β-LACTAMASES IN *FRANCISELLA NOVICIDA*

by

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By

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Bachelor of Science
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Dedication

I dedicate this thesis to my family: my parents, two older brothers, two sisters-in-law, and my dog Rosie.

Acknowledgements

I would like to thank my family. They have been so supportive of me while I have been taking classes for my master's degree. I am grateful that my family is still willing to listen to me talk about my research, even if they do not understand a word of it. I would also like to thank my fellow lab mates, as they have helped teach me techniques in the laboratory and I have been able to bounce ideas off them. Lastly, I would like to thank Dr. van Hoek, Dr. Froelich, and Dr. Hakami in my committee who have been of invaluable help by giving me feedback in my research.

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Abstract

β-LACTAMASES IN *FRANCISELLA NOVICIDA*

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The bacterium *Francisella tularensis* is a versatile bacterium. It is able to infect multiple hosts through multiple transmission routes, including the bite of an arthropod, ingestion, inhalation, and contact through the eyes, causing tularemia. In the 1950s, it was developed as a biowarfare agent as it can cause infection with a low dosage of bacterial cells within the host. *F. tularensis* is resistant to penicillins and there is a possibility that it will acquire resistance to the current antibiotics used to treat tularemia. While studying the bacterium's resistance to penicillin, researchers have discovered that *F. tularensis* produces β-lactamases to hydrolyze the drug before it can destroy the bacterial cell wall. *F. tularensis* has two β-lactamase genes in its genome: FTT_0681c (bla1) and FTT_0611c (bla2). However, *F. tularensis* is a biosafety level 3 (BSL-3) organism, so it requires special resources and training of the researchers to study this bacterium. *Francisella novicida* U112 has homologous genes to *F. tularensis* Schu S4 (FTN_1002 and FTT_0681c (bla1), FTN_1072 and FTT_0611c (bla2), and FTN_1227 and

FTT_0783), so it could be used as a model for the BSL-3 organism. Understanding more about the β-lactamase enzyme produced by *F. novicida* will allow for a greater understanding of the molecular mechanisms of *F. tularensis*' antibiotic resistance and whether it can express carbapenemase activity. Increasing knowledge of this bacterium allows researchers to prepare for alternative treatment options if *F. tularensis* becomes resistant to the current antibiotic treatments.

Chapter 1: Introduction

1.1 Background on *Francisella tularensis*

One of the most infectious organisms in the world is the bacterium *Francisella tularensis*. This organism is the cause of tularemia, also known as "rabbit fever". It can infect a wide range of hosts, such as amphibians, birds, and mammals [1], with rabbits being the preferred host. *F. tularensis* mostly travels between hosts through the bite of a mosquito or tick into the blood stream, causing the host to develop ulcers at the site of transmission. Other modes of transmission are through ingestion, inhalation, eyes, etc. Inhalation is the most dangerous mode of transmission, as it can cause a mortality rate of 30-60% with 50 or less organisms if left untreated [2,3].

After an incubation time of 3 to 6 days, a human will experience flu-like symptoms, pneumoniae, delirium or shock if *F. tularensis* was inhaled [4]. This bacterium is phagocytized through host immune cells (such as macrophages, dendritic cells, and neutrophils) to travel to lymphoid tissues, and then to host organs (especially the lungs, spleen, or liver) [4,5]. Proliferation within the lungs can cause respiratory tularemia regardless of initial mode of transmission, and proliferation in systemic organs can cause host cell damage, increased secretion of cytokines, and death by "cytokine storm" [3,5]. Host immune response to *F. tularensis* is slow and weak, and changes depending on the

mode of transmission [5,6]. Luckily, *F. tularensis* has not been reported to have a person-to-person mode of transmission.

1.11 *Francisella tularensis* as a biowarfare agent

Francisella tularensis was discovered in 1911 in Tulare County, California and studied extensively by Edward Francis, after both of which the organism was named. The subspecies *tularensis, novicida*, *philomiragia*, *mediastica* and *holarctica* were discovered and classified later [4]. *F. tularensis* was produced as a biowarfare agent by the Russian and American offensive biological weapons programs in the 1950s. *Francisella tularensis* does not have an FDA approved vaccine and is a Biosafety level 3 (BSL-3) organism, so it requires specialized containment facilities to study this organism. The anthrax attacks into 2001 renewed interest in *Francisella tularensis* as a biological weapon. Research continues to be conducted to understand *F. tularensis* and develop new vaccines and therapeutics in case it is used in a biological weapons attack [7].

Francisella novicida is classified as a BSL-2 organism as it rarely causes disease in humans and is only associated with water and soil, so it is used as a substitute to study *Francisella tularensis* as it has homologous genes with the fully human virulent strain [1].

1.12 Francisella tularensis and antibiotics

Since its discovery, several antibiotics have been shown to combat the effects of tularemia infection. Aminoglycosides were the first treatment discovered to be effective against *F. tularensis* in the 1940s, which are effective in killing the bacteria by inhibiting protein synthesis [8,9]. However, both the streptomycin and gentamicin aminoglycosides can cause side effects with prolonged administration, and streptomycin is an intramuscular injection, which can be painful [10,11]. The next type of antibiotic found to be able to treat tularemia were the the bacteriostatic tetracyclines which inhibit the synthesis of proteins [9,12]. However, *F. tularensis* is able to become resistant against these antibiotics and this can cause a relapse of tularemia in the patient if the antibiotic is used for less than 14 days [13]. The most recent class of antibiotic found to be useful are the bactericidal fluoroquinolones, which were very effective intracellularly by inhibiting nucleic acid synthesis via DNA Gyrase inhibition. All of these antibiotics are still used in treatment today with the aminoglycosides used for severe cases, and quinolones and tetracyclines used for mild to moderate cases [14]. **Table 1** below shows the effectiveness of these antibiotics listed as Minimum Inhibitory Concentration (MIC) results from previously published research. The low numbers indicate that the *Francisella* family is not resistant to these antibiotics. Treatments using penicillins do not work against *F. tularensis*, as it is naturally resistant against penicillin [9].

