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THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE GRENOBLE ALPES

Spécialité : **Modèles, méthodes et algorithmes en biologie,
santé et environnement**

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préparée au sein du **Laboratoire TIMC**

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Modèles cellulaires appliqués à la compréhension de l'épidémiologie et de la pathogénicité de *Francisella tularensis*.

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RESUME

Modèles cellulaires appliqués à la compréhension de l'épidémiologie et de la pathogénicité de *Francisella tularensis*.

Francisella tularensis est la bactérie responsable de la tularémie. La contamination humaine peut survenir par contact avec un animal infecté, par piqûre d'un arthropode, par contact avec le réservoir environnemental hydro-tellurique ou par ingestion d'eau ou d'aliments contaminés. Les sources de contamination à partir de l'environnement hydro-tellurique ainsi que les mécanismes de survie dans cet environnement sont peu caractérisés. Ainsi, il est nécessaire de développer les connaissances à ce sujet, d'autant plus que *F. tularensis* est considéré comme un agent potentiel de bioterrorisme.

La première partie de ce travail a consisté en la réalisation d'une revue de la littérature sur le lien entre *F. tularensis* et l'environnement aquatique. Ceci a permis de mettre en évidence que la contamination de l'Homme par cette bactérie à partir du réservoir aquatique pouvait survenir par ingestion ou utilisation d'eau contaminée, par piqûre de moustique ou suite à des activités récréatives en milieu aquatique. Concernant les modes de survie dans l'environnement aquatique, il apparaît que *F. tularensis* puisse survivre dans l'eau de plusieurs façons : sous forme planctonique avec possible évolution vers une forme viable mais non cultivable (VBNC), par l'intermédiaire de la production de biofilm, par infection de larves de moustiques et par interaction avec les amibes. Cependant peu de travaux ont porté sur l'étude de ces mécanismes et leurs résultats sont parfois contradictoires.

Ainsi, la deuxième partie de ce travail a visé à préciser les interactions entre les amibes et *F. tularensis* en comparaison à *F. novicida* et *F. philomiragia*, deux bactéries du genre *Francisella* de réservoir aquatique et de pathogénicité faible. Dans un modèle de screening Amoeba Plate Test (APT), toutes les espèces de *Francisella* testées résistaient à la lyse par les amibes. Dans des modèles d'infection en milieu pauvre l'ensemble des *Francisella* testées ne présentaient pas de multiplication intra-amibienne. Enfin, dans un modèle de co-culture, les amibes favorisaient la survie des trois espèces de *Francisella* sur une période de 16 jours. Les mécanismes impliqués dans cette survie favorisée en présence d'amibes pourraient être la production par les amibes de composés favorisant la croissance

bactérienne pour *F. tularensis* et *F. novicida* mais aussi des interactions de contact pour *F. novicida*. Ces travaux démontrent que les amibes participent probablement à la survie des bactéries du genre *Francisella* dans l'environnement aquatique, y compris *F. tularensis*.

Un troisième volet de ce travail de thèse a évalué la prévalence de la contamination par *F. tularensis* de l'environnement aquatique dans une région française présentant une augmentation de l'incidence de la tularémie. Pour ce faire, 87 échantillons d'eau ont été prélevés, au cours de deux campagnes de prélèvements, et ont été analysés par PCR en temps réel. Parmi les 57 échantillons de la première campagne, 15 (26,3 %) étaient positifs pour *Francisella* sp., neuf (15,8 %) pour *F. tularensis* et/ou *F. novicida* et quatre (7,0 %) pour *F. tularensis* subsp. *holarctica*. Ces proportions étaient de 25/30 (83,3 %), 24/30 (80,0 %) et 4/30 (13,3 %) pour la seconde campagne. Ces travaux indiquent que la contamination de l'environnement aquatique par l'ADN de *F. tularensis* est relativement fréquente et que l'environnement aquatique pourrait participer au maintien de *F. tularensis* et éventuellement à l'augmentation de l'incidence de la tularémie dans cette région.

Enfin, deux travaux plus appliqués ont permis de décrire le premier cas humain d'infection à *F. salinarina*, espèce initialement isolée de l'environnement aquatique et d'évaluer les performances du kit Biotaxis, un kit de qPCR commercial permettant la détection de *F. tularensis* sur échantillons cliniques et environnementaux.

Ces travaux ont permis de mettre en évidence que l'environnement aquatique occupe une place centrale dans l'écologie de *F. tularensis*.

Mots clés : Tularémie, *Francisella tularensis*, *Francisella* sp., environnement aquatique, amibes, écologie.

ABSTRACT

Cellular models for investigation of *Francisella tularensis* epidemiology and pathogenicity

Francisella tularensis is the bacterium responsible for tularemia. Human contamination can occur through contact with an infected animal, arthropod bite, after contact with the hydro-telluric environment or after ingestion of contaminated water or food. However, the ways of human contamination from hydro-telluric environment as well as the mechanisms of survival in this environment are poorly characterised. Thus, it is mandatory to improve knowledge about these topics, especially because *F. tularensis* is classified as a potential agent for bioterrorism.

The first part of this work was to performed a literature review about *F. tularensis* and aquatic environment. This allowed to highlight that water-born tularemia can occur after consumption or use of contaminated water, after bite of in infected mosquito, or after recreational aquatic activities. The mechanisms of *F. tularensis* survival in water may include survival in a planktonic form in water with the possible transition to a viable but nonculturable (VBNC) state, the formation of biofilms, infection of mosquito larvae, and interaction with free-living amoebae. However, few studies about these mechanisms are available and some of them present conflicting results.

Indeed, the second part of this work aimed to deeply investigate the interactions between *F. tularensis* and amoebae, in comparison to *F. novicida* and *F. philomiragia* which are aquatic bacteria of low virulence. In amoeba plate screening tests (APT), all the *Francisella* species tested resisted the attack by amoebae. In infection model in poor culture medium, all the *Francisella* species tested did not displayed intra-amoebic growth. In co-culture models, the amoebae favoured survival of the three *Francisella* species over 16 days. This enhanced survival was likely dependent on amoeba-excreted compounds for *F. novicida* and for *F. tularensis* and on direct contact between bacteria and amoebae for *F. novicida*. Overall, our results demonstrate that amoebae likely promote *Francisella* survival in aquatic environments, including *F. tularensis*.

A third step of this work aimed to assess prevalence of *F. tularensis* DNA in the natural aquatic environment in a French region where tularemia indigence increased. In this aim, 87 water samples were collected during two sampling campaigns and were analysed

using a combination of real-time PCR assays. Among 57 water samples of the first campaign, 15 (26.3%) were positive for *Francisella* sp., nine (15.8%) for *F. tularensis* and/or *F. novicida*, and four (7.0%) for *F. tularensis* subsp. *holarctica*. The ratios were 25/30 (83.3%), 24/30 (80.0%), and 4/30 (13.3%) for the second campaign. Thus, these results revealed a high prevalence of *F. tularensis* DNA in the studied aquatic environment. This aquatic environment could therefore be involved in the maintenance of *F. tularensis* and possibly in the endemicity of tularemia in this region.

Altogether, these works have shown that the aquatic environment is central to the ecology of *F. tularensis*.

Finally, two more applied works were performed. One described the first human case of *F. salinarina* infection, a species initially isolated from the aquatic environment. Another evaluated the performances of the Biotoxis kit, a commercial qPCR kit for the detection of *F. tularensis* in clinical and environmental samples.

Keywords: Tularemia, *Francisella tularensis*, *Francisella* sp., aquatic environment, amoebae, ecology.

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LISTE DES ABREVIATIONS

ADN	Acide DésoxyriboNucléique
canINDEL	insertion/délétion canonique
CDC	Centers for Disease Control and Prevention
CNR	Centre National de Référence
DL100	Dose Létale 100
DO	Déclaration Obligatoire
ELISA	Enzyme-Linked Immunosorbent Assay
FLE	<i>Francisella</i> Like Endosymbiontes
FNI	<i>Francisella novicida</i> Island
FPI	<i>Francisella</i> Pathogenicity Island
GBP	Protéines Guanylate-Binding
IFI	Immunofluorescence Indirecte
LNSB2-3	Laboratoire de Niveau de Sécurité Biologique (2 ou 3)
LPS	Lipopolysaccharide
LVS	Live Vaccine Strain
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation Time Of Flight
MAT	Micro-Agglutination Test
MLST	MultiLocus Sequence Typing
MLVA	MultiLocus Variable-number tandem repeat Analysis
OMS	Organisation Mondiale de la Santé
PCR	Polymerase Chain Reaction
RD	Région de Différence
SELDI-TOF	Surface Enhanced Laser Desorption Ionization – Time of Flight
SNP	Single Nucleotide Polymorphism
T6SS	Système de Sécrétion de Type VI
TLR	Toll Like Receptors
UFC	Unité Formant Colonie
VBNC	Viable But Not Culturable : viable mais non cultivable

INTRODUCTION GENERALE

I. *Francisella tularensis*, agent de la tularémie et autres bactéries du genre *Francisella*

1. Historique de la maladie

Depuis plusieurs siècles, dans l'hémisphère Nord, étaient décrits des cas de maladie des lemmings ou des lièvres (1). La bactérie responsable fut authentifiée pour la première fois en 1911, puis isolée en 1912, par McCoy, chez des rongeurs présentant une symptomatologie proche de la peste dans le comté de Tulare en Californie et fut nommée par celui-ci *Bacterium tularense* (2). Le premier cas humain vérifié de tularémie a été décrit dans l'Ohio en 1914 par Wherry et Lamb qui isolèrent la bactérie chez un commis de cuisine souffrant d'une conjonctivite associée à des adénopathies et de la fièvre (3). Un second cas fut décrit chez une fermière présentant une symptomatologie similaire et les lapins furent suspectés comme source potentielle de contamination (4). En 1919, Edward Francis, supposa que les cas de fièvre prolongée survenant après piqûre de taon chez les habitants du comté de Millard en Utah devaient être dus à la même bactérie que celle isolée par McCoy (5). Il nomma cette infection « Tularémie ». Edward Francis travailla ensuite toute sa carrière sur la tularémie : il développa un milieu de culture glucosé supplémenté avec du sang et de la cystéine permettant l'isolement de la bactérie ; il mit au point un test sérologique de diagnostic ; il démontra la transmission de cet agent infectieux par les taons, les puces des rongeurs et lagomorphes, les tiques ou par contact avec un animal infecté. Francis décrivit de très nombreux cas humains et également la forte contagiosité pour le personnel de laboratoire (6,7). En parallèle, au Japon, Hachiro Ohara décrivait une pathologie humaine similaire transmise par contact avec les lièvres. Les deux chercheurs collaborèrent pour démontrer que les deux pathologies étaient dues à la même bactérie (8).

Dans les années 1920s la bactérie fut transitoirement classée dans le genre *Pasteurella* sur la base d'analyses sérologiques. Finalement, dans les années 1950-60s, il fut déterminé par hybridation ADN/ADN que cette bactérie n'appartenait pas au genre *Pasteurella* mais à un nouveau genre appelé *Francisella* (9). Ainsi, la bactérie responsable de la tularémie fut nommée *F. tularensis*.

2. Francisella tularensis, un agent potentiel de bioterrorisme

Selon le Center for Disease Control and Prevention (CDC), *F. tularensis* est un des six agents biologiques appartenant à la classe A des agents potentiels de bioterrorisme, c'est-à-dire comme le plus susceptible d'être utilisé comme une arme biologique (10). Les 5 autres agents sont les bactéries *Bacillus anthracis* (agent du charbon), *Yersinia pestis* (agent de la peste) et *Clostridium botulinum* par le biais de sa toxine (agent du botulisme) ainsi que les virus Variola major (agent de la variole) et les Filovirus et Arenavirus (agents des fièvres hémorragiques).

Cette classification de *F. tularensis* en tant qu'agent de bioterrorisme se justifie par les caractéristiques de cet agent infectieux (11):

- Cette bactérie, en particulier les souches de type A, est hautement infectieuse pour l'Homme et de nombreuses espèces animales, 10 suffisant pour induire une infection par voie inhalée notamment ;
- Elle peut être aisément dispersée par aérosol, induisant la forme pulmonaire de la maladie, qui est l'une des plus sévères ;
- La bactérie peut survivre dans l'environnement hydro-tellurique de manière prolongée conduisant à un grand nombre de cas secondaires ;
- Il n'existe pas de vaccin internationalement validé contre la tularémie ;
- Les options thérapeutiques sont réduites et il est possible de créer *in vitro* des souches antibio-résistantes.

L'Organisation Mondiale de la Santé (OMS), a développé un modèle permettant de prédire l'impact des attaques bioterroristes. Concernant la tularémie, il a été déterminé que l'aérosolisation de 50 kg de bactéries sur une zone métropolitaine avec une population de 5 millions d'habitants induiraient 250 000 malades et 19 000 morts. L'épidémie initiale persisterait plusieurs semaines et des nouveaux cas seraient enregistrés pendant plusieurs mois (12). Le CDC a évalué le coût d'une telle épidémie à 5,4 milliards de dollars pour 100 000 individus atteints (13).

En ex-URSS, aux Etats Unis et au Japon, des programmes militaires ont porté sur l'utilisation de *F. tularensis* en tant qu'arme biologique, avant et pendant la deuxième guerre mondiale (1).

Après l'attaque des enveloppes piégées au charbon aux Etats-Unis en 2001 (14), la surveillance de la tularémie est devenue d'intérêt prioritaire. En France, la déclaration obligatoire (DO) de la tularémie, qui avait été abolie en 1986, a été rétablie en 2002. Un Centre National de Référence (CNR) de la tularémie a été créé, actuellement hébergé au sein du laboratoire de Bactériologie du CHU Grenoble Alpes.

3. Taxonomie

La taxonomie du genre *Francisella* est en permanente évolution, encore plus ces dix dernières années et cela pour deux raisons : les découvertes régulières de nouvelles espèces et l'avènement du séquençage génome complet qui remanie la phylogénie.

3.1. F. tularensis

Il y a 3 sous-espèces au sein de l'espèce *F. tularensis*, distinguées par leurs caractéristiques métaboliques et leur virulence : *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* et *F. tularensis* subsp. *mediasiatica*.

3.1.1. F. tularensis subsp. tularensis

Les souches de *F. tularensis* subsp. *tularensis* sont aussi appelées souches de Type A. Cette sous-espèce n'a été isolée d'infections humaines qu'en Amérique du Nord où elle représente 70 % des cas de tularémie (15). Cependant des souches de Type A ont également été isolées chez des arthropodes en Slovaquie et en Autriche mais leur analyse génomique a révélé qu'elles étaient très proches de la souche de laboratoire Schu S4, laissant supposer la contamination de l'environnement à partir d'un laboratoire (16). Les souches de type A sont hautement virulentes pour l'Homme et induisent des formes sévères de tularémie. Cette

sous-espèce présente une haute contagiosité puisque 10 bactéries peuvent induire une infection humaine après injection sous-cutanée et 25 bactéries après inhalation (17,18).

3.1.2. *F. tularensis* subsp. *holarctica*

Ces souches sont également appelées souches de type B. Cette sous-espèce se retrouve dans tous les pays de l'Hémisphère Nord (1) et a également été récemment isolée d'une infection humaine en Australie (19). Les doses infectieuses pour cette sous-espèce ne sont pas clairement définies. Bien que moins virulentes que les souches de types A, les souches de type B induisent la tularémie. La sous-espèce *F. tularensis* subsp. *holarctica* est également divisée en biovars. Les souches isolées au Japon, présentant des caractéristiques biochimiques propres (fermentation du glycérol), appartiennent au biovar *japonica* (20). Les souches non japonaises, si elles sont sensibles à l'érythromycine sont qualifiées de biovar I ery^S et si elles sont résistantes à l'érythromycine de biovar II ery^R (20).

3.1.3. *F. tularensis* subsp. *mediasiatica*

Cette sous-espèce a uniquement été isolée en Asie centrale et en ex-URSS chez des lagomorphes, des rongeurs et des tiques (21). Les souches de cette sous-espèce sont assez peu caractérisées mais semblent présenter une virulence pour les animaux similaire aux souches de Type B. *Francisella tularensis* subsp. *mediasiatica* n'a jamais été isolée chez l'Homme (1).

3.2. *F. novicida*

Cette espèce présente des exigences métaboliques moindres en comparaison de *F. tularensis*. Cependant, l'analyse des acides gras et du profil ADN la rapproche de l'espèce *F. tularensis* (22) avec laquelle elle partage plus de 97 % d'homologie du génome (23). Ainsi ces souches sont considérées par certains auteurs comme une sous-espèce de *F. tularensis* et

par d'autres comme une espèce à part entière (24,25). Néanmoins, les dernières études phylogénétiques préconisent de considérer cette bactérie comme une espèce à part entière (26). Dans ce document, nous parlerons de *F. novicida*. Ces souches semblent présenter un réservoir aquatique uniquement puisqu'elles n'ont jamais été isolées d'arthropodes ou d'animaux (27). Elles présentent une virulence faible chez l'Homme. En effet, de rares infections humaines à *F. novicida* ont été rapportées, essentiellement chez des patients immunodéprimés, après contamination d'origine aquatique (27). Des souches très proches de cette espèce, caractérisées comme des *F. novicida*-like, ont été également isolées au cours d'infections humaines, suite à une contamination aquatique (28,29).

3.3. *F. philomiragia*

F. philomiragia est une espèce de réservoir majoritairement hydrique, de faible virulence. De rares cas d'infections humaines liées à cette bactérie sont décrits, chez des patients immunodéprimés, notamment au cours de la granulomatose septique chronique, après exposition d'origine aquatique ou chez des patients ayant subi une quasi-noyade (30–37).

3.4. Autres espèces de *Francisella* impliquées en pathologie humaine

F. hispaniensis, caractérisée pour la première fois en 2003, a été décrite à trois reprises comme responsable d'infection humaine, en Espagne (38,39), en Australie (40) et en Chine (41). Pour deux des patients, la source de contamination a pu être identifiée et était d'origine aquatique.

F. opportunistica a été décrite comme responsable d'infection chez trois patients immunodéprimés aux USA, en 2005, 2006 et 2014, dont la source de contamination n'a pas été identifiée (42,43).

Enfin, une infection cutanée due à une nouvelle espèce de *Francisella*, proche des espèces marines, a été décrite aux USA, chez un patient diabétique, après immersion d'une plaie dans de l'eau saumâtre (44,45).

3.5. Autres espèces de *Francisella* non décrites comme agents d'infections humaines

D'autres espèces de *Francisella* sont connues mais non décrites jusqu'à présent comme agents d'infections humaines.

Parmi elles, certaines sont des pathogènes de poissons ou de mollusques telles que *F. orientalis*, *F. noatunensis* (46,47), *F. halioticida* (48) et *F. marina* (49).

D'autres espèces de *Francisella* sont isolées de l'environnement aquatique, telles que *F. salina*, *F. uliginis* et *F. salimarina* isolées d'eau de mer (50–52). *F. frigiditurris* et *F. guangzhouensis* ont été isolées de tours de refroidissement mais cette dernière a ensuite été transférée dans le genre *Allofrancisella* (51,53–56) avec *A. inopinata* et *A. frigidaquae* également isolées de tours aérorefrigérantes (55).

D'autres espèces sont nommées *Francisella* Like Endosymbiontes (FLE). Elles sont des endosymbiontes d'arthropodes et présentent une grande similarité dans la séquence de l'ARNr 16s avec les bactéries du genre *Francisella* (57). Ainsi, la bactérie *Wolbachia persica*, endosymbionte de tique a récemment été reclassée dans le genre *Francisella*, en tant que *F. persica* (58). Les *Francisella* peuvent aussi être des endosymbiontes de ciliés marins, tels que *F. endociliophora* (59) et *F. adeliensis* (60) (aussi appelée *Parafrancisella adeliensis* (26)).

Enfin, un nouveau genre bactérien, proche de *Francisella* a été récemment décrit, avec comme seul représentant *Pseudofrancisella aestuarii*, isolé d'eau de mer (61).

4. Caractéristiques phénotypiques de *F. tularensis*

F. tularensis est un cocco-bacille à Gram négatif de petite taille (0,2 µm de large, 0,2 à 0,7 µm de long) (Figure 1A). Lors de l'observation de frottis de tissus infectés l'aspect de la bactérie peut être pléiomorphe, parfois plus coccoïde que bacillaire. Cette bactérie est immobile, aérobic stricte, non sporulée, non productrice de toxine et de gaz (62). *F. tularensis* possède une capsule polysaccharidique impliquée dans la virulence bactérienne (63). Elle est indole négative, uréase négative, oxydase négative et catalase faiblement positive (42).

F. tularensis est une bactérie de culture exigeante. Elle est notamment auxotrophe pour la cystéine ce qui rend nécessaire l'utilisation de milieux enrichis pour la cultiver. Les géloses au sang cuit « chocolat » enrichies en vitamines « Polyvitex » (BioMérieux®) sont utilisées en pratique courante au laboratoire de bactériologie médicale pour cultiver cette bactérie. Des géloses « Mueller-Hinton modifiées » enrichies en glucose (0,1 %), cystéine (0,1 %) et pyrophosphate de fer (0,025 %) peuvent également être fabriquées. Concernant la culture en milieu liquide, le milieu Chamberlain, le bouillon « Mueller-Hinton modifié » et le « Schaedler K3 » (BioMérieux®) peuvent être utilisés. Des bouillons tels que le milieu cœur-cerveau (BHI) ou trypticase soja peuvent être utilisés à condition d'être supplémentés avec du Polyvitex®. Les conditions d'incubation optimales sont une incubation à 37°C en atmosphère aérobie enrichie avec 5 % de CO₂, jusqu'à 10 jours. Après au minimum deux à quatre jours d'incubation, de petites colonies grisâtres sont observées sur gélose (Figure 1B) (64).

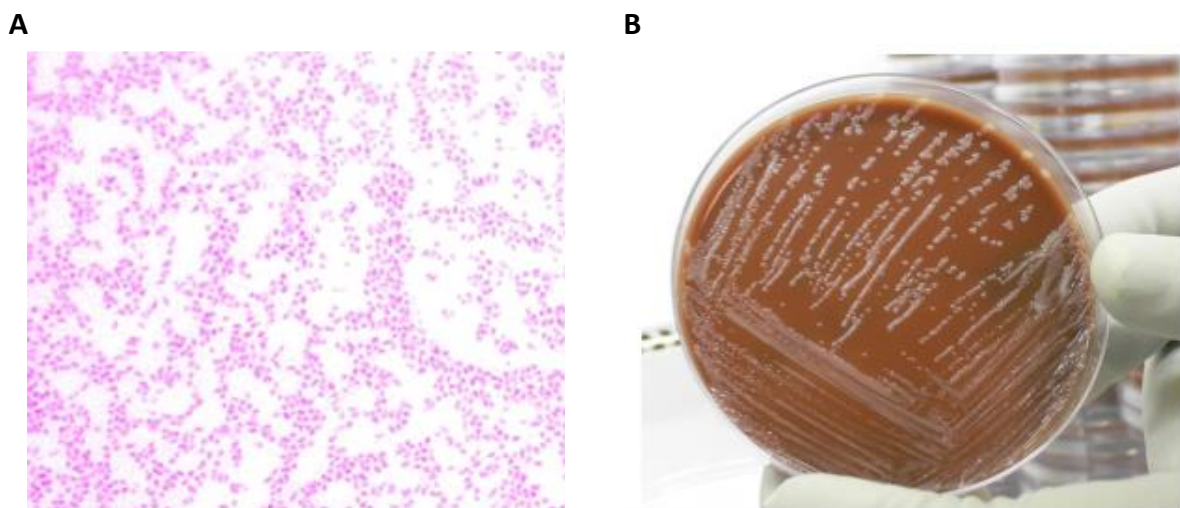


Figure 1. Caractéristiques phénotypiques de *F. tularensis*
A. Coloration de Gram : petits cocco-bacilles à Gram négatif
B. Culture sur gélose chocolat PVX® : petites colonies grisâtres

5. Caractéristiques génotypiques de *F. tularensis*

Le premier génome complet de *Francisella* séquencé a été celui de la souche Schu S4 de *F. tularensis* subsp. *tularensis*, en 2005 (65). Il se compose d'un chromosome circulaire de

1 892 819 paires de bases dont 1 804 séquences codantes (incluant plus de 10 % de pseudo-gènes) (65). Le G+C % est faible (32,9 %) (65).

En 2006, le génome de la souche OSU18 de *F. tularensis* subsp. *holarctica* a été séquencé (66). Il comprend 1 895 727 paires de bases et 1934 séquences codantes (incluant plus de 10 % de pseudo-gènes) (66). Le G+C % est de 32,16 % (66). Il existe une grande homologie de séquence entre les souches de Type A et B, proche de 99 % (66). La principale différence entre ces souches est la grande quantité de réarrangements génomiques observée chez les souches de Type A par rapport aux souches de Type B. La grande majorité de ces réarrangements est due à des recombinaisons homologues survenant entre deux séquences d'insertions, *ISFtu1* et *ISFtu2* (66).

Enfin, la souche U112 de *F. novicida* a été séquencée en 2007 et a révélé une homologie de 97,8 % avec la souche LVS et de 98,1 % avec Schu S4 (67). La quantité de pseudo-gènes chez *F. novicida* était plus de 10 fois inférieure à celle des souches de Type A et B ce qui correspond à un plus petit nombre de gènes inactivés. Ceci peut probablement en partie expliquer le caractère moins fastidieux de *F. novicida* et sa virulence moindre (27).

Les avancées technologiques récentes en biologie moléculaire ont permis le typage et l'étude phylogénétique et phylogéographiques des souches de *F. tularensis*. Différentes techniques de typages moléculaires peuvent être utilisées. Parmi elles, l'électrophorèse en champ pulsé, le polymorphisme de longueur de fragments amplifiés, l'analyse multi-locus de séquences répétées en tandem en nombre variable (MLVA), l'analyse des régions de différence (RD), le typage par séquence multi-locus (MLST) et le ribotypage. Aujourd'hui, le séquençage de génome complet avec analyse des single nucleotide polymorphisms (SNPs) et parfois des insertions/délétions canoniques (canINDELs) est la référence (68).

F. tularensis subsp. *tularensis* se divise en deux clades génétiquement différents, A1 (divisible en deux sous-clades a et b) et A2 (divisible en quatre sous-clades) (68).

Les souches de *F. tularensis* subsp. *holarctica* sont divisées en un plus grand nombre de clades. La plupart des souches européennes appartiennent aux clades B6 et B13 (69). Plus précisément, la majorité des souches d'Europe de l'Ouest, appartiennent au sous-clade B.FTNT002-00 (inclus dans le clade B6) et les souches d'Europe centrale et de l'Est au clade B13, lui-même divisé en de nombreux sous-clades (69). Toutes les souches françaises

responsables de cas humains appartiennent au clade B.FTNF002–00 et sont caractérisées par une délétion caractéristique de 1,59 kb dans la région de différence RD₂₃ (70).

6. Pouvoir pathogène de *F. tularensis*

6.1. Virulence

La virulence des souches de *F. tularensis* diffère entre les sous-espèces *tularensis* et *holarctica*. Des différences de virulence existent également au sein de la sous-espèce *tularensis*.

Chez la souris et le cochon d'Inde, après injection sous-cutanée, la dose létale tuant 100 % des individus (DL100) est d'une bactérie, à la fois pour *F. tularensis* subsp. *tularensis* et subsp. *holarctica* cependant les animaux meurent plus rapidement lorsqu'ils sont infectés par la sous-espèce *tularensis* (71,72). En modèle murin, les variations du délai de mortalité existent aussi entre les clades de *F. tularensis* subsp. *tularensis*. En effet, les souris infectées par des souches de *F. tularensis* subsp. *tularensis* A1b meurent plus rapidement que celles infectées par des souches A1a ou A2 (73).

La différence de virulence entre les deux sous-espèces de *F. tularensis* est plus marquée chez le lapin qui semble plus résistant à *F. tularensis* que la souris et le cochon d'Inde. Chez ce mammifère, après injection sous-cutanée, la DL100 est d'une bactérie pour *F. tularensis* subsp. *tularensis* alors qu'elle est de 10⁹ bactéries pour *F. tularensis* subsp. *holarctica* (71,72).

6.2. Cycle de vie intracellulaire chez l'Homme et principaux facteurs de virulence

F. tularensis est une bactérie intracellulaire facultative, capable d'infecter et de se multiplier dans de nombreux types cellulaires mais qui peut également exister sous forme extracellulaire dans le sang des hôtes infectés (74). La principale cellule cible de cette bactérie est le macrophage mais d'autres cellules phagocytaires (cellules dendritiques,

monocytes, polynucléaires neutrophiles) ainsi que les hépatocytes et les cellules pulmonaires peuvent être infectés (75).

Nous allons détailler ici le cycle de vie intracellulaire de *F. tularensis* dans les macrophages. Les principales étapes sont l'attachement bactérien suivi de l'internalisation, l'échappement du phagosome, la réplication et la survie dans le cytosol puis la libération de la bactérie et la diffusion aux autres cellules (75).

6.2.1 Adhésion et invasion

L'attachement bactérien aux cellules est médié par des protéines de membrane et par les pili de type IV (63). Différents types de récepteurs cellulaires peuvent être impliqués dans l'internalisation de *F. tularensis* par les macrophages, par exemple les récepteurs du complément CR3 (CD11b/CD18), les récepteurs mannose, les récepteurs scavenger A (SRA), les FcγRs et les nucléolines de surface (76).

Comme pour la plupart des bactéries intracellulaires, l'internalisation de *F. tularensis* se fait par polymérisation de l'actine, mais d'une manière unique, par formation d'une large boucle ou de pseudopodes (Figure 2, A) (77).

La capsule et le lipopolysaccharide (LPS) de *F. tularensis*, de par leur structure particulière, n'activent pas les récepteurs Toll-like de type 4 (TLR4) ce qui permet à la bactérie d'éviter la reconnaissance par le système immunitaire de l'hôte. Cependant, les lipoprotéines bactériennes activent les TLR2 qui sont ainsi les premiers TLR impliqués dans la réponse inflammatoire à l'infection par *F. tularensis* (63).

6.2.2 Etape phagosomale

Dans le macrophage, *F. tularensis* réside dans un grand phagosome (Figure 2, B) pendant 30 minutes à 4 heures. Pendant son séjour dans le phagosome, *F. tularensis* est capable de résister à des systèmes de défense antimicrobiens de l'hôte, tels que les peptides antimicrobiens et les espèces réactives de l'oxygène produites par la NADPH oxydase, par inhibition de celle-ci et par production d'enzymes de lutte contre le stress oxydant (78,79).

Des études indiquent que le compartiment contenant *F. tularensis* est acidifié avant l'échappement au phagosome (80) alors que d'autres études indiquent que le phagosome de *F. tularensis* résiste à l'acidification (81). Quoiqu'il en soit, le phagosome ne parvient pas à acquérir les éléments du lysosome (75).

Francisella tularensis possède un cluster de gènes, appelé *Francisella* Pathogenicity Island (FPI), codant notamment un système de sécrétion de type VI (T6SS). Ce T6SS ainsi que d'autres gènes exprimés par le FPI, jouent un rôle majeur dans la virulence bactérienne en permettant à *F. tularensis* d'échapper au phagosome (Figure 2, C) (82). *F. tularensis* possède deux copies du FPI alors que *F. novicida* et *F. philomiragia* n'en présentent qu'une (83,84). *Francisella novicida* possède un autre cluster de gènes, similaire au FPI, appelé le FNI (pour *Francisella novicida* Island), qui code un autre T6SS, qui ne semble pas impliqué dans la virulence bactérienne dans les macrophages et dont la fonction est pour le moment inconnue (83,85).

6.2.3 Etape cytoplasmique

Une fois dans le cytosol de la cellule hôte, *F. tularensis* se multiplie. Cette multiplication est dépendante de l'utilisation par la bactérie des acides aminés de la cellule hôte comme principal apport nutritionnel, on parle de « virulence nutritionnelle » (Figure 2, D) (86). Les protéines impliquées dans l'acquisition du fer sont également impliquées dans la virulence bactérienne (87).

Certaines études indiquent que la bactérie stimulerait l'internalisation dans des capsules autophagiques afin de favoriser sa croissance (88) alors que d'autres travaux semblent indiquer que la bactérie cherche à éviter l'autophagie par la cellule hôte (Figure 2, E) (75).

Les protéines guanylate-binding (GBPs) sont un système clef de l'hôte qui restreint la réplication bactérienne intra-cytoplasmique, directement en bloquant la réplication de *Francisella* et en induisant la lyse de la bactérie et indirectement, en induisant l'activation de l'inflammasome AIM2 qui, à son tour, induit l'élimination de *Francisella* de la niche répliquative (89).

6.2.4 Diffusion aux autres cellules

La dissémination de *F. tularensis* aux cellules adjacentes peut survenir de plusieurs façons :

- Soit après relargage des bactéries dans le milieu extracellulaire suite à la lyse de la cellule infectée par apoptose ou pyroptose (75) (Figure 2, F)
- Soit par un mécanisme de trogocytose, de découverte récente. Dans ce cas, la bactérie ne passe pas par le compartiment extracellulaire. Il se produit un transfert de la bactérie de la cellule infectée vers une cellule saine au cours d'un contact entre ces deux cellules (90) (Figure 2, G).

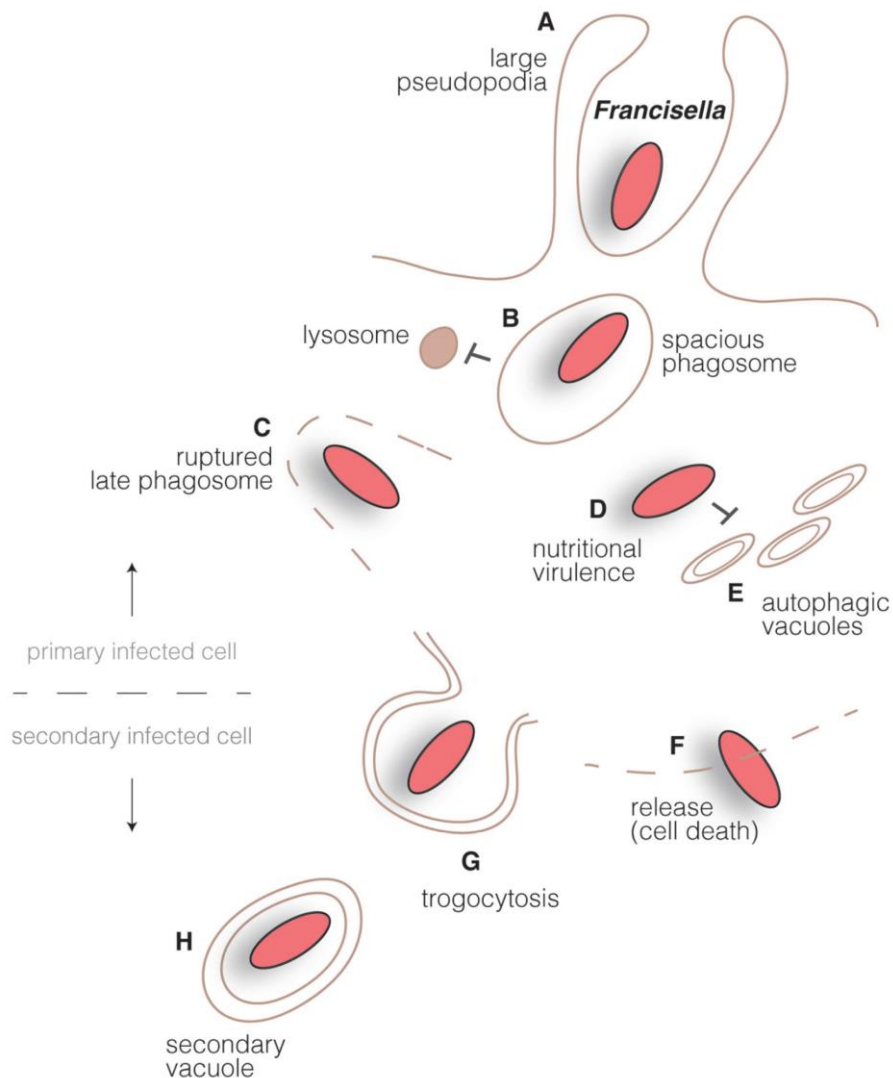


Figure 2. Cycle de vie intracellulaire de *F. tularensis* (d'après Pizzaro-Cerdá *et al.* 2016 (91))

Large pseudopodia : large pseudopode ; lysosome : lysosome ; spacious phagosome : grand phagosome ; ruptured late phagosome : rupture du phagosome ; nutritional virulence : virulence nutritionnelle ; autophagic vacuoles : caspusles autophagiques ; release (cell death) : relargage (mort cellulaire) ; trogocytosis : trogocytose ; secondary vacuole : vacuole secondaire.

7. Réservoirs et modes de transmission à l'Homme

7.1. Réservoirs de *F. tularensis*

Francisella tularensis présente un large réservoir animal. La bactérie peut infecter de nombreuses espèces d'animaux sauvages (92). Les petits rongeurs (en particulier les rats, souris, écureuils, castors, ragondins et rats laveurs) et les lagomorphes (en particulier les lièvres et les lapins) sont fréquemment infectés et développent le plus souvent des infections aiguës fatales (92,93).

Les arthropodes sont également des réservoirs de *F. tularensis*. Les tiques sont très fréquemment la source d'infections humaines dans l'hémisphère Nord (92,94). Les moustiques sont responsables d'épidémies estivales de tularémie en Suède et en Finlande (95). Chez ces deux arthropodes, l'infection des différents stades évolutifs et la transmission trans-stadiale de la bactérie a été démontrée (96,97). Cependant, il n'a pas été observé de transmission trans-ovarienne de *F. tularensis* chez la tique (98). Ceci laisse supposer que les arthropodes soient d'avantage des vecteurs, permettant le transfert du pathogène, plutôt qu'un réservoir durable. D'autres arthropodes sont également vecteurs de la maladie, tels que les taons (94).

Enfin, bien que peu caractérisé, l'environnement hydrotellurique semble être un réservoir significatif de la bactérie. En effet, *F. tularensis* peut être isolée de prélèvement d'eau et l'environnement aquatique est régulièrement identifié comme la source d'infection humaine (92). De plus, de très nombreuses espèces de *Francisella* (décrites précédemment dans ce manuscrit) sont d'origine aquatique et les nouvelles technologies de phylogénie indiquent que l'ancêtre commun des bactéries du genre *Francisella* serait d'origine aquatique (99).

Entre ces trois réservoirs, un cycle de vie de *F. tularensis* peut être proposé, tel que décrit par Maurin et Gyuranecz sur la Figure 3. Néanmoins, ce cycle, en particulier son versant aquatique, n'est probablement pas complètement élucidé. En effet, *F. tularensis* semble également pouvoir, dans l'eau, former du biofilm (100,101), interagir avec les amibes (102) ainsi que survivre directement dans l'eau pendant plusieurs mois, d'abord sous forme viable puis sous forme viable mais non cultivable (VBNC) (103,104).

