition,
278 sequencing of *gyrA* and *gyrB* in Fno1 and Fno2 lineages at different passages clearly showed
279 that MIC continued to increase after appearance of mutations in these FQ target genes.
280 Together, these data suggest that mutational events could occur in other genes. The genomic
281 analysis of the four lineages highly resistant to FQs (passage 14) is summarized Table 2. An
282 interesting feature found in the Fno2 lineage is a mutation of the topoisomerase IV gene
283 encoding ParE. However, sequencing data revealed that this mutation occurs at the final stage
284 of antibiotic exposure (passage 14), as previously reported for the *F. tularensis* lineage (11).
285 These data revealed that, besides changes in the DNA gyrases, another characteristic shared
286 between all these isolates was a mutation within the promoter of the FTN_0480 gene. Other
287 mutations detected were specific for each clone. Their putative impact on FQ resistance is
288 discussed below.

289 **DISCUSSION**

290 Here we successfully produced the recombinant GyrA and GyrB subunits found in FQ-
291 resistant and -susceptible lineages of *F. novicida*. All affinity-purified proteins were
292 enzymatically active, as shown by the capacity of reconstituted complexes to promote
293 supercoiling of relaxed pBR322. This specific *in vitro* DNA supercoiling assay has never

294 been reported before for *Francisella* proteins and allowed us to clarify the role of the different
295 mutations on the FQ susceptibility of these bacterial pathogens.

296 One of the main features of *F. novicida* lineages exposed to FQs is a substitution affecting
297 the D87 residue located in QRDR-A that has been associated with acquired quinolone
298 resistance in several bacterial species, including *E. coli* and *M. tuberculosis* (30)
299 (corresponding residue D94; see supplementary file, Fig. S2). The work presented here
300 clearly demonstrates that this residue is also a hot-spot quinolone resistance target in
301 *Francisella* strains. Its replacement by glycine or tyrosine indeed confers a high-level
302 resistance to mutated recombinant DNA gyrases from *F. novicida*, a result in agreement with
303 structural studies showing that these substitutions adversely affect the interactions with FQs
304 by modifying the geometric properties of the QBP and by displacing the conserved water
305 molecules involved in Mg²⁺ ion preservation, which is essential for FQ activity (13). The
306 functional analysis of other *F. novicida* mutated GyrA subunits revealed that the P43H
307 substitution, never reported before, also contributes to FQ resistance, although to a lesser
308 extent. The capacity of mutations outside QRDR-A to confer a small increase in MIC for
309 ciprofloxacin and gatifloxacin was previously described in *E. coli* (A51V) (31). The
310 molecular modeling of the structure of the functional core of *F. novicida* DNA gyrase
311 presented here shows that the residue P43 that is conserved in several pathogens (Fig. S2) is
312 located in close proximity to the catalytic tyrosine residues and to DNA and could thus play a
313 role in the interaction between FQs and their target. In contrast, the deletion within C-
314 terminus of GyrA (Δ E524, Δ S525) is not associated with resistance to ciprofloxacin. DNA
315 supercoiling assays also showed that GyrB mutations are associated with a high-level FQ
316 resistance in *F. novicida*. Thus, insertion of the residue P466 in the QRDR-B alter the FQ
317 inhibitory effect against supercoiling as efficiently as the QRDR-A D87 substitution. While
318 inactive on its own, the D487E, Δ K488 mutation indirectly contributes to the acquisition of a

319 high level of FQ resistance in double GyrA/GyrB mutants, as confirmed by complementation
320 experiments carried out on mutated lineages of *F. novicida* resistant to FQs.

321 In Gram-negative bacteria, resistance to FQs has been predominantly attributed to GyrA
322 mutations, GyrB mutations being less commonly encountered and often found secondary to
323 alterations in GyrA (14). In Gram-positive bacteria, depending on the FQs used, different
324 levels of inhibitory activity against DNA gyrase and topoisomerase IV were observed, but in
325 several cases topoisomerase IV seems to be the more sensitive enzyme (32). In this case, the
326 C subunit of topoisomerase IV (ParC) is considered as the primary target of FQs, whereas
327 mutations affecting the E subunit (ParE) occur at a lower frequency and as second-step
328 mutations.

329 Here, we identified for the first time mutations in GyrB that are associated with a high
330 level of resistance and that represent the first step in selection for decreased susceptibility to
331 FQs. This unusual order in which GyrB is altered before GyrA is consistent with our data on
332 topoisomerase IV. While in the *F. novicida* lineages examined here, topoisomerase IV
333 mutation is a rare event that occurs at the late stage of FQ exposure and seems to play a minor
334 role in the development of FQ resistance, it should be noted that the ParE substitution
335 observed in the lineage Fno2 is not preceded by ParC changes. Such an unusual first-step
336 mutation in GyrB was confirmed following a single-step selection with ciprofloxacin on
337 plates *in vitro* (Table S3). Thus of the fifteen independent single-step mutants selected, five
338 had a QRDR-A mutation and 10 shared a GyrB substitution including 1 isolate with P466
339 insertion and 7 isolate with a substitution of the S465 GyrB residue. Interestingly, a first-step
340 mutation in GyrB was also recently evidenced in a FQ-resistant *Salmonella enterica* isolate
341 from an infected patient (33). From this interesting, it can be hypothesized that the mutation
342 pattern observed in *F. novicida* is not based on the *in vitro* selection protocol but is likely to
343 be observed *in vivo*. The preponderant role of GyrB in FQ resistance highlighted here fits well

344 with the recently proposed inverted binding mode of FQs to gyrase-DNA complexes (34).
345 Using *Mycobacterium smegmatis* as a model, Mustaev *et al.* indeed proposed a role for E466
346 or K447 GyrB residues as an alternative way to ensure stabilization of the drug-enzyme-DNA
347 complex known to be mediated through the magnesium-water bridge with GyrA residues 83
348 and 87.

349 Overall, our findings provide new insights into the molecular mechanisms of FQ resistance
350 in *F. novicida*. We noted some discrepancy between the IC₅₀ of reconstituted DNA gyrase and
351 the MIC of resistant bacterial lineages, most particularly concerning Fno3 and Fno4 mutants,
352 suggesting that other mechanisms, independent of GyrA or GyrB mutations, contribute to
353 resistance, as is the case for several human pathogens (35, 36). Genome sequencing of the
354 FQ-resistant *F. novicida* isolates identified, in each of the four lineages, a mutation within the
355 promoter of the FTN_0480 gene encoding the 110-amino acid Fev protein weakly
356 homologous to the MerR family of transcription factors and characterized as an essential
357 regulator of virulence in *Francisella* (37). Given that reports on the incidence of promoter
358 mutations in *Francisella* are lacking, it is difficult to speculate on the consequence of this
359 mutation on resistance to FQ. With this exception, the mutations detected are specific for each
360 lineage. A set of three to five targets is mutated after antibiotic exposure, leading to several
361 interrupted coding sequences. Concerning Fno3, for which the IC₅₀ against DNA supercoiling
362 is low compared to the bacterial MIC, nonsense mutations are present in *pilB* and *pilE* genes
363 required for the expression of functional type IV pili (38) as well as in FTN_1029 coding for
364 a protein involved in the isoprenoid biosynthesis pathway. Interestingly, this metabolic
365 pathway, known to be involved in major cellular processes, including cell wall biosynthesis or
366 the regulation of membrane fluidity (39), has been described as a promising target for the
367 development of novel antimicrobial drugs in *F. tularensis* (40). Another nonsense mutation is
368 found in Fno3 *wbtQ* that contributes to the biosynthesis of the *F. novicida* O-antigen involved

369 in bacterial resistance to macrolides (41). The *wbtH* targeted in the Fno2 lineage belongs to
370 the same gene cluster. Overall, we noted that several mutations target genes encoding proteins
371 located at the membrane or involved in transmembrane transport that could be impaired in
372 their functions, leading to changes in antibiotic susceptibility. The erythromycin and
373 doxycycline cross-resistance patterns of *F. novicida* lineages highly resistant to FQs reported
374 in the Sutura *et al.* paper (11) reinforce the hypothesis of alterations in outer membrane
375 permeability. While the precise consequences of such mutations remain to be investigated, it
376 is tempting to speculate that they are somewhere relevant for the emergence of *F. novicida*
377 mutants that are highly resistant to FQs. This hypothesis reinforced by the published data
378 from Jaing *et al.*, who identified proteins possibly involved in *F. tularensis* ciprofloxacin
379 resistance through a careful analysis of both microarrays and next-generation sequencing data
380 from 11 isolates that had acquired resistance *in vitro* upon exposure to increasing antibiotic
381 concentrations (19). Besides the GyrA and GyrB substitutions, three identified *F. tularensis*
382 LVS mutated targets are also mutated in *F. novicida*, including *fupA* (FTL_0439/FTN_0444)
383 coding for the protein responsible for siderophore-mediated iron acquisition and thought to be
384 involved in bacterial virulence (42, 43), *wbtQ* (FTL_0601/FTN_0430), as discussed above, as
385 well as an efflux pump-encoding gene (FTL-1547/FTN_1609).

386 In summary, while the whole-genome analyses of resistant clones suggest that the response
387 to FQ exposure might be much more complex than initially thought, we have functionally
388 characterized for the first time DNA gyrase mutations through which FQ resistance emerges
389 in *F. novicida*.

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521 **TRANSPARENCY DECLARATIONS**

522 The authors have nothing to declare.

523

524 **FIGURE LEGENDS**

525 FIG. 1 Supercoiling activity of reconstituted *F. novicida* DNA gyrase complex. Recombinant
526 GyrA and GyrB subunits from *F. novicida* (250 ng each) were assayed for DNA supercoiling
527 activity for 30 min at 37°C using 0.15 µg relaxed pBR322 as substrate and in presence of
528 different concentrations of MgCl₂, ATP and KGlu, as indicated. The DNA product was
529 analyzed on a 0.8% agarose gel. OC and SC indicate Open Circular and Supercoiled forms of
530 pBR322, respectively.

531

532 FIG. 2 Ciprofloxacin inhibits DNA supercoiling and promotes DNA cleavage induced by
533 wild-type and mutated DNA gyrases from *F. novicida*. The inhibitory activity of increasing
534 concentrations of ciprofloxacin was measured for 30 min at 37°C against DNA supercoiling
535 activity induced by the DNA gyrase complexes reconstituted with recombinant (A) *F.*
536 *novicida* wild-type GyrA and GyrB or (B) mutated GyrA (D87Y) and GyrB (+466) found in
537 the resistant Fno1 lineage. Lane A, relaxed pBR322 and GyrA (2U); lane B, relaxed pBR322
538 and GyrB (2U); lane 3, pBR322 linearized by BamHI digestion; lanes 4–11, supercoiling
539 activity obtained in presence of increasing concentrations of ciprofloxacin in comparison with
540 the positive control (2U each subunit) without an antibiotic. The gels are representative of at
541 least three distinct experiments. (C) Ciprofloxacin sensitivity expressed as IC₅₀ values was
542 measured on DNA gyrase complexes sharing mutations in GyrA (white columns), GyrB
543 (hatched columns) or both (dotted columns). (D, E, F) Same legend as above using
544 supercoiled pBR322 incubated for 1 h at 25°C in presence of fixed amounts of recombinant
545 proteins (125 nM) and increasing concentrations of ciprofloxacin. Fragmented DNA was
546 detected following addition of proteinase K and SDS. Data represent the mean ± SEM of
547 three to nine distinct experiments conducted with two different batches of recombinant
548 proteins. (*) indicates a *P*-value < 0.05.

549 FIG. 3 Three-dimensional structure modeling of the *F. novicida* QRDR. Left panel: front
550 view of the complex with the Toprim domains represented in magenta and pink, the breakage-
551 reunion domains in blue and DNA in transparent grey. The black arrow indicates the location
552 of the QBP of the catalytic core. Right panel: top-view zoom of the QBP. The catalytic Y122
553 residues are colored in yellow and residues at positions critical for FQ resistance are
554 indicated.

555

556 FIG. 4 Stepwise appearance of GyrA/GyrB mutations and FQ resistance. The QRDR-A and
557 the mutated GyrB regions from initial lineages and resistant clones of *F. novicida* saved at
558 different passages during the course of the antibiotic exposure were sequenced using specific
559 primers (Table S1) to check the appearance of the mutations indicated by a black symbol and
560 arrows on the graph. Susceptibility to ciprofloxacin was also evaluated before and after
561 acquisition of mutations; MICs are represented as the MIC modal value. (A) Lineage Fno1;
562 (B) lineage Fno2.

563 TABLE 1 Characteristics of the *F. novicida* lineages used in this study

564

Lineage	MIC (mg/L)	Amino acid mutations in		Reference
		GyrA	GyrB	
Fno_WT	0.064	No mutation	No mutation	CIP56.12
Fno1	128	D87Y	+P466	Sutera <i>et al.</i> 2014
Fno2	128	D87G	D487E, Δ K488	Sutera <i>et al.</i> 2014
Fno3	32	Δ E524, Δ S525	No mutation	Sutera <i>et al.</i> 2014
Fno4	64	P43H	No mutation	Sutera <i>et al.</i> 2014

565

566 The bacteria used in this study include the FQ-sensitive parental strain of *F. novicida* U112
567 (Fno_WT) and four lineages (Fno1 to Fno4) that acquired a high FQ resistance level
568 associated with mutations in GyrA and GyrB after 14 subcultures in presence of antibiotics
569 (11). The ciprofloxacin MICs were measured from the bacterial clone selected for whole
570 genome sequencing at the final stage of the evolution procedure, using Mueller-Hinton 2
571 broth supplemented with 2% PolyViteX® as described (11).

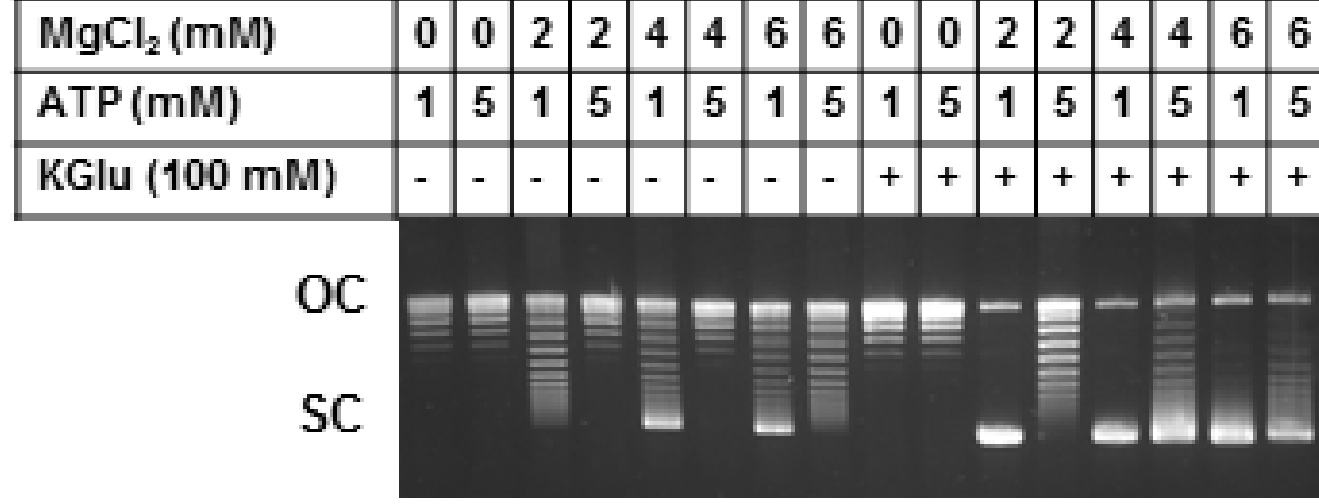
572 TABLE 2 SNPs and indels identified by whole genome sequencing of the highly resistant evolved lineages

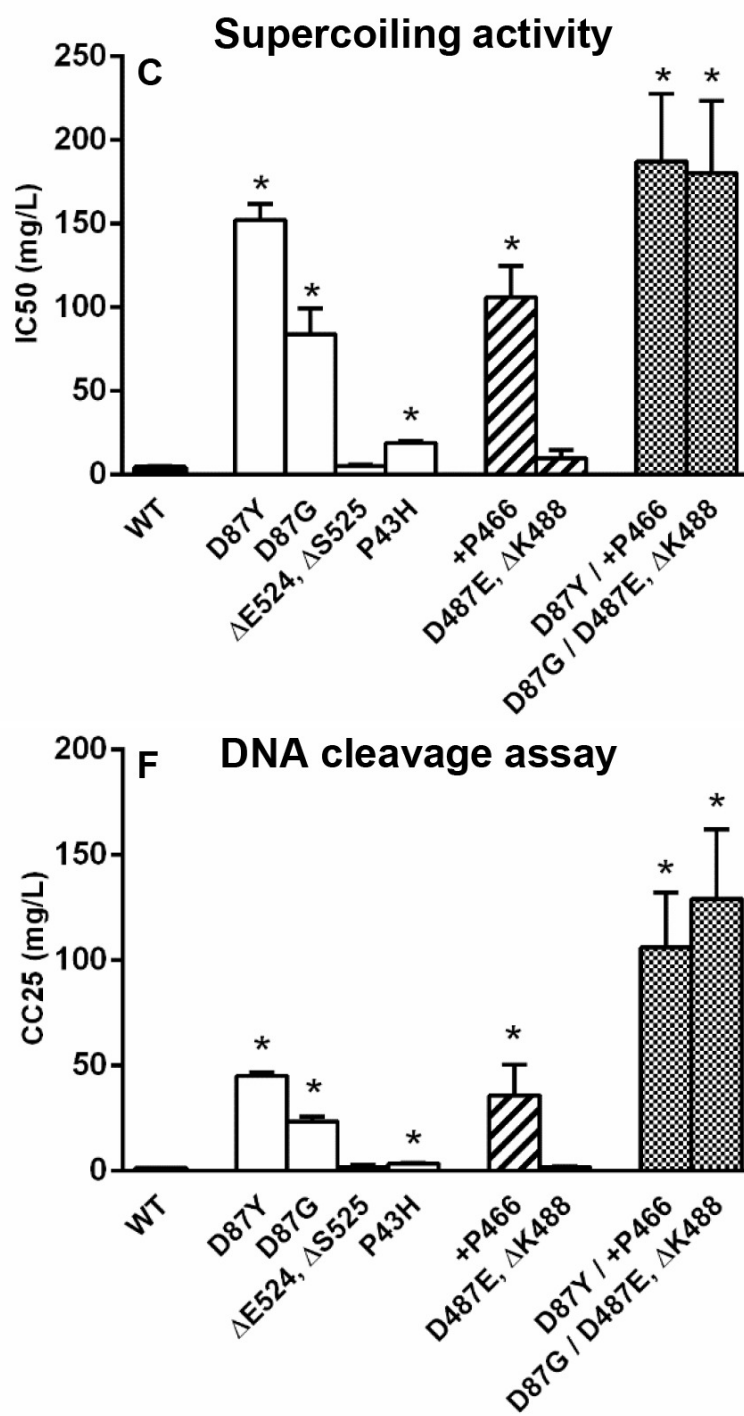
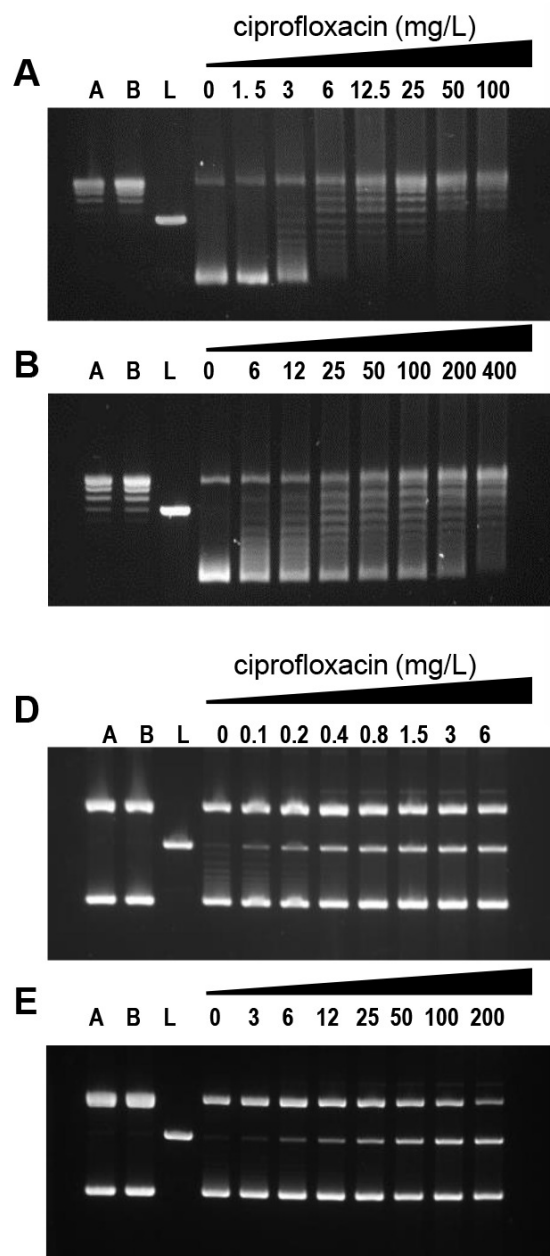
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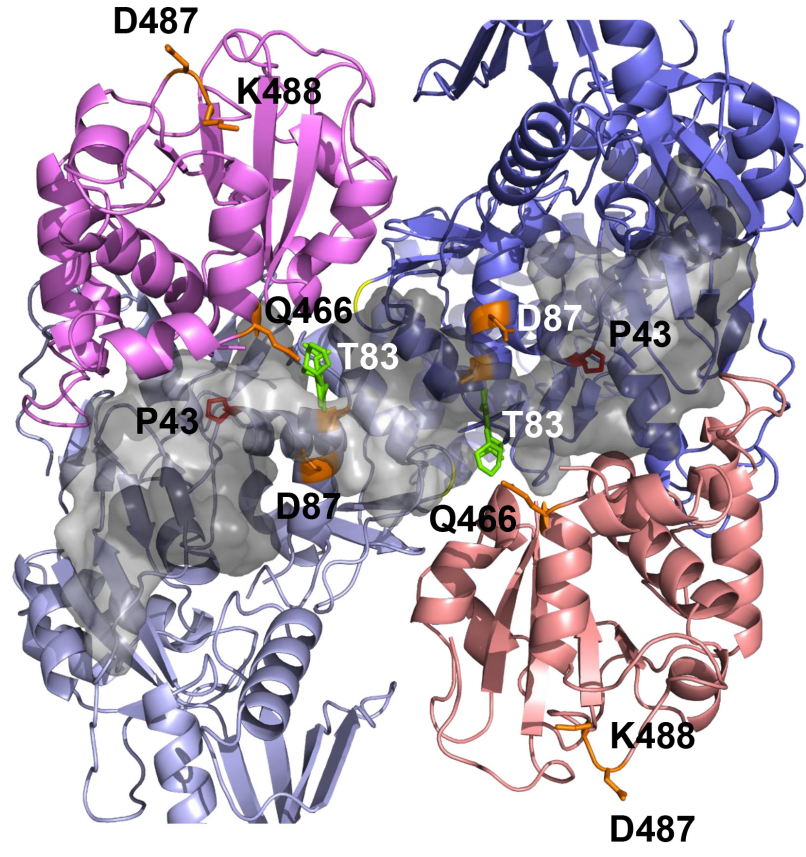
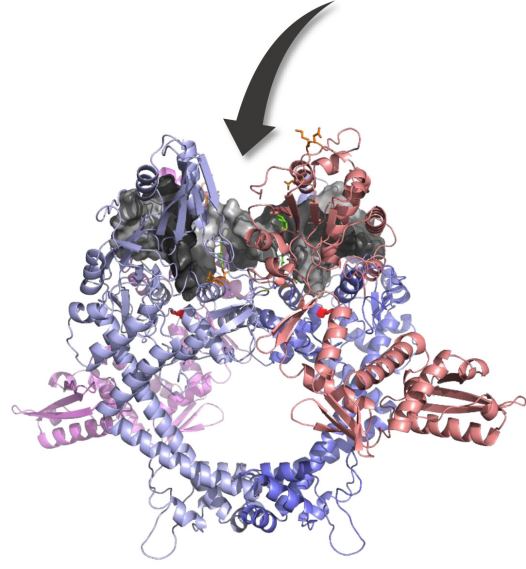
Lineage	GyrA mutations	GyrB mutations	Other mutations identified in the genome of sequenced mutants				
			Gene number	Gene name	Description	Type of mutation	Consequences
Fno1	D87Y	+P466	FTN_0480	<i>fevR</i>	Francisella effector of virulence regulation	In point mutation in the promoter	Unknown
			FTN_1418	<i>manC</i>	Mannose-1-phosphate guanylyltransferase	Insertion	STOP codon 38
			FTN_1510	<i>secB2</i>	Preprotein translocase, subunit B	Deletion	STOP codon 89
			FTN_1652	<i>atpB</i>	ATP synthase	Deletion	STOP codon 10
Fno2	D87G	D487E, ΔK488	FTN_0402	<i>aroC</i>	Chorismate synthase	Deletion	STOP codon 140
			FTN_0480	<i>fevR</i>	Francisella effector of virulence regulation	In point mutation in the promoter	Unknown
			FTN_1083	-	Hypothetical protein	In point mutation	D128N
			FTN_1421	<i>wbtH</i>	Glutamine amidotransferase/asparagine synthase	Deletion	STOP codon 313
			FTN_1550	<i>parE</i>	Topoisomerase IV, subunit B	In point mutation	P742S
			FTN_1610	-	RND efflux transporter, AcrB/AcrD/AcrF family	In point mutation	Y320H
Fno3	ΔE524, ΔS525	-	FTN_0444*	<i>fupA</i>	Fer utilization protein	Deletion	STOP codon 39
			FTN_0480	<i>fevR</i>	Francisella effector of virulence regulation	In point mutation in the promoter	Unknown
			FTN_0913	<i>rpoD</i>	RNA polymerase sigma-70 factor	Deletion	Deletion D52
			FTN_1029	-	Isoprenoid biosynthesis protein with amidotransferase-like domain	Insertion	STOP codon 43
			FTN_1115	<i>pilB</i>	Type IV pili ATPase	Insertion	STOP codon 180
			FTN_1430*	<i>wbtQ</i>	Aminotransferase	Deletion	STOP codon 145
Fno4	P43H	-	FTN_0070	<i>pilE</i>	Type IV pili, pilus assembly protein	Deletion	STOP codon 117
			FTN_0480	<i>fevR</i>	Francisella effector of virulence regulation	In point mutation in the promoter	Unknown
			FTN_0504	-	Lysine decarboxylase	Insertion	STOP codon 612
			FTN_1471	<i>pcs</i>	(CDP-alcohol) phosphatidyltransferase	Deletion	STOP codon 92
			FTN_1609*	-	MexH family multidrug efflux RND transporter periplasmic adaptor	Deletion	Deletion Y101

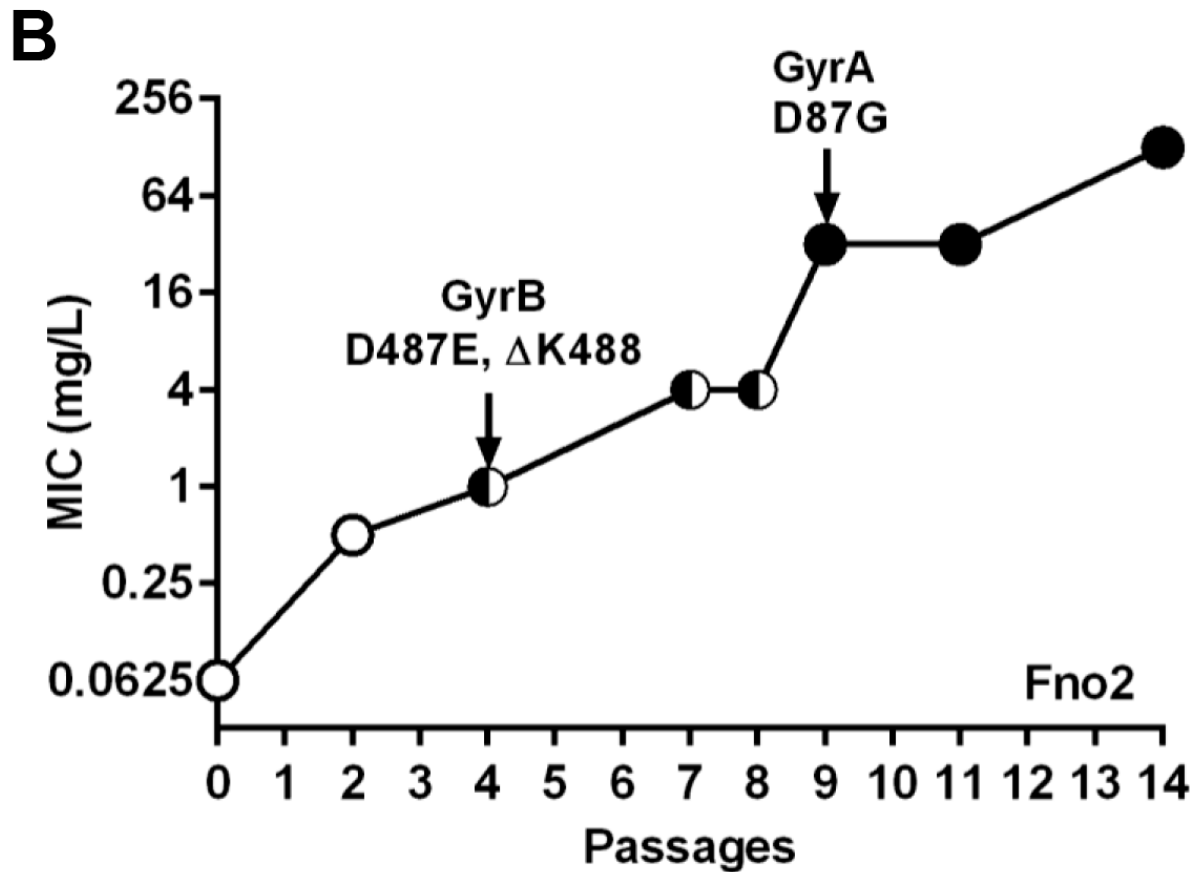
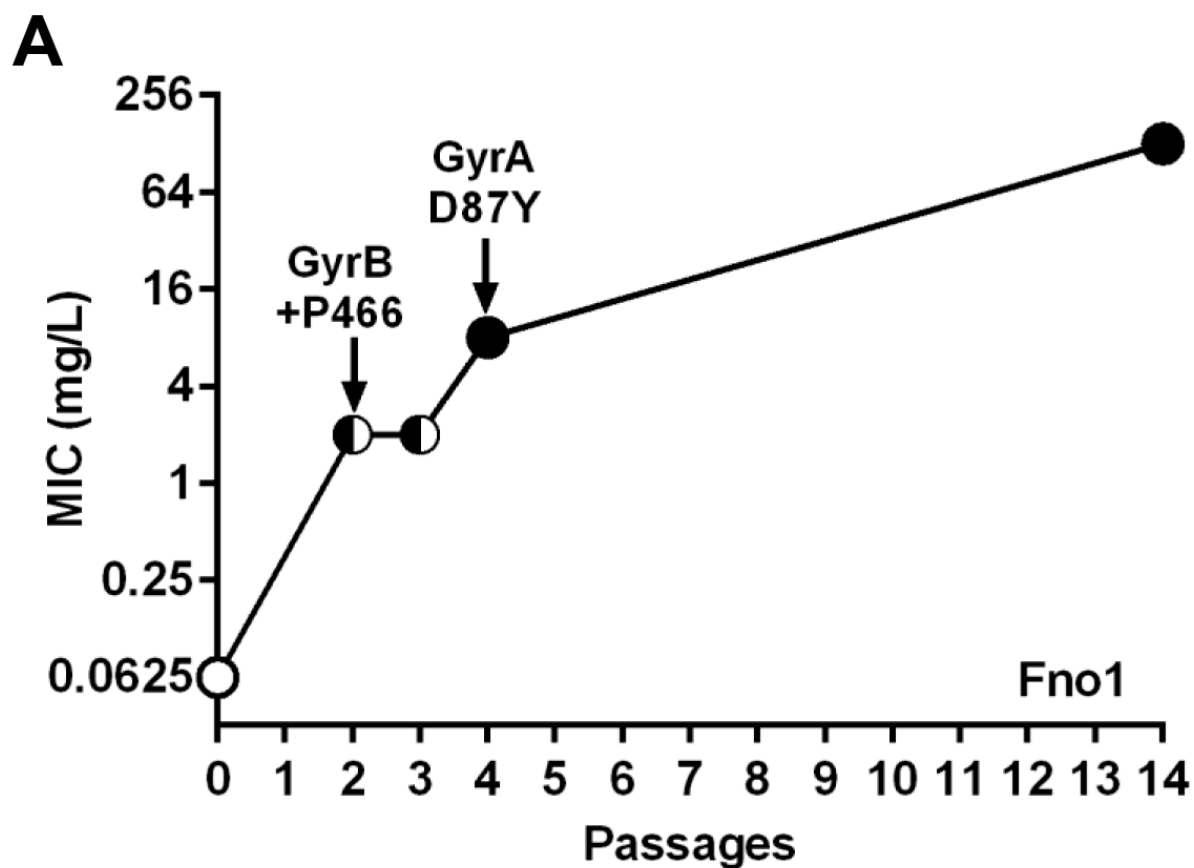
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575 *indicates genes found to be containing mutations in ciprofloxacin-resistant *F. tularensis* isolates (19)







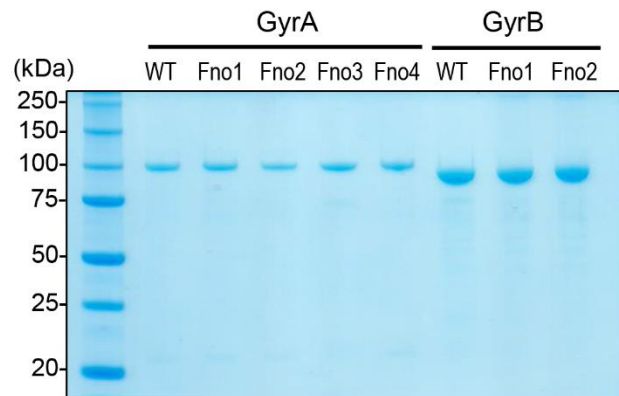


1 Supplementary Files

2 FIG S1 SDS-PAGE analysis of purified *F. novicida* DNA gyrases. Recombinant proteins expressed in *E. coli* and purified by Ni-NTA affinity
3 chromatography were resolved on SDS-PAGE (10%) and stained with Coomassie blue. Values on the left indicate molecular weights of the marker
4 proteins.

5 This figure shows that the apparent molecular weights of GyrA and GyrB are of around 100 and 93 kDa that corresponds to their expected molecular
6 masses. For each construct, the protein was purified to >90% purity.