Table 1. Minimum Inhibitory Concentration (MICs) in the *Francisella* family. The MIC indicates the lowest concentration of antibiotic (ug/ml) is needed to inhibit bacterial growth. The lower numbers indicate that only a small amount of each drug is needed to prevent *Francisella* from growing. The *Francisella* family does not have resistance to these antibiotics. Information obtained from [14-17].

Antibiotics	**MIC data (µg/ml)**						
	F. tularensis	*F. holarctica* (multiple strains)	*F. holarctica LVS*	*F. hispaniensis*	*F. mediasiatica*	*F. novicida*	*F. philomiragia*
Gentamycin	≤0.25-0.25	<0.03-16	0.06	≤0.25	≤0.25	≤0.25	≤0.25
Streptomycin	≤2	≤2-4		≤2	≤2	≤2	≤2
Levofloxacin	0.031-0.125	0.016-0.125	0.016	≤0.031	≤ 0.031-0.063	0.063	≤0.031-0.063
Tetracycline	≤ 0.125-0.5	0.125-8		2	0.25-2	1-2	1-4

Recently, *Francisella* strains have been tested for their susceptibility to various carbapenem antibiotics, especially imipenem and meropenem. **Table 2** below summarizes all the reports testing carbapenem resistance in different *Francisella* strains and samples. The data shows that *Francisella* has mixed resistance to carbapenem antibiotics, as there is a wide range in the MIC values.

Table 2. Minimum Inhibitory Concentrations (MICs) against the *Francisella* family with monobactam and carbapenem antibiotics. The wide range in numbers indicates that there are inconclusive results of the antibiotics on the various *Francisella* strains. Information obtained from [9, 14-23]

Antibiotics	MIC data µg/ml				
	F. tularensis	*F. holarctica* (multiple strains)	*F. holarctica LVS*	*F. holarctica biovar japonica*	*F. philomiragia*
Aztreonam	0.5 to >256	4-32	0.063	0.75 to >256	4 to 32
Imipenem	0.047 to >64	16 to >128	0.25		≤0.25 to 2
Meropenem	0.094 to >64	1 to >128	0.031- >32		≤0.25
Ertapenem	≤0.06 to >64	2-16	≤0.008		≤0.25 to 1
Doripenem			0.031		≤0.25 to 1
Faropenem	0.12 to 16				

1.2 Beta-lactams

Penicillin-binding proteins (PBP)s are enzymes that help create the cell wall of a bacterium for its survival [24,25]. The first beta-lactam antibiotic penicillin prevents the PBP from properly forming the cell wall of the bacterium, resulting in an unstable cell wall and bacterial death. The beta-lactam structure is a ring that consists of three carbons and a nitrogen double-

bonded to an oxygen, as shown in red in **Figure 1** below [9,26]. Carbapenems are a category of beta-lactam antibiotics that are considered broad-spectrum antibiotics that can kill both gram-positive and gram-negative bacteria [27].

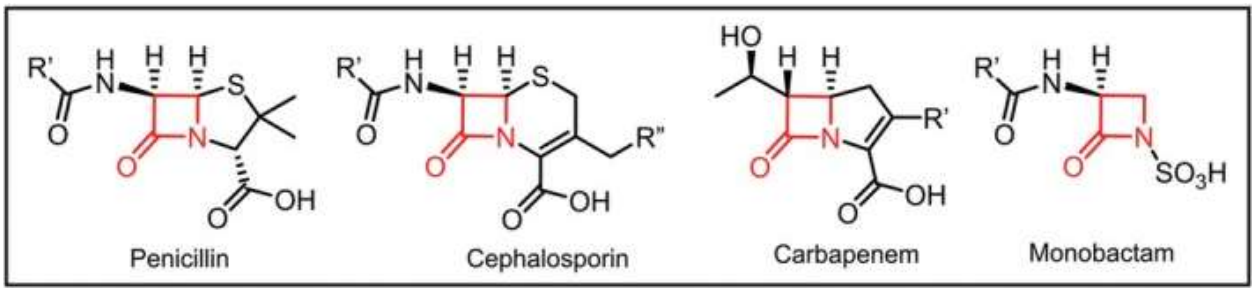

Figure 1. The four categories of beta-lactam antibiotics (Figure obtained from 26). The beta-lactam ring is shown in red. Penicillin is the first beta-lactam antibiotic discovered and the other three (cephalosporin, carbapenem, and monobactam) are specific types of beta-lactam antibiotics.