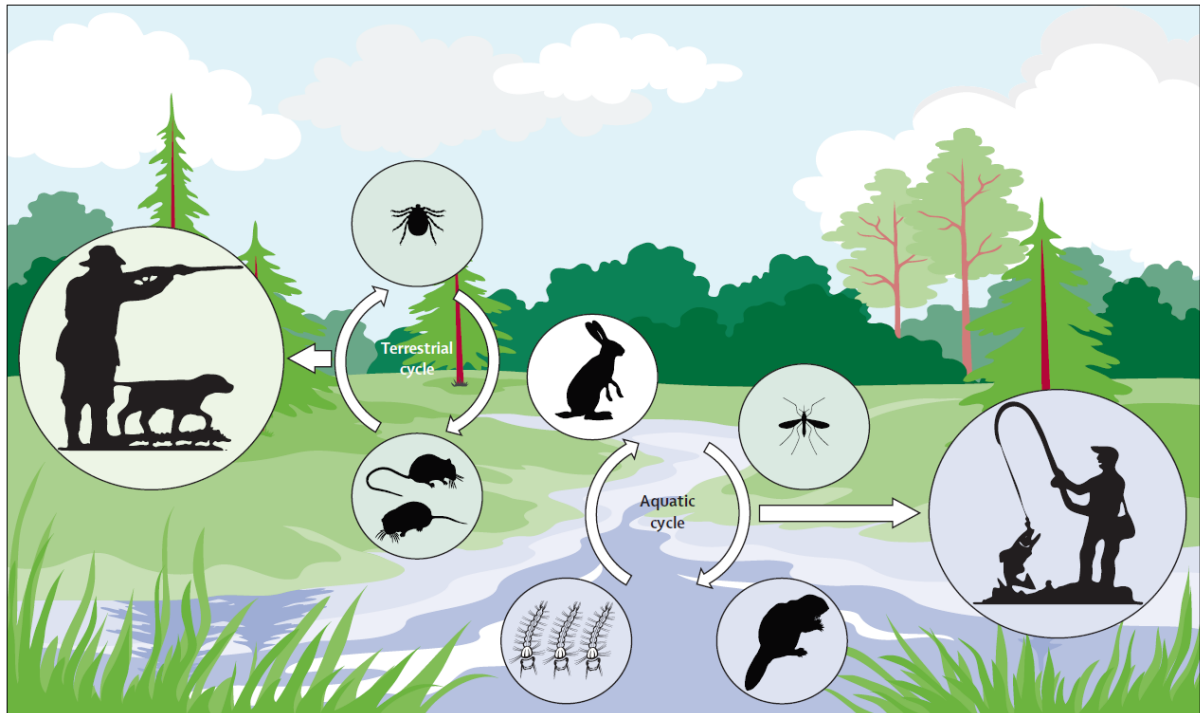


Figure 3. Cycle de vie de *F. tularensis* (d'après Maurin et Gyuranecz. 2016 (92))

7.2. Modes de transmission à l'Homme

L'infection humaine par *F. tularensis* peut survenir à partir des trois réservoirs décrits précédemment : animaux, arthropodes et environnement hydrotellurique. L'infection humaine peut survenir par pénétration à travers la peau (saine ou lésée par des égratignures ou des plaies), pénétration conjonctivale, ingestion ou inhalation (92).

La transmission à partir du réservoir animal peut survenir suite à la manipulation d'un animal infecté, à l'ingestion de viande insuffisamment cuite d'un animal infecté, à la morsure d'un animal infecté ou à l'inhalation de poussières contaminées par les déjections ou le cadavre des animaux contaminés (92). Les lagomorphes sauvages sont les plus fréquemment responsables d'infection humaines. Plus rarement, les animaux domestiques (chats, chiens, ...) et les animaux d'élevage peuvent aussi être infectés par *F. tularensis* et être la cause d'infections humaines (92). La tularémie est donc une zoonose, c'est-à-dire une maladie qui se transmet de l'animal à l'Homme.

La transmission à partir des arthropodes résulte de la morsure ou de la piqûre de ces derniers (92).

Enfin, la transmission à partir de l'environnement hydrotellurique peut survenir après ingestion d'eau contaminée par la bactérie, après contact avec de l'eau contaminée au cours d'activités de baignade ou de pêche, ou après contact avec des végétaux ou de la boue contaminés (92).

Ces modes de transmission permettent d'expliquer pourquoi les personnes les plus touchées par la tularémie sont les chasseurs, les fermiers et les personnels travaillant en forêt et dans les abattoirs. Ainsi, la tularémie est considérée comme une maladie professionnelle dans ces professions.

La transmission interhumaine n'a jamais été documentée (92) à l'exception de deux cas de transmission suite à la transplantation de deux patients par des reins contaminés issus d'un même donneur (105).

8. Clinique de la tularémie humaine

Quelle que soit la porte d'entrée, la période d'incubation de la maladie est en moyenne de 3 à 5 jours (92). Les symptômes initiaux sont aspécifiques, pseudo-grippaux : fièvre, frissons, asthénie, douleurs articulaires et musculaires (92). Six formes cliniques majeures de la tularémie sont décrites, dépendant essentiellement de la porte d'entrée. La forme ulcéro-ganglionnaire, survenant après pénétration cutanée de la bactérie, correspond à une lésion locale au point de pénétration de la bactérie, se transformant en ulcération nécrotique d'évolution chronique, associée à des adénopathies régionales, se situant sur le territoire de drainage de la lésion, qui peuvent suppurer, nécroser et scléroser (92). La forme ganglionnaire, survenant également après pénétration cutanée, correspond à des adénopathies sans lésion cutanée (92). La forme oculo-ganglionnaire survient après pénétration oculaire de la bactérie. Elle correspond à une conjonctivite associée à des adénopathies régionales (92). La forme oropharyngée survient essentiellement après ingestion de la bactérie et correspond à une stomatite ou une pharyngite ou une angine, associée à des adénopathies cervicales et rétropharyngées (92). La forme pleuro-pulmonaire peut survenir après inhalation d'un aérosol contaminé ou secondairement, après dissémination sanguine de la bactérie. Elle se manifeste par une toux sèche, une bronchiolite ou une pleuro-pneumonie avec des adénopathies hilaires (92) ou par une pneumonie aigue

grave. Enfin, la forme typhoïdique correspond à une bactériémie à *F. tularensis*, quelle que soit la porte d'entrée de la bactérie. Les manifestations en sont une forte fièvre, parfois associée à des troubles digestifs et neurologiques. La bactériémie peut évoluer vers un choc septique (92). Les formes pleuro-pulmonaires et typhoïdiques sont des formes plus sévères, potentiellement mortelles (92). La figure 4 présente des photographies des manifestations courantes de la tularémie : ulcère cutané et adénopathie.

Le pronostic de la tularémie dépend de nombreux paramètres : forme clinique, terrain du patient, délais d'instauration d'une antibiothérapie efficace, sous-espèce voire clade infectant le patient. En Europe, où seule la sous-espèce *holarctica* est impliquée dans la tularémie, le taux de létalité est inférieur à 1 % (92). Aux USA, où les sous-espèces *holarctica* et *tularensis* coexistent, la létalité globale est de 3 à 5 % et peut monter jusqu'à 30 % en cas de forme pleuro-pulmonaire (92). Toujours aux USA, il a été démontré que les taux de mortalité varient en fonction de la sous-espèce mais aussi du clade au sein de la sous-espèce : 24 % de mortalité pour les souches A1b, 4 % pour les souches A1a, 0 % pour les A2 et 7 % pour les souches de type B (106).

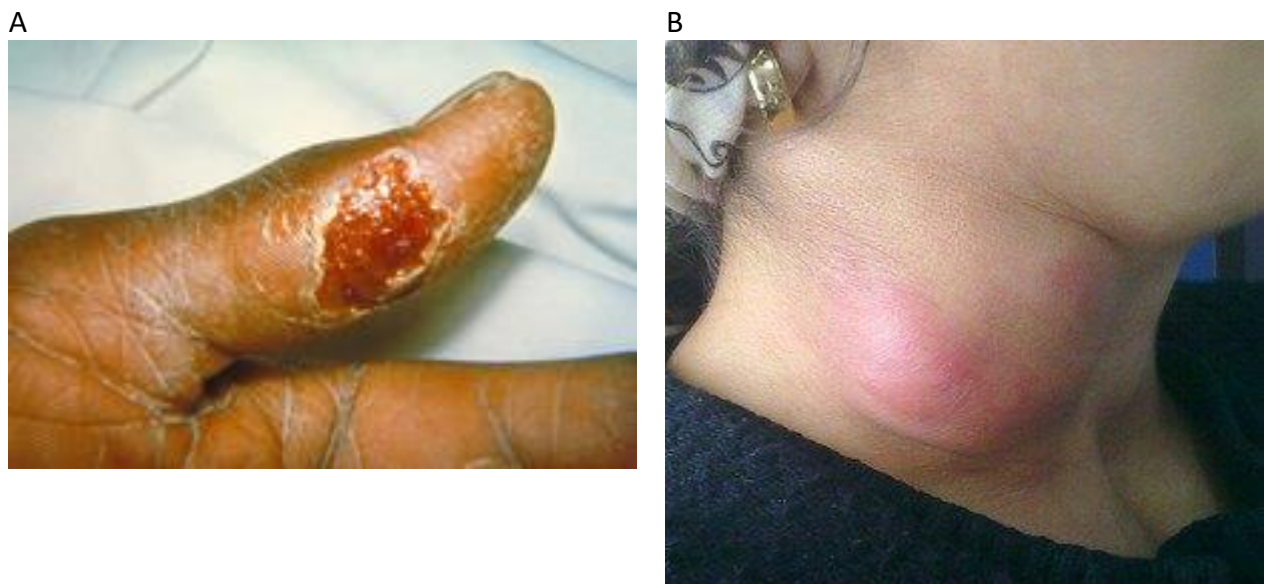


Figure 4. Exemples de manifestations cliniques de la tularémie.

A. Ulcère cutané d'une tularémie ulcéro-ganglionnaire (d'après Centers for Disease Control and Prevention (107)).

B. Adénopathie cervicale d'une tularémie oropharyngée (d'après Tunser *et al.* 2014 (108)).

9. Epidémiologie de la tularémie humaine

9.1. Epidémiologie mondiale de la tularémie

La tularémie est largement décrite dans l'Hémisphère Nord, et cela sur les trois continents. En Amérique du Nord, la tularémie est implantée au Canada et aux Etats-Unis d'Amérique. Dans ce dernier pays, les cas humains de tularémie sont dus à *F. tularensis* subsp. *tularensis*, pour la majorité, et à *F. tularensis* subsp. *holarctica* (109). Le taux d'incidence moyen entre 2001 et 2010 était de 0,041 cas pour 100 000 habitants (soit 1208 cas rapportés sur cette période de neuf ans correspondant à une médiane de 126,5 cas par an) et la grande majorité des cas survenait entre mai et septembre, correspondant à la période de présence des arthropodes et des activités humaines en extérieur (109).

En Europe, la tularémie est due uniquement à *F. tularensis* subsp. *holarctica*. Des cas de tularémie ont été décrits dans presque tous les pays entre 2015 et 2019 avec un taux d'incidence global en 2019 de 0,3 cas pour 100 000 habitants (soit 1463 cas rapportés sur l'année) (110). Sur la même période, l'Islande, l'Irlande, le Luxembourg, l'Albanie, la Grèce et Chypre n'ont pas rapporté de cas et le Liechtenstein et le Danemark n'ont pas participé (110). En Europe, les pays de plus forte incidence sont les pays scandinaves, notamment en lien avec une forte transmission par les moustiques (110) mais aussi le Kosovo suite à la détérioration des conditions sanitaires après la guerre du Kosovo (111). Néanmoins, même dans les pays européens où la tularémie n'a pas été rapportée, les cas importés restent possibles. En Europe, comme aux USA, l'épidémiologie annuelle est saisonnière avec une plus forte incidence en été (110). Cette saisonnalité est très marquée dans les pays scandinaves, pays de forte incidence où les épidémies estivales sont liées majoritairement aux piqûres de moustiques, mais est pratiquement absente en Europe centrale.

En Asie, la tularémie est également largement décrite, due à *F. tularensis* subsp. *holarctica*. On retrouve cette pathologie sur tout le continent asiatique, que ce soit dans la partie est du continent (Nord et Ouest de la Chine, Japon), dans sa partie centrale (Kazakhstan, Ouzbékistan, Turkménistan), en Russie, en Asie occidentale (Iran, Turquie) et dans les pays du Caucase (Arménie, Azerbaïdjan) (112).

Dans l'hémisphère Sud, la tularémie est classiquement absente. Néanmoins, elle a été formellement décrite en Australie, en 2011 pour la première fois : il s'agissait d'une infection à *F. tularensis* subsp. *holarctica* après morsure par un opossum (19). Peu de données sont disponibles en ce qui concerne les pays en développement de l'hémisphère Sud qui sont pour l'instant considérés comme indemnes de cette maladie. Cependant, une étude sérologique menée au Kenya a détecté des anticorps dirigés contre *F. tularensis* chez 3,7 % (soit 27 patients) des patients testés mais aucune souche n'a pu être isolée pour confirmer la tularémie (113). Une publication décrit une bactériémie à *F. tularensis* au Soudan mais la souche bactérienne en cause n'a pas été formellement identifiée comme une *Francisella* remettant en cause cette description (114). Les moyens diagnostiques étant limités dans la majorité des pays de l'hémisphère Sud, la recherche de *F. tularensis* y est peu, voire pas, réalisée, ce qui limite la capacité à conclure sur la présence de la maladie dans ces parties du monde.

9.2. Epidémiologie française de la tularémie

En France, la tularémie étant une maladie à déclaration obligatoire, son épidémiologie est relativement bien connue et Santé Publique France produit un rapport épidémiologie annuel. Le tableau 1 résume les principales caractéristiques des cas de tularémie recensés en France entre 2014 et 2018 (115). Il ressort de ces données que la tularémie est une maladie rare, avec, en France, environ 50 à 100 cas annuels. Elle touche toutes les tranches d'âges de la population et beaucoup plus fréquemment les hommes que les femmes, en raison des sources d'exposition, plus fréquemment rencontrées chez les hommes. Les formes ganglionnaires et ulcéro-ganglionnaires de la maladie sont les plus fréquentes, suivies par les formes pleuro-pulmonaires. Les sources d'exposition retrouvent, en France, les animaux, les tiques, les loisirs de plein air et l'exposition professionnelle au travers de divers métiers, notamment le travail dans les espaces verts, le travail au contact des animaux, le travail dans les égouts, dans les abattoirs ou dans les restaurants cuisinant du gibier.

Tableau 1. Epidémiologie de la tularémie en France (d'après Santé Publique France (115)).

		2014	2015	2016	2017	2018
Nombre de cas		57	87	99	47	133
Ages (médiane)		1 - 85 ans (49 ans)	1 - 92 ans (50 ans)	2 - 86 ans (48 ans)	22 - 81 ans (56 ans)	3 - 91 ans (54 ans)
Sexe ratio H/F		3,8	2,6	2,8	2,4	3,4
Forme clinique	Ganglionnaire (%)	49	49	43	47	39
	Ulcéro- ganglionnaire (%)	35	25	25	34	27
	Oro-pharyngée (%)	0	3	6	6	3
	Oculo- ganglionnaire (%)	0	0	2	0	0
	Pleuro-pulmonaire (%)	10	16	10	2	21
	Typhoïdique (%)	5	6	13	11	9
	Otite (%)	1	0	0	0	0
Exposition à risque (Plusieurs réponses possibles)	Lièvres/lapins (%)	35	50	38	40	20
	Sangliers/cervidés (%)	7	26	8	17	5,7
	Rongeurs (%)	19	8	22	30	17
	Morsures de tiques (%)	18	15	17	21	15
	Expositions professionnelles à risque* (%)	14	14	24	23	21
	Loisirs de plein air (%)	75	75	69	64	64

* agriculteurs, travailleurs de la filière bois, vétérinaire, égoutier, employé de zoo, cuisinier travaillant le gibier, personnel des abattoirs, paysagiste, garde-forestier, garde-chasse, employé de scierie, moniteur de canyoning

10. Diagnostic biologique de la tularémie

Le diagnostic de tularémie est difficile à suspecter car cette maladie est rare et les signes cliniques sont peu évocateurs et protéiformes. C'est pourquoi, la recherche par le clinicien,

au cours de l'interrogatoire du patient, d'un contexte épidémiologique évocateur est capitale.

Le diagnostic biologique de tularémie peut être réalisé de différentes façons : sérologie, isolement de la souche en culture et biologie moléculaire.

Dans le cadre de la surveillance de la tularémie, des définitions de cas confirmés et probables ont été établies. En France, ces définitions ont été élaborées par Santé Publique France (115).

Un cas confirmé de tularémie correspond à un tableau clinique évocateur associé à :

- l'isolement de *F. tularensis* à partir de prélèvements cliniques
- ou la multiplication par quatre au moins du titre d'anticorps ou une séroconversion entre un sérum prélevé en phase aiguë et un sérum prélevé 15 jours plus tard ou en phase de convalescence
- ou une PCR positive à partir d'un prélèvement clinique

Un cas probable de tularémie correspond à un tableau clinique évocateur associé à la mise en évidence d'anticorps spécifiques dans un seul sérum avec un titre supérieur ou égal au seuil du laboratoire.

Les définitions de cas varient entre les pays, par exemple, aux Etats-Unis, une PCR positive à partir d'un prélèvement clinique classe le cas en probable et non en confirmé (109).

10.1. Sérologie

La sérologie consiste à rechercher la production d'anticorps dirigés contre la bactérie. Il s'agit d'une méthode de diagnostic indirect. C'est la méthode la plus utilisée pour le diagnostic de la tularémie (116). Les anticorps spécifiques, IgM et IgG, dirigés contre *F. tularensis* apparaissent 2-3 semaines après le début des symptômes avec un pic 4-6 semaines après (116). Puis, en général les titres d'anticorps déclinent lentement au cours du temps. Cependant, des taux d'anticorps résiduels (à la fois IgG et plus rarement IgM) peuvent persister pendant plusieurs années (116). Ces taux résiduels d'anticorps rendent

l'interprétation des sérologies de la tularémie délicate. C'est pourquoi le diagnostic de tularémie peut être confirmé seulement lorsqu'une séroconversion ou une multiplication par quatre ou plus des taux d'anticorps est observée entre deux sérums. Des réactions croisées avec d'autres espèces bactériennes (telles que *Brucella* ou *Yersinia*) sont possibles, pouvant conduire à des résultats faussement positifs (116). Enfin, il ne faut pas perdre de vue que des réactions faussement négatives sont également possibles avec la sérologie, en cas de non production d'une réponse anticorps chez le patient (rare, mais possible, chez l'immunodéprimé notamment) ou de prélèvement sanguin réalisé pendant la fenêtre sérologique c'est-à-dire entre le début des symptômes et l'apparition des anticorps (116).

Plusieurs techniques de sérologie peuvent être utilisées pour le diagnostic de la tularémie. Les techniques d'agglutinations peuvent être utilisées. La plus connue est la microagglutination (MAT, pour microagglutination test). Il s'agit de technique « maison », c'est à dire que la préparation des réactifs (y compris les antigènes) et le test sont réalisés par le laboratoire lui-même, sans kit commercial. Cette technique détecte principalement des IgM, bien que les IgG participent également à la réaction d'agglutination (116). La sensibilité et la spécificité de la technique sont dépendantes du seuil fixé par le laboratoire. Si le seuil est fixé bas, la sensibilité sera forte mais la spécificité sera faible (par exemple, sensibilité de 82,4-100 % pour un seuil à 10-20) alors que si le seuil est fixé haut la sensibilité sera plus faible mais la spécificité sera meilleure (par exemple, sensibilité de 50-97,6 % pour un seuil à 80-160) (116). Ainsi, l'OMS préconise un seuil de 128 pour la technique MAT (117). Un autre inconvénient de la technique MAT est son manque de sensibilité pour les sérums en phase précoce de la maladie (116).

Les techniques d'immunofluorescence indirecte (IFI, pour indirect immunofluorescence) permettent de détecter et de titrer indépendamment les IgG et les IgM (116). Ici encore, il s'agit de techniques « maison ». Un autre inconvénient de ces techniques est la subjectivité et la difficulté de la lecture visuelle de la fluorescence par l'opérateur. La sensibilité et la spécificité des techniques IFI sont similaires à celles des techniques MAT (116). Comme les techniques MAT, les techniques IFI manquent de sensibilité pour les sérums prélevés en phase précoce de la maladie (116).

Enfin, des techniques ELISA et de chimiluminescence ont été développées récemment et présentent l'avantage d'être disponibles sous forme de kits commerciaux et de pouvoir être

réalisées de manière totalement automatisée. Ces tests recherchent indépendamment les IgG et les IgM (116). Ils sont très sensibles et permettent un diagnostic plus précoce que les autres techniques sérologiques (116). Cependant, ils sont moins spécifiques et peuvent produire des résultats faussement positifs (116).

D'autres techniques sérologiques, telles que l'immunochromatographie ou l'immunoblot, existent pour le diagnostic de la tularémie, mais sont peu utilisées (116).

Les limitations de différents tests sérologiques font qu'aucun d'entre eux ne peut être considéré comme une référence. Une stratégie est donc d'associer ces tests. Par exemple, au CNR Français de la tularémie, il a été choisi de commencer par un test de chimiluminescence, automatisable et de forte sensibilité. Puis les sérums positifs en chimiluminescence sont testés par une technique IFI « maison » plus spécifique, afin de confirmer ou d'infirmer le diagnostic.

10.2. Isolement de la souche en culture

L'isolement de *F. tularensis* à partir de prélèvements cliniques est difficile à obtenir en raison du caractère fastidieux de cette bactérie. Moins de 10 % des patients pour lesquels un diagnostic de tularémie est posé bénéficient d'un isolement de la souche en culture (118,119). La bactérie peut être isolée à partir de prélèvement de sang, de ganglions, d'ulcères cutanés, de lésions conjonctivales ou pharyngées, de prélèvements respiratoires ou de toutes autres localisations plus rares.

Francisella tularensis étant une bactérie hautement pathogène et visée par la réglementation sur les micro-organismes et toxines hautement pathogènes (MOT), elle doit être manipulée en laboratoire de niveau de sécurité biologique 3 (LNSB3), avec des équipements de protection adaptés, prévenant une contamination des manipulateurs et de l'environnement (92) (Figure 5). Cependant, les symptômes de la tularémie étant aspécifiques, ce diagnostic n'est parfois pas suspecté en première intention et les cultures sont alors manipulées en LNSB2 (119).



Figure 5. Equipements de protection pour le travail en laboratoire de niveau de sécurité biologique 3.

F. tularensis est une bactérie exigeante, dont la croissance nécessite des milieux riches, tels que la gélose au sang cuit « chocolat » enrichie en vitamines « Polyvitex », incubés en atmosphère aérobie enrichie avec 5 % de CO₂, à 37°C. Après au minimum 2 à 4 jours d'incubation, de petites colonies grisâtres sont observées sur gélose. L'identification bactérienne peut être réalisée de plusieurs manières. Une technique ancienne est l'étude des caractères biochimiques de la bactérie, par des systèmes de galeries manuelles ou automatisées. Ces techniques permettent l'identification à l'espèce de *F. tularensis* mais ne permettent pas toujours la distinction des sous-espèces (120). Les techniques d'identification biochimiques ne sont presque plus utilisées dans les laboratoires de biologie médicale depuis l'avènement, il y a quelques années, de l'identification bactérienne par spectrométrie de masse MALDI-TOF (Matrix Assisted Laser Desorption Ionisation - Time of Flight Mass Spectrometry). La spectrométrie de masse MALDI-TOF, basée sur l'étude du spectre protéique de la bactérie, permet l'obtention de l'identification de colonies bactériennes en quelques minutes. Cette technique permet l'identification de *F. tularensis*, à condition que la bactérie soit incluse dans la base de données de l'automate, ce qui commence à se généraliser dans les bases de données commerciales (121). Des bases de

données « maison » peuvent être développées, afin notamment d'inclure d'autres espèces du genre *Francisella* et d'essayer de distinguer les sous-espèces *holarctica* et *tularensis* (121). La distinction de ces deux sous-espèces a été réalisée par technique SELDI-TOF (Surface Enhanced Laser Desorption Ionization – Time of Flight Mass Spectrometry), non disponible en routine dans les laboratoires de biologie médicale (122) et semble également envisageable en MALDI-TOF (123). Enfin, les colonies bactériennes peuvent être identifiées à l'espèce, à la sous-espèce voire typées génétiquement, par technique de biologie moléculaire. Cependant, les sets de PCR adaptés à l'identification de *F. tularensis* ne sont disponibles que dans peu de laboratoires de biologie médicale.

L'antibiogramme des souches de *F. tularensis* isolées de prélèvements biologiques n'est pas réalisé en routine car, à ce jour, aucune résistance acquise aux antibiotiques d'intérêt clinique n'a été rapportée en pratique clinique.

10.3. Biologie moléculaire

La PCR peut être utilisée sur colonies bactériennes (comme expliqué ci-dessus) ou directement sur prélèvement biologique.

La recherche par PCR de *F. tularensis* sur prélèvement biologique présente l'intérêt d'être plus sensible que la culture et de permettre un diagnostic plus précoce que la sérologie (92). De nombreuses cibles sont décrites : *ISFtu2*, *tul4*, 23 kDa, *fopA*, ... (92). Il est cependant primordial de connaître leur spécificité et leur sensibilité afin d'interpréter avec fiabilité les résultats de PCR.

La PCR *ISFtu2*, ciblant la séquence d'insertion *ISFtu2*, présente l'intérêt d'être très sensible car cette séquence d'insertion est présente en multi-copies dans le génome de *F. tularensis* (12-17 copies chez la sous-espèce *tularensis*, 26-30 copies chez la sous-espèce *holarctica*). Cependant cette cible est présente chez d'autres espèces de *Francisella* (6-18 copies chez *F. novicida*, 1-2 copies chez *F. philomiragia*) (26,124). Des analyses in silico ont ainsi démontré que la PCR *ISFtu2* détectait également *F. novicida* et *F. philomiragia* mais ne détectait par d'autres espèces de *Francisella* telles que *F. opportunistica* et les *Francisella* endosymbiontes (26). Ceci pose peu de problèmes lors de l'analyse de prélèvements humains, car les

infections humaines à des *Francisella* autres que *F. tularensis* sont rares et associées à des contextes cliniques et épidémiologiques particuliers. Cependant ceci pose des problèmes lors de l'analyse de prélèvements environnementaux puisque, dans ces derniers, de multiples espèces de *Francisella* peuvent être présentes.

La PCR Tul4 cible le gène *tul4* (également appelé *lpaA*), codant une protéine de surface, présent en une seule copie chez *F. tularensis* et *F. novicida*. Bien que des études aient réalisé des contrôles et conclu que *F. philomiragia* et les autres espèces de *Francisella* n'étaient pas détectées par cette PCR (124), des études *in silico* ont montré un léger manque de spécificité pour certaines souches de *F. philomiragia* mais pas pour les autres espèces (26).

La PCR 23kDa cible le gène *23kDa* codant une protéine impliquée dans l'infection des macrophages. Elle détecte *F. tularensis*, *F. novicida* et certaines souches de *F. philomiragia* (124).

La PCR fopA cible le gène *fopA* codant une protéine de surface et détecte *F. tularensis*, *F. novicida* et *F. philomiragia* (124). Sa sensibilité apparaît inférieure à celles des autres PCR *Francisella* (124).

Des PCR (parfois associées au séquençage), permettent ensuite la distinction des sous-espèces *holarctica* et *tularensis*. Par exemple, la PCR « Type B » cible une jonction entre la séquence d'insertion ISFtu2 et une région flanquante en 3', qui est retrouvée seulement chez *F. tularensis* subsp. *holarctica* (125).

En raison des différences de performance de ces PCR, une stratégie est de les combiner. Par exemple, au CNR Français de la tularémie, sur les prélèvements cliniques suspects de tularémie, une PCR ISFtu2 est d'abord réalisée, suivie, si elle est positive, d'une PCR « Type B ».

11. Prise en charge thérapeutique et prévention

11.1. Traitement de la tularémie

Les classes d'antibiotiques actives sur *F. tularensis* sont peu nombreuses. Les bêta-lactamines sont inactives sur cette bactérie en raison de la production d'une bêta-lactamase par *F. tularensis* et de leur faible pénétration dans les cellules eucaryotes (126). Les aminosides, les tétracyclines et les fluoroquinolones sont très actifs *in vitro* sur *F. tularensis* (126). Néanmoins, les tétracyclines ne sont que bactériostatiques et leur efficacité est inférieure à celle des fluoroquinolones pour le traitement de la tularémie (126). La rifampicine est également active *in vitro* (126). Les macrolides sont modérément actifs voire totalement inactifs sur les souches de *F. tularensis* subsp. *holarctica* de biovar 2 qui sont naturellement résistantes aux macrolides (126). Les phénicolés et le cotrimoxazole sont modérément actifs *in vitro* (126).

Selon l'OMS, les cas légers à modérés de tularémie seront traités par fluoroquinolone (lévofloxacine 500 mg une fois par jour ou ciprofloxacine 500 mg deux fois par jour) ou tétracycline (doxycycline, 100 mg deux fois par jour), par voie orale, pendant deux semaines (117).

Des études plus récentes recommandent trois semaines de traitement, en particulier pour la doxycycline ou lorsque le diagnostic est tardif (127,128). De plus, certains auteurs préconisent d'utiliser préférentiellement les fluoroquinolones par rapport à la doxycycline (128).

Concernant les cas sévères de tularémie, l'OMS préconise l'hospitalisation et un traitement par aminoside par voie parentérale (gentamicine 5 mg/kg IV une ou deux fois par jour ou à défaut streptomycine lorsqu'elle est disponible) pendant 10 jours ou plus (117).

Ici encore, des travaux plus récents suggèrent qu'une bi-antibiothérapie par aminoside et fluoroquinolone devrait être préférée pour le traitement des tularémies sévères (128).

En cas de tularémie chez une femme enceinte ou un enfant, une problématique est que les trois classes d'antibiotiques recommandées pour le traitement de la tularémie sont contre-indiquées dans ces populations. Néanmoins, la balance bénéfice-risque est en faveur d'un traitement par aminoside ou par ciprofloxacine (117).

Aucune résistance aux trois familles d'antibiotiques utilisables pour le traitement n'a été décrite en pratique clinique (128). Cependant, des souches résistantes aux fluoroquinolones et aux macrolides ont été générées *in vitro* (129,130).

Les échecs du traitement antibiotique et les rechutes sont fréquents chez les patients diagnostiqués tardivement ou ayant une adénopathie suppurée (119). Les fluoroquinolones sont associées à des taux de rechutes plus faibles que les tétracyclines (5–10 % vs 10–15 %) (92). Dans ces situations, l'exérèse chirurgicale de l'adénopathie est nécessaire.

11.2. Prévention de la tularémie

Une souche de *F. tularensis* subsp. *holarctica* de virulence atténuée, appelée LVS (Live Vaccine Strain) a été utilisée précédemment comme vaccin mais a été abandonnée en raison d'une faible efficacité et de craintes quant à la réversion de la virulence de la souche (131). Depuis, des recherches portent sur le développement de nouveaux vaccins, sans résultat pour le moment (131).

Ainsi, la prévention de la tularémie repose essentiellement sur la réduction des contacts humains avec les sources de contamination de la tularémie, dans la mesure du possible.

//. Bactéries et amibes

1. Les amibes libres

Les amibes libres sont des organismes unicellulaires eucaryotes du règne des protozoaires, présentes partout à travers le monde (132). Elles vivent de façon indépendante dans le sol ou l'eau et ne nécessitent ni hôte humain ni animal. Dans le sol, les amibes libres sont plus abondantes à l'interface entre le sol et les plantes car les plantes sont le support de nombreuses bactéries et champignons que les amibes utilisent pour se nourrir (132). Dans l'eau, les espèces disposant de flagelles peuvent nager tandis que les espèces démunies de

ces organelles s'attachent à des particules de matière (132). Ainsi, les amibes libres vivent le plus souvent au sein de biofilms ou à l'interface eau-sol, eau-air ou eau-plantes (132). Les amibes libres vivent dans l'eau douce mais aussi, pour certaines espèces telles qu'*Acanthamoeba*, dans l'eau de mer (132). Certaines espèces d'amibes libres, telles que *Vermamoeba (Hartmanella)*, *Naegleria* et *Acanthamoeba* peuvent être isolées de réseaux d'eau à usage humain tels que l'eau de boisson, les tours aéroréfrigérantes, les piscines, les réseaux d'eau sanitaires des maisons et hôpitaux (132).

Les amibes libres présentent, en général, deux stades évolutifs : la forme trophozoïte et la forme kystique (132). Le trophozoïte est la forme active métaboliquement, qui se nourrit et qui se multiplie (132). Les amibes libres se nourrissent essentiellement de bactéries, de champignons et d'algues. L'alimentation se fait via un processus de phagocytose (132). Lorsque les nutriments viennent à manquer et/ou lorsque les conditions environnementales deviennent défavorables, le trophozoïte évolue vers une forme kystique, forme de résistance (132). A l'inverse, lorsque les conditions s'améliorent, le kyste évolue vers une forme trophozoïte (132).

Les amibes libres ne provoquent que rarement des infections humaines. Les espèces pathogènes de l'Homme appartiennent aux genres *Naegleria*, *Acanthamoeba* et *Balamuthia* (133). Elles peuvent causer notamment des kératites et des méningo-encéphalites (133).

2. Micro-organismes résistants aux amibes libres

Certains virus, champignons ou bactéries présentent la capacité de résister à la destruction par les amibes libres (132). Soit ils ne sont pas internalisés par les amibes soit ils sont capables, après internalisation, de survivre, voire de se multiplier et de ressortir des amibes (132).

Il existe une grande diversité de bactéries résistantes aux amibes. Certaines sont des bactéries intracellulaires obligatoires (telles que *Coxiella burnetii*), d'autres sont intracellulaires facultatives (telles que *Listeria monocytogenes* ou *Mycobacterium avium*) et certaines sont mêmes des bactéries non connues comme capables de pénétrer dans les

cellules eucaryotes (telles que *Burkholderia cepacia* ou *Pseudomonas aeruginosa*) (132). Certaines bactéries résistantes aux amibes sont des bactéries pathogènes pour l'Homme (telles que *Chlamydia pneumoniae*), d'autres sont des pathogènes émergents (telles que *Simkania negevensis*) et certaines bactéries résistantes aux amibes ne sont pas pathogènes pour l'Homme (telles que *Bradyrhizobium japonicum*) (132). Enfin, parmi les bactéries résistantes aux amibes, certaines sont cytopathogènes pour les amibes, c'est à dire capables de lyser les amibes (telles que *Legionella pneumophila*) (132). A l'inverse, d'autres bactéries résistantes aux amibes n'induisent pas de dommage à l'amibe hôte (telles que *Parachlamydia acanthamoeba*) (132), on parle alors d'endosymbionte, définissant une cohabitation harmonieuse de deux partenaires non apparentés, dont l'un vit dans le corps de l'autre (134).

Nous allons par la suite, prendre l'exemple de deux bactéries, *L. pneumophila* et *P. aeruginosa* pour décrire la diversité des relations entre bactéries résistantes aux amibes et amibes libres.

Legionella pneumophila est une bactérie intra-cellulaire facultative, agent d'une pneumonie grave chez l'Homme, la légionellose. Dans le milieu aquatique, la bactérie survit dans deux réservoirs : le biofilm et les amibes. Il a d'abord été démontré que cette bactérie était capable d'infecter les amibes, de s'y multiplier, puis de lyser l'amibe pour s'en extraire (135). Par la suite, il a été découvert que les amibes étaient en réalité un élément clé de la multiplication de la bactérie en milieu aquatique, bien que celle-ci soit capable de survivre dans les biofilms en l'absence d'amibe (136). La bactérie est également capable de survivre dans l'amibe enkystée, ce qui assure sa protection en cas de dégradation des conditions environnementales (132). La co-évolution de *L. pneumophila* avec les amibes est telle, que la bactérie a acquis, par des échanges de génomes, des gènes amibiens, lui conférant notamment des facteurs de virulence et de résistance additionnels (132).

Pseudomonas aeruginosa est une bactérie extracellulaire, régulièrement incriminée en pathologie humaine, agent d'infections opportunistes telles que des pneumonies, des infections urinaires ou des infections de plaies. Cette bactérie peut être utilisée comme source de nourriture par les amibes libres lors de cultures xéniques (132). Pourtant, il a été démontré *in vitro* que *P. aeruginosa* pouvait inhiber la croissance d'amibes (137). De plus, des amibes infectées par *P. aeruginosa* ont été isolées de réseaux d'eaux sanitaires (138).

Ainsi, la relation entre amibes libres et *P. aeruginosa* est complexe, la bactérie pouvant à la fois être sensible ou résistante aux amibes.

3. *Francisella tularensis* et amibes libres

Les études concernant l'interaction de *Francisella* sp. avec les amibes sont peu nombreuses et présentent des résultats parfois contradictoires.

Une étude a mis en évidence que *F. tularensis* pouvait pénétrer dans l'amibe *A. castellanii* puis sortir de celle-ci (139). Par la suite, d'autres travaux ont décrit la multiplication de *F. tularensis* subsp. *tularensis* dans *A. castellanii* 24 heures après l'infection (102). *F. novicida* et *F. philomiragia*, bactéries de réservoir aquatique, ont également démontré dans plusieurs études une capacité à se multiplier dans les amibes *V. vermiformis*, *A. castellanii* ou *Dictyostelium discoideum* (102,140–144). *Francisella noatunensis*, pathogène de poisson, apparaît également capable d'infecter et de se multiplier dans *D. discoideum* (145).

D'autres travaux suggèrent une survie favorisée de *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* et *F. novicida* en co-culture avec les amibes *A. castellanii*, *A. polyphaga*, *V. vermiformis* ou *Ochromonas danica* (146–148). Ces auteurs indiquent, que dans les modèles de co-culture, les bactéries se localisent à l'intérieur ou à l'extérieur des amibes, ce qui suggère que l'amélioration de la survie bactérienne pourrait passer par des mécanismes intra- et/ou extra-cellulaires (146–148). D'ailleurs, il a été démontré que le surnageant de culture de l'amibe *A. palestinensis*, une fois déplété des amibes, favorisait la multiplication de *F. tularensis* (149).

Par opposition à ce qui est décrit pour *L. pneumophila*, la culture en présence d'amibes de *F. novicida* n'augmentait pas sa virulence (142) ni sa résistance aux désinfectants (150).

TRAVAUX PERSONNELS

F. tularensis présente trois grands types de réservoirs potentiels, à partir desquels l'Homme peut se contaminer : les animaux, les arthropodes et l'environnement hydrotellurique. Ce dernier réservoir est peu caractérisé. Tout d'abord les modes de contamination de l'Homme à partir de l'environnement aquatique, en dehors de l'eau de boisson, ne sont que peu définis. Ensuite, les mécanismes de survie de *F. tularensis* dans l'environnement aquatique ne sont que partiellement élucidés. Ajouté à cela, ces dernières années, de nombreuses nouvelles espèces de *Francisella* ont été découvertes. Celles-ci ont été isolées à partir de la faune aquatique ou de l'eau elle-même. D'autres ont été isolées d'infections humaines, avec dans tous les cas où la source de contamination a pu être identifiée, une relation avec l'environnement hydrique.

Tout ceci laisse à penser que l'environnement aquatique présente une position centrale dans l'épidémiologie de la tularémie, qui nécessite d'être clarifiée.

Ainsi, les objectifs de mon travail de thèse étaient en lien avec ces problématiques.

Dans un premier temps, j'ai réalisé une revue de la littérature concernant le lien entre *Francisella* sp. et l'environnement aquatique afin de résumer et d'organiser l'ensemble des connaissances actuelles sur le sujet.

Dans un second temps, dans une partie expérimentale fondamentale, j'ai évalué si les amibes participaient à la survie environnementale de *Francisella* sp.

Ajouté à cela, au cours d'une étude de terrain, j'ai évalué la prévalence de la contamination par *F. tularensis* de l'environnement aquatique dans une région française de forte incidence de la tularémie.

En parallèle de ces travaux, j'ai participé à la caractérisation d'une souche de *Francisella* non *tularensis* isolée d'une infection humaine et probablement d'origine aquatique.

Enfin, j'ai évalué les performances concernant la détection de *F. tularensis* d'un kit commercial de PCR multiplexe ciblant des agents de bioterrorisme, qui pourrait s'avérer utile pour la recherche à haut débit de *F. tularensis* dans l'environnement aquatique.

TRAVAIL N°1 : Tularemia as a waterborne disease: a review

(Article publié, Emerging Microbes and Infections, 2019)

Il est supposé que l'environnement hydrotellurique constitue un réservoir de *F. tularensis*. Les modes de contamination de l'Homme à partir de ce réservoir, en dehors de l'ingestion d'eau contaminée, sont peu définis. De la même manière, les mécanismes par lesquels la bactérie survit dans l'eau sont peu caractérisés. Ainsi, j'ai commencé mon travail de thèse par la réalisation d'une revue de la littérature concernant le lien entre *F. tularensis* et l'environnement aquatique. Pour ce faire, j'ai inclus tous les articles, publiés dans la littérature anglophone entre 1998 et 2018 et indexés dans PubMed, traitant du sujet.