7



8

9

17 TABLE S1 List of primers used in this study

gene	primer name	nucleotide sequence
<i>Cloning in pDESt-17 expression vector</i>		
<i>gyrA</i>	Fno_gyrA_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGTTCTATAATTACTAAAGAGTC
	Fno_gyrA_Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTATTCCTCTGCACAGTAACATCAG
<i>gyrB</i>	Fno_gyrB_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAGGGTTCTGAGAATAAAGCTTATGACTC
	Fno_gyrB_Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTAACGTCCAAGTTAACGAC
<i>Screening, PCR amplification and sequencing</i>		
	attB1	ACAAGTTTGTACAAAAAAGCAGGCT
	attB2	ACCACTTTGTACAAGAAAGCTGGGT
<i>gyrA</i>	Fno_gyrA_F	as above
	FnoGyrA_598R	TATCAATAAGAGCTATAGTACC
	Fno_gyrA_Rev	as above
<i>gyrB</i>	Fno_gyrB_F	see above
	FnoGyrB_839F	ATCCACAAAGAGATGGTGG
	Fno_gyrB_Rev	as above
<i>parE</i>	Fno_ParE_1267Fw	GGAGGTTCTGCTAAACAAGCAC
	Fno_ParE_1516Rv	ACATTGCACATAGTAGTGTGCG
<i>Cloning in pFNLT6, screening and sequencing</i>		
<i>gyrB</i>	GyrB_FnoF_NotI	aattaaGCGGCCGCATGTCTGAGAATAAAGCTTATGACTC
	GyrB_FnoR_AgeI	aattaaACCGGTTTAAACGTCCAAGTTAACGACATTAAGAGC
	GyrB308F	TTATTTTGACGGTGTTCATGCAGG
	GyrB956F	AACAGGTGAAGATACACGCGAAGG
	GyrB1698F	TATAGGTCTAGAAGGCGCGTG
	pFNLT6_3925F	AATATCTAGACTTGCAAGAGCTTGG
	pFNLT6_5403R	AATGCACGCAAATACATACCTGCC

18

19 TABLE S2 Purification conditions for GyrA and GyrB proteins used in this study

20

Proteins	Nucleotide changes	AA changes	Optimized purification conditions		
			cells	induction	Lysis buffer
GyrA_Fno wild-type	-	-	C41(DE3)	ON 18°C	Tris 50mM pH 8.8 - NaCl 500mM - imidazole 10 mM - CHAPS 1%
GyrA_Fno1	g259t	D87Y	BL21 (DE3)	ON 16°C	Tris 50mM pH 8.8 - NaCl 500mM - imidazole 10 mM - CHAPS 1%
GyrA_Fno2	a260g	D87G	C41(DE3)	ON 16°C	Tris 50mM pH 8.8 - NaCl 500mM - imidazole 10 mM - CHAPS 1%
GyrA_Fno3	Δ gagtca 1570-1575	Δ E524, Δ S525	C41(DE3)	ON 16°C	Tris 50mM pH 8.8 - NaCl 500mM - imidazole 10 mM - lysozyme 0.25 mg/ml - DTT
GyrA_Fno4	c128a	P43H	C41(DE3)	ON 16°C	Tris 50mM pH 8.8 - NaCl 500mM - imidazole 10 mM - lysozyme 0.25 mg/ml - DTT
GyrB_Fno wild-type	-	-	BL21 (DE3)	4h 37°C	Tris 50mM pH 8.8 - NaCl 500mM - imidazole 10 mM - CHAPS 1%
GyrB_Fno1	+ctc 1396-1398	+P466	BL21 (DE3)	ON 16°C	Tris 50mM pH 8.8 - NaCl 500mM - imidazole 10 mM - CHAPS 1%
GyrB_Fno2	Δ taa 1460-1462	D487E, Δ 488K	BL21 (DE3)	4h 37°C	Tris 50mM pH 8.8 - NaCl 500mM - imidazole 10 mM - CHAPS 1%

21

22

23

24

25 TABLE S3 Concentrations of ciprofloxacin inhibiting supercoiling activity (IC50) and stabilizing the cleavable-complex formation (CC 25) by
 26 the recombinant wild-type or mutant DNA gyrases from *F. novicida*.

27
 28

Lineage	GyrA mutations	GyrB mutations	IC50 (mg/L)	CC25 (mg/L)
Wild-type	No mutation	No mutation	4.11 ± 0.91	0.9 ± 0.28
Fno1	D87Y	+P466	186.9 ± 40.69	105.9 ± 26.28
-	D87Y	No mutation	152.3 ± 9.2	44.84 ± 1.67
-	No mutation	+P466	105.09 ± 18.86	35.74 ± 14.58
Fno2	D87G	D487E, ΔK488	181.1 ± 43.09	129 ± 33.05
-	D87G	No mutation	83.82 ± 15.2	23.39 ± 2.438
-	No mutation	D487E, ΔK488	9.6 ± 1.07	1.667 ±
Fno3	ΔE524, ΔS52	No mutation	4.98 ± 0.65	1.673 ±
Fno4	P43H	No mutation	18.9 ± 1.07	3.293 ± 0.2945

29

30

31 TABLE S4 GyrA and GyrB mutations identified in ciprofloxacin resistant mutants obtained starting from a same single *F. novicida* colony and
 32 through a one-round selection protocol on enriched MHB agar plates containing 0.075 µg/mL Cipro as described by Jaing *et al* (1). Fifteen
 33 resistant colonies were picked from the plates and propagated in enriched MH broth with 0.05 µg/ml of antibiotic before sequencing of the
 34 QRDR-A and QRDR-B regions using primers described in Table (Fno_gyrA_F / FnoGyrA_598 and FnoGyrB_839F / Fno_gyrB_Rev).

35

Mutant	GyrA		GyrB	
	Nucleotide change	AA change	Nucleotide change	AA change
Fno_OS1	c248t	T83I	No mutation	No mutation
Fno_OS2	No mutation	No mutation	+ctc 1396-1398	+P466
Fno_OS3	No mutation	No mutation	c1394t	S465F
Fno_OS4	No mutation	No mutation	c1394t	S465F
Fno_OS5	No mutation	No mutation	c1394t	S465F
Fno_OS6	c248t	T83I	No mutation	No mutation
Fno_OS7	No mutation	No mutation	c1394a	S465Y
Fno_OS8	g259a	D87N	No mutation	No mutation
Fno_OS9	No mutation	No mutation	a1780t	I594F
Fno_OS10	No mutation	No mutation	g1430a	C477Y
Fno_OS11	g259t	D87Y	a1020g	No mutation
Fno_OS12	No mutation	No mutation	c1394a	S465Y
Fno_OS13	No mutation	No mutation	t1393c	S465P
Fno_OS14	c248t	T83I	No mutation	No mutation
Fno_OS15	No mutation	No mutation	t1393c	S465P
Number of mutants		5		10

36

37

38 1. **Jaing CJ, McLoughlin KS, Thissen JB, Zemla A, Gardner SN, Vergez LM, Bourguet F, Mabery S, Fofanov VY, Koshinsky H, Jackson PJ.** 2016.
 39 Identification of Genome-Wide Mutations in Ciprofloxacin-Resistant *F. tularensis* LVS Using Whole Genome Tiling Arrays and Next Generation
 40 Sequencing. *PLoS One* **11**:e0163458.

41

3.2. Publication 4 : *In vitro* and *in vivo* evaluation of fluoroquinolone resistance associated with DNA gyrase mutations in *Francisella tularensis*, including in tularaemia patients with treatment failure.

(Soumis à *International Journal of Antimicrobial Agents*)

Après avoir démontré que la sélection de mutants résistants de *F. tularensis* aux fluoroquinolones était possible *in vitro* nous avons identifié les principales mutations de résistances aux fluoroquinolones chez cet agent pathogène et démontré qu'elles étaient situées sur les gènes codant pour les sous-unités A et B de l'ADN gyrase de *F. tularensis*. En dehors des mutations classiques en position 83 et 87 dans le QRDR de la sous unité GyrA, nous avons validé le rôle dans la résistance aux fluoroquinolones chez *Francisella*, des mutations P43H de la sous unité GyrA et +P466 et D487E-ΔK488 de la sous unité GyrB. Nous avons voulu rechercher dans une quatrième étude, la présence des mutations identifiées *in vitro* par des techniques de biologie moléculaire, sur des souches mais surtout directement sur des prélèvements de patients atteints de tularémie, en situation d'échec thérapeutique sous fluoroquinolones. En effet, des situations cliniques propices au développement de résistance *in vivo* sont théoriquement possible. La présence d'un fort inoculum bactérien (en cas de diagnostic tardif) ou la diffusion insuffisante de l'antibiotique au site de l'infection peuvent être à l'origine de concentrations sub-inhibitrices en antibiotique au site de l'infection, favorables à la sélection de souches résistantes.

Nous avons ainsi analysé 42 souches et 82 échantillons de patients réceptionnés au CNR des *Francisella* et liés à 104 cas confirmés de tularémie. Parmi ces cas, l'analyse rétrospective des dossiers cliniques des patients et des informations transmises au CNR, nous a permis d'identifier 32 patients (30,7%) en situation d'échec thérapeutique sous fluoroquinolone. Ces situations d'échec étaient définies par :

- l'absence d'amélioration, la détérioration ou la récurrence des symptômes des patients
- une culture bactérienne positive malgré un traitement antibiotique bien conduit d'au moins 7 jours
- la suppuration d'une ou plusieurs adénopathies, nécessitant une résection chirurgicale
- la persistance d'un abcès cutané ou sous-cutané nécessitant une résection chirurgicale
- la présence de complications telles qu'une pleurésie ou une méningite

La recherche de mutations de résistance sur ces échantillons a été réalisée selon plusieurs approches. Nous avons tout d'abord mis au point une PCR en temps réel ciblant les mutations de résistance les plus fréquentes, situés en position 83 et 87 du QRDR de la sous-unité GyrA.

Puis nous avons séquencé la totalité du QRDR des gènes *gyrA* et *gyrB* afin de rechercher les autres mutations préalablement identifiées *in vitro* ou de mettre en évidence des mutations différentes *in vivo*, au sein des QRDR de ces deux gènes.

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

4

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22

23 **Abbreviations**

24 FQs : fluoroquinolones ;

25

26 **ABSTRACT**

27 The fluoroquinolones (FQ) are highly effective for treating tularaemia, a zoonosis caused by
28 *Francisella tularensis*, but failures and relapses remain frequent in patients with treatment
29 delay or immunocompromised status. FQ-resistant strains of *F. tularensis* harboring
30 mutations in the quinolone resistance determining region (QRDR) of *gyrA* and *gyrB*, the
31 genes encoding the subunits A and B of DNA gyrase, have been selected *in vitro*. Such
32 mutants have never been isolated from humans, which could result from the difficulty to
33 culture this microorganism. In this study, we assessed the presence of FQ-resistant mutants of
34 *F. tularensis* in tularaemia patients using combined culture- and PCR-based approaches.
35 We analyzed 42 *F. tularensis* strains and 82 tissue samples collected from 104 tularaemia
36 cases, including 32 (30.7%) with FQ treatment failure or relapse. Forty *F. tularensis* strains
37 and 55 clinical samples were obtained before any FQ treatment, while 2 strains and 15 tissue
38 samples were collected after treatment. FQ resistance was evaluated by the minimum
39 inhibitory concentration (MIC) for the bacterial strains, and by newly developed PCR-based
40 methods targeting the *gyrA* and *gyrB* QRDRs for both the bacterial strains and the clinical
41 samples. As compared to FQ-susceptible controls, none of the *F. tularensis* strains displayed
42 an increased MIC. Neither *gyrA* nor *gyrB* QRDR mutation was found in bacterial strains and
43 tissue samples tested including those from patients with FQ treatment failure or relapse.
44 Further phenotypic and genetic resistance traits should be explored to explain the poor clinical
45 response to FQ treatment in such tularaemia patients.

46

47 **Key words:** *Francisella tularensis*; tularaemia; fluoroquinolones; antibiotic resistance; DNA
48 gyrase; treatment failure

49 **Abbreviations:** FQ, fluoroquinolones; QRDR, quinolone resistance determining region;

50

51 INTRODUCTION

52 *Francisella tularensis* is a Gram-negative, facultative intracellular bacterium responsible for
53 the zoonosis tularaemia [1] and classified as a category A bioterrorism agent by the CDC [2].
54 Human infections are caused by *F. tularensis* subsp. *tularensis* (type A) in North America and
55 subsp. *holarctica* (type B) throughout the northern hemisphere [3]. The clinical symptoms of
56 tularaemia are classically grouped into six clinical forms [3–5]: the ulceroglandular and
57 glandular forms, a regional lymphadenopathy with a skin inoculation lesion only detectable in
58 the former presentation; the oculoglandular and oropharyngeal forms, respectively a
59 conjunctivitis or a pharyngitis with a regional lymphadenopathy; the pneumonic form, a
60 pneumonia occurring after inhalation of contaminated aerosols or haematogenous spread of
61 bacteria from other infection sites; and the typhoidal form, a systemic disease mimicking
62 typhoid. Complications include lymph node suppuration in approximately 30% of patients
63 with lymphadenopathy [3,6,7] and *F. tularensis* bacteraemia with possible secondary
64 infectious sites and severe sepsis [3–5]. Mortality rates vary from less than 1% for type B
65 infections in Europe [7], to up to 30% for type A pneumonia cases reported in the USA [8].
66 The fluoroquinolones (FQ) and the tetracyclines are considered first-line drugs for treatment
67 of mild to moderate severity cases, while the aminoglycosides (streptomycin or gentamicin)
68 are advocated for severe infections [1,4,5]. The FQ are bactericidal against *F. tularensis* in
69 axenic media [9–12], in infected cells [13,14], and in animal models [15–17]. They are highly
70 effective when administered early in the course of disease [18–20]. In contrast, treatment
71 failures and relapses are still observed in 5-30% of patients with treatment delay,
72 complications (e.g., suppurated lymphadenopathy) and/or altered immune status [21,22]. We
73 hypothesized that poor clinical response to FQ treatment might be caused by FQ-resistance
74 acquisition in *F. tularensis*. Bacterial resistance to these antibiotics is mainly related to
75 mutations in genes encoding type II topoisomerases (DNA gyrase and topoisomerase IV) and

76 overexpression of efflux pumps [23]. The DNA gyrase, which is described as the main FQ
77 target in Gram-negative bacteria is encoded by *gyrA* and *gyrB*, while topoisomerase IV is
78 encoded by *parC* and *parE*. FQ resistance may occur through single mutations in a specific
79 region of these genes, referred as the Quinolone-Resistance Determining Region (QRDR). *In*
80 *vitro* *F. tularensis* strains resistant to FQ were thus selected owing to mutations in
81 topoisomerases II encoding genes [24–26], the first mutations occurring in *gyrA* and *gyrB*
82 [24]. No FQ resistance has been evidenced so far in natural strains of *F. tularensis* [9–
83 12,22,27–29], but this fastidious bacterium is rarely isolated from tularaemia patients. In the
84 present study, we assessed the possibility to detect FQ-resistant *F. tularensis* strains in
85 humans using combined culture- and PCR-based approaches.

86

87 MATERIAL AND METHODS

88 1. Patients and clinical specimens

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

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

101 Group A includes 42 cases (9 females, 33 males), mainly occurring as sporadic infections
102 (39/42) between 2004 and 2014, for which a *F. tularensis* strain was isolated before any
103 antibiotic treatment (except 2 patients already under FQ treatment) (Table 1). The clinical
104 forms were ulceroglandular (12 cases), glandular (3), oropharyngeal (7, including 3 family
105 cases), oculoglandular (2), pneumonic (8) and typhoidal (10).

106 Group B includes 70 cases for which 82 clinical samples tested positive for *F. tularensis*
107 DNA using a qPCR-*ISFtu2* assay targeting the insertion sequence *Ftu2* of this species (Table
108 1).[7] The clinical forms were ulceroglandular (22 cases), glandular (24), oropharyngeal (16),
109 oculoglandular (4), pneumonic (3) and typhoidal (1). Group B patients were split into
110 subgroups B1 and B2, respectively for patients who received or not FQ (mostly ciprofloxacin)
111 before clinical sample collection (Table 1). One group B1 patient and seven group B2 patients
112 also belong to group A.

113

114 2. Bacterial strains

115 We used five *Francisella* sp. strains with low virulence in humans (Table 2): *F. tularensis*
116 subsp. *holarctica* Live Vaccine strain (FthLVS, NCTC 10857, provided by the Institut de
117 recherche biomédicale des armées, Grenoble, France); *F. novicida* strain CIP56.12 (Centre de
118 Ressources Biologiques de l'Institut Pasteur, Paris, France); and 3 FQ resistant mutants, either
119 derived from FthLVS (Fth1P14 and Fth2P14) or from *F. novicida* CIP56.12 (Fno1P14).[24]

120 The specificity of the qPCR_{FtgyrA} assay was evaluated using 34 strains outside the
121 *Francisella* genus (Table 3). All cultures were performed in a biosafety level 3 (BSL3)
122 laboratory.

123

124 3. MIC determination

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

133

134 **4. Real-time PCR assay for detection of *F. tularensis gyrA* mutations (qPCR_{FtgyrA})**

135 The qPCR_{FtgyrA} assay was developed according to the MIQE guidelines [33].

136 DNA was extracted from heat-inactivated bacterial suspensions (0.5 McFarland turbidity,
137 95°C for 30 min), using the QIAmp DNA mini kit (Qiagen, Courtaboeuf, France). The
138 clinical samples were first homogenized using the MagNA Lyser instrument (Roche
139 Diagnostics, Meylan, France), and DNA extracted as above.

140 Primers and dual-labelled hybridization probes were designed using the Roche LightCycler
141 Probe Design software (Roche Diagnostics), to allow amplification of a 219-bp portion of the
142 QRDR-A (nucleic acids 235–267, amino acids 79–88) in *F. tularensis* and *F. novicida* (Table
143 4). The qPCR_{FtgyrA} assay was performed using the LightCycler 2.0 apparatus (Roche
144 Diagnostics) and the LightCycler FastStart DNA Master HybProbe reaction mix (Roche
145 Diagnostics). DNA amplification and melting curve analyses were performed using the
146 LightCycler 4.1 software (Roche Diagnostics). Each run included a non-template control
147 (NTC, PCR-grade water), and 4 positive controls (FthLVS strain, and the FQ-resistant clones
148 Fth1P14, Fth2P14 and Fno1P14).

149 The linearity, limit of detection (LOD) and efficiency of the qPCR_{FtgyrA} assay were
150 evaluated by testing ten-fold serial dilutions (2.5 to 2.5 10⁸ genome units per test) of a
151 calibrated FthLVS suspension in PCR-grade water, in the presence or not of human DNA
152 (300–350 ng) extracted from sterile vascular tissue. The experiments were performed in
153 triplicate. The intra-assay and inter-assay coefficients of variability of the melting
154 temperatures (T_m) were determined using results obtained in the different runs for the
155 positive controls (FthLVS strain, and the FQ-resistant clones Fth1P14, Fth2P14 and
156 Fno1P14). Diagnostic sensitivity and specificity of the qPCR_{FtGyrA} assay were evaluated by
157 testing strains belonging to *F. tularensis* or *F. novicida* species or to genera outside this
158 bacterial phylum. A low signal in the qPCR_{FtgyrA} assay being obtained for some clinical
159 specimens, a nested-PCR protocol was also established: a PCR amplification run was first
160 performed using primers used for *gyrA* sequencing (see below); the amplified products were
161 then purified on MNSU 030 plates (Merck Millipore, Billerica, USA), concentrated 2.5 times,
162 and retested with the qPCR_{FtgyrA} assay.

163

164 **5. PCR amplification and sequencing of the QRDR-A and QRDR-B.**

165 Regions encompassing the QRDR-A and the QRDR-B (QRDR of *gyrB*) of human *F.*
166 *tularensis* isolates were amplified and sequenced as previously described [24]. The primers
167 were designed to amplify a 740-bp fragment of *gyrA* or a 773-bp fragment of *gyrB* (Table 4).
168 PCR products were sequenced using the same primers and the Dye terminator method, with
169 the DTCS Quick Start kit (Beckman Coulter, Villepinte, France) and a Ceq 8000 apparatus
170 (Beckman Coulter).

171 For patients with FQ treatment failure or relapse, we evaluated the presence of *F. tularensis*
172 QRDR-A or QRDR-B mutations directly from the clinical samples. To improve the PCR
173 sensitivity, we performed two consecutive amplification runs. For QRDR-A, PCR

174 amplification and sequencing were performed using the same primers as for qPCR*FtgyrA*
175 (Table 4). For QRDR-B, PCR amplification and sequencing were performed using primers
176 designed to amplify a 435 bp fragment of *gyrB* (Table 4). The first PCR reaction was
177 performed using Q5®High Fidelity DNA Polymerase (New England Biolabs, Evry, France),
178 while the second PCR run used TAKARA Premix Ex Taq HS Version (TAKRR030A,
179 Ozyme, St Quentin-en-Yvelines, France). PCR sequencing was performed by Eurofins
180 (Ebersberg, Germany).

181

182 **6. Correlation between treatment failures and relapses, and QRDR-A or QRDR-B** 183 **mutations.**

184 For each group of patients, we evaluated the number of treatment failures and relapses as well
185 as their occurrence with respect to any FQ treatment. Treatment failures and relapses
186 (recurrence of symptoms four weeks or more after apparent clinical cure) were recorded in
187 patients who received an appropriate treatment according to the WHO recommendations,[1]
188 with the occurrence of any of the following conditions: 1/ no improvement, worsening or
189 recurrence of clinical symptoms, 2/ a positive culture after completion of at least seven days
190 of antibiotic treatment; 3/ lymph node suppuration needing surgical resection; 4/ a cutaneous
191 or subcutaneous abscess needing surgical resection; and 5/ other complications (including
192 pleurisy and meningitis). A FQ treatment failure or relapse was recorded when the above
193 conditions occurred while the patient had already received a FQ treatment (ofloxacin,
194 levofloxacin, ciprofloxacin or moxifloxacin).

195

196 **RESULTS**

197 **1. Evaluation of the performance of the qPCR*FtgyrA* assay**

198 We first evaluated the ability of the qPCR F_{tgyrA} assay to amplify the QRDR-A. Such
199 amplification was obtained from all *F. tularensis* subsp. *holartica* and *F. novicida* strains
200 tested, but not from the 34 strains not belonging to *Francisella* spp. (data not shown)
201 indicating that this assay is 100% sensitive and specific. The LOD of the qPCR F_{tgyrA} assay
202 for both the FthLVS and *F. novicida* strains was 250 genome units (GU) per reaction. The
203 quantification cycles (C_q) and bacterial loads (log₁₀ GU per test) showed a linear relationship
204 over 5 log₁₀ units, between 250 GU/test and 2.5×10⁷ GU/test. The efficiency of the
205 qPCR F_{tgyrA} was 95.7% ($y = -3.43 x + 41.1$, $r^2=0.99$). The LOD and efficiency of the
206 qPCR F_{tgyrA} assay were not altered in the presence of human DNA.

207 We then evaluated the ability of the qPCR F_{tgyrA} assay to detect and distinguish the *gyrA*
208 mutations using the control strains FthLVS, Fth1P14, Fth2P14 and Fno1P14. The intra- and
209 inter-assay variabilities of the T_m determined for these strains were 0.25% and 0.44%,
210 respectively. For each QRDR-A genotype, we calculated the mean ± 3 SD of the T_m obtained
211 for 12 replicates (Table 5). No overlap was observed between the T_m intervals of the different
212 wild-type and mutated QRDR-A. The target T_m and their confidence intervals were then used
213 as interpretative criteria of qPCR F_{tgyrA} tests (Table 5). We also determined that the FthLVS
214 and Fth1P14 strains can be differentiated by their T_m in a mixed population at 1:10 ratio or
215 higher. At a ratio lower than 1:10, the minority population can no longer be detected.

216 Surprisingly, concerning the FQ-susceptible strains of *F. tularensis*, a low DNA load
217 (<26,000 GU/test for FthLVS) was associated with a bimodal melting curve with a second
218 melting peak between 61.2 and 63.1 °C. To overcome this problem, we established a more
219 accurate nested PCR protocol allowing reversion of the bimodal melting curves to a single
220 peak with the expected T_m value.

221

222 **2. Phenotypic and genetic traits of the 42 clinical *F. tularensis* subsp. *holarctica***
223 **isolates**

224 As illustrated Table 2, the 42 clinical isolates of *F. tularensis* were susceptible to FQs with
225 MIC90 values of 0.064 mg/L, 0.064 mg/L and 0.125 mg/L for ciprofloxacin, levofloxacin and
226 moxifloxacin, respectively. MIC values were ≤ 2 mg/L for erythromycin compatible with
227 biovar 1 of *F. tularensis* subsp. *holarctica*. The 42 strains displayed a single qPCR F_{tgyrA}
228 melting peak (66.8-67.7°C) in favor of a wild-type QRDR-A. The QRDR-A and QRDR-B of
229 these strains was the same as those of the FThLVS (Figure 1).

230

231 **3. qPCR F_{tgyrA} , and QRDRs testing for clinical samples.**

232 *F. tularensis* QRDR-A was detected by qPCR F_{tgyrA} in 62 (75.6%) of the 82 samples tested,
233 with a single melting peak (T_m , 66.6-67.4°C) for 8 samples compatible with a wild-type
234 QRDR-A nucleotidic sequence. A bimodal melting curve suggesting low *F. tularensis* DNA
235 loads was observed for the remaining 54 specimens. Hence, the mean C_p for the single and
236 bimodal melting curves were 25.7 and 33.2 cycles, respectively ($p=0.001$).

237 To increase the sensitivity of the QRDR-A detection in clinical samples, we retested 74
238 clinical samples using a nested-PCR protocol, including the 20 negative-qPCR F_{tgyrA}
239 samples and the 54 specimens with a bimodal melting curve. Under such conditions a QRDR-
240 A was detected for 64 (86.5%) of these 74 specimens, and a single melting peak (66.8-
241 67.6°C) compatible with a wild-type QRDR-A was obtained for 60 (93.7%), while a bimodal
242 curve was still obtained for the remaining four specimens.

243 Direct PCR amplification and sequencing of the QRDR-A and QRDR-B was obtained for 16
244 clinical samples from 14 group B1 patients, but no mutation was identified.

245

246 **4. Correlation between treatment failures and relapses, and QRDR-A or QRDR-B**
247 **mutations.**

248 Treatment failures or relapses were recorded in 11 (26.2%) of the 42 group A patients.
249 Treatment failures correspond to persistent *F. tularensis* bacteremia after eight days of
250 ofloxacin treatment for one patient, and lymph node suppuration after 2 weeks of
251 ciprofloxacin treatment in another one. All group B1 patients were considered as FQ
252 treatment failures or relapses, since clinical samples were collected following FQ therapy
253 because of lymph node suppuration (14/15) or persistent fever (one case). Among group B2
254 patients, 40/55 (72.7%) experienced treatment failures or relapses with lymph node
255 suppuration (39/40), psoas abscess (one case) or pleurisy (one case). However, only 16 out of
256 40 patients received a FQ treatment (15 lymph node suppurations and one psoas abscess).
257 Obviously, no correlation could be established between QRDR-A or QRDR-B mutations and
258 treatment failures or relapses.

259
260 **DISCUSSION**

261 Although treatment failures and relapses are still frequently observed in tularaemia patients
262 receiving a tetracycline or a FQ [4,5], no acquired resistance to these antibiotics has been
263 characterized so far in natural strains of *F. tularensis* [9–12,28,29]. Only a human isolate with
264 subtle increase in levofloxacin MIC was recently reported [34]. This could be related to
265 difficulties to isolate *F. tularensis* from tularaemia patients, especially after antibiotics have
266 been administrated [4,5]. In France, a *F. tularensis* subsp. *holarctica* strains was isolated in
267 only 42 of the 400 tularaemia cases notified to the Institut de Veille Sanitaire (InVS) during
268 the past decade, i.e. 10.5% [35].

270 Here, we assessed the presence of FQ-resistant mutants of *F. tularensis* in infected patients by
271 testing two hypotheses: 1/ human infection with a FQ-resistant strain of *F. tularensis* from
272 animal or environmental sources; and 2/ *in vivo* selection of FQ-resistant mutants of *F.*
273 *tularensis* during antibiotic therapy. Our strategy focused on the detection of DNA gyrase
274 mutants and more accurately on mutations at the “hot spot” amino acids 83 and 87 of the *F.*
275 *tularensis* QRDR-A. Previous *in vitro* experiments indeed revealed that such mutations that
276 occur at the early stage of FQ exposure were associated with high-level resistance in
277 *Francisella* [24–26, 36]. The presence of such mutants was investigated through both
278 phenotypic and genetic tests performed on our collection of *F. tularensis* strains and clinical
279 samples collected from tularaemia patients before or after FQ therapy. Accordingly, we
280 developed a qPCR_{FtgyrA} assay able to detect and to distinguish the *gyrA83* and *gyrA87*
281 mutations as well as their combination, even starting from clinical samples with low *F.*
282 *tularensis* DNA load.

283

284 To explore the hypothesis of human infection with FQ-resistant strains of *F. tularensis*, we
285 tested 42 clinical isolates collected in 42 tularaemia patients at the early stage of their disease,
286 and before any antibiotic treatment in 40 cases. All the 42 *F. tularensis* strains were
287 susceptible to FQs with MICs in the range of those previously reported [9–12]. Without
288 exception they displayed a wild-type QRDR-A melting curve profile with the qPCR_{FtgyrA}
289 assay in agreement with wild-type QRDR-A and QRDR-B sequences. Because 11 (26.2%)
290 out of the 42 group A patients suffered from treatment failures and relapses during the course
291 of their disease, our results clearly show that the clinical response to antibiotic treatment
292 could not be predicted by antibiotic resistance in the infecting *F. tularensis* strains. The
293 reservoir of *F. tularensis* tularaemia is mainly represented by wild fauna, including hares,

294 small rodents and ticks [3]. This reservoir is usually not in contact with antibiotics, especially
295 synthetic ones such as the FQs.

296

297 The second hypothesis, based on the *in vivo* selection of FQ-resistant mutants of *F. tularensis*,
298 was evaluated by testing 82 clinical samples collected from 70 tularaemia cases for diagnostic
299 confirmation and/or because of the need for surgical removal of suppurated tissues. Here, to
300 check for the presence of resistant strains in patients in the absence of growing bacteria, we
301 optimized a new molecular tool allowing amplification and sequencing of the DNA gyrase
302 regions associated with FQ resistance in *Francisella* strains [24-26]. Using a similar
303 approach, we recently demonstrated the *in vivo* selection of FQ-resistant mutants of
304 *Legionella pneumophila* in legionellosis patients with poor clinical response to these
305 antibiotics [37]. Detection of *F. tularensis* *gyrA83* or *gyrA87* mutations was more likely in
306 clinical samples from group B1 compared to group B2 patients, because only the former
307 received a FQ treatment before sampling. All these samples contained *F. tularensis* subsp.
308 *holarctica* DNA, as evidenced by a positive qPCR targeting the *ISFtu2* and amplification and
309 sequencing of the 16S-23S rRNA intergenic region [7]. FQ treatment failures or relapses were
310 recorded in 15 group B1 patients and 16 group B2 patients. Altogether, the qPCR_{FtgyrA}
311 melting profiles correspond to a wild-type QRDR-A for 72 (87.8%) of the 82 clinical
312 samples, including 32 samples from patients experiencing FQ treatment failure or relapse. We
313 found bimodal melting curves for samples containing small amounts of *F. tularensis* DNA
314 while a single-peak profiles suggestive of wild-type QRDR-A alleles was observed using a
315 nested-PCR protocol. Because the qPCR_{FtgyrA} assay could detect fewer than 10% *gyrA*
316 QRDR mutants in a mixed *F. tularensis* wild type/mutated population, the presence of such
317 mutants in tissue samples at a clinically significant level was unlikely. We further evaluated
318 the presence of QRDR mutations in 16 clinical samples from 14 group B1 patients, but only

319 wild-type genotypes were detected. Altogether, our results show that, in contrast to bacteria
320 exposed to FQs *in vitro*, the *F. tularensis* genomic DNA from tissue samples of treated
321 patients is devoid of QRDR mutations. This strongly suggests that treatment failures and
322 relapses observed in tularaemia patients treated with these antibiotics are not resulting from
323 mutational events within the FQ hot-spot target DNA gyrase. We recently evidenced that the
324 *in vitro* exposure of *F. tularensis* to FQ induce mutations in DNA gyrase residues located
325 outside QRDR [24], but also in other genomic target (unpublished data) that merits further
326 investigations. Also, efflux pumps have been recently described in *F. tularensis* [38], and
327 their potential role in antibiotic resistance should be evaluated. Further experiments are thus
328 needed to explore all these possibilities before claiming that antibiotic resistance does not
329 occur *in vivo* in *F. tularensis*.

330 Interestingly, among our collection of 42 clinical strains of *F. tularensis* obtained during the
331 2006-2016 period, only 3 isolates (two for group A and one for group B1 patients) were
332 obtained after FQ therapy, representing 7.1% of the isolated strains but only 0.75% of the 400
333 tularaemia cases reported to the InVS during the same period. This confirms that the
334 administration of FQs in tularaemia patients is likely to compromise further cultivation of *F.*
335 *tularensis*. This may not result from the presence of FQs in the tested clinical samples since
336 many of them were collected several weeks after withdrawal of the antibiotic therapy. It could
337 also be speculated that FQ exposure induces changes in bacterial cell structure or in metabolic
338 activity leading to persistent or dormant bacteria [39]. A low metabolic activity and dormancy
339 in bacteria have been previously associated with reduced susceptibility to antibiotics. The
340 failure to cultivate resistant strains from human samples was emphasized by Sandoz et al. [40]
341 for *Chlamydia* species, and the authors suggested that it can result from a low recovery rate of
342 bacteria from various infection sites as well as from a slower growth or the potential
343 instability of resistant isolates *in vitro*. In accordance with these hypotheses, culture of *F.*

344 *tularensis* from lymph node tissue collected in patients with chronic suppurated
345 lymphadenopathy remain most often negative, although huge amount of DNA of this
346 pathogen is often detected in these samples by quantitative real-time PCR (unpublished data).

347

348 **CONCLUSIONS**

349 Our aim was to explore the possible detection of *F. tularensis* DNA gyrase mutants in
350 tularaemia patients, especially in case of treatment failures and relapses after FQ treatment.
351 Because a *F. tularensis* isolation is rare and usually obtained before antibiotic treatment
352 administration, our strategy was mainly based upon molecular testing of clinical samples
353 collected in tularaemia patients during the course of their disease. In contrast to what was
354 observed *in vitro* [24] these results show that DNA gyrase mutants could not be detected *in*
355 *vivo*, including in patients with FQ treatment failure. Our study shows that DNA gyrase
356 mutations that are major determinants in FQ resistance in most other bacterial species, are
357 unlikely to explain treatment failures in tularaemia patients. Further hypotheses should be
358 explored, including other genetic mechanisms leading to FQ resistance, but also other *in vivo*
359 alterations of *F. tularensis* phenotypes (reduced metabolism, dormancy) that may be
360 associated with antibiotic resistance.

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372 report; and in the decision to submit the article for publication.

373

374 **TRANSPARENCY DECLARATIONS**

375 None to declare.

376

377 **AUTHORS' CONTRIBUTION**

378 All authors designed the experiments and participated in data acquisition, analysis and
379 interpretation; MM, VS and PR drafted the article, and all authors made an important
380 contribution in revising it and approved its final version; VS founded the bacterial lineages
381 from the LVS strain; VS and GH developed the qPCR_{FtgyrA} assay and tested the clinical
382 strains and human samples; VS and YC performed antibiotic susceptibility testing; VS and
383 YC performed PCR amplification and DNA sequencing experiments.