1.3 Beta-lactamases

Bacterial resistance to penicillins and other beta-lactams (such as carbapenems) is caused by the production of beta-lactamase enzymes (and carbapenemase enzymes). Beta-lactamases are enzymes produced by bacteria to become resistant against both environmental and clinical beta-lactams [28]. Beta-lactamases are classified using Ambler's molecular classifying system, in which they are divided into four categories based on their amino acid sequence (Class A, B, C, and D) and the antibiotics that they are resistant against [29]. Class A, C, and D have a serine group at the active site that, when activated, hydrolyzes the beta-lactam antibiotics [30]. Class B beta-lactamases, also called

metallo-beta-lactamases (MBLs), use zinc to inactivate the beta-lactam antibiotics instead of a serine group as the other classes of beta-lactamases [3]. According to the Ambler classification, Class A has S-X-X-K at position 70, S-D-N at position 130, and K-T-G at position 234 in the genome sequence [31,32]. Class C contains S-X-S-K at position 64, Y-S-N at position 150, and K-T-G at position 314; Class D contains S-X-X-K at position 70, Y-G-N at position 144, and K-T-G at position 214 [31]. Carbapenemases have two cysteine residues at position 69 (which is before the serine active site at position 70) and position 238 (which is after the K-T-G motif) that form a disulfide bridge [21, 32]. Another classification of beta-lactamases are extended-spectrum beta-lactamases (ESBLs). These enzymes hydrolyze and cause resistance to third generation cephalosporin antibiotics [33].

1.31 Beta-lactamases in the *Francisella* family

Beta-lactamases are encoded by the *bla* gene. In *Francisella tularensis*, there are two genes with the annotation of *bla* on the chromosome: FTT_0681c (*bla1*) and FTT_0611c (*bla2*) [9]. *F. tularensis*, *F. philomiragia*, *F. holarctica*, and *F. novicida* have homologous genes and site of replication in the chromosome indicating a common ancestor for all the species [34]. As such, beta-lactamases have been found in other subspecies of *Francisella tularensis*: class A beta-lactamase with weak carbapenemase activity in *Francisella holarctica* FTU-1 and a class A carbapenemase in *Francisella philomiragia* FPH-1, which are 77% identical to each other [21,22]. Previous research has also identified

beta-lactamases in *F. novicida* (strains Fx1, Fx2, and U112), but has not yet confirmed the classification of beta-lactamase produced [35-38].

1.32 Beta-lactamases in *Francisella novicida*

Three genes (FTN_1002, FTN_1072, and FTN_1227) have been suggested for the location of beta-lactamase production in *Francisella novicida*. FTN_1002 has 90% protein sequence identity homology with the *F. tularensis* gene FTT_0681c (bla1), as shown in **Figure 2**, FTN_1072 has 90% protein sequence identity homology with FTT_0611c (bla2) as shown in **Figure 3**, and FTN_1227 has 90% protein sequence identity homology with FTT_0783 (annotated as having functions similar to a metallo-beta-lactamase) as shown in **Figure 4** [39]. Sequence information was obtained online [39]. In **Figure 3**, FTN_1072 and FTT_0611c (*bla2*) both have S-V-F-K at position 61 and K-T-G at position 226, showing that the classification of the β-lactamase produced is Class A. FTN_ 1072 and FTT_0611c both have cysteine residues at positions 60 and 230, which is a characteristic of all carbapenemases. However, FTT_0611c has **S-D-N** at position 122, while FTN_1072 has **S-D-S** at position 122. This discrepancy in the amino acid sequence might cause a change in the effect of the two β-lactamases. In summary, FTN_1002 (*bla1*) and FTN_1227 do not have the motifs of Class A, C, or D β-lactamase, so it is unlikely that *Francisella novicida* produces a β-lactamase of those classifications with either of those genes. However, it is likely that FTN_1072 (*bla2*) could be a Class A carbapenemase.

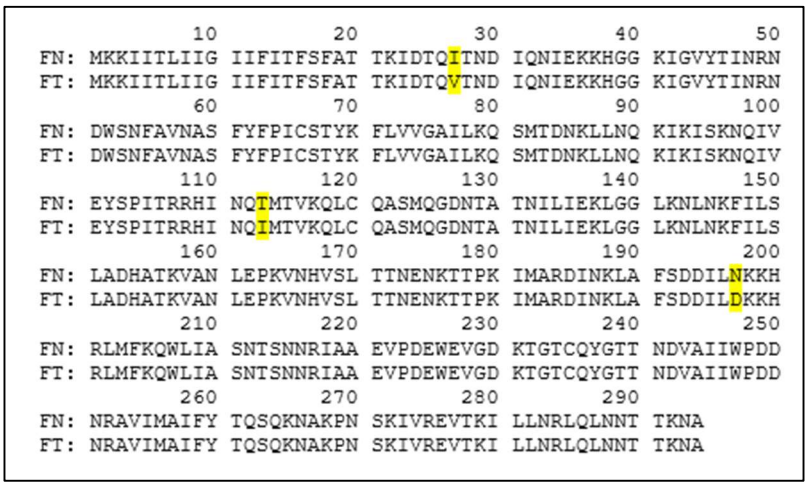

Figure 2. Sequence comparison between FTN_1002 and FTT_0681c (*bla1*). Highlighted marks are differences in the sequences. FN: *F. novicida* U112, FT: *F. tularensis* Schu S4.

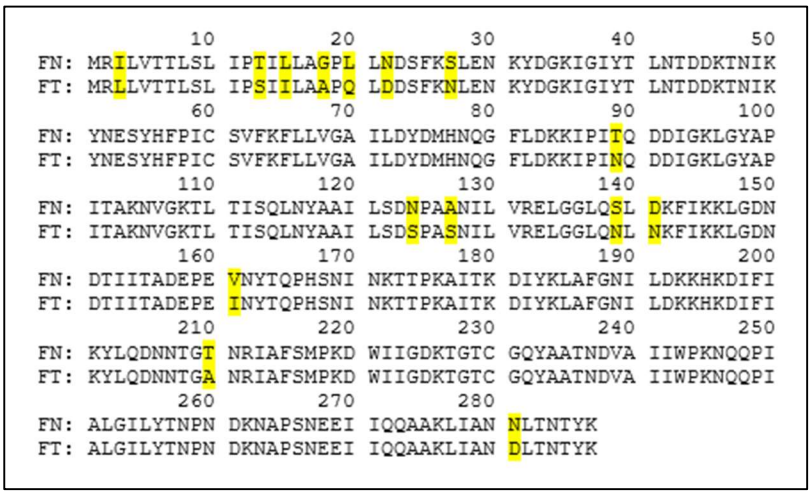

Figure 3. Sequence comparison between FTN_1072 and FTT_0611c (*bla2*). Highlighted marks are differences in the sequences. FN: *F. novicida* U112, FT: *F. tularensis* Schu S4.