Tout d'abord, cette revue a permis de caractériser les modes de contamination des cas de tularémie d'origine aquatique :

- Comme cela était connu, le mode de contamination principal à partir de l'environnement aquatique est l'ingestion d'eau contaminée. Ce mode de contamination entraîne principalement des formes de tularémie oro-pharyngées bien qu'occasionnellement des formes ganglionnaires, ulcéro-ganglionnaires, oculo-ganglionnaires, typhoïdiques et pleuro-pulmonaires aient également été décrites après ingestion d'eau. Les sources d'eau incriminées peuvent être les réseaux d'eau domestiques, en particulier lorsqu'ils sont insuffisamment entretenus ou décontaminés, l'eau de source ou l'eau des puits. La contamination par ingestion d'eau est très fréquente en Turquie où elle est à l'origine d'épidémies pouvant parfois toucher plus de 100 personnes. Ce mode de contamination a également été responsable d'épidémies de tularémie dans des pays voisins de la Turquie que sont le Kosovo, la Bulgarie, la Géorgie et la Macédoine. Dans ces pays, les réseaux d'eaux sanitaires peuvent être contaminés, de même que l'eau de source et l'eau des puits. En Scandinavie, plus précisément en Suède et en Norvège, des cas sporadiques ou des petites épidémies de tularémie sont dus à l'ingestion d'eau provenant de puits privés. Enfin, ce mode de contamination a été ponctuellement décrit en Italie et en Allemagne.
- Un autre mode de contamination lié à l'eau repose sur les piqûres de moustiques, qui se contaminent au stade larvaire dans l'eau. Ce mode de contamination est endémique en Suède et en Finlande, entraînant de larges épidémies estivales de tularémie, dans les régions boisées et humides, principalement de forme ulcéro-ganglionnaire. En Allemagne, un cas de tularémie a également été attribué à une

piqûre de moustique, suggérant que ce mode de contamination pourrait diffuser au-delà de la Suède et de la Finlande.

- Enfin, dans le monde entier, des expositions aquatiques diverses ont été décrites comme source de cas de tularémie. Des blessures cutanées lors de la pêche, les quasi-noyades, le canyoning et la nage ont conduit à des cas de tularémie de diverses formes, en Europe, en Turquie et en Amérique du Nord.

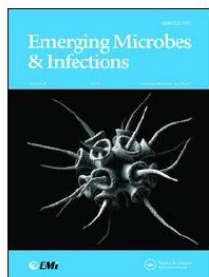
Ajouté à cela, des infections humaines à des bactéries du genre *Francisella* autres que *F. tularensis* peuvent occasionnellement survenir, le plus souvent après contamination aquatique. C'est essentiellement le cas de *F. philomiragia* et *F. novicida*, dont le réservoir aquatique est clairement établi, qui peuvent causer des infections opportunistes après contamination aquatique.

Concernant les mécanismes de survie de *F. tularensis* dans l'eau, les travaux publiés suggèrent plusieurs possibilités :

- *F. tularensis* apparaît capable de survivre sous forme planctonique dans de l'eau pendant plusieurs jours à plusieurs mois. Selon certaines études, la bactérie se maintient initialement sous forme cultivable puis évolue vers une forme viable mais non cultivable (VBNC). Ces observations expérimentales sont en adéquation avec de nombreuses études de terrain qui ont détecté *F. tularensis* dans des échantillons d'eau environnementale, par PCR ou plus rarement par culture.
- Il a également été démontré expérimentalement que *F. tularensis* est capable de former du biofilm, ce qui pourrait participer à sa survie en milieu aquatique.
- Des expérimentations *in vitro* ont également démontré que *F. tularensis* pourrait se multiplier dans les amibes alors que d'autres études suggèrent plutôt une absence de multiplication de la bactérie dans ces protozoaires mais une survie prolongée lors de co-culture entre ces deux micro-organismes.
- Enfin, les larves de moustiques, s'infectant dans l'eau, pourraient également constituer un réservoir de *F. tularensis*.

L'ensemble des connaissances regroupées dans cette revue de la littérature a très clairement mis en évidence le lien fort existant entre *F. tularensis* et l'eau. Les modes de

contamination à partir du réservoir aquatique semblent bien décrits. Cependant, les mécanismes de survie de *F. tularensis* dans l'environnement aquatique doivent encore être clarifiés.



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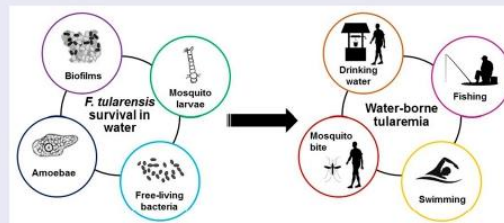
Tularemia as a waterborne disease: a review

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ABSTRACT

Francisella tularensis is a Gram-negative, intracellular bacterium causing the zoonosis tularemia. This highly infectious microorganism is considered a potential biological threat agent. Humans are usually infected through direct contact with the animal reservoir and tick bites. However, tularemia cases also occur after contact with a contaminated hydro-telluric environment. Water-borne tularemia outbreaks and sporadic cases have occurred worldwide in the last decades, with specific clinical and epidemiological traits. These infections represent a major public health and military challenge. Human contaminations have occurred through consumption or use of *F. tularensis*-contaminated water, and various aquatic activities such as swimming, canyoning and fishing. In addition, in Sweden and Finland, mosquitoes are primary vectors of tularemia due to infection of mosquito larvae in contaminated aquatic environments. The mechanisms of *F. tularensis* survival in water may include the formation of biofilms, interactions with free-living amoebae, and the transition to a 'viable but nonculturable' state, but the relative contribution of these possible mechanisms remains unknown. Many new aquatic species of *Francisella* have been characterized in recent years. *F. tularensis* likely shares with these species an ability of long-term survival in the aquatic environment, which has to be considered in terms of tularemia surveillance and control.



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KEYWORDS Tularemia; *Francisella tularensis*; *Francisella* species; waterborne diseases; aquatic environment; mosquitoes; amoeba; bacterial biofilms

Introduction

Francisella tularensis is a small, Gram-negative coccobacillus and the causative agent of the zoonosis tularemia [1]. Because it is highly infectious and can be spread by aerosols, this microorganism is classified in the category A of potential agents of biological threat by the US Centers for Disease Control and Prevention (CDC) [2]. The species *F. tularensis* is classically divided into four subspecies: *F. tularensis* subsp. *tularensis* (Type A strains), *F. tularensis* subsp. *holarctica* (Type B strains), *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida*, which may be considered a different species (*F. novicida*) because it is an aquatic bacterium of low virulence in humans [3,4]. Type A and type B strains of *F. tularensis* are the two etiological agents of tularemia. The former subspecies is mainly localized in North America, although

it has been occasionally detected in arthropods in Slovakia and Austria [5]. The latter one is found throughout the Northern Hemisphere [1], but has recently been detected in Australia [6].

F. tularensis has multiple reservoirs. Firstly, this bacterium can infect a multitude of animal species, including lagomorphs and small rodents, which are the primary sources of human infections [1,7,8]. Secondly, *Ixodidae* ticks are vectors but also a probable reservoir of *F. tularensis* owing to the transstadial transmission of this pathogen in these arthropods [1,8]. Mosquitoes and deer flies can also transmit *F. tularensis* to humans and animals in specific areas, but are not considered long-term reservoirs of this pathogen [1,8]. Finally, a hydro-telluric reservoir of *F. tularensis* is suggested by numerous studies but has not been extensively characterized [1,8]. Water-borne

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tularemia was first described in the 1930s in the USSR [9]. Human infection with *F. tularensis* may occur from these different reservoirs, and the clinical presentation of tularemia is primarily related to the mode of infection. Six major clinical forms of tularemia are classically recognized. The most frequent route of contamination is through the skin, through contact with an infected animal (especially hares in hunters) or arthropod bites. The ulceroglandular form of tularemia combines a skin ulcer at the site of *F. tularensis* inoculation and regional lymphadenopathy. The glandular form corresponds to regional lymphadenopathy without any visible skin lesion. Infection with *F. tularensis* through the conjunctiva (e.g. hand to eye contamination) or the oral route (ingestion of contaminated water or food) correspond to the oculoglandular and oropharyngeal forms, respectively. The involvement of lungs through inhalation of infected aerosols or hematogenous spread of bacteria corresponds to the pneumonic form. Finally, whatever the portal of entry of bacteria, severe sepsis often associated with confusion and *F. tularensis* bacteremia corresponds to the typhoidal form. The present review summarizes the literature data on human tularemia cases infected from aquatic sources, detection of *F. tularensis* in aquatic environments, and potential mechanisms of *F. tularensis* survival in water environments.

Search strategy and selection criteria

Data on tularemia cases related to aquatic sources, on the presence of *Francisella* species in water environments, and the mechanisms of survival of these bacteria in water environments were collected from the English literature in the PubMed database. Only articles published in the last two decades (1998–2018) were included. They were extracted using the keywords ‘tularemia’ or ‘*Francisella*’ and ‘case report’ or ‘water’ or ‘mosquito’ or ‘biofilms’ or ‘amoeba.’ In addition, data on other aquatic *Francisella* species were added, including *F. novivida*, *F. philomiragia*, *F. halioticida*, *F. hispaniensis*, *F. noatunensis*, *F. salina*, *F. frigiditurris*, *Allofrancisella* (formerly *Francisella*) *gangzhouensis*, *F. marina*, *F. ulginis* and *F. endociliophora*.

Tularemia of aquatic sources

Tularemia and drinking water

In the last 20 years, tularemia cases linked to drinking water have been reported in Turkey, Kosovo, Bulgaria, Georgia, Macedonia, Norway, Sweden, Italy, and Germany (Table 1).

In Turkey, tularemia reemerged in 1988 in the form of water-borne outbreaks of oropharyngeal tularemia cases [10]. Between 1988 and 2018, 28 tularemia

outbreaks and non-outbreak tularemia cases linked to consumption of contaminated water were reported in this country [11–36]. The Turkish outbreaks were frequently of large scale, involving more than one hundred people for some of them [11–15]. Patients were almost exclusively suffering from the oropharyngeal form of tularemia, in accordance with the oral route of contamination with *F. tularensis*. In addition, cases were observed in the whole population, with a male/female sex ratio close to 1 or even lower, and both children and adults involved [11–19,31,33–35]. This is in sharp contrast to countries where infections usually occur through contact with animals or tick bites, and therefore predominate in middle-aged men due to more frequent occupational or leisure exposures [8]. Less frequently, exposure to contaminated water resulted in glandular [12,15], oculoglandular [14,15,19,25,26], and pneumonic [24] forms of tularemia. In some reports, *Francisella* species or more specifically *F. tularensis* were detected by PCR or culture in the suspected water sources [12,15–20,24,32]. Moreover, the same *F. tularensis* genotypes were detected concomitantly from water sources and related tularemia patients in some studies [24,32,37], confirming the epidemiological link between drinking water and human infection. In the majority of cases, the source of contamination was spring water, water from the community water supplies or water from wells. These water sources were not or inadequately disinfected. Contamination of the water by infected animal carcasses or excreta was the most likely hypothesis [10]. Although *F. tularensis* subsp. *holarctica* was identified only for some of these outbreaks, only type B strains have been associated with human infections in Europe and Asia.

In Kosovo, two huge oropharyngeal tularemia outbreaks occurred in 1999–2000 and 2001–2002, each one involving more than two hundred patients [38,39]. It was not possible to establish if the primary source of infection was contaminated water or food, but it can be assumed that both were involved in the human transmission of *F. tularensis* [38,39]. These outbreaks were likely a collateral damage of the Kosovo war, which ended in 1999 and left the country with bad sanitary conditions. Indeed, people reported that mice and rats invaded their villages during this period, which may correlate with contamination of water wells and cellars with *F. tularensis*. This bacterium was detected in a field mouse recovered from a water well in an affected village [39].

A long-lasting outbreak occurred between 1997 and 2005 in Bulgaria, involving 285 people [40]. *F. tularensis* was isolated from water samples collected from private wells. Thus, the route of human contamination was likely water consumption. The majority of patients suffered from the oropharyngeal form of tularemia [40]. Similarly, in Georgia, a tularemia outbreak involving 26 patients occurred in 2006 [41], with a

Table 1. Tularemia outbreaks and sporadic cases related to drinking water.

Country	Year of occurrence	Number of cases	Clinical forms (n)	Source of infection (water detection of <i>F. tularensis</i>)	<i>F. tularensis</i> subsp. involved	Reference
Bulgaria	1997–2005	285	OP (275), UG (6), OG (4)	Drinking water mainly from private wells (culture, pos)	<i>holarctica</i>	[40]
Georgia	2006	26	OP (21), GL (5)	Using water from the community water supply (culture, pos)	NS	[41]
Germany	2007	1	OP	Drinking surface water (NS)	<i>holarctica</i>	[49]
Italy	1988	24	OP (12), OG (1), AS (11)	Drinking water (NS)	NS	[48]
Kosovo	1999–2000	> 200	OP	Drinking water or eating food (Ag, neg)	NS	[39]
	2001–2002	> 200	OP	Drinking water or eating food (Ag, neg)	NS	[38]
Macedonia	2015	13	NS	Drinking water (NS)	NS	[42]
Norway	1997	8	OP or UG	Drinking water from wells (one well with a lemming carcass PCR-positive for <i>F. tularensis</i>) (PCR, pos)	NS	[43]
	2006	9	OP (5), GL (3), UN (1)	Drinking water from private wells or eating snow (PCR, pos)	NS	[44]
	2011	39	OP (21), UG or GL (10), PN (2), TY (2), AS (3), UN (1)	Drinking water from private wells or stream (PCR, pos)	NS	[45]
Sweden	NS	1	OP	Drinking water from a well (NS)	<i>holarctica</i>	[46]
	2013	6	OP	Drinking water from a private well (culture, pos)	NS	[47]
Turkey	1988–1998	205	OP (83%), OG (8%), UN (9%)	Drinking water (NS)	NS	[14]
	NS	1	OP	Drinking water from a well (culture, neg)	NS	[29]
	2001	14	OP	Drinking village pipe water (culture, neg)	NS	[35]
	2005	5	OP	Drinking natural spring water (PCR, neg)	NS	[30]
	2005	10	OP (7), AS (3)	Drinking spring water (culture, neg and PCR, pos)	NS	[20]
	2004–2005	54	OP	Drinking water (PCR, neg)	NS	[34]
	2004–2005	39	OP	Drinking water (NS)	NS	[36]
	NS	2	OP	Drinking water (NS)	NS	[28]
	2005	11	OP (8), OG (3)	Drinking village fountain water (PCR, pos)	NS	[19]
	2004–2005	145	OP	Drinking spring water (NS)	<i>holarctica</i>	[13]
	2005	70	OP mostly	Drinking natural spring water (culture, neg)	NS	[33]
	2004	86	OP mostly	Drinking water from a rivulet (PCR, pos)	NS	[17]
	2004–2005	135	OP	Drinking natural spring water (PCR, neg)	NS	[11]
	2000	22	OP (19), UG (3)	Drinking spring water (PCR positive for <i>Francisella</i> spp.)	NS	[18]
	2005–2006	58	OP	Drinking natural spring water (PCR, pos)	<i>holarctica</i>	[16]
	NS	1	OG	Drinking and washing face with spring water (NS)	NS	[26]
	NS	3	OP	Drinking spring water (NS)	<i>holarctica</i>	[27]
	NS	4	OP	Drinking water (NS)	NS	[23]
	2011	2	PN (with bacteremia)	Drinking water (culture, pos)	<i>holarctica</i>	[24]
	2010	4	OP (3), OP and OG (1)	Drinking (3) or exposure (1) to natural spring water (NS)	NS	[25]
	2009–2011	139	OP (74%), GL (15.8%), OG (5%)	Drinking spring water (PCR, pos)	NS	[15]
	NS	2	OP (with abdominal lymphadenopathy)	Drinking natural spring water (NS)	NS	[21]
	NS	3	OP	Drinking natural spring water (NS)	NS	[22]
	2010–2012	110	OP or GL	Drinking water (PCR, pos)	NS	[12]
	2013	55	OP	Drinking tap water contaminated by surface water (culture, neg)	NS	[31]
	2008, 2009 and 2012	89, 54 and 35	OP	Drinking water (culture, pos)	NS	[32]

(NS) Not specified; Clinical forms: ulceroglandular (UG), glandular (GL), oropharyngeal (OP), oculoglandular (OG), pneumonic (PN), typhoidal (TY), asymptomatic (AS), and unknown form (UN); (Ag) *F. tularensis* antigen detection; (pos) positive, (neg) negative.

predominance of oropharyngeal cases. The water source was the community water supply from which *F. tularensis* was isolated [41]. Finally, a small outbreak involving 13 patients was described in 2015 in Macedonia [42].

Water-borne tularemia cases are also reported in Scandinavia. In Norway, three small outbreaks, involving eight to 39 people, linked to the consumption of contaminated water were reported over 20 years [43–45]. Most of the patients suffered from oropharyngeal tularemia [43–45], although ulceroglandular, glandular, pneumonic and typhoidal forms were also observed [43–45]. The source of these infections was water collected from private water wells, as confirmed by

PCR-detection of *F. tularensis* DNA [43–45]. *F. tularensis*-infected rodent carcasses probably contaminated these water wells. In one report, a lemming carcass retrieved from the contaminated water well was PCR-positive for *F. tularensis* [43]. The involved *F. tularensis* subspecies was not identified, but only type B strains are found in this country. In Sweden, a small outbreak and sporadic cases of oropharyngeal tularemia were also associated with the consumption of contaminated water from private water wells [46,47].

Tularemia cases related to *F. tularensis*-contaminated water consumption have been occasionally observed in other countries. In Italy, in 1988, a tularemia outbreak linked to the use of water collected from

an aqueduct involved 24 people living in the same village, most of which developed oropharyngeal tularemia [48]. More recently, a German oropharyngeal case of tularemia was suspected to be associated with drinking of non-purified surface water during a holiday in Bavaria [49].

Tularemia and mosquitoes

In Sweden and Finland, tularemia is mainly transmitted through mosquito bites. Large tularemia outbreaks, frequently involving more than one hundred people, have occurred almost annually in Sweden during the last two decades [46,50–54]. These outbreaks mainly occurred in central Sweden (counties of Örebro, Stockholm, Södermanland, Västmanland, Värmland, and Dalarna), in wet and woody areas of the Boreal Forest, during the late summer period, because of the mosquito vector habitat and seasonality [46,50–54]. A large scale epidemiological study in Sweden over 29 years identified that tularemia incidence was positively correlated with the presence of lakes and rivers [55]. Similarly, outbreak modeling demonstrated that tularemia in Sweden is concentrated in a few high-risk regions, with a high incidence in summer likely related to the population dynamics of the mosquito vector [56]. Due to this specific mode of transmission, the ulceroglandular form of tularemia is predominant in Sweden. In addition, cases occur in the exposed population regardless of age, but more frequently in men than women probably because of more outdoor activities in men [46,50,51,53]. Swedish mosquitoes collected in Örebro were PCR-positive for *F. tularensis* subsp. *holarctica*, still arguing their role in tularemia transmission in this country [57].

Tularemia is also predominantly a mosquito-borne disease in Finland, where several tularemia outbreaks [58] or sporadic cases [59,60] have been linked with this mode of transmission. Here again, tularemia is a seasonal disease occurring during late summer, and ulceroglandular tularemia is the primary clinical presentation [58,61].

The potential role of mosquitoes in the transmission of *F. tularensis* to humans has been occasionally reported in other countries, such as Germany [62].

All these reports involved *F. tularensis* subsp. *holarctica* as they occurred in Europe. Table 2 summarizes the literature reports of mosquito-borne tularemia cases. Interestingly, *F. tularensis* DNA was detected in Alaskan mosquitoes, suggesting that tularemia could be a mosquito-borne disease in this U.S. state [63].

Tularemia of other aquatic sources

All around the world, human contamination with *F. tularensis* has occasionally occurred through other types of water exposure (Table 3).

Tularemia cases were reported after near-drowning accidents in France [64] and Finland [65]. For these two cases, patients developed a pneumonic form of the disease after inhalation of contaminated water. For the French case, contamination occurred after inhalation of freshwater and was caused by *F. tularensis* subsp. *holarctica*. The involved subspecies and water salinity were not specified for the Finnish case. Four tularemia cases reported in Turkey were related to swimming activities [66,67]. Three of these cases were oropharyngeal forms that developed after swimming in the same lake [67]. The fourth patient suffered from a glandular form after swimming in a natural aquatic environment in a valley [66]. Otitis caused by *F. tularensis* subsp. *holarctica* were reported in three patients (two from France, one from Germany) after canyoning in the same river in France, between 2009 and 2014 [68]. Otitis likely occurred after exposure to *F. tularensis* contaminated water, although the source was not specifically identified [68].

Tularemia cases also occurred after handling *F. tularensis*-contaminated aquatic animals [69–71]. A small tularemia outbreak involving 19 patients occurred in Spain after crayfish fishing [69]. The patients developed a glandular or ulceroglandular form of the disease through skin injuries while catching or cleaning red swamp crayfishes in the same river. Cases were grouped over three weeks after which fishing was prohibited [69]. The source of contamination was confirmed by PCR-detection of *F. tularensis* DNA in one crayfish and two water samples from the river [69]. In Canada, a patient

Table 2. Tularemia outbreaks and sporadic cases related to mosquito bites.

Country	Year of occurrence	Number of cases	Clinical forms	<i>F. tularensis</i> subsp. involved	Reference
Finland	2000	1	UG	NS	[59]
	2007	50	UG mostly	<i>holarctica</i>	[58]
	2010	1	UG	NS	[60]
Germany	NS	1	UG	<i>holarctica</i>	[62]
Sweden	1981, 1995, 1999, 2000, 2002, 2003, and 2004	349	UG mostly	<i>holarctica</i>	[50]
	2000–2004	278	UG	<i>holarctica</i>	[46,51]
	2000	105	UG mostly	NS	[52]
	2003	475	NS	NS	[53]
	2006	90	NS	NS	[54]

(NS) Not specified; Clinical forms: ulceroglandular (UG).

Table 3. Tularemia outbreaks and sporadic cases related to other aquatic sources.

Country	Year of occurrence	Number of cases	Clinical forms (n)	Source of infection	<i>F. tularensis</i> subsp. involved	Reference
Canada	NS	1	UG	Suspicion of finger injury while cleaning freshwater fishes	NS	[70]
Finland	NS	1	PN	Near-drowning accident	NS	[65]
France	NS	1	PN with bacteremia	Near-drowning accident while fishing in a river	<i>holarctica</i>	[64]
Spain	2008–2014	3	Otomastoiditis	Canyoneering in the same river	<i>holarctica</i>	[68]
	1998	19	UG and GL	Crayfish fishing in a river	<i>holarctica</i>	[69]
Turkey	2007	3	OP	Swimming in the same lake	NS	[67]
	2010	1	GL	Swimming in freshwater	NS	[66]
USA	2000–2006 (Martha's Vineyard island)	59	PN (38), UG or GL (9), TY (2), OP (1)	Water environmental source likely	<i>tularensis</i>	[73,74]
	2016	1	UG	Finger injury while fishing in a freshwater lake.	<i>holarctica</i>	[71]

(NS) Not specified; Clinical forms: ulceroglandular (UG), glandular (GL), oropharyngeal (OP), pneumonic (PN), and typhoidal (TY).

developed ulceroglandular tularemia after a finger injury while cleaning pike and pickerel, which are freshwater fishes [70]. Similarly, in the USA, a patient contracted an ulceroglandular tularemia due to *F. tularensis* subsp. *holarctica* after a finger injury while fishing on a freshwater lake [71].

The particular ecology of Martha's Vineyard (Massachusetts), an island in the east coast of the USA should be highlighted. Two outbreaks of pneumonic tularemia occurred on this island, the first in 1978 [72] and the second from 2000 to 2006 [73,74]. *F. tularensis* subsp. *tularensis* was isolated from one dead patient and rabbits [73,75]. According to a case-control study, landscaping activities, especially lawn mowing and brush-cutting, were the most likely sources of exposure to *F. tularensis* through aerosols from environmental material [73,76]. However, the environmental reservoir of *F. tularensis* could not be characterized. Skunks and raccoons were found to be seropositive for *F. tularensis*, but other animal species tested had not or very rarely been infected with this pathogen [75]. A low prevalence of infection and a high degree of *Francisella* genetic diversity in ticks argued against the role of these arthropods as

a source of human contamination [77]. In contrast, the role of the aquatic environment as a long-term reservoir of *F. tularensis* was strongly suspected. PCR detection of *Francisella* sp. DNA was frequently reported from brackish-water samples collected on the island [78].

The role of brackish water as a potential reservoir of *F. tularensis* is supported by a more recent study in Nunavik territory (Canada) demonstrating that tularemia seroprevalence in the human population was positively correlated with residence near the coast [79].

Other *Francisella* species and aquatic reservoir

The genus *Francisella* includes many other species with a proven or probable aquatic habitat. Genetic analyses have suggested that the ancestral *Francisella* species originated in a marine habitat [80]. Here, we review water-borne human infections caused by *Francisella* species other than *F. tularensis* (Table 4). Some of these species are opportunistic pathogens mainly

Table 4. Human infections caused by *Francisella* species other than *F. tularensis* related to aquatic sources.

Country	Year of occurrence	Number of cases	Clinical forms	Source of infection	<i>F. tularensis</i> subsp. involved	Reference
Australia	NS	1	UG	Cut in the toe in brackish water	<i>F. novicida</i> -like	[99]
	NS	1	NS, bacteremia	Cut with a fishhook while fishing	<i>F. hispaniensis</i>	[105]
Canada	NS	1	OP and PN	Skin abrasion from a saltwater crab	<i>F. philomiragia</i>	[84]
Spain	NS	1	OP	Holiday activities in the Mediterranean sea	<i>F. philomiragia</i>	[83]
Turkey	NS	1	TY	Swimming in the sea and taking mud baths	<i>F. philomiragia</i>	[81]
USA	1977–1985	5	PN, bacteremia	Near-drowning accident in saltwater or brackish water	<i>F. philomiragia</i>	[86]
	1995	1	NS, bacteremia	Use of water from a private well for food preparation and bathing	<i>F. novicida</i> -like	[100]
	NS	1	NS, bacteremia	Working in the brackish water of Assawoman Bay in Maryland	<i>F. philomiragia</i>	[85]
	NS	1	TY	Practice of jet-ski in a bay in the Atlantic Ocean	<i>F. philomiragia</i>	[82]
	NS	1	NS, bacteremia	Near-drowning accident while surfing in the Atlantic Ocean	<i>F. novicida</i>	[97]
	2001	1	NS	Exposure to hot spring water near a salt-lake	<i>F. novicida</i>	[89]
	NS	1	UG	Suspicion of infection through contact of an open wound in brackish water	Species close to <i>F. haliotida</i>	[113]
	2011	3	NS, bacteremia	Consumption of ice from ice machines	<i>F. novicida</i>	[98]

(NS) Not specified; Clinical forms: ulceroglandular (UG), glandular (GL), oropharyngeal (OP), oculoglandular (OG), pneumonic (PN), and typhoidal (TY).

causing diseases in immunocompromised patients exposed to an aquatic environment. Among them, infections caused by *F. philomiragia* or *F. novicida* have been most frequently reported.

Less than 20 human infections with *F. philomiragia* have been published in the English literature [81–88]. These infections occurred in healthy individuals that have survived a near-drowning accident [86] or in immunocompromised patients (especially people suffering from chronic granulomatous disease) after exposure to an aquatic environment [81–86]. Interestingly, human contamination usually occurred after exposure to salt- or brackish-water [81–86]. Infections with *F. philomiragia* most often occurred during recreational activities in sea, ocean or bay connected to the ocean [81–83,85,86]. A young patient was contaminated after a skin lesion caused by a saltwater crab [84]. In a review of 14 cases, Wenger *et al.* showed that most *F. philomiragia* infections occurred in patients living within 50 miles of a salt-water coastline [86], again suggesting a clear association between salt-water exposure and *F. philomiragia* infections.

The geographical distribution of *F. philomiragia* is probably wide as human infections with this species have been described in the USA [82,85,86], Canada [84], Europe [83] and Turkey [81]. Frequent detection of *F. philomiragia* in water samples by culture or PCR indicates that the aquatic environment is likely the primary reservoir of this bacterium [78,89–93]. In two studies, in Norway [92] and the USA [78], *F. philomiragia* was only found in salt- or brackish-water but not in fresh-water, suggesting that water salinity is a major element in the natural life cycle of *F. philomiragia*. However, this species was also isolated from spring water near a salt lake in the USA [89] and cooling towers in China [91], reflecting its distribution in different aquatic reservoirs. *F. philomiragia* DNA was also detected in ballast water from cargo traveling all around the world [90], which might be a mode of diffusion of this species at the global scale. Although the aquatic reservoir appears to be predominant, *F. philomiragia* was isolated in a sick muskrat and in brackish water surrounding it [94] suggesting that a mammal reservoir may exist for this bacterium. *F. philomiragia* was also detected in *Dermacentor* ticks [95].

F. novicida is also a rare human pathogen. A dozen cases have been published in the English literature so far [96]. For all cases with an identified mode of contamination, an aquatic source was involved [89,97–100]. In the USA, a case of *F. novicida* bacteremia occurred after a near-drowning accident in the Atlantic Ocean [97], another case after exposure to hot spring water near a salt lake [89], and the last one after exposure to water from a private water well [100]. A striking outbreak of *F. novicida* bacteremia occurred among inmates in the USA after consumption of ice

from ice machines from which *F. novicida* DNA was detected by PCR [98]. Finally, in Australia, a patient developed an ulceroglandular form of infection with *F. novicida* after cutting himself in brackish water [99]. Most of the patients suffering from *F. novicida* infection were immunocompromised or had underlying health conditions [98,100]. The identification of *F. novicida* has never been reported in animals or arthropods [96]. The only known reservoir of this bacterium is the aquatic environment as attested by its repeated isolation from water samples [78,89,101,102]. As for *F. philomiragia*, salinity seems to impact *F. novicida* survival in water, as this bacterium was detected by culture or PCR only in sea-water [101,102], brackish-water [78] and spring water near a salt lake [89].

Other *Francisella* species have been rarely associated with human infections originating from aquatic sources. *F. hispaniensis* was first isolated in 2003 from a Spanish patient suffering from bacteremia [103,104]. The source of contamination was not identified [103]. However, a few years later, *F. hispaniensis* was isolated in the blood of an Australian immunocompromised patient after he cut himself with a fishhook while fishing [105].

The genus *Francisella* also includes species that are pathogenic for marine animals. *F. noatunensis* subsp. *orientalis* and *Francisella noatunensis* subsp. *noatunensis* are widely described as warm- and cold-water fish pathogens, responsible for ‘piscine francisellosis.’ This disease causes high morbidity and mortality in many fish species worldwide and is responsible for economic losses in aquaculture [106–109]. *F. noatunensis* does not seem to be pathogenic in humans [106]. *F. halioticida* can infect *Haliotis* mollusks (abalones) [110,111] and *F. marina* sp. nov. was recently identified as causing disease in Spotted Rose Snapper fishes [112]. Interestingly, a novel *Francisella* species very close to *F. halioticida* was isolated in the USA, in a diabetic patient, from an infected skin wound developed after contact with brackish water [113]. Finally, other *Francisella* species are endosymbionts of marine ciliates such as *F. endociliophora* [114].

Over the past ten years, new *Francisella* species have been isolated from the aquatic environment. *F. salina* and *F. uliginis* were isolated from sea-water in the USA [101,102]. Water from cooling towers also seems to be a reservoir of *Francisella* species as attested by recent isolation from these air conditioning systems of *F. frigiditurrus* in the USA [102] and *F. guangzhouensis* in China and Germany [91,115–117]. These two latter species have been transferred to the new genus *Allofrancisella* [115].

Table 5 summaries studies dealing with the detection of *Francisella* species in water samples, either using culture, PCR or both methods. It shows the broad spectrum of *Francisella* species found in aquatic reservoirs.

Regarding PCR methods, it is important to notice that *Francisella* species that are not yet characterized may not be PCR-amplified from water samples due to the use of inadequate primers [78]. Even if there is PCR amplification with a new species, it may not be accurately identified as a novel species due to a lack of resolution within the utilized amplicon. Full identification of a novel species requires whole genome sequencing.

Mechanisms of *F. tularensis* survival in water environments

The high frequency of water-borne tularemia cases implies the persistence of *F. tularensis* in the aquatic environment. However, the mechanisms of *F. tularensis* survival in this environment have not been elucidated so far.

Long-term survival of *F. tularensis* in water

Experimental studies suggest long-term survival of *F. tularensis* in various water environments. Several authors described the survival of *F. tularensis* in water microcosm from 1 to 70 days [118–120]. *F. tularensis* survival seems to be influenced by both water temperature [118] and salinity [119]. Gilbert and Rose observed that *F. tularensis* subsp. *holarctica* remains cultivable after a stay in water for one day at 5°C or 25°C, but up to 28 days at 8°C [118]. Berrada and Telford showed that both type A and type B strains of *F. tularensis* remain cultivable after a stay of 8–10 days in fresh-water, but 30–42 days in brackish-water, both at 21°C [119]. In another study, *F. tularensis* subsp. *holarctica* remained cultivable after a stay in fresh water at 8°C up to 70 days [120].

Interestingly, when *F. tularensis* became uncultivable on agar plates, bacteria could still be detected and were metabolically active in water [118,120]. This phenomenon was observed by Gilbert and Rose [118] and Forsman *et al.* [120] for *F. tularensis* subsp. *holarctica*. The latter authors described the persistence of metabolic activity for *F. tularensis* in water up to 140 days [120]. This state is defined as ‘viable but non-culturable’ (VBNC) and could be responsible for long-term survival of bacteria in the water environment. The VBNC state has been defined as a state from which bacterial cells cannot be cultured but maintain a metabolic activity and cellular integrity [118]. In addition, the VBNC state may be reversible, as bacteria may become cultivable under certain conditions. This reversion in the ability to grow on acellular media is called ‘resuscitation’ of VBNC bacteria. The VBNC state has been described for a wide range of bacteria such as *Vibrio* sp. [121,122], *Campylobacter* sp. [123], *Escherichia coli* [122] and *Legionella pneumophila* [124]. Depending on the bacterial species, the infectious nature and pathogenic potential of VBNC cells

are variable, as well as the ways of their resuscitation. Forsman *et al.* reported that *F. tularensis* VBNC cells were no longer virulent in mice, and could not be resuscitated [120]. Finally, a recent study demonstrated that *F. tularensis* subsp. *holarctica* possesses a mechanosensitive channel that protects this bacterium from hypo-osmotic shock when it is released from an infected animal to water [125].

Long-term survival in water has also been reported for other *Francisella* species. Berrada and Telford showed that *F. novicida* (like *F. tularensis*) remains cultivable after a stay of up to 30–42 days in brackish-water, at 21°C [119]. The fish-pathogen *Francisella* species are able to survive in water in the absence of a suitable fish host. Indeed, *F. noatunensis* subsp. *orientalis* remains cultivable after a stay up to 2 days in freshwater and up to 3 days in seawater [126]. *F. noatunensis* subsp. *noatunensis* remains cultivable after up to 12 days in freshwater and up to 50 days in seawater [127]. Interestingly, *F. noatunensis* was also able to enter in a VBNC state after a period of stay in the water, and these VBNC were not pathogenic to cods [127].

Long-term survival of *F. tularensis* in natural aquatic environments is suggested by a number of studies detecting this bacterium by culture or PCR in environmental water samples [12,16,17,19,20,32,40,41,43,44,47,78,128–132]. Not surprisingly, long-term detection of *F. tularensis* in water environments was reported in countries where water-borne tularemia cases are frequent and predominant. In Turkey, two environmental studies identified *F. tularensis* subsp. *holarctica* in water samples collected from the aquatic environment or from village water supply systems highlighting the role of different water sources as common and persistent reservoirs of *F. tularensis* in this country [32,130]. In Sweden, *F. tularensis* subsp. *holarctica* was PCR-detected in water samples collected during outbreak and non-outbreak periods, in tularemia endemic areas [131]. However, *F. tularensis* was also detected from water samples in countries where tularemia is not or rarely a water-borne disease [128,129,132]. Hightower *et al.* isolated *F. tularensis* strains from water in Ukraine and considered that the aquatic environment was the third main *F. tularensis* reservoir after arthropods and mammals in this country [129]. In Germany, following the reemergence of tularemia in 2004, an ecological study in outbreak areas found a river water sample PCR-positive for *F. tularensis* suggesting a natural aquatic reservoir for this bacterium [132]. In the Netherlands, tularemia also re-emerged in 2011 leading to the surveillance of *F. tularensis* prevalence in the environment [128]. Surface water samples were PCR-positive for *F. tularensis* subsp. *holarctica* in 10% of the randomly collected samples and 88% of the samples collected in areas where tularemia cases among hares or humans had been reported [128]. These studies show the almost

Table 5. Detection of *Francisella* species in water samples.

Country	Year of sampling	Type of water samples (n)	Testing methods	Findings (n samples)	Reference
China	2008	Cooling towers (NS)	Culture and strain identification by fatty acid analysis, and 16S rRNA, 23S rRNAs, <i>recA</i> , <i>rpoA</i> , <i>rpoB</i> , <i>rpoD</i> , <i>rpoH</i> , <i>groEL</i> , <i>dnaK</i> , <i>gyrB</i> , <i>sdhA</i> , and <i>fopA</i> genes sequencing	<i>F. guangzhouensis</i> (4)	[117]
	2009–2011	Cooling towers (312)	Culture and strain identification by 16S rRNA, <i>rpoB</i> and <i>sdhA</i> genes sequencing	<i>Francisella</i> strains phylogenetically close to <i>F. philomiragia</i> (1) or <i>F. guangzhouensis</i> (8)	[91]
	2008 and after	Cooling towers (NS)	Culture and strain identification by mass spectrometry, fatty acid analysis, and 16S rRNA, <i>rpoB</i> , <i>mdhA</i> , and <i>sdhA</i> genes sequencing	<i>Francisella</i> strains phylogenetically close to <i>F. guangzhouensis</i> (5). Description of <i>Allofrancisella inopinata</i> gen. nov., sp. nov. and <i>A. frigidaquae</i> sp. nov.; transfer of <i>F. guangzhouensis</i> to <i>A. guangzhouensis</i> comb. nov.	[115]
Germany	2005–2006	NS (28)	PCR targeting 16S rRNA and <i>fopA</i> genes	<i>F. tularensis</i> (1)	[132]
	2012	Cooling tower (NS)	Culture and strain identification by 16S rRNA, <i>fopA</i> , <i>gyrA</i> , <i>rpoA</i> , <i>groEL</i> , <i>sdhA</i> , and <i>dnaK</i> genes sequencing	<i>Francisella</i> strain phylogenetically close to <i>F. guangzhouensis</i> (1)	[116]
Netherlands	2013–2017	Surface water collected from areas with reported human or hare tularemia cases (127) or unrelated to recent tularemia cases (339)	PCR targeting <i>ISFtu2</i> and <i>fopA</i> genes	<i>F. tularensis</i> in 88% of the case-related samples and in 10% of the randomly collected samples	[128]
Norway	2010	Seawater (149) or freshwater (64)	PCR sequencing of 16S rRNA; and for positive samples <i>sdhA</i> and <i>purCD</i> PCR	<i>Francisella</i> sp. in seawater (38) but not in freshwater samples. <i>F. philomiragia</i> -related species mostly.	[92]
Sweden	2003–2005	Surface water (341)	PCR sequencing of <i>lpaA</i> , 16S rDNA, <i>lpaA</i> , and FIM19InDel	<i>F. tularensis</i> (108), mainly subsp. <i>holarctica</i> , rarely subsp. <i>mediasiatica</i>	[131]
Turkey	2008–2009	Rivers, spring waters or village fountains in tularemia-endemic areas (154)	Culture and strains identification by 16S rRNA gene sequencing; and PCR targeting <i>ISFtu2</i>	<i>F. tularensis</i> subsp. <i>holarctica</i> isolation (4); or <i>F. tularensis</i> positive PCR <i>ISFtu2</i> (17)	[130]
Ukraine	1941–2008	NS (NS)	Culture	<i>F. tularensis</i> (393)	[129]
USA	2003	NS (23)	PCR sequencing of 16S rDNA; and for positive samples <i>ISFtu2</i> , <i>23 kDa</i> , <i>tul4</i> , <i>fopA</i> and <i>sdhA</i> PCR	<i>F. philomiragia</i> (1)	[93]
	NS	Seawater (NS)	Culture (CHAB-PACCV medium) and strains identification by PCR sequencing of 16S rRNA and <i>sdhA</i> genes	<i>F. philomiragia</i> -like (2) and <i>F. novicida</i> -like (1). The two <i>F. philomiragia</i> -like strains were latter characterized as new <i>Francisella</i> species by Challacombe et al.: <i>F. salina</i> and <i>F. uliginis</i> .	[101,102]
	2005–2007 (Martha's Vineyard island)	Fresh-water (35) or brackish-water (42)	Culture and PCR targeting 16S rRNA gene; for positive samples <i>sdhA</i> , <i>tul4</i> , <i>ISFtu2</i> , and <i>fopA</i> PCR	No positive fresh-water samples. <i>Francisella</i> DNA detected by PCR in brackish-water samples (19). Mainly <i>F. philomiragia</i> ; few <i>F. novicida</i> - or <i>F. tularensis</i> -like strains. <i>F. philomiragia</i> grown from one brackish-water sample.	[78]
	NS	Hot or cold spring waters near a salt lake (NS)	Culture and strain identification by fatty acid analyses, ribotyping and 16S rRNA gene sequencing	<i>F. philomiragia</i> and <i>F. novicida</i> (NS)	[89]
Cargo ships*	NS	Cooling tower (NS)	NS	<i>F. frigiditurris</i> (NS)	[102]
	2007–2008	Ballast water from 5 general cargo ships (NS)	PCR sequencing of 16S rRNA gene	<i>F. philomiragia</i> and <i>F. noatunensis</i> in ballast water from 4 cargo ships	[90]

(NS) Not specified; * Cargo ships from Columbia, Republic of the Congo, USA, Canada, and Iran.

constant presence of *F. tularensis* in randomly collected water samples at different time periods. This observation can only be explained by repeated contaminations of the aquatic environment, particularly from the animal reservoir during epizootics, but also by the persistence of these bacteria in the water environment during non-epizootic periods.