384



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- 491
- 492

493 **Figure 1.** Nucleotide (*gyrA* and *gyrB*) and amino acid (*GyrA* and *GyrB*) sequences of the
494 QRDR of sub-units A and B of DNA gyrase, respectively. *F. tularensis* subsp. *holarctica*
495 LVS (FthLVS) is taken as a FQs-susceptible reference strain. Fth1P14 and Fth2P14 are FQs-
496 resistant *gyrA* mutants derived from *F. tularensis* LVS strain. Samples correspond to the 42 *F.*
497 *tularensis* isolates and 16 clinical samples from 14 group B1 patients for which the QRDR
498 and QRDRB were determined. Hot spots for FQs resistance are indicated for *GyrA* and for
499 *GyrB* in *E. coli*. FthLVS mutants were previously described by our team [14] but do not
500 correspond to known hot spot positions for FQ resistance.
501

<i>gyrA</i>	FthLVS	GTCCATCGTCGTGTACTTTTTGCAATGAATGAACTATCAAATTATTACAATAGACCATAT
	Patients	GTCCATCGTCGTGTACTTTTTGCAATGAATGAACTATCAAATTATTACAATAGACCATAT
	Fth1P14	GTCCATCGTCGTGTACTTTTTGCAATGAATGAACTATCAAATTATTACAATAGACCATAT
	Fth2P14	GTCCATCGTCGTGTACTTTTTGCAATGAATGAACTATCAAATTATTACAATAGACCATAT
	FthLVS	AAAAAGTCTGCAAGGGTTGTCGGTGATGTAATCGGTAAATATCACCCCTCATGGAGATACA
	Patients	AAAAAGTCTGCAAGGGTTGTCGGTGATGTAATCGGTAAATATCACCCCTCATGGAGATACA
	Fth1P14	AAAAAGTCTGCAAGGGTTGTCGGTGATGTAATCGGTAAATATCACCCCTCATGGAGATATA
	Fth2P14	AAAAAGTCTGCAAGGGTTGTCGGTGATGTAATCGGTAAATATCACCCCTCATGGAGATAAA
	FthLVS	GCTGTTTACGATACTATTGTGTCAGAATGGCACAACCTTTTTTCATTGCGCTAT
	Patients	GCTGTTTACGATACTATTGTGTCAGAATGGCACAACCTTTTTTCATTGCGCTAT
	Fth1P14	GCTGTTTACGATACTATTGTGTCAGAATGGCACAACCTTTTTTCATTGCGCTAT
	Fth2P14	GCTGTTTACTATACTATTGTGTCAGAATGGCACAACCTTTTTTCATTGCGCTAT
<i>GyrA</i>	FthLVS	VHRRVLFAMNELSNYYNRPYKKSARVVGDVIGKYHPHGDIAVYDTIVRMAQPFSLRY
	Patients	VHRRVLFAMNELSNYYNRPYKKSARVVGDVIGKYHPHGDIAVYDTIVRMAQPFSLRY
	Fth1P14	VHRRVLFAMNELSNYYNRPYKKSARVVGDVIGKYHPHGDIAVYDTIVRMAQPFSLRY
	Fth2P14	VHRRVLFAMNELSNYYNRPYKKSARVVGDVIGKYHPHGDIAVYDTIVRMAQPFSLRY
		 GyrA83 GyrA87
<i>gyrB</i>	FthLVS	GAGGGAGACTCTGCAGGAGGTTTCAGCAAAGCAGGCTCGTGATCGTAAAACGCAGGCGATT
	Patients	GAGGGAGACTCTGCAGGAGGTTTCAGCAAAGCAGGCTCGTGATCGTAAAACGCAGGCGATT
	FthLVS	TTGCCACTAAAAGGTAAGATTCTCAATGTTGAGAAAACACGTTTTTGATAAAAATGCTTGGT
	Patients	TTGCCACTAAAAGGTAAGATTCTCAATGTTGAGAAAACACGTTTTTGATAAAAATGCTTGGT
	FthLVS	TCTCAAGAGGTAGCTACGCTCATTAAAGCTCTAGGTTGTGG
	Patients	TCTCAAGAGGTAGCTACGCTCATTAAAGCTCTAGGTTGTGG
<i>GyrB</i>	FthLVS	EGDSAGGSAKQARDRKTQAILPLKGIKLNVEKARFDKMLGSEQEVATLIKALGC
	Patients	EGDSAGGSAKQARDRKTQAILPLKGIKLNVEKARFDKMLGSEQEVATLIKALGC
		 <i>E. coli</i> hot spots FthLVS mutants

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503

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

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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
	Lymph node tissue	6	9	39
Clinical sample for isolation or DNA detection of <i>F. tularensis</i>	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

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509 **Table 2: Fluoroquinolone MICs (mg/L) for *F. tularensis* strains tested in this study.**
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Strains	Ciprofloxacin	Levofloxacin	Moxifloxacin
<i>F. tularensis</i> subsp. <i>holarctica</i> live vaccine strain (FthLVS)	0.016	0.016	0.032
<i>Francisella novicida</i> strain CIP 56.12	0.064	0.064	0.125
Fth1P14 (<i>gyrA83</i> mutation, T83I amino acid change)	32	32	32
Fth2P14 (<i>gyrA83</i> and <i>gyrA87</i> mutations, T83K and D87Y amino acid changes)	32	32	64
Fno1P14 (<i>gyrA87</i> mutation, D87Y amino acid change)	32	32	64
5 LVS-derived lineages with <i>gyrA83</i> mutation (MIC _p)	0.25–1		
5 LVS-derived lineages with <i>gyrA87</i> mutation (MIC _p)	0.125–0.5		
42 clinical strains of <i>F. tularensis</i> subsp. <i>holarctica</i>	0.016–0.064	0.032–0.064	0.125–0.25

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513 **Table 3. Strains outside the *Francisella* genus and used in this study for qPCR*FtgyrA***
514 **specificity testing.**

515

Groups (n)	Species
<i>Staphylococcus</i> (13)	<i>Staphylococcus aureus</i> , <i>S. warneri</i> , <i>S. sciuri</i> , <i>S. epidermidis</i> , <i>S. caprae</i> , <i>S. lugdunensis</i> , <i>S. capitis</i> , <i>S. auricularis</i> , <i>S. schleiferi</i> , <i>S. hominis</i> , <i>S. haemolyticus</i> , <i>S. cohnii</i> and <i>S. intermedius</i>
<i>Streptococcus</i> (3)	<i>Streptococcus pneumoniae</i> , <i>S. pyogenes</i> and <i>S. mitis</i>
<i>Enterobacteriaceae</i> (4)	<i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Proteus mirabilis</i> and <i>Serratia marcescens</i>
<i>Mycobacterium</i> (6)	<i>Mycobacterium tuberculosis</i> , <i>M. avium</i> , <i>M. intracellulare</i> , <i>M. abscessus</i> , <i>M. gordonae</i> and <i>M. xenopi</i>
Others (8)	<i>Enterococcus faecalis</i> , <i>Corynebacterium striatum</i> , <i>Micrococcus luteus</i> , <i>Propionibacterium acnes</i> , <i>Bacillus subtilis</i> , <i>Haemophilus influenza</i> , <i>Pseudomonas aeruginosa</i> , and <i>Bartonella henselae</i>

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523 **Table 4: Primers and probes for DNA sequencing and real-time PCR assay**

Name	DNA sequence (5' to 3')	Target gene, amplicon size	Annealing temperature
Fth/Fno <i>gyrA1</i> -Fw*	aggaaaatctggagctggaa	<i>gyrA</i> , 740 bp	50.5 °C
Fth/Fno <i>gyrA1</i> -Rv*	tgaatggattgctaaagagtgc		
Fth/Fno <i>gyrB3</i> -Fw	agcttctcaggatacgtt	<i>gyrB</i> , 773 bp	49 °C
Fth/Fno <i>gyrB3</i> -Rv			
qPCR F _t <i>gyrA</i> -Fw	cctcgtcgtgtacttttgc	<i>gyrA</i> , 219 bp	60 °C
qPCR F _t <i>gyrA</i> -Rv	ggagaatcaccatcaacagaacc		
qPCR F _t <i>gyrA</i> Anchor probe	gggttgctcggatgtaatcggtaaatc-Fluorescein		
qPCR F _t <i>gyrA</i> Sensor probe	LC640-cctcatggagatacagctgtttacgatacta-P		
Fth <i>gyrB</i> -Fw1232	gccaagaaaagatccggca	<i>gyrB</i> , 435 bp	63°C
Fth <i>gyrB</i> -Rv1596	attctgccaagcatcttcatctt		

524 *: Fw and Rv stand for forward and reverse primers, respectively.

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528 **Table 5: Interpretative criteria used for detection of QRDR-A mutations, as determined**
529 **in 12 independent replicates.**

	FthLVS	Fth1P14	Fth2P14	Fno1P14
Mean Tm (°C)	67.4	62.9	58.5	65.4
Standard deviation	0.3	0.3	0.3	0.3
Lower interpretative Tm*	66.5	62	57.7	64.5
Higher interpretative Tm*	68.4	63.7	59.4	66.2

530 * Lower and higher interpretative Tm correspond to mean Tm \pm 3 SD.

**4. NOUVEAUX COMPOSES DE
SYNTHESE ANTIBACTERIENS
DE STRUCTURE BIS-
INDOLIQUE**

4.1 Publication 5 : Bis-indolic compounds as potential new therapeutic alternatives for tularaemia

La dernière partie de cette thèse a pour but de rechercher de nouveaux composés antibactériens actifs vis-à-vis de *F. tularensis*, non encore commercialisés, afin de pouvoir identifier des molécules potentiellement plus efficaces que les traitements actuels, permettant de réduire les taux d'échecs thérapeutiques observés. La recherche de molécules possédant une structure différente des antibiotiques actuellement disponibles en thérapeutique est privilégiée afin de mettre en évidence une nouvelle classe d'antibiotiques et de ne pas être soumis aux mécanismes de résistances bactériens existants. Dans ce contexte, une collaboration fructueuse avec le Département de Chimie Moléculaire (DCM) de l'Université Grenoble Alpes, nous a permis d'identifier de nouveaux composés de synthèse de structure bis-indolique possédant une activité antibactérienne. Ces composés bromés présentent une structure originale proche de celles d'alkaloïdes isolés d'éponges marines de la classe des spongotines, des topsentines et des hamacanthines. (252) Les composés testés ont été obtenus suite au développement par le DCM de voies de rétrosynthèse des composés naturels précédemment cités, afin d'en obtenir de plus fortes quantités que celles qui sont extractibles à partir des éponges marines. (252–254) Ces développements ont fait l'objet de trois brevets d'invention. (255–257)

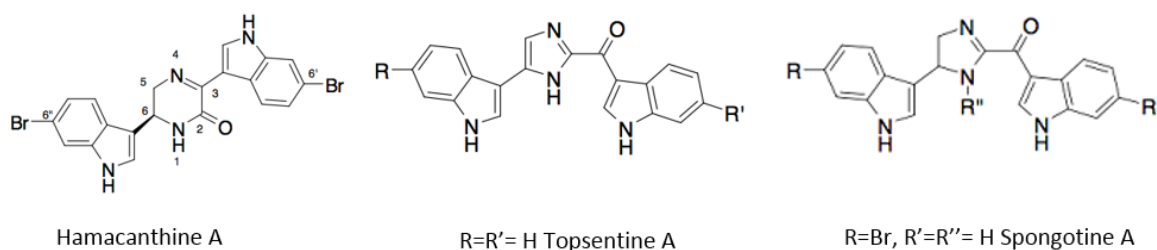


Figure 13 : Structures des Hamacanthines, Topsentines et Spongotines (D'après Caspar, 2013 (252))

Des modifications structurales du noyau bis-indolique de base, relié par une fonction amide ou alpha-céto-amide, a conduit aux 4 composés évalués dans cette étude. L'activité de ces composés a été évaluée par la mesure de leur CMI selon les techniques de référence en microdilution en milieu liquide dans du bouillon de Mueller Hinton ajusté en cation et

supplémenté de 2% de Polyvitex[®] vis-à-vis de 41 souches de *F. tularensis* subsp. *holarctica* de la collection du CNR des *Francisella* ainsi que vis-à-vis d'une souche de référence de *F. tularensis* subsp. *holarctica* LVS, de *F. novicida* et de *F. philomiragia*. La mesure de leur concentration minimale bactéricide et la détermination de leur cinétique de bactéricidie sur 48h ont également été réalisées, en comparaison avec un antibiotique bactéricide, la gentamicine, et un antibiotique bactériostatique, la doxycycline.



Bis-indolic compounds as potential new therapeutic alternatives for tularaemia

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Francisella tularensis is the etiological agent of tularaemia and a CDC class A biological threat agent. Few antibiotic classes are currently useful in treating tularaemia, including the aminoglycosides gentamicin and streptomycin, fluoroquinolones, and tetracyclines. However, treatment failures and relapses remain frequent and *F. tularensis* strains resistant to antibiotics have been easily selected *in vitro*. In this study, we evaluated the activity of new synthetic bis-indole derivatives against this pathogen. Minimum inhibitory concentrations (MICs) of four compounds (dcm01 to dcm04) were determined for the reference strains *F. tularensis* subsp. *holarctica* LVS NCTC10857, *F. tularensis* subsp. *novicida* CIP56.12 and *F. philomiragia* ATCC25015, and for 41 clinical strains of *F. tularensis* subsp. *holarctica* isolated in France. Minimal bactericidal concentrations (MBCs) were determined for the dcm02 and dcm04 compounds for the LVS and two clinical strains. Killing curves were also determined for the same three strains exposed to dcm04. All tested bis-indole compounds were bacteriostatic against *F. tularensis* subsp. *holarctica* strains, with a MIC₉₀ of 8 µg/mL for dcm01, dcm02, and dcm03, and 2 µg/mL for dcm04. Only one strain was resistant to both dcm01 and dcm03, with MICs > 32 µg/mL. In contrast, *F. tularensis* subsp. *novicida* was resistant to all derivatives and *F. philomiragia* was only susceptible to dcm02 and dcm04, with MICs of 16 and 4 µg/mL, respectively. MBC and killing curve experiments revealed significant bactericidal activity (i.e., 3-log reduction of the bacterial inoculum) of the dcm02 and dcm04 compounds only for the LVS strain. In conclusion, we have identified novel synthetic bis-indole compounds that are active against *F. tularensis* subsp. *holarctica*. They may be drug candidates for the development of new therapeutic alternatives for tularaemia treatment. Their further characterization is needed, especially identification of their bacterial targets.

Keywords: tularaemia, *Francisella tularensis*, bis-indolic compounds, antibacterial activity

INTRODUCTION

Francisella tularensis, the agent of the zoonosis tularaemia, may cause severe to fatal human infections. This intracellular, Gram-negative bacterium is highly infectious for humans and many animal species. No human-to-human transmission has been described so far and human infection may occur through direct contact with infected animals, ingestion of contaminated meat or water, arthropod bites, contact with contaminated environments and laboratory exposure to *F. tularensis* cultures (Dennis et al., 2001; Maurin et al., 2011). *F. tularensis* is a class A biological threat agent according to the CDC (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). The highly virulent *F. tularensis* subsp. *tularensis* strains (Jellison type A) are located in North America, whereas *F. tularensis* subsp. *holarctica* strains (Jellison type B) are found throughout the northern hemisphere. In Europe, tularaemia cases are often sporadic (Maurin et al., 2011), but outbreaks have recently been reported in many countries, including in Spain, Norway and Sweden (Pérez-Castrillón et al., 2001; Larssen et al., 2011; Rydén et al., 2012). The first-line therapy of tularaemia is based on a reduced number of antibiotics,

including the aminoglycosides (gentamicin and streptomycin), the tetracyclines (e.g., doxycycline), and the fluoroquinolones (e.g., ciprofloxacin) (Johansson et al., 2002). Treatment duration is usually 7–10 days for gentamicin and ciprofloxacin, and 2–3 weeks for doxycycline. However, high rates of failure and relapse are observed in tularaemia patients, especially when treatment is delayed and/or lymph node suppuration occurs (Rotem et al., 2012).

The aminoglycosides such as gentamicin and streptomycin have a bactericidal activity against *F. tularensis* *in vitro*, and their use in tularaemia patients is associated with almost 100% cure rates (Kaya et al., 2011; Rotem et al., 2012). However, they are nephro- and ototoxic and can only be administered parenterally (Tärnvik and Chu, 2007). Gentamicin is currently used in many countries where streptomycin is no longer available. However, treatment failures with this antibiotic have recently been reported in 11 paediatric patients with oropharyngeal tularaemia in Turkey, with successful recovery after switching to streptomycin (Kaya et al., 2011). Doxycycline can be administered orally, with few side effects. However, the tetracyclines are contraindicated in

children under 8 years of age and in pregnant women because of the risk of permanent staining of the dental enamel and bone toxicity in the foetus (Tärnvik and Chu, 2007; Kaya et al., 2011). Treatment with this bacteriostatic antibiotic is associated with higher relapse rates as compared to aminoglycosides and fluoroquinolones, especially when treatment is delayed and/or of short duration (Dennis et al., 2001; Tärnvik and Chu, 2007). Doxycycline is administered for a minimum of 14 days (Dennis et al., 2001; Tärnvik and Chu, 2007). The fluoroquinolones (especially ciprofloxacin and levofloxacin) are preferred as first-line drugs for treatment of tularaemia cases of mild to moderate severity (Johansson et al., 2002). They are bactericidal against *F. tularensis in vitro*, orally administrable, and have few side effects. They can be administered to young children but not to pregnant women (Johansson et al., 2000; Dennis et al., 2001; Tärnvik and Chu, 2007; Kaya et al., 2011). Ciprofloxacin is recommended as first-line drug in case *F. tularensis* is used as a biological weapon (Dennis et al., 2001; Rotem et al., 2012).

Other antibiotics such as the beta-lactams, the macrolides, cotrimoxazole, chloramphenicol, and rifampicin are not recommended for treatment of tularaemia. Beta-lactams are not effective both because they are inactivated by the class A beta-lactamase produced by *F. tularensis* (Antunes et al., 2012) and they are poorly effective against the intracellular form of this pathogen (Maurin et al., 2000). The macrolides are considered unreliable for treatment of tularaemia because most *F. tularensis* strains have natural high-level resistance to these antibiotics. Only azithromycin may be a possible alternative in pregnant women infected with type B biovar I strains (Dentan et al., 2013). Chloramphenicol and cotrimoxazole are poorly effective *in vitro* and potentially associated with severe side effects (Tärnvik and Chu, 2007). Rifampicin is active against *F. tularensis in vitro*, but its use as a monotherapy is usually associated with rapid selection of resistant mutants.

No natural strains of *F. tularensis* with acquired resistance to gentamicin, fluoroquinolones or doxycycline have been isolated so far. However, *in vitro* experiments have shown that mutants resistant to fluoroquinolones, rifampicin or macrolides can be selected easily (Tärnvik and Chu, 2007; Gestin et al., 2010; Sutura et al., 2014). At present, treatment failures and relapses are considered to be primarily related to delayed administration of appropriate antibiotic therapy rather than *in vivo* selection of antibiotic-resistant mutants (Dennis et al., 2001; Johansson et al., 2002; Kaya et al., 2011; Rotem et al., 2012). However, the bioengineering of genetically modified strains of *F. tularensis* resistant to first-line drugs for use as a biological warfare agent is a major concern. Thus, innovative antibiotics with original structures and bacterial targets, active against this highly virulent pathogen, would be beneficial not only to improve treatment efficacy in tularaemia patients, but also to reinforce our preparedness against the misuse of antibiotic-resistant *F. tularensis* strains.

We recently identified synthetic bis-indole derivatives as new antistaphylococcal compounds with preserved activity against multi-drug resistant strains of *Staphylococcus aureus*, including MRSA strains (Denis et al., 2013a,b). In this study, we evaluated the activity of four of the leading compounds against clinical isolates of *F. tularensis* subsp. *holarctica*.

MATERIALS AND METHODS

BIS-INDOLIC COMPOUNDS AND ANTIBIOTICS

The four bis-indolic compounds evaluated in this study (dcm01, dcm02, dcm03, and dcm04) were synthesized by the DCM (Département de Chimie Moléculaire, Université Joseph Fourier Grenoble-1, Grenoble, France) according to previously published protocols (Denis et al., 2013a,b). The structures of the tested bis-indole compounds are presented on **Figure 1**. We also used gentamicin (Panpharma, Fougères, France) and doxycycline (Sigma-Aldrich, Lyon, France) as controls. Stock solutions of the bis-indolic compounds were prepared at 12.8 g/L in 100% DMSO (Sigma-Aldrich, Lyon, France) and stock solutions of gentamicin and doxycycline were prepared in sterile distilled water. All were kept frozen at -80°C until used.

BACTERIAL STRAINS

All experiments were conducted in a biosafety level 3 laboratory. The use of *F. tularensis* strains was carried out under the approval of ANSM (Agence nationale de sécurité du médicament et des produits de santé). We tested 41 isolates (Ft1–Ft41) of *F. tularensis* subsp. *holarctica* identified to the subspecies level in our laboratory (French reference center for *Francisella*) by amplification and sequencing of the intergenic 16S-23S rRNA region (Maurin et al., 2011). Four isolates were obtained from dead hares and the 37 others from human samples (**Table 1**). The clinical strains corresponded to independent and sporadic tularaemia cases occurring throughout France between 2004 and 2013 (Maurin et al., 2011). We also tested reference strains including *F. tularensis* subsp. *holarctica* LVS NCTC10857, *F. tularensis* subsp. *novicida* CIP56.12 and *F. philomiragia* ATCC25015. *S. aureus* ATCC29213 was tested as a control strain susceptible to the tested bis-indole compounds (Denis et al., 2013a,b). The reference bacterial strains were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) or the Collection of the Pasteur Institute (CIP, Centre de Ressource Biologique de l'Institut Pasteur, Paris, France). All strains are kept frozen in cryotubes (MastDiagnostic, Amiens, France) at -80°C . When needed, they are grown on chocolate agar supplemented

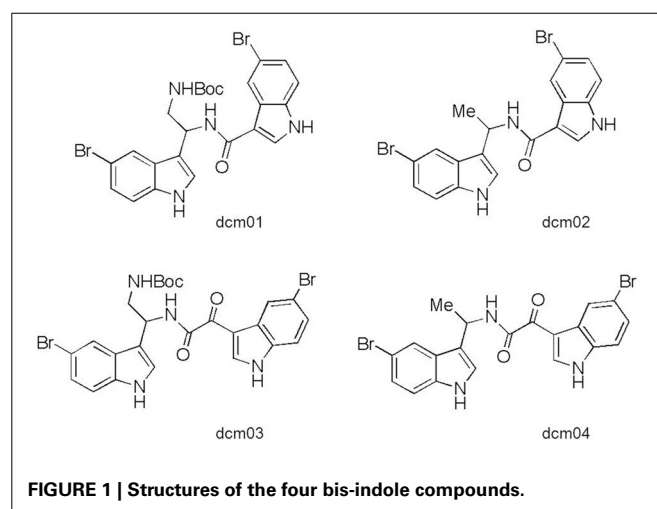


Table 1 | Sources of the 41 isolates of *F. tularensis* subsp. *holarctica* (Ft1 to Ft41) used in this study.

Strain	Host	Year of isolation	Clinical sample
Ft1	Animal	UNK	Hare tissue
Ft2	Animal	UNK	Hare tissue
Ft3	Animal	UNK	Hare tissue
Ft4	Animal	UNK	Hare tissue
Ft5	Human	2004	Blood culture
Ft6	Human	2007	Blood culture
Ft7	Human	2006	Conjunctivitis
Ft8	Human	2007	Cutaneous ulcer
Ft9	Human	2007	Blood culture
Ft10	Human	2008	Mediastinal lymph node
Ft11	Human	2008	Pharynx
Ft12	Human	2008	Pharynx
Ft13	Human	2008	Pharynx
Ft14	Human	2008	Blood culture
Ft15	Human	2008	Cerebrospinal fluid
Ft16	Human	2008	UNK
Ft17	Human	2008	UNK
Ft18	Human	2008	Blood culture
Ft19	Human	2008	Blood culture
Ft20	Human	2008	Skin ulcer
Ft21	Human	2008	Conjunctivitis
Ft22	Human	2009	Whitlow
Ft23	Human	2009	Middle ear
Ft24	Human	2009	Lymph node
Ft25	Human	2010	Blood culture
Ft26	Human	2010	Blood culture
Ft27	Human	2010	Blood culture
Ft28	Human	2010	Lymph node
Ft29	Human	2010	Blood culture
Ft30	Human	2011	Lymph node
Ft31	Human	2011	UNK
Ft32	Human	2012	UNK
Ft33	Human	2011	Blood culture
Ft34	Human	2012	Blood culture
Ft35	Human	2012	Finger abscess
Ft36	Human	2012	UNK
Ft37	Human	2010	Blood culture
Ft38	Human	2012	Blood culture
Ft39	Human	2012	Whitlow
Ft40	Human	2012	Pleural fluid
Ft41	Human	2013	Pleural fluid

UNK, unknown.

with Polyvitex® (CHA-PVX medium, bioMérieux, Marcy l'Etoile, France) at 37°C in a 5% CO₂-enriched atmosphere.

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATIONS

Minimum inhibitory concentrations (MICs) of the four bis-indolic compounds were determined against *S. aureus* ATCC29213, *F. philomiragia* ATCC25015 and all *F. tularensis* strains, using a broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI;

M07-A8 Vol. 29, No. 2). Mueller-Hinton 2 broth supplemented with 2% PolyViteX® (MH2-PVX, bioMérieux, Marcy l'Etoile, France) was used as the antibiotic susceptibility testing medium for *F. tularensis* strains because of their fastidious nature. MH2 alone was used for other species. One row of a 96-well microtiter plate was filled with 75 µL of twofold serial dilutions of the tested bis-indolic compound in MH2-PVX medium, so as to obtain final bis-indolic concentrations ranging from 0.06 to 32 µg/mL in 0.5% DMSO. A bacterial inoculum (75 µL per well, 5×10^5 CFU/mL of final inoculum) was then added to each well. Antibiotic free cultures containing 0.5% DMSO were used as DMSO toxicity controls. MH2-PVX medium with 0.5% DMSO served as a negative control. Microplates were incubated at 37°C in a 5% CO₂ atmosphere. The MICs were read after 18 h culture incubation for *S. aureus* ATCC29213 and 48 h for *F. tularensis* strains. MICs corresponded to the minimum bis-indolic compound concentration that allowed complete inhibition of visual growth of bacteria. Experiments were conducted at least twice to confirm results. Following the same procedure but without DMSO, the MICs of gentamicin and doxycycline were determined against *F. tularensis* subsp. *holarctica* Ft6 and Ft24 strains and the control strain *S. aureus* ATCC29213.

DETERMINATION OF THE MINIMUM BACTERICIDAL CONCENTRATIONS

Minimum bactericidal concentrations (MBCs) were determined in triplicate experiments following CLSI recommendations (CLSI, M26-A, Vol. 19, No. 18), for the two most active compounds (dcm02 and dcm04) against three *F. tularensis* subsp. *holarctica* strains: the LVS strain and the two clinical strains Ft6 and Ft24. We used the same microdilution broth method described for MIC determination, but the primary bacterial inoculum was 10^6 CFU/mL. MBCs of gentamicin and doxycycline were determined in parallel as a bactericidal and a bacteriostatic control, respectively. After 48 h incubation at 37°C in a 5% CO₂ atmosphere, MBCs were determined by plating 50 µL of tenfold serial dilutions of the bacterial suspensions of wells with no visible growth, and of the antibiotic-free control well, onto CHA-PVX medium. CFU counts were determined after 72 h incubation of the plates at 37°C, in a 5% CO₂ atmosphere. The detection limit was 20 CFU/mL. The MBC corresponded to the minimal antibiotic concentration which resulted in at least 99.9% reduction of the primary bacterial inoculum (i.e., 3 log₁₀ reduction of bacterial titers).

TIME-KILL CURVES

Time-kill curves were determined for the LVS, Ft6, and Ft24 strains and the leading dcm04 compound. The primary inoculum calibrated at 10^6 CFU/mL was prepared in MH2-PVX medium and split into five 5-mL aliquots: one drug-free control; three others receiving 4, 8, and 16 times, respectively, the MIC of dcm04 for the tested strain, with 0.5% final concentration of DMSO in all three aliquots; and the last one receiving eight times the MIC of gentamicin for the tested strain, used as a positive control. Sterile MH2-PVX medium with 0.5% DMSO served as a negative control. Cultures were incubated 48 h at 37°C in 5% CO₂. At 0, 6, 12, 24, 36, and 48 h of incubation, a 50-µL aliquot was taken from

Table 2 | MICs ($\mu\text{g/mL}$) of *Francisella* strains for the four bis-indolic compounds: dcm01, dcm02, dcm03, and dcm04.

Bis-indole compound	<i>F. tularensis</i> subsp. <i>holarctica</i>			LVS	subsp. <i>novicida</i>	<i>F. philomiragia</i>
	FT1 to Ft41		MIC		CIP 56.12	ATCC25015
	MIC range	MIC ₅₀			MIC ₉₀	MIC
dcm01	2–>32	4	8	2	>32	>32
dcm02	4–8	4	8	2	32	16
dcm03	2–>32	4	8	2	>32	>32
dcm04	2–4	2	2	1	>32	4

each culture after shaking. Then 50 μL of ten-fold serial dilutions of each aliquot was plated on CHA-PVX medium. CFU counts were determined after 72 h incubation of the plates at 37°C in 5% CO₂. The detection limit was 20 CFU/mL. A 3- \log_{10} or more reduction of the primary bacterial inoculum at any incubation time was considered a significant bactericidal effect. Experiments were conducted at least twice to confirm the results.

STATISTICAL ANALYSIS

A statistically significant decrease of viable bacterial counts in MBC assays was evaluated by one-tailed Student t-test using Statview® software. For each antibiotic concentration tested, we compared the bacterial count obtained after 48 h of incubation of cultures to the primary inoculum [i.e., $\log(N/N_0)$] and to a 3-log reduction cutoff. Significance was defined as a p -value < 0.05.

RESULTS

ALL TESTED SYNTHETIC BIS-INDOLIC COMPOUNDS ARE ACTIVE AGAINST *F. TULARENSIS* SUBSP. *HOLARCTICA* BUT NOT *F. TULARENSIS* SUBSP. *NOVICIDA*

MICs are represented in Tables 2, 3. Almost all *F. tularensis* strains tested were susceptible to the four bis-indole derivatives. In contrast, the Ft5 strain was susceptible to dcm02 and dcm04 (MIC = 8 and 2 $\mu\text{g/mL}$, respectively), but resistant to dcm01 and dcm03 (MICs > 32 $\mu\text{g/mL}$). Dcm04 was the most active bis-indole compound with MICs ranging from 2 to 4 $\mu\text{g/mL}$ and a MIC₉₀ of 2 $\mu\text{g/mL}$. The MIC₉₀ of the three other compounds was 8 mg/L. It should be noted that prolonged incubation of cultures only increased MICs by one dilution for some compounds. In comparison, the MIC of gentamicin against the LVS, Ft6 and Ft24 strains was 0.25 $\mu\text{g/mL}$ and the MIC of doxycycline was 0.125 $\mu\text{g/mL}$ against LVS and 0.25 $\mu\text{g/mL}$ against the Ft6 and Ft24 strains. As for control strains, the *F. tularensis* subsp. *holarctica* LVS strain was susceptible to the four bis-indole derivatives with a MIC of 1–2 $\mu\text{g/mL}$. In contrast, the reference *F. tularensis* subsp. *novicida* strain and the reference *F. philomiragia* strain were more resistant to these compounds. MICs of all bis-indole derivatives were ≥ 32 $\mu\text{g/mL}$ for *F. tularensis* subsp. *novicida*, whereas dcm02 and dcm04 displayed lower MICs (16 and 4 $\mu\text{g/mL}$, respectively) against *F. philomiragia*.

MBC DETERMINATION AND TIME-KILL STUDIES REVEALED A BACTERICIDAL ACTIVITY AGAINST THE LVS STRAIN BUT ONLY BACTERIOSTATIC ACTIVITY AGAINST THE Ft6 AND Ft24 STRAINS

The bactericidal activities of the bis-indole compounds, gentamicin and doxycycline, were determined for the LVS, Ft6, and Ft24

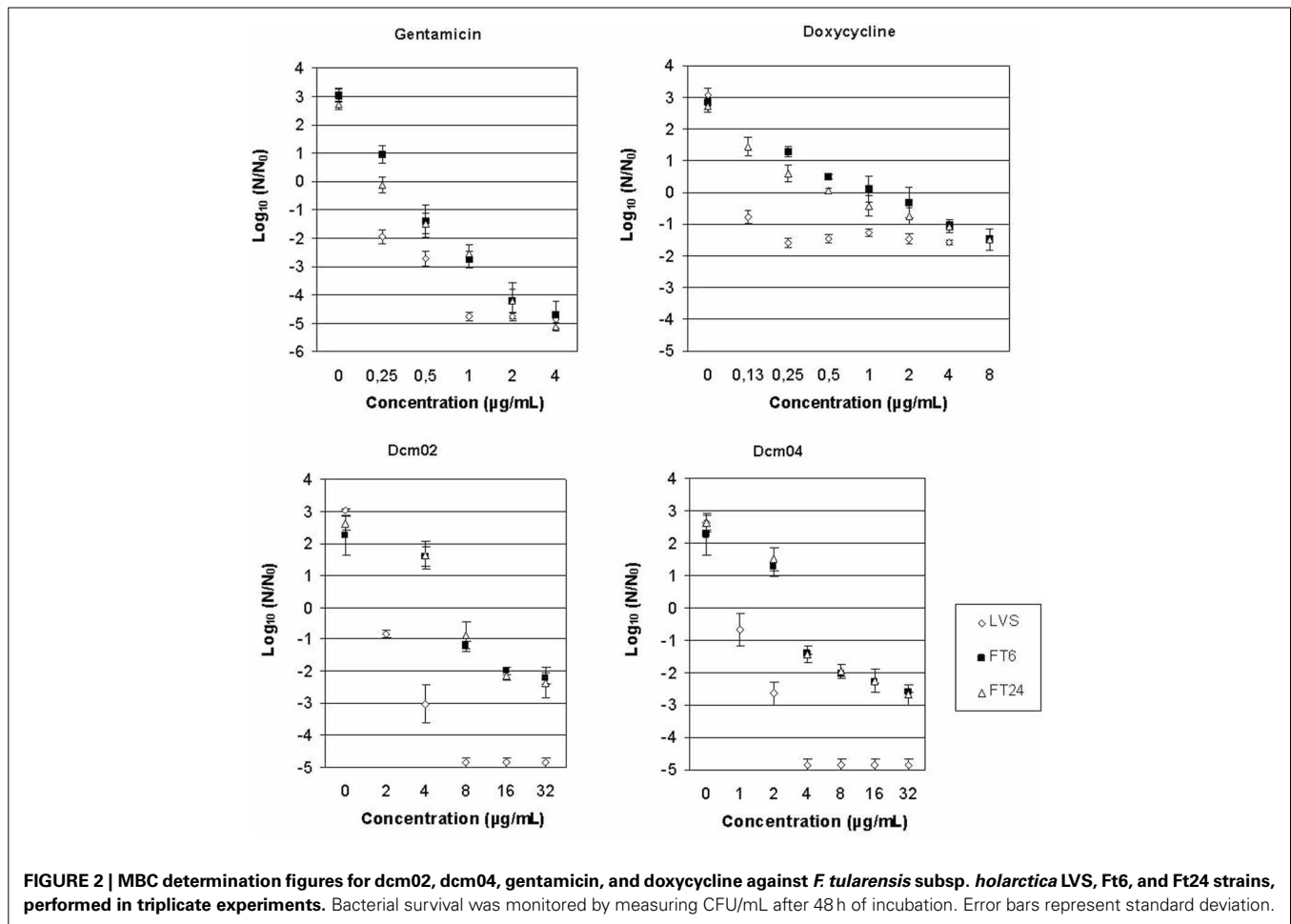
Table 3 | MICs ($\mu\text{g/mL}$) and MBCs ($\mu\text{g/mL}$) of the tree *F. tularensis* subsp. *holarctica* strains used for time-kill studies and MBC determination.