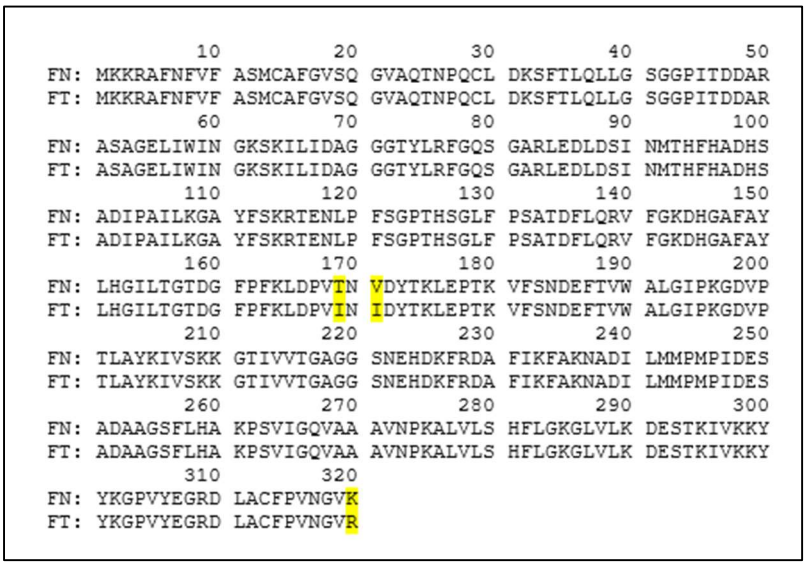

Figure 4. Sequence comparison between FTN_1227 and FTT_0783c (MBL-like gene). Highlighted marks are differences in the sequences. FN: *F. novicida* U112, FT: *F. tularensis* Schu S4.

Previous research has shown that the *F. tularensis* Schu S4 strain and its homologues produce beta-lactamases. One study found two β-lactamase genes in *F. holarctica* live-vaccine strain (LVS) that were 99% identical to *F. tularensis* genes. The FTT_0681c (*bla1*) was homologous to $bla1_{LVS}$ and FTT_0611c (*bla2*) was homologous to $bla2_{LVS}$ [21]. The results of this study showed that $bla1_{LVS}$ was a nonfunctional β-lactamase and $bla2_{LVS}$ was only effective against penicillin-class antibiotics. Therefore, it is likely that *F. novicida* will produce similar results. The *F. novicida bla1* homologue (FTN_1002) might produce a nonfunctional β-lactamase and the *bla2* homologue (FTN_1072) might produce a β-lactamase that is only effective against penicillin-class antibiotics.

1.4 Problem Statement (Hypothesis)

Francisella tularensis is a dangerous organism in the way that it can infect the host through multiple routes and with very few bacteria via aerosol. There are already antibiotics that are successful as treatment, but there is no FDA approved vaccine [23,40]. Finding out if a carbapenemase is produced will increase knowledge of what antibiotics could be effective against *F. tularensis* should it become resistant to the current antibiotics used for treatment. Since *F. novicida* is used as a model for studying *F. tularensis,* if *F. novicida* produces a carbapenemase, researchers will have the knowledge that carbapenem antibiotics are not to be used as treatment for tularemia. Classifying the β-lactamase produced by the bacterium will allow for better understanding of what antibiotics could be effective against *F. tularensis*.

Chapter 2: Materials/ Methods

2.1 Bacteria and Media

F. novicida U112 was obtained from ATCC. The three mutants of FTN_1002, two mutants of FTN_1072, and two mutants of FTN_1227 were obtained from the mutant library from ATCC with information obtained online [41]. Mutants were plated on tryptic soy agar with 0.1% cysteine (TSA-C; L-Cysteine hydrochloride monohydrate 99%; LOT number: T14D018) from the mutant library and grew overnight at 37°C. A few colonies were taken from the plate using a 10µl loop and suspended in 5ml of tryptic soy broth (TSB; Ref number: 211825; LOT number: 0287614) containing 0.1% cysteine and grew overnight in a shaking incubator at 37°C. Stocks were made by taking 0.5ml of the broth and adding 0.5ml of 40% glycerol into microcentrifuge tubes. The stocks were frozen at -80°C.

Plates for antibiotic susceptibility were made from Mueller-Hinton Agar (MHA; Ref number: 211438; LOT number: 2046738) containing 0.1% cysteine. Each plate contained 25ml of agar for a depth of 4mm. Ertapenem (Ref number: CT1761B; LOT number: 3164399), Meropenem (Ref number: CT0774B; LOT number: 3235577), Imipenem (Ref number: CT0455B; LOT number: 1865520), and Aztreonam (Ref number: CT0264B; LOT number: 1846386) antibiotic discs were obtained from Oxoid Ltd.

Doripenem (Ref number: 67348; LOT number: 64393912) antibiotic discs were obtained from Bio-Rad Laboratories.