Table 5 summarizes studies dealing with *F. tularensis* detection in water environments. PCR was more effective than culture for detection of *F. tularensis* in water samples, possibly because of a low bacterial inoculum, the encroachment of *F. tularensis* by other bacterial species, or a VBNC state of bacteria. However, PCR results should be interpreted with caution since it has been shown that *F. tularensis* specific primers (such as those targeting *foxA* or *tulA* genes) may also amplify DNA from *Francisella* species other than the tularemia agents [78].

Survival in biofilms

An essential mechanism for survival and persistence of bacteria in the water environment is biofilm formation. Biofilms are defined as naturally formed adherent communities of bacteria within an extracellular polymeric matrix [133]. A number of bacterial species, such as *Vibrio cholerae* [134], *Legionella pneumophila* [135], *Helicobacter pylori* [136] and *Pseudomonas aeruginosa* [137] form biofilms to promote their survival under environmental water conditions. *In vitro* studies have demonstrated that both *F. tularensis* subsp. *holarctica* [138,139] and *F. tularensis* subsp. *tularensis* [139] can form biofilms. The aquatic *Francisella* species, *F. novicida* [139–141] and *F. philomiragia* [142] have also been capable of biofilm formation experimentally. *F. novicida* was also demonstrated to be able to form biofilm in chitin surface, the second most abundant biopolymer in nature, providing the structure of arthropods, insects, and fungi [139]. *F. philomiragia* was shown to form more biofilm at 25°C than at 37°C, which is compatible with its natural aquatic reservoir [142]. The fish pathogen *F. noatunensis* was also demonstrated to form biofilm *in vivo*. [143]. Until now, biofilm formation has not been associated with virulence in *Francisella* species [133]. Thus, biofilm formation is most likely a way of environmental survival and persistence in these species [133]. To our knowledge, *Francisella* biofilm has never been described in natural environmental water microcosm.

Survival in amoebae

Free-living amoebae are ubiquitous organisms in soil and water environments. Several human pathogens, such as *L. pneumophila* and some *Mycobacterium* species resist phagocytosis and digestion by the free-living amoebae and may survive in water environment

inside amoebae [144]. Moreover, these bacteria may survive for long periods in amoeba cysts [144]. This mode of survival may apply to *Francisella* species, especially *F. tularensis*. Multiple experimental studies have focused on the interaction between *Francisella* species and several amoeba species [145–154]. Berdal *et al.* demonstrated that *F. tularensis* could penetrate in the amoeba *Acanthamoeba castellanii* and be released from it [152]. Then, other authors described the multiplication of *F. tularensis* subsp. *tularensis* in *A. castellanii* 24 h post-infection [150]. *F. novicida* and *F. philomiragia* were also able to multiply within *Hartmanella vermiformis* and *A. castellanii* [142,146,148,150,151]. *F. noatunensis* was also able to infect and replicate within the amoeba *Dictostelium discoideum* [149]. *Francisella* cells were localized within vacuoles in amoeba trophozoites [146] but were also able to survive in amoebal cysts for several weeks [150]. This latter finding suggested that amoeba could be a long-term reservoir of *Francisella* spp. in water environments. Other authors described an enhanced survival of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* and *F. novicida* in co-culture with amoebae such as *A. castellanii*, *A. polyphaga*, *Vermamoeba vermiformis* or *Ochromonas danica* [145,147,153]. These authors described the presence of *Francisella* sp. inside and outside the amoeba in the co-culture model, suggesting that bacterial survival could be related to intra-amoebal replication, to favorable interaction between extracellular bacteria and amoeba, or both [145,147,153]. Interestingly, Gustafsson *et al.* demonstrated that growth supernatant of *A. palestinensis* (without the presence of amoebae) increased multiplication of *F. tularensis* [154]. After five days in co-culture with amoeba, *F. tularensis* subsp. *holarctica* was also shown to enter in a VBNC state [147]. In contrast to *L. pneumophila*, passage through amoebae did not increase *Francisella novicida* virulence [148]. Despite some disagreements about interaction mechanisms, all these reports argue that *Francisella* spp. are resistant to free-living amoeba and that protists may contribute to the survival of *Francisella* sp. in the water environment. However, to our knowledge, *Francisella* species have never been detected within amoebae in environmental water samples, in contrast to *L. pneumophila* and *Mycobacterium* spp. [155].

Survival in mosquito larvae

Mosquito larvae may also represent a long-term *F. tularensis* reservoir in the aquatic environment. It has been shown that these larvae can ingest *F. tularensis* subsp. *holarctica* that are present in water and ingested bacteria survive throughout the different maturation stages of these arthropods up to adult mosquitoes [57,63,138,156,157]. The fact that

mosquito-borne tularemia cases have occurred over years in Sweden and Finland should be considered a further evidence of the existence of a long-term aquatic reservoir of *F. tularensis*. However, it should be notified that mosquitoes could also be infected at the adult stage after a blood meal on an infected host [57]; in this case tularaemia is not water-borne.

Discussion

Although water-borne tularemia was first described in the 1930s [9], this route of human contamination has been largely underestimated. Tularemia cases linked to the aquatic reservoir are common and can occur as large epidemics. Thus, tularemia is a major public health problem in countries where water-borne tularemia cases predominate. These cases may occur through consumption of *F. tularensis*-contaminated drinking water, such as in Turkey [11–36] and its neighboring countries [38–42], and in Norway [43–45] where large-scale tularemia outbreaks caused by *F. tularensis* subsp. *holarctica* are regularly reported. Drinking water as a source of human infections with type B strains of *F. tularensis* have also been occasionally encountered in Sweden [46,47] and central Europe [48,49]. Human infections have occurred after consumption of contaminated water from the community water supplies, especially in countries with bad sanitary conditions, old water networks and inappropriate water treatment. Consumption of unsanitized surface water or well water was also a source of contamination. Consequently, it is of primary interest for practitioners to keep in mind that tularemia may correspond to sub-acute or chronic pharyngitis associated with cervical lymphadenopathy, especially in patients living or traveling in countries where water-borne tularemia cases are frequent. In addition, medical questioning regarding tularemia exposure should include a statement about unsanitized water consumption. Until now, this mode of human contamination has been observed in restricted areas and only linked to *F. tularensis* subsp. *holarctica*. However, similar cases could occur throughout the Northern hemisphere where type B strains are encountered. *F. tularensis* subsp. *tularensis* has also been associated with the water environment [72–74], and could also cause tularemia cases related to drinking water.

Mosquito-borne tularemia is also related to contamination of the aquatic environment by *F. tularensis*. Tularemia outbreaks caused by *F. tularensis* subsp. *holarctica* in Sweden [46,50–54] and Finland [58–60] are primarily related to mosquito bites. Because of skin inoculation of bacteria, the ulceroglandular form of tularemia predominates. Infections usually occur during the warm season at the time of maximum activity of mosquitoes.

Scandinavian practitioners are now particularly aware of this situation and usually diagnose tularemia early in the course of the disease. In contrast, this mode of transmission is most often unknown by physicians in other parts of the world, and mosquito-borne tularemia cases could be missed in patients returning from Scandinavian countries. Also, potential transmission of tularemia through mosquito bites outside Scandinavia has not been thoroughly evaluated. A probable autochthonous mosquito-borne tularemia case was reported in Germany [62]. In Alaska, mosquitoes tested positive for *F. tularensis* DNA [63] arguing that these arthropods could also transmit tularemia to humans in this US state. In this time of global warming, mosquito vectors could spread to new geographic areas, leading to a rise in mosquito-borne tularemia cases [158].

Finally, tularemia cases may occur through other types of aquatic exposure such as near drowning accident [64,65], swimming [66,67], canyoning [68] and fishing activities [69–71], due to penetration of *F. tularensis* through the skin, conjunctiva, or digestive and respiratory tracts. These cases have been reported in Europe [64,65,68,69], Turkey [66,67] and North America [70,71], suggesting a wide aquatic distribution of *F. tularensis*. Type B strains of *F. tularensis* were likely involved in all these cases, except one case occurring in Canada for which type A and type B strains could be involved [70]. These data suggest that *F. tularensis* subsp. *holarctica* could be more frequently associated with aquatic reservoirs than *F. tularensis* subsp. *tularensis*. There is currently no explanation regarding the preferential association of type B strains with water. Williamson *et al.* demonstrated that type B strains can resist to hypoosmotic shock when released into water [125]. It would be interesting to compare the osmotic shock resistance of type A versus type B strains. Noticeably, almost all human contaminations occurred after contact with fresh-water, suggesting that *F. tularensis* may better survive in such aquatic environment. *F. tularensis* subsp. *tularensis* may also infect humans through water exposure, as attested by the particular ecology of the Martha's Vineyard island in the USA [72–74]. In this case, brackish-water was considered as a potential reservoir of *F. tularensis* [78]. Altogether, available data indicate that human contamination with *F. tularensis* may occur from a wide diversity of aquatic sources and activities.

Francisella species other than *F. tularensis* are primarily considered aquatic bacteria. *F. philomiragia* and *F. novicida* have been occasionally involved in human infections, especially in immunocompromised patients, but also in specific situations such as near-drowning accident [81–86,89,97–100]. Infections caused by these species were frequently associated



Figure 1. Potential aquatic sources of human infections with *Francisella tularensis*. *Francisella tularensis* is released into water from animals. The bacterium is able to survive in water (W), in mosquito larvae (L), in biofilms (B), or in cooperation with amoeba (A). Human can be contaminated from the aquatic reservoir by drinking contaminated water (D), after a mosquito bite (M), or during swimming (S) and fishing (F) activities.

with exposure to brackish-water or salt-water [81–86,89,97,99]. In line with this observation, *F. philomiragia* and *F. novicida* have been frequently isolated from brackish- and salt-water samples [78,89,90,92,101,102]. In recent years, novel *Francisella* species have been detected in environmental or clinical samples, the majority of them being associated with an aquatic reservoir. Among them, *F. hispaniensis* [105] and *F. halioticida* [113] are rare human pathogens. These findings strongly suggest that the *Francisella* species are mainly aquatic bacteria.

Several mechanisms could be involved in the survival of *F. tularensis* in aquatic environments. Experimental and epidemiological studies have demonstrated that both *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* can survive for long periods in water microcosms [32,78,118–120,125,128–132]. Interestingly, after a long stay in water, *F. tularensis* has been shown experimentally to evolve to a VBNC state [118,120], which could account for long-term survival of this bacterium in water. Both type A and type B strains of *F. tularensis* can form biofilms *in vitro* [138,139], another potential survival mechanism of these bacteria in aquatic environments. Experimentally, both subspecies are able to multiply in amoebae or at least interact with these protozoa to enhance their survival [145,147,150,152,153]. Finally, *F. tularensis* subsp. *holarctica* is also able to infect mosquito larvae *in vitro* and survive during larvae maturation up to the adult stage [57,63,138,156,157]. It should be mentioned, however, that all these mechanisms have been evaluated experimentally, but not yet confirmed in natural water environments. It is very likely that all these mechanisms exist and are entangled. Infected animals and animal carcasses may contaminate

water environments, in which protozoa, mosquito larvae and biofilm communities may become contaminated with *F. tularensis* and serve as reservoirs for this bacterium.

In conclusion, our goal was to demonstrate that *F. tularensis* is likely able to survive for prolonged periods in various aquatic environments, which likely constitute a significant reservoir for this bacterium. Figure 1 summarizes the probable tularemia aquatic cycle as it can be pictured from current literature data. In terms of public health, it is important to remember that providing people with access to safe drinking water via treatment of municipal and private sources remains a priority. Better characterization and control of the aquatic reservoir of *F. tularensis* would also be of tremendous importance following a bioterrorist attack. Following a bioterrorist attack, *F. tularensis* could survive for months in the environment, leading to a high number of secondary tularemia cases. Water and mosquito reservoirs should be monitored in the overall tularemia surveillance, in addition to the wildlife reservoir.

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**TRAVAIL N°2 : Amoebae can promote the
survival of *Francisella* species in the
aquatic environment**

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Le travail de revue précédemment réalisé a mis en évidence un manque de connaissances concernant les mécanismes de survie de *F. tularensis* dans l'environnement aquatique. Ainsi, j'ai poursuivi mon travail de thèse sur un versant expérimental ayant pour objectif de définir si les amibes participaient à la survie environnementale de l'agent de la tularémie. En effet, peu d'études sur le sujet étaient disponibles et leurs résultats étaient partiellement contradictoires.

Afin de répondre à cette problématique, j'ai établi successivement plusieurs modèles d'interactions entre les amibes du genre *Acanthamoeba* et plusieurs souches de *Francisella*, dont une souche virulente de *F. tularensis* isolée en clinique.

Le premier modèle utilisé était le modèle simplifié Amoeba Plate Test (APT). Ce modèle étudie la capacité d'une souche bactérienne, déposée sur une gélose recouverte d'un tapis amibien confluent, à échapper à la prédation des amibes afin de traverser le tapis amibien et de se développer sur la gélose. Ce modèle a mis en évidence une résistance de l'ensemble des souches de *Francisella* testées aux amibes. L'espèce aquatique *F. philomiragia* présentait la plus forte capacité de croissance sur le tapis amibien. *Francisella novicida* et les souches cliniques virulentes de *F. tularensis* présentaient une capacité de croissance intermédiaire. Enfin, la souche vaccinale LVS de *F. tularensis* présentait la capacité de croissance la plus faible.

Le modèle APT ayant mis en évidence la capacité des bactéries du genre *Francisella* à résister aux amibes, j'ai donc cherché à mieux définir les interactions impliquées. Pour ce faire, j'ai poursuivi mes expérimentations par des tests d'infection, visant à déterminer si les bactéries du genre *Francisella* pouvaient se multiplier à l'intérieur des amibes. Le principe des tests d'infection est d'infecter un tapis amibien par une souche de *Francisella* puis d'éliminer les bactéries extra-cellulaires après l'étape d'infection, afin de monitorer uniquement l'évolution des bactéries intra-amibiennes. J'ai tout d'abord réalisé un modèle d'infection préalablement décrit dans la littérature, utilisant comme surnageant des cultures un milieu riche, le milieu PYG sans glucose (PYG/wg). Dans le modèle d'infection en milieu PYG/wg, j'ai observé une forte multiplication intra-amibienne de *F. philomiragia*, *F. novicida* et de la souche clinique Ft6 de *F. tularensis*. A l'inverse, la souche LVS ne présentait pas de multiplication intra-amibienne. Cependant, des tests préliminaires m'avaient permis de mettre en évidence que les bactéries utilisées étaient capables de multiplication en milieu

PYG/wg. En effet, les numérations bactériennes extra-cellulaires au cours des tests d'infection en PYG/wg étaient superposables aux numérations des bactéries intra-cellulaires, rendant difficilement exploitables les résultats de ces tests. C'est pourquoi, j'ai par la suite décidé de réaliser des tests d'infection en milieu plus pauvre, le milieu SM (Starvation Medium), dans lequel j'avais préalablement vérifié que les bactéries du genre *Francisella* ne pouvaient pas se multiplier extra-cellulairement. Les tests d'infection en milieu SM ont révélé une absence de multiplication intra-amibienne de *F. philomiragia*, *F. novicida*, Ft6 et LVS. Les numérations de bactéries intra-amibiennes décroissaient progressivement jusqu'à devenir indétectables entre un et sept jours après l'infection.

N'ayant pas mis en évidence de multiplication intra-amibienne des bactéries du genre *Francisella*, j'ai poursuivi mes expérimentations par des modèles de co-culture visant à déterminer si les amibes pouvaient favoriser la survie des bactéries du genre *Francisella*, que ce soit via des interactions intra ou extracellulaires. Ce modèle est d'ailleurs plus représentatif des interactions survenant dans le milieu aquatique naturel. Les tests de co-culture étaient réalisés par mise en contact d'un tapis amibien avec une souche de *Francisella* sans destruction des bactéries extra-cellulaires, en milieu SM. Sur 16 jours, la numération bactérienne totale (c'est-à-dire extra-amibienne et intra-amibienne) était monitorée régulièrement. Dans ce modèle de co-culture, *F. novicida* et *F. philomiragia* ont démontré une survie favorisée par la présence d'amibes. En effet, lorsque la bactérie était seule dans le milieu de culture, sa numération décroissait pendant 16 jours alors qu'en présence d'amibes, la numération bactérienne se maintenait, voire augmentait sur 16 jours. A l'inverse, pour *F. tularensis* Ft6, en présence ou en l'absence d'amibe la numération bactérienne déclinait progressivement sur 16 jours.

La survie prolongée de *F. philomiragia* et de *F. novicida* en présence d'amibes pouvait s'expliquer soit par une interaction de « contact » entre ces deux micro-organismes soit par l'excrétion par les amibes de composés favorisant la survie des bactéries et/ou la dégradation de composés toxiques par les protozoaires. Afin d'investiguer ces hypothèses, j'ai reproduit les tests de co-culture en séparant les bactéries et les amibes par un insert. Ainsi, les bactéries et les amibes ne pouvaient plus entrer en contact, cependant elles partageaient toujours le même milieu de culture. La survie favorisée de *F. novicida* en présence d'amibes était partiellement perdue lorsque les bactéries et les amibes étaient séparées par un insert bien que les numérations bactériennes restaient significativement

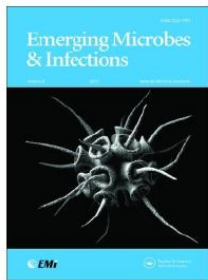
supérieures avec insert que lorsque les bactéries étaient seules dans le milieu. Ceci suggère que la survie prolongée de *F. novicida* en présence d'amibes est à la fois due à des interactions de « contact » et à la production par les amibes de composés favorisant la croissance bactérienne. Les expérimentations avec inserts pour *F. philomiragia* conduisaient à des résultats non reproductibles ne permettant pas de conclure. Bien que les co-cultures de Ft6 avec les amibes n'avaient pas permis d'observer une survie favorisée par les amibes, j'ai tout de même réalisé les co-cultures avec insert pour cette souche bactérienne. De manière surprenante, lorsque Ft6 était séparée des amibes par un insert, la survie bactérienne était largement favorisée. Une hypothèse pour expliquer ce phénomène serait que la réplication de Ft6 étant relativement lente, lorsque la bactérie est en contact avec les amibes, elle est phagocytée plus rapidement qu'elle ne se réplique mais lorsque la bactérie est séparée des amibes par l'insert, elle n'est plus phagocytée et elle bénéficie de composés sécrétés par les amibes qui favorisent sa croissance.

Pour finir, j'ai étudié le rôle du système de sécrétion de type 6 (T6SS) de *Francisella* dans les interactions avec les amibes. Pour rappel, le T6SS est un facteur de virulence majeur de *F. tularensis*, indispensable à la réplication dans les macrophages. Pour ce faire, j'ai comparé, dans les différents modèles précédemment décrits, le comportement d'une souche sauvage de *F. novicida* et d'une souche knock-out pour ce système, générée par l'équipe du Dr Thomas Henry à Lyon. En APT, en infection en PYG/wg, en infection en SM et en co-culture en SM, les deux souches se sont comportées de manière identique, suggérant que le T6SS n'était pas impliqué dans les interactions avec les amibes.

Mes travaux personnels ont été complétés par l'équipe croate du Pr. Marina Santic, qui travaille également sur l'étude des interactions entre les bactéries du genre *Francisella* et les amibes. L'équipe du Pr. Santic a réalisé des co-cultures de *F. novicida* et d'amibes dans de l'eau de source à température ambiante pendant 30 jours afin d'être au plus près des conditions environnementales. Dans ce modèle, il a été observé une survie prolongée de *F. novicida* en présence d'amibes par rapport à la bactérie seule dans le même milieu, de même qu'un maintien de l'intégrité bactérienne en présence d'amibes.

Ce travail expérimental a permis de préciser les relations entre les bactéries du genre *Francisella*, y compris des souches virulentes cliniques de *F. tularensis*, et les amibes. Selon mes travaux, *F. philomiragia*, *F. novicida* et *F. tularensis* ne se multiplient pas à

l'intérieur des amibes mais leur survie est favorisée par la présence de ces protozoaires. Ainsi, il existe très probablement une relation de commensalisme entre ces deux micro-organismes, suggérant que les amibes sont potentiellement impliquées dans la survie dans le milieu aquatique des *Francisella*, y compris *F. tularensis*. Néanmoins, les variations observées entre les espèces du genre *Francisella* ainsi que les observations parfois contradictoires entre les études laissent à penser que les amibes ne sont probablement pas le seul support de la survie aquatique des *Francisella*. Ainsi, la capacité des *Francisella* de former et de survivre dans les biofilms et la capacité de survie sous forme planctonique, potentiellement sous forme viable mais non cultivable (VBNC) doivent également être plus précisément investiguées.



Amoebae can promote the survival of *Francisella* species in the aquatic environment

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Amoebae can promote the survival of *Francisella* species in the aquatic environment

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ABSTRACT

Francisella tularensis, a tier 1 select agent, is the causative bacterium of tularemia, a zoonosis with a large animal reservoir. However, *F. tularensis*, like many other *Francisella* species, is assumed to have an aquatic reservoir. The mechanisms of *Francisella* species persistence in surface water remain poorly characterized. In this study, we deeply investigated the long-term interactions of the tularemia agent *F. tularensis* subsp. *holarctica*, *F. novicida* or *F. philomiragia* with amoebae of the *Acanthamoeba* species. In amoeba plate screening tests, all the *Francisella* species tested resisted the attack by amoebae. In *in vitro* infection models, intra-amoebic growth of *Francisella* varied according to the involved bacterial species and strains, but also the amoeba culture medium used. In co-culture models, the amoebae favoured *Francisella* survival over 16 days, which was likely dependent on direct contact between bacteria and amoebae for *F. novicida* and on amoeba-excreted compounds for *F. novicida* and for *F. tularensis*. In a spring water co-culture model, amoebae again enhanced *F. novicida* survival and preserved bacterial morphology. Overall, our results demonstrate that amoebae likely promote *Francisella* survival in aquatic environments, including the tularemia agent *F. tularensis*. However, bacteria-amoebae interactions are complex and depend on the *Francisella* species considered.



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Introduction

Francisella tularensis is a Gram-negative, facultative intracellular bacterium, causing the potentially life-threatening zoonosis tularemia. This microorganism is classified as a category A potential agent of biological threat by the US Centers for Disease Control and Prevention (CDC) [1]. Taxonomically, the *F. tularensis* species is divided into four subspecies. *F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B) are the etiological agents of tularemia. *F. tularensis* subsp. *mediasia-tica* is restricted to central Asia and Russia, and has never been isolated from humans. *F. tularensis* subsp. *novicida* is an aquatic bacterium of low virulence in humans, also considered a different species, *F. novicida* [2,3]. For clarity, in our study we will differentiate *F. tularensis* (type A and type B tularemia agents) from the aquatic bacterium *F. novicida*. *F. tularensis* can infect a wide range of animals, which constitute the primary reservoir of this species

[4]. Ticks and other arthropods (especially mosquitoes) are vectors of this pathogen [4]. More recently, the hydro-telluric environment has been highlighted as a probable major reservoir of this zoonotic agent and as a source of human infections [5]. Although *F. tularensis* has been detected in natural water sources [5], the role of the aquatic environment as a reservoir of this species remains to be firmly established. Furthermore, the mechanisms and conditions of *F. tularensis* long-term survival in this environment have to be characterized. Some intracellular bacteria such as *Legionella pneumophila* and *Mycobacterium avium* persist in natural ecosystem by using free living amoebae as a multiplication niche and reservoir [6]. Amoebae such as *Acanthamoeba* and *Vermamoeba* species are ubiquitous in water and soil [7]. A few experimental studies have investigated the relationships between *Francisella* sp. and amoebae, with contradictory results [8–14]. Most of these studies have involved the live vaccine strain (LVS) of

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F. tularensis, *F. novicida*, *F. philomiragia*, or *F. noatunensis* [8–14]. Only two studies have evaluated the interactions with amoebae of virulent *F. tularensis* type A strains [8,14] and only one with a virulent *F. tularensis* type B strain [14].

In mammalian cells, it is well established that *Francisella* species multiplies intracellularly thanks to an atypical Type VI secretion system (T6SS) encoded in the *Francisella* Pathogenicity Island (FPI) [15–17]. *F. tularensis* possesses two almost identical copies of the FPI in its genome, while *F. novicida* and *F. philomiragia* only possess a single copy [17,18]. *F. novicida* harbours another genetic locus, that we termed FNI, likely encoding a different T6SS of unknown function [17,19]. The role of *Francisella* T6SSs in persistence/replication within amoebae remains unclear [9,11,12,20].

In the present study, we conducted a detailed characterization of the interactions of three *Francisella* species, including a virulent type B strain, with two amoebic species in order to further characterize the potential role of amoebae in the long-term survival of these species in the aquatic environment.

Materials and methods

Bacterial strains

The following strains of *Francisella* sp. were used (Table S1): the reference strains *F. philomiragia* ATCC 25015, *F. novicida* U112 (CIP 56.12), and *F. tularensis* subsp. *holarctica* LVS NCTC 10857; one clinical strain of *F. philomiragia* (Ft47); six clinical strains of *F. tularensis* subsp. *holarctica* (Ft5, Ft6, Ft7, Ft46, Ft62, Ft74), *F. novicida* Δ FPI mutant [21], *F. novicida* Δ FNI mutant [19], and the *F. novicida* Δ FPI Δ FNI double mutant. Clinical strains of *F. tularensis* subsp. *holarctica* were isolated from French patients suffering from the different tularemia forms. It was previously shown that all French clinical strains belong to clade B.44 and display low genetic diversity (genomes sequences of these clinical strains are available at DDBJ/EMBL/GenBank under the BioProject number PRJNA551589) [22]. Therefore, for most experiments, the Ft6 strain was used as representative of clinical strains. Clinical strain of *F. philomiragia* (Ft47) was isolated from a French patient suffering from a systemic infection with bacteraemia. All *Francisella* strains used in this study are owned by the French National Reference Center for *Francisella* (Grenoble University Hospital, Grenoble, France). Specific authorizations were obtained from the Agence Nationale de Sécurité du Médicament et des produits de santé (ANSM, authorization number ADE-103892019-7) for *F. tularensis* strains. For experiments, *Francisella* sp. strains were grown on chocolate agar media supplemented with

PolyViteX[®] (bioMérieux, Marcy-l'Étoile, France), incubated at 35°C in a 5% CO₂-enriched atmosphere for one day for *F. philomiragia* and *F. novicida* strains, and two days for *F. tularensis* strains. Experiments were all conducted in a biosafety level 3 (BSL3) laboratory. The *F. novicida* Δ FPI Δ FNI double mutant (U112 Δ F_{FTN_1309-1325::aphA}, Δ F_{FTN_0037-0054::FRTsc}) was generated in the wild-type U112 strain by transforming the Δ FNI mutant with genomic DNA from the Δ FPI mutant using chemical transformation. Briefly, the Δ FNI mutant grown in Tryptic Soy Broth (TSB), 0.1% L-cys and 0.4% glucose until an O.D.600 nm of 0.9 was washed once and 10-fold concentrated in chemical transformation buffer (Tris 50 mM, NaCl 270 mM, MgSO₄ 25 mM, CaCl₂ 20 mM, L-Arg 2 mM, L-His 1 mM, L-Met 2 mM, L-Asp 3 mM, Spermine 0.2 mM; MnCl₂ 35 mM, pH6.8). 500 μ l of the obtained bacterial solution was incubated for 20 min at 37°C with shaking 100 rpm with 1 μ g gDNA from the Δ FPI strain (Δ F_{FTN_1309-1325::aphA}). 1 ml TSB, 0.1% L-cys and 0.4% glucose was then added to the bacteria which were further incubated for 2 h at 37°C before plating on selective plates (Tryptic Soy Agar (TSA), 0.1% L-cys, 10 μ g/ml Kanamycin). Following clonal isolation, gene deletions were verified by PCR on gDNA using FPI and FNI flanking primers. Other bacterial strains used as controls included *Staphylococcus epidermidis* ATCC 12228 and *Legionella pneumophila* CIP107629 T. *S. epidermidis* ATCC 12228 was grown on sheep blood agar medium (bioMérieux, Marcy-l'Étoile, France) and *L. pneumophila* on Buffered Charcoal Yeast Extract (BCYE) medium (Oxoid, Cambridge, UK).

Amoebae and amoebae culture media

We used *Acanthamoeba castellanii* (Neff) and *A. polyphaga* (Linc-AP1) strains. Amoebae were grown axenically in peptone-yeast extract-glucose medium (PYG, Eurobio, France) at 27°C, in 75-cm² cell culture flasks (Falcon, Corning Incorporated, Life Sciences, Durham, USA). Peptone-yeast extract-glucose medium (PYG = ATCC medium 712) is a rich culture medium (2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO₄ · 7H₂O, 0.4 mM CaCl₂, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH₄)₂(SO₄)₂ · 6H₂O, 2.5 mM Na₂HPO₄ · 7H₂O, 2.5 mM KH₂PO₄) supporting *Acanthamoeba* sp. living in trophozoite form and multiplication.

For experiments, several amoebae culture media were used. PYG without glucose medium (PYG/wg) presents the same composition as PYG medium except that it does not contain glucose. This medium supports *Acanthamoeba* sp. survival in trophozoite form for 16 days but does not allow amoebae multiplication (data not shown). Starvation medium (SM) is a less nutritive medium (1 mM Na₂HPO₄, 1 mM

KH_2PO_4 , 0.016 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.027 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 mM NaCl, 0.005 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.2% yeast extract, 1.8% glucose) [23] allowing *Acanthamoeba* sp. survival in trophozoite form for 16 days without amoebae multiplication (data not shown). Page's amoeba saline (PAS = ATCC medium 1323) is a poor medium (1 mM Na_2HPO_4 , 1 mM KH_2PO_4 , 0.016 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.027 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 mM NaCl) allowing *Acanthamoeba* sp. survival in cystic form without multiplication.

Amoeba plate tests (APT)

This test allows evaluation of interactions of bacteria with an amoebae monolayer established on the surface of an agar plate [10,24]. APT were performed using *A. castellanii* or *A. polyphaga*, for *F. tularensis* (LVS, and clinical Ft5, Ft6, Ft7, Ft46, Ft62, and Ft74 type B strains), *F. philomiragia* (ATCC 25015, or Ft47 clinical strain), and *F. novicida* U112.

Amoebae grown in PYG medium in 75-cm² cell culture flask were harvested at 90% confluence by rapping the flask to bring amoebae into suspension. Amoebae were counted, centrifuged 10 min at 1000 g, and resuspended in fresh PYG medium at a concentration of 2.67×10^6 cells/ml. The amoebae suspension (1.5 ml) was spread on chocolate agar plates and allowed to dry 1–2 h to form an amoebae monolayer on the surface of the agar medium. For each bacterial strain tested, a suspension of 10^9 CFU/ml was prepared in Phosphate-Buffered Saline (PBS, Thermo Fisher Scientific). Tenfold serial dilutions (from 10^9 to 10^6 CFU/ml) of this suspension were prepared, and 10 μl of each dilution was inoculated on the amoebae monolayer. The agar plates were then incubated at 30°C for 10 days, and examined daily for bacterial growth. As a bacterial growth control, chocolate agar plates without amoeba were inoculated with the same bacterial suspensions. For negative and positive APT controls, *S. epidermidis* ATCC 1228 and *L. pneumophila* CIP107629 T respectively were inoculated to amoebae monolayers using the same protocol except that BCYE agar plates were used for *L. pneumophila*. For *Francisella* strains, the results of APTs were evaluated visually and semi-quantitatively according to the lowest bacterial inoculum allowing colony formation and the intensity of the bacterial growth (equal or inferior) in comparison to the amoeba-free growth control. *Francisella* growth on the amoeba monolayer was also compared to that of *L. pneumophila* on the same protozoa. Experiments were performed twice.

Growth of *Francisella* strains in amoebae culture media

We tested the capacity of *F. philomiragia* ATCC 25015, *F. novicida* U112, and the LVS and Ft6 strains

of *F. tularensis* to grow in the amoebae culture media PYG, PYG/wg, SM, and PAS, in the absence of amoeba. Each medium was inoculated with 10^5 CFU/ml of the tested bacterial strain, spread in 24-well plates and then incubated at 27°C. At different time intervals over a 16-day period, an aliquot of each culture medium was sampled, serially diluted and spread onto chocolate agar plates to determine CFU counts. Experiments were performed twice, each time in triplicate.

Amoebae infection with *Francisella* models

We evaluated intra-amoebic growth of *Francisella* strains (*F. philomiragia* ATCC 25015, *F. novicida* U112, and *F. tularensis* LVS and Ft6 strains) in *A. castellanii* or *A. polyphaga*. Two models were evaluated either using PYG/wg or SM media as the amoebae culture supernatant.

For the first model, a suspension of 5.3×10^5 cells/ml in PYG/wg medium was prepared for each amoeba strain tested, using the same procedure described for APT experiments. Then, the amoebae suspensions were dispensed in five 24-well plates (950 μL per well, each plate containing five wells for each amoeba strain), and amoebae were allowed to adhere for 1 h at 27°C. For each *Francisella* sp. strain tested, a bacterial suspension (50 μl of 10^8 CFU/ml in PYG/wg) was added to wells containing either *A. castellanii* or *A. polyphaga* (in triplicate) to obtain a multiplicity of infection (MOI) of 10. The plates were centrifuged at 1000 g for 20 min to optimize contact between bacteria and amoebae, and incubated 40 min at 27°C. Then, the culture supernatant was removed, the amoebae layer was washed three times with PAS, and new PYG/wg medium containing 100 $\mu\text{g}/\text{ml}$ of gentamicin was added to remove non-phagocytized bacteria. The plates were further incubated 1 h at 27°C. The amoebae monolayers were then washed three times with PAS and finally incubated in new PYG/wg medium at 27°C. This time of the experiment corresponded to T0. Plates were incubated during seven days at 27°C. CFU counts were determined at T0, day one (D1), D2, D5 and D7 of incubation of cell cultures (one plate per each time). Therefore, the culture supernatant of each well was removed and amoebae were lysed with 1% saponin for 10 min at 27°C followed by vigorous pipetting. The amoebic lysate was serially diluted and spread onto chocolate agar plates to determine intra-amoebic CFU counts. In parallel, the removed supernatant was also serially diluted and spread onto chocolate agar plates to determine extra-amoebic CFU counts. The bacterial detection limit was 10 CFU/ml.

For the second model, the same procedure as above was performed, but SM was used instead of PYG/wg and extra-amoebic CFU counts were not determined.

Experiments were performed twice, each time in triplicate.

Amoebae and Francisella co-culture models

We developed two co-culture models to evaluate growth of *Francisella* strains in the presence of amoebae, with or without direct contact of bacteria with these protozoa.

In the first model, *A. castellanii* or *A. polyphaga* were infected with *F. philomiragia* ATCC 25015, *F. novicida* U112, or *F. tularensis* Ft6 strains, using the same procedure as above and SM as the amoeba culture medium. However, killing of non-phagocytized bacteria using gentamicin was not performed. The plates were incubated at 27°C for 16 days. At T0, D2, D7, D12 and D16, amoebae were lysed by addition of 1 ml of 2% saponin during 10 min at 27°C and vigorous pipetting without removing the amoebae supernatant. The lysates were serially diluted and spread onto chocolate agar plates in order to determine the total (i.e. intra- and extra-amoebic) CFU counts. Control wells without amoeba were prepared using the same protocol. The bacterial detection limit was 20 CFU/ml.

In the second model, co-culture conditions were the same as above, but bacteria and amoebae were separated by a cell culture insert with a pore size of 0.4 µm (Millicell Merck Millipore, Darmstadt, Germany). Therefore, there was no direct contact between these microorganisms, but possible interactions due to a shared culture medium. In co-culture with inserts model, at each time point listed previously, supernatant above the insert was sampled, serially diluted and spread onto chocolate agar plates in order to determine CFU counts. After this step, insert was removed and 1 ml of 2% saponin was added on the amoebae supernatant for 10 min at 27°C followed by vigorous pipetting. The lysate was spread onto chocolate agar plate in order to check for the absence of bacteria.

Experiments were performed twice, each time in triplicate.

Amoebae survival

During infection and co-culture experiments, wells with infected or uninfected amoebae were incubated in similar conditions. At each time point, amoebic morphology and adherence of infected and uninfected amoebae were checked under a microscope. After amoebae detachment through vigorous pipetting, determination of the percentage of mortality were performed using trypan blue dye in a counting chamber. In addition, for co-culture experiments that lasted for 16 days, total amoebae counting was also performed throughout the experiment.

Immunofluorescence detection of Francisella in infected amoebae

At different time points of the infection experiments in PYG/wg or SM media, infection of amoebae with *Francisella* strains was evaluated by immunofluorescence and confocal microscopy. Infection experiments were performed as described above in 24-well plates, except that a 12 mm microscopy slide (SPL Life Sciences, Korea) was added in each well at the beginning of the experiments. At the different incubation times previously indicated, microscopy slides were removed from wells, fixed with 3.7% paraformaldehyde for 20 min at room temperature and then washed with PBS. Amoebae were permeabilized with PBS-Triton X-100 0.2% for 10 min at room temperature followed by two PBS washes. Blocking was performed with 3% bovine serum albumin for 30 min at room temperature. Rabbit anti-*Francisella tularensis* primary antibody (# TC-7005, Tetracore, Rockville, USA) diluted 1:100 in PBS-BSA 0.3% was added for 1 h in humid chamber at room temperature followed by three washes with PBS-BSA 0.3%-Tween 0.01%. This anti-*Francisella tularensis* antibody was shown to also label *F. philomiragia* (data not shown). Then, AlexaFluor 594 goat-anti-rabbit secondary antibody (# A-11012, Invitrogen, Thermo Fisher Scientific, Rockford, USA) diluted 1:1000 in PBS-BSA 0.3%, together with AlexaFluor 488 phalloidin (Invitrogen, Thermo Fisher Scientific, Rockford, USA) were added for 1 h at obscurity in a humid chamber at room temperature, respectively for *Francisella* and amoebae actin staining. Slides were then washed with PBS-BSA 0.3%-Tween 0.01% twice, then with PBS twice. They were mounted with ProLong Glass (Invitrogen, Thermo Fisher Scientific, Rockford, USA) and examined by immunofluorescent microscopy (on a Nikon Eclipse TS100) and confocal microscopy. Confocal microscopy was performed using a confocal laser scanning microscope (CLSM, LSM710, Zeiss, Jena, Germany) equipped with a Plan-Apochromat 63×/1.40 Oil DIC M27 objective.