Bacterial strain	MIC (MBC)			
	dcm02	dcm04	Gentamicin	Doxycycline
<i>F. tularensis</i> subsp. <i>holarctica</i> Ft6	4	2	0.25 (2)	0.25
<i>F. tularensis</i> subsp. <i>holarctica</i> Ft24	4	2	0.25 (2)	0.25
<i>F. tularensis</i> subsp. <i>holarctica</i> LVS	2 (4)	1 (4)	0.25 (2)	0.125

strains (Figures 2, 3). As expected, gentamicin displayed bactericidal activity against the three strains (> 3 \log_{10} reduction of the primary bacterial inoculum, $p < 0.01$), with MBCs of 1 $\mu\text{g/mL}$ for the LVS strain and 2 $\mu\text{g/mL}$ for Ft6 and Ft24 strains. As for doxycycline, a significant reduction of the primary bacterial inoculum was observed (i.e., between 1 and 2 \log_{10} at MIC \times 64 for the LVS strain and MIC \times 32 for the Ft6 and Ft24 strains; $p < 0.01$) but the 3-log reduction cutoff was not reached.

MBCs of the dcm02 and dcm04 compounds (4 $\mu\text{g/mL}$ for both compounds) were only two or four times their respective MICs for the LVS strain (Table 3), respectively. In contrast, MBCs could not be determined for the dcm02 and dcm04 compounds against the Ft6 and Ft24 strains. Here again reduction of the primary bacterial inoculum was significant (2-log reduction at concentrations up to MIC \times 8 for dcm02 and MIC \times 16 for dcm04, $p < 0.01$) but did not reach the 3-log cutoff. Higher concentrations of these compounds could not be tested because of their poor solubility.

Time-kill studies revealed a 3- \log_{10} reduction of the primary inoculum of the LVS strain after 12 h incubation for gentamicin (MIC \times 8) and 24 h for dcm04 (MIC \times 4). As for dcm04, the same bactericidal kinetics were observed at 4, 8, and 16 times the MIC of this compound for the LVS strain. As for the Ft6 and Ft24 strains, we observed a progressive decrease of the bacterial load over the first 48 h of contact with dcm04 (Figure 3), but a 3- \log_{10} reduction of the primary bacterial inoculum was never reached. Thus, the dcm02 and dcm04 compounds were only bacteriostatic against the clinical strains of *F. tularensis* subsp. *holarctica*.



DISCUSSION

Among the currently developed therapeutic alternatives for tularaemia, two promising original classes of compounds have recently been identified. First, substituted diphenyl ethers have demonstrated potent inhibition of *ftuFabI* Enoyl-acyl carrier protein reductase (England et al., 2009). This enzyme, absent in human cells, plays a key role in the type II fatty acid biosynthesis and has proved to be a useful target for growth inhibition of various pathogens such as *Mycobacterium tuberculosis*, *S. aureus* and *Plasmodium falciparum* (England et al., 2009; Lu et al., 2009; Hevener et al., 2012; Mehboob et al., 2012; Kingry et al., 2013). The leading compound SBPT04 has an MIC of $0.16 \pm 0.06 \mu\text{g/mL}$ against *F. tularensis* LVS and Schu4 strains and also has a bactericidal activity with a MBC of $0.25 \mu\text{g/mL}$. In a murine model of *F. tularensis* infection, this compound cleared bacteria by day 4 of treatment, without any relapse the following 30 days post-treatment (England et al., 2009). Secondly, screening of a library of more than 1000 2,5,6- and 2,5,7-trisubstituted benzimidazoles identified 21 leading derivatives exhibiting MICs between 0.35 and $48.6 \mu\text{g/mL}$ against the *F. tularensis* LVS strain. Their bacterial target remains uncharacterized, but these compounds may block polymerization of FtsZ, which is a homolog of tubulin/microtubule proteins found in eukaryotes, thus interfering with cell division processes (Kumar et al., 2013).

Here, we report that bis-indole derivatives in which the two indole groups are linked either with an amide (dcm01 and dcm02) or an α -keto-amide (dcm03 and dcm04) central linker exhibit antimicrobial activity against *F. tularensis* subsp. *holarctica*. These compounds were previously characterized as anti-staphylococcal drugs active against methicillin-resistant, vancomycin-intermediate, and fluoroquinolone-resistant *S. aureus* strains (Denis et al., 2013a,b). In this study, the 24 bis-indolic molecules evaluated were inactive against Gram-negative bacteria, including enterobacterial species (*Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Enterobacter cloacae*), *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The MICs of two bis-indolic derivatives were lower ($16 \mu\text{g/mL}$) against *Haemophilus influenzae*. The cytotoxicity of the four bis-indole compounds we tested against *F. tularensis* strains was previously evaluated using three different cell lines: KB (human mouth carcinoma), MCR5 (human lung fibroblast) and HCT116 (human colon tumor) (Denis et al., 2013a,b). The IC₅₀ determined using the HCT116 cell line were 1–5 times higher than the MICs found for *F. tularensis* strains.

We found a significant bacteriostatic activity of these tested bis-indole derivatives against 41 strains of *F. tularensis* subsp. *holarctica* isolated in France. Dcm04 appeared to be the most

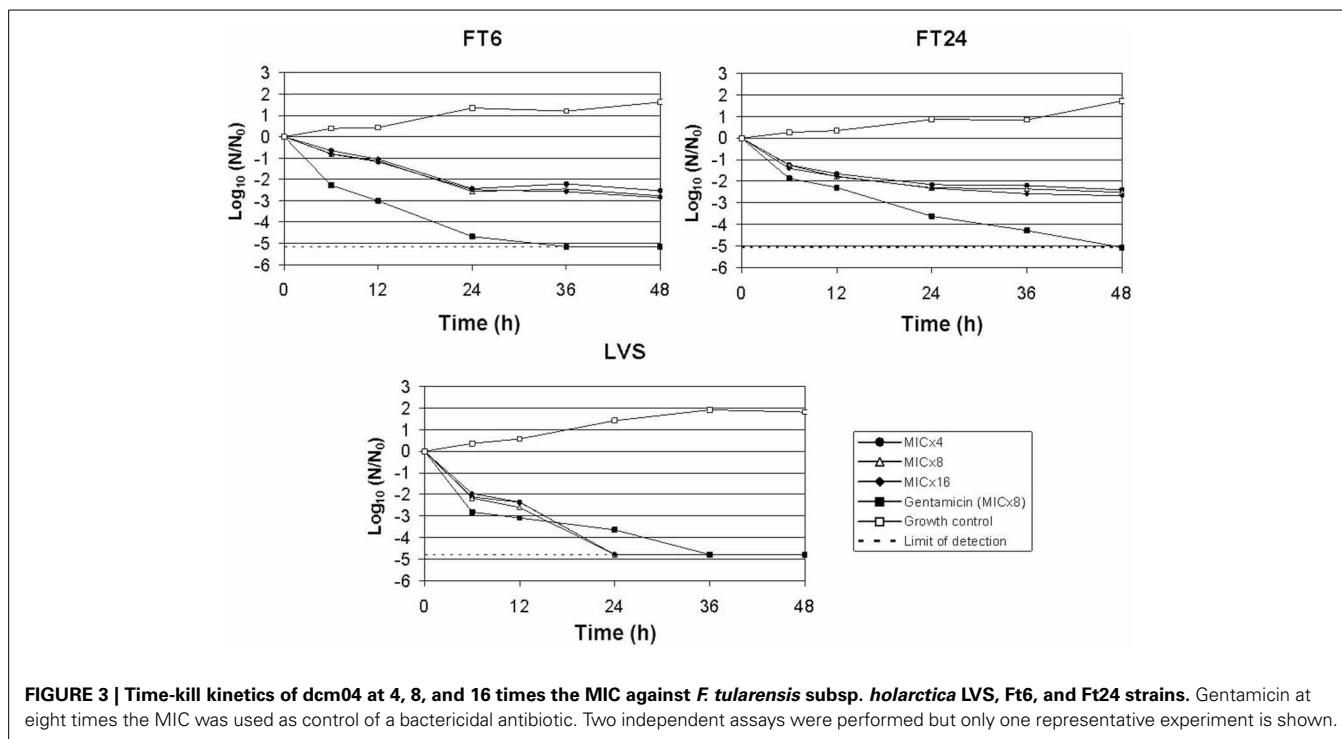


FIGURE 3 | Time-kill kinetics of dcm04 at 4, 8, and 16 times the MIC against *F. tularensis* subsp. *holarctica* LVS, Ft6, and Ft24 strains. Gentamicin at eight times the MIC was used as control of a bactericidal antibiotic. Two independent assays were performed but only one representative experiment is shown.

effective compound with a MIC₉₀ of 2 µg/mL. The MICs ranged from 2 to 16 µg/mL when considering all four bis-indole compounds, except for a single strain that displayed higher MICs for the dcm01 and dcm03 compounds. The variations in antibiotic activities between the four compounds (especially between dcm01 and dcm03 vs. dcm02 and dcm04) may be related to differences in chemical structure, especially the presence of a large CH₂NHBoc chemical group in dcm01 and dcm03, whereas it is replaced by a methyl in dcm02 and dcm04. This large chemical group may limit access of dcm01 and dcm03 to their bacterial target or limit their penetration within bacteria. Surprisingly, the dcm02 and dcm04 compounds displayed a bactericidal activity against the virulence-attenuated LVS strain, but not the Ft6 and Ft24 clinical strains of *F. tularensis* subsp. *holarctica*. This was demonstrated both by MBC determinations and in killing curve experiments. The bactericidal activity of dcm04 against the LVS strain was not concentration-dependent but time-dependent. Hopefully, further structural optimization of these bis-indolic compounds and identification of their bacterial targets may enable us to obtain the same bactericidal activity for clinical strains of *F. tularensis* subsp. *holarctica*.

The activity of the bis-indole compounds also showed *Francisella* species and subspecies specificity, since these compounds were active against *F. tularensis* subsp. *holarctica*, only partially active (dcm02 and dcm04) against *F. philomiragia* and inactive against *F. tularensis* subsp. *novicida*. As a result, *F. tularensis* subsp. *novicida* cannot be used as an experimental model to identify the bacterial targets of these compounds, nor to evaluate the *in vivo* activity of the bis-indoles. Comparison of the complete genomes of *F. tularensis* subsp. *holarctica* LVS, OSU18, and FSC200, and that of *F. tularensis* subsp. *tularensis* Schu

S4, previously identified a relatively limited number of specific genetic alterations (Petrosino et al., 2006; Rohmer et al., 2006) in the attenuated LVS strain. This might help in further investigations to search for an antibacterial target of these compounds in *F. tularensis*, or at least to explain the differences observed in susceptibility to the bis-indoles. Working hypotheses may include an increased affinity of the bis-indoles for their bacterial target in the LVS strain, a reduced penetration of the bis-indole in the clinical strains as compared to the LVS strain, partial inactivation of the bis-indoles in the clinical strains but not in the LVS strain, an escape pathway to the action of the bis-indoles in clinical strains but not in the LVS strain, and a lower efflux of these molecules in the LVS strain. Another hypothesis is that the bis-indole compounds are more active against the LVS strain because it belongs to the type B biovar II strains of *F. tularensis*, whereas the 41 clinical strains belong to biovar I of this sub-species. Biovar II strains naturally resistant to erythromycin are found in Central and Eastern Europe, and Asia (Kudelina and Olsufiev, 1980), whereas only the erythromycin-susceptible biovar I strains are found in France. We did not evaluate the activity of the bis-indoles against type A *F. tularensis* strains. Testing the activity of these compounds against a larger panel of *F. tularensis* strains will be needed to assess potential variability in susceptibility among different sub-species and biovars.

In conclusion, we have identified novel synthetic bis-indole compounds active against *F. tularensis* subsp. *holarctica* but not the closely related bacteria *F. tularensis* subsp. *novicida* and *F. philomiragia*. These compounds may be drug candidates for the development of new therapeutic alternatives for tularaemia treatment. Their bacterial targets remain to be characterized.

AUTHOR CONTRIBUTIONS

Research project design: Yvan Caspar, Max Maurin. Experiments: Yvan Caspar, Vivien Sutura, Sandrine Boisset. Writing: Yvan Caspar, Max Maurin.

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4.2 Publication 6 : Novel synthetic bis-indolic derivatives with antistaphylococcal activity, including against MRSA and VISA strains.

L'évaluation de ces nouveaux composés bis-indoliques de synthèse s'est poursuivie par la détermination de leur spectre d'activité antibactérienne. Ces molécules se sont avérées posséder un spectre assez large vis-à-vis de plusieurs espèces de bactéries à Gram positif appartenant notamment aux genres *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Bacillus* et *Listeria*. En revanche, aucune autre espèce bactérienne à Gram négatif en dehors des *Francisella* n'a présenté de sensibilité à ces dérivés bis-indoliques (*Enterobacteriaceae*, *Pseudomonas*, *Acinetobacter*, *Haemophilus*) (Données non encore publiées). Cela laisse penser que ces composés ont soit du mal à traverser la membrane externe des bactéries à Gram négatif (résistance par imperméabilité), soit plus probablement, sont soumis à des mécanismes d'efflux au sein des bactéries à Gram négatifs. L'absence ou la faible présence de systèmes d'efflux au sein de *F. tularensis*, peut expliquer l'activité de ces composés bis-indoliques sur cette bactérie, par analogie à l'activité modérée observée par exemple avec le linézolide sur *F. tularensis*, alors qu'il s'agit également d'un antibiotique utilisé pour le traitement d'infections liées à des bactéries à Gram positif. L'activité la plus forte de ces composés bis-indoliques a été observée vis-à-vis des staphylocoques, sans aucune diminution d'activité vis-à-vis de souche multi-résistantes de *S. aureus*, que ce soit des souches résistantes à la méticilline ou intermédiaires à la vancomycine, comme le montre ce dernier article. L'étude de ces composés s'est donc poursuivi en premier lieu sur des souches de staphylocoques, de manipulation plus aisée que les *Francisella* car sont des bactéries de croissance plus rapide et surtout ne nécessitant pas d'être manipulées en LNSB3. La détermination des CMI de 24 composés bis-indoliques de synthèse vis-à-vis de 13 souches de *S. aureus* et *S. epidermidis* présentant des mécanismes de résistance variés a été réalisée. Puis l'activité des trois composés les plus actifs a été vérifiée vis-à-vis de 39 souches de staphylocoques à coagulase négative isolées d'hémocultures. L'activité bactéricide de ces composés a été mesurée par la réalisation de courbes de bactéricidie sur 24h. L'évaluation en parallèle de 24 composés a permis de réaliser une étude de relation structure-activité afin de déterminer les groupements chimiques importants pour l'activité antibactérienne de ces composés. Enfin cette étude a permis de d'obtenir de premières données de solubilité, de liaison aux protéines plasmatique, de cytotoxicité et de fréquence d'apparition de mutations de résistance pour les composés les plus actifs, en vue d'étudier ces paramètres indispensables au développement d'un nouvel agent antibactérien.

Novel synthetic bis-indolic derivatives with antistaphylococcal activity, including against MRSA and VISA strains

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Objectives: We report the synthesis, antibacterial activity and toxicity of 24 bis-indolic derivatives obtained during the development of new ways of synthesis of marine bis-indole alkaloids from the spongotone, topsentin and hamacanthin classes.

Methods: Innovative ways of synthesis and further structural optimizations led to bis-indoles presenting either the 1-(1*H*-indol-3'-yl)-1,2-diaminoethane unit or the 1-(1*H*-indol-3-yl)ethanamine unit. MIC determination was performed for reference and clinical strains of *Staphylococcus aureus* and CoNS species. MBC, time–kill kinetics, solubility, hydrophobicity index, plasma protein-binding and cytotoxicity assays were performed for lead compounds. Inhibition of the *S. aureus* NorA efflux pump was also tested for bis-indoles with no antistaphylococcal activity.

Results: Lead compounds were active against both *S. aureus* and CoNS species, with MICs between 1 and 4 mg/L. Importantly, the same MICs were found for MRSA and vancomycin-intermediate *S. aureus* strains. Early concentration-dependent bactericidal activity was observed for lead derivatives. Compounds with no intrinsic antibacterial activity could inhibit the *S. aureus* NorA efflux pump, which is involved in resistance to fluoroquinolones. At 0.5 mg/L, the most effective compound led to an 8-fold reduction of the ciprofloxacin MIC for the SA-1199B *S. aureus* strain, which overexpresses NorA. However, the bis-indole compounds displayed a high hydrophobicity index and high plasma protein binding, which significantly reduced antibacterial activity.

Conclusions: We have synthesized and characterized novel bis-indole derivatives as promising candidates for the development of new antistaphylococcal treatments, with preserved activity against MDR *S. aureus* strains.

Keywords: antibiotics, drug development, drug susceptibility testing, staphylococci, efflux pumps, bis-indoles

Introduction

Since the discovery of penicillin G in the early 1940s, antibiotics have become the cornerstone of treatment of bacterial infections.¹ However, despite the development of numerous antibiotic classes, multidrug resistance to antibiotics now occasionally leads to therapeutic impasses,^{2,3} especially in hospital settings where

bacteria face high antibiotic selection pressure. Among MDR bacteria, *Staphylococcus aureus* is able to resist antibiotics through several mechanisms: (i) the production of enzymes that inactivate antibiotics (e.g. penicillinase and transferases); (ii) the modification of natural targets of antibiotics, as observed for the *mecA* gene that codes for a novel PBP (PBP2a) resistant to the action of almost all β -lactams in MRSA strains; and (iii) the

overproduction of efflux systems such as the multidrug NorA efflux pump involved in resistance to quinolones and fluoroquinolones.^{4–6} Antibiotic treatment of infections caused by *S. aureus* strains with acquired resistance to the first-line antistaphylococcal drugs, especially β -lactams (MRSA strains) and glycopeptides [vancomycin-intermediate *S. aureus* (VISA) strains and vancomycin-resistant *S. aureus* strains], is highly challenging. Moreover, infections caused by MRSA strains or *S. aureus* strains with reduced susceptibility to vancomycin (MIC >1 mg/L) are associated with a poorer outcome.^{7,8} There is an urgent need to develop new drugs active against these MDR *S. aureus* strains.

Over the last six decades, the search for new antibiotic compounds has been supported by the pharmaceutical industry, with continuous development of new drugs derived from known antibiotic classes. Because of similar structural features between old and new compounds, resistance to the latter has rapidly emerged. Therefore, pharmaceutical companies have currently lost interest in this therapeutic field,^{3,9} owing to the poor return on investments. This is particularly true for Gram-negative MDR bacteria, whereas several anti-MRSA agents are still in the pipeline.¹⁰ Measures to overcome bacterial resistance to antibiotics may include three complementary approaches: (i) preserve the antibiotic activity of the more recently developed molecules by optimizing their use in humans and animals; (ii) inhibit known bacterial resistance mechanisms in order to restore the activity of inactivated antibiotics; and (iii) develop novel compounds active against MDR bacteria, ideally displaying original chemical structures, in order to delay the emergence of new resistance mechanisms.² Since the 1970s, only two new antibiotic classes have been developed and approved:^{1,11} the oxazolidinones (e.g. linezolid, Pfizer), which inhibit protein synthesis by binding to the peptidyl transferase centre of the bacterial ribosome;¹² and the lipoglycopeptides (e.g. daptomycin, Cubist Pharmaceuticals), which act through membrane depolarization and disturbance of membrane-associated processes, leading to bacterial cell death.^{13,14} Unfortunately, resistance mechanisms to these two antibiotic classes have already emerged in *Staphylococcus* strains.^{15,16}

Because most currently available antibiotics were first extracted from terrestrial microorganisms during the last two decades, the search for original bioactive molecules has focused on marine bio-organisms. Among them, sponges appear to be one of the richest phyla in toxicogenic species because of their ability

to produce a wide variety of secondary metabolites. In particular, bis-indole alkaloids have shown cytotoxic, antitumoral, antiviral, antiparasitic, antifungal, anti-inflammatory and antibacterial activities.¹⁷ These molecules present unique structures with two indole units linked either by an imidazole or a piperazine core. Indeed, the central linker is an α -carbonylimidazoline for spongotines, an α -carbonylimidazole for topsentins and a 5,6-dihydropyrazinone for hamacanthins A (Figure 1; molecules **1**, **2** and **3**, respectively).^{17–19} The search for innovative methods of chemical synthesis of spongotines, topsentins and hamacanthins highlighted that these molecules include the 1-(1*H*-indol-3'-yl)-1,2-diaminoethane unit **4** (Figure 1).^{20,21} This common key scaffold is not easily accessible via conventional synthetic methodologies. We developed an original access to the first derivatives of this key unit, using a reaction between an α -amino nitron and the appropriate indolic core, paving the way to spongotines, topsentins and hamacanthins A.^{20,21} These innovative protocols led to a large number of original structures, sharing either an indolic or a bis-indolic scaffold.¹⁸

In the meantime, compounds of DCM's chemical library were screened against various biological targets, including bacterial strains, in order to evaluate their potential activities. Owing to the great diversity of compounds of this chemical library, the chances of finding bioactive molecules were increased. Indeed, mono-indolic molecules with intrinsic antibacterial activity against *S. aureus* and other *Staphylococcus* species or inhibiting the *S. aureus* NorA multidrug efflux pump were discovered.^{18,20–23} Due to their original structures and antibacterial activities, these compounds were patented²⁴ and further structural optimizations allowed us to obtain new molecules with a bis-indolic scaffold linked either by an amide or an α -keto-amide group.^{25,26} This study reports the original ways of synthesis, antistaphylococcal activities, physicochemical properties and cytotoxicity of these new families of bis-indolic compounds.

Materials and methods

Synthetic derivatives

Twenty-four bis-indolic molecules were synthesized (Table 1 as well as the Supplementary data, available at JAC Online, for compound **12**). Their preparation (Figures S1–S6) as well as their characterization data are

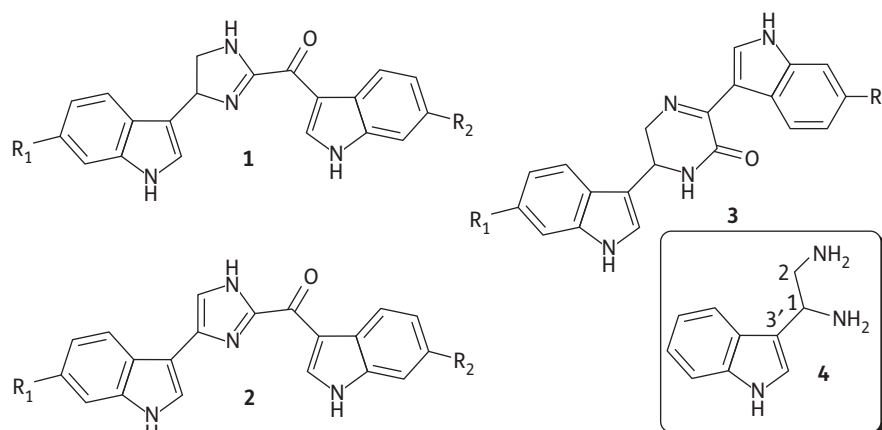
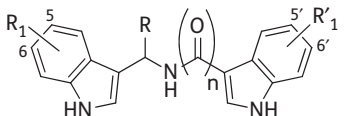


Figure 1. Chemical structures of spongotines **1**, topsentins **2** and hamacanthins A **3** and 1-(1*H*-indol-3'-yl)-1,2-diaminoethane unit **4**.

Table 1. Structures and overall yields of synthesized bis-indolic compounds


Compound	n	R	R ₁	R' ₁	Overall yield (%)
9a	1	CH ₂ NHBoc	H	H	40
9b	1	CH ₂ NHBoc	5-Br	H	73
9c	1	CH ₂ NHBoc	H	5'-Br	72
9d	1	CH ₂ NHBoc	6-Br	H	65
9e	1	CH ₂ NHBoc	H	6'-Br	76
9f	1	CH ₂ NHBoc	5-Br	5'-Br	68
9g	1	CH ₂ NHBoc	5-Br	6'-Br	65
9h	1	CH ₂ NHBoc	6-Br	5'-Br	52
9i	1	CH ₂ NHBoc	6-Br	6'-Br	47
9j	1	CH ₂ NHBoc	5-Cl	5'-Cl	75
9k	1	CH ₂ NHBoc	5-Cl	6'-Cl	62
10a	1	Me	5-Br	5'-Br	76
10b	1	Me	5-Br	5'-F	50
14a	1	CO ₂ Me	5-Br	5'-Br	61
14b	1	CO ₂ H	5-Br	5'-Br	57
14c	1	CH ₂ OH	5-Br	5'-Br	56
15a	1	CONH(CH ₂) ₂ OH	5-Br	5'-Br	46
15b	1	CONH(CH ₂) ₄ OH	5-Br	5'-Br	40
15c	1	CONH(CH ₂) ₄ NH ₂	5-Br	5'-Br	36
17a	2	CH ₂ NHBoc	H	H	97
17b	2	CH ₂ NHBoc	H	6'-Br	90
17c	2	CH ₂ NHBoc	5-Br	5'-Br	62
17d	2	Me	5-Br	5'-Br	63

See the Supplementary data for compound **12** structure.

described in the Supplementary data. Then, stock solutions of these compounds were prepared by diluting powders in pure DMSO (Sigma–Aldrich, Saint-Quentin Fallavier, France) at a concentration of 25.6 g/L. Working solutions of the bis-indolic compounds were prepared in bacterial growth medium so as to obtain final concentrations of DMSO <5%, which was verified to be non-toxic for the bacterial strains tested.

Bacterial strains

Reference bacterial strains, including ATCC strains (ATCC, Manassas, VA, USA) and CIP strains (Centre de Ressources Biologiques de l'Institut Pasteur, Paris, France) were purchased. These included: six MSSA strains (ATCC 25923, ATCC 29213, ATCC 9144, ATCC 6538, CIP 65.6 and CIP 103428); two MRSA strains (CIP 65.25 and ATCC 33592); one VISA strain (ATCC 106414); two methicillin-susceptible *Staphylococcus epidermidis* strains (ATCC 12228 and CIP 81.55); two methicillin-resistant *S. epidermidis* strains (CIP 103627 and ATCC 49461); and one *Staphylococcus sciuri* strain (ATCC 29061). MSSA476 and MRSA252 strains, whose genomes have been entirely sequenced, were kindly provided by the French reference centre for *Staphylococcus* (Lyon, France). We also used 39 clinical strains of *Staphylococcus* species isolated from human blood samples, including 2 *Staphylococcus arlettae*, 10 *Staphylococcus capitis*, 1 *S. epidermidis*, 7 *Staphylococcus haemolyticus*, 8 *Staphylococcus hominis*, 1 *Staphylococcus lugdunensis*, 1 *Staphylococcus massiliensis*, 2 *Staphylococcus pettenkoferi*, 2 *Staphylococcus saprophyticus*, 1 *Staphylococcus simulans* and 4

Staphylococcus warneri. Identification at the species level for these clinical strains was obtained by amplification and sequencing of the *tuf* gene according to previously published protocols.²⁷ The fluoroquinolone-resistant *S. aureus* SA-1199B strain, which overexpresses the chromosomal *norA* gene encoding the NorA efflux pump and also harbours an A116E mutation in *grlA*,²⁸ was generously provided by G. W. Kaatz (University of Michigan). This strain displays a ciprofloxacin MIC of 16 mg/L, whereas its parental counterpart (SA-1199) has an MIC of 0.25 mg/L.

Determination of MICs

MICs of the 24 bis-indolic compounds were determined for all *S. aureus* strains described above except MSSA476 and MRSA252 and for *S. epidermidis* strains ATCC 12228, CIP 81.55 and CIP 103627. A broth microdilution method was used, as recommended by the CLSI (M07-A8) using Mueller–Hinton 2 (MH2) broth (bioMérieux, Marcy-l'Étoile, France) as the experimental medium. For all those bacterial strains, two rows of a 96-well microtitre plate were filled with a bacterial inoculum (180 µL per well, 5 × 10⁵ cfu/mL of final inoculum) and 20 µL of 2-fold serial dilutions of a stock solution of the tested bis-indolic compound in MH2 broth with 10% DMSO, so as to obtain final concentrations ranging from 0.5 to 128 mg/L. For some of the compounds, antibiotic suspensions at 64 and 128 mg/L were cloudy, suggesting that the solubility limit was reached and the antibiotic concentration could have been overestimated. Drug-free cultures served as growth controls. Cultures containing DMSO at final concentrations found in working solutions of the tested bis-indolic compounds were used as DMSO toxicity controls. Microplates were incubated at 37°C in ambient air. MICs were read after 24 h of culture incubation and corresponded to the minimum concentration of the bis-indolic compound that allowed complete inhibition of visual growth of bacteria. The determined MICs of ciprofloxacin and gentamicin were used as positive controls. Experiments were performed at least twice for each bis-indolic compound to confirm results.

MICs of three lead compounds for a large number of *Staphylococcus* species

A second assay was built in order to evaluate the activity of three lead compounds (**9f**, **17c** and **17d**) against a larger number of *Staphylococcus* species. To improve the solubility of the bis-indoles at high concentrations, two rows of a 96-well microtitre plate were filled with a bacterial inoculum (192 µL per well, 5 × 10⁵ cfu/mL of final inoculum) and 2-fold serial dilutions of the tested bis-indolic compound in pure DMSO (8 µL per well) so as to obtain final concentrations ranging from 0.5 to 32 mg/L, with <5% DMSO. Growth controls and DMSO toxicity controls were added as previously described. The antistaphylococcal activity of lead compounds was determined against seven reference strains, including the previously tested *S. aureus* ATCC 106414, *S. aureus* SA-1199B and *S. epidermidis* ATCC 12228 strains, taken as controls, and strains MSSA476, MRSA252, *S. epidermidis* ATCC 49461 and *S. sciuri* ATCC 29061. We also tested 39 clinical strains belonging to different *Staphylococcus* species. Microplates were incubated at 37°C in ambient air and MICs were read after 24 h of incubation. For each experiment, we checked that MICs obtained for the control strains were within 0.5–2× expected MICs. Experiments were conducted at least in duplicate for each strain.

Evaluation of plasma protein binding using MIC protocols

To seek potential plasma protein binding of our lead compounds, **9f**, **14a**, **17c** and **17d**, the MICs were determined (using the second protocol described above) for the *S. aureus* ATCC 29213 strain in the presence of 0%, 1%, 5% or 10% of decomplexed FCS (Gibco®, Life Technologies, Saint Aubin, France). The bis-indole derivatives were tested at concentrations ranging from 0.03 to 32 mg/L. Gentamicin (0.03–16 mg/L) was used

as a positive control. The MIC assays were performed in triplicate to confirm results.

Determination of MBCs

MBCs were determined for the lead compounds **10a**, **14a** and **17d** and gentamicin (control) for *S. aureus* ATCC 29213 according to the CLSI (formerly NCCLS) M26-A microdilution procedure.²⁹ Briefly, a protocol closely related to MIC determination was applied, except that the primary inoculum was prepared from bacterial cultures in the logarithmic phase of growth. After 24 h of incubation of the plates at 37°C in ambient air, the MBCs were determined after stirring by inoculation of 10 µL of the bacterial suspension from each well with no visible growth (concentration \geq MIC) on MH2 agar plates (bioMérieux). cfu counts were determined after 24–48 h of incubation of the plates at 37°C in ambient air and compared with CLSI M26-A tables. The MBC corresponds to the minimum antibiotic concentration that results in \geq 99.9% reduction of the primary bacterial load (i.e. 3 log₁₀ reduction of bacterial titres). MBC experiments were conducted in triplicate to confirm results.

Determination of time–kill curves

Time–kill curves were measured for the lead compounds **9f**, **10a**, **14a**, **17c** and **17d** against *S. aureus* ATCC 29213 and ATCC 25923 according to the CLSI M26-A procedure.²⁹ For each *S. aureus* strain, an overnight culture in MH2 broth was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard. Then, 100 µL of this suspension was added to 10 mL of MH2 broth (pre-warmed to 37°C). This mixture was incubated at 37°C for 4 h before being diluted to 1/65 in 40 mL of pre-warmed MH2 broth, so as to obtain 5×10^5 cfu/mL of a primary inoculum. The primary inoculum was split into seven 5 mL aliquots in glass tubes, one serving as an antibiotic-free growth control (containing 4% DMSO) and the six others receiving, respectively, **9f**, **10a**, **14a**, **17c**, **17d** (in 4% DMSO) or gentamicin (with no DMSO) at eight times their respective MIC for the tested strain. Sterile MH2 broth with 4% DMSO served as a sterility control. Cultures were incubated for 24 h at 37°C. At 0, 2, 4, 8 and 24 h of incubation, a 50 µL aliquot was taken from each culture after shaking. Then, 50 µL of 10-fold serial dilutions in MH2 broth of each aliquot were plated onto MH2 agar plates. cfu counts were determined after 24–48 h of incubation of the plates at 37°C in ambient air. A significant bactericidal effect corresponded to a ≥ 3 log₁₀ reduction of the primary bacterial inoculum at any time of incubation. For compound **17d**, we repeated the experiment at 2 \times , 4 \times and 8 \times MIC for *S. aureus* ATCC 29213 and ATCC 25923 strains to test for concentration-dependent time–kill kinetics. All time–kill kinetics assays were performed in triplicate. The results are expressed as the mean of the three replicates \pm SD. The statistical significance of the reduction of cfu counts compared with the primary inoculum was determined at various exposure times of bacteria to the bis-indoles or gentamicin, using a one-tailed Student's *t*-test and a *P* value < 0.05 .

Because we observed bacterial regrowth at 24 h incubation time, we checked the presence of resistant mutants in the cultures exposed to **17d** at 2 \times , 4 \times and 8 \times MIC. Subcultures (50 µL) were performed on MH2 and the bacterial colonies obtained were tested for **17d** MICs (two replicates per subculture) at the same time as the reference strains.

Determination of single-step resistance frequency

The frequency of spontaneous mutants resistant to one of the three lead compounds (**9f**, **17c** and **17d**) was determined in duplicate experiments. A bacterial suspension (10^9 – 10^{10} cfu/mL) of MSSA476 or MRSA252 was inoculated (100 µL) onto MH2 agar plates containing 4 \times , 8 \times or 16 \times MIC of the bis-indolic compound tested. After 48 h of incubation of the plates at 37°C in ambient air, the colonies were counted. We confirmed that the isolated colonies corresponded to resistant bacteria by determination of their MICs using the broth microdilution method. Resistance was defined

as an MIC that was $\geq 4 \times$ MIC for the parental strain. The frequency of single-step resistance to a given compound was calculated by the ratio of the number of confirmed resistant colonies to the total number of colonies obtained in a drug-free control.

Physicochemical analysis

Measurements of solubility, the chromatographic hydrophobicity index (CHI; which estimates the log D and thus the hydrophobicity of compounds) and plasma protein binding were conducted for compounds **9f**, **14a**, **14c**, **17c** and **17d** at TechMed^{ILL} (ESBS, Illkirch, France) according to previously published protocols.^{30,31} For complete details, refer to www.pcbis.fr and the Supplementary data.

All solubility, CHI and plasma protein-binding assays were performed in duplicate. Results are expressed as the mean \pm SD.

Determination of NorA efflux pump inhibition

Compounds showing no intrinsic antibacterial activity were the only ones to be tested for NorA efflux pump inhibition. This activity was determined for four bis-indolic compounds (**9a**, **12**, **14b** and **17a**) by testing their capacity to restore the activity of ciprofloxacin against the SA-1199B strain. Various concentrations of the bis-indolic compounds were tested in combination with three different subinhibitory concentrations of ciprofloxacin: 4 mg/L (MIC/4), 2 mg/L (MIC/8) or 1 mg/L (MIC/16). For each combination, the minimum concentration of the bis-indolic compound that restored the bacteriostatic activity of ciprofloxacin due to NorA efflux pump inhibition was recorded as the epi-MIC.