Cation-adjusted Mueller-Hinton Broth (CAMHB; Ref number: 212322; LOT number: 1242967) was used for the minimum inhibitory concentration (MIC) tests. BBL IsoVitaleX Enrichment (Ref number: 211876; LOT number: 9035837) was added to the CAMHB and pH adjusted to 7.1 ± 0.1. Penicillin G Na Salt (CAS Number: 69-57-8) was obtained from MP Biomedicals, Aztreonam (J62887; LOT number S06C032) was obtained from Thermo Scientific, Meropenem Trihydrate (CAS number: 119478-56-7; LOT number: YCY8L-BF), Biapenem (HY-13573) was obtained from MedChemExpress, and Doripenem hydrate (Z482777) was obtained from Selleck Chem. Multiple imipenem antibiotics were used: imipenem monohydrate (744312) obtained from Fischer and imipenem monohydrate (I0160) obtained from Sigma.

2.2 Minimum Inhibitory Concentration (MIC) Test

The minimum inhibitory concentration (MIC) test is used to determine the concentration of antibiotic needed to kill or inhibit the growth of a bacterium. The CLSI standard protocol for testing *Francisella* is as follows [23]: 50µl of cation-adjusted Mueller-Hinton broth (CAMHB) containing 2% IsoVitalex (pH adjusted to 7.1 ± 0.1) was added to each well of a 96-well plate, and the antibiotic was serially diluted 2-fold in the broth. The bacterial inoculum was prepared by adding bacterial stock to TSB-C and grown

overnight at 37°C. The inoculum was then diluted to have a concentration of 1 x 10^6 CFU/ml, and 50µl was added to each well for a final inoculum of approximately 5×10^4 CFU/well (5×10^5 CFU/ml). The 96-well plate was incubated overnight at 37°C and read at 600nm at about 21 hours. Depending on the growth of the bacterium, it can be classified as sensitive (S), intermediate (I), or resistant (R) to each antibiotic [42]. Gentamicin was used as a positive control, as *F. tularensis* is susceptible to aminoglycoside antibiotics [23,40].

2.3 Antibiotic susceptibility testing

Each bacterium was plated on Chocolate Agar and left to grow overnight at 37°C. Colonies were taken from each plate and put into a TSB-C solution until the McFarland read 0.5. A lawn was streaked on Mueller Hinton Agar plates containing 0.1% Cysteine (MHA+C) using the Kirby-Bauer method, and each antibiotic disc was placed on each plate. After incubating at 37°C for 24 hours, the zones of inhibition were recorded in millimeters.

2.4 Modified Hodge Test (MHT)

The Modified Hodge Test is an experiment that tests for the presence of carbapenemases [43]. For this method, the indicator organism *E. coli* ATCC 25922 was plated on MHA+C as a lawn at a reading of 0.5 McFarland. The next step was to put an imipenem, meropenem, or ertapenem antibiotic disc in the center of the plate. The last step was to streak one line of controls (*K. pneumoniae* ATCC BAA-1705 carbapenemase positive and *K. pneumoniae* ATCC BAA-1706 carbapenemase negative) with the bacterium of interest (*F. novicida* strain U112*)* from the center where the antibiotic disc is to the edge of the plate. A cotton-tipped indicator was dipped into a solution reading of 1.5 McFarland to create the streak. The plate was incubated for 24 hours, and the zone of inhibition was examined. If the organism of interest produces a carbapenemase, then the zone of inhibition will not be completely round and will have a "clover-leaf" shape (as shown in 1 and 3 in **Figure 5** below) as the indicator organism has been allowed to grow [44]. If the organism of interest does not produce a carbapenemase, then the zone of inhibition will be round as normal (as shown in 2 in **Figure 5** below) as the indicator organism has been inhibited [44].]

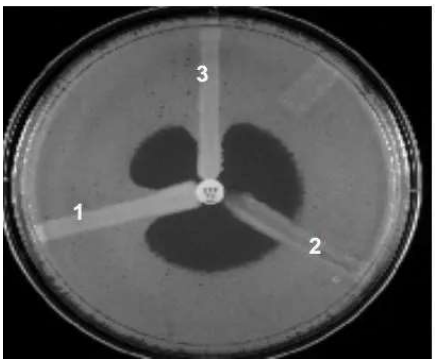

Figure 5. Sample of Modified Hodge Test. (Figure obtained from 44). 1 indicates the positive control, 2 indicates the negative control, and 3 indicates the bacterial strain tested (with positive results).

2.5 CarbaNP Test

The CarbaNP tests for the presence of a beta-lactamase through a pH indicator. When a beta-lactamase hydrolyzes the beta-lactam ring of a beta-lactam antibiotic, the pH value decreases. The CarbaNP test has two solutions: Solution A (aqueous phenol red 0.05% + SO_4Zn 10mM + 0.1% (v/v) of Triton X-100), adjusted to a pH value of 7.8 and Solution B (Solution A + 6 mg/ml imipenem) [45]. Nine microcentrifuge tubes contained 100µl of Solution A and nine microcentrifuge tubes contained 100µl of Solution B. The bacterium of interest (and controls) was plated on Chocolate Agar and grow overnight at 37°C. A 1µl loopful of the bacteria was added to each microcentrifuge tube and vortexed. The tubes were incubated at 37°C for 2 hours and were checked periodically for any color change. If *F. novicida* produces a beta-lactamase, the phenol red indicator in the Solution B will

change from red to yellow due to the decreased pH value. If *F. novicida* does not produce a beta-lactamase, the phenol red indicator in Solution B will remain red [46].