Study of the T6SS impact on Francisella and amoebae interactions

The role of the *F. novicida* T6SS in amoebae interactions was evaluated using the previously described models. APT were performed for *A. castellanii* and either wild-type (WT) *F. novicida* U112, *F. novicida* ΔFPI, *F. novicida* ΔFNI, or *F. novicida* ΔFPIΔFNI. Infections in PYG/wg and SM were performed for *A. polyphaga* and either WT *F. novicida* U112 or *F. novicida* ΔFPIΔFNI. Co-culture without inserts were performed for *A. polyphaga* and WT *F. novicida* U112 or *F. novicida* ΔFPIΔFNI. Experiments were performed twice, each time in triplicate.

Amoebae and Francisella co-cultures in spring water

Spring water (pH 7.5) was collected from a large permanent spring in Croatia and then autoclaved at 121°C for 15 min. *F. novicida* U112 and *A. castellanii* were inoculated in 1 L of spring water in glass bottles, with shaking, and capped with cap half-loosened, at room temperature. Amoebae and bacteria concentrations were of: 10^6 *F. novicida*/ml alone, 10^7 *F. novicida*/ml alone, 10^6 *F. novicida*/ml together with 10^7 *A. castellanii*/ml, 10^7 *F. novicida*/ml together with 10^6 *A. castellanii*/ml, 10^6 *A. castellanii*/ml alone and 10^7 *A. castellanii*/ml alone. Two hours after inoculation, most of the amoebae were adhered to the bottle, the rest were floating in the water. Growth kinetics of bacteria and amoebae were followed every five days in a period of 30 days. The suspensions were homogenized by shaking before each sampling. The number of *F. novicida* at each time point was determined by plating serial dilutions on BCYE agar plates. In order to determine the number of amoeba cells, at each time point 1 ml of water sample was transferred to a 24 well plate, and left for 2 h at room temperature to allow amoebae to adhere. It was followed by analysing the samples using light microscopy. Experiments were performed twice, each time in triplicate.

During these experiments in spring water, morphology and structure of *F. novicida*, after 15 days incubation alone in spring water or in co-incubation with *A. castellanii* in spring water was studied by TEM in comparison to control bacteria grown on BCYE agar. Bacteria were prepared for TEM by negative staining. Bacterial suspension was applied to the Carbon Coated 200 mesh Cooper Grid for 2 min, and drown off from the edge of the grid with filter paper. After that, the grid was stained using 10 µl of 2% phosphotungstic acid for 1 min and again drained with the filter paper. The grid was placed directly into the grid box and allowed to air dry before observation. By transmission electron microscopy on a Zeiss 902A, we observed morphology of bacteria, including their size, shape and density. Ten fields for each sample were randomly photographed.

Statistical analysis

A two-tailed Student t-test was used to compare bacterial loads in the different experimental models, using a significance level of 0.05.

Results

Amoeba plate tests

The APT was first performed as a screening assay of interactions between a monolayer of amoebae and *Francisella* sp strains. Figure S1 shows some visual

results of the APT tests. All *Francisella* strains tested were able to pass through the amoebae monolayer forming bacterial colonies, demonstrating resistance of these bacteria to the predatory properties of amoebae. *F. philomiragia* (ATCC 25015 and Ft47) growth was observed at the 10^6 CFU/ml dilution with the same intensity with or without amoebae and similar to the *L. pneumophila* control. For the clinical strains of *F. tularensis*, and *F. novicida* U112, growth was observed at the 10^6 CFU/ml dilution with or without amoebae but with inferior intensity in the presence of amoebae and compared to the *L. pneumophila* control. Finally, for the LVS strain, growth on the amoebae monolayer was observed at the 10^9 CFU/ml dilution (3 log inferior to the amoeba-free control and the *L. pneumophila* control).

Growth of Francisella strains in amoebae culture media

In order to define the most suitable supernatant medium for further evaluations of *Francisella* and amoebae interactions, we evaluated the growth of *F. tularensis* LVS and Ft6 strains, *F. philomiragia* ATCC 25015, and *F. novicida* U112 in the amoebae culture media PYG, PYG without glucose (PYG/wg), starvation medium (SM), and PAS. In the absence of amoeba, the four bacterial strains showed strong growth in PYG and PYG/wg media, with an increase of more than 4 log of CFU counts after 16 days of incubation of cultures (Figure S2). In contrast, these strains were unable to grow in SM or PAS media, with a progressive decrease in CFU counts up to undetectable level within 5–7 days for PAS and 5–16 days for SM (Figure S2).

Amoebae infection with Francisella in PYG/wg

Since APT revealed interactions between *Francisella* sp. and amoebae, we needed to better characterize these interactions. In this aim, we first used an infection model in a rich culture medium (i.e. PYG/wg) previously described in the literature [9,11] but with longer incubation period. Infections of *A. castellanii* or *A. polyphaga* with *F. philomiragia* ATCC 25015, *F. novicida* U112, or *F. tularensis* (LVS or Ft6 strains) were performed.

During the first two days of incubation of cultures, we observed a significant increase in intracellular CFU counts for *F. philomiragia* in *A. polyphaga* (3.04 log CFU/ml, $p < 0.01$) and *A. castellanii* (3.46 log CFU/ml, $p < 0.001$) and for *F. novicida* in *A. polyphaga* (1.50 log CFU/ml, $p < 0.001$) and *A. castellanii* (1.73 log CFU/ml, $p < 0.05$). Then, a progressive decrease (for *F. philomiragia*) or stagnation (for *F. novicida*) of intra-amoebic CFU counts was observed the following five days. CFU counts of both *Francisella* strains in

the amoebae culture supernatant evolved similarly to the intra-amoebic CFU counts with difference between them at each time point of one log or less (Figure 1).

As for *F. tularensis* Ft6, we observed a weaker and slower increase in CFU counts within the amoebic compartment, with a peak at five days of incubation, also more marked for *A. polyphaga* (2.22 log CFU/ml, $p < 0.01$) than for *A. castellanii* (1.07 log CFU/ml, not significant (NS)). Here again, CFU counts in culture supernatant evolved similarly to the intra-amoebic CFU counts with less than one log difference between them at each time point (Figure 1).

In contrast, the LVS strain did not display any intra- or extra-amoebic growth, but a progressive decline in intra-amoebic CFU counts during the seven days of the experiments, of 2.91 log CFU/ml ($p < 0.01$) for *A. polyphaga* and to undetectable level for *A. castellanii* ($p < 0.001$) (Figure 1).

For the four bacterial strains tested, microscopic observations of infected and uninfected amoebae showed conservation of adherence and maintenance in trophozoite form of these protozoa at each time point of the experiments and amoebae mortality rates were inferior to 10% (data not shown).

Amoebae infection with *Francisella* in SM

In the absence of amoebae, we observed that PYG/wg supported a progressive growth of *Francisella* strains over time, whereas SM did not. Indeed, to prevent any extracellular growth of *Francisella* that may interfere with intra-amoebic CFU counts monitoring, we then developed an infection model in poor culture medium (i.e. SM), precluding extracellular growth of these bacteria. *A. castellanii* or *A. polyphaga* were infected with *F. philomiragia* ATCC 25015, *F. novicida* U112, or *F. tularensis* (LVS or Ft6 strains) using the same procedure as above but SM as the culture supernatant.

In this model, a rapid decrease of intra-amoebic CFU counts was observed up to undetectable level within one to seven days of incubation of cultures depending on the *Francisella* sp. strain considered (Figure 2).

For the four bacterial strains tested, microscopic observations of infected and uninfected amoebae showed conservation of adherence and maintenance in trophozoite form of these protozoa at each time point of the experiments and amoebae mortality rates were inferior to 10% (data not shown).

Amoebae and *Francisella* co-cultures in SM

Since APT revealed that *Francisella* sp. resisted to amoebae and infection in SM did not suggested intra-amoebic survival of *Francisella* sp., we developed

co-culture models in order to evaluate if amoebae could promote *Francisella* sp. survival whether intracellular or extracellular. In co-culture model, after the infection step, extra-cellular bacteria were not killed. This model was supposed to be closer to the interactions between amoebae and bacteria that may occur in nature. Co-cultures of *A. castellanii* or *A. polyphaga* with *F. tularensis* Ft6, *F. philomiragia* ATCC 25015 or *F. novicida* U112 were established using SM as the amoebae culture medium.

We observed an enhanced survival of both *F. philomiragia* and *F. novicida* in the presence of *A. castellanii* or *A. polyphaga*, compared to amoeba-free SM medium (Figure 3). The *F. philomiragia* and *F. novicida* CFU counts declined during the first seven to 12 days of experiments, both in the presence of *A. castellanii* (3.99 log CFU/ml, $p < 0.001$; and 3.73 log CFU/ml, $p < 0.001$, respectively) or *A. polyphaga* (1.19 log CFU/ml, $p < 0.001$; and 1.08 log CFU/ml, $p < 0.01$, respectively) (Figure 3). However, we then observed an increase in *F. philomiragia* CFU counts between days 12 and 16 for *A. castellanii* (1.97 log CFU/ml, NS), and days 7 and 16 for *A. polyphaga* (2.28 log CFU/ml, $p < 0.05$). Between days 7 and 16, *F. novicida* CFU counts significantly increased in *A. castellanii* (2.93 log CFU/ml, $p < 0.001$), and remained relatively stable in *A. polyphaga* (Figure 3). In contrast, CFU counts steadily and significantly decreased in the absence of amoebae, to undetectable level for *F. philomiragia* ($p < 0.01$), and by 5.19 log CFU/ml ($p < 0.001$) after 16 days for *F. novicida* (Figure 3). Therefore, there was a clear persistence of both *F. philomiragia* and *F. novicida* in the presence of either amoeba species, compared to amoeba-free culture conditions. For *F. novicida*, the difference in CFU counts at D16 was 4.32 log CFU/ml ($p < 0.001$) between amoeba-free and *A. castellanii* cultures, and 3.93 log CFU/ml ($p < 0.001$) between amoeba-free and *A. polyphaga* cultures. For *F. philomiragia*, the difference in CFU counts between amoeba-free and either *A. castellanii* or *A. polyphaga* cultures was more than four log (p could not be evaluated).

For *F. tularensis* Ft6, CFU counts progressively and similarly decreased between T0 and D16 in the presence or absence of amoebae: 3.53 log CFU/ml reduction ($p < 0.001$) in the presence of *A. castellanii*; 1.88 log CFU/ml ($p < 0.001$) with *A. polyphaga*; and 3.27 CFU/ml ($p < 0.001$) without amoebae (Figure 3). At day 16 of incubation, CFU counts were not statistically different between Ft6 alone and Ft6 with *A. castellanii* and was slightly superior for Ft6 with *A. polyphaga* than for Ft6 alone ($p < 0.05$) (Figure 3).

For the three bacterial strains tested, microscopic observations of infected and uninfected amoebae showed conservation of adherence and maintenance in trophozoite form of these protozoa. Total amoebae

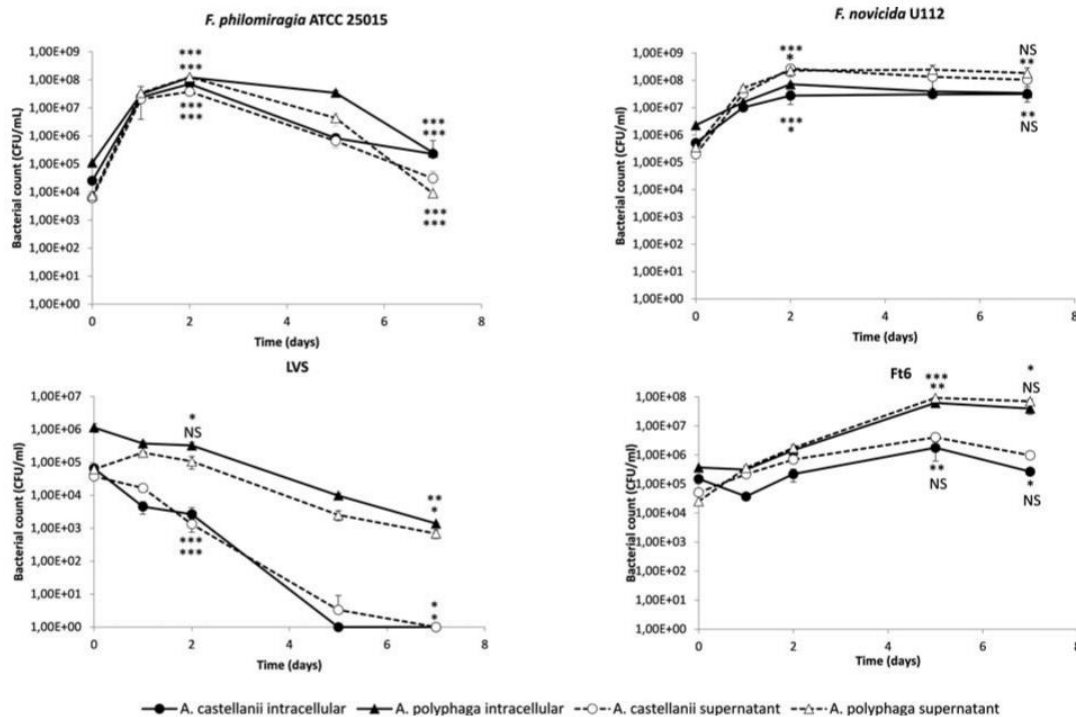


Figure 1. Infection of *A. castellanii* and *A. polyphaga* by *Francisella* sp. in PYG/wg. The figure shows one experiment made in triplicate. Similar results were obtained in a second experiment. The error bars represent standard deviations. Comparison of T0 versus D2 (or D5 for Ft6), and D2 (or D5 for Ft6) versus D7. NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

counts varied from 3×10^5 – 8×10^5 amoebae/ml and amoebae mortality rates were inferior to 10% (data not shown).

Amoebae and *Francisella* co-cultures in SM in the presence of inserts

We tried to further investigate the mechanisms implicated in the enhanced survival of *F. novicida* U112 and *F. philomiragia* ATCC 25015 in co-culture with amoebae. In this aim, we established new co-cultures of these species in the presence of *A. castellanii* or *A. polyphaga*. However, direct contact between bacteria and amoebae was prevented by using cell culture inserts, while these microorganisms still shared the same culture medium.

Enhanced survival of *F. novicida* in the presence of either of the two amoebae species was partially lost when bacteria and amoebae were separated by an insert. *F. novicida* CFU counts progressively decreased between T0 and D16 in wells with inserts (3.56 log CFU/ml reduction ($p < 0.001$) for *A. castellanii* and 2.91 CFU/ml reduction ($p < 0.01$) for *A. polyphaga*) (Figure 4). For each amoeba species, CFU counts in wells with versus without inserts were significantly different at D16 ($p < 0.01$ and $p < 0.001$ for *A. castellanii* and *A. polyphaga*, respectively) (Figure 4). However, bacterial counts at D16 in wells with

amoebae and inserts were significantly higher than in wells with bacteria alone ($p < 0.05$ for *A. castellanii* and inserts and $p < 0.001$ *A. polyphaga* and inserts).

F. philomiragia ATCC 25015 co-culture experiments with inserts gave non reproducible results within and between experiments and thus were not included in this study.

Although Ft6 survival was not favoured by the presence of amoebae, we performed co-cultures with insert for this bacterial strain. Surprisingly, *F. tularensis* Ft6 better survived when it was separated from the amoebae compared to co-cultures without inserts. When bacteria were separated from amoebae by an insert, CFU counts decreased during the first seven days (0.61 log CFU/ml ($p < 0.01$) reduction for *A. castellanii* and 1.54 log CFU/ml reduction ($p < 0.001$) for *A. polyphaga*) and then increased up to 16 days (1.66 log CFU/ml increase ($p < 0.05$) for *A. castellanii* and 2.74 log CFU/ml increase ($p < 0.05$) for *A. polyphaga*), for both amoebae species (Figure 4). CFU counts were significantly higher in wells with insert than in wells without inserts at D16 ($p < 0.05$ both for *A. castellanii* and *A. polyphaga*) (Figure 4).

For the two bacterial strains, no bacteria were obtained from the amoebae supernatants lysates demonstrating that bacteria did not pass through the insert during the 16 days of the co-culture experiment.

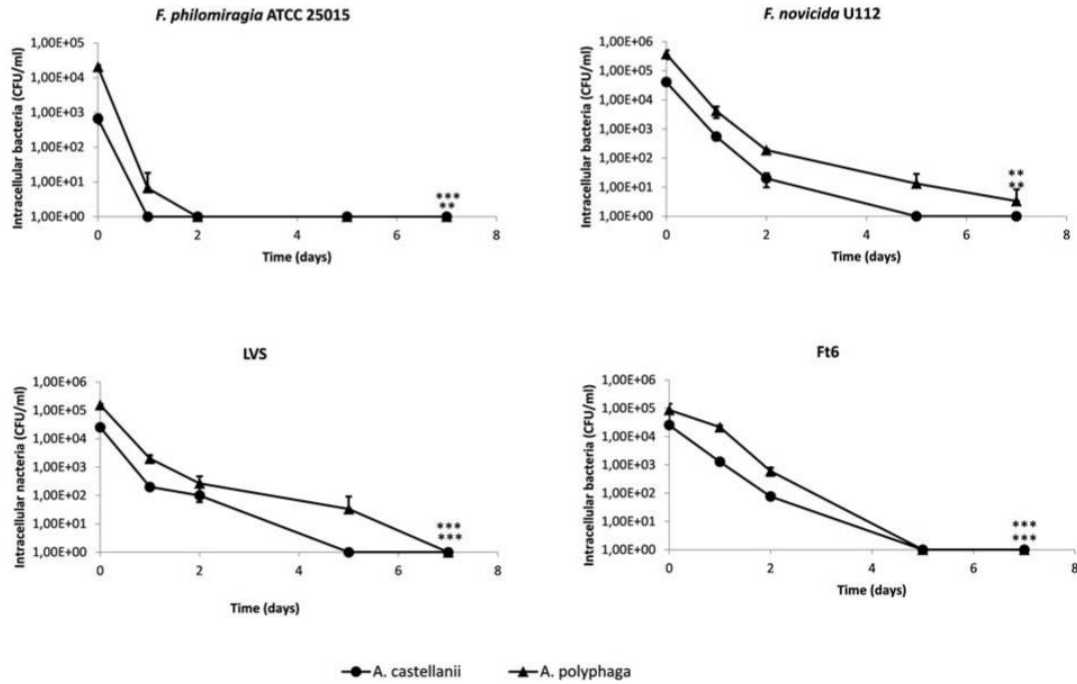


Figure 2. Infection of *A. castellanii* and *A. polyphaga* by *Francisella* sp. in SM. The figure shows one experiment made in triplicate. Similar results were obtained in a second experiment. The error bars represent standard deviations. Comparison of T0 versus D7. NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

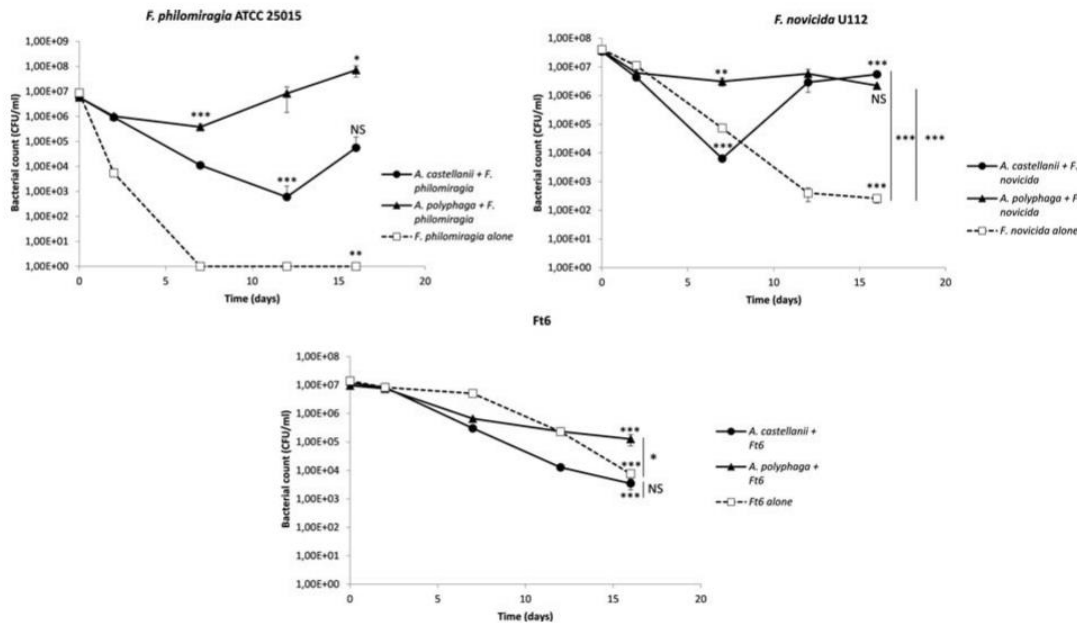


Figure 3. Co-culture of *A. castellanii* and *A. polyphaga* with *Francisella* sp. in SM. The figure shows one experiment made in triplicate. Similar results were obtained in a second experiment. The error bars represent standard deviations. Comparison of T0 versus D7, D12 or D16; or D16 versus D7 or D12. Comparison of bacteria with amoebae and bacteria alone at D16. NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Immunofluorescence detection of *Francisella* in infected amoebae

Immunofluorescence staining and confocal microscopy were performed for the two amoebae

species and the four *Francisella* strains at T0, D2 and D7 of infection in PYG/wg, and at T0 and D7 of infection in SM. Confocal microscopy confirmed that the four *Francisella* strains tested were located inside

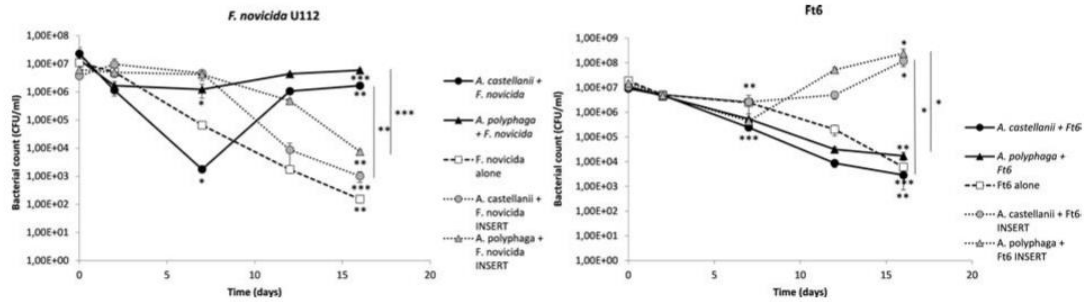


Figure 4. Co-culture with inserts of *A. castellanii* and *A. polyphaga* with *Francisella* sp. in SM. The figure shows one experiment made in triplicate. Similar results were obtained in a second experiment. The error bars represent standard deviations. Comparison of T0 versus D7 or D16; or D7 vs D16. Comparison of bacteria with amoebae separated or not by an insert at D16. NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

A. polyphaga or *A. castellanii* amoebae. No major variation was observed over time in PYG/wg, whereas bacteria disappeared at D7 in SM. Figure 5 shows confocal microscopy of *A. polyphaga* infected with *F. tularensis* Ft6, either in PYG/wg at T0, D2 and D7, or in SM at T0.

Study of the T6SS impact on *Francisella* and amoebae interactions

In order to evaluate the role of the T6SS on *F. novicida* interactions with amoebae, we tested T6SS-deleted mutants compared to the wild-type (WT) U112 strain of *F. novicida* in the previously described models.

In APT model, *F. novicida* Δ FPI, *F. novicida* Δ FNI, and *F. novicida* Δ FPI Δ FNI gave the same results with *A. castellanii* compared to the WT strain (Figure S1).

During infection in PYG/wg of *A. polyphaga*, the intra-amoebic counts of *F. novicida* WT and Δ FPI Δ FNI strains evolved similarly although subtle but significant differences were observed at D2 and D7. There was a raise in CFU counts during two or five days, followed by a relative stabilization (Figure S3). Here again, CFU counts evolved similarly inside and outside amoebae, and were not significantly different between the mutant and WT strains over time (Figure S3).

During infection in SM of *A. polyphaga*, the intra-amoebic CFU counts of *F. novicida* WT and

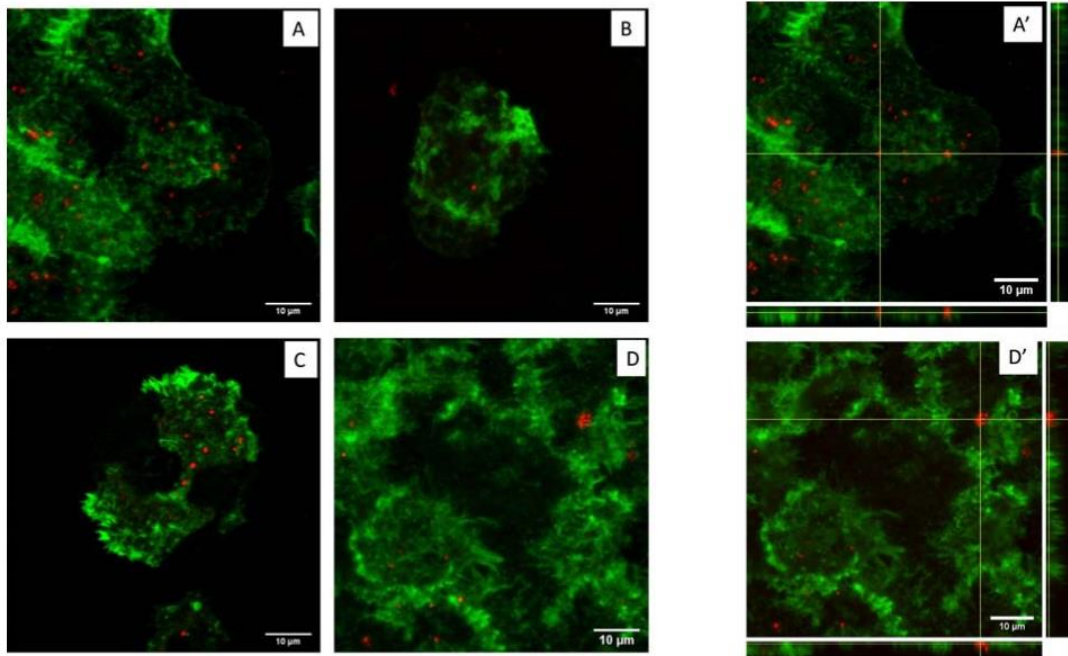


Figure 5. Confocal microscopy of *A. polyphaga* infected by *F. tularensis* Ft6. (A) T0 in PYG/wg; (B) D2 in PYG/wg; (C) D7 in PYG/wg; (D) T0 in SM; No more bacteria were observed inside amoeba at D7 in SM (data not shown). A') orthogonal view of T0 in PYG/wg; D') orthogonal view of T0 in SM. Green: actin (phalloidin Alexa Fluor 488). Red: *Francisella* sp. (rabbit primary antibody against *Francisella*, goat secondary antibody against rabbit Alexa Fluor 594).

Δ FPI Δ FNI decreased progressively and similarly over time despite bacterial counts slightly higher for *F. novicida* Δ FPI Δ FNI (Figure S3).

Finally, during co-culture without inserts, *A. polyphaga* enhanced the survival of either *F. novicida* WT or *F. novicida* Δ FPI Δ FNI in a similar manner. In contrast, the bacterial count of these two strains progressively and similarly decreased over time in amoeba-free medium (Figure S3).

Amoebae and Francisella co-culture in spring water

To better mimic the natural conditions of *Francisella* sp. survival in aquatic environments, *F. novicida* U112 and *A. castellanii* co-cultures were performed in spring water and at room temperature, during 30 days.

In co-culture with amoebae, *F. novicida* showed enhanced survival compared to amoeba-free water samples, regardless of doses. During the observed period, bacterial counts in co-culture were higher in comparison to samples with no amoebae ($p < 0.05$ at D5, D10, D15, and D20, NS at D25 and D30 for *F. novicida* at 10^6 CFU/ml and $p < 0.01$ at D5, D10, and D15, $p < 0.001$ at D20, $p < 0.01$ at D25 and NS at D30 for *F. novicida* at 10^7 CFU/ml) (Figure 6(A)). Moreover, *F. novicida* survived for a longer period of time in the presence of amoebae.

Unlike previous models, in the spring water co-culture model, amoebic counts were not stable during the experiments and decreased progressively. Besides, in co-culture models, amoebic counts decreased slightly faster in comparison to control water samples with no bacteria (NS at D5, $p < 0.01$ at D10, $p < 0.01$ at D15, $p < 0.001$ at D20, NS at D25 and D30 for *A. castellanii* at 10^6 cells/ml and NS at D5, $p < 0.05$ at D10, $p < 0.001$ at D15, $p < 0.01$ at D20, $p < 0.05$ at D25 and NS at D30 for *A. castellanii* at 10^7 cells/ml) (Figure 6(B)).

Morphology of *F. novicida*, after 15 days incubation alone in spring water or in co-incubation with *A. castellanii* in spring water was also studied by

transmission electron microscopy (TEM). Multiple morphological changes of *F. novicida* cells were observed after 15 days incubation in amoeba-free spring water. Bacteria showed disorganized cytoplasm separated from the cell wall, with multiple clumping and highly undefined cell wall. In contrast, *F. novicida* co-cultured with amoebae in spring water were roundly shaped and showed well preserved cell structures with smooth and intact cell wall, similar to the control bacteria grown on BCYE agar (Figure 7).

Discussion

The aim of our study was to evaluate *in vitro* survival or even proliferation of *Francisella* species in the presence of amoebae over an extended period. This could help to understand their long-term survival in natural aquatic environments. Therefore, we set-up different models of interactions between *F. tularensis*, *F. philomiragia*, or *F. novicida* with two amoeba species, *A. castellanii* and *A. polyphaga*.

We first evaluated the interactions between *Francisella* and amoebae using the Amoeba Plate Test (APT). This screening test was developed by Albers *et al.* [24] to study the interactions between *L. pneumophila* and amoebae [24]. The APT evaluates the ability of a specific bacterial strain to pass through a layer of amoebae to form colonies on an agar plate. A negative APT usually indicates that the tested bacterium is unable to kill amoebae either directly or through intra-amoebic multiplication. Conversely, a positive test usually indicates that the bacterium is able to resist the attack by amoebae and possibly multiply within these protozoa [6]. Interestingly, we observed a positive APT for all tested *Francisella* strains but with varying results. We observed a strong growth with *F. philomiragia*, a medium growth with *F. novicida* and the clinical strains of *F. tularensis*, and the weakest growth with the LVS strain. As for *F. philomiragia*, our results are in agreement with those reported by Verhoeven *et al.* [10] showing higher resistance of

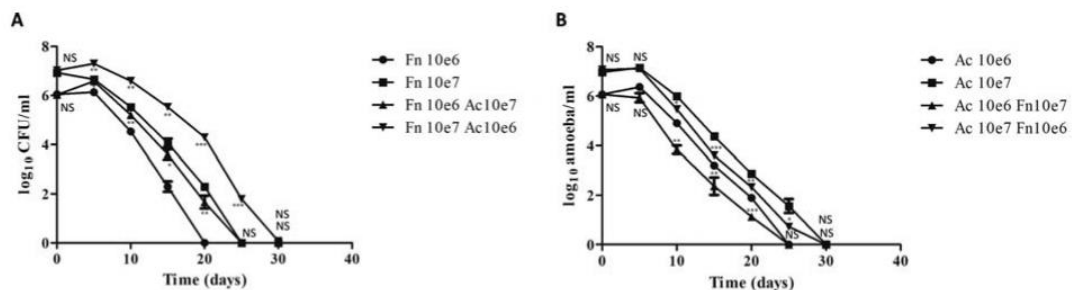


Figure 6. Growth kinetics of *F. novicida* (A) and *A. castellanii* (B) in spring water. The figure shows one experiment made in triplicate. Similar results were obtained in a second experiment. The error bars represent standard deviations. Comparison at each time point of (A) *F. novicida* with or without amoebae (B) *A. castellanii* with or without bacteria. NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Fn: *F. novicida*; Ac: *A. castellanii*

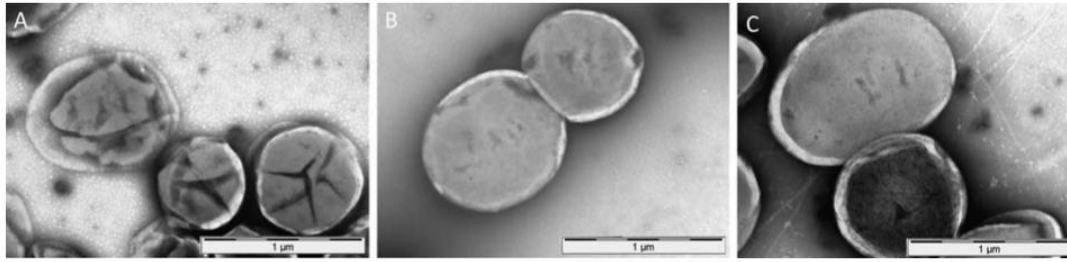


Figure 7. Morphology and structure of *F. novicida*, after 15 days incubation in spring water (A), co-incubation with *A. castellanii* in spring water (B), and as a control after growth on BCYE agar (C) by TEM.

F. philomiragia compared to *F. novicida* in an *A. castellanii* APT model and those of Thelaus *et al.* [25] showing that *F. philomiragia* was the least edible *Francisella* species for the ciliate *Tetrahymena pyriformis*. Regarding *F. tularensis* subsp. *holarctica* strains, LVS was less efficient to resist to amoebae than the clinical strains. This might be due to the attenuated virulence of this vaccine strain. Interestingly, the six clinical strains of *F. tularensis* subsp. *holarctica* displayed similar results, suggesting a homogenous amoeba resistance between strains of this subspecies, correlating with the low genetic diversity of French Type B strains [22]. Overall, the APT demonstrated some interactions between *Francisella* sp. and amoebae, with variations between *Francisella* species that could suggest different degree of adaptation to protozoan predation, as previously suggested by Thelaus *et al.* [25].

To better characterize interactions between *Francisella* and amoebae, we then used different cell models: 1/ an infection model in rich culture medium previously described in the literature [9,11] but with longer incubation period; 2/ an infection model in poor culture medium developed to prevent any extracellular growth of *Francisella*; and 3/ co-culture models in which amoebae and *Francisella* were allowed to interact through direct contact or not.

We first evaluated the ability of *Francisella* to multiply in amoeba culture media (PYG, PYG/wg, SM and PAS) in the absence of these protozoa. In models evaluating the ability of bacteria to grow inside eukaryotic cells, the culture supernatant should not support their extracellular growth. *F. philomiragia*, *F. novicida*, and *F. tularensis* Ft6 and LVS strains displayed a strong growth in PYG and PYG/wg. This observation was previously reported by El-Etr *et al.* [8]. Because we incubated our cultures for up to 16 days, we considered that an extracellular growth of bacteria may lead to erroneous results. Thus, in our experiments, we did not use the PYG and PYG/wg media but rather the SM medium for which no bacterial growth was observed.

In the infection in PYG/wg model, use as control, we observed a two- to four-log increase in CFU counts of *F. novicida*, *F. philomiragia* and Ft6 strains within amoebae during the first two or five days of infection

(Figure 1). Confocal microscopy confirmed that bacteria were present inside the amoebae (Figure 5). These findings were similar to those previously reported by Santic *et al.* [9] for *F. novicida* and *A. castellanii* or *V. vermiformis*. Yet, the next days of the experiments we observed a stagnation or reduction in intra-amoebic CFU counts. However, extracellular CFU counts overlaid intracellular CFU counts. The LVS strain did not exhibit any CFU increase in the intra- or extra-amoebic compartments, while this bacterium was able to grow in amoeba-free PYG/wg. This result suggests that the LVS strain was phagocytosed and digested by the amoebae, which may be correlated to the low virulence of this vaccine strain. Because of extra-amoebic growth of most *Francisella* strains tested, we considered this model not suitable for long-term evaluation of the intra-amoebic growth of these bacteria.

The infection in SM model was designed to allow only intra-amoebic growth of *Francisella* strains, by using SM as the culture medium. The SM supported the survival of *A. castellanii* and *A. polyphaga* for 16 days in trophozoite forms (data not shown), but did not allow growth of the tested *Francisella* sp. strains (Figure S2). In this model, no intra-amoebic replication of *F. novicida*, *F. philomiragia*, LVS and Ft6 was observed. A rapid decrease of intra-amoebic CFU counts was observed (Figure 2). Confocal microscopy confirmed that *Francisella* sp. were localised inside the amoebae at T0, but then disappeared at D7 post infection (Figure 5), a result correlating with reduction in CFU counts. El-Etr *et al.* also reported a decrease in intracellular counts of LVS and some clinical strains of *F. tularensis* subsp. *tularensis*, in an infection model using a poor culture medium (High-salt buffer) [8]. However, in the same study, an increase in intra-amoebic CFU counts was observed for *F. novicida* U112 and others *F. tularensis* subsp. *tularensis* clinical strains [8] suggesting that intra-amoebic growth could depend on the *Francisella* species and strain studied and also on experimental protocol used.

Since APT revealed that *Francisella* sp. resisted to amoebae and infection in SM did not suggested intra-amoebic survival of *Francisella* sp., we developed co-culture models. The aim of the co-culture model

was to evaluate if amoebae could promote *Francisella* sp. survival whether intracellular or extracellular. Enhanced survival of *F. philomiragia* and *F. novicida* was observed in the presence of amoebae, while we found a rapid loss of cultivability of these bacteria alone in amoeba-free medium (Figure 3). Buse *et al.* also showed that several amoebae species enhanced bacterial survival but did not enable multiplication of *F. novicida*, LVS and type-A and type-B clinical strains [14]. Unlike this study, we observed a real multiplication of both *F. novicida* and *F. philomiragia* after seven to 12 days of incubation (Figure 3). However, Buse *et al.* stopped their experimentations after 10 days of co-incubation, maybe just before bacterial multiplication onset [14]. This suggested that amoebae could promote the survival of these two *Francisella* species. Two mechanisms should be considered to explain this enhanced survival in the presence of amoebae: 1/ a direct “physical” interaction between bacteria and amoebae; 2/ the production of nutritive elements by amoebae in the culture supernatant that could be useful for *Francisella* survival and/or a degradation of toxic compounds by amoebae. In order to investigate these two hypotheses, we repeated the same co-culture experiments with the addition of inserts to physically separate bacteria from amoebae while allowing them to share the same medium. Enhanced survival of *F. novicida* was partially lost when an insert separated amoebae and bacteria (Figure 4). This result suggested that enhanced survival of *F. novicida* in co-culture with amoebae could be partly dependent on direct contact between bacteria and protozoa but also on amoeba-excreted compounds. Experiments with *F. philomiragia* gave too heterogeneous results within and between experiments to draw any conclusion.

Although the Ft6 strain did not survive in amoeba co-cultures without insert, we observed an enhanced survival of this strain when separated from the amoebae (Figure 4). We can hypothesize that, because *F. tularensis* strains (including Ft6) have a lower growth rate than *F. philomiragia* and *F. novicida*, phagocytosis by amoebae may outcompete bacterial growth in co-culture models without insert leading to a reduction in bacterial CFU counts. In contrast, when amoebae cannot phagocytize Ft6 because of the insert, this bacterium may benefit from amoeba-excreted compounds. This differs slightly from the observations of Buse *et al.* [14] who reported an enhanced survival of a *F. tularensis* type B strain in contact with amoebae, which may indicate that results are dependent on the bacterial strain used or on the experimental protocol. Verhoeven *et al.* reported that the inoculation of *F. philomiragia* ATCC 25015 in PYG medium preconditioned by *A. castellanii* (i.e. growth supernatant of *A. castellanii* from which the amoebae were removed) reduced biofilm production

and increased bacterial growth [10]. Gustafsson *et al.* also demonstrated the same positive effect of culture media preconditioned by *A. palestinensis* on the growth of *F. tularensis* LVS [26]. These two studies suggested that several *Francisella* species could benefit from elements excreted by amoebae during co-culture with these protozoans.

Co-culture models of *F. novicida* with *A. castellanii* were then performed in a spring water environment and at room temperature to be as close as possible from natural ecosystem. In this model, amoebae again favoured *F. novicida* survival. Indeed, bacterial counts were higher in the presence of amoebae compared to amoeba-free spring water and survival time of *F. novicida* was longer in the presence of amoebae (Figure 6(A)). In addition, bacterial morphology was preserved in presence of amoebae compared to amoeba-free spring water (Figure 7).