Bis-indolic compounds were dissolved in DMSO at 10 mg/mL and then further diluted in MH2 broth. A 96-well microplate was filled with a SA-1199B suspension at 10^6 cfu/mL. Using a Biomek 2000 handling robot (Beckman Coulter, Villepinte, France), the bis-indolic compounds were dispensed so as to obtain final 2-fold serial concentrations ranging from 0.125 to 128 mg/L. For each bis-indolic concentration tested, ciprofloxacin was added at a final concentration of 4, 2 or 1 mg/L, respectively. Bacterial growth was determined both visually and using a microplate reader at a 620 nm absorption wavelength, after 24 h of incubation of the plates at 37°C. Cultures free of bis-indolic compounds but containing 5 µL of DMSO were used as growth controls. Positive controls included sub-inhibitory (4 mg/L) and inhibitory (64 mg/L) concentrations of ciprofloxacin for SA-1199B. All experiments were performed in duplicate.

Cytotoxicity determination

The *in vitro* cytotoxicity of 20 bis-indolic compounds (**9a–k**, **10a**, **12**, **14a–c** and **17a–d**) was assayed on three different cell lines: KB (human mouth carcinoma), MCR5 (human lung fibroblast) and HCT116 (human colon tumour) (Table S1). Cell monolayers were prepared in 96-well tissue culture microplates and grown in 200 µL of complete medium for 24 h. Then, using a Biomek 3000 apparatus (Beckman-Coulter), 2 µL of a stock solution in DMSO of the bis-indolic compound tested was added to the cell culture supernatant to obtain a final concentration of 10^{-5} or 10^{-6} M. Cell cultures free of bis-indolic compound but with the same concentration of DMSO (1% of final volume) served as DMSO toxicity controls. After 72 h of incubation of cell cultures, MTS reagent (Promega, Lyon, France) was added and incubated for 3 h at 37°C. Absorbance at 490 nm of aqueous soluble formazan product was monitored and the results were expressed as the percentage of cellular growth inhibition calculated according to the $[1 - (OD_{490} \text{ treated} / OD_{490} \text{ control})] \times 100$ ratio, determined in triplicate experiments. In a separate experiment, using a similar protocol, the IC₅₀ (i.e. the concentration of compound inhibiting 50% of cellular growth) was determined in duplicate against the HCT116 cell line for compounds **9b**, **9c**, **9f**, **9g**, **9h**, **9i**, **9j**, **10a**, **12**, **15c**, **17c** and **17d**, tested at concentrations ranging from 0.005 to 100 µM (Table 2).

Table 2. MICs (mg/L) of bis-indolic derivatives for 13 *Staphylococcus* reference strains and IC₅₀s for the HCT116 cell line

Gram-positive strains	MIC (mg/L)																IC ₅₀ HCT116 (mg/L, replicate 1)	IC ₅₀ HCT116 (mg/L, replicate 2)									
	9a	9b	9c	9d	9e	9f	9g	9h	9i	9j	9k	10a	10b	12	14a	14b			14c	14d	15a	15b	15c	17a	17b	17c	17d
<i>S. aureus</i> ATCC 25923	>128	8	8	8	16	2	2	1	2	4	4	4	16	>128	4	>32	4	32	32	32	8	>128	4	2	2	0.25	0.25
<i>S. aureus</i> ATCC 29213	>128	8	8	8	8	2	2	1	2	≤2	4	4	16	>128	4	>32	4	32	16	8	>128	2	1	2	0.25	0.25	
<i>S. aureus</i> ATCC 9144	—	8	8	8	8	1	2	1	2	4	4	4	16	>128	4	>32	4	32	32	8	>128	4	1	1	0.13	—	
<i>S. aureus</i> ATCC 6538	—	8	8	8	8	1	2	1	2	4	4	2	16	>128	4	>32	4	32	16	8	>128	4	1	1	0.063	—	
<i>S. aureus</i> CIP 65.6	—	8	8	8	16	1	2	2	1	4	4	2	>32	>128	4	>32	4	>32	32	8	>128	4	1	2	0.13	—	
<i>S. aureus</i> CIP 103428	—	8	8	8	8	1	1	2	1	4	4	2	16	>128	4	>32	4	32	16	8	>128	4	2	1	0.13	—	
<i>S. aureus</i> CIP 65.25 (MRSA)	—	8	8	8	8	2	1	1	1	4	4	2	16	>128	4	>32	4	32	32	8	>128	4	2	1	0.13	—	
<i>S. aureus</i> ATCC 33592 (MRSA)	—	8	8	8	32	2	2	2	1	4	4	2	16	>128	4	>32	4	32	32	8	>128	4	1	1	0.13	—	
<i>S. aureus</i> ATCC 106414 (VISA)	—	8	8	8	8	2	1	1	1	4	4	2	16	>128	4	>32	4	32	32	8	>128	4	1	1	>4	—	
<i>S. aureus</i> SA-1199B (FQR)	—	8	8	8	8	2	2	2	1	4	4	2	—	>128	4	>32	8	—	—	—	—	—	—	2	16	—	
<i>S. aureus</i> ATCC 12228	>128	8	8	16	8	2	1	1	2	4	4	2	16	>128	4	>32	4	32	32	8	>128	4	1	2	0.13	0.0625	
<i>S. epidermidis</i> ATCC 81155	—	8	8	16	8	2	2	1	2	4	4	2	16	>128	4	>32	4	32	>32	8	>128	8	2	2	0.063	—	
<i>S. epidermidis</i> CIP 103627 (MRSE)	—	8	8	16	—	1	2	2	2	4	8	4	16	>128	4	>32	4	32	32	8	>128	>16	2	2	0.063	—	
IC ₅₀ HCT116 (mg/L, replicate 1)	7.5	4.0	5.6	2.7	3.0	3.2	1.9	1.1	2.1	2.9	2.9	4.5	6.0	6.0	29.7	29.7	29.7	29.7	29.7	29.7	29.7	2.7	4.9	2.7	4.9	—	
IC ₅₀ HCT116 (mg/L, replicate 2)	5.6	2.7	2.9	2.7	2.9	3.5	2.4	1.2	1.8	4.5	4.5	7.7	7.7	7.7	24.0	24.0	24.0	24.0	24.0	24.0	24.0	3.7	5.0	3.7	5.0	—	

MRSE, methicillin-resistant *S. epidermidis*; FQR, fluoroquinolone resistant; —, not determined. Controls: ciprofloxacin (CIP) and gentamicin (GEN).

Results

Synthesis of bis-indolic compounds

The structures of the bis-indolic compounds synthesized are original compared with those of natural alkaloids, especially those found in marine sponges. Twenty-four bis-indolic molecules, including 20 with an amide group linker (**9a–k**, **10a**, **10b**, **12**, **14a–c** and **15a–c**) and four with an α -keto-amide linker (**17a–d**), were prepared (Table 1 and Figures S1–S6; see the Supplementary data for complete synthesis details). The synthesis of two of them (**17a** and **17b**) was previously described.²⁰

Lead bis-indolic compounds exhibit potent antistaphylococcal activity, including against MDR *S. aureus* strains

MICs of the 24 bis-indolic compounds were determined for 10 reference *S. aureus* strains including two MRSA strains and one VISA strain and for three reference *S. epidermidis* strains including a methicillin-resistant strain. Four derivatives (**9a**, **12**, **14b** and **17a**) had no proper antibacterial activity against any of the bacterial strains tested. In contrast, the remaining 20 compounds were active against all *Staphylococcus* strains (Table 2). However, MICs ranged from 1 to 32 mg/L for the various compounds, the most active being **9f**, **9g**, **9h**, **9i**, **17c** and **17d**, with MICs ranging from 1 to 2 mg/L. Interestingly, MICs of the different bis-indolic compounds remained unchanged when testing *S. aureus* strains resistant to currently available antibiotics, including β -lactams (MRSA strain), glycopeptides (VISA strain) and fluoroquinolones (SA-1199B strain). For the three lead compounds **9f**, **17c** and **17d**, we further tested one MSSA, one MRSA and 41 CoNS strains belonging to 12 different species (Table 3). All these strains were susceptible to **9f**, **17c** and **17d** with MICs of 1–4 mg/L.

MBCs and time-kill studies revealed early bactericidal activity followed by bacterial regrowth

The bactericidal activity of bis-indoles was evaluated by time-kill kinetic studies for compounds **9f**, **10a**, **14a**, **17c** and **17d** against *S. aureus* ATCC 29213 and ATCC 25923 strains (Figure 2a and b). A significant reduction in cfu counts was found for all compounds against both strains and a 3 log reduction of cfu counts was found for compounds **10a**, **14a** and **17d** after 8 h of culture incubation. We then observed bacterial regrowth after 24 h of culture incubation. In contrast, MBC determination revealed that compounds **10a**, **14a** and **17d** allowed a 99.9% reduction of the initial bacterial load (5×10^5 cfu/mL) after 24 h of incubation for *S. aureus* ATCC 29213 strain at 8, 8–16 and 16–32 mg/L, respectively. For compounds **9f** and **17c**, time-kill kinetics only revealed a 1–2-fold reduction of cfu counts within the first 4–8 h of incubation.

For compound **17d**, time-kill kinetics were also performed at 2 \times , 4 \times and 8 \times MIC for *S. aureus* ATCC 29213 and ATCC 25923 strains in order to distinguish time-dependent from concentration-dependent bactericidal activity. As shown in Figure 2(c and d), **17d** displayed a concentration-dependent bactericidal effect, with again a maximum activity after 8 h of culture incubation.

Table 3. Confirmation of broad-range antistaphylococcal activity by MIC determination of lead compounds **9f**, **17c** and **17d** against two *S. aureus* strains and 41 strains of CoNS species, including 39 strains isolated from human blood cultures

Species (no. of strains)	MIC (mg/L)		
	9f	17c	17d
<i>S. arlettae</i> (2)	4	1–2	2
<i>S. aureus</i> MRSA252	2	1	2
<i>S. aureus</i> MSSA476	2	1	2
<i>S. capitis</i> (10)	4	1–2	1–2
<i>S. epidermidis</i>	4	1	1
<i>S. epidermidis</i> ATCC 49461	4	1	1
<i>S. haemolyticus</i> (7)	4	1–2	2–4
<i>S. hominis</i> (8)	4	1–2	1–4 (2)
<i>S. lugdunensis</i>	4	1	2
<i>S. massiliensis</i>	2	1	1
<i>S. pettenkoferi</i> (2)	2–4	1–2	1–2
<i>S. saprophyticus</i> (2)	2	1	2
<i>S. sciuri</i> ATCC 29061	4	2	4
<i>S. simulans</i>	4	1	2
<i>S. warneri</i> (4)	2–4	1–2	2

When MICs were split between two or more dilutions, the mode value is indicated in bold.

Bacterial regrowth was observed at 24 h incubation time, but MICs determined for selected colonies obtained from these bacterial populations revealed no selection of resistance to **17d**. MICs for subcultured bacteria were the same as those for the parental *S. aureus* strains (i.e. 2 mg/L).

No resistant mutants were selected by the single-step resistance selection study

Spontaneous resistance frequency was determined in duplicate for the three lead compounds (**9f**, **17c** and **17d**) against one MSSA strain and one MRSA strain. Although rare colonies had grown after 24 h of incubation of bacterial suspensions on MH2 agar plates containing 4×, 8× or 16× MIC for the corresponding *S. aureus* strain, none of them corresponded to resistant mutants, as confirmed by MIC determination. Thus, spontaneous single-step resistance frequencies were $<10^{-9}$ for the three compounds tested against both MSSA and MRSA strains.

Evaluation of plasma protein binding and physicochemical analysis of lead compounds revealed high hydrophobicity, poor solubility and high plasma protein binding

To better guide further structural optimization and apprehend protein binding of our synthetic bis-indole compounds, the effect of 1%–10% decomplexed FCS on MIC values was evaluated for lead compounds **9f**, **14a**, **17c** and **17d**. Overall, a 1–16-fold

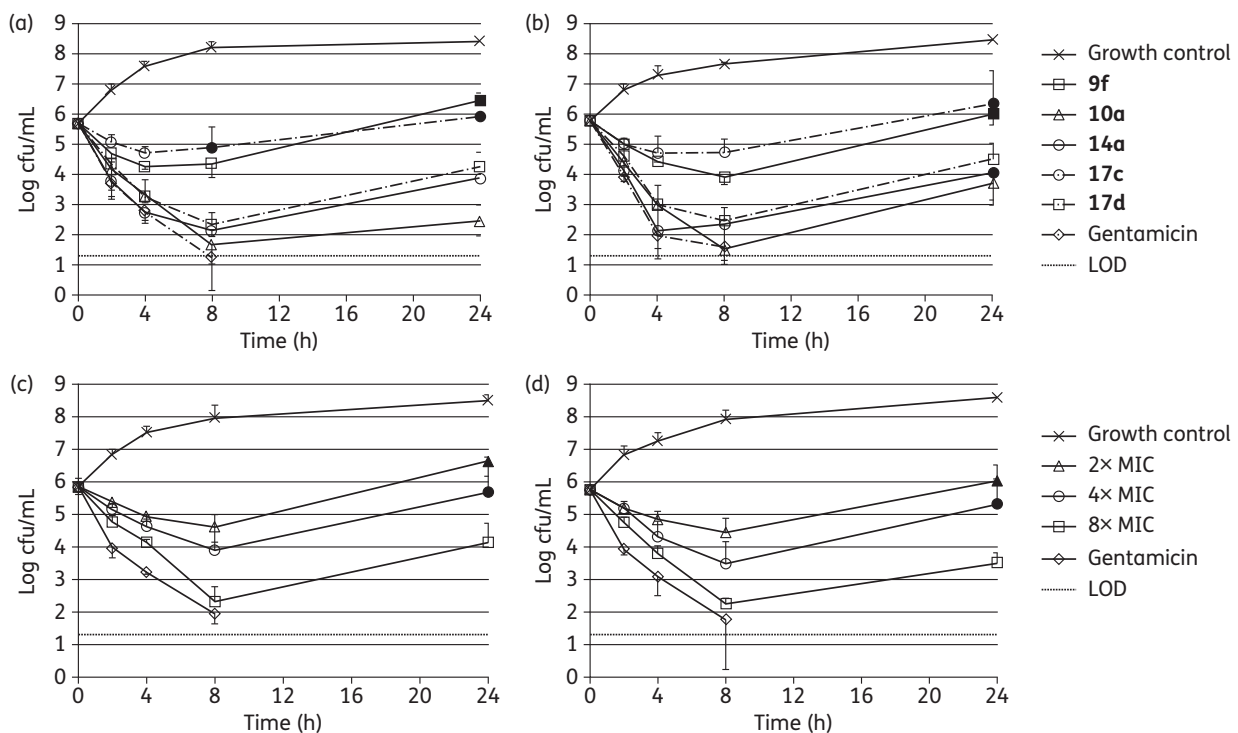


Figure 2. Time-kill kinetics of lead bis-indoles **9f**, **10a**, **14a**, **17c** and **17d** at 8× MIC (in 4% DMSO) for *S. aureus* (a) ATCC 29213 and (b) ATCC 25923 strains. Time-kill kinetics of bis-indole **17d** at 2×, 4× and 8× MIC (in 4% DMSO) for *S. aureus* (c) ATCC 29213 and (d) ATCC 25923 strains. Gentamicin at 8× MIC and a growth control without antibiotic, but with 4% DMSO, were added to all experiments. Results are presented as the mean of a triplicate experiment \pm SD. A significant decrease in viable bacterial counts compared with the primary bacterial inoculum was defined as a *P* value <0.05 and corresponds to open symbols. A non-significant decrease corresponds to filled symbols. LOD, limit of detection.

Table 4. MICs of lead compounds for *S. aureus* ATCC 29213 strain in the presence of 0%–10% decomplexed FCS

Compound	MIC (mg/L)			
	0% FCS	1% FCS	5% FCS	10% FCS
9f	2	4	16	32
17c	1	2	8	16
17d	2	4	16	32
14a	4	8	32	>32
Gentamicin	0.25	0.25	0.125	0.125

Table 5. Solubility, hydrophobicity and plasma protein binding of lead compounds

Compound	Hydrophobicity		Solubility, concentration (μM) \pm SD	Plasma protein binding, % \pm SD
	CHI \pm SD	log D CHI		
9f	93.8 \pm 0.3	3.58	2.3 \pm 0.1	100 \pm 0
14a	87.8 \pm 0.6	3.27	6.0 \pm 0.1	100 \pm 1
14c	74.8 \pm 0.9	2.61	30.3 \pm 0.4	100 \pm 1
17c	102.4 \pm 0.3	4.03	2.6 \pm 1.2	100 \pm 0
17d	99.0 \pm 0.9	3.85	1.2 \pm 0.1	100 \pm 0

increase in MIC values was observed when adding 1%–10% FCS (Table 4), meaning that these lead compounds probably strongly bind to plasma proteins. To confirm these results and further evaluate the physicochemical properties of the lead derivatives, the exact protein-binding percentage was measured as well as solubility at physiological blood pH and estimated log D via CHI determination. As expected, the solubility of the lead compounds ranged from only 1.2 to 30.3 μM at pH 7.4, log D was between 2.61 and 4.03 and the whole fraction of our compounds was bound to plasma protein when tested at 100 μM (Table 5).

Determination of inhibitory activity of the multidrug efflux pump *NorA*

The four bis-indolic derivatives **9a**, **12**, **14b** and **17a**, showing no intrinsic antibacterial activity against *S. aureus* strains ATCC 25923 and SA-1199B (i.e. MIC >32 mg/L), potentiated ciprofloxacin activity against *S. aureus* SA-1199B. The ciprofloxacin MIC for SA-1199B was reduced 4-fold (from 16 to 4 mg/L) when this antibiotic was tested in combination with 32 mg/L of compound **14b**, but only 0.25 mg/L of compounds **9a** or **12** (Table 6). Compound **17a**, a bis-indolic derivative possessing an α -keto-amide linker, decreased the ciprofloxacin MIC to 4 and 2 mg/L for SA-1199B when tested at 0.5 and 2 mg/L, respectively. The non-brominated derivatives **9a** and **17a** were therefore as efficient as the methylated indole compound **12**. The latter derivative had no proper antibacterial activity against SA-1199B, whereas its non-methylated analogue **9f** displayed a 2 mg/L MIC for this strain. Thanks to the presence of the carboxylic function, compound

Table 6. Inhibitory activity of bis-indole compounds against the *S. aureus* *NorA* efflux pump

Ciprofloxacin (mg/L)	epi-MIC ^a (mg/L) of the bis-indole compounds			
	9a	12	14b	17a
1	2	4	128	ND
2	0.5	1	64	2
4	0.25	0.25	32	0.5

ND, not determined.

^aMIC of the bis-indole compounds tested that could restore the bacteriostatic activity of ciprofloxacin used at 1, 2 or 4 mg/L against SA-1199B (MIC of ciprofloxacin = 16 mg/L).

14b was completely soluble whilst inactive at 128 mg/L when used alone. However, higher concentrations of compound **14b** were necessary to significantly decrease the ciprofloxacin MIC. In the presence of 1 mg/L of ciprofloxacin, as high as 128 mg/L compound **14b** was necessary to inhibit bacterial growth.

Structure–activity relationship (SAR) study

We tried to determine the pharmacophore involved in the antistaphylococcal activity of the bis-indolic compounds (see the Supplementary data for detailed SAR analysis).

In summary, the most effective compounds contained in their structure two indole cores with a free indolic nitrogen, with either an amide or an α -keto-amide linker, each indolic core carrying one bromine atom at position 5 or 6. However, higher antibacterial activity correlated with increased cytotoxicity for many compounds.

Discussion

The search for efficient therapeutic alternatives against MDR bacteria, and more specifically MRSA strains, is a scientific and medical priority.^{2,8,32} Various marine invertebrates including bryozoans, coelenterates, tunicates and sponges synthesize indole-containing alkaloids.^{18,19} Among them, bis-indole alkaloids from the spongione, topsentin and hamacanthin classes were isolated from many sponge species, including *Topsentia* sp., *Hamacantha* sp., *Spongosorites* sp. and *Rhaphisia lacazei*.^{18,19,33–37} Previous studies have shown that these compounds are active against various bacterial species, including *Bacillus subtilis*, *Micrococcus luteus*, MSSA, MRSA, *Proteus vulgaris* and *Salmonella enterica* Typhimurium, but not *Escherichia coli*.^{19,38} The most active compounds were deoxytopsentin, bromotopsentin, hamacanthin A, *trans*-4,5-dihydrohamacanthin A, hamacanthin B and 6''-debrohamacanthin A. They exhibited MICs of 4–32 mg/L for MSSA and MRSA, 1–32 mg/L for *P. vulgaris* and 4–64 mg/L for *S. enterica* Typhimurium.^{19,38}

During the synthesis of some of those marine bis-indole alkaloids, we obtained some mono-indolic compounds displaying good antibacterial activities against *Staphylococcus* species, with MICs of 8–32 mg/L for the most active compounds. This

activity was maintained against MDR MRSA and VISA strains.²² SAR studies highlighted the importance of the presence of a halogen atom at position C-5 or C-6 of the indole core and of the ethanamine side chain (-CH₂NH₂ or -CH₂NHBoc) for the antibacterial activity.²² Using a disconnection approach, further synthetic developments of bis-indole alkaloids from 1-(1*H*-indol-3-yl) ethanamine derivatives led to a set of bis-indolic compounds with either a central amide or α -keto-amide linker.^{20–22} In the present study, we have demonstrated that these compounds display potent activities against *S. aureus* and CoNS species with the lead compounds having MICs of 1–4 mg/L. Moreover, the antistaphylococcal activity of the bis-indolic compounds was not altered by common *Staphylococcus* sp. resistance mechanisms, including resistance to methicillin (through the *mecA* gene encoding PBP2a), glycopeptides (VISA strains) or fluoroquinolones (through NorA overexpression). The bis-indoles displayed a bactericidal effect against *S. aureus* strains after 8 h of exposure, although it was more pronounced for compounds **10a**, **14a** and **17d**. However, after 8 h bacterial regrowth was observed. According to the CLSI M26-A guidelines, regrowth can be due to: (i) selection of resistant mutants; (ii) drug inactivation over time in incubated cultures; and (iii) bacterial adhesion to the glass tubes used in time–kill assays leading to artefactual growth of susceptible bacteria.²⁹ The first hypothesis was ruled out by demonstrating that **17d** MICs were unchanged for surviving bacteria. Inactivation of the bis-indoles after 24 h of incubation at 37°C also seems unlikely since a $>3 \log_{10}$ reduction in cfu counts was observed in MBC experiments after the same incubation time. Therefore, the bacterial regrowth observed for time–kill experiments could be artefactual. Especially, the poor correlation between MBC and time–kill experiments at 24 h incubation time may be related to different experimental conditions, including very different reaction volumes and bacterial growth containers (polystyrene microplates versus glass tubes, respectively).

A preliminary SAR analysis showed that the concomitant presence of a bromine atom at the C-5, C-5', C-6 or C-6' position of the indole cores and of the CH₂NHBoc side chain in the linker made it possible to obtain antibacterial activities similar to those of the mono-indolic derivatives previously described. Replacement of the CH₂NHBoc chain with a carboxylic acid, an ester, a methyl alcohol or an alkyl amide group decreased or abolished antibacterial activities. Compared with mono-indolic derivatives, the bis-indole compounds showed a substantial increase in antibacterial activities when one bromine atom was added to each of the two indole cores. Improved antistaphylococcal activity was obtained with two atoms of bromine, whereas the introduction of atoms of fluorine or chlorine in place of bromine atom(s) led to MICs being increased 2–8-fold. Finally, we demonstrated the importance of the free nitrogen atom of the second indole core. Indeed, the N-methylated derivative was completely inactive. This could result from the loss of opportunities for hydrogen bonds between this amine function in the N-methylated derivative and its bacterial target, likely necessary to inhibit bacterial growth. It was also surprising to note that the replacement of the CH₂NHBoc side chain by a methyl group did not alter the antibacterial activity of the synthetic di-brominated bis-indole compounds. Further structural optimizations are still required, however, to improve their solubility at physiological pH, to obtain a higher fraction of unbound compound and to reduce their cytotoxicity while enhancing their antibacterial activity.

The mechanism(s) of action and bacterial target(s) of our bis-indole derivatives have not yet been elucidated, but a number of hypotheses can be highlighted. In the literature, a few molecules with a bis-indole scaffold (other than natural marine bis-indole alkaloids) are mentioned as antibacterial agents, including bis-(imidazolylindole) derivatives with broad-spectrum antibacterial activity against Gram-positive (i.e. *Staphylococcus*, *Enterococcus*, *Streptococcus* and *Clostridium* species) and Gram-negative (i.e. Enterobacteriaceae, *Acinetobacter*, *Pseudomonas*, *Burkholderia* and *Haemophilus* species) bacteria^{39,40} and bis-indoles active against MDR bacteria such as MRSA and vancomycin-resistant *Enterococcus* species,^{39,40} *Mycobacterium tuberculosis*⁴¹ and *Acinetobacter baumannii*.⁴² Sharing some structural features with molecules that bind to the minor groove of duplex DNA, these compounds may act through DNA synthesis inhibition with higher affinity for bacterial DNA than for mammalian DNA.³⁹ However, their rapid bactericidal effect, broad-spectrum activity and planar structure able to bind DNA probably indicate a different mechanism of action as compared with our bis-indole compounds. In another study, aryl-3,3'-bis(indolyl)methanes displaying weak antibacterial activity have been described, with no mention of their potential mechanism of action.⁴³ Very recently, antibacterial activity against *E. coli* and *B. subtilis* of synthetic bis-indole molecules with -CO-NH-NH-CO- and -CO-CO-NH-NH-CO- linkers was identified. They inhibit the interaction between RNA polymerase and σ factors, which is essential to initiate bacterial transcription properly and efficiently.⁴⁴

As for the mode of action of the bis-indole compounds isolated from marine sponges, the first hypothesis focused on inhibition of virulence-associated protein sortase A (SrtA), a membrane-associated transpeptidase modulating the adhesion ability of *S. aureus* to host tissue.⁴⁵ However, in 2011, Zoraghi et al.³⁸ demonstrated that SrtA is not essential for the growth and viability of *S. aureus* and that specific SrtA inhibitors did not reduce bacterial growth. They also identified spongatine A, *cis*-3,4-dihydrohamacanthin B and bromodeoxytopsentin isolated from *Topsentia pachastrelloides* as potent inhibitors of MRSA pyruvate kinase (PK), an essential hub in the MRSA interactome and thus an advantageous target for development of new therapeutics.^{38,46} Measured IC₅₀ values were under the nanomolar level for MRSA PK and compounds displayed ≥ 166 –600-fold selectivity towards bacterial versus human PK isoforms, owing to structural differences in protein sequences and structures.^{38,43,46,47} Cocrystallization of MRSA PK with inhibitor molecules located the binding pocket in a small interface of the PK tetramer. Through salt bridges and hydrophobic interactions, bis-indole compounds may block PK in a rigid conformation, preventing the enzyme from passing from its inactive to active state.^{38,46,47} Evidence that our synthetic bis-indole derivatives may share the same antibacterial mechanism as the bis-indole alkaloids of sponges (i.e. inhibit MRSA PK) is also highlighted by the antistaphylococcal spectra and the structural similarities observed between compounds presented in this study and the previously identified MRSA PK inhibitors.^{46,48,49} On the other hand, various observations require clarification: e.g. Zoraghi et al.³⁸ reported that bromotopsentin did not inhibit MRSA PK, although its activity against MRSA strains was described by Oh et al.¹⁹ as similar to or 2-fold higher than that of bromodeoxytopsentin, a compound described as a potent MRSA PK inhibitor.³⁸ Moreover, in 2012, Kumar et al.⁴⁹

synthesized chemical compounds with high antistaphylococcal activity despite weak inhibition properties against MRSA PK, possibly because of their particular Z-configuration, and concluded that there was a poor correlation between anti-MRSA and PK inhibitory activities. This was also highlighted in a more recent paper from Kumar *et al.*,⁴⁸ who developed compounds with low IC₅₀ against MRSA PK but high MICs. The authors suggested that the poor correlation between MRSA PK inhibitory activity and MIC values for *S. aureus* may be due to differences in solubility, cellular penetration or efflux of their bis-indole compounds.⁴⁸ The authors also demonstrated that the bactericidal activity of their lead compounds was dependent on the presence of the *pyk* gene in the tested bacterial strain but did not require PK metabolic activity. A bacteriostatic activity was preserved against an *S. aureus* strain in which the *pyk* gene had been deleted.

We recently demonstrated that some of our lead bis-indole compounds are also active against *Francisella tularensis* subsp. *holarctica*,⁵⁰ a highly virulent, facultative intracellular, Gram-negative bacillus. Therefore, the antibacterial spectrum of these compounds may not be limited to *Staphylococcus* species. The mode of action of the bis-indole compounds, either natural or synthetic, still has to be fully characterized. It can be hypothesized that these compounds block MRSA PK in an inactive state, but also alter non-enzymatic functions of this hub protein. Alternatively, there may be another bacterial target explaining *pyk*-independent bacteriostatic activity. It should be emphasized that SAR studies should be conducted on the basis of MIC values and not on the basis of the IC₅₀ for MRSA PK.

Bacterial resistance to fluoroquinolones is mainly related to mutations in the genes coding for DNA gyrase and topoisomerase IV, the natural targets of these antibiotics, and/or to overexpression of multidrug efflux pumps.^{28,51,52} In the latter case, efflux pump inhibitors (EPIs) can partially restore the activity of the fluoroquinolones. Moreover, EPIs can reduce the emergence of fluoroquinolone-resistant mutants.⁵¹ Characterization of new EPIs is mainly based on high-throughput screening of existing chemical libraries. Some of our synthetic bis-indole compounds displayed no proper antibacterial activity, but high inhibitory properties, against the multidrug efflux pump NorA, a member of the major facilitator superfamily whose overexpression plays a major role in fluoroquinolone resistance in *S. aureus*. To our knowledge, the structure of the NorA efflux pump has not been resolved, but this protein is considered to present a large hydrophobic binding site.²⁸ Previous indole-containing NorA EPIs have been described, including reserpine and 5-nitro-2-phenyl-1*H*-indole (INF55). The latter has recently been combined with berberine in a single molecule resulting in a >382-fold increase in berberine antibacterial activity against the NorA-overexpressing *S. aureus* strain SA-K2378.⁵³ We previously demonstrated that mono-indolic aldonitrone carrying a halogen atom at the C-5 position and a CH₂NHBoc alkyl chain were potent NorA EPIs, with an activity 4–5-fold higher than that of the reference compound reserpine.²³ In this study, we observed a 4-fold reduction of the ciprofloxacin MIC when adding one of the most active bis-indole compounds **9a** or **12** at 0.5 and 1 mg/L, respectively. The restoration of the activity of ciprofloxacin was proportional to the concentration of the bis-indole derivative tested, confirming a synergistic effect between the two compounds. The combination of a fluoroquinolone and a bis-indole NorA-EPI may represent an advantageous therapeutic alternative for infections caused

by NorA-overexpressing *S. aureus* strains. This combination may also better prevent the clinical emergence of high-level fluoroquinolone-resistant strains. The bis-indole derivatives with proper antibacterial activity could not be tested for potential NorA EPI activity, but these compounds may cumulate both properties. The present results correlate with those of Kumar *et al.*,⁴⁸ which showed that the MIC of some of their bis-indole compounds was reduced 4–32-fold for *S. aureus* when adding verapamil (an inhibitor of the ABC class of efflux pumps) at 200 mg/L to the culture medium. This suggests that the bis-indole compounds and verapamil are substrates of the same efflux pumps. The activation of such efflux pumps may explain why the bis-indole compounds are only transiently bactericidal against *S. aureus in vitro*.

In contrast to mono-indole EPIs, the SAR studies for bis-indole NorA EPIs did not show better antibacterial activity when a halogen was added at any position on the indole core, as observed for EPI compounds **9a** and **12**. Moreover, the acidic function of EPI **14b** correlated with a strong loss of NorA EPI potency. This was probably related to the introduction of a negative charge via deprotonation of the acidic function, no longer fitting with the hydrophobic pocket of the active site of the NorA efflux pump. Further ethidium bromide efflux inhibition assays will enable us to compare the inhibition potency of our lead EPIs with other potent NorA EPIs.

In conclusion, we synthesized bis-indolic compounds with original structures as compared with the natural bis-indole alkaloids of marine sponges. Among them, we identified derivatives with antistaphylococcal properties, including against MRSA and VISA strains, but also derivatives with inhibitory activity against the *S. aureus* NorA efflux pump. All these derivatives are promising candidates for development of new antistaphylococcal treatments that are active against MDR *S. aureus*. The bacterial target(s) of these bis-indolic compounds remains to be characterized. Further structural optimization is needed to tentatively increase the solubility and activity of these compounds and reduce their cytotoxicity.

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Transparency declarations

None to declare.

Supplementary data

Supplementary data, including Figures S1 to S6 and Table S1, are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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Supplemental Material

Novel synthetic bis-indolic derivatives with antistaphylococcal activity, including against MRSA and VISA strains

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Chemistry – General informations

Purchased reagents and solvents were used without purification except those indicated below. Tetrahydrofuran, diethylether and toluene were refluxed over sodium-benzophenone and then distilled. Dichloromethane, acetonitrile, *N,N*-dimethylformamide, methanol, pyridine and triethylamine were dried by refluxing over CaH₂ and then distilled. Acetyl chloride was distilled prior use. Reactions were monitored by thin layer chromatography (TLC) using commercial aluminium-backed silica gel plates (Merck G 60 F254). TLC spots were viewed under ultraviolet light and by heating the plate after treatment with either a 0.5% solution of ninhydrine in 3% ethanolic acetic acid or a 2% solution of potassium permanganate in 7% aqueous sodium carbonate. *N*-Hydroxylamines were detected with a 1% triphenyl tetrazolium chloride (TTC) in ethanol followed by alkaline treatment with an aqueous solution of sodium hydroxide (red color). Product purification by gravity column chromatography was performed using Macherey-Nagel Silica Gel 60 (70-230 mesh). Infrared (IR) spectra were recorded on a Nicolet Impact-400 Fourier transform infrared spectrometer (FTIR) or on a Nicolet Magna-550 Fourier transform infrared spectrometer (FTIR) equipped with an ATR (Attenuated Total Reflection) device and the data are reported in reciprocal centimeters (cm⁻¹). ¹H NMR and ¹³C NMR spectra were run on Bruker Advance300 spectrometer. Chemical shifts for ¹H spectra are values determined from the internal solvent signal [CD₃OD (δ 3.31 ppm), (CD₃)₂CO (δ 2.05 ppm) and DMSO-d₆ (δ 2.5 ppm)] or from tetramethylsilane in CDCl₃ (δ 0.00 ppm), and are reported as follows: chemical shifts (ppm), multiplicity, coupling constants (Hz), and integration. The following abbreviations are used for the multiplicities: s: singlet, d: doublet, dd: doublet of doublet, ddd: doublet of doublet of doublet, t: triplet, dt: doublet of triplet, q: quadruplet, m: multiplet, br s: broad singlet. Chemical shifts for ¹³C NMR spectra are determined from the internal solvent signal. Low-resolution mass spectra (LRMS) were recorded on a ThermoFinnigan PolarisQ ion-trap spectrometer using DCI (ammonia/isobutane 63:37) or on a Bruker Esquire 3000 plus (ESI) at DCM. High-resolution mass spectra (HRMS) were performed on a Bruker maXis mass spectrometer by the Fédération de Recherche ICOA/CBM (FR2708) platform, Orléans, France. Microwave irradiation experiments were conducted in a CEM Discover S-Class apparatus (single mode technology).