2.6 Clavulanic Acid Method

The clavulanic acid method tests for the presence of ESBLs within an organism. ESBLs are able to hydrolyze third-generation cephalosporins (such as cefotaxime and ceftazidime) and aztreonam, yet are inhibited by clavulanic acid [47]. For this method, the organism of interest (in this case *F. novicida*) was plated onto MHA+C with the Kirby-Bauer method at a 0.5 McFarland reading to achieve a lawn of the bacterium of interest. Antibiotic discs were placed on the plates 3-5 minutes after the lawn has been plated. On one plate, the antibiotic discs were cefotaxime and cefotaxime with clavulanic acid; a second plate had ceftazidime and ceftazidime with clavulanic acid. If *F. novicida* grows in the presence of the third-generation cephalosporins, but is inhibited by their clavulanic acid counterparts, then *F. novicida* produces an ESBL. If there is an increase in the zone of inhibition diameter of ≥5 mm when the clavulanic acid is used in addition to the antibiotic disc over only using the antibiotic disc, then the organism of interest produces an ESBL [48].

2.7 Modified carbapenem inactivation method (mCIM) + EDTA

The modified carbapenem inactivation method (mCIM) is a more accurate method than the MHT for identifying for the presence of carbapenemases, but it still cannot determine the type of carbapenemase that is present [49]. However, when EDTA (or dipicolinic acid) is added to the test, the EDTA can block metallo-beta-lactamase (MBL) activity by preventing the carbapenemase from binding to zinc. For this test, 1µl loopful of the bacterium of interest and controls were put into 2ml solutions of TSB-C and TSB-C + 0.5 M EDTA. Meropenem antibiotic discs were added to the solutions and incubated at 37°C for 4 hours. Before incubation was complete, a 0.5 McF of the *E. coli* ATCC 25922 indicator organism was plated as a lawn on MHA + C plates. The meropenem antibiotic discs were placed on the plates and incubated at 37°C for 24 hours. If the mCIM has a zone size of ≥19mm, then the test is negative for carbapenemase. If the zone is 6-15mm, then the test is considered positive for carbapenemase. If the mCIM + EDTA is ≥5mm than the mCIM, then the test is positive for metallo-carbapenemase production. If the mCIM + EDTA is <4 mm than the mCIM, then the test is negative for metallo-carbapenemase production.

Chapter 3: Results

3.1 Previous data

A previous student in the van Hoek lab, Cody Weisenen, has demonstrated that the MIC of *F. novicida* against the following antibiotics is as shown in **Supplementary Table A** in **Appendix A**. This Table shows that the wild-type *F. novicida* strain is resistant to all of the mono-bactam and carbapenem antibiotics used. The FTN_1072 mutants (which are homologous to FTT_0611c (*bla2*)), were shown to be regain susceptibility to the Biapenem, Doripenem and Meropenem carbapenem antibiotics used for the MIC test. This data suggests that the enzyme encoded by this gene contributes to its resistance. However, these FTN_1072 *bla2* mutants continued to be highly resistant to Imipenem and Aztreonam, so FTN_1072 did not contribute to that effect. Finally, some increased sensitivity of *F. novicida* to Biapenem, Doripenem and Meropenem carbapenem antibiotics was found to result from mutants in the other two genes FTN_1002 (bla1) and FTN_1227 (MBL), but did not bring the mutants into the "sensitive" range.

3.2 *F. novicida* and MIC test

While verifying this previous data, *F. novicida* showed no resistance to the carbapenem antibiotics. The MIC test determines the concentration of antibiotic needed (ug/ml) to inhibit the growth of the bacterium of interest. **Table 3** below shows that *F. novicida* had resistance to penicillin, intermediate resistance to Aztreonam, and was sensitive to the carbapenem antibiotics. The FTN_1002 and FTN_1227 mutants showed similar results to the wild type strain. This means that these mutants do not encode the gene for carbapenem resistance. The FTN_1072 mutant showed less resistance to penicillin and aztreonam antibiotics, but has the same result as the wild type for the carbapenem antibiotics. This means that this mutant might encode the gene for beta-lactam and mono-bactam resistance, but not for carbapenem resistance.

Table 3. Minimum Inhibitory Concentrations (MICs) of *F. novicida* U112, mutants, and controls with different antibiotics. The antibiotic concentrations are in µg/ml.

Bacterium		Antibiotic					
Gene locus	**Fn mutant tested**	**Penicillin**	**Aztreonam**	**Imipenem**	**Meropenem**	**Biapenem**	**Doripenem**
***F. novicida* U112**		>256	32	2	1	1	1
FTN_1002 blaA (*bla1*) FTT_0681c homologue	tnfn1_pw060419p02q192 tnfn1_pw060328p04q109 tnfn1_pw060328p04q112	>256 >256 >256	16 16 32	1 1 1	0.5 0.5 2	1 1 4	1 2 2
FTN_1072 bla2 FTT_0611c homologue	tnfn1_pw060323p06q181 tnfn1_pw060418p01q102	8 64	4 8	0.25 1	1 0.5	1 1	0.5 0.5
FTN_1227 MBL FTT_0783 homologue	tnfn1_pw060329p01q182 tnfn1_pw060420p02q133	>256 >256	32 32	1 1	2 2	4 2	2 2
***E. coli* ATCC 25922**		128	0.5	0.5	0.25	1	0.25
***K. pneumoniae* BAA-1705**		>256	>256	256	256	256	128
***K. pneumoniae* BAA-1706**		>256	2	32	32	64	32

3.3 *F. novicida* and Kirby Bauer

To verify the results of the MIC assays, Kirby-Bauer antibiotic susceptibility tests were performed on *F. novicida* U112 and the FTN mutants, as shown in **Table 4** below. The principle of this assay is that antibiotics in the disc will spread throughout the agar, inhibiting growth of the bacterium that is plated on top of the agar. The smaller zone diameter means that the bacterium has more resistance to the antibiotic disc, while a larger zone diameter means that the bacterium has less resistance (is susceptible) to the antibiotic disc. *F. novicida* has a smaller zone for aztreonam and a larger zone for the carbapenem antibiotics. This means that *F. novicida* has resistance to aztreonam but is susceptible to carbapenem antibiotics. The FTN mutants show the same results as *F. novicida* for the carbapenem antibiotics, with varying results for aztreonam. FTN_1072 shows the largest zone for the aztreonam antibiotic. This means that this mutant has the least resistance towards aztreonam.