In all the models tested, we did not observe any difference between WT *F. novicida* and a *F. novicida* strain deleted of the two loci encoding T6SS, strongly suggesting that *F. novicida* does not rely on its T6SSs to interact with amoebae. This result is in sharp contrast with the key role of the FPI-encoded T6SS in promoting bacterial replication in mammalian cells [17]. However, this fits well with our infection and co-culture experiments results that suggest an absence of *Francisella* sp. replication inside amoebae but a *Francisella* sp. survival and multiplication in the presence of amoebae, probably extracellularly.

Overall, data available in the literature on the interactions between *Francisella* sp. and amoebae are conflicting. Some studies described the multiplication of *F. noatunensis*, *F. philomiragia*, *F. novicida*, LVS, and clinical strains of *F. tularensis* subsp. *tularensis* inside or in association with amoebae such as *A. castellanii*, *V. vermiformis* and *Dictyostelium discoideum* [8–13]. Conversely, other publications reported a lack of multiplication of *F. novicida*, LVS or type A and type B clinical strains [8,14]. However, these studies used very different experimental protocols, especially in terms of amoebae culture media. Previous works reporting multiplication of *Francisella* strains inside or in the presence of amoebae for 12 or 20 days have used rich media such as PYG medium (ATCC medium 712) [10,11]. For such models, it may be difficult to distinguish intra- from extra-amoebic growth of bacteria, and increase in CFU counts should be interpreted cautiously. To overcome this potential bias, we decided to work in SM medium, which did not support growth of *Francisella* strains used in this study. We evaluated for the first time the interactions of amoebae with several virulent clinical strains of *F. tularensis* subsp. *holarctica*. Although more tedious, these models can be considered more relevant to study ecological aspects of this major human pathogen compared to the attenuated LVS strain or strains belonging

to other *Francisella* species. Hence, very different results were obtained in this study between the studied *Francisella* species, but also between LVS and clinical strains of *F. tularensis* subsp. *holarctica*.

In our models, *F. philomiragia*, *F. novicida* and virulent type B strain of *F. tularensis* did not multiply inside amoebae, but could survive and multiply in an environment containing amoebae suggesting a potential commensal relationship between these two microorganisms. The interaction conditions established in our co-culture models likely occur in the aquatic environment where amoebae are widespread [7]. We found that *F. novicida* and *F. philomiragia* survive in the presence of amoebae. These two species are considered to have a primary aquatic reservoir, which fits well with the water-borne nature of the rare human infections caused by these two pathogens [5]. We also demonstrated that fully virulent *F. tularensis* strains may benefit from the presence of amoebae to survive in the environment. This may partly explain why human tularemia cases are occasionally associated with water-born transmission [5]. However, the variable ability of *Francisella* species to interact with amoebae, as described in the literature, also suggests that amoebae should not be considered the only reservoir of these bacteria in the aquatic environment. Other mechanisms potentially involved may include survival of *Francisella* sp. in biofilms, in mosquitoes larvae, and in water as viable but non-culturable (VBNC) forms [5] and have to be further investigated.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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TRAVAIL N°3 : Presence of *Francisella tularensis* subsp. *holarctica* DNA in the aquatic environment in France

(article soumis)

Le travail de revue de la littérature m'a permis de recenser plusieurs études ayant recherché *Francisella* sp. dans des échantillons d'eau environnementale. Ces études ont été réalisées en Turquie, aux Pays-Bas, en Allemagne, en Ukraine, en Suède, en Norvège et aux USA mais jamais en France.

Ainsi, dans une troisième partie de mon travail de thèse j'ai participé à une étude de terrain visant à évaluer la prévalence de la contamination par *F. tularensis* des eaux de surface dans un territoire français ayant enregistré une augmentation de l'incidence de la tularémie.

En 2018, un doublement du taux d'incidence de la tularémie a été enregistrée dans les régions Pays de la Loire, Bretagne et Nouvelle Aquitaine, associée à une augmentation de la fréquence des formes pleuro-pulmonaires de tularémie. La source de contamination n'a pas pu être identifiée pour la majorité des patients.

Nous avons réalisé deux campagnes de prélèvements d'échantillons d'eau environnementale dans cette zone géographique, en juillet 2019 et janvier 2020, permettant la collection de 87 échantillons d'eau environnementale de diverses natures (eaux de mer, rivières, lacs, ruisseaux...). Ces échantillons d'eau ont été analysés par un set de trois qPCR permettant respectivement la détection de *Francisella* sp. (qPCR-ISFtu2), *F. tularensis* et *F. novicida* (qPCR-Tul4) ou *F. tularensis* subsp. *holarctica* (qPCR-Type B). En parallèle, la salinité, la température et la présence d'amibes du genre *Acanthamoeba* ont également été étudiées sur ces échantillons.

Parmi les 57 échantillons recueillis lors de la première campagne de prélèvement, 15 (26,3 %, IC 95% [14,9-37,7 %]) étaient positifs pour *Francisella* sp., neuf (15,8 %, IC 95% [6,3-25,3 %]) pour *F. tularensis* et/ou *F. novicida* et quatre (7,0 %, IC 95% [0,4-13,6 %]) pour *F. tularensis* subsp. *holarctica*. Les échantillons positifs pour *Francisella* sp. incluaient à la fois des échantillons d'eau de mer, d'eau saumâtre et d'eau douce sans que l'impact de la salinité sur la présence de *Francisella* sp. ne puisse être évalué en raison d'une trop faible proportion d'échantillons d'eau salée. La température et la présence d'amibes n'est pas apparue comme corrélée à la présence de *Francisella* sp. dans les échantillons d'eau.

Lors de la seconde campagne de prélèvement, 30 échantillons d'eau ont été prélevés provenant de 12 sites précédemment positifs pour *Francisella* sp. et de 18 sites

précédemment négatifs. L'analyse a révélé 25/30 (83,3 %) positifs pour *Francisella* sp., 24/30 (80,0 %) pour *F. tularensis* et/ou *F. novicida* et 4/30 (13,3 %) pour *F. tularensis* subsp. *holarctica*. Un site a été positif pour *F. tularensis* subsp. *holarctica* lors des deux campagnes.

Ces résultats traduisent que la contamination des eaux environnementales par *Francisella* sp., y compris, *F. tularensis* est possible et relativement fréquente. Nous avons pu constater lors de nos prélèvements que les zones concernées étaient des zones agricoles entrecoupées de petits plans d'eau dans lesquels était pompée de l'eau ensuite dispersée sur les cultures à des fins d'arrosage. Nous avons émis l'hypothèse que la génération d'aérosols d'eau contaminée pourrait être la cause de l'inhalation de la bactérie et des cas de tularémie observés, majoritairement de forme pleuro-pulmonaire. Cette hypothèse reste cependant à vérifier, tout d'abord en comparant les géotypes de souches environnementale et clinique et en vérifiant l'infectiosité des *F. tularensis* détectée dans l'eau.

1 **Presence of *Francisella tularensis* subsp. *holarctica* DNA in the aquatic environment in**
2 **France**

3

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16

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22 **Abstract**

23 In 2018, the incidence of tularemia increased twofold in the West of France, with many
24 pneumonic forms, suggesting environmental sources of infection. We investigated the
25 presence of *Francisella tularensis* subsp. *holarctica* and other *Francisella* species DNA in the
26 natural aquatic environment of this geographic area. Two sampling campaigns, in July 2019
27 and January 2020, allowed the collection of 87 waters samples. Using a combination of real-
28 time PCR assays, we tested the presence of either *Francisella* sp., *F. tularensis*/*F. novicida*,
29 and *F. tularensis* subsp. *holarctica*, the only tularemia agent in Europe. Among 57 water
30 samples of the first campaign, 15 (26.3%) were positive for *Francisella* sp., nine (15.8%) for
31 *F. tularensis* and/or *F. novicida*, and four (7.0%) for *F. tularensis* subsp. *holarctica*. Ratios
32 were 25/30 (83.3%), 24/30 (80.0%), and 4/30 (13.3%) for the second campaign. Among the
33 thirty sites sampled during the two campaigns, nine were positive for *Francisella* sp. both
34 times, seven for *F. tularensis* and/or *F. novicida*, and one for *F. tularensis* subsp. *holarctica*.
35 Altogether, our study reveals a high prevalence of *Francisella* sp. DNA (including the
36 tularemia agent) in the studied aquatic environment. This aquatic environment could therefore
37 participate in the endemicity of tularemia in West of France.

38

39 **Keywords:** Tularemia, *Francisella tularensis*, zoonosis, water, France, environment and
40 public health

41

42 **Introduction**

43 *Francisella tularensis* is a Gram-negative bacterium causing the zoonosis tularemia. It
44 is a highly virulent human pathogen classified in the category A of potential agents of
45 biological threat by the US Centers for Disease Control and Prevention (CDC) [1].
46 Classically, four subspecies of *F. tularensis* are recognized. Only two are associated with
47 human tularemia: *F. tularensis* subsp. *tularensis* (type A), the most virulent, only present in
48 North America; and *F. tularensis* subsp. *holarctica* (type B), less virulent, located in all
49 northern hemisphere and Australia [2,3]. *F. tularensis* subsp. *mediasiatica* is restricted to
50 central Asia and Russia, and has never been associated with human infections [4]. *F.*
51 *tularensis* subsp. *novicida*, is an aquatic bacterium with low virulence in humans [5] and can
52 also be considered a different species, *F. novicida* [6,7]. Based on the most recent
53 phylogenetic studies, we choose in the article to considered *F. novicida* as a species [8]. Other
54 *Francisella* species are occasionally associated with human infections, like the aquatic
55 bacterium *F. philomiragia* [5]. In the past twenty years, multiple new environmental
56 *Francisella* species were described, all of them isolated from water or from aquatic fauna [8].
57 Six major clinical forms of tularemia are recognized depending on the mode of
58 contamination. The most frequent are the ulceroglandular and glandular forms, corresponding
59 to a regional lymphadenopathy, with or without a skin inoculation lesion. The oculoglandular
60 and oropharyngeal forms correspond to regional lymphadenopathy with conjunctivitis or
61 pharyngitis, respectively. Two systemic forms, which may be associated with fatality rates up
62 to 30%, are recognized: the pneumonic form and the typhoidal form, severe sepsis resembling
63 typhoid [9]. The primary sources of human infections are wildlife animals (especially
64 lagomorphs and small rodents) and arthropods (ticks, mosquitoes, and rarely biting flies) [9].
65 Infections through contact with a contaminated hydro-telluric environment are still poorly
66 defined [9]. However, tularemia can also be a waterborne infection occurring after aquatic

67 activities (e.g., fishing, swimming, and canyoning) or through drinking *F. tularensis*-
68 contaminated water [5]. Several modes of *Francisella* survival in water are presumed:
69 survival as planktonic form, through interactions with amoebae, in mosquito larvae, or in
70 biofilms [5].

71 Several studies have reported the detection of *Francisella* sp. in environmental water
72 samples, including in Turkey [10,11], the Netherlands [12], Germany [13], Ukraine [14],
73 Sweden [15], Norway [16], and the USA [17–20]. To our knowledge, no similar study has
74 been previously conducted in France.

75 In 2018, tularemia incidence increased twofold in France, with 21% of pneumonic cases,
76 while this clinical form usually represents less than 10% of reported tularemia cases (data
77 from santepubliquefrance.fr). Seventy-nine out of 113 patients with a known address lived in
78 the West of France, especially in Pays de la Loire, Bretagne, and the North of Nouvelle
79 Aquitaine. These infections were sporadic and spread over 65 000 km² areas. Although most
80 of the patients were farmers, their mode of infection was not identified (data from
81 santepubliquefrance.fr). In 2019 and 2020, we carried out a study on the presence of *F.*
82 *tularensis* DNA in natural aquatic environments located in Pays de la Loire region. Our goal
83 was to demonstrate that surface waters could represent a reservoir of this bacterium in France
84 and a potential source of human infections.

85

86 **Materials and methods**

87 *Sample Collection and DNA extraction*

88 Environmental water samples (2 to 4 L) were collected in Pays de la Loire region in natural
89 aquatic sites in areas where most tularemia cases had occurred. Fifty-seven water samples
90 were collected in July 2019 (Figure S1). A second field study was carried out in the same
91 geographic areas in January 2020, during which 30 water samples were collected, including

92 12 from previously *Francisella* PCR-positive sites and 18 from negative sites (Figure S2). A
93 wide diversity of water sites were sampled : ocean, rivulets, rivers, canals, ponds, lakes, and
94 dams (see results for more details). For each sampling site, the GPS coordinates, temperature,
95 and salinity of the collected water samples were recorded. These samples were transferred to
96 our laboratory within two days and stored at 4°C before testing. For PCR experiments,
97 filtration of one liter of each water sample was performed using 0.22 µm or 0.45 µm pore size
98 filters (ThermoFisher Scientific, Waltham, US) depending on turbidity. Total DNA was then
99 extracted from the filters using the Nuclomag DNA/RNA Water kit (Macherey-Nagel,
100 Hoerd, France).

101

102 *Francisella* species detection

103 To identify *Francisella*-positive samples and characterize the species or subspecies present,
104 we used three real-time PCR (qPCR) employed for *Francisella* identification or tularemia
105 diagnosis. These assays allowed detection of respectively *Francisella* sp. (ISFtu2-qPCR), *F.*
106 *tularensis* (i.e., *F. tularensis* subsp. *tularensis*, subsp. *holarctica*, and subsp. *mediasiatica*) and
107 *F. novicida* (Tul4-qPCR) and *F. tularensis* subsp. *holarctica* (Type B-qPCR) (Table 1). Each
108 PCR mixture (20 µl) contained 10 µl of TaqMan Fast Advanced PCR Master Mix kit
109 (ThermoFisher Scientific, Waltham, US), 0.4 µl of each 10 µM primers, 0.4 µl of 2µM
110 probe, 3.8 µl of water and 5 µl of 10 ng/µl diluted DNA. The thermocycling program was the
111 same for the three qPCR tests: 50°C for 2 min, 95°C for 2 min, 45 cycles at 95°C for 3 sec
112 and 60°C for 30 sec, using the Ligthcycler 480 (Roche Diagnostics, Meylan, France). All
113 qPCR tests were performed in duplicate. The qPCRs were considered positive for a specific
114 sample if the two duplicate tests were positive, with a cycle threshold (C_t) value ≤ 36 for
115 ISFtu2-qPCR, and < 40 for Tul4- and Type B-qPCR tests. In case of inconsistent results
116 within the duplicate, sample was tested a third time to conclude. Controls were carried out

117 using DNA extracts from strains (*Serratia marcescens* CIP 103551, *Streptococcus equi*
118 ATCC 43079, *F. philomiragia* ATCC 25015, *F. noatunensis* LMG 23800, *F. novicida* U112
119 and clinical strain Ft92 of *F. tularensis* subsp. *holarctica*) and with DNA extracts from *F.*
120 *tularensis* subsp. *holarctica* LVS NCTC 10857 spiked water samples at concentrations
121 ranging from 0.1 to 10,000 CFU/l (Table 2). Our *F. tularensis* collection has been approved
122 by the Agence Nationale de Sécurité du Médicament et des Produits de santé (France)
123 (ANSM, authorization number ADE-103892019-7).

124

125 *Amebae detection*

126 *Acanthamoeba* sp. DNA was detected using a qPCR (Acanth-qPCR) targeting a 180 bp
127 fragment of the 18S rDNA, as previously described [21] (Table 1). Each PCR mixture (20 µl)
128 contained 10 µl of TaqMan Fast Advanced PCR Master Mix kit (ThermoFisher Scientific,
129 Waltham, US), 0.4 µl of each 10 µM primers, 0.4 µl of 2µM probe, 3.8 µl of water and 5 µl
130 of 10 ng/µl diluted DNA. The qPCR settings were as follows: 50°C for 2 min, 95°C for 2 min,
131 followed by 45 cycles of 95°C for 15 sec, 55°C for 30 sec, and 60°C for 30 sec, on a
132 LightCycler 480 (Roche Diagnostics, Meylan, France). Each water sample was tested in
133 duplicate or triplicate for inconsistent results. Water samples were considered positive for
134 *Acanthamoeba* sp. if the two duplicate tests were positive with a C_t value < 40.

135

136 *Statistical analysis*

137 Mann-Whitney and Khi2 tests were used to compare the influence of temperature and
138 presence of *Acanthamoeba* sp. on *Francisella* sp. detection, using a significance level of 0.05.
139 Statistical analyzes were not carried out on the second campaign because the sampling sites
140 were chosen according to the first campaign results, introducing a bias.

141

142 **Results**

143 *qPCR controls*

144 Regarding the bacterial strains controls, the ISFtu2-qPCR gave a strongly positive signal for
145 *F. tularensis* subsp. *holarctica* and *F. novicida*. A weaker signal (C_t of 36) was obtained for
146 *F. noatunensis* and *F. philomiragia*. However, non-*Francisella* strains tested also gave a weak
147 amplification signal ($C_t > 36$) that was abolished when testing lower DNA concentrations
148 (data not shown). Indeed, we choose to consider ISFtu2-qPCR as positive when C_t was
149 inferior or equal to 36. Tul4-qPCR gave a strong positive signal for *F. tularensis* subsp.
150 *holarctica* and *F. novicida* and was negative for all the other strains tested. Finally, Type B-
151 qPCR was only positive for *F. tularensis* subsp. *holarctica*.

152 Regarding the *F. tularensis* subsp. *holarctica* LVS NCTC 10857 spiked water samples, when
153 applying our defined thresholds, sensitivity of our qPCR-assay was of 1 CFU/l for ISFtu2-
154 qPCR, 10 CFU/l for Tul4-qPCR and 100 CFU/l for Type B-qPCR. qPCR controls results are
155 presented in table 2.

156

157 *First field study*

158 Regarding the first field study, 57 water samples were collected: one from the ocean,
159 two from canals near the ocean, one from a river near the ocean, one from a pond near the
160 ocean, 30 from ponds, 12 from rivers (some of them were sampled at several points), two from
161 rivulets, three from lakes, three from dams and two from canals. Fifteen (26.3%) out of 57
162 collected water samples were positive for ISFtu2-qPCR, indicating the presence of
163 *Francisella* sp. at the corresponding sampling sites. Among the 15 samples positive for
164 ISFtu2-qPCR, nine were also positive for Tul4-qPCR, indicating the presence of *F. tularensis*,
165 or *F. novicida*, or both. Four samples were positive for the three qPCR tests, including Type
166 B-qPCR, confirming the presence of *F. tularensis* subsp. *holarctica* (Figures 1 and S1). One

167 river was found contaminated at two sampling sites. Characteristics of the positive water
168 samples are presented in table S1. None of the ISFtu2-qPCR negative samples gave an
169 amplification signal with the Tul4- or Type B-qPCR tests.

170 Salinity was evaluated for 12 of the 15 ISFtu2-qPCR-positive samples and ranged
171 from 1 g/L to 38 g/L. Indeed, the ISFtu2-qPCR-positive samples corresponded either to salt-
172 water (3 samples), brackish-water (1 sample), or freshwater (8 samples). Salinity was
173 evaluated for 19 of the 42 ISFtu2-qPCR-negative samples and ranged from 1 g/L to 46 g/L.
174 The impact of salinity on *Francisella* sp. detection could not be evaluated due to too few salt-
175 and brackish-water samples. The water temperature of the 15 ISFtu2-qPCR-positive samples
176 ranged from 18.2°C to 27.8°C, and that of the 42 ISFtu2-qPCR-negative samples from 16.2°C
177 to 29.9°C. There was no significant difference between the average temperature of ISFtu2-
178 qPCR-positive and -negative samples ($p = 0.809$). *Acanthamoeba* sp. DNA was detected in 38
179 (67.9%) of the 56 tested surface water samples. For these samples, seven (18.4%) were
180 positive for ISFtu2-qPCR, and 31 (81.6%) were negative. For the 18 *Acanthamoeba*-negative
181 samples, seven (38.9%) were positive for ISFtu2-qPCR, and 11 (61.1%) were negative. There
182 was no significant correlation between the distribution of positive or negative samples for
183 ISFTu2-qPCR and *Acanthamoeba*'s presence or absence ($p = 0.063$).

184

185 *Second field study*

186 Regarding the second field study that was conducted mainly around the locations that
187 were positive during the first field study, the ISFtu2-qPCR was positive for 25/30 (83.3%)
188 samples, the Tul4-qPCR for 24/30 (80.0%), and the Type B-qPCR for 4/30 (13.3%) (Figures
189 1 and S2). Two rivers were found contaminated at three or more sampling sites. Here again,
190 none of the ISFtu2-qPCR negative samples gave an amplification signal with the Tul4- or
191 Type B-qPCR tests. The salinity ranged from 1 g/L to 12 g/L for the 25 ISFtu2-qPCR-positive

192 samples, and 1 g/L to 31 g/L for the five ISFtu2-qPCR-negative samples. The temperature
193 ranged from 7.0°C to 13.6°C for the 25 ISFtu2-qPCR-positive samples and 6.6°C to 13.2°C
194 for the five ISFtu2-qPCR-negative samples. Finally, 29/30 (96.7%) water samples collected
195 during this second campaign were positive for *Acanthamoeba* sp. PCR.

196 Of the 12 sites that were ISFtu2-qPCR positive during the first water sampling campaign, and
197 re-sampled during the second campaign, nine were still positive for this qPCR test, suggesting
198 the persistence of *Francisella* sp. DNA over time or a regular recontamination of the
199 environment or both. For the Tul4-qPCR, seven of the nine previously positive sites were still
200 positive during the second campaign. Finally, of the four sites Type B-qPCR positive during
201 the first water sampling campaign, one remained positive.

202 Furthermore, among the 18 water samples that were negative for the ISFtu2-qPCR test during
203 the first campaign, 16 tested positive for this qPCR during the second campaign. Of these 16
204 samples, 15 also tested positive for Tul4-qPCR, and two for both Tul4- and Type B-qPCRs.
205 Characteristics of the positive water samples are presented in table S1.

206

207 **Discussion**

208 A significant increase of tularemia cases was experienced in 2018 in France. A
209 doubling of the disease incidence was observed in the Western part of the country compared
210 to previous years. Because of a the rise in the pneumonic forms of the disease (21 % of cases
211 in 2018 vs 10% the previous years), inhalation of *F. tularensis* contaminated aerosols could be
212 suspected as one of the significant sources of human infections. As these pneumonic
213 tularemia cases occurred throughout the whole year, the long-term environmental reservoir of
214 this bacterium was hypothesized.

215 This study aimed to assess the prevalence and the spatial and temporal distribution of
216 *F. tularensis* in surface waters. For this purpose, two sampling campaigns were performed six

217 months apart, in July 2019 and January 2020, to collect natural environmental water sources
218 in the geographic area where most tularemia cases occurred.

219 A dozen studies have evaluated the presence of *Francisella* sp. in aquatic
220 environments [10–20]. The previous studies on environmental water samples mainly detected
221 *F. philomiragia* or *F. novicida* [16–20], which are well-known aquatic bacteria [5]. Few
222 environmental studies identified *F. tularensis* in water samples [10–15]. Most of these
223 previous studies performed on environmental water samples relied on PCR assays [10–13,15–
224 17,19], while few studies used culture methods [10,14,18–20]. Isolation of *F. tularensis* from
225 environmental water samples has been only rarely reported [10,14]. Environmental surface
226 water samples are contaminated by a wide diversity of bacteria that grow faster than the
227 fastidious *Francisella*, thus preventing the latter's isolation. Consequently, we did not try to
228 culture our water samples, although it would have been interesting to correlate PCR and
229 culture results. PCR-based methods seems more suitable for detecting *Francisella* in
230 environmental samples. However, a wide diversity of *Francisella* species are present in the
231 environment, including many water-species (e.g. *F. ulginis*, *F. salminarina*, *F. salina*),
232 fishes- and molluscs-associated species (e.g. *F. noatunensis*, *F. orientalis*, *F. halioticida*, *F.*
233 *marina*) and endocytobionts of marine ciliates (e.g. *F. endociliophora* and *F. adeliensis*) [8].
234 Consequently, the choice of PCR targets is critical for the nature of *Francisella* species
235 identified. Thus, we performed a set of three real-time PCR tests allowing to discriminate
236 between *Francisella* species and subspecies. The ISFtu2-qPCR assay targets the insertion
237 element-like sequence ISFtu2, which is present in multiple copies in the *F. tularensis* (i.e., *F.*
238 *tularensis* subsp. *tularensis*, subsp. *holarctica* and subsp. *mediastitica*) and *F. novicida*
239 genome (i.e., 12-17 copies for subsp. *tularensis*, 26-30 copies for subsp. *holarctica*, and 6-18
240 copies for *F. novicida*) and thus improves the sensibility of the qPCR. This DNA target is
241 present in other *Francisella* species (i.e., 1-2 copies of ISFtu2 in *F. philomiragia* genome)

242 [8,22]. Consequently, qPCR results from PCR targeting the *ISFtu2* sequence should be
243 interpreted cautiously because it could detect a wide diversity of *Francisella* species. This
244 was observed with our ISFtu2-qPCR controls which demonstrated both detection of *F.*
245 *philomiragia* and *F. noatunensis*. Öhrman *et al.*, by in silico approaches, demonstrated that
246 ISFtu2-qPCR can detect *F. novicida* and *F. philomiragia* but does not detect other
247 *Francisella* species such as *F. opportunistica* and *Francisella* endocytobionts [8].
248 Consequently the ISFtu2-qPCR is a *Francisella* sp. PCR with the limitation that not all the
249 described *Francisella* species are detected. The Tul4-qPCR assay targets a simple copy gene,
250 which can be found in *F. tularensis* and in *F. novicida* [22]. Although our Tul4-qPCR
251 controls and those of Versage *et al.* [22] concluded that *F. philomiragia* and other *Francisella*
252 near neighbour were not amplified by this assay, Öhrman *et al.*, by in silico approaches found
253 that *F. philomiragia* can occasionally be amplified by the Tul4-qPCR [8]. Finally, the Type B-
254 qPCR test targets a specific junction between *ISFtu2* and a flanking 3' region, which is only
255 found in *F. tularensis* subsp. *holarctica* [23], the exclusive tularemia agent described in
256 Europe [9]. Thus, our multi-step PCR approach, ISFtu2-, Tul4- and Type B-qPCR, allowed
257 reliable detection of respectively *Francisella* sp., *F. tularensis*/*F. novivida*, and *F. tularensis*
258 subsp. *holarctica*.

259 Regarding the first collection campaign, 26.3% of samples were positive only for
260 ISFtu2-qPCR, 15.8% for ISFtu2 and Tul4-qPCR, and 7.0% for ISFtu2, Tul4, and Type B-
261 qPCR. To be as stringent as possible, these samples were classified as containing *Francisella*
262 sp., *F. tularensis* and/or *F. novicida*, or *F. tularensis* subsp. *holarctica*, respectively. Because
263 qPCR targeting the multi-copy *ISFtu2* is more sensitive than the two other qPCR tests,
264 samples with only a positive ISFtu2-qPCR could also correspond to *F. tularensis* or even *F.*
265 *tularensis* subsp. *holarctica*. Therefore, a 7% prevalence of water samples contaminated with
266 *F. tularensis* subsp. *holarctica* should be considered a minimum in this study. The effect of

267 salinity on *Francisella* sp. detection could not be evaluated because of too little salt- and
268 brackish- water samples included. However, *Francisella* sp. were detected in fresh, brackish,
269 and salt-water indicating that these bacteria could contaminate a wide water type diversity.
270 Similarly, in the Netherland, Janse *et al.* identified *Francisella* sp. in fresh-, brackish- and
271 salt-water samples [12]. Interestingly, in Norway, Duodu *et al.* found *Francisella* sp. in 44
272 sea-water samples, while all 128 freshwater samples were free of *Francisella* sp. [16]. In our
273 study, no significant difference in water temperature was observed between *Francisella* sp.
274 positive and negative samples. In Norway, Duodu *et al.* also observed that water temperature
275 had no effect on *Francisella* sp. presence in water, but they only found *Francisella* sp.
276 positive samples south of Arctic Circle, suggesting that climate could have an impact on
277 *Francisella* sp. presence in the aquatic environment [16].

278 The second collection campaign aimed to assess the possible persistence of
279 *Francisella* sp. in previously investigated aquatic sites. Therefore, 30 of the previously
280 collected sites were re-sampled, including 12 positive for *Francisella* sp. and 18 that were
281 negative but located close to positive sampling points. Among the 12 samples positive for
282 ISFtu2-qPCR during the first campaign, nine (75.0%) were still positive during the second
283 campaign. As for the Tul4- and Type B-qPCR, 7/9 and 1/4 of previously positive sites were
284 still positive during the second campaign. Thus, one water sample was positive for the
285 tularemia agent *F. tularensis* subsp. *holarctica* both in July 2019 and in January 2020. These
286 results indicate either the persistence of *Francisella* sp. in the corresponding surface water
287 sites for several months or repeated contamination over time of these aquatic environments or
288 both. Each of these situations corresponds to a high risk of occurrence of human
289 contamination from these aquatic reservoirs. Interestingly, 16/18 (88.9%) of the previously
290 *Francisella* sp.-negative sites were ISFtu2-qPCR positive during the second campaign,
291 arguing that *Francisella* sp. contamination of the aquatic environment can be variable in time

292 and space and might spread over time. These observed variations impel that repeated
293 sampling is required for *Francisella* environmental monitoring. Overall, these results
294 highlight that *Francisella* sp. contamination of water environment is recurrent whatever the
295 season.

296 In total, there was respectively 26.3% and 83.3% of ISFtu2-qPCR positive samples in
297 the first collection campaign and the second collection campaign. These percentages of
298 ISFtu2-qPCR positive samples are high and indicate that *Francisella* sp. contamination of the
299 environment is widespread. Janse *et al.* previously described in the Netherlands that 88% of
300 water samples were positive for *Francisella* sp. in areas with reported tularemia cases, and
301 10% in randomly collected sites [12]. Broman *et al.*, in Sweden, collected 341 fresh surface
302 water samples during three years and found 108 (32%) samples positives for *F. tularensis*
303 [15]. In contrast, other studies reported a lower prevalence of *Francisella* sp. in the
304 environment. Kaysser *et al.* in Germany sampled 28 freshwater sites and found one (4%) PCR
305 positive sample [13]. Simşek *et al.* collected 154 water samples from rivers, spring water, and
306 village fountains after tularemia outbreaks in Turkey and detected *F. tularensis* subsp.
307 *holarctica* by culture in four samples and by PCR in 17 (11%) samples [10]. However, it
308 would have been interesting to include in this study samples from a control location with
309 lower tularemia incidence in order to compare frequency of contamination by *F. tularensis*
310 DNA .

311 Altogether, our study in the West of France demonstrates that the presence of *F.*
312 *tularensis* subsp. *holarctica* DNA in surface waters is frequent and may be recurrent in areas
313 close to human tularemia cases. Although the presence of *F. tularensis* DNA in surface water
314 samples is not evidence of human contamination, the presence of this bacterium over a wide
315 range of time and space could favor the occurrence of human infections in the corresponding
316 geographic area. Indeed, the high number of pneumonic tularemia cases in 2018 strongly

317 suggested an environmental mode of contamination. Although aerosols inhalation from
318 contaminated soil is considered the primary source of pneumonic tularemia [24,25], the
319 persistence of *F. tularensis* in the aquatic environment has been proposed to explain
320 waterborne tularemia cases [5]. Waterborne tularemia cases classically occur through drinking
321 contaminated water or direct contact with contaminated water sources, especially during
322 recreational activities such as fishing and swimming, or through consumption of such non-
323 potable water [5]. However, contact or consumption of contaminated water usually leads to
324 skin or oropharyngeal inoculation of *F. tularensis*, thus, ulceroglandular or oropharyngeal
325 forms of tularemia. We hypothesize another water-related human contamination leading to the
326 disease's pulmonary form through aerosols formed from contaminated surface water. Indeed,
327 the 2018 tularemia cases occurred in areas with extensive agricultural activities, and most
328 patients were farmers. We noted that most of the crop fields of the studied areas were irrigated
329 by sprinklers using surface waters, which could generate large aerosols.

330 The widespread detection of the tularemia agent *F. tularensis* subsp. *holarctica* in
331 surface water raises the question of how this bacterium can survive and persist in such aquatic
332 environments and why it does not infect thousands of people according to the high virulence
333 of this bacterium. Experimental studies have shown that *F. tularensis* can survive in water
334 microcosms for months [26–29], firstly in a culturable form and then in a potentially viable
335 but non-culturable form (VBNC) [26,28]. Long-term planktonic forms of *F. tularensis* subsp.
336 *holarctica* have been found to be still virulent in mice, whereas similar forms of *F. tularensis*
337 subsp. *tularensis* did not [29]. VBNC forms of *F. tularensis* subsp. *holarctica* appeared to be
338 no longer virulent in mice [28]. This possible loss of virulence over time in aquatic
339 environments could explain the relatively low number of tularemia cases related to aquatic
340 habitats in endemic areas compared to the virulence of the bacteria in its virulent state. In our
341 study, the DNA detection of the tularemia agent does not allow us to determine the detected

342 bacteria's infectivity. Several *in vitro* experimental studies have shown that *F. tularensis* can
343 survive in water through interaction with amoebae [30–33]. We detected DNA from
344 *Acanthamoeba* sp., which are ubiquitous environmental amoebae [34], in 38/56 (67.9%) water
345 samples of the first collection campaign, and 29/30 (96.7%) of water samples of the second
346 collection campaign. However, no correlation was found between the presence of
347 *Acanthamoeba* and *Francisella* DNA in water samples. There are conflicting studies about the
348 capacity of *F. tularensis* to produce biofilms to survive in water [29,35]. Finally, this
349 bacterium was shown to be able to survive within mosquitoes larvae [36]. While the tularemia
350 agent aquatic cycle is not yet fully characterized, our work demonstrates that *F. tularensis*
351 subsp. *holarctica* DNA can be found in the aquatic environment.

352 In conclusion, our study demonstrates, for the first time, the presence of *F. tularensis*
353 subsp. *holarctica* DNA in surface water in France. Although contaminated water as a source
354 of pneumonic forms of human tularemia remains to be firmly established, the monitoring of
355 *F. tularensis* contamination of surface water should be considered for public health
356 surveillance of tularemia.

357

358 **Author contributions**

359 CDB and AH conceived the idea, collected samples, realized experiences and wrote
360 manuscript. JP collected samples. IP collected tularemia cases data. YC collected tularemia
361 cases data and revised manuscript. MM obtained funding, conceived the idea, collected
362 samples and revised manuscript. All authors read and approved the submitted version.

363

364 **Conflict of interest**

365 The authors declare that they have no conflict of interest.

366

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370

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492

493 **Table 1. Primers and probes used for *Francisella* sp. and *Acanthamoeba* sp. detection.**

qPCR (target)	Primers / probes	5' to 3' DNA sequence	Amplicon size	Species detected	Reference
ISFtu2-qPCR (ISFtu2 insertion sequence element)	Forward	ttgtagatcagttggggataac	97 bp	<i>Francisella</i> sp.	[22]
	Reverse	tgagtttacctctgacaacaatattc			
	Probe	FAM-aaatccatgctatgactg atgcttaggtaatcca-BHQ1			
Tul4-qPCR (<i>tul4</i> gene)	Forward	attacaatggcaggtccaga	91 bp	<i>F. tularensis</i> and <i>F.</i> <i>novicida</i>	[22]
	Reverse	tgccaagttttatgcttct			
	Probe	FAM-ttctaagtccatgatacaag ctccaataactaag-BHQ1			
Type B-qPCR (junction between ISFtu2 and a specific flanking 3' region)	Forward	cttgacttttattggctactgagaaact	144 bp	<i>F. tularensis</i> subsp. <i>holarctica</i>	[23]
	Reverse	cttgcttggttgtaaatatagtggaa			
	Probe	FAM-Acctagtcaacc*t*caag acttttagtaatgggaatgtca-BHQ1 Internal quencher			
Acanth-qPCR (<i>Acanthamoeba</i> sp. 18S rDNA)	Forward	cccagatcgtttaccgtgaa	180 bp	<i>Acanthamoeba</i> sp.	[21]
	Reverse	taaataatgcccccaactatcc			
	Probe	FAM-ctgccaccgaatacattagcatgg- BHQ1			

494

495 **Table 2. qPCR controls.**

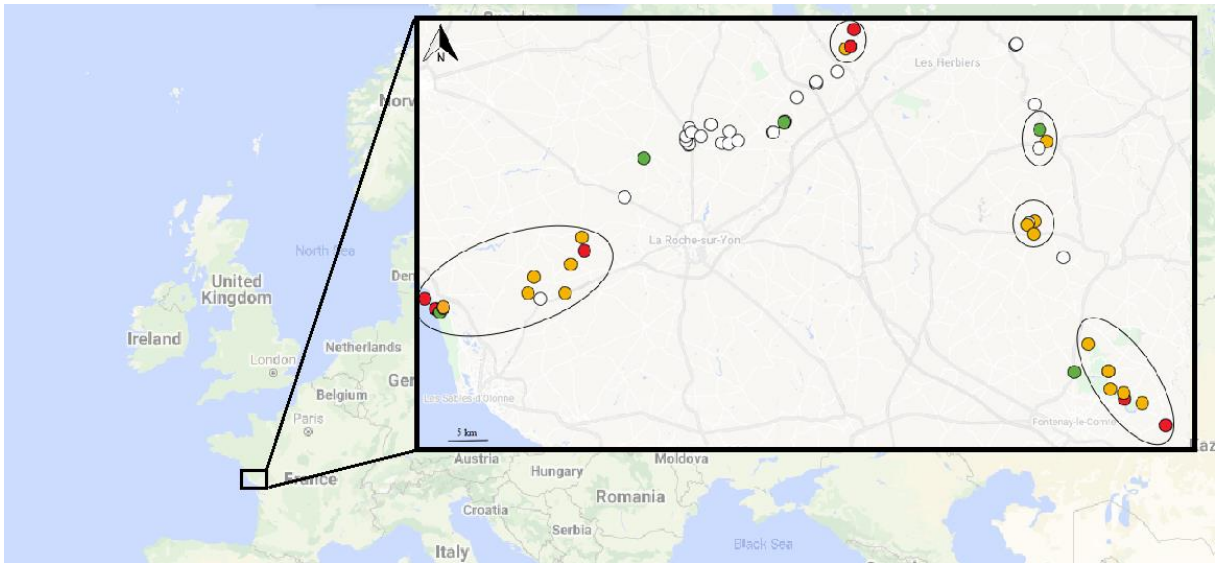
DNA extracts	ISFtu2-qPCR (C _t)	Tul4-qPCR (C _t)	Type B-qPCR (C _t)
<i>Serratia marcescens</i> CIP 103551*	37	Negative	Negative
<i>Streptococcus equi</i> ATCC 43079*	37	Negative	Negative
<i>F. noatunensis</i> LMG 23800*	36	Negative	Negative
<i>F. philomiragia</i> ATCC 25015*	36	Negative	Negative
<i>F. novicida</i> U112*	12	16	Negative
Clinical strain Ft92 of <i>F. tularensis</i> subsp. <i>holarctica</i> *	12	15	16
LVS spiked water 0.1 CFU/l	38	Negative	Negative
LVS spiked water 1 CFU/l	36	Negative	Negative
LVS spiked water 10 CFU/l	36	37	Negative
LVS spiked water 100 CFU/l	34	38	38
LVS spiked water 1,000 CFU/l	27	33	33
LVS spiked water 10,000 CFU/l	25	30	30

496 * 50 ng of DNA introduced in the PCR mix

497

498

499 **Figure 1. *Francisella* sp. detection in surface water samples collected during both**
500 **campaigns.**



501
502 For each site, DNA was extracted from water, and three q-PCR tests were performed (ISFtu2-
503 qPCR for *Francisella* sp., Tul4-qPCR for *F. tularensis*, and Type B-qPCR for *F. tularensis*
504 subsp. *holarctica*). Disk: water sampling site. White disk: sample with no positive qPCR.
505 Green disk: sample with positive ISFtu2-qPCR in the first campaign or the second campaign
506 or both, and negative Tul4 and Type B-qPCR. Orange disk: sample with positive ISFtu2 and
507 Tul4-qPCR in the first campaign or the second campaign or both and negative Type B-qPCR.
508 Red disk: sample with positive ISFtu2, Tul4, and Type B-qPCR in the first campaign or the
509 second campaign or both. Circles indicate sites where water samples were collected during
510 the two campaigns.