Experimental procedures and characterization data for products

Synthesis of bis-indolic compounds 9a-k and 10a,b (Figure 2)

1H-Indole-3-carboxylic acids 8a-f

1H-Indole-3-carboxylic acids **8a-f** are commercially available. 5-Halo-*1H*-indole-3-carboxylic acids **8b-f** were prepared in a two-step sequence according to the method reported by Katner.³⁴

Mono-indolic amines 7a-d

Indolic compounds **7a**, **7b** and **7c** were prepared as previously described.²²

tert-Butyl 2-amino-2-(5-chloro-*1H*-indol-3-yl)ethylcarbamate **7d**

The synthesis of the *tert*-butyl 2-(5-chloro-*1H*-indol-3-yl)-2-(hydroxyamino)ethylcarbamate was previously described.²⁴ To a stirred solution of this compound (326 mg, 1.0 mmol) in 5 mL of methanol was added 1.7 mL of a 15% aqueous solution of titanium trichloride (339 mg, 2.2 mmol). The resulting mixture was stirred at room temperature during 30 min. A large excess of a 20% aqueous solution of NaOH saturated with NaCl was added. Methanol was then removed under vacuum and the crude mixture was extracted with EtOAc (3x20 mL).

The combined organic layers were washed with H₂O and brine, dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum. Pure amine **7d** was obtained as a white solid (288 mg, 0.93 mmol). Yield: 93%. ¹H NMR (300 MHz, CD₃OD): δ = 1.41 (s, 9H), 3.39-3.45 (m, 2H), 4.30-4.34 (m, 1H), 7.06 (d, *J* = 7.8 Hz, 1H), 7.28-7.33 (m, 2H), 7.66 (s, 1H) ppm. ¹³C NMR (75.5 MHz, CD₃OD): δ = 28.8 (3C), 48.7, 49.3, 80.2 (C), 113.6, 117.2, 119.2, 122.8, 124.5, 125.7, 128.6, 136.5, 156.5 ppm. LRMS (ESI⁺): *m/z* (%) = 310 (13) [(M+H)⁺], 237 (100) [(M-C₄H₉O+H)⁺]. HRMS (ESI⁺) calcd for C₂₂H₂₇ClN₃O₃ [(M+H)⁺]: 416.1735. Found: 416.1735.

1-(5-Bromo-1H-indol-3-yl)ethanamine 7e

The synthesis of the *N*-[1-(5-bromo-1*H*-indol-3-yl)ethyl]hydroxylamine was reported.²⁴ LRMS (ESI⁻): *m/z* = 253 and 255 [(M-H)⁻]. HRMS (ESI⁺) calcd for C₁₀H₁₀BrN₂O: 252.9971 and 254.9951. Found: 252.9974 and 254.9955 [(M+H-H₂)⁺] (loss of H₂). In a round-bottomed flask, *N*-[1-(5-bromo-1*H*-indol-3-yl)ethyl]hydroxylamine (780 mg, 3.06 mmol) was dissolved in methanol (10 mL). A 20% weight aqueous solution of TiCl₃ (4.3 mL, 6.73 mmol) was added dropwise at room temperature. During the addition, the mixture became black and then turned colorless rapidly. After stirring for 30 minutes, the mixture was poured in a 20% weight aqueous solution of NaOH saturated with NaCl. The resulting solution was extracted three times with ethyl acetate. The organic layers were washed with water and brine, dried over anhydrous MgSO₄ and concentrated. The desired indolic amine **7e** (670 mg, 2.80 mmol) was obtained without any further purification as a brown solid. Yield: 92%. ¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.38 (d, *J* = 6.8 Hz, 3H, CH₃), 2.16 (br s, 2H, NH₂), 4.25 (q, *J* = 6.8 Hz, 1H, CH), 7.15 (dd, *J* = 1.6 and 8.6 Hz, 1H, CH), 7.23 (s, 1H, CH), 7.30 (d, *J* = 8.6 Hz, 1H, CH), 7.84 (s, 1H, CH), 11.00 (br s, 1H, NH) ppm. ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 25.0 (CH₃), 43.4 (CH), 110.6 (C), 113.2 (CH), 119.2 (C), 121.4 (CH), 122.6 (CH), 123.1 (CH), 127.5 (C), 135.1 (C) ppm. LRMS (ESI⁻): *m/z* = 237 and 239 [(M-H)⁻].

Bis-indolic compounds 9a-k and 10a,b

General procedure

In a flask, indolic diamine **7** (1 equiv.) was dissolved in THF or CH₂Cl₂. To this solution, 1*H*-indole-3-carboxylic acid **8** (1 equiv.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) or dicyclohexylcarbodiimide (DCC) (1.1 equiv.) were added. The mixture was stirred for 20 hours at room temperature. The homogeneous crude material was then diluted in ethyl acetate (30 mL). The organic layer was washed twice with 1N aqueous HCl and once with brine. The organic layer was dried over anhydrous MgSO₄ and concentrated under vacuum. The crude material was purified by flash chromatography (EtOAc/pentane) to afford the desired product **9** or **10**.

tert-Butyl [2-(1H-indol-3-yl)-2-(1H-indol-3-yl-carboxamido)ethyl] carbamate 9a

Prepared according to the above general procedure from indolic diamine **7a** (100 mg, 0.364 mmol), 1*H*-indole-3-carboxylic acid **8a** (59 mg, 0.366 mmol) and EDCI (71 μL, 0.400 mmol) in THF (5 mL). After purification, the compound **9a** (60 mg, 0.143 mmol, 40%) was obtained as a white solid. ¹H NMR (300 MHz, (CD₃)₂CO): δ = 1.36 (s, 9H, C(CH₃)₃), 3.70-3.75 (m, 2H, CH₂), 5.70-5.76 (m, 1H, CHN), 6.28 (s, 1H, NHBoc), 7.01 (t, *J* = 7.5 Hz, 1H, H_{indole}), 7.08-7.18 (m, 3H, H_{indole}), 7.35-7.45 (m, 4H, H_{indole} + NH), 7.79 (d, *J* = 8.0 Hz, 1H, H_{indole}), 7.98 (s, 1H, H_{indole}), 8.29 (d, *J* = 7.4 Hz, 1H, H_{indole}), 10.13 (br s, 1H, NH), 10.64 (br s, 1H, NH) ppm. ¹³C NMR (75.5 MHz, DMSO-*d*₆): δ = 28.2 (C(CH₃)₃), 44.5 (CH₂), 45.2 (CHN), 77.7 (C(CH₃)₃), 110.7, 111.3, 111.7, 115.1, 118.4, 118.9, 120.2, 121.0, 121.7, 122.3, 126.2, 126.4, 127.8, 136.1, 136.2, 156.0 (C=O), 164.1 (C=O) ppm. LRMS (ESI⁺): *m/z* = 441 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₇N₄O₃ [(M+H)⁺]: 419.2078. Found: 419.2078.

tert*-Butyl [2-(5-bromo-1*H*-indol-3-yl)-2-(1*H*-indol-3-yl-carboxamido)ethyl]carbamate **9b*

Prepared according to the above general procedure from indolic diamine **7b** (60 mg, 0.169 mmol), 1*H*-indole-3-carboxylic acid **8a** (27 mg, 0.169 mmol) and DCC (38 mg, 0.186 mmol) in CH₂Cl₂ (5 mL). After purification, the compound **9b** (62 mg, 0.125 mmol, 73%) was obtained as a white solid. Mp: 198°C decomp. (EtOAc/cyclohexane). ¹H NMR (400 MHz, CD₃OD): δ = 1.35 (s, 9H), 3.58-3.70 (m, 2H), 5.56-5.60 (m, 1H), 7.12-7.19 (m, 3H), 7.27 (d, *J* = 8.6 Hz, 1H), 7.32 (s, 1H), 7.40 (d, *J* = 7.4 Hz, 1H), 7.90-7.92 (m, 2H), 8.11 (d, *J* = 7.6 Hz, 1H) ppm. ¹³C NMR (100 MHz, CD₃OD): δ = 28.7 (C(CH₃)₃), 45.7 (CH₂), 48.4 (CHN), 80.3 (C(CH₃)₃), 111.9, 112.7, 113.3, 114.0, 115.5, 121.8, 122.0, 122.5, 123.4, 124.8, 125.4, 127.1, 129.3, 129.4, 136.8, 138.1, 159.1, 168.2 ppm. IR (ATR): 3411, 3392, 3370, 3328, 3289, 1657, 1619, 1536, 1489, 1458, 1169, 732 cm⁻¹. LRMS (ESI⁺): *m/z* = 497 and 499 [(M+H)⁺], 519 and 521 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₅N₄O₃BrNa [(M+Na)⁺]: 519.1002 and 521.0985. Found: 519.1005 and 521.0983.

tert*-Butyl [2-(1*H*-indol-3-yl)-2-(5-bromo-1*H*-indol-3-yl-carboxamido)ethyl] carbamate **9c*

Prepared according to the above general procedure from indolic diamine **7a** (150 mg, 0.545 mmol), 5-bromo-1*H*-indole-3-carboxylic acid **8b** (131 mg, 0.545 mmol) and EDCI (110 μL, 0.599 mmol) in THF (8 mL). After purification, the compound **9c** (195 mg, 0.392 mmol, 72%) was obtained as a white solid. Mp: 183°C (EtOAc/pentane). ¹H NMR (300 MHz, CD₃OD): δ = 1.35 (s, 9H), 3.60-3.74 (m, 2H), 5.62-5.66 (m, 1H), 6.99-7.05 (m, 1H), 7.08-7.13 (m, 1H), 7.25-7.31 (m, 4H), 7.71-7.74 (m, 1H), 7.91 (s, 1H), 8.32 (s, 1H) ppm. ¹³C NMR (75.5 MHz, CD₃OD): δ = 28.7 (C(CH₃)₃), 45.7 (CH₂), 48.6 (CHN), 80.3 (C(CH₃)₃), 111.7, 112.4, 114.4, 115.4, 119.9, 120.1, 122.7, 123.2, 124.7, 126.3, 127.7, 129.2, 130.2, 136.7, 138.3, 159.1, 167.6 ppm. IR (ATR): 3374, 3309, 1728, 1652, 1626, 1533, 1455, 1371, 1355, 1293, 1256, 1166, 739 cm⁻¹. LRMS (ESI⁺): *m/z* = 519 and 521 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₅N₄O₃BrNa [(M+Na)⁺]: 519.1002 and 521.0985. Found: 519.1004 and 521.0982.

tert*-Butyl [2-(6-bromo-1*H*-indol-3-yl)-2-(1*H*-indolyl-3-carboxamido)ethyl] carbamate **9d*

Prepared according to the above general procedure from indolic diamine **7c** (60 mg, 0.169 mmol), 1*H*-indole-3-carboxylic acid **8a** and EDCI (33 μL, 0.186 mmol) in THF (4 mL). After purification, the compound **9d** (55 mg, 0.111 mmol, 65%) was obtained as a white solid. Mp: 207°C decomp. (EtOAc/cyclohexane). ¹H NMR (300 MHz, CD₃OD): δ = 1.34 (s, 9H), 3.63-3.67 (m, 2H), 5.59-5.64 (m, 1H), 7.10-7.19 (m, 3H), 7.29 (s, 1H), 7.39-7.42 (m, 1H), 7.51 (d, *J* = 1.5 Hz, 1H), 7.65 (d, *J* = 8.5 Hz, 1H), 7.89 (s, 1H), 8.08-8.11 (m, 1H) ppm. ¹³C NMR (75.5 MHz, CD₃OD): δ = 28.7 (C(CH₃)₃), 45.6 (CH₂), 48.4 (CHN), 80.3 (C(CH₃)₃), 111.9, 112.8, 115.3, 116.0, 116.1, 121.4, 121.8, 122.0, 123.1, 123.4, 124.1, 126.6, 127.1, 129.3, 138.1, 139.0, 159.1, 168.2 ppm. IR (ATR): 3400, 3367, 3309, 2983, 2936, 1655, 1625, 1536, 1496, 1365, 1289, 1163, 807, 734 cm⁻¹. LRMS (ESI⁺): *m/z* = 497 and 499 [(M+H)⁺], 519 and 521 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₅N₄O₃BrNa [(M+Na)⁺]: 519.1002 and 521.0985. Found: 519.1005 and 521.0983.

tert*-Butyl [2-(1*H*-indol-3-yl)-2-(6-bromo-1*H*-indolyl-3-carboxamido)ethyl] carbamate **9e*

Prepared according to the above general procedure from indolic diamine **7a** (60 mg, 0.218 mmol), 6-bromo-1*H*-indole-3-carboxylic acid **8c** (53 mg, 0.218 mmol) and EDCI (45 μL, 0.248 mmol) in THF (4 mL). After purification, the compound **9e** (83 mg, 0.167 mmol, 77%) was obtained as a white solid. Mp: 215°C (EtOAc/pentane). ¹H NMR (400 MHz, CD₃OD): δ = 1.35 (s, 9H), 3.63-3.72 (m, 2H), 5.62-5.65 (m, 1H), 7.01 (t, *J* = 7.4 Hz, 1H), 7.10 (t, *J* = 7.4 Hz, 1H), 7.24 (d, *J* = 8.6 Hz, 1H), 7.28 (s, 1H), 7.35 (d, *J* = 8.0 Hz, 1H), 7.58 (br s, 1H), 7.72 (d, *J* = 7.9 Hz, 1H), 7.89 (s, 1H), 8.03 (d, *J* = 8.6 Hz, 1H) ppm. ¹³C NMR (75.5 MHz, CD₃OD): δ = 28.7 (C(CH₃)₃), 45.7 (CH₂), 48.6 (CHN), 80.2

(C(CH₃)₃), 112.2 (C), 112.4 (CH), 115.3 (C), 115.6 (CH), 116.8 (C), 119.9 (CH), 120.0 (CH), 122.7 (CH), 123.1 (CH), 123.4 (CH), 125.0 (CH), 126.3 (C), 127.6 (C), 129.8 (CH), 138.3 (C), 138.9 (C), 159.1 (C), 167.6 (C) ppm. IR (ATR): 3410, 3392, 3370, 3324, 3292, 2977, 1655, 1626, 1535, 1496, 1160, 737 cm⁻¹. LRMS (ESI⁺): *m/z* = 519 and 521 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₅N₄O₃BrNa [(M+Na)⁺]: 519.1002 and 521.0985. Found: 519.1004 and 521.0982.

tert*-Butyl [2-(5-bromo-1*H*-indol-3-yl)-2-(5-bromo-1*H*-indolyl-3-carboxamido)ethyl] carbamate **9f*

Prepared according to the above general procedure from indolic diamine **7b** (60 mg, 0.169 mmol), 5-bromo-1*H*-indole-3-carboxylic acid **8b** (41 mg, 0.169 mmol) and EDCI (33 μL, 0.186 mmol) in THF (5 mL). After purification, the compound **9f** (67 mg, 0.116 mmol, 68%) was obtained as a white solid. Mp: 225°C (EtOAc/pentane). ¹H NMR (300 MHz, CD₃OD): δ = 1.35 (s, 9H), 3.56-3.71 (m, 2H), 5.55-5.59 (m, 1H), 7.18 (dd, *J* = 1.8 and 8.6 Hz, 1H), 7.25-7.34 (m, 4H), 7.89-7.92 (m, 2H), 8.31 (br s, 1H) ppm. ¹³C NMR (75.5 MHz, CD₃OD): δ = 28.7 (C(CH₃)₃), 45.6 (CH₂), 48.3 (CHN), 80.3 (C(CH₃)₃), 111.6, 113.3, 114.0, 114.3, 115.3, 122.5, 124.6, 124.7, 125.4, 126.3, 129.1, 129.4, 130.1, 136.7, 138.8, 159.0, 167.5 ppm. IR (ATR): 3429, 3376, 3296, 2985, 1729, 1647, 1625, 1525, 1457, 1361, 1254, 1164, 887, 790 cm⁻¹. LRMS (ESI⁺): *m/z* = 597, 599 and 601 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₄N₄O₃Br₂Na [(M+Na)⁺]: 597.0107, 599.0089 and 601.0073. Found: 597.0114, 599.0091 and 601.0071.

tert*-Butyl [2-(5-bromo-1*H*-indol-3-yl)-2-(6-bromo-1*H*-indolyl-3-carboxamido)ethyl] carbamate **9g*

Prepared according to the above general procedure from indolic diamine **7b** (80 mg, 0.226 mmol), 6-bromo-1*H*-indole-3-carboxylic acid **8c** (54 mg, 0.226 mmol) and EDCI (45 μL, 0.248 mmol) in THF (5 mL). After purification, the compound **9g** (84 mg, 0.146 mmol, 65%) was obtained as a white solid. Mp: 203°C (EtOAc/cyclohexane). ¹H NMR (300 MHz, CD₃OD): δ = 1.35 (s, 9H), 3.58-3.69 (m, 2H), 5.55-5.58 (m, 1H), 7.18-7.27 (m, 3H), 7.29 (s, 1H), 7.58 (s, 1H), 7.90 (s, 2H), 8.03 (d, *J* = 8.5 Hz, 1H) ppm. ¹³C NMR (75.5 MHz, CD₃OD): δ = 28.7 (C(CH₃)₃), 45.6 (CH₂), 48.3 (CHN), 80.3 (C(CH₃)₃), 112.2, 113.3, 114.0, 115.4, 115.6, 116.8, 122.5, 123.4, 124.7, 125.1, 125.4, 126.5, 129.4, 129.8, 136.8, 138.8, 159.1, 167.6 ppm. IR (ATR): 3359, 3293, 2977, 1663, 1626, 1533, 1448, 1277, 1167 cm⁻¹. LRMS (ESI⁺): *m/z* = 597, 599 and 601 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₄N₄O₃Br₂Na [(M+Na)⁺]: 597.0107, 599.0089 and 601.0073. Found: 597.0113, 599.0090 and 601.0071.

tert*-Butyl [2-(6-bromo-1*H*-indol-3-yl)-2-(5-bromo-1*H*-indolyl-3-carboxamido)ethyl] carbamate **9h*

Prepared according to the above general procedure from indolic diamine **7c** (40 mg, 0.113 mmol), 5-bromo-1*H*-indole-3-carboxylic acid **8b** (27 mg, 0.113 mmol) and EDCI (22 μL, 0.124 mmol) in THF (3 mL). After purification, the compound **9h** (34 mg, 0.059 mmol, 52%) was obtained as a white solid. Mp: 227°C (EtOAc/pentane). ¹H NMR (300 MHz, CD₃OD): δ = 1.35 (s, 9H), 3.61-3.71 (m, 2H), 5.59-5.65 (m, 1H), 7.12 (dd, *J* = 1.7 and 8.5 Hz, 1H), 7.24-7.34 (m, 3H), 7.51 (d, *J* = 1.5 Hz, 1H), 7.64 (d, *J* = 8.5 Hz, 1H), 7.92 (s, 1H), 8.32 (br s, 1H) ppm. ¹³C NMR (75.5 MHz, CD₃OD): δ = 28.7 (C(CH₃)₃), 45.6 (CH₂), 48.2 (CHN), 80.3 (C(CH₃)₃), 111.6, 114.3, 115.3, 115.3, 115.8, 116.1, 121.4, 123.2, 124.1, 124.6, 126.3, 126.6, 129.1, 130.1, 136.7, 139.0, 159.1, 167.5 ppm. IR (ATR): 3364, 3293, 2978, 2933, 1652, 1628, 1530, 1455, 1290, 1253, 1162, 800, 788 cm⁻¹. LRMS (ESI⁺): *m/z* = 597, 599 and 601 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₄N₄O₃Br₂Na [(M+Na)⁺]: 597.0107, 599.0089 and 601.0073. Found: 597.0113, 599.0091 and 601.0071.

tert-Butyl [2-(6-bromo-1H-indol-3-yl)-2-(6-bromo-1H-indolyl-3-carboxamido)ethyl] carbamate 9i

Prepared according to the above general procedure from indolic diamine **7c** (70 mg, 0.198 mmol), 6-bromo-1H-indole-3-carboxylic acid **8c** (47 mg, 0.198 mmol) and EDCI (38 μ L, 0.217 mmol) in THF (4 mL). After purification, the compound **9i** (54 mg, 0.094 mmol, 47%) was obtained as a white solid. Mp: 231°C (EtOAc/pentane). ¹H NMR (300 MHz, CD₃OD): δ = 1.35 (s, 9H), 3.60-3.71 (m, 2H), 5.58-5.63 (m, 1H), 7.12 (dd, J = 1.5 and 8.5 Hz, 1H), 7.24 (dd, J = 1.5 and 8.6 Hz, 1H), 7.29 (s, 1H), 7.51 (d, J = 1.5 Hz, 1H), 7.57 (d, J = 1.7 Hz, 1H), 7.63 (d, J = 8.5 Hz, 1H), 7.89 (s, 1H), 8.03 (d, J = 8.6 Hz, 1H) ppm. ¹³C NMR (75.5 MHz, CD₃OD): δ = 28.7 (C(CH₃)₃), 45.6 (CH₂), 48.4 (CHN), 80.3 (C(CH₃)₃), 112.2, 115.3, 115.7, 115.9, 116.1, 116.9, 121.4, 123.2, 123.5, 124.1, 125.1, 126.3, 126.7, 129.8, 138.9, 139.1, 159.1, 167.6 ppm. IR (ATR): 3436, 3365, 3303, 2982, 2939, 1727, 1650, 1626, 1531, 1451, 1360, 1255, 1161, 840, 802 cm⁻¹. LRMS (ESI⁺): m/z = 597, 599 and 601 [(M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₄H₂₄N₄O₃Br₂Na [(M+Na)⁺]: 597.0107, 599.0089 and 601.0073. Found: 597.0113, 599.0091 and 601.0071.

tert-Butyl [2-(5-chloro-1H-indol-3-yl)-2-(5-chloro-1H-indolyl-3-carboxamido)ethyl] carbamate 9j

Prepared according to the above general procedure from indolic diamine **7d** (93 mg, 0.300 mmol), 5-chloro-1H-indole-3-carboxylic acid **8d** (65 mg, 0.330 mmol) and EDCI (51 mg, 0.330 mmol) in CH₂Cl₂ (5 mL). After purification, the compound **9j** (110 mg, 0.226 mmol, 75%) was obtained as beige foam. ¹H NMR (300 MHz, CDCl₃): δ = 1.36 (s, 9H), 3.56-3.60 (m, 2H), 5.22-5.28 (m, 1H), 5.38-5.43 (m, 1H), 6.88-7.20 (m, 6H), 7.46-7.52 (m, 2H), 8.13 (s, 1H), 8.99 (s, 1H), 9.58 (s, 1H) ppm. ¹³C NMR (75.5 MHz, CDCl₃): δ = 28.0 (3C), 44.6, 47.9, 79.8, 110.4, 112.4, 112.6, 113.7, 118.1, 120.1, 122.1, 122.8, 123.5, 124.9, 126.4, 126.7, 126.9, 128.8, 134.7, 134.8, 157.7, 165.8 ppm. IR (neat): 3410, 3290, 2975, 2930, 1685, 1625, 1535, 1450, 1365, 1160, 895, 795 cm⁻¹. LRMS (ESI⁺): m/z (%) = 509 (39) [(M+Na)⁺], 237 (100). HRMS (ESI⁺) calcd for C₂₄H₂₅Cl₂N₄O₃ [(M+H)⁺]: 487.1298 and 489.1274. Found: 487.1294 and 489.1269.

tert-Butyl [2-(5-chloro-1H-indol-3-yl)-2-(6-chloro-1H-indole-3-carboxamido)ethyl] carbamate 9k

Prepared according to the above general procedure from indolic diamine **7d** (93 mg, 0.300 mmol), 6-chloro-1H-indole-3-carboxylic acid **8e** (65 mg, 0.330 mmol) and EDCI (51 mg, 0.330 mmol) in CH₂Cl₂ (5 mL). After purification, the compound **9k** (90 mg, 0.185 mmol, 62%) was obtained as beige foam. ¹H NMR (300 MHz, CDCl₃): δ = 1.36 (s, 9H), 3.57-3.63 (m, 2H), 5.22-5.28 (m, 1H), 5.40-5.45 (m, 1H), 6.89-7.19 (m, 6H), 7.42-7.54 (m, 2H), 7.96-8.01 (s, 1H), 9.01 (s, 1H), 9.61 (s, 1H) ppm. ¹³C NMR (75.5 MHz, CDCl₃): δ = 27.7 (3C), 44.1, 47.3, 79.4, 110.5, 111.3, 112.2, 113.4, 117.9, 121.2, 121.3, 121.7, 123.3, 123.8, 124.6, 126.7, 128.0, 128.3, 134.8, 136.7, 157.5, 165.8 ppm. IR (neat): 3410, 3270, 2970, 2930, 1685, 1620, 1530, 1445, 1365, 1160, 850, 795 cm⁻¹. LRMS (ESI⁺): m/z (%) = 509 (32) [(M+Na)⁺], 237 (100). HRMS (ESI⁺) calcd for C₂₄H₂₅Cl₂N₄O₃ [(M+H)⁺]: 487.1298 and 489.1274. Found: 487.1296 and 489.1270.

5-Bromo-N-[1-(5-bromo-1H-indol-3-yl)ethyl]-1H-indole-3-carboxamide 10a

Prepared according to the above general procedure from indolic amine **7e** (150 mg, 0.627 mmol), 5-bromo-1H-indole-3-carboxylic acid **8b** (150 mg, 0.627 mmol) and EDCI (107 mg, 0.690 mmol) in THF (10 mL). After purification, the compound **10a** (220 mg, 0.477 mmol, 76%) was obtained as a white solid. Mp: 144°C. ¹H NMR (400 MHz, DMSO-d₆): δ = 1.61 (d, J = 6.8 Hz, 3H, CH₃), 5.49-5.56 (m, 1H, CH), 7.17 (dd, J = 1.6 and 8.4 Hz, 1H, CH), 7.27 (dd, J = 2.0 and 8.8 Hz, 1H, CH), 7.32-7.41 (m, 3H, CH), 7.82 (s, 1H, CH), 8.12 (d, J = 2.4 Hz, 1H, NH), 8.16 (d, J = 8.4 Hz, 1H, CH), 8.38 (s, 1H, CH), 11.11

(br s, 1H, NH), 11.70 (br s, 1H, NH) ppm. ^{13}C NMR (100 MHz, DMSO- d_6): δ = 20.9 (CH₃), 40.0 (CH), 110.2 (C), 111.0 (C), 113.0 (C), 113.3 (CH), 113.7 (CH), 118.1 (C), 121.3 (CH), 123.3 (CH), 123.4 (CH), 123.7 (CH), 124.2 (CH), 127.8 (C), 128.2 (C), 128.8 (CH), 134.7 (C), 135.0 (C), 163.2 (C) ppm. IR (ATR): 3298, 3160, 2986, 1592, 1537, 1445, 1205 cm^{-1} . LRMS (ESI⁻): m/z = 458, 460 and 462 [(M-H)⁻]. HRMS (ESI⁺) calcd for C₁₉H₁₆Br₂N₃O [(M+H)⁺]: 459.9655, 461.9635 and 463.9617. Found: 459.9646, 461.9631 and 463.9612.

N*-[1-(5-Bromo-1*H*-indol-3-yl)ethyl]-5-fluoro-1*H*-indole-3-carboxamide **10b*

Prepared according to the above general procedure from indolic amine **7e** (72 mg, 0.300 mmol), 5-fluoro-1*H*-indole-3-carboxylic acid **8f** (59 mg, 0.330 mmol) and EDCI (51 mg, 0.330 mmol) in CH₂Cl₂ (5 mL). After purification, the compound **10b** (60 mg, 0.150 mmol, 50%) was obtained as beige foam. Yield: 50%. ^1H NMR (300 MHz, CD₃OD-DMSO- d_6): δ = 1.60 (d, J = 6.8 Hz, 3H), 5.49 (q, J = 6.8 Hz, 1H), 6.87 (dt, J = 2.6 and 9.0 Hz, 1H), 6.99 (dt, J = 2.6 and 9.0 Hz, 1H), 7.09 (dd, J = 1.9 and 8.7 Hz, 1H), 7.19-7.23 (m, 2H), 7.44 (dd, J = 4.4 and 9.0 Hz, 1H), 7.65 (dd, J = 2.6 and 9.6 Hz, 1H), 7.74 (d, J = 7.7 Hz, 3H), 8.18 (s, 1H) ppm. ^{13}C NMR (75.5 MHz, CD₃OD-DMSO- d_6): δ = 21.3, 42.3, 106.9 (d, J = 25.1 Hz), 111.9 (d, J = 26.6 Hz), 112.3, 112.8, 114.0 (d, J = 9.8 Hz), 121.2, 121.4, 121.5, 124.6, 126.2, 127.5, 134.7, 134.9, 135.1, 160.0 (d, J = 235.1 Hz), 168.8, 175.5 ppm. IR (neat): 3455, 3240, 2975, 2870, 1740, 1595, 1530, 1460, 1425, 1205, 1170, 1080, 1005, 930, 850, 760 cm^{-1} . LRMS (ESI⁺): m/z = 422 and 424 [(M+Na)⁺], 101. HRMS (ESI⁺) calcd for C₁₉H₁₆BrFN₃O [(M+H)⁺]: 400.0455 and 402.0437. Found: 400.0452 and 402.0434.

Alternative synthesis of indolic amine **7e** (Figure S1)

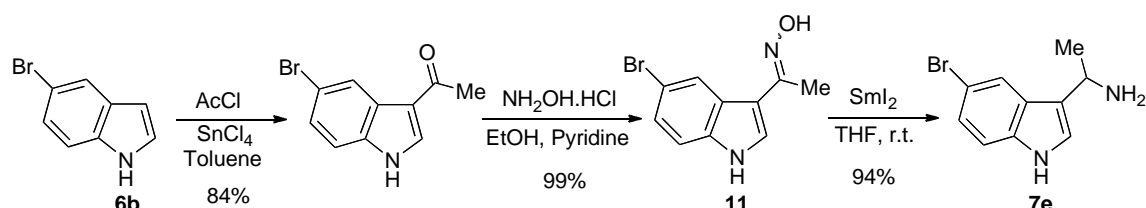


Figure S1 Synthesis of the primary indolic amine **7e**.

1-(5-Bromo-1*H*-indol-3-yl)ethanone

The synthesis of this compound was already described.³⁵

1-(5-Bromo-1*H*-indol-3-yl)ethanone oxime **11**

The oxime was synthesized according to a previously described procedure.³⁷

Hydroxylamine hydrochloride (834 mg, 12.0 mmol) was added to a stirred solution of 3-acetyl-5-bromoindole (952 mg, 4.00 mmol) and pyridine (0.967 mL, 948 mg, 12.0 mmol) in 20 mL of ethanol. The resulting mixture was stirred at reflux for 2 hours and then ethanol was evaporated. Water (50 mL) was added and then the mixture was extracted with EtOAc (3x30 mL). The organic layers were washed with water, brine, dried over anhydrous MgSO₄, filtered and concentrated under vacuum. Pure oxime **11** (1.00 g, 3.95 mmol) was obtained as colourless oil and was used before further purification. Yield: 99%. ^1H NMR (300 MHz, CD₃OD): δ = 2.22 (s, 3H), 7.18-7.28 (m, 2H), 7.45 (s, 1H), 8.37 (s, 1H) ppm.

1-(5-Bromo-1*H*-indol-3-yl)ethanamine **7e**

To a stirred and carefully deoxygenated solution of indolic oxime **11** (633 mg, 2.5 mmol) and H₂O (720 mg, 40.0 mmol, 16 equiv.) in 10 mL of THF, a 0.1 M solution of SmI₂ (110 mL, 11.0 mmol, 4.4 equiv.) was added at room temperature under argon. After 10 minutes, a saturated solution of Na₂S₂O₃ (20 mL) and NaHCO₃ (20 mL) was added, followed by EtOAc (50 mL). After extraction, the organic layer was washed with brine, dried over anhydrous

Na₂SO₄, filtered, and concentrated. Pure amine **7e** (560 mg, 2.34 mmol) was obtained as a white solid. Yield: 94%.

Synthesis of the bis-indole amide **12** (Figure S2)

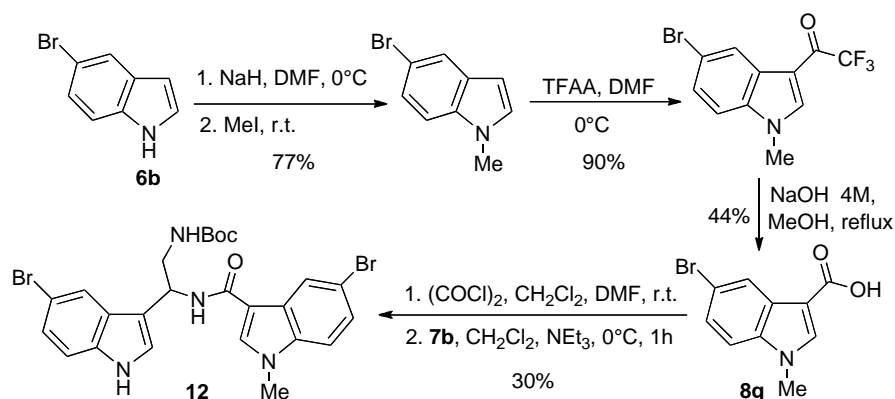


Figure S2 Synthesis of the bis-indole amide **12**.

5-Bromo-1-methyl-1H-indole

This compound was synthesized according to a previously described procedure.^a

In an oven-dried flask under argon, 5-bromoindole **6b** (1.50 g, 7.65 mmol) was dissolved in anhydrous DMF (15 mL). After cooling the mixture to 0°C, sodium hydride (367 mg, 9.18 mmol, 60% in oil) was added. The mixture was stirred at 0°C for 30 minutes. Then, methyl iodide (0.525 mL, 1.196 g, 8.42 mmol) was added. The resulting mixture was then stirred at room temperature for 5 hours and quenched with water. After dilution in ethyl acetate and separation, the organic layer was washed three times with brine, dried over anhydrous MgSO₄ and concentrated under vacuum. The desired product (1.24 g, 5.90 mmol) was obtained as a yellow solid without further purification. Yield: 77%. ¹H NMR (300 MHz, CDCl₃): δ = 3.79 (s, 3H, CH₃), 6.42 (d, *J* = 3.3 Hz, 1H, CH), 7.03 (d, *J* = 3.3 Hz, 1H, CH), 7.18 (d, *J* = 8.7 Hz, 1H, CH), 7.25-7.31 (m, 1H, CH), 7.74 (d, *J* = 1.8 Hz, 1H, CH) ppm.

1-(5-Bromo-1-methyl-1H-indol-3-yl)-2,2,2-trifluoroethanone

This compound was prepared according to the method reported by Katner.³⁴

In an oven-dried flask under argon, 5-bromo-1-methyl-1H-indole (1.24 g, 5.90 mmol) was dissolved in anhydrous DMF (6 mL). The mixture was cooled to 0°C and trifluoroacetic anhydride (1.23 mL, 1.860 g, 8.86 mmol) was added dropwise. The mixture was stirred at 0°C for 3.5 hours then quenched with water. The crude mixture was filtered to afford a solid. This solid was washed twice with water and dissolved in ethyl acetate. The organic layer was washed with a saturated aqueous NaHCO₃ solution followed by brine, dried over anhydrous MgSO₄ and evaporated. The desired product (1.62 g, 5.29 mmol) was obtained as a solid without further purification. Yield: 90%. ¹H NMR (300 MHz, CDCl₃): δ = 3.90 (s, 3H, CH₃), 7.26 (d, *J* = 8.7 Hz, 1H, CH), 7.49 (dd, *J* = 2.1 and 8.7 Hz, 1H, CH), 7.88 (d, *J* = 1.5 Hz, 1H, CH), 8.56 (d, *J* = 2.1 Hz, 1H, CH) ppm. HRMS (ESI⁺) calcd for C₁₁H₈BrF₃NO [(M+H)⁺]: 305.9736 and 307.9716. Found: 305.9739 and 307.9717.