Table 4. Antibiotic susceptibility test of *F. novicida*, mutants, and controls. The zones of inhibition were measured in millimeters (mm).

Bacterium		Antibiotic Discs				
Gene locus	**Fn Mutant tested**	**Aztreonam**	**Imipenem**	**Meropenem**	**Doripenem**	**Ertapenem**
***F. novicida* U112**		12.8 ± 1.6	30.1 ± 1.4	25.1 ± 2.2	28.1 ± 0.9	25.3 ± 1
FTN_1002 (*bla1*) FTT_0681c homologue	tnfn1_pw060419p02q192	11.9 ± 0.5	30.6 ± 1.4	21.9 ± 0.8	29.6 ± 0.7	23.8 ± 0.9
	tnfn1_pw060328p04q109	13.8 ± 0.8	31.8 ± 1	24 ± 1.1	32 ± 0.8	23.5 ± 1.3
	tnfn1_pw060328p04q112	15 ± 0.7	31.4 ± 1.4	24.1 ± 1.1	31 ± 0.5	24.5 ± 0.6
FTN_1072 (*bla2*) FTT_0611c homologue	tnfn1_pw060323p06q181	19.4 ± 0.5	33.7 ± 1.1	20.6 ± 1.2	30.2 ± 1.2	24.3 ± 1
	tnfn1_pw060418p01q102	20.3 ± 0.5	30.1 ± 1.6	22.5 ± 0.6	26.8 ± 1	22.8 ± 0.6
FTN_1227 FTT_0783 homologue	tnfn1_pw060329p01q182	14.7 ± 0.4	32.4 ± 1	26.2 ± 1	32.1 ± 0.9	23.4 ± 0.8
	tnfn1_pw060420p02q133	14.2 ± 0.5	32 ± 0.7	25.2 ± 0.6	31.2 ± 0.9	24.3 ± 0.6
***KP* BAA-1705 Carbapenemase Positive**		No Zone	13.2 ± 0.8	8.8 ± 0.3	16.2 ± 0.9	10.2 ± 0.8
***KP* BAA-1706 Carbapenemase Negative**		20.9 ± 0.5	18.1 ± 1.3	13.8 ± 0.6	21.1 ± 0.4	12.7 ± 0.4

The antibiotic susceptibility tests have an inverse relationship to the MIC test. The higher the MIC number, the smaller the zone of inhibition. The lower the MIC number, the larger the zone of inhibition.

3.4 *F. novicida* and the Modified Hodge Test

The Modified Hodge Test (MHT) was conducted to verify the data from the MIC and susceptibility tests as a visual representation of the results. **Figure 6** below shows two sets of plates (Chocolate Agar and MHA+C) with *F. novicida* U112 as the experimental streak. The figure shows that the positive and negative controls are functioning properly in changing the shape of the zone of inhibition (positive control creates the "clover leaf" shape; negative control has no change over the shape). The *F. novicida* streak does not show a change in the shape of the zone of inhibition for any of the carbapenem antibiotics used for the test (imipenem, meropenem, and ertapenem). This means that *F. novicida* does not rescue the *E. coli* indicator organism by hydrolyzing the carbapenem antibiotics through the production of carbapenemase.