Supplementary Figures and Table

Table S1. Characteristics of *Francisella* sp. positive water samples

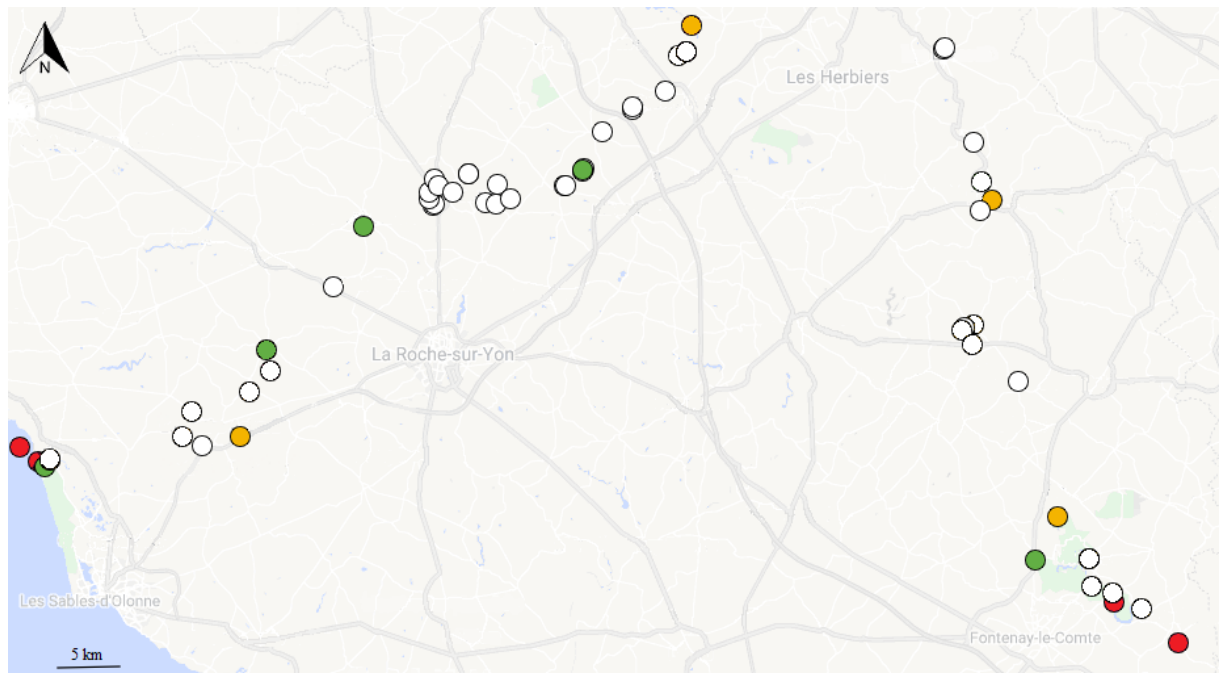
Site of water sampling	Type of water sample	First campaign						Second campaign					
		T °C	NaCl (g/L)	ISFtu2-qPCR (average Ct)	Tul4-qPCR (average Ct)	Type B-qPCR (average Ct)	Acant-qPCR	T °C	NaCl (g/L)	ISFtu2-qPCR (average Ct)	Tul4-qPCR (average Ct)	Type B-qPCR (average Ct)	Acant-qPCR
J1P1	Ocean	21.0	37	+ (31.1)	+ (36.5)	+ (35.8)	+	11.0	31				+
J1P2	Canal near ocean	25.6	16	+ (32.7)	+ (36.4)	+ (36.9)		11.6	2				+
J1P3	Canal near ocean	27.2	38	+ (35.6)				13.2	3				+
J1P4	River near ocean	23.8	37	+ (32.1)	+ (36.8)		NP	7.0	6	+ (35.4)	+ (37.3)		+
J1P5	Pond near ocean	27.8	46					13.6	12	+ (33.2)	+ (34.4)		+
J1P6	Pond	27.1	2					12.6	2	+ (34.9)	+ (37.6)		+
J1P7	Pond	29.9	1					12.2	3	+ (31.8)	+ (35.9)		+
J1P9	Pond	27.8	2	+ (36.0)	+ (37.6)			12.4	2	+ (33.3)	+ (36.6)		+
J1P11	Pond	28.8	1					9.2	2	+ (33.5)	+ (37.1)		
J1P12A	Pond	18.6	2					12.9	2	+ (32.5)	+ (35.7)	+ (38.2)	+
J1P13	River	18.7	2	+ (35.4)				12.7	2	+ (33.5)	+ (38.1)		+
J2P1	River	18.8	NP	+ (36.0)			+	NS	NS	NS	NS	NS	NS
J2P16SB	River	18.2	NP	+ (35.0)			+	NS	NS	NS	NS	NS	NS
J2P22	Pond	25.9	NP					11.8	1	+ (33.3)	+ (36.5)		+
J2P23A	Pond	21	2				+	13.3	2	+ (33.5)	+ (36.4)		+

J2P23B	River	23.7	NP				+	13.0	1	+ (30.9)	+ (34.7)	+ (39.4)	+
J2P24	Pond	22.3	2	+ (35.6)	+ (38.4)			13.1	2	+ (33.9)	+ (37.2)	+ (38.8)	+
J2P42	Pond	23.7	3				+	9.0	2	+ (34.1)			+
J2P43	Lake	24.4	2	+ (36.0)	+ (38.4)		+	12.3	1	+ (33.3)	+ (36.4)		+
J2P52A	Pond	24.7	1				+	12.0	2	+ (33.5)	+ (36.4)		+
J2P53SA	Pond	23.2	2					10.6	2	+ (35.1)	+ (37.7)		+
J2P54A	Pond	24.4	1				+	11.6	1	+ (32.4)	+ (35.9)		+
J2P55SA	Pond	20.0	NP	+ (35.7)			+	NS	NS	NS	NS	NS	NS
J3P1	Dam on river*	20.3	2	+ (32.8)	+ (37.0)			11.3	2	+ (32.7)	+ (37.4)		+
J3P6SA	River*	25.6	1	+ (34.5)				12.3	2	+ (33.6)	+ (37.8)		+
J3P6SB	River*	21.6	2				+	12.3	2	+ (34.5)	+ (37.2)		+
J3P7	River£	21.6	1				+	10.6	1	+ (35.1)	+ (37.3)		+
J3P8	Dam£	25.6	1	+ (32.0)	+ (35.5)	+ (37.4)	+	13.0	2	+ (35.1)	+ (38.4)		+
J3P8SA	River£	20.1	1					11.6	2	+ (34.6)	+ (37.1)		+
J3P9	River£	23.3	2				+	12.3	2	+ (34.5)	+ (36.5)		+
J3P9SA	River	19.7	2	+ (31.9)	+ (37.5)	+ (36.5)	+	13.0	2	+ (32.9)	+ (34.9)	+ (37.6)	+

For ISFtu2-qPCR : + = positive in duplicate with Ct ≤ 36 ; For Tul4-qPCR, Type B-qPCR and Acanth-qPCR : + = positive in duplicate ; NP =

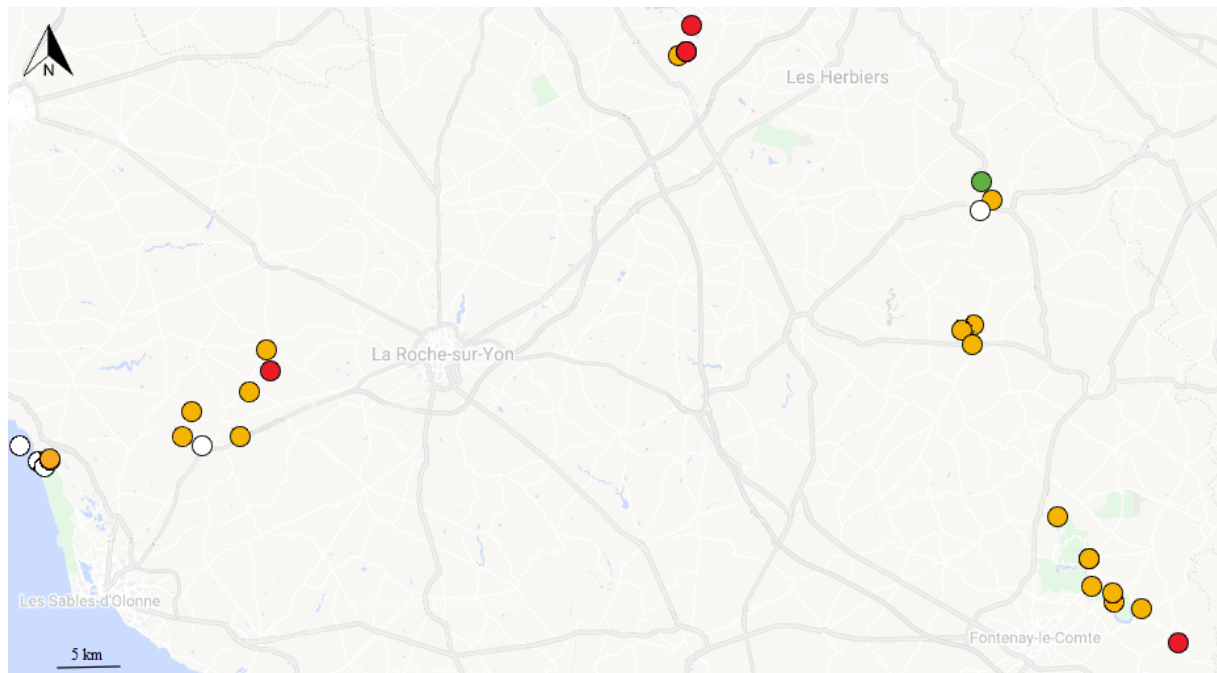
not performed ; NP = not performed ; NS = not sampled in the second campaign ; * and £: samples from the same river

Figure S1. *Francisella* sp. detection in surface water samples collected during July 2019.



For each site DNA were extracted from water and three q-PCR were performed (ISFtu2-qPCR for *Francisella* sp., Tul4-qPCR for *F. tularensis*, and Type B-qPCR for *F. tularensis* subsp. *holarctica*). White disk: sample with no positive qPCR. Green disk: sample positive for ISFtu2-qPCR and negative for Tul4 and Type B-qPCR. Orange disk: sample positive for ISFtu2 and Tul4-qPCR and negative for Type B-qPCR. Red disk: sample positive for the three qPCR tests.

Figure S2. *Francisella sp.* detection in surface water samples collected during January 2020.



For each site DNA were extracted from water and three q-PCR were performed (ISFtu2-qPCR for *Francisella sp.*, Tul4-qPCR for *F. tularensis*, and Type B-qPCR for *F. tularensis* subsp. *holarctica*). White disk: sample with no positive qPCR. Green disk: sample positive for ISFtu2-qPCR and negative for Tul4 and Type B-qPCR. Orange disk: sample positive for ISFtu2 and Tul4-qPCR and negative for Type B-qPCR. Red disk: sample positive for the three qPCR tests.

**TRAVAIL N°4 : Ulceroglandular infection
and bacteremia caused by *Francisella
salinarum* in an immunocompromised
patient, France
(article soumis)**

Lors de mon travail de thèse, réalisé au sein du laboratoire de Bactériologie et Hygiène Hospitalière du CHU Grenoble Alpes, Centre National de Référence de la Tularémie, j'ai pu participer à la caractérisation biochimique et génétique d'une souche de *Francisella* non *tularensis* impliquée dans une infection humaine.

La mission du CNR de la Tularémie est d'apporter son expertise et de participer au développement des connaissances dans le cadre de l'épidémiologie, de la physiopathologie, du diagnostic et du traitement de la tularémie.

En l'occurrence, le CHU de Poitiers, a adressé au CNR de la tularémie une souche bactérienne (CHUGA-F75) identifiée comme une *Francisella* sp., responsable d'un ulcère cutané et d'une bactériémie chez un patient immunodéprimé. J'ai participé à la caractérisation de cette souche.

Tout d'abord, j'ai étudié les caractères biochimiques et culturels de cette souche bactérienne à l'aide des méthodes utilisées en routine dans les laboratoires de microbiologie. La souche CHUGA-F75 s'est avérée être capable de croissance sur milieu chocolat enrichie en vitamines Polyvitex, sur gélose au sang mais aussi sur milieux moins nutritifs, tels que la gélose trypticase soja. Ainsi cette souche présentait des exigences nutritionnelles moindres que *F. tularensis*. La souche CHUGA-F75 était également halotolérante, c'est-à-dire capable de croissance dans des milieux riches en sel, ce qui la distingue de nouveau de *F. tularensis*. Concernant le typage biochimique, la souche CHUGA-F75 était oxydase positive, par opposition à *F. tularensis*.

Dans un second temps, le génome complet de la souche CHUGA-F75 a été séquencé par technologie de séquençage nouvelle génération de 2^{ème} (Illumina MiSeq) et 3^{ème} génération (Oxford Nanopore MinION). J'ai ensuite participé à l'analyse bio-informatique de la souche. L'assemblage de la souche a permis de circulariser un génome de 1 940 863 paires de bases, plus gros que celui de *F. tularensis*. L'analyse taxonomique de la souche a permis de mettre en évidence qu'elle appartenait à un groupe de trois espèces de *Francisella* récemment décrites : *F. salimarina*, *F. marina* et *F. salina*, qui représentent en réalité probablement une unique espèce bactérienne. L'hybridation AND-ADN, étudiée in silico, entre la souche CHUGA-F75 et les trois souches *F. salimarina/marina/salina* était supérieure à 80 % et la différence de GC % (pourcentage de guanine et de cytosine dans l'ADN) inférieure à 1 %. Ceci permet de conclure que la souche CHUGA-F75 appartient à l'espèce *F. salimarina/marina/salina*. L'espèce *F. salimarina* étant la seule validée par le Code

International de Nomenclature des procaryotes, nous avons choisi de qualifier la souche CHUGA-F75 de *F. salimarina*.

Francisella marina a été décrite comme responsable d'infection chez des poissons d'eau de mer (vivaneau rose, *Lutjanus guttatus*) en Amérique centrale. *Francisella salimarina* a été isolée d'eau de mer côtière en Chine. *Francisella salina* a été isolée d'eau de mer et d'algues prélevées sur la côte Est des États-Unis. Ainsi, il s'agirait du premier cas humain d'infection à *F. salimarina*. La source d'infection du patient n'a pas pu être identifiée. Cependant, au vu des réservoirs aquatiques marins décrits préalablement pour cette espèce bactérienne, une source de contamination aquatique peut être envisagée.

Ainsi, ce cas clinique, comme le reste de mon travail de thèse, vient rappeler le fort lien entre *Francisella* sp. et environnement aquatique.

Running Title: First *Francisella salimarina* human infection.

1 **Keywords:** *Francisella salimarina*, *Francisella* species, opportunistic infections, France.

2

3 **Title:** Ulceroglandular infection and bacteremia caused by *Francisella salimarina* in an
4 immunocompromised patient, France.

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17 * These two first authors contributed equally to this work.

18

19 **Abstract – 50 words**

20 While *Francisella tularensis* is a well-known highly virulent bacterium causing
21 tularemia in humans, several other *Francisella* species have been associated with sporadic
22 human infections. Here we describe the first human infection caused by the recently identified
23 *Francisella salimarina* species resulting in a cutaneous infection with bacteremia in an
24 immunocompromised patient.

25 **Text – 1289 words**

26 The taxonomy of the genus *Francisella* has been revised recently into four clades to
27 reorder genetically similar isolates and new species described in the last two decades (1). The
28 two subspecies of *F. tularensis*, *F. tularensis* subsp. *tularensis* (Type A strains) and *F.*
29 *tularensis* subsp. *holarctica* (Type B strains) causing the potentially life threatening disease
30 tularemia, belong to clade 1 (1). They are mainly transmitted to human through contact with
31 the animal reservoir, arthropod bites or contact with a contaminated hydro-telluric
32 environment (2). Depending on the portal of entry of the bacteria, several clinical forms of
33 tularemia are described: glandular, ulceroglandular, oropharyngeal, oculoglandular,
34 pneumonic or typhoidal (2). Among clade 2 species, the single species that has occasionally
35 been isolated from human so far is *F. philomiragia*, an opportunistic bacterium of aquatic
36 reservoir causing rare human infections in immunocompromised patients or after near-
37 drowning accidents (2). Here we describe the first human infection due to another clade 2
38 species related to aquatic environments: *F. salimarina*.

39 **The Patient**

40 On early June 2017, a 76-year-old man was diagnosed with an acute myelomonocytic
41 leukemia and admitted to the Poitiers University Hospital, France. On June 28, a first line of
42 chemotherapy consisting in subcutaneous azacitidine was started for seven days. After three
43 days of chemotherapy, piperacillin-tazobactam was introduced for five days because of febrile
44 aplasia. The patient was then discharged with a prophylactic antibiotic treatment of
45 sulfamethoxazole/trimethoprim (800/160 mg, three times a week). On July 26, the second
46 azacitidine cure was not administered because the patient presented again with febrile aplasia.
47 Physical examination revealed skin lesions on two left hand fingers that had appeared 3 weeks
48 earlier. These lesions were erythematous and crusty, but not purulent. They were associated
49 with a left axillary lymphadenopathy. Antibiotic treatment with piperacillin-tazobactam and

50 teicoplanin was started, switched to imipenem/cilastatin and daptomycin after 5 days because
51 of poor clinical response. Aerobic blood cultures performed on patient's admission were
52 positive on July 31st and Gram stain showed a small Gram-negative coccobacillus. Antibiotic
53 treatment was changed for cefepime administered for three days. MALDI-TOF mass
54 spectrometry (Vitek[®] MS) did not succeed to identify the bacteria. The isolated bacterial
55 strain was then identified as a *Francisella* species by 16s rDNA amplification and sequencing.
56 Because of persistence of fever and worsening of skin lesions, a cutaneous biopsy was
57 performed, from which the same *Francisella* sp. strain was isolated. Doxycycline (100 mg
58 twice a day) was administrated to the patient for eight days followed by
59 sulfamethoxazole/trimethoprim leading to apyrexia. He lived in a small town 30 kilometers
60 from the Atlantic Ocean. He did not travel abroad recently and had no pet.

61 The *Francisella* sp. strain (referred as CHUGA-F75) was sent to the French National
62 Reference Centre for *Francisella* (FNRCF) for further characterization. The strain was strictly
63 aerobic and grew well on chocolate agar plus Isovitalex, blood agar, and tryptic soy agar,
64 yielding grey mucoid colonies after 24 h of incubation at 35°C in 5% CO₂, but not on
65 Drigalski agar. Biochemical testing revealed a positive oxidase, weakly positive catalase and
66 a negative urease test. The strain was also halotolerant as it could grow in modified Mueller-
67 Hinton broth with up to 8% NaCl. ISFtu2, Tul4, and Type B real-time PCR tests, allowing
68 detection of respectively most *Francisella* sp., *F. tularensis*, and *F. tularensis* subsp.
69 *holarctica*, were all negative for DNA extracted from this strain (3,4). Species identification
70 could not be obtained using MALDI-TOF mass spectrometry, either with the routine database
71 (MBT IVD Library DB-7171) or the Biotox[®] database (MBT SR Library) from Bruker
72 Daltonics[®], or our own (FNRCF) database containing *F. tularensis*, *F. novicida* and *F.*
73 *philomiragia* species (5). Therefore, whole genome sequencing of the strain was performed
74 using 2nd and 3rd next generation sequencing platforms (Illumina MiSeq and Oxford Nanopore

75 MinION). Hybrid assembly of the sequencing data using Unicycler software on the Galaxy
76 web platform (<https://usegalaxy.org/>) allowed circularization of a 1,940,863 bp bacterial
77 chromosome. Whole-genome based identification of the strain was assessed using state-of-
78 the-art genome-based taxonomy via the Type Strain Genome Server (<https://tygs.dsmz.de/>)
79 (6) which clustered the CHUGA-F75 strain in the same branch as the *F. salimarina* SYSU
80 SYW-1, the *F. marina* E95-16, and the *F. salina* TX07-7308 strains, probably representing
81 the same species because of a high genetic homology although different species names have
82 been published (7–9). Digital DNA-DNA hybridization superior to 80% and difference in
83 GC% inferior to one percent between the CHUGA-F75 strain and the three *F.*
84 *salimarina/marina/salina* strains confirmed belonging of CHUGA-F75 isolate to the same
85 species. As the only validly published species name according to The International Code of
86 Nomenclature of prokaryotes is *F. salimarina*, we identified CHUGA-F75 as responsible for
87 the first case of human infection with *F. salimarina*.

88 Using the broth microdilution method in cation adjusted Mueller-Hinton broth as
89 recommended by the Clinical and Laboratory Standards Institute (CLSI), the CHUGA-F75
90 strain was sensitive to gentamicin (MIC = 0.125 mg/L), doxycycline (MIC = 1 mg/L) and
91 ciprofloxacin (MIC = 0.016 mg/L). It was resistant to sulfamethoxazole/trimethoprim (MIC =
92 32 mg/L).

93 **Discussion**

94 Altogether, these phenotypic and whole genome data confirmed that the CHUGA-F75
95 strain is a *F. salimarina* strain. Its recovery in pure culture from the blood and from the skin
96 ulcer supports the fact that this bacterium was responsible for the severe illness of the patient.
97 So far, *F. marina*, was only described as responsible for systemic disease in fishes (cultured
98 spotted rose snapper, *Lutjanus guttatus*) in Central America, while four *F. salimarina* strains
99 have been isolated from costal seawater in Guangdong Province in China and one strain of *F.*

100 *salina* has been grown from brackish seawater and seaweed off the Gulf coast of Galveston in
101 the United States (7–9). Thus, this species likely has a large distribution and can cause
102 opportunistic human infections. The source of contamination was not identified in this case
103 but is likely environmental.

104 This report, like previous descriptions of human infections due to emergent
105 *Francisella* species, highlight that environmental or fish-related *Francisella* species could be
106 responsible for opportunistic infections in humans, resembling tularemia. *F. novicida* is the
107 closest species to *F. tularensis* in the *Francisella* phylogenetic tree while having mainly an
108 aquatic reservoir (1). It is of low virulence in humans, causing occasional infections in
109 immunocompromised patients (2). Two bacteremia and one ulceroglandular infection due to
110 *F. novicida*-like organisms were described in two immunocompromised patients in the US,
111 probably acquired through contact with contaminated water, and after a cut in the toe in
112 brackish water in one patient in Australia, respectively (10,11). Other *Francisella* species
113 belonging to clade 1 with reported human infections are *F. opportunistica* and *F.*
114 *hispaniensis*. *F. opportunistica* infection was reported in three distinct US patients: one with
115 meningitis, two with bacteremia, both immunocompromised (12). A *F. hispaniensis* strain
116 was isolated from blood and urine of an immunocompetent patient in Spain (13) followed by
117 the description of two *F. hispaniensis* strains causing bacteremia in an Australian
118 immunocompromised patient after he cut himself while fishing (14) and in an
119 immunocompetent Chinese patient after a deep-sea dive (15). Finally, a new *Francisella*
120 species belonging to clade 4 and closed to marine species was responsible for a cutaneous
121 infection developed after contact of an open wound in brackish water in an
122 immunocompromised US patient (16).

123 The rise of pathologies requiring immunosuppressive treatments and the democratization of
124 whole genome sequencing technics will probably lead to an increasing discovery of emergent

125 *Francisella* species and more generally of a wide diversity of opportunistic bacteria.
126 Microbiologists should be aware that small Gram-negative coccobacillus which are not
127 identified by mass spectrometry could be an environmental *Francisella* sp strain, especially if
128 identified from an infected patient after exposure to aquatic environments.

129 The complete genome sequence of the CHUGA-F75 strain has been deposited in GenBank
130 under the accession number CP076680.

131 **Conflict of interest**

132 The authors declare that they have no conflict of interest.

133 **Acknowledgments**

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135 present work (ANR-17-ASTR-0024).

136 **About the authors**

137 Aurélie Hennebique is a clinical microbiologist in the bacteriology laboratory of Grenoble
138 University Hospital, France, that also hosts the French National Reference Center for
139 *Francisella tularensis*. Her primary research interests include the tularemia agent with an
140 emphasis on its mechanisms of environmental survival in water, susceptibility to antibiotics
141 and virulence.

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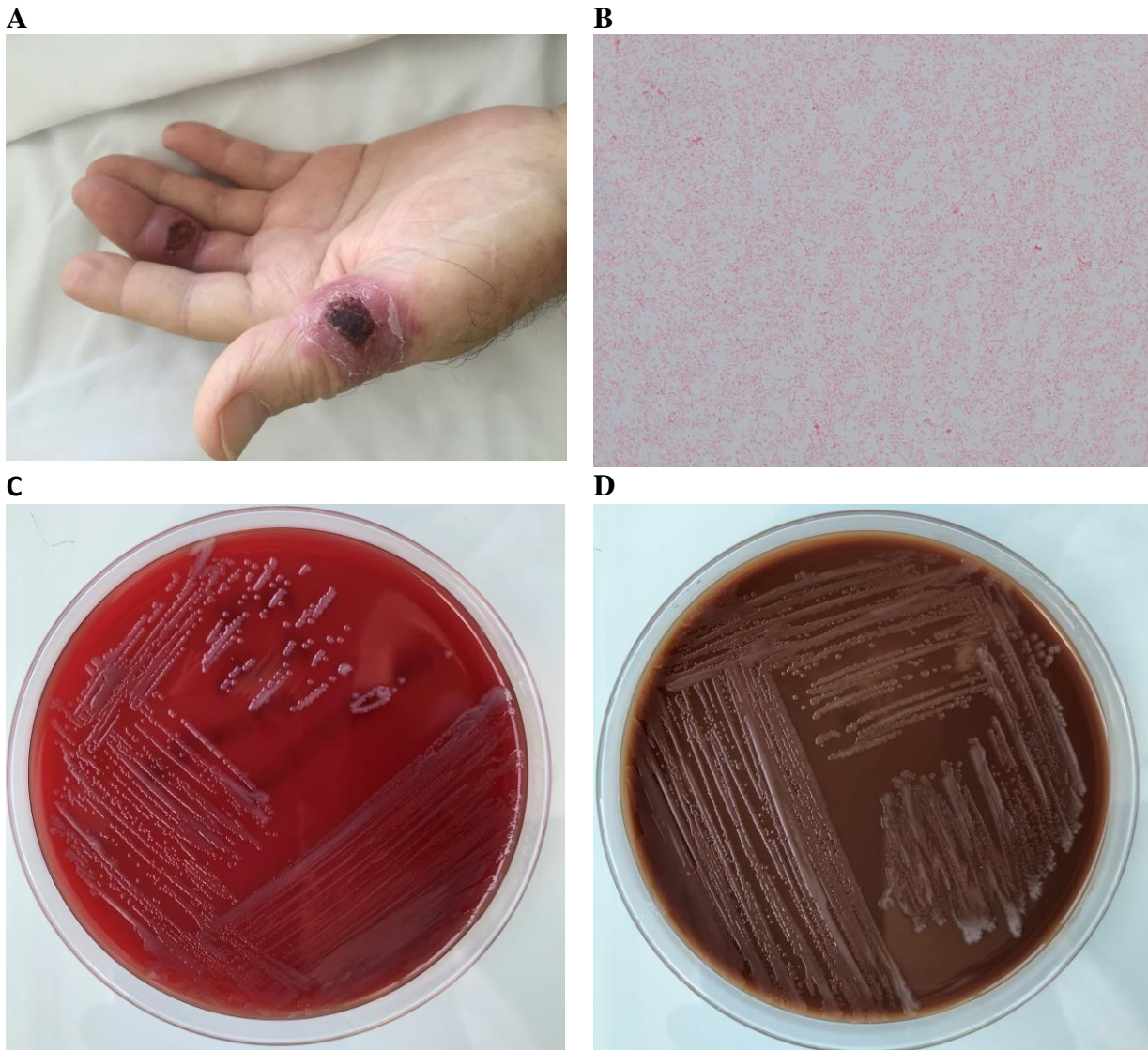
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197

198

199 **Figure 1: Skin ulcers and bacteremia in an immunocompromised patient and isolated**
200 **bacteria morphology.**

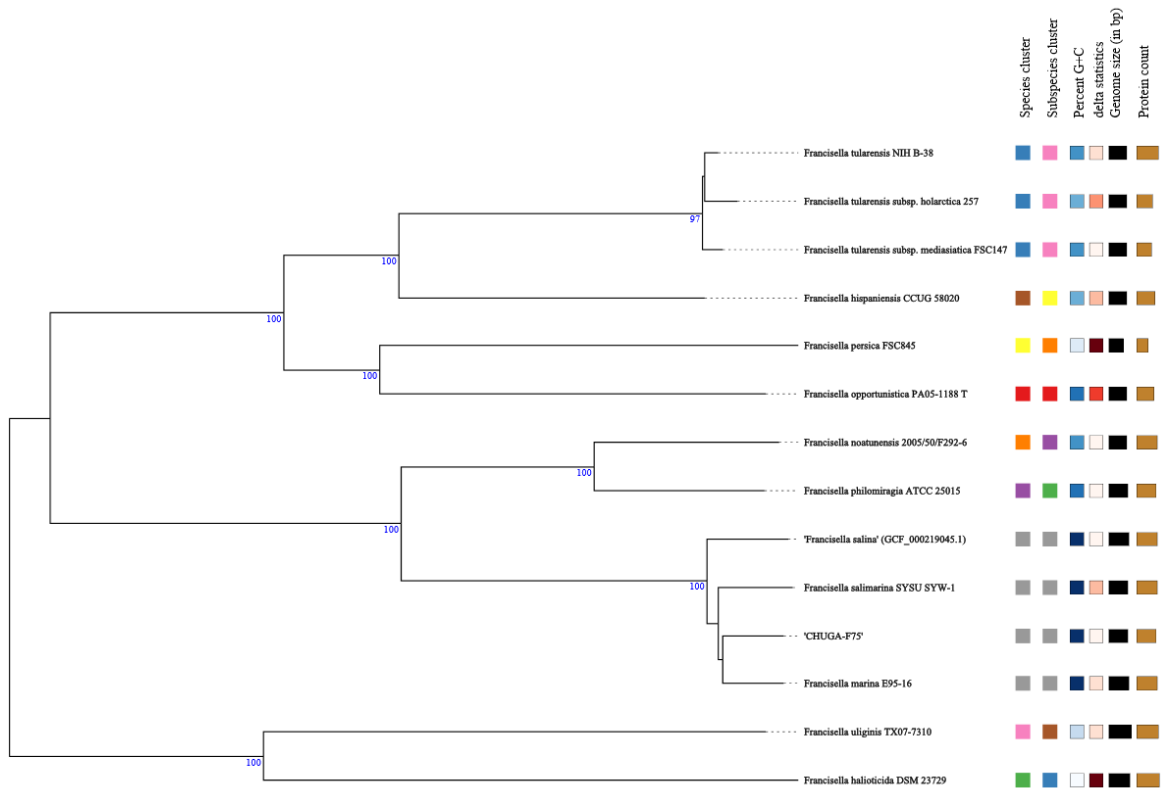


201
202 A) Skin lesion on two left hand fingers. B) Small Gram-negative coccobacillus isolated from
203 blood and skin lesions. C) Growth on blood agar after two days of incubation at 35°C in 5%
204 CO₂. D) Growth on chocolate agar after two days of incubation at 35°C in 5% CO₂.

205

206

207 **Figure 2: Genome-scale GBDP (Genome Blast Distance Phylogeny) tree of CHUGA-F75**
 208 **strain.**



209
 210 Whole-genome based identification of the strain was assessed using the Type Strain Genome
 211 Server (<https://tygs.dsmz.de/>)

**TRAVAIL N°5 : Evaluation of the Biotosis
qPCR Detection Kit for *Francisella
tularensis* Detection in Clinical and
Environmental Samples**

(Article publié, Journal of Clinical Microbiology, 2020)

Francisella tularensis est un agent potentiel de bioterrorisme. Ainsi, des méthodes de recherche rapides et fiables de cet agent infectieux doivent être disponibles dans les laboratoires de biologie médicale ainsi que dans les laboratoires vétérinaires et environnementaux. La recherche de *F. tularensis* dans des échantillons peut s'effectuer par culture ou par biologie moléculaire. Les techniques de culture ne sont pas adaptées à une recherche en urgence de la bactérie pour plusieurs raisons : 1/ cette bactérie est de croissance lente, les délais de croissance sont de deux à quatre jours minimum ; 2/ cette bactérie est de nature fastidieuse, par exemple son isolement à partir de prélèvements biologiques est obtenu dans moins de 10 % des cas ; 3/ en cas de prélèvements polymicrobiens, tels que les prélèvements environnementaux, l'isolement de la bactérie devient encore plus incertain car des bactéries de croissance plus rapide peuvent envahir les milieux de cultures. Ainsi, les techniques de PCR sont plus adaptées pour du diagnostic rapide puisque des résultats de PCR peuvent être rendus en quelques heures. Cependant, très peu de laboratoires disposent d'amorces et de sondes pour la recherche de *F. tularensis*. Ainsi, des kits de PCR commerciaux, permettant une recherche rapide et fiable de *F. tularensis*, sont indispensables. Une exigence supplémentaire dans le cadre de la surveillance environnementale de la tularémie, voire dans le cadre du bioterrorisme, est de pouvoir tester rapidement un très grand nombre d'échantillons.

L'objectif de ce travail a été d'évaluer les performances sur la détection de *F. tularensis* d'un kit commercial de PCR, le kit Biotoxis (Bertin Bioreagent, Montigny-le-Bretonneux, France). Il s'agit d'un kit de PCR multiplexe en temps réel, recherchant trois agents de bioterrorisme : *Yersinia pestis*, *Bacillus anthracis* et *F. tularensis*. J'ai ainsi comparé les performances du kit Biotoxis à celles de PCR utilisées en routine au Centre National de Référence (CNR) de la tularémie : la qPCR-ISFtu2 (ciblant la séquence d'insertion ISFtu2, présente en multi-copies chez *F. tularensis* et *F. novicida* et en une ou deux copies chez *F. philomiragia*), la qPCR-Tul4 (ciblant le gène *tul4*, présent en une seule copie chez *F. tularensis* et *F. novicida*) et la qPCR-Type B (ciblant une jonction entre la séquence d'insertion ISFtu2 et une région flanquante en 3', retrouvée seulement chez *F. tularensis* subsp. *holarctica*).

J'ai commencé par évaluer les performances du kit Biotoxis sur des souches bactériennes. Le kit Biotoxis a amplifié 87/87 souches de *F. tularensis* subsp. *holarctica* mais aussi la souche de *F. novicida*. Il n'a pas amplifié la souche de *F. philomiragia* ni les souches appartenant à d'autres espèces bactériennes.

Concernant l'analyse de 31 échantillons biologiques d'origine humaine, le kit Biotoxis a présenté une sensibilité comprise entre 90,32 % et 96,55 %, en comparaison aux qPCR-ISFtu2 et -Type B respectivement. Les 30 échantillons biologiques caractérisés négatifs pour la tularémie au CNR n'ont pas conduit à une amplification avec le kit Biotoxis.

L'analyse d'échantillons d'eau artificiellement contaminés par *F. tularensis* a mis en évidence un seuil de détection du kit Biotoxis de 1 000 UFC/litre par opposition à 100 UFC/litre pour la qPCR-Type B, 10 UFC/litre pour la qPCT-Tul4 et 0,1 UFC/litre pour la qPCR-ISFtu2.

Concernant l'analyse de 57 échantillons d'eau environnementale collectés en zone d'épidémie de tularémie, le kit Biotoxis a identifié comme positifs pour *F. tularensis* 6/15 échantillons positifs en qPCR-ISFtu2 et 4/4 échantillons positifs en qPCR-Type B.

En conclusion, le kit Biotoxis a démontré de bonnes performances en termes de sensibilité et de spécificité. Cependant l'amplification de *F. novicida*, bactérie de faible pathogénicité pour l'Homme et de réservoir hydrique, doit être connue des utilisateurs et peut poser problème pour l'interprétation de résultats de prélèvements environnementaux. La sensibilité moindre du kit Biotoxis par rapport aux qPCR de référence pourrait s'expliquer par le nombre de copies du gène cible utilisé dans le kit Biotoxis et par le caractère multiplexe de ce kit.

Le kit Biotoxis a également démontré sa capacité de tester un grand nombre d'échantillons rapidement, de par son format en plaque et une excellente simplicité d'utilisation. Ceci le rend utilisable par tous les laboratoires disposant d'un équipement de qPCR.

Ce travail a donc permis d'évaluer un kit commercial de PCR *F. tularensis* en comparaison aux PCR de référence utilisées au CNR. Ce travail de recherche appliqué fait partie des missions d'expertise d'un CNR, afin d'aider les laboratoires non référents dans le choix de leurs outils diagnostiques.



Evaluation of the Biotoxis qPCR Detection Kit for *Francisella tularensis* Detection in Clinical and Environmental Samples

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ABSTRACT Rapid and reliable detection and identification of *Francisella tularensis* (a tier 1 select agent) are of primary interest for both medical and biological threat surveillance purposes. The Biotoxis qPCR detection kit is a real-time quantitative PCR (qPCR) assay designed for the detection of *Bacillus anthracis*, *Yersinia pestis*, and *F. tularensis* in environmental or biological samples. Here, we evaluated its performance for detecting *F. tularensis* in comparison to previously validated qPCR assays. The Biotoxis qPCR was positive for 87/87 *F. tularensis* subsp. *holarctica* (type B) strains but also for *F. tularensis* subsp. *novicida*. It was negative for *Francisella philomiragia* and 24/24 strains belonging to other bacterial species. For 31 tularemia clinical specimens, the Biotoxis qPCR displayed a sensitivity between 90.32% and 96.55%, compared to qPCR tests targeting ISFtu2 or a type B-specific DNA sequence, respectively. All 30 nontularemia clinical specimens were Biotoxis qPCR negative. For water samples, the Biotoxis qPCR limit of detection was 1,000 CFU/liter of *F. tularensis*. For 57 environmental water samples collected in France, the Biotoxis qPCR was positive for 6/15 samples positive for ISFtu2 qPCR and 4/4 positive for type B qPCR. In conclusion, the Biotoxis qPCR detection kit demonstrated good performances for *F. tularensis* detection in various biological and environmental samples, although cross-amplification of *F. tularensis* subsp. *novicida* must be considered. This plate format assay could be useful to test a large number of clinical or environmental specimens, especially in the context of natural or intentional tularemia outbreaks.

KEYWORDS *Francisella tularensis*, diagnosis, real-time PCR, tularemia

Francisella tularensis is a small Gram-negative bacterium causing the zoonotic disease tularemia (1, 2). More strictly, among the four subspecies of *F. tularensis*, only two are currently associated with human and animal tularemia cases: *Francisella tularensis* subsp. *tularensis* (type A strains), in North America, and *Francisella tularensis* subsp. *holarctica* (type B strains) in the whole Northern Hemisphere and southern Australia (1–3). *Francisella tularensis* subsp. *mediasiatica* has been detected in arthropods and rodents in central Asia and Russia (1) but never associated with human infections. *Francisella tularensis* subsp. *novicida* (also referred to as *F. novicida*) is an aquatic bacterium, rarely responsible for opportunistic human diseases (4). Other aquatic *Francisella* species have been described, including *Francisella philomiragia*, another rare opportunistic human pathogen (4).

F. tularensis can infect a wide range of vertebrate species (especially lagomorphs and small rodents) and arthropods (including *Ixodidae* ticks and mosquitoes) (1, 2). This bacterium is also able to survive for prolonged periods in the hydrotelluric environment (1, 2, 4). *F. tularensis* can contaminate humans through contact with infected animals, arthropod bites, exposure to contaminated environments, or ingestion of contami-

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nated food or water (1, 2). Several clinical forms of tularemia exist, mainly depending on the portal of entry of the bacteria. The ulceroglandular and glandular forms correspond to localized lymphadenopathy with or without a skin ulcer, respectively. The oropharyngeal form combines pharyngitis and cervical lymphadenopathy. The oculoglandular form usually corresponds to conjunctivitis with periauricular lymphadenopathy. The pneumonic form (pneumonia or pleuropneumonia) usually occurs through the inhalation of contaminated aerosols. The typhoidal form is severe sepsis, often with confusion, but with no inoculation lesion or lymphadenopathy (1, 2). Because of its ability to be spread by aerosols, its low infectious dose, and high virulence, *F. tularensis* is classified in category A of potential biological threat agents by the U.S. Centers for Disease Control and Prevention (CDC) (5). Pneumonic tularemia cases caused by the most virulent type A strains are associated with up to 30% mortality rates (1, 2, 5).