5-Bromo-1-methyl-1H-indole-3-carboxylic acid **8g**

This compound is already described.^b

^a Johanson MB, Kerr MA. Direct functionalization of indoles: Copper-catalyzed malonyl carbenoid insertions. *Org. Lett.* 2010; **12**:4956-59.

A flask was charged with 1-(5-bromo-1-methyl-1*H*-indol-3-yl)-2,2,2-trifluoroethanone (1.53 g, 5.03 mmol) and an aqueous solution of 4 M sodium hydroxide (40 mL). The mixture was refluxed for 2.5 hours. Methanol (10 mL) was added to the mixture, which was refluxed overnight, then carefully acidified with 6M aqueous HCl. During the acidification, the carboxylic acid precipitated off the mixture. The resulting suspension was filtered. The solid was washed with water and dissolved in a mixture of ethyl acetate/methanol (about 9/1). The combined organic layers were dried over anhydrous MgSO₄ and evaporated under vacuum. The desired product **8g** (560 mg, 2.20 mmol) was obtained as a beige solid. Yield: 44%. ¹H NMR (300 MHz, DMSO-*d*₆): δ = 3.84 (s, 3H, CH₃), 7.38 (dd, *J* = 2.1 and 8.7 Hz, 1H, CH), 7.52 (d, *J* = 8.7 Hz, 1H, CH), 8.08 (s, 1H, CH), 8.13 (d, *J* = 2.1 Hz, 1H, CH), 12.13 (br s, 1H, OH) ppm. ¹³C NMR (75.5 MHz, DMSO-*d*₆): δ = 33.1 (CH₃), 105.7 (C), 112.8 (CH), 114.2 (C), 122.7 (CH), 124.6 (CH), 128.0 (C), 135.7 (C), 137.1 (CH), 165.1 (C) ppm. HRMS (ESI⁺) calcd for C₁₀H₉BrN₂O [(M+H)⁺]: 253.9811 and 255.9791. Found: 253.9814 and 255.9794.

tert*-Butyl (2-(5-bromo-1-methyl-1*H*-indole-3-carboxamido)-2-(5-bromo-1*H*-indol-3-yl)ethyl) carbamate **12*

In an oven-dried flask under argon, the carboxylic acid **8g** (100 mg, 0.390 mmol) was dissolved in dry dichloromethane (3 mL) and few drops of dry DMF. The sluggish solution was cooled to 0°C and freshly distilled oxalyl chloride (60 mg, 0.470 mmol) was added dropwise. The mixture was then stirred for 4 hours at room temperature and directly evaporated with toluene under reduced pressure. The desired acid chloride was obtained as a yellow solid and used directly for the next step.

In an oven-dried flask under argon, the amine **7b** (70 mg, 0.198 mmol) and triethylamine (32 mg, 0.316 mmol) were dissolved in 2 mL of dry dichloromethane. After cooling this solution to 0°C, the acid chloride previously obtained (65 mg, 0.237 mmol) was added and the mixture is stirred at 0°C for one hour. The reaction was then quenched with saturated NaHCO₃ solution and extracted twice with ethyl acetate. The organic layers were combined, washed with water, dried over anhydrous MgSO₄ and concentrated. After purification by flash chromatography (EtOAc/pentane, 1/1 until neat EtOAc), the desired product **12** (35 mg, 0.059 mmol) was obtained as a white solid. Yield: 30%. Mp: 233°C. ¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.32 (s, 9H, CH₃), 3.40-3.54 (m, 2H, CH₂), 3.81 (s, 3H, CH₃), 5.44-5.50 (m, 1H, CH), 6.92 (br s, 1H, NH), 7.17 (dd, *J* = 2.0 and 8.4 Hz, 1H, CH), 7.30-7.37 (m, 3H, CH and NH), 7.48 (d, *J* = 8.8 Hz, 1H, CH), 7.84 (s, 1H, CH), 8.04- 8.07 (m, 2H, CH and NH), 8.30 (s, 1H, CH), 11.13 (br s, 1H, NH) ppm. ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 28.1 (CH₃), 33.0 (CH₃), 44.2 (CH₂), 45.1 (CH), 77.6 (C), 109.1 (C), 111.7 (C), 112.3 (CH), 113.3 (C), 113.4 (CH), 114.9 (C), 121.1 (CH), 123.2 (CH), 123.3 (CH), 124.0 (CH), 124.3 (CH), 128.19 (C), 128.23 (C), 132.9 (CH), 134.7 (C), 135.3 (C), 155.8 (C), 163.2 (C) ppm. IR (ATR): 3365, 3271, 1668, 1609, 1529, 1513, 1466, 1231, 1156 cm⁻¹. HRMS (ESI⁺) calcd for C₂₅H₂₇Br₂N₄O₃ [(M+H)⁺]: 589.0444, 591.0426 and 593.0410. Found: 589.0436, 591.0419 and 593.0404.

Synthesis of the bis-indole 14a-c possessing hydrophilic groups (Figure 3)

Methyl 2-(5-bromo-1*H*-indol-3-yl)-2-oxoacetate^c

^b Duflos M, Nourrisson M-R, Brelet J, Courant J, LeBaut G, Grimaud N, Petit J-Y. *N*-Pyridinyl-indole-3-(alkyl)carboxamides and derivatives as potential systemic and topical inflammation inhibitors. *Eur J Med Chem* 2001; **36**:545-53.

^c Ilovich O, Billauer H, Dotan S, Mishani E. Labeled 3-aryl-4-indolylmaleimide derivatives and their potential as angiogenic PET biomarkers. *Bioorg. Med. Chem.* 2010; **18**: 612-20.

An oven-dried flask was charged with 5-bromoindole **6b** (4.00 g, 20.4 mmol) and anhydrous diethyl ether (60 mL) under argon. This solution was cooled to 0°C and freshly distilled oxalyl chloride (2.10 mL, 24.5 mmol) was added. The solution turned rapidly yellow. After stirring for 1.5 hours at 0°C, the mixture was quenched by addition of methanol (16.4 mL, 408 mmol) at 0°C and stirred for 10 minutes at room temperature. The solution was concentrated and the yellow powder was washed twice with diethyl ether. The yellow solid was then dried under vacuum to afford the desired product (4.68 g, 16.6 mmol) without any further purification. Yield: 81%. ¹H NMR (300 MHz, DMSO-d₆): δ = 3.89 (s, 3H, CH₃), 7.43 (dd, *J* = 1.6 and 8.8 Hz, 1H, CH), 7.54 (d, *J* = 8.8 Hz, 1H, CH), 8.29 (d, *J* = 1.6 Hz, 1H, CH), 8.50 (d, *J* = 3.3 Hz, 1H, CH), 12.66 (br s, 1H, NH) ppm. ¹³C NMR (75.5 MHz, DMSO-d₆): δ = 52.5 (CH₃), 111.8 (C), 114.8 (CH), 115.5 (C), 123.2 (CH), 126.4 (CH), 127.3 (C), 135.4 (C), 139.2 (CH), 163.3 (C), 178.3 (C) ppm. LRMS (ESI⁻): *m/z* = 280 and 282 [(M-H)⁻]. HRMS (ESI⁺) calcd for C₁₁H₉BrNO₃ [(M+H)⁺]: 281.9760 and 283.9741. Found: 281.9762 and 283.9742.

Methyl 2-(5-bromo-1*H*-indol-3-yl)-2-(hydroxyimino)acetate

A vial was charged with the keto-ester previously obtained (600 mg, 2.13 mmol), methanol (10 mL), water (4 mL), sodium acetate (1.74 g, 21.27 mmol) and hydroxylamine hydrochloride (1.47 g, 21.27 mmol). The vial was sealed and heated at 100°C under microwave irradiation for 45 minutes. The resulting brown solution was diluted with ethyl acetate, washed with a 1M aqueous solution of HCl and water. The organic layer was dried over anhydrous MgSO₄ and concentrated under vacuum. The crude material was purified by flash chromatography (EtOAc/pentane, 4/6) to afford the desired product (342 mg, 1.15 mmol) as a red-brown solid (2 isomers, ratio, 1/1). Yield: 54%. **Isomer 1.** ¹H NMR (400 MHz, DMSO-d₆): δ = 3.83 (s, 3H, CH₃), 7.27 (d, *J* = 8.8 Hz, 1H, CH), 7.42 (d, *J* = 8.8 Hz, 1H, CH), 7.50 (s, 1H, CH), 8.02 (s, 1H, CH), 11.87 (br s, 1H, NH), 12.2 (s, 1H, OH) ppm. ¹³C NMR (100 MHz, DMSO-d₆): δ = 62.1 (CH₃), 116.5 (C), 122.2 (CH), 123.8 (C), 132.9 (CH), 134.0 (CH), 136.7 (C), 141.4 (CH), 144.1 (C), 157.2 (C), 175.1 (C) ppm. **Isomer 2.** ¹H NMR (400 MHz, DMSO-d₆): δ = 3.87 (s, 3H, CH₃), 7.32 (d, *J* = 8.8 Hz, 1H, CH), 7.42 (d, *J* = 8.8 Hz, 1H, CH), 7.55 (s, 1H, CH), 8.13 (d, *J* = 1.6 Hz, 1H, CH), 11.31 (s, 1H, OH), 11.79 (br s, 1H, NH) ppm. ¹³C NMR (100 MHz, DMSO-d₆): δ = 62.3 (CH₃), 113.5 (C), 122.9 (CH), 124.0 (C), 133.5 (CH), 135.0 (CH), 135.4 (C), 138.7 (CH), 145.5 (C), 153.4 (C), 174.1 (C) ppm. IR (ATR): 3359, 2953, 1719, 1607, 1531, 1419, 1235, 1106 cm⁻¹. LRMS (ESI⁻): *m/z* = 295 and 297 [(M-H)⁻]. HRMS (ESI⁺) calcd for C₁₁H₁₀BrN₂O₃ [(M+H)⁺]: 296.9869 and 298.9850. Found: 296.9850 and 298.9852.

Methyl 2-amino-2-(5-bromo-1*H*-indol-3-yl)acetate **13**

The indolic oxime previously obtained (1.67 g, 5.62 mmol) was dissolved in THF (30 mL). This solution was then added to a cold mixture (0°C) of acetic acid (55 mL) and water (55 mL). Once the temperature of the mixture was cooled to 0°C, zinc dust (3.67 g, 56.23 mmol) was slowly added (over 15 minutes). After addition of zinc, the mixture was stirred for one hour at 0°C. During the reaction, the color of the mixture was changed from red to green. Ethyl acetate (80 mL) was then added to the solution and the acetic acid was quenched by addition of potassium carbonate. The basic (pH = 8–9) aqueous layer was extracted twice with ethyl acetate. The organic layers were combined, washed with brine, dried over anhydrous MgSO₄ and concentrated under vacuum. The residue was washed twice with pentane and dried under vacuum to afford the desired amine **13** (1.17 g, 4.13 mmol) as a brown solid. Yield: 74%. ¹H NMR (400 MHz, DMSO-d₆): δ = 2.21 (br s, 2H, NH₂), 3.60 (s, 3H, CH₃), 4.76 (s, 1H, CH), 7.19 (d, *J* = 8.8 Hz, 1H, CH), 7.33 (d, *J* = 8.8 Hz, 1H, CH), 7.36 (d, *J* = 2.0 Hz, 1H, CH), 7.80 (d, *J* = 2.0 Hz, 1H, CH), 11.20 (br s, 1H, NH) ppm. ¹³C NMR (100 MHz, DMSO-d₆): δ = 51.3 (CH), 51.6 (CH₃), 111.2 (C), 113.4 (CH), 114.3

(C), 121.3 (CH), 123.5 (CH), 124.7 (CH), 127.3 (C), 134.9 (C), 174.7 (C) ppm. LRMS (ESI⁻): m/z = 281 and 283 [(M-H)⁻]. HRMS (ESI⁺) calcd for C₁₁H₉BrN₂O₂ [(MH-NH₃)⁺]: 265.9811 and 267.9792. Found: 265.9815 and 267.9795.

Methyl 2-(5-bromo-1H-indol-3-yl)-2-(5-bromo-1H-indole-3-carboxamido)acetate 14a

In a flask, the amine **13** (1.13 g, 4.00 mmol) was dissolved in 50 mL of THF. To this solution, 5-bromo-1H-indole-3-carboxylic acid **8b** (862 mg, 3.60 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI, 680 mg, 4.39 mmol) were added. The reacting mixture was stirred overnight at room temperature. The crude material was then diluted in ethyl acetate. The organic layer was washed twice with a 1N aqueous solution of HCl and once with brine. The organic layer was dried over anhydrous MgSO₄ and concentrated. The crude material was purified by flash chromatography (EtOAc/pentane, 1/1 then 7/3 and pure EtOAc) to afford the desired product **14a** as a white solid. Yield: 75%. The solid was washed with pentane to remove traces of ethyl acetate (1.10 g, 2.18 mmol). Yield: 61%. Mp: 126°C. ¹H NMR (300 MHz, DMSO-d₆): δ = 3.67 (s, 3H, CH₃), 5.82 (d, J = 6.0 Hz, 1H, CH), 7.24 (dd, J = 1.8 and 6.6 Hz, 1H, CH), 7.28 (dd, J = 2.1 and 8.7 Hz, 1H, CH), 7.39 (d, J = 8.7 Hz, 1H, CH), 7.41 (d, J = 8.7 Hz, 1H, CH), 7.53 (d, J = 2.4 Hz, 1H, CH), 7.80 (d, J = 2.1 Hz, 1H, CH), 8.29 (d, J = 2.4 Hz, 1H, CH), 8.33 (d, J = 1.8 Hz, 1H, CH), 8.55 (d, J = 6.0 Hz, 1H, NH), 11.41 (br s, 1H, NH), 11.78 (br s, 1H, NH) ppm. ¹³C NMR (75.5 MHz, DMSO-d₆): δ = 49.3 (CH), 51.9 (OCH₃), 108.9 (C), 109.0 (C), 111.6 (C), 113.2 (C), 113.6 (CH), 113.8 (CH), 121.2 (CH), 123.2 (CH), 123.9 (CH), 124.4 (CH), 126.5 (CH), 127.7 (C), 128.2 (C), 130.1 (CH), 134.7 (C), 134.9 (C), 164.2 (C), 171.6 (C) ppm. IR (ATR): 3223, 3031, 1752, 1704, 1597, 1537, 1435, 1372, 1204 cm⁻¹. LRMS (ESI⁻): m/z = 501, 502, 503, 504 and 505 [(M-H)⁻]. HRMS (ESI⁺) calcd for C₂₀H₁₆Br₂N₃O₃ [(M+H)⁺]: 503.9553, 505.9534, and 507.9517. Found: 503.9547, 505.9529 and 507.9510.

2-(5-Bromo-1H-indol-3-yl)-2-(5-bromo-1H-indole-3-carboxamido)acetic acid 14b

The ester **14a** (400 mg, 0.79 mmol) was dissolved in a mixture of methanol/water (8.0 mL / 5.3 mL). Lithium hydroxide monohydrate (133 mg, 3.20 mmol) was then added and the resulting mixture was stirred for 105 min at 40°C. The reaction was then quenched with 27 mL of a 1M aqueous solution of HCl and extracted three times with ethyl acetate. The organic layers were combined, washed with brine, dried over anhydrous MgSO₄ and evaporated under vacuum. A brownish solid was obtained, then washed with pentane to remove traces of ethyl acetate. The desired carboxylic acid **14b** (370 mg, 0.75 mmol) was obtained without further purification as a beige solid. Yield: 94%. Mp: 195°C. ¹H NMR (400 MHz, DMSO-d₆): δ = 5.78 (d, J = 6.8 Hz, 1H, CH), 7.24 (dd, J = 2.0 and 8.8 Hz, 1H, CH), 7.28 (dd, J = 2.0 and 8.8 Hz, 1H, CH), 7.39 (d, J = 8.8 Hz, 1H, CH), 7.41 (d, J = 8.8 Hz, 1H, CH), 7.53 (d, J = 2.4 Hz, 1H, CH), 7.84 (d, J = 2.0 Hz, 1H, CH), 8.31 (d, J = 2.4 Hz, 1H, CH), 8.35 (d, J = 2.0 Hz, 1H, CH), 8.46 (d, J = 6.8 Hz, 1H, NH), 11.37 (br s, 1H, NH), 11.78 (br s, 1H, NH), 12.54 (br s, 1H, OH) ppm. ¹³C NMR (100 MHz, DMSO-d₆): δ = 49.2 (CH), 109.2 (C), 109.9 (C), 111.5 (C), 113.2 (C), 113.6 (CH), 113.8 (CH), 121.5 (CH), 123.2 (CH), 123.8 (CH), 124.4 (CH), 126.4 (CH), 127.8 (C), 128.2 (C), 130.0 (CH), 134.7 (C), 134.9 (C), 164.1 (C), 172.6 (C) ppm. IR (ATR): 3406, 3268, 2974, 1715, 1614, 1529, 1446, 1187, 1098 cm⁻¹. LRMS (ESI⁻): m/z = 488, 489, 490, 491 and 492 [(M-H)⁻]. HRMS (ESI⁺) calcd for C₁₉H₁₄Br₂N₃O₃ [(M+H)⁺]: 489.9396, 491.9377 and 493.9360. Found: 489.9392, 491.9374 and 493.9360.

5-Bromo-N-(1-(5-bromo-1H-indol-3-yl)-2-hydroxyethyl)-1H-indole-3-carboxamide 14c

The ester **14a** (150 mg, 0.30 mmol) was dissolved in a mixture of THF/methanol (5.0 mL / 1.0 mL). To this solution, NaBH₄ (114 mg, 3.00 mmol) was added portionwise. The mixture was refluxed for 3 hours then diluted in ethyl acetate. The organic layer was washed with water and brine, dried over anhydrous MgSO₄ and concentrated. The residue was washed with pentane to afford the pure desired alcohol **14c** (130 mg, 0.274 mmol) as a white powder.

Yield: 92%. Mp: 155°C. ¹H NMR (300 MHz, DMSO-d₆): δ = 3.84 (t, *J* = 6.3 Hz, 2H, CH₂), 4.87 (t, *J* = 6.3 Hz, 1H, OH), 5.42 (ddd, *J* = 6.3, 6.3 and 8.4 Hz, 1H, CH), 7.16 (dd, *J* = 1.8 and 8.7 Hz, 1H, CH), 7.26 (dd, *J* = 2.1 and 8.4 Hz, 1H, CH), 7.33 (d, *J* = 8.4 Hz, 1H, CH), 7.37 (s, 1H, CH), 7.40 (d, *J* = 8.7 Hz, 1H, CH), 7.86 (d, *J* = 1.8 Hz, 1H, CH), 8.06 (d, *J* = 1.8 Hz, 1H, NH), 8.17 (s, 1H, CH), 8.33 (d, *J* = 2.1 Hz, 1H, CH), 11.12 (br s, 1H, NH), 11.71 (br s, 1H, NH) ppm. ¹³C NMR (75.5 MHz, DMSO-d₆): δ = 47.3 (CH), 63.8 (CH₂), 110.2 (C), 111.0 (C), 113.0 (C), 113.2 (CH), 113.7 (CH), 114.7 (C), 121.2 (CH), 123.2 (CH), 123.3 (CH), 124.2 (CH), 124.4 (CH), 128.1 (C), 128.2 (C), 129.0 (CH), 134.7 (C), 134.8 (C), 163.7 (C) ppm. IR (ATR): 3282, 2948, 1708, 1593, 1541, 1432, 1255, 1204, 1041 cm⁻¹. LRMS (ESI⁻): *m/z* = 474, 476 and 478 [(M-H)⁻]. HRMS (ESI⁺) calcd for C₁₉H₁₆Br₂N₃O₂ [(M+H)⁺]: 475.9604, 477.9584 and 479.9567. Found: 475.9600, 477.9580 and 479.9561.

Synthesis of bis-indole molecules 15a-c, alkylated on the amide linker (Figure 4)

5-Bromo-*N*-(1-(5-bromo-1*H*-indol-3-yl)-2-((2-hydroxyethyl)amino)-2-oxoethyl)-1*H*-indole-3-carboxamide 15a

A vial was charged with the ester **14a** (50 mg, 0.100 mmol), potassium carbonate (27 mg, 0.200 mmol), excess of ethanolamine (1.00 g, 16.4 mmol) and acetonitrile (1 mL). The mixture was heated at 115°C for 15 minutes under microwave irradiation. The crude material was diluted in ethyl acetate, washed three times with water, dried over anhydrous MgSO₄ and concentrated. The residue was recrystallized in methanol to afford a white solid **15a** (40 mg, 0.075 mmol). Yield: 75%. Mp: 164°C. ¹H NMR (300 MHz, DMSO-d₆): δ = 3.15-3.24 (m, 2H, CH₂), 3.40-3.46 (m, 2H, CH₂), 4.65 (t, *J* = 5.4 Hz, 1H, OH), 5.92 (d, *J* = 7.8 Hz, 1H, CH), 7.19 (dd, *J* = 2.1 and 8.7 Hz, 1H, CH), 7.27 (dd, *J* = 2.1 and 8.7 Hz, 1H, CH), 7.33-7.41 (m, 3H, CH), 7.86 (d, *J* = 1.8 Hz, 1H, CH), 8.15 (t, *J* = 6.0 Hz, 1H, NH), 8.24 (d, *J* = 7.8 Hz, 1H, NH), 8.28 (s, 1H, CH), 8.32 (d, *J* = 2.1 Hz, 1H, CH), 11.23 (s, 1H, NH), 11.76 (br s, 1H, NH) ppm. ¹³C NMR (75.5 MHz, DMSO-d₆): δ = 41.6 (CH₂), 49.2 (CH), 59.6 (CH₂), 109.4 (C), 111.3 (C), 112.2 (C), 113.2 (C), 113.4 (CH), 113.8 (C), 121.4 (CH), 123.1 (CH), 123.5 (CH), 124.3 (CH), 125.7 (CH), 127.9 (C), 128.1 (C), 129.8 (CH), 134.7 (C), 134.8 (C), 163.6 (C), 170.6 (C) ppm. IR (ATR): 3420, 3230, 2935, 1644, 1605, 1531, 1495, 1455, 1194 cm⁻¹. HRMS (ESI⁺) calcd for C₂₁H₁₉Br₂N₄O₃ [(M+H)⁺]: 532.9818, 534.9799 and 536.9783. Found: 532.9812, 534.9793 and 536.9776.

5-Bromo-*N*-(1-(5-bromo-1*H*-indol-3-yl)-2-((4-hydroxybutyl)amino)-2-oxoethyl)-1*H*-indole-3-carboxamide 15b

A vial was charged with the ester **14a** (50 mg, 0.100 mmol), potassium carbonate (27 mg, 0.200 mmol), excess of 4-aminobutanol (1.00 g, 11.2 mmol) and acetonitrile (1 mL). The mixture was heated at 115°C for 15 minutes under microwave irradiation. The crude material was diluted in ethyl acetate, washed three times with water, dried over MgSO₄ and concentrated. The residue was recrystallized in methanol/EtOAc to afford a white solid **15b** (37 mg, 0.066 mmol). Yield: 66%. Mp: 187°C. ¹H NMR (400 MHz, DMSO-d₆): δ = 1.36-1.47 (m, 4H, CH₂), 3.08-3.13 (m, 2H, CH₂), 3.30-3.38 (m, 2H, CH₂), 4.33 (br s, 1H, OH), 5.89 (d, *J* = 8.0 Hz, 1H, CH), 7.19 (dd, *J* = 2.1 and 8.7 Hz, 1H, CH), 7.27 (dd, *J* = 2.1 and 8.7 Hz, 1H, CH), 7.33-7.41 (m, 3H, CH), 7.87 (d, *J* = 1.8 Hz, 1H, CH), 8.15 (t, *J* = 6.0 Hz, 1H, NH), 8.20 (d, *J* = 7.8 Hz, 1H, NH), 8.28 (s, 1H, CH), 8.32 (d, *J* = 2.1 Hz, 1H, CH), 11.22 (br s, 1H, NH), 11.75 (br s, 1H, NH) ppm. ¹³C NMR (100 MHz, DMSO-d₆): δ = 25.6 (CH₂), 29.7 (CH₂), 39.3 (CH₂), 49.2 (CH), 60.3 (CH₂), 109.4 (C), 111.3 (C), 112.3 (C), 113.1 (C), 113.4 (CH), 113.7 (CH), 121.4 (CH), 123.1 (CH), 123.5 (CH), 124.3 (CH), 125.6 (CH), 127.8 (C), 128.1 (C), 129.8 (CH), 134.6 (C), 134.8 (C), 163.5 (C), 170.2 (C) ppm. IR (ATR): 3396, 3246, 2932, 1615, 1526, 1446, 1194 cm⁻¹. HRMS (ESI⁺) calcd for C₂₃H₂₃Br₂N₄O₃ [(M+H)⁺]: 561.0131, 563.0112 and 565.0096. Found: 561.0125, 563.0107 and 565.0090.

N*-2-((4-Aminobutyl)amino)-1-(5-bromo-1*H*-indol-3-yl)-2-oxoethyl)-5-bromo-1*H*-indole-3-carboxamide **15c*

A vial was charged with the ester **14a** (60 mg, 0.119 mmol), potassium carbonate (58 mg, 0.214 mmol), excess of 1,4-diaminobutane (1.20 g, 13.6 mmol) and acetonitrile (1.5 mL). The mixture was heated at 115°C for 15 minutes under microwave irradiation. The crude material was diluted in ethyl acetate, washed three times with water, dried over anhydrous MgSO₄ and concentrated to afford the product **15c** (39 mg, 0.07 mmol) as a white solid. Yield: 59%. Mp: 205°C. ¹H NMR (300 MHz, DMSO-*d*₆): δ = 1.17-1.33 (m, 2H, CH₂), 1.33-1.47 (m, 2H, CH₂), 2.43-2.52 (m, 2H, CH₂), 3.05-3.14 (m, 2H, CH₂), 5.89 (d, *J* = 7.8 Hz, 1H, CH), 7.19 (dd, *J* = 1.8 and 8.7 Hz, 1H, CH), 7.27 (dd, *J* = 1.8 and 8.7 Hz, 1H, CH), 7.33-7.41 (m, 4H, CH), 7.88 (d, *J* = 1.8 Hz, 1H, CH), 8.14-8.24 (m, 2H, CH and NH), 8.28-8.32 (m, 2H, CH), 11.22 (br s, 1H, NH) ppm. IR (ATR): 3256, 2928, 2869, 1610, 1531, 1444, 1205 cm⁻¹. HRMS (ESI⁺) calcd for C₂₃H₂₄Br₂N₅O₂ [(M+H)⁺]: 560.0291, 562.0272 and 564.0256. Found: 560.0285, 562.0267 and 564.0248.

Synthesis of bis-indole compounds **17a-d** (Figure 5)

2-(1*H*-Indol-3-yl)-2-oxoacetyl chlorides (16a-c**)**

The 2-(1*H*-indol-3-yl)-2-oxoacetyl chlorides **16a-c** were already described.^{22,23,40}

Synthesis of α-keto-amides **17a-d**

tert*-Butyl 2-(2-(1*H*-indol-3-yl)-2-oxoacetamido)-2-(1*H*-indol-3-yl)ethyl)carbamate **17a*

This compound was already described.²² LRMS (ESI⁺): *m/z* = 469 [(M+Na)⁺], 485 [(M+K)⁺], 915 [(2M+Na)⁺]. HRMS (ESI⁺) calcd for C₂₅H₂₇N₄O₄ [(M+H)⁺]: 447.2027. Found: 447.2025.

tert*-Butyl 2-(2-(6-bromo-1*H*-indol-3-yl)-2-oxoacetamido)-2-(1*H*-indol-3-yl)ethyl)carbamate **17b*

This compound was already described.²² HRMS (ESI⁺) calcd for C₂₅H₂₆BrN₄O₄ [(M+H)⁺]: 525.1132 and 527.1115. Found: 525.1126 and 527.1108.

tert*-Butyl 2-(2-(5-bromo-1*H*-indol-3-yl)-2-(2-(5-bromo-1*H*-indol-3-yl)-2-oxoacetamido)ethyl)carbamate **17c*

In an oven-dried flask under argon, the indolic primary amine **7b** (170 mg, 0.480 mmol) and triethylamine (77 mg, 0.768 mmol) were dissolved in 5 mL of dry dichloromethane and 1 mL of dry THF. After cooling this solution to 0°C, the acid chloride **16b** (165 mg, 0.576 mmol) was added and the mixture was stirred at 0°C for one hour. The reaction was then quenched with an aqueous saturated solution of NaHCO₃ and diluted with ethyl acetate. The organic layer was washed with saturated NaHCO₃ and water, dried over anhydrous MgSO₄ and concentrated. The crude material was purified by flash chromatography (EtOAc/pentane, 7/3) to afford the desired product **17c** (180 mg, 0.300 mmol) as a white solid. Yield: 62%. Mp: 207°C. ¹H NMR (400 MHz, DMSO-*d*₆): δ = 1.34 (s, 9H, CH₃), 3.45-3.52 (m, 2H, CH₂), 5.34-5.40 (m, 1H, CH), 6.98 (t, *J* = 5.6 Hz, 1H, NH), 7.19 (d, *J* = 8.4 Hz, 1H, CH), 7.31 (d, *J* = 8.8 Hz, 1H, CH), 7.38-7.42 (m, 2H, CH), 7.51 (d, *J* = 8.4 Hz, 1H, CH), 7.84 (s, 1H, CH), 8.34 (d, *J* = 1.6 Hz, 1H, CH), 8.74 (s, 1H, CH), 8.92 (d, *J* = 8.8 Hz, 1H, NH), 11.19 (br s, 1H, NH), 12.39 (br s, 1H, NH) ppm. ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 28.1 (CH₃), 43.8 (CH₂), 45.6 (CH), 77.7 (C), 111.2 (C), 111.6 (C), 113.4 (CH), 113.6 (C), 114.6 (CH), 115.2 (C), 120.9 (CH), 123.2 (CH), 123.5 (CH), 124.5 (CH), 125.9 (CH), 127.9 (C), 128.0 (C), 134.7 (C), 135.0 (C), 139.2 (CH), 155.8 (C), 162.7 (C), 182.1 (C) ppm. IR (ATR): 3480, 3332, 3175, 1672, 1615, 1494, 1425, 1286, 1226, 1149 cm⁻¹. HRMS (ESI⁺) calcd for C₂₅H₂₅Br₂N₄O₄ [(M+H)⁺]: 603.0237, 605.0218 and 607.0203. Found: 603.0228, 605.0210 and 607.0191.

2-(5-Bromo-1*H*-indol-3-yl)-*N*-(1-(5-bromo-1*H*-indol-3-yl)ethyl)-2-oxoacetamide **17d**

In a dry flask under argon, the indolic amine **11** (100 mg, 0.420 mmol) and triethylamine (67 mg, 0.670 mmol) were dissolved in 3 mL of dry dichloromethane. After cooling this solution to 0°C, the acid chloride **16b** (143 mg, 0.502 mmol) was added and the mixture was stirred at 0°C for 30 minutes. The reaction was then quenched with an aqueous saturated solution of NaHCO₃ and diluted with ethyl acetate. The organic layer was washed with saturated NaHCO₃ and water, dried over anhydrous MgSO₄ and concentrated. The crude material was purified by flash chromatography (EtOAc/pentane, 1/1) to afford the desired product **17d** (130 mg, 0.266 mmol) as a white solid. Yield: 63%. Mp: 141°C. ¹H NMR (300 MHz, DMSO-d₆): δ = 1.62 (d, *J* = 6.9 Hz, 3H, CH₃), 5.34-5.44 (m, 1H, CH), 7.17 (d, *J* = 8.4 Hz, 1H, CH), 7.32-7.41 (m, 3H, CH), 7.52 (d, *J* = 8.4 Hz, 1H, CH), 7.86 (s, 1H, CH), 8.34 (d, *J* = 2.1 Hz, 1H, CH), 8.74 (s, 1H, CH), 8.94 (d, *J* = 8.8 Hz, 1H, NH), 11.15 (br s, 1H, NH), 12.41 (br s, 1H, NH) ppm. ¹³C NMR (75.5 MHz, DMSO-d₆): δ = 20.3 (CH₃), 40.6 (CH), 111.2 (C), 111.7 (C), 113.4 (CH), 114.6 (CH), 115.2 (C), 116.9 (C), 121.1 (CH), 123.2 (CH), 123.5 (CH), 124.1 (CH), 126.0 (CH), 127.5 (C), 127.8 (C), 134.97 (C), 135.03 (C), 139.1 (CH), 162.5 (C), 182.6 (C) ppm. IR (ATR): 3270, 2975, 1665, 1614, 1494, 1418, 1229, 1124 cm⁻¹. HRMS (ESI⁺) calcd for C₂₀H₁₆Br₂N₄O₄ [(M+H)⁺]: 487.9604, 489.9584 and 491.9567. Found: 487.9598, 489.9580 and 491.9569.

Cytotoxicity assays

Cytotoxicity assays performed on KB, MCR5 and HCT116 cell lines (Table S1)

Table S1 Results of cytotoxicity assays performed on KB (human mouth carcinoma), MCR5 (human lung fibroblast) and HCT116 (human colon tumor) cell lines, expressed as the percentage of cellular growth inhibition of bis-indolic compounds tested at 10^{-5} M (10^{-6} M).

Compounds	KB	MRC5	HCT116
	10^{-5} M (10^{-6} M)	10^{-5} M (10^{-6} M)	10^{-5} M (10^{-6} M)
9a	18±1 (16±1)	14±1 (2±7)	22±5 (11±2)
9b	41±6 (0±4)	8±5 (7±4)	30±2 (0±9)
9c	46±8 (0±16)	16±9 (0±5)	42±3 (11±2)
9d	67±3 (0±9)	73±6 (0±1)	73±3 (9±5)
9e	99±1 (0±22)	92±6 (0±9)	92±1 (2±5)
9f	97±1 (0±5)	91±10 (0±8)	89±9 (0±9)
9g	98±1 (0±10)	83±10 (0±6)	86±2 (9±4)
9h	100±1 (0±3)	92±1 (0±16)	100±3 (0±1)
9i	100±1 (3±1)	91±7 (10±5)	91±1 (4±7)
9j	87±6 (4±8)	82±4 (0±9)	80±9 (0±5)
9k	92±4 (2±4)	99±1 (0±3)	97±6 (0±4)
10a	13±3 (0±5)	36±4 (7±4)	12±1 (0±3)
10b	ND	ND	ND
12	52±2 (2±7)	23±6 (12±3)	35±3 (8±3)
14a	51±1 (0±2)	28±9 (0±1)	51±5 (0±5)
14b	0±1 (0±4)	3±3 (3±2)	0±4 (0±2)
14c	1±4 (0±3)	6±1 (0±2)	12±5 (0±5)
15c	ND	ND	ND
17a	97±1 (33±8)	100±2 (10±5)	96±1 (53±4)
17b	100±1 (4±3)	98±1 (0±3)	97±1 (0±13)
17c	76±5 (0±7)	51±9 (0±3)	58±4 (0±9)
17d	91±3 (0±11)	85±2 (6±3)	80±3 (10±6)

ND: not determined

5. CONCLUSION ET PERSPECTIVES

Comme nous avons pu le voir dans l'introduction de cette thèse, les taux d'échecs thérapeutiques et de rechutes après traitement antibiotique chez les patients atteints de tularémie demeurent élevés, peuvent aller jusqu'à près de 40% dans certaines études, selon la classe d'antibiotique concernée. Nos investigations sur les causes potentielles de ces échecs et notre volonté de rechercher des stratégies thérapeutiques alternatives amènent aux conclusions suivantes. Notre revue de la littérature a tout d'abord permis de confirmer l'absence de souches résistantes vis-à-vis des classes d'antibiotiques utilisées pour le traitement de première intention de la tularémie (aminosides, fluoroquinolones, et doxycycline). Nous avons colligé l'ensemble des données de sensibilité de *F. tularensis* aux antibiotiques disponibles à ce jour, déterminées à la fois *in vitro* et en modèle animal. La première observation qui découle de cette revue de la littérature est le manque de standardisation des techniques de détermination de la sensibilité aux antibiotiques concernant l'espèce *F. tularensis*, entre les pays et les études. On retrouve ainsi des données obtenues à la fois par la technique de microdilution en milieu liquide, par la technique E-test® en bandelette de diffusion en milieu gélosé de réalisation beaucoup plus aisée, et par la technique de diffusion en agar. La technique de référence recommandée à l'heure actuelle est la microdilution en milieu liquide utilisant du bouillon de Mueller-Hinton supplémenté avec 2% de facteurs de croissance de type Polyvitex®. Les techniques alternatives (notamment l'utilisation de bandelettes E-test®) ne peuvent pas être formellement recommandées à l'heure actuelle devant l'absence de données expérimentales suffisantes, évaluant la corrélation de ces techniques alternatives avec la technique de référence. Cependant la dispersion des CMI semble faible entre les différentes techniques. Nous envisageons de comparer ces méthodes dans des études ultérieures. En cas de résultats concluants, la technique en bandelette E-test pourrait fournir une alternative rapide et simple pour l'étude de la sensibilité d'une souche isolée sporadiquement par rapport à la microdilution en milieu liquide qui est une technique plus chronophage, plus fastidieuse et plus à risque pour le personnel de laboratoire. Cette dernière resterait néanmoins la technique de référence, qui serait réalisée sur de plus grandes séries de souches dans les Centres de Référence.

En terme d'activité antibactérienne, les fluoroquinolones se sont révélées être les antibiotiques possédant les CMI les plus basses et l'activité intracellulaire la plus rapide par

rapport à la gentamicine, dont l'effet bactéricide intracellulaire nécessite au minimum 48h, et la doxycycline qui n'est que bactériostatique. L'activité bactéricide des fluoroquinolones a été confirmée *in vitro* et en modèle animal. Les données issues des modèles animaux ont permis de confirmer les observations cliniques quant à l'infériorité de la doxycycline par rapport à la ciprofloxacine pour le traitement des formes modérées de tularémie, notamment en cas de délai avant instauration d'une antibiothérapie efficace, ce qui en pratique est souvent le cas. En modèle murin, la doxycycline est associée à davantage d'échecs thérapeutiques en comparaison avec la ciprofloxacine, lorsque le traitement est débuté 24 à 72h post-infection. Les données en modèle animal confirment également l'importance de la durée du traitement et de l'utilisation d'antibiotiques bactéricides afin d'éviter la persistance de l'infection. En outre, la revue de la littérature a permis d'identifier des pistes thérapeutiques intéressantes (azithromycine, télithromycine), notamment pour le traitement des cas de tularémie liés aux souches de biovar I de *F. tularensis* subsp. *holarctica*. Les souches de biovar I semblent devoir être catégorisées « intermédiaires » pour les macrolides, mais l'azithromycine a montré une bonne efficacité en modèle cellulaire. Il faut cependant garder à l'esprit que la concentration intracellulaire de l'azithromycine est plus importante dans les polynucléaires que dans d'autres types cellulaires. Il manque donc à ce stade une étude en modèle animal pour confirmer ces données et pouvoir envisager plus sereinement l'utilisation de ces molécules, en cas de contre-indication aux aminosides, aux fluoroquinolones et aux tétracyclines, comme cela peut s'observer en cas de grossesse. La télithromycine semble à ce titre encore plus intéressante que l'azithromycine de par ses CMI plus basses et son activité bactéricide intracellulaire réduisant de deux à trois \log_{10} l'inoculum bactérien en 72h.

Le faible nombre de données publiées concernant la sensibilité des souches françaises de *F. tularensis* subsp. *holarctica*, dont toutes appartiennent à la lignée clonale B. FTNF002-00 présente en Europe de l'Ouest, nous a conduit à mener une étude sur la collection de souches du CNR des *Francisella*. Cette étude a démontré l'absence de souches résistantes aux fluoroquinolones, à la gentamicine et à la doxycycline en France. Par le biais de cet article, de nouvelles recommandations thérapeutiques pour le traitement de la tularémie ont été proposées. Premièrement l'affirmation formelle de la supériorité des fluoroquinolones sur la doxycycline pour le traitement des formes modérées de tularémie avec un positionnement de

la doxycycline uniquement en cas de contre-indication aux fluoroquinolones. Deuxièmement la proposition d'une bithérapie pour les formes sévères de tularémie, associant la ciprofloxacine pendant 10 à 14 jours et la gentamicine en monodose journalière pendant uniquement 5 à 7 jours, afin de : 1/limiter la néphrotoxicité du traitement par gentamicine, 2/de fournir une activité bactéricide intracellulaire pendant les 48 premières heures de traitement pendant lesquelles la diffusion intracellulaire de la gentamicine est très faible, et 3/de permettre un relai par voie orale par fluoroquinolone lors de l'amélioration clinique du patient. Dans cette étude, l'intérêt de la télithromycine a été confirmé, avec plus de la moitié des souches évaluées catégorisées sensibles à cet antibiotique en se basant sur les concentrations critiques définies pour *Haemophilus influenzae*. Son étude doit être approfondie par l'évaluation de son activité antibactérienne vis à vis des souches de biovar I en modèle animal. Elle pourrait fournir une alternative thérapeutique encore plus intéressante que l'azithromycine en cas de contre-indications aux molécules recommandées actuellement pour le traitement de la tularémie. Enfin, cette étude nous a permis de proposer des concentrations critiques épidémiologiques pour une meilleure détection de potentielles apparitions de résistance aux fluoroquinolones, à la gentamicine et à la doxycycline chez *F. tularensis*.

L'absence d'isolement de souches résistantes n'a pas permis de fournir une explication quant aux échecs thérapeutiques observés, alors que la génération de souches résistantes est rapide et facile *in vitro*. Nous avons alors voulu rechercher des phénomènes de résistance bactérienne potentiels par des techniques de biologie moléculaire, directement sur des prélèvements de patients, en se limitant dans cette thèse à rechercher des mutations de résistance aux fluoroquinolones. Dans un premier article, nous avons validé l'impact fonctionnel de mutations de résistance générées *in vitro* sur des lignées de *F. novicida*, utilisées comme modèle avirulent pour l'étude de la résistance aux fluoroquinolones chez *F. tularensis*. Parmi les mutations étudiées, celles en position 87 du QRDR de la sous-unité GyrA, ainsi que la mutation +P466 dans le QRDR de la sous-unité GyrB semblent avoir le plus d'impact sur le niveau de résistance aux fluoroquinolones chez *F. novicida*. Des mutations sur ces mêmes positions ainsi qu'en position 83 de la sous-unité GyrA sont décrites chez *F. tularensis*. Par ailleurs nous avons validé l'impact sur la résistance aux fluoroquinolones de

mutations situées en dehors des QRDR des gènes *gyrA* et *gyrB*. La mutation P43H au niveau de la sous-unité GyrA inhibe de façon significative mais plus modérée l'activité des fluoroquinolones sur le surenroulement et le relâchement de l'ADN. La mutation D487E- Δ K488 en dehors du QRDR de la sous-unité GyrB ne semble en revanche pas entraîner de résistance aux fluoroquinolones en elle-même, mais potentialise la résistance liée à l'acquisition d'une deuxième mutation en position 87 de la sous-unité GyrA. Il est donc malgré tout intéressant de la rechercher. De plus cette étude a permis de mettre en évidence que chez *Francisella*, l'apparition de mutations sur le gène *gyrB* pouvait s'avérer plus précoce que l'acquisition de mutations sur le gène *gyrA*. En revanche, la recherche de mutations de résistance dans le QRDR des gènes *gyrA* et *gyrB* chez 32 patients en situation d'échec thérapeutique après traitement par une fluoroquinolone n'a pas permis de mettre en évidence des mutations de résistance *in vivo* dans ces deux séquences génomiques. Les causes des échecs cliniques observés, en ce qui concerne les fluoroquinolones, demeurent donc inexplicables.

Plusieurs hypothèses et pistes d'exploration futures sont envisageables. Un des facteurs importants pour le succès thérapeutique semble être le délai avant l'instauration d'une antibiothérapie efficace, notamment en cas d'atteinte ganglionnaire. Des différences significatives ont été observées lorsque le traitement est débuté dans les trois semaines après l'apparition des premiers signes cliniques par rapport aux traitements débutés plus tardivement. L'amélioration de la connaissance de la maladie par les médecins généralistes français pourrait donc permettre de diminuer les taux d'échec thérapeutiques. Peu de données sont disponibles en France concernant les traitements utilisés en fonction de la sévérité ou du type de forme clinique de la maladie. De même, l'évolution des patients en fonction de l'antibiotique utilisé et du délai avant mise en route de l'antibiothérapie est mal connue en France. Une étude nationale réalisée dans le cadre du Centre National de Référence des *Francisella*, semble opportune pour répondre à ces questions.

La deuxième hypothèse pour expliquer ces échecs reste la présence de souches résistantes *in vivo* bien que non détectées à ce jour, comme cela a été le cas jusqu'à récemment pour *Legionella pneumophila*. La sensibilité insuffisante des techniques de culture ou de PCR conventionnelles pourrait être à l'origine de ces résultats. L'amélioration des conditions de

culture de *F. tularensis* (utilisation de flacons d'hémoculture, mise au point d'un milieu de culture spécifique) sont des pistes à explorer afin de pouvoir isoler les souches résistantes malgré la diminution de leur taux de croissance, lié au coût répliatif de l'acquisition de mutations de résistances. L'alternative possible est de détecter ces mutations par biologie moléculaire directement dans les prélèvements de patients en échec thérapeutique, sans nécessité de culture de la souche. Dans cette hypothèse de souches résistantes, soit les mutations de résistance sont situées dans les zones de mutations décrites *in vitro* (par exemple dans les QRDR des gènes *gyrA* et *gyrB* pour ce qui est des fluoroquinolones). Des techniques plus sensibles comme la PCR digitale, ciblant des mutations ponctuelles particulières, pourraient alors être envisageables. Soit les mutations de résistance sont situées en dehors des zones étudiées (par exemple en dehors des QRDR des gènes *gyrA* et *gyrB* pour ce qui est des fluoroquinolones). La solution serait alors de rechercher ces mutations par séquençage de génomes bactériens complets en utilisant des techniques de séquençage de nouvelle génération directement sur les prélèvements de patients. Dans ce dernier cas, cela nécessiterait cependant de traiter au préalable l'échantillon par à une technique permettant d'augmenter le ratio ADN bactérien/ADN humain dans le prélèvement afin d'obtenir une sensibilité de détection suffisante des mutations par le biais d'une profondeur de séquençage suffisante.

Les autres pistes à explorer pour expliquer ces échecs thérapeutiques sont diverses. La recherche de bactéries quiescentes ou persistantes, c'est à dire ayant une activité métabolique très réduite, est une piste d'intérêt car les antibiotiques utilisés pour le traitement de la tularémie ne sont actifs que sur les bactéries métaboliquement actives. Les bactéries persistantes sont des variants phénotypiques présents au sein d'une population bactérienne, qui se mettent dans un état de dormance lorsqu'elles font face à divers stress, notamment en cas de traitement antibiotique. (258) Les antibiotiques agissant sur les grandes fonctions métaboliques de la bactérie (synthèse des protéines, transcription de l'ARN, répliation de l'ADN, synthèse du peptidoglycane), la présence de cellules persistantes s'accompagne d'un phénomène dit de tolérance aux antibiotiques, malgré l'absence de mutations de résistance. De plus la réduction de l'activité métabolique des bactéries peut être à l'origine d'une diminution de la pénétration des antibiotiques à l'intérieur de la bactérie

notamment si ceux-ci pénètrent par des systèmes de transports actifs. La proportion de cellules persistantes au sein de la population bactérienne à l'origine de l'infection est contrôlée par les voies de signalisation de la réponse des bactéries au stress et par la voie SOS ainsi que par certaines alarmones comme le (p)ppGpp. (258) A l'arrêt de l'antibiothérapie, les cellules quiescentes sont capables de se réactiver, entraînant alors des phénomènes d'échec thérapeutiques. La présence de bactéries persistantes au site de l'infection et tolérantes au traitement antibiotiques est donc une hypothèse très intéressante à investiguer dans de futurs travaux. Une autre possibilité est la formation de biofilm par *F. tularensis* au site de l'infection, permettant à la bactérie de limiter l'action des traitements antibiotiques et de persister à l'intérieur du biofilm. La formation de biofilm *in vitro* a déjà été démontrée pour *F. tularensis* subsp. *tularensis* SchuS4, *F. tularensis* subsp. *holarctica* LVS, *F. novicida* et *F. philomiragia*. (259) Cette formation de biofilm pourrait être impliquée dans la persistance environnementale de la bactérie mais également être à l'origine d'échec thérapeutique en cas de formation de biofilm *in vivo* au site de l'infection, notamment en cas d'adénopathie traitée tardivement. La formation de biofilm peut également permettre à la bactérie d'entrer dans un état viable mais non cultivable, ce qui pourrait expliquer certaines des difficultés à isoler la bactérie en cas d'échec thérapeutique. Enfin une dernière hypothèse ne peut être écartée concernant certaines situations d'échec thérapeutique. Il s'agit de la mauvaise observance des patients lors de leur traitement de 10 à 14 jours par fluoroquinolones ou de 14 à 21 jours par doxycycline.

Dans la dernière partie de cette thèse, nous avons évalué l'activité antibactérienne de nouveaux composés bis-indoliques de synthèse, de structure proche de celles d'alcaloïdes isolés d'éponges marines. La recherche de nouveaux composés antibiotiques est une priorité de l'organisation mondiale de la santé car très peu d'antibiotiques possédant un mécanisme d'action original et non soumis aux mécanismes de résistances connus à l'heure actuelle, ont été développés au cours des trois dernières décennies. *F. tularensis* ne fait pas partie de la liste des bactéries pathogènes prioritaires pour l'OMS, mais les taux d'échec cliniques rapportés avec les traitements actuels restent trop élevés, justifiant la recherche d'alternatives thérapeutiques. En revanche plusieurs bactéries potentiellement multi-résistantes, en particulier les staphylocoques dorés résistants à la méticilline (MRSA) ou

intermédiaires à la vancomycine (VISA) ainsi que les *E. faecium* résistants à la vancomycine sont catégorisés comme des pathogènes prioritaires pour l'OMS en ce qui concerne la recherche de nouvelles possibilités thérapeutiques. Les composés bis-indoliques de synthèse évalués et développés en collaboration avec le Département de Chimie Moléculaire de l'Université Grenoble Alpes, se sont avérés posséder un spectre antibactérien principalement dirigé contre les bactéries à Gram positif, tout en démontrant une activité antibactérienne vis-à-vis de *F. tularensis*. En particulier, le composé dcm04 a présenté une CMI₉₀ à 2 mg/L vis-à-vis de *F. tularensis* et des staphylocoques, avec une activité identique sur les souches multi-résistantes de *S. aureus*. Les composés bis-indoliques les plus actifs se sont révélés bactéricides vis-à-vis des staphylocoques et de la souche LVS de *F. tularensis*, mais uniquement bactériostatiques sur les souches cliniques de *F. tularensis*. En revanche, ces composés sont peu solubles en milieu aqueux et ont une forte affinité pour les protéines plasmatiques, ce qui appelle des développements futurs. La question qui vient dès lors à l'esprit est celle de la cible bactérienne potentielle de ces molécules. Des investigations sont actuellement en cours pour répondre à cette question. Pour des raisons de conditions expérimentales plus aisées avec *S. aureus* par rapport à *F. tularensis*, notamment l'absence de manipulation en LNSB3, le modèle expérimental choisi pour la détermination de la cible s'est porté sur *S. aureus*. Des lignées de *S. aureus* résistantes à certains de ces composés bis-indoliques ont été générées *in vitro* selon la même méthodologie que celle employée par Sutura et al, à partir de souches de *S. aureus* dont le génome complet a été séquencé. (224) Comme observé pour les fluoroquinolones, les mutations de résistance apparaissent fréquemment dans les gènes codant les cibles des antibiotiques. Bien qu'aucune mutation de résistance n'ait pu être obtenue en « single step » (Article 6), quelques rares mutants résistants ont pu être isolés par sélection en multi-passages en présence de concentrations sub-inhibitrices d'antibiotiques. La poursuite du projet consiste à re-séquencer ces souches résistance pour rechercher des mutations de résistance et ainsi identifier le gène cible potentiel. Par ailleurs, des tentatives d'amélioration de la solubilité des composés les plus actifs sont en cours, notamment par l'ajout de groupement hydrophiles sur les structures existantes, afin d'obtenir des composés hydrosolubles, tout en conservant voire augmentant leur activité antibactérienne et leur spectre. Cette augmentation de la solubilité est un prérequis à l'analyse future de ces composés en modèle cellulaire puis en modèle animal.

Les perspectives de ce travail sont donc multiples et exaltantes, dépassant le seul cadre de la tularémie. De nombreuses questions restent en suspens pour diminuer les échecs thérapeutiques dans les cas de tularémie mais plusieurs pistes devront être explorées, dont l'une conduira peut-être au développement d'une nouvelle famille d'antibiotiques.

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ANNEXES

Annexe 1 : Composition des géloses chocolat et du mélange PolyViteX™

Annexe 2 : Fiche de déclaration obligatoire de la tularémie

Gélose Chocolat (CHOCO-F)/ Mélange PolyViteX™ (PVX-M)

IVD

Isolement des bactéries exigeantes

INTRODUCTION ET OBJET DU TEST

La gélose Chocolat s'utilise additionnée du mélange PolyViteX. Ce milieu est destiné à la culture et à l'isolement des souches exigeantes appartenant aux genres *Neisseria*, *Haemophilus*, *Streptococcus* (*S. pneumoniae*).

PRINCIPE

Ce milieu est composé d'une base nutritive enrichie en facteur X (hémine) apporté par l'hémoglobine.

Les facteurs de croissance indispensables, comme le facteur V (NAD), sont apportés par le mélange PolyViteX (1, 2).

La sélectivité de la gélose Chocolat additionnée de PolyViteX peut être assurée par l'addition du mélange VCN, pour la recherche des *Neisseria* (*N. meningitidis* et *N. gonorrhoeae*).

PRESENTATION

Références commercialisées séparément

	Milieu prêt à l'emploi
REF 41 536	Gélose Chocolat
	Coffret de 6 flacons de 100 ml

	Mélange PolyViteX
REF 55 651	R1 : 4 x →1 ml (lyophilisé) R2 : 4 x 1 ml de solvant 1 notice fournie dans le coffret ou téléchargeable sur www.biomerieux.com/techlib
REF 55 652	R1 : 4 x →10 ml (lyophilisé) R2 : 4 x 10 ml de solvant 1 notice fournie dans le coffret ou téléchargeable sur www.biomerieux.com/techlib

COMPOSITION

Ces milieux peuvent être ajustés et/ou supplémentés en fonction des critères de performances imposés :

Gélose Chocolat**Formule théorique**

Peptone de caséine (bovin)	7,5 g
Peptone de viande (bovin ou porcin)	7,5 g
Amidon de maïs.....	1 g
Phosphate dipotassique	4 g
Chlorure de sodium	5 g
Hémoglobine (bovin).....	10 g
Agar.....	10 g
Eau purifiée	1 l

pH 7,2

Mélange PolyViteX

Formule théorique en mg/l après addition à 100 ml de base :

Vitamine B12.....	0,1
L-Glutamine.....	100
Adénine.....	10
Chlorhydrate de guanine	0,3
Acide para-aminobenzoïque.....	0,13
L-Cystine.....	11
Diphosphopyridine nucléotide oxydé.....	2,5
Coccarboxylase	1,04
Nitrate ferrique	0,2
Chlorhydrate de thiamine	0,03
Chlorhydrate de cystéine.....	259

MATERIEL NECESSAIRE MAIS NON FOURNI

- Boîtes de Petri stériles.
- Bains-marie.
- Générateurs d'atmosphère contrôlée.
- Jarres.
- Etuve bactériologique.
- Ou
- Enceintes thermorégulées à atmosphère contrôlée.

REACTIF COMPLEMENTAIRE

- Mélange VCN (Réf. 55 663 ou 55 664).

PRECAUTIONS D'UTILISATION

- **Pour diagnostic *in vitro* uniquement.**
- **Pour usage professionnel uniquement.**
- Ce coffret contient des composants d'origine animale. La maîtrise de l'origine et/ou de l'état sanitaire des animaux ne pouvant garantir de façon absolue que ces produits ne contiennent aucun agent pathogène transmissible, il est recommandé de les manipuler avec les précautions d'usage relatives aux produits potentiellement infectieux (ne pas ingérer, ne pas inhaler).
- Les prélèvements, cultures bactériennes et produits ensemencés doivent être considérés comme potentiellement infectieux et doivent être manipulés de façon appropriée. Les techniques aseptiques et les précautions usuelles de manipulation pour le groupe bactérien étudié doivent être respectées tout au long de la manipulation; se référer à "CLSI® M29-A, *Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline* – Révision en vigueur". Pour informations complémentaires sur les précautions de manipulation, se référer à "Biosafety in Microbiological and Biomedical Laboratories – CDC/NIH - Dernière édition", ou à la réglementation en vigueur dans le pays d'utilisation.
- Les milieux de culture ne doivent pas être utilisés comme matériau ou composant de fabrication.
- Ne pas utiliser les réactifs après la date de péremption.
- Ne pas utiliser des flacons présentant une suspicion de contamination.
- Avant utilisation, s'assurer de l'intégrité des ergots d'inviolabilité de la capsule des flacons et de l'intégrité des bouchons.
- Après régénération et supplémentation, le flacon doit être intégralement réparti en boîtes (le milieu ne pouvant pas être liquéfié plusieurs fois).
- Les performances présentées ont été obtenues avec la méthodologie indiquée dans cette notice. Toute déviation de méthodologie peut modifier les résultats.
- L'interprétation des résultats du test doit être faite en tenant compte du contexte clinique, de l'origine du prélèvement, des aspects macro et microscopiques et éventuellement des résultats d'autres tests.

CONDITIONS DE STOCKAGE

- **Les flacons de gélose se conservent entre 2°C et 25°C dans leur coffret jusqu'à la date de péremption.**
- **Les flacons de mélange se conservent entre 2°C et 8°C dans leur coffret jusqu'à la date de péremption.**

- Après répartition en boîtes de Petri, les milieux supplémentés en PolyViteX™ se conservent 1 semaine à 2 - 8°C.
- Après reconstitution du mélange PolyViteX :
 - Le flacon de 1 ml est utilisé extemporanément.
 - Le flacon de 10 ml se conserve 1 semaine à 2-8°C.

ECHANTILLONS

Les prélèvements peuvent être de toute nature et sont directement ensemencés sur la gélose.

Il convient de respecter les bonnes pratiques en terme de prélèvements et de transport, adaptées à chaque type de prélèvement (3).

Ce milieu peut être utilisé pour le repiquage des souches bactériennes afin d'obtenir des cultures pures.

MODE OPERATOIRE

Préparation du mélange PolyViteX :

1. Laisser les flacons revenir à température ambiante.
2. Reprendre aseptiquement le flacon de réactif lyophilisé (R1) par un volume de solvant (R2) mesuré précisément (1 ml pour la réf. 55 651, 10 ml pour la réf. 55 652).
3. Agiter pour assurer une dissolution complète.

Préparation des boîtes de Petri :

1. Desserrer au préalable la capsule du flacon de gélose.
2. Mettre le flacon à régénérer dans un bain marie sécurisé à environ 50°C, monter la température jusqu'à 95°C et laisser fondre la gélose (environ 45 minutes).
3. Homogénéiser après fermeture de la capsule (utiliser des gants de protection contre les risques thermiques):
4. Laisser les flacons à température ambiante au moins 15 secondes avant de les transférer dans un bain d'eau thermostaté à environ 45-50°C. Maintenir les flacons à cette température jusqu'au moment de l'utilisation.
5. Ajouter 1 ml du contenu du flacon de mélange PolyViteX dans 100 ml de gélose Chocolat.

Remarque : l'incorporation éventuelle du mélange sélectif VCN est réalisée simultanément à l'addition du mélange PolyViteX (se reporter à la notice correspondante pour la préparation de ce mélange).

6. Homogénéiser et répartir en boîtes de Petri (à raison de 18 à 20 ml par boîte).
7. Après reprise et refroidissement du milieu, placer les boîtes à 2 - 8°C.

Ensemencement et incubation :

1. Laisser les boîtes revenir à température ambiante.
2. Ensemencer le prélèvement dès son arrivée au laboratoire.
3. Placer la boîte en atmosphère appropriée en utilisant éventuellement des générateurs d'atmosphère contrôlée.
4. Incuber à l'étuve, couvercle en bas, à 37°C. Le choix de la température et de l'atmosphère d'incubation est de la responsabilité de l'utilisateur en fonction de l'application et des normes en vigueur.

La durée d'incubation varie selon le type de prélèvement et la nature des micro-organismes recherchés. Les cultures sont examinées généralement après 24 à 48 heures d'incubation. Dans certains cas, il peut être nécessaire de prolonger l'incubation.

LECTURE ET INTERPRETATION

- Après incubation, observer la croissance bactérienne.
- L'identification du ou des micro-organismes isolés doit être réalisée grâce à des tests complémentaires.

CONTROLE DE QUALITE

Protocole :

La fertilité de la gélose Chocolat additionnée de mélange PolyViteX peut être testée vis-à-vis des souches suivantes incubées sous atmosphère enrichie en CO₂ :

- *Neisseria gonorrhoeae* ATCC® 43069
- *Haemophilus influenzae* ATCC® 10211

Résultats attendus :

Souche	Résultat à 33-37°C
<i>Neisseria gonorrhoeae</i> ATCC® 43069	Croissance en 48 heures
<i>Haemophilus influenzae</i> ATCC® 10211	

Remarque :

Il est de la responsabilité de l'utilisateur de prendre en compte la nature de l'application et la législation locale en vigueur pour la mise en oeuvre du contrôle de qualité (fréquence, nombre de souches, température d'incubation...).

LIMITES DU TEST

- Le développement est fonction des exigences propres à chaque micro-organisme. Il est donc possible que certaines souches ayant des exigences spécifiques ne se développent pas.
- En fonction des prélèvements analysés et selon les micro-organismes recherchés, il est recommandé d'associer la gélose Chocolat PolyViteX avec des milieux complémentaires (milieux sélectifs) (1).

PERFORMANCES

Les performances ont été évaluées, à 37°C sur la gélose Chocolat additionnée du mélange PolyViteX, sur 38 souches bactériennes (*N. gonorrhoeae*, *N. meningitidis*, *Haemophilus*, *Streptococcus pneumoniae*, autres bactéries Gram (+) et Gram (-)) et 2 levures (*Candida* et *Saccharomyces*).

Fertilité :

Toutes les souches se sont développées en 24 heures, à l'exception d'une souche d'*Haemophilus equigenitalis* qui s'est développée en 48 heures.

ELIMINATION DES DECHETS

Les réactifs non utilisés peuvent être éliminés comme déchets non dangereux.








Éliminer les réactifs utilisés ainsi que les matériels à usage unique contaminés en suivant les procédures relatives aux produits infectieux ou potentiellement infectieux.

Il incombe à chaque laboratoire de gérer les déchets et les effluents qu'il produit selon leur nature et leur dangerosité, et d'en assurer (ou faire assurer) le traitement et l'élimination selon les réglementations applicables.

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TABLE DES SYMBOLES

Symbole	Signification
	Référence du catalogue
	Dispositif médical de diagnostic <i>in vitro</i>
	Fabricant
	Limites de température
	Utiliser jusqu'à
	Code du lot
	Consulter les instructions d'utilisation

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Résumé

La tularémie est une zoonose liée à la bactérie *Francisella tularensis*, hautement pathogène pour l'homme. La sous espèce la plus virulente, *F. tularensis* subsp. *tularensis*, est retrouvée uniquement en Amérique du Nord, alors que la sous-espèce *F. tularensis* subsp. *holarctica* est présente dans tout l'hémisphère Nord. En France toutes les souches appartiennent au biovar I de la sous-espèce *holarctica* et plus précisément au groupe phylogénétique B.FTNF002-00. Bien que rarement grave en France, la tularémie pose le problème de taux d'échecs thérapeutiques élevés, jusqu'à 25% en cas de traitement par ciprofloxacine ou gentamicine, et 35% pour la doxycycline. Les causes de ces échecs ne sont pas bien élucidées à l'heure actuelle. L'analyse de la littérature ainsi que la détermination de la sensibilité de 59 souches françaises de *F. tularensis* subsp. *holarctica* à 18 antibiotiques, confirment qu'aucune souche isolée à ce jour ne présente de résistance acquise à ces trois familles d'antibiotiques, qui représentent le traitement de première ligne de la tularémie. Les fluoroquinolones (en particulier la ciprofloxacine et la lévofloxacine) présentent concentrations minimales inhibitrices les plus basses, devant la gentamicine et la doxycycline. Les données disponibles *in vitro* et en modèle animal étant corrélées aux données humaines en termes d'efficacité et de taux d'échecs thérapeutiques, il semble néanmoins préférable de positionner la ciprofloxacine en première ligne pour le traitement des formes modérées de tularémie et de limiter l'utilisation de la doxycycline aux cas de contre-indication aux fluoroquinolones. L'azithromycine et la téli-thromycine ont été identifiées comme des alternatives thérapeutiques envisageables en cas d'infection par une souche de biovar I de *F. tularensis* subsp. *holarctica* lorsqu'existe une contre-indication aux traitements de première ligne. Des études en modèles animaux restent néanmoins nécessaires pour conforter ces dernières observations. La sélection *in vitro* de souches résistantes aux fluoroquinolones est possible, ce qui suggère la possibilité d'émergence de mutants résistants *in vivo* pour expliquer les taux d'échec thérapeutiques. Les principales mutations de résistance aux fluoroquinolones chez *F. tularensis* sont observées au niveau des gènes *gyrA* et *gyrB* codant pour les topoisomérases de type II. L'impact fonctionnel de mutations de résistances aux fluoroquinolones a été caractérisé *in vitro* chez *F. novicida*, pris comme modèle de bactérie avirulente proche de *F. tularensis*. L'activité de superenroulement et de clivage de l'ADN en présence de fluoroquinolones a été déterminée suite à la reconstruction *in vitro* de complexes GyrA/GyrB fonctionnels. La résistance aux fluoroquinolones était la plus forte en cas de mutation D87G/D87Y pour la sous-unité GyrA ou +P466 pour la sous-unité GyrB. La mutation P43H située en dehors du QRDR de GyrA est à l'origine d'un plus faible niveau de résistance. La mutation D487R-ΔK488 en dehors du QRDR de GyrB ne confère pas de résistance intrinsèque mais potentialise l'effet d'une mutation D87G concomitante. En revanche, l'identification de mutations de résistance *in vivo* au sein des QRDR des gènes *gyrA* et *gyrB* chez des patients en situation d'échec thérapeutique traités par une fluoroquinolone est demeurée négative. Enfin, notre recherche a permis d'identifier de nouveaux composés de synthèse de structure bis-indolique possédant des activités antibactériennes. Ces composés sont bactériostatiques vis-à-vis de *F. tularensis* mais bactéricides vis-à-vis des staphylocoques y compris vis-à-vis de souches multi-résistantes de *Staphylococcus aureus* avec des CM₁₉₀ évaluées à 2mg/L chez *F. tularensis* et *S. aureus* pour le composé le plus actif. La faible solubilité de ces composés en milieu aqueux, leur forte liaison aux protéines plasmatiques ainsi que la recherche de leur mécanisme d'action original appellent néanmoins de nombreux développements futurs.

Mots Clés : Nouvelles stratégies thérapeutiques, *Francisella tularensis*, Composés bis-indoliques, Tularémie, Résistance bactérienne

Abstract

Tularemia is a zoonosis caused by the highly pathogenic bacterium *Francisella tularensis*. The most virulent subspecies, *F. tularensis* subsp. *tularensis*, is found only in North America while the subspecies *F. tularensis* subsp. *holarctica* is present in the whole Northern hemisphere. In France, all strains belong to the biovar I of the subspecies *holarctica* and more specifically to the phylogenetic subclade B.FTNF002-00. Although tularemia is usually not a severe disease in France, many patients suffer from therapeutic failures despite receiving an appropriate treatment. These treatments failures are observed in up to 25% of patients treated with ciprofloxacin or gentamicin, and up to 35% if patients treated with doxycycline. The causes of those therapeutic failures remain poorly elucidated. Analysis of the literature and determination of the susceptibility of 59 French *F. tularensis* subsp. *holarctica* strains to 18 antibiotics confirmed that to date, no strain with acquired resistance to any of the first-line antibiotics used for treatment of tularemia have been isolated. The fluoroquinolones (in particular ciprofloxacin and levofloxacin) exhibit the lowest minimal inhibitory concentrations, compared to gentamicin and doxycycline. Data obtained *in vitro* and in animal models are concordant with human data concerning the efficacy of antibiotics and therapeutic failure rates. Thus, we advocate the use of ciprofloxacin as first-line treatment for mild form of tularemia, and the use of doxycycline only as a second-line treatment in patients with contraindications to fluoroquinolones. Azithromycin and telithromycin may also be considered as potential therapeutic alternatives for tularemia cases caused by biovar I strains of the subspecies *holarctica*, but only for patients with contraindications to first-line antibiotics. Further data in animal models are however required to consolidate our *in vitro* data. The *in vitro* selection of fluoroquinolone-resistant strains of *F. tularensis* has been reported. This suggests that the *in vivo* selection of such resistant mutants may occur. *In vitro*, the main fluoroquinolone resistance mutations occur in the *gyrA* and *gyrB* genes that encode type II topoisomerases of *F. tularensis*. We have characterized the functional impact of such mutations in avirulent *F. novicida* strains, taken as a surrogate of *F. tularensis*. Supercoiling and DNA cleavage activity of GyrA/GyrB complexes reconstituted *in vitro* have been determined in the presence of fluoroquinolones. Fluoroquinolone resistance level was the highest in strains with a D87G/D87Y mutation in the GyrA subunit or +P466 mutation in the GyrB subunit. The mutation P43H located outside the GyrA Quinolone-Resistance-Determining-Region (QRDR) conferred significant but lower fluoroquinolone resistance. The mutation D487R-ΔK488 also outside GyrB QRDR did not cause fluoroquinolone resistance by itself, but increased the resistance level in case of concomitant D87G mutation. No mutation could be identified *in vivo* in the QRDR of *gyrA* and *gyrB* genes amplified from clinical samples collected in patients treated with a fluoroquinolone, although some of them experienced therapeutic failure. Finally, while searching for new antibiotic compounds, we identified new synthetic bis-indolic derivatives with antibacterial activity. Lead compounds were only bacteriostatic against *F. tularensis* but bactericidal against staphylococci including against multi-drug-resistant *Staphylococcus aureus*. MIC₉₀ were measured at 2mg/L for *F. tularensis* and *S. aureus* strains for the most active compound. However, many developments are still required to improve their solubility in water, decrease their plasma proteins binding and elucidate their original mechanism of action.

Keywords : New therapeutic strategies, *Francisella tularensis*, Bis-indolic compounds, tularemia, antibiotic resistance