Tularemia diagnosis is currently based on serological methods, and *F. tularensis* detection is performed by culture or PCR-based methods (2). However, this fastidious and slow-growing bacterium is usually isolated from less than 10% of tularemia patients (2). Serological methods can detect significant antibody titers only 2 to 3 weeks after disease onset (2, 6). Therefore, real-time PCR tests are currently the most effective method for detecting *F. tularensis* in human, animal, or environmental samples (2). Although many in-house qPCR tests have been developed, a user-friendly commercial test allowing rapid, accurate, and standardized detection of *F. tularensis* in various types of samples is a high priority.

The Biotoxis quantitative PCR (qPCR) detection kit is a plate-format TaqMan probe-based real-time PCR assay designed for the combined detection of *Bacillus anthracis*, *Yersinia pestis*, and *F. tularensis* DNA in various sample types. In the present study, we evaluated this kit's performance for detection and identification of *F. tularensis* in DNA extracts from bacterial strains, clinical samples, and environmental water samples, in comparison to previously validated PCR tests used routinely in our laboratory.

MATERIALS AND METHODS

Biotoxis qPCR assay. The Biotoxis qPCR detection kit (Bertin Bioreagent, Montigny-le-Bretonneux, France) was carried out according to the manufacturer's instructions. Briefly, each of the PCR mixtures (25 μ l) contained 12.5 μ l of the qPCR mix, 3.75 μ l of primer and probe mix, 3.75 μ l of water, and 5 μ l of DNA sample (at various concentrations according to sample type; see below). The PCR was performed on a LightCycler 480 instrument II (Roche) with an initial enzyme activation step of 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 30 s. The results were analyzed at the endpoint.

***Francisella tularensis* reference qPCR assays.** We compared results obtained with the Biotoxis qPCR detection kit with those obtained with three previously validated qPCR methods, which are routinely used at the French National Reference Center for *Francisella* (French NRCF) (Grenoble University Hospital, Grenoble, France). One qPCR test (referred to as ISFtu2 qPCR) targets the ISFtu2 insertion sequence present in multiple copies in the *F. tularensis* genome (7). The second one (Tul4 qPCR) targets the gene encoding the single-copy Tul4 surface protein-encoding gene (7). The last one (type B qPCR) targets a DNA fragment located between ISFtu2 and a flanking 3' region and is specific for *F. tularensis* subsp. *holarctica* (8).

These three qPCR tests were performed using the same protocol. Each of the PCR mixtures (20 μ l) contained 10 μ l of 2 \times TaqMan Fast Advanced PCR master mix (Applied Biosystems, Thermo Fisher Scientific, Vilnius, Lithuania), 0.4 μ l of each primer at 10 μ M, 0.4 μ l of 2 μ M probe, 3.8 μ l of water, and 5 μ l of DNA sample (at various concentrations according to sample type; see below). The PCRs were run on a LightCycler 480 instrument II (Roche) with an initial enzyme activation step of 50°C for 2 min and 95°C for 2 min, followed by 45 cycles of denaturation at 95°C for 3 s and annealing and extension at 60°C for 30 s. The results were analyzed at the endpoint.

DNA extraction methods. For bacterial strains and clinical samples, DNA extraction was obtained using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France), according to the manufacturer's recommendations.

For artificial and environmental water samples, 1 liter of water was filtered through 0.22- μ m or 0.45- μ m filters, and DNA was extracted from the filter using the NucleoMag DNA/RNA water kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's recommendations.

Concentrations of bacterial DNA extracts and environmental water DNA extracts were then assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

Bacterial strains. We tested 90 *Francisella* sp. strains, including 86 clinical strains of *F. tularensis* subsp. *holarctica*, one clinical strain of *F. philomiragia*, and the reference strains *F. philomiragia* ATCC 25015, *F. novicida* CIP 56.12, and *F. tularensis* subsp. *holarctica* LVS NCTC 10857 (Table 1). Clinical strains

TABLE 1 Bacterial strains used in this study

Species	Strain
<i>F. tularensis</i> subsp. <i>holarctica</i>	Clinical strains (86 strains, including Ft5 to -46, Ft48 to -65, Ft67 to -74, Ft76 to -80, Ft83 to -89, Ft91, and Ft92 to -96)
	LVS NCTC 10857
<i>F. novicida</i>	CIP 56.12
<i>F. philomiragia</i>	Clinical strain Ft47
	ATCC 25015
<i>Corynebacterium jeikeium</i>	CIP 8251
<i>Staphylococcus epidermidis</i>	CIP 103627
<i>Staphylococcus aureus</i>	ATCC 6538
<i>Streptococcus agalactiae</i>	ATCC 12400
<i>Streptococcus pneumoniae</i>	ATCC 49619
<i>Streptococcus uberis</i>	ATCC 9727
<i>Streptococcus salivarius</i>	Clinical strain
<i>Streptococcus mitis</i>	CIP 103335
<i>Streptococcus oralis</i>	Clinical strain
<i>Enterococcus faecalis</i>	ATCC 29212
<i>Enterococcus faecium</i>	CIP 5432
<i>Bacillus subtilis</i>	ATCC 6633
<i>Haemophilus influenzae</i>	ATCC 49766
<i>Acinetobacter baumannii</i>	ATCC 19606
<i>Escherichia coli</i>	ATCC 25922
<i>Enterobacter cloacae</i>	ATCC 13047
<i>Klebsiella pneumoniae</i>	ATCC 35657
<i>Staphylococcus sciuri</i>	ATCC 29061
<i>Stenotrophomonas maltophilia</i>	ATCC 17666
<i>Pseudomonas aeruginosa</i>	CIP 5933
<i>Streptococcus pyogenes</i>	CIP 104226
<i>Neisseria elongata</i>	Clinical strain
<i>Moraxella catarrhalis</i>	Clinical strain
<i>Serratia marcescens</i>	CIP 103551

of *Francisella* sp. were previously identified at the French NRCF. *F. philomiragia* was identified by whole 16S rRNA gene sequencing (9). *F. tularensis* subsp. *holarctica* was identified at the subspecies level by combining a positive ISFtu2 or Tul4 qPCR test and either a positive type B qPCR or PCR amplification and sequencing of the expected 16S-23S intergenic region (10). The French NRCF owns all the above strains, and specific authorizations were obtained from the Agence Nationale de Sécurité du Médicament et des Produits de Santé (ANSM; authorization number ADE-103892019-7). In addition, 24 reference or clinical strains belonging to bacterial species other than *Francisella* sp. were used (Table 1).

Bacteria were grown on chocolate agar medium supplemented with PolyViteX (bioMérieux, Marcy-l'Etoile, France) or on sheep blood agar medium (bioMérieux, Marcy-l'Etoile, France) at 35°C in a 5% CO₂-enriched atmosphere for 1 or 2 days. *Francisella tularensis* strains were grown in a biosafety level 3 laboratory.

DNA extracts from *Francisella* sp. strains were prepared at 10 ng/μl for analysis with the Biotoxis qPCR detection kit. DNA extracts from four clinical strains of *F. tularensis* subsp. *holarctica*, one clinical strain of *F. philomiragia* (*F. philomiragia* ATCC 25015), and *F. novicida* CIP 56.12 were used to compare cycle threshold (C_t) values obtained with the Biotoxis qPCR to those of the ISFtu2, Tul4, and type B qPCR tests (Table 2). For non-*Francisella* strains, three DNA extract concentrations were used (0.1, 1, and 10 ng/μl).

Clinical specimens. For sensitivity purposes, we tested 31 clinical samples (mainly lymph node and respiratory samples) collected for routine medical care in 30 tularemia patients, including 29 confirmed cases and one probable case (Table 3). These samples were received at the French NRCF between 2018

TABLE 2 Biotoxis qPCR testing of bacterial strains

Strain ^a	Result (C _t) in qPCR			
	Biotoxis	ISFtu2	Tul4	Type B
<i>F. tularensis</i> type B Ft41	Positive (20)	Positive (14)	Positive (19)	Positive (19)
<i>F. tularensis</i> type B Ft54	Positive (20)	Positive (13)	Positive (17)	Positive (18)
<i>F. tularensis</i> type B Ft72	Positive (19)	Positive (13)	Positive (17)	Positive (18)
<i>F. tularensis</i> type B Ft92	Positive (18)	Positive (12)	Positive (15)	Positive (16)
<i>F. novicida</i> CIP 56.12	Positive (18)	Positive (12)	Positive (16)	Negative
<i>F. philomiragia</i> Ft47	Negative	Positive (26)	Negative	Negative
<i>F. philomiragia</i> ATCC 25015	Negative	Positive (36)	Negative	Negative

^aDNA extracts were standardized at 10 ng/μl.

TABLE 3 Biotoxis qPCR testing of 31 clinical samples from 30 tularemia patients^a

Sample	Type	Clinical form	Result of:			
			Serology/culture	qPCR (C _T) ^b		
				ISFtu2	Type B	Biotoxis
S1	LN	GL	POS/NA	POS (27.97)	POS (31.96)	POS (34.50)
S2 ^c	Peritoneal tissue	INT	POS/NEG	POS (29.35)	POS (35.06)	POS (32.59)
S3 ^c	Peritoneal liquid	INT	POS/NEG	POS (28.22)	POS (32.57)	POS (35.05)
S4	LN	GL	POS/NEG	POS, 1/100 (31.98)	POS, 1/100 (39.71)	POS, 1/100 (38.06)
S5	LN	PNE	POS/NEG	POS (20.47)	POS (28.89)	POS (27.37)
S6	BAL	PNE	POS/NA	POS (30.48)	POS (34.65)	POS (37.29)
S7	BAL	PNE	POS/NA	POS (28.64)	POS (33.06)	POS (35.66)
S8	LN	GL	POS/NEG	POS (27.35)	POS (34.82)	POS (32.60)
S9	LN	PNE	POS/NEG	POS (30.62)	POS (35.17)	POS (39.06)
S10	Tracheal aspirate	PNE	NA/NEG	POS (30.88)	POS (37.02)	POS (37.63)
S11	LN	GL	NA/NEG	POS (30.55)	POS (34.66)	POS (36.23)
S12	LN	GL	POS/NEG	POS (23.56)	POS (32.92)	POS (30.22)
S13	LN	GL	NEG/NEG	POS (32.14)	POS (36.43)	POS (38.76)
S14	LN	GL	SCV/NEG	POS (33.85)	POS (38.25)	NEG
S15	LN	GL	SCV/NEG	POS (30.56)	POS (35.02)	POS (34.90)
S16	Sputum	PNE	NA/NA	POS (29.64)	POS (36.09)	POS (36.04)
S17	LN	UG	POS/NEG	POS (27.10)	POS (33.76)	POS (32.23)
S18	LN	GL	NA/NEG	POS (26.24)	POS (30.64)	POS (30.09)
S19	LN	GL	POS/NEG	POS (28.50)	POS (34.76)	POS (31.90)
S20	LN	PNE	POS/NA	POS (36.61)	NEG	NEG
S21	Knee prosthesis	OA	POS/POS	POS (30.94)	POS (35.89)	POS (37.19)
S22	LN	UG	POS/NEG	POS (23.82)	POS (29.68)	POS (30.79)
S23	LN	UG	NA/NEG	POS (25.29)	POS (29.53)	POS (28.71)
S24	LN	OG	NA/NEG	POS (28.70)	POS (35.76)	POS (35.80)
S25	LN	OP	POS/NEG	POS (24.18)	POS (30.14)	POS (31.69)
S26	LN	UG	POS/NEG	POS (30.11)	POS (35.57)	POS (35.77)
S27	LN	GL	FFR/NEG	POS (34.85)	NEG	NEG
S28	LN	GL	POS/NEG	POS (25.50)	POS (30.33)	POS (31.91)
S29	LN	GL	POS/NEG	POS (29.46)	POS (33.56)	POS (35.86)
S30	LN	PNE	NA/NEG	POS (32.82)	POS (35.83)	POS (37.96)
S31	LN	UG	NA/NEG	POS (24.72)	POS (31.98)	POS (31.25)

^aUG, ulceroglandular; GL, glandular; PNE, pneumonic; OG, oculoglandular; OP, oropharyngeal; INT, intestinal; OA, osteoarticular; LN, lymph node; BAL, bronchoalveolar lavage; NA, not available; NEG, negative; POS, positive; SCV, seroconversion; FFR, a fourfold rise in antibody titers.

^bDNA extracts were tested pure or at the specified dilution.

^cSamples from the same patient.

and 2019 for tularemia diagnostic confirmation. A tularemia confirmed case was defined as an instance of clinically compatible disease with detection of *F. tularensis* from any clinical specimen by culture or PCR (ISFtu2 or type B qPCR, C_T ≤ 35), seroconversion, or a 4-fold (or greater) change in titers of serum antibody to *F. tularensis* antigen between acute- and convalescent-phase sera. A probable case was defined as an instance of a clinically compatible disease with a single positive serum sample. According to our laboratory's routine procedure, all clinical samples were first tested using ISFtu2 qPCR for *F. tularensis* detection (7). Positive samples were further tested using type B qPCR for the identification of subspecies *F. tularensis* subsp. *holarctica* (8). When possible, *F. tularensis* culture was also performed.

These samples were analyzed by the Biotoxis qPCR detection kit retrospectively. DNA extracts were analyzed pure and diluted to 1/10 and 1/100. Samples were considered positive if at least one of the three DNA dilutions tested was positive. Negative results were duplicated for confirmation.

For specificity purposes, we also tested 30 clinical samples from patients for which the French NRCF excluded tularemia diagnosis because of negative diagnostic tests for *F. tularensis*, including eight samples positive for *Bartonella henselae* by PCR. All these samples were collected as part of routine patient care and analyzed retrospectively using the Biotoxis qPCR detection kit.

Environmental samples. We first determined the limit of detection (LOD) of the Biotoxis qPCR using *F. tularensis*-spiked water samples. For this purpose, a water sample collected in the environment was first sterilized by filtration on a 0.22- μ m filter. Several aliquots were then inoculated with *F. tularensis* subsp. *holarctica* LVS NCTC 10857 strain at concentrations ranging from 0.1 to 10,000 CFU/liter. One liter of each aliquot was filtered through a 0.22- μ m filter, and DNA was extracted from the filter. The DNA extracts were tested using the Biotoxis qPCR detection kit (in triplicate) and by ISFtu2, Tul4, and type B qPCRs, which were used as controls.

We then tested 57 surface water samples collected in France in 2019 from various aquatic environments. One liter of each sample was passed through a 0.45- or 0.22- μ m filter, from which DNA was extracted. DNA extracts were then adjusted to 10 ng/ μ l and tested in duplicate by the ISFtu2, Tul4, and type B qPCR tests. These qPCR tests were considered positive only when both duplicate tests displayed a C_T of ≤36 for ISFtu2 qPCR or a C_T of <40 for Tul4 and type B qPCRs. However, the presence of *F.*

tularensis DNA was considered confirmed only for water samples with a positive type B qPCR test, which can specifically detect *F. tularensis* subsp. *holarctica* strains found in France. Water samples were then tested using the Biotoxis qPCR detection kit, and results were compared to those of the three previous qPCR tests.

RESULTS

Bacterial strains. The Biotoxis qPCR gave a strongly positive signal (C_T values between 15 and 22) for the 86 clinical strains of *F. tularensis* subsp. *holarctica* and for the reference strain *F. tularensis* subsp. *holarctica* LVS NCTC 10857, corresponding to a 100% sensitivity for the tested type B tularemia strains. DNA extracts from four clinical strains of *F. tularensis* subsp. *holarctica* were tested to determine the analytical sensitivity of the Biotoxis qPCR, compared to those of the ISFtu2, Tul4, and type B qPCR tests. The C_T ranges for these four samples were 18 to 20 for the Biotoxis qPCR, 12 to 14 for ISFtu2 qPCR, 15 to 19 for Tul4 qPCR, and 16 to 19 for type B qPCR (Table 2).

The Biotoxis qPCR also gave a strongly positive signal for *F. novicida* CIP 56.12, like the ISFtu2 and Tul4 qPCR tests (7). As expected, type B qPCR was negative for this strain (Table 2). Biotoxis qPCR did not amplify the two *F. philomiragia* strains tested. The ISFtu2 qPCR gave a weak signal for this species, while Tul4 and type B qPCRs were negative (Table 2) (7).

The Biotoxis qPCR was negative for 21 non-*Francisella* strains but gave a weak signal (C_T between 34 and 37 at 10 ng/ μ l of DNA) for *Streptococcus salivarius*, *Pseudomonas aeruginosa* CIP 5933, and *Neisseria elongata* strains. For these three strains, testing lower DNA concentrations (0.1 to 1 ng/ μ l) abolished the amplification signal. Overall, the Biotoxis qPCR displayed a 96.30% specificity for the 27 non-type B strains tested owing to the cross-amplification of *F. novicida*.

Clinical samples. The 31 clinical samples collected from 30 tularemia patients included 24 lymph nodes, four respiratory samples, two peritoneal samples, and one osteoarticular sample (Table 3).

Tests were positive for 31/31 clinical samples for ISFtu2 qPCR, 29/31 for type B qPCR, and 28/31 for Biotoxis qPCR (Table 3). Therefore, the Biotoxis qPCR test's sensitivity could be evaluated at 90.32% and 96.55% compared to ISFtu2 and type B qPCR tests, respectively. The C_T ranged from 20.47 to 36.61 for ISFtu2 qPCR (with 30/31 tests with a C_T of ≤ 35), 28.89 to 39.71 (20/31 tests with a C_T of ≤ 35) for type B qPCR, and 27.37 to 39.06 (15/31 tests with a C_T of ≤ 35) for Biotoxis qPCR (Table 3).

The Biotoxis qPCR was positive for 27/31 pure DNA extracts (with C_T values ranging from 27.37 to 39.06), 19/31 DNA extracts diluted 1/10 (C_T , 29.85 to 39.43), and 13/31 DNA extracts diluted 1/100 (C_T , 33.26 to 39.26). Interestingly, sample S4 was positive by ISFtu2 qPCR, type B qPCR, and Biotoxis qPCR only when diluted 1/100, suggesting the presence of PCR inhibitors.

The 30 clinical samples from patients for which tularemia diagnosis was excluded corresponded to 14 lymph nodes, six blood samples, five abscess samples, one respiratory sample, and four biopsy samples. The Biotoxis qPCR gave negative results for these 30 clinical samples, including the eight patients with bartonellosis, corresponding to a 100% specificity.

Environmental samples. Water samples artificially inoculated with *F. tularensis* subsp. *holarctica* LVS NCTC 10857 from 0.1 to 10,000 CFU/liter were analyzed by Biotoxis qPCR and by ISFtu2, Tul4, and type B qPCRs. Biotoxis qPCR detected *F. tularensis* from 1,000 CFU/liter (with a C_T at 34.47). It was less sensitive than type B qPCR (positive from 100 CFU/liter with a C_T at 38.23), Tul4 qPCR (positive from 10 CFU/liter with a C_T at 36.96), and ISFtu2 qPCR (positive from 0.1 CFU/liter with a C_T at 37.57).

The 57 environmental water samples collected in France were previously tested using the ISFtu2, Tul4, and type B qPCR tests. Fifteen were positive by ISFtu2 qPCR, nine were positive by both ISFtu2 qPCR and Tul4 qPCR, and four were positive by ISFtu2 qPCR, Tul4 qPCR, and type B qPCRs. The Biotoxis qPCR was positive for six (40%) of the 15 samples that were positive by ISFtu2 qPCR, five of the nine samples that were positive by both ISFtu2 and Tul4 qPCR, and the four samples that were positive by

TABLE 4 Biotoxis qPCR testing of 57 environmental water samples

Result of qPCR				No. of water samples
ISFtu2	Tul4	Type B	Biotoxis (C_T)	
Positive	Positive	Positive	Positive (35.52–39.53)	4
Positive	Positive	Negative	Positive (38.23)	1
Positive	Negative	Negative	Positive (39.58)	1
Positive	Positive	Negative	Negative	4
Positive	Negative	Negative	Negative	5
Negative	Negative	Negative	Negative	42

ISFtu2, Tul4, and type B qPCRs (Table 4). The Biotoxis qPCR was negative for all 42 samples that were negative by the ISFtu2, Tul4, and type B qPCRs (Table 4).

DISCUSSION

In this study, we evaluated the commercial Biotoxis qPCR detection kit's performances for the detection of *F. tularensis* in clinical and water samples, compared to previously validated qPCR tests recommended by the World Health Organization for tularemia diagnosis (11).

We first evaluated this kit's ability to detect clinical and reference strains of *F. tularensis* previously identified in our laboratory. The 86 clinical strains and the reference LVS strain of *F. tularensis* subsp. *holarctica* gave strong amplification signals with the Biotoxis qPCR (C_T between 15 and 22 for a DNA extract standardized at 10 ng/ μ l). Similar C_T values were obtained with the qPCR tests targeting the single-copy Tul4 or type B-specific DNA sequences. In contrast, lower C_T values were obtained with the multicopy ISFtu2 target (26 to 30 copies in *F. tularensis* subsp. *holarctica* genome [7]) (Table 2). To evaluate the specificity of the Biotoxis qPCR, we tested two *F. philomiragia* strains, one *F. novicida* strain, and 24 strains not belonging to *Francisella* species. The Biotoxis qPCR gave a strongly positive signal for the reference strain of *F. novicida*. This result was not unexpected, since *F. novicida* (also referred to as *F. tularensis* subsp. *novicida*) has $\geq 97.7\%$ similarity at the genome level to *F. tularensis* (12). The same cross-amplification has been described for the ISFtu2 and Tul4 qPCR tests (7). Differentiating these two closely related microorganisms using a qPCR test remains highly challenging. However, such cross-amplification currently has little impact on tularemia diagnosis, because human infections with *F. novicida* are rare and associated with clinical and epidemiological contexts different from those of tularemia (4). Additional diagnostic tests are usually performed for tularemia diagnostic confirmation and differentiation of type A and type B infections (11). Because *F. novicida* is an aquatic bacterium, the situation is different when environmental water samples are tested. In this case, the Biotoxis qPCR test is not sufficiently discriminating. A more specific test must be performed for accurate differentiation between *F. novicida* and *F. tularensis*. Therefore, in the context of bioterrorism, the Biotoxis qPCR kit is currently not specific enough for rapid and accurate confirmation of the presence of *F. tularensis* in environmental samples.

The Biotoxis qPCR was negative for *F. philomiragia*, confirming the absence of cross-amplification of this other aquatic bacterium. The Biotoxis qPCR was also negative for 21 non-*Francisella* species tested, representing common human pathogens. When testing high DNA concentrations (10 ng/ μ l), we observed a weak qPCR signal ($C_T \geq 34$) for the *S. salivarius*, *P. aeruginosa*, and *N. elongata* strains. Using lower DNA concentrations could eliminate such nonspecific signals. It should be recommended that DNA concentrations of 0.1 ng/ μ l be used when DNA extracts from bacterial strains are being tested, to avoid nonspecific amplification.

We did not include *F. tularensis* subsp. *tularensis* (type A) strains because we do not possess such strains in the French NRCF. The genes classically used to target *F. tularensis* (i.e., the ISFtu2 element, the 23-kDa-protein gene, *fopA*, and *tu14*) detect all four *F. tularensis* subspecies (7). Although the identity of the Biotoxis qPCR detection kit's targeted genes is proprietary, the manufacturer validated during the development

process that this test can detect both type A and type B strains. This kit's performance for type A strains in different sample types will have to be checked by further studies.

As for clinical specimens, we tested 31 samples previously collected from 30 tularemia patients. At the time of diagnosis, all samples were positive for the ISFtu2 qPCR (C_T range of 20.47 to 36.61) and 29/31 for type B qPCR (C_T range of 28.89 to 39.71). The Biotoxis qPCR was positive for 28/31 clinical samples, with C_T values ranging from 27.37 to 39.06 (Table 3). Therefore, the Biotoxis qPCR test's sensitivity could be evaluated at 90.32% and 96.55% compared to ISFtu2 and type B qPCR tests, respectively. The *F. tularensis* subsp. *holarctica* genome contains 26 to 30 copies of ISFtu2 (7), explaining the higher sensitivity of the ISFtu2 qPCR compared to the Tul4 and type B qPCR tests, which target a single DNA copy. However, the ISFtu2 insertion sequence is also found in the genomes of other *Francisella* species, including *F. novicida* and *F. philomiragia* (7). The Biotoxis qPCR displayed sensitivity similar to that of the type B qPCR. C_T values higher than 35 were found for 9/29 samples for type B qPCR and 13/28 for Biotoxis qPCR. Such high C_T values likely reflected the low *F. tularensis* inoculum and the presence of PCR inhibitors in the tested clinical samples. However, for the Biotoxis qPCR test, no C_T is specified by the manufacturer. Overall, this test's sensitivity could be considered very satisfactory, taking into account its multiplex nature. In addition, clinical samples were stored at -80°C for 1 to 2 years before Biotoxis qPCR testing, which could have slightly altered DNA quality. Regarding specificity, no amplification was observed for the 30 *F. tularensis*-free clinical specimens with this kit.

When testing water samples artificially contaminated with *F. tularensis* subsp. *holarctica*, we found LODs of 1,000 CFU/liter for the Biotoxis qPCR, 100 CFU/liter for type B qPCR, 10 CFU/liter for Tul4 qPCR, and 0.1 CFU/liter for ISFtu2 qPCR. Here again, the lower LOD of ISFtu2 qPCR could be explained by this target's multicopy nature (7). The higher LOD of the Biotoxis qPCR compared to those of the Tul4 and type B qPCR assays could be related to the multiplex nature of this commercial test. Multiplexed qPCR tests usually display lower analytical sensitivities than their simplex counterparts do (13).

We then tested 57 environmental water samples collected from natural aquatic environments. The Biotoxis qPCR was positive for 6/15 samples that were positive by ISFtu2 qPCR, 5/9 Tul4 qPCR-positive samples, and 4/4 type B qPCR-positive samples (Table 4). As expected, the Biotoxis qPCR did not detect some samples that were positive in the ISFtu2 and Tul4 qPCR tests. However, only samples positive in the highly specific type B qPCR test could be considered genuinely contaminated with *F. tularensis* (type B strain). The same samples tested positive with the Biotoxis qPCR test. This result likely indicates the higher specificity of these two tests to detect *F. tularensis* DNA in aquatic environments. It should be noted that high C_T values were found for most qPCR-positive water samples, reflecting low bacterial loads, as previously reported in other environmental studies (14). A much higher bacterial load would be expected in a bioterrorist attack context. It would have been interesting to culture these environmental water samples to correlate PCR and culture results. However, we did not try to culture them, since it would be tedious because of (i) the fastidious nature of *F. tularensis* and (ii) the high quantity of contaminant bacteria in these samples.

The Biotoxis qPCR detection kit is designed to perform multiple tests in a 96-well-plate format. Unlike other commercial products, such as the BioFire FilmArray Biothreat panel, it is not suitable for unit sample analysis. Consequently, the Biotoxis qPCR detection kit appears to be more appropriate for testing many biological or environmental samples at a moderate cost. In our hands, this kit displayed equivalent specificity but a slightly lower sensitivity compared to *F. tularensis* reference qPCR assays. However, the purpose of the Biotoxis kit is to simultaneously detect the presence of *F. tularensis*, *Y. pestis*, and *B. anthracis* in a bioterrorism context. It is a ready-to-use kit that can be handled in all laboratories equipped with a qPCR apparatus, without need for specific expertise. We did not evaluate the Biotoxis qPCR detection kit's performances for the detection of *B. anthracis* and *Y. pestis*. The kit was validated for all three pathogens by the manufacturer during the development process. However, further studies will be needed to assess the sensitivity and specificity of the Biotoxis kit for

detection of *Y. pestis* and *B. anthracis*. Consequently, our study does not enable us to state the usefulness of the Biotoxis qPCR kit in the context of bioterrorism. Apart from the investigation of a suspected bioterrorist attack, we believe that this test could be useful for rapid exploration of the potential sources of human infections during natural tularemia outbreaks.

In conclusion, the Biotoxis qPCR detection kit displayed good performance for detecting *F. tularensis* in clinical specimens and environmental water samples. However, the cross-amplification of *F. novicida* should be taken into account. This kit was easy to use, and results were available within 60 min. It can be useful for the rapid detection of *F. tularensis* DNA in many clinical samples, especially in the context of a tularemia outbreak. It can also be used to detect *F. tularensis* in a large number of environmental samples. The Biotoxis qPCR detection kit was designed primarily for simultaneous detection of *F. tularensis*, *B. anthracis*, and *Y. pestis* in a bioterrorism context. Further studies are needed to assess the performance of this kit for detection of the two latter pathogens.

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We declare that we have no conflicts of interest.

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CONCLUSION ET PERSPECTIVES

La tularémie est une maladie infectieuse peu fréquente mais potentiellement grave. Ajouté à cela, la bactérie *F. tularensis* est catégorisée comme un agent potentiel de bioterrorisme. Cela impose de parfaitement maîtriser les connaissances relatives à cet agent infectieux en termes d'épidémiologie, de physiopathologie, de diagnostic et de traitement. L'objectif de ma thèse portait essentiellement sur l'amélioration des connaissances concernant l'épidémiologie de *F. tularensis*. Cette bactérie présente de multiples réservoirs. Les réservoirs animaux et arthropodes sont relativement bien caractérisés. Un réservoir moins étudié est le réservoir aquatique. Ainsi, le but de ma thèse était d'étoffer les connaissances concernant les cas de tularémie contractés à partir du réservoir aquatique et de préciser les modalités de survie de *F. tularensis* dans l'eau, en particulier au sein du réservoir ambien. Ce travail de thèse s'inscrivait dans le cadre d'un projet de recherche plus vaste, le projet TULAMIBE, financé par l'ANR ASTRID, qui avait pour objectif l'évaluation des conditions et des mécanismes de survie prolongée de *F. tularensis* dans l'environnement et la proposition de contre-mesures.

J'ai commencé mon travail de thèse par un exercice bibliographique en réalisant une revue de la littérature à propos du lien entre *F. tularensis* et environnement aquatique. Ceci m'a permis de mettre en évidence, dans une première partie de la revue, les modes de contamination par *F. tularensis* à partir du réservoir hydrique. Ils peuvent être classés en trois grandes catégories : ingestion d'eau contaminée, piqûre de moustique et activités récréatives en milieu aquatique, telles que la pêche, la nage et le canyoning. La précision des modes d'acquisition possibles de la tularémie à partir du réservoir aquatique pourra notamment permettre de systématiser les connaissances et de faire plus facilement évoquer une tularémie à un clinicien confronté à une symptomatologie pouvant évoquer la tularémie dans un contexte d'exposition ne relevant pas des habituels contacts avec des animaux ou morsures de tiques.

Une autre partie de ce travail de revue a visé à synthétiser les connaissances actuelles concernant les modes de survie de *F. tularensis* dans l'eau. Les expérimentations ont démontré que la bactérie était capable de survivre dans l'eau sous forme planctonique pendant plusieurs mois. Certains travaux décrivent une capacité d'évolution de la bactérie dans l'eau vers une forme viable mais non cultivable (VBNC). *Francisella tularensis* a également été démontrée capable de former du biofilm, ce qui pourrait participer à sa survie

en milieu aquatique. D'autres travaux décrivent que l'association aux amibes pourrait participer à la survie aquatique de *F. tularensis*. Enfin, l'infection des larves de moustiques pourrait également constituer un mode de survie dans l'eau de la bactérie. Cette synthèse des connaissances concernant les modes de survie de *F. tularensis* dans l'eau a permis de mettre en évidence que les données actuelles étaient peu nombreuses et souvent contradictoires. Ceci nous a permis de mettre en évidence des axes de recherches à développer dans le cadre du projet TULAMIBE : la survie par interaction avec les amibes, objet de mon travail de thèse fondamentale ; la survie par une évolution dans l'eau vers une forme VBNC, travail de thèse d'une autre doctorante du laboratoire, Camille Brunet, et afin, la survie dans les biofilms, étudiée par une autre chercheuse du laboratoire, Patricia Renesto.

Ainsi, la deuxième partie de mon travail de thèse a consisté en un versant expérimental fondamental, ayant pour objectif de définir si les amibes participaient à la survie aquatique de *F. tularensis* et par quels mécanismes.

Pour ce faire, j'ai établi successivement plusieurs modèles d'interactions entre les amibes du genre *Acanthamoeba* et plusieurs souches de *Francisella*, dont une souche clinique virulente de *F. tularensis* subsp. *holarctica*.

En modèle Amoeba Plate Test (APT), j'ai pu déterminer que *F. tularensis* était capable de croître sur une gélose recouverte d'un tapis amibien, attestant de la résistance de cette bactérie aux amibes. Afin de préciser les interactions entre *F. tularensis* et les amibes, j'ai réalisé des modèles d'infection de tapis amibiens par la bactérie. Les infections réalisées en utilisant des milieux de culture ne permettant pas la croissance extracellulaire de *F. tularensis*, ont conclu à une absence de multiplication de cette bactérie dans les amibes et à une survie intra-amibienne de l'ordre de quelques jours. J'ai poursuivi mes expérimentations par des modèles de co-culture d'amibes et de bactéries afin de déterminer si les amibes pouvaient favoriser la survie de *F. tularensis* que ce soit via des interactions intra ou extracellulaires. Il s'est ainsi avéré que lorsque *F. tularensis* était cultivée dans un milieu de culture contenant des amibes et en n'autorisant pas de contact direct entre les amibes et les bactéries, *F. tularensis* était capable de se multiplier alors que la bactérie seule dans le milieu de culture décroissait. L'hypothèse évoquée serait que *F. tularensis* bénéficie de composés sécrétés par les amibes qui favorisent sa croissance.

Ce travail expérimental a permis de préciser les relations entre *F. tularensis* et les amibes. Selon mes résultats, *F. tularensis* ne se multiplie pas à l'intérieur des amibes mais sa survie est favorisée par la présence de ces protozoaires. Ainsi les amibes pourraient participer à la survie de *F. tularensis* dans l'environnement aquatique. Néanmoins, les données de la littérature concernant l'interaction des amibes avec *F. tularensis* restent contradictoires. Il serait pertinent de poursuivre les expérimentations :

- en incluant un plus grand nombre de souches cliniques virulentes de *F. tularensis* subsp. *tularensis* et subsp. *holarctica*. En effet, les études réalisées jusqu'alors ont principalement utilisé des espèces de *Francisella* non *tularensis* ou la souche vaccinale atténuée LVS de *F. tularensis*.
- en utilisant des milieux de culture ne permettant pas la croissance extra-amibienne de *F. tularensis*, ce qui peut biaiser l'interprétation des résultats, voire en utilisant de l'eau environnementale pour être au plus proche des conditions naturelles.

De plus, les amibes ne sont assurément pas les uniques vecteurs de la survie environnementale de *F. tularensis*. Ainsi, la capacité des *F. tularensis* à former et à survivre dans les biofilms et la capacité de survie sous forme planctonique, potentiellement sous forme VBNC doivent également être plus précisément investiguées. Ces travaux sont en cours dans notre laboratoire.

Enfin, la mise en évidence de la capacité de survie prolongée de *F. tularensis* dans l'eau est particulièrement inquiétante dans le contexte du bioterrorisme. Ainsi, une perspective serait de développer des contre-mesures pouvant permettre de réduire la contamination d'un environnement aquatique par cet agent infectieux. Ainsi, mes travaux de recherche à venir porteront sur l'étude des moyens utilisables pour décontaminer un environnement aquatique contaminé. J'envisage notamment d'étudier l'effet des biocides, des rayonnements ultra-violet, de l'ozone et de la filtration.

Mon travail de revue m'a permis de mettre en évidence que *F. tularensis* semble capable de survie prolongée dans le milieu aquatique. Plusieurs études ont pu retrouver la bactérie, ou plus souvent son ADN, dans des prélèvements d'eau environnementale. Aucune étude de ce type n'avait jusqu'alors été réalisée en France. Ainsi, dans une troisième partie de mon travail de thèse j'ai conduit une étude de terrain visant à évaluer la prévalence de la

contamination par *F. tularensis* des eaux de surfaces dans un territoire français ayant enregistré un grand nombre de cas de tularémie.

Deux campagnes de prélèvements ont été réalisées dans l'Ouest dans la France, en juillet 2019 et janvier 2020, permettant la collection de 87 échantillons d'eau environnementale de diverses natures (eaux de mer, rivières, lacs, ruisseaux...). Ces échantillons d'eau ont été analysés par un set de trois qPCR permettant respectivement la détection de *Francisella* sp. (qPCR-ISFtu2), *F. tularensis* et *F. novicida* (qPCR-Tul4) ou *F. tularensis* subsp. *holarctica* (qPCR-Type B). Sept pour cent (4/57) des échantillons d'eau étaient positifs pour *F. tularensis* subsp. *holarctica* lors de la première campagne de prélèvement et 13,3 % (4/30) lors de la seconde campagne. Un site a été retrouvé contaminé par l'ADN de cette bactérie lors des deux campagnes. Ces résultats, traduisent, comme ceux de la littérature, que la contamination des eaux de surfaces par *F. tularensis* est possible et relativement fréquente, démontrant une fois de plus que l'environnement aquatique occupe une place majeure dans le cycle de vie de *F. tularensis*. Cependant la détection de l'ADN de *F. tularensis* dans l'environnement ne permet pas d'affirmer que ce dernier est la cause de l'augmentation des cas de tularémie dans l'Ouest de de le France. Une perspective de ce travail serait de génotyper les échantillons positifs pour *F. tularensis* subsp. *holarctica* afin de les comparer aux génotypes des souches isolées de patients infectés dans cette région. Au cours d'une campagne de prélèvement future, il serait également pertinent d'essayer de cultiver les souches de *F. tularensis*, en utilisant des milieux de culture sélectifs.

Je suis pharmacien biologiste médical, j'ai réalisé cette thèse en parallèle d'une activité de biologiste au laboratoire de Bactériologie du CHU Grenoble Alpes. Ainsi, j'ai pu participer à des activités de recherche appliquée dans le cadre des missions du CNR de la tularémie, basé au CHU Grenoble Alpes. Les deux travaux suivants sont les résultats de cette activité de recherche appliquée.

J'ai participé à la caractérisation d'une souche de *Francisella* non *tularensis* isolée au CHU de Poitiers chez un patient immunodéprimé ayant présenté une bactériémie à point de départ cutané. J'ai réalisé la caractérisation biochimique et génétique de cette souche. Ceci a permis de définir qu'il s'agissait d'une souche de *F. salinarina*. Cette espèce bactérienne avait pour l'instant été isolée d'eau de mer et de poissons. Il s'agissait donc du premier cas

d'infection humaine due à cette espèce bactérienne. Bien que, chez ce patient, la source de contamination n'ait pas pu être identifiée, le lien avec mon sujet de thèse n'est pas loin, car au vu des origines primaires de *F. salinarum*, une contamination au contact du réservoir aquatique peut être fortement suspectée. Concernant ce travail, la perspective est de rédiger un second article proposant une description biochimique et génétique approfondie de la souche, en comparaison à *F. tularensis*.

Enfin, j'ai participé à l'évaluation des performances pour la détection de *F. tularensis* d'un kit commercial de PCR multiplexe, le kit Biotoxis, ciblant trois agents de bioterrorisme : *Y. pestis*, *B. anthracis* et *F. tularensis*. L'évaluation des performances de ce kit a été réalisée sur souches bactériennes, sur prélèvements biologiques et sur prélèvements environnementaux. Le kit a démontré de bonnes sensibilité et spécificité avec néanmoins la détection croisée de *F. novicida* qui doit être connue et prise en compte lors de l'analyse de prélèvements environnementaux. Ici encore, le lien avec mon sujet de thèse est présent, puisque ce kit est destiné en priorité à l'analyse de prélèvements cliniques ou environnementaux dans un contexte d'attaque bioterroriste.

En conclusion, ce travail de thèse a permis de préciser les modes de contamination par *F. tularensis* à partir de l'environnement aquatique ainsi que les mécanismes de survie de cette bactérie dans l'environnement hydrique, en particulier par interaction avec les amibes. Les données relatives au rôle des biofilms et des VBNC dans la survie aquatique de *F. tularensis* sont en cours d'acquisition et/ou déjà publiées. L'ensemble de ces résultats permettra d'actualiser le cycle de vie de *F. tularensis* en plaçant l'environnement hydrotellurique au cœur de ce dernier.

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