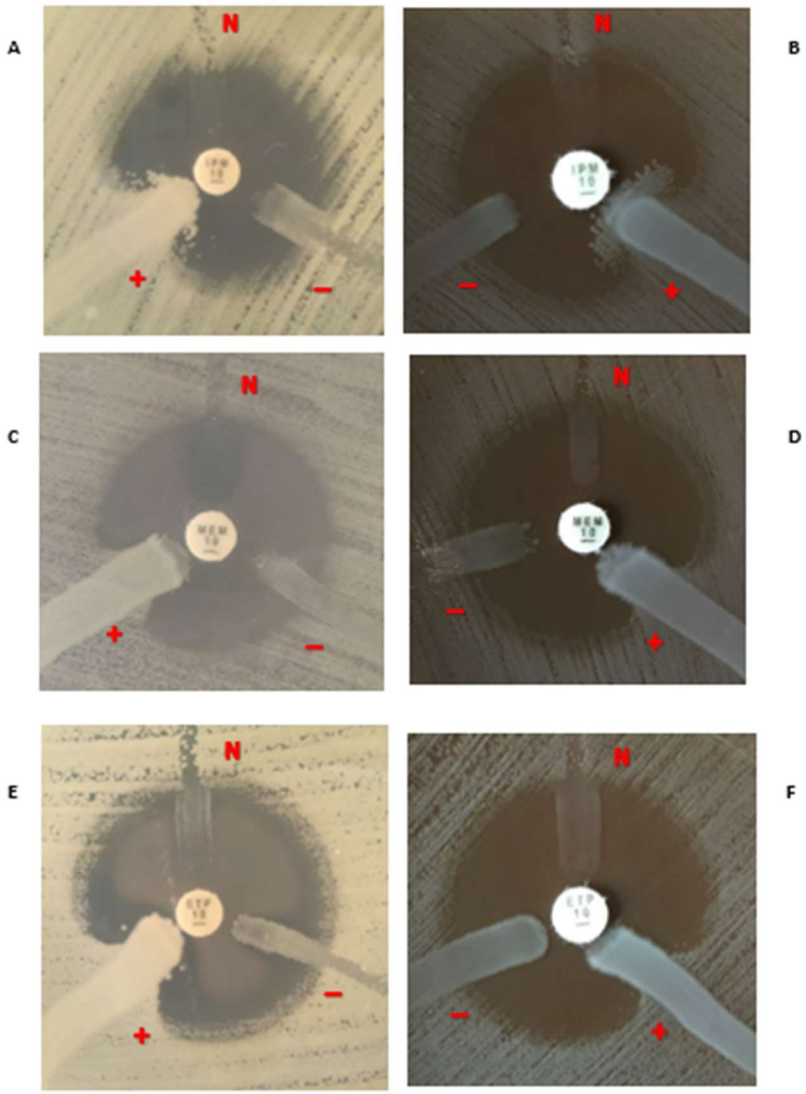

Figure 6. **Results from MHT.** Experiments A, C, and E were performed on MHA+C. B, D, and F were performed on Chocolate Agar to enhance *F. novicida* growth. A/B: IMP: Imipenem, C/D: MEM: Meropenem, E/F: ETP: Ertapenem.

N: *F. novicida* streak, +: *KP* BAA-1705 carbapenemase positive streak, -: *KP* BAA-1706 carbapenemase negative streak.

3.5 *F. novicida* and the CarbaNP test

The CarbaNP test was another test performed with visual results in the form of a color change. Hydrolysis of the imipenem antibiotic resulted in a color change from red to yellow. **Figure 7** below shows the results of the CarbaNP test. Solutions A and B were incubated for 2 hours as a quality control and had a slight color change in Solution B to a red-orange color. The positive control resulted in an obvious color change from red to yellow, while the negative control and *F. novicida* did not result in any color change. These results show that there was no hydrolysis of imipenem in either the negative control or *F. novicida*, as there was no color change from red to yellow. This means that *F. novicida* does not produce a carbapenemase to hydrolyze imipenem.

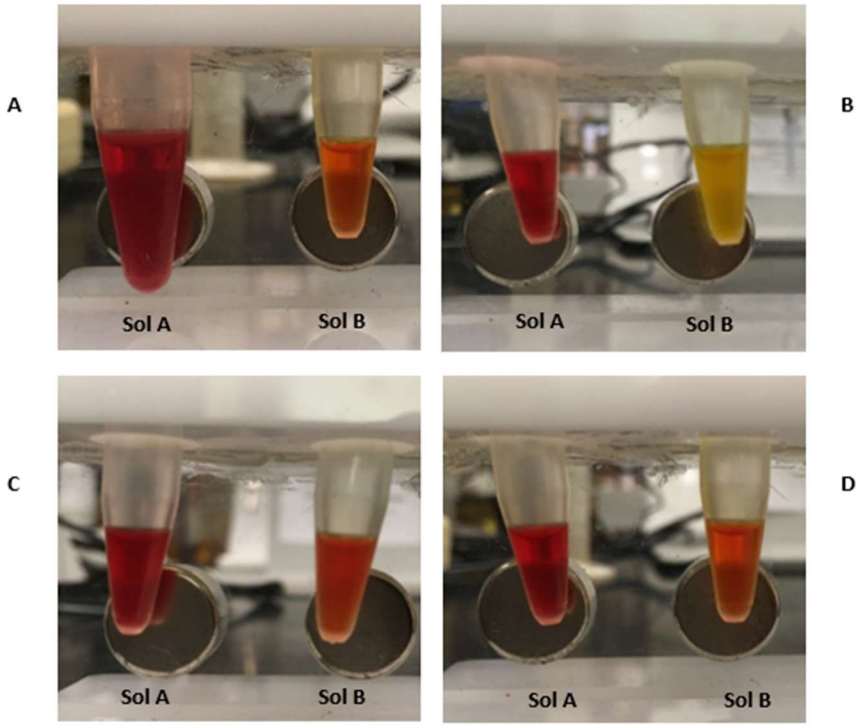

Figure 7. Results from the CarbaNP Test. Sol A: Solution A; Sol B: Solution B (contains imipenem). A: Solutions without bacteria. B: *KP* BAA-1705 carbapenemase positive control. C: *KP* BAA-1706 carbapenemase negative control. D: *F. novicida*.

3.6 *F. novicida* and the clavulanic acid method

The clavulanic acid method tests for the presence of an extended-spectrum beta-lactamase (ESBL). The zones of inhibition of cefotaxime and ceftazidime were measured with and without clavulanic acid. The results of this test are shown in **Table 5** below. There was less than 5mm difference in the zones of inhibition between cefotaxime and cefotaxime/clavulanic acid and ceftazidime and ceftazidime/clavulanic acid, so *F. novicida* does not produce an ESBL.

Table 5. Zones of Inhibition measured to determine ESBL production. The zones are measure in millimeters (mm).

Antibiotic disc	Zone of Inhibition
Cefotaxime	18 ± 5
Cefotaxime/ Clavulanic Acid	18.6 ± 3.5
Ceftazidime	26.9 ± 1.9
Ceftazidime/ Clavulanic Acid	26.7 ± 1.9

3.7 *F. novicida* and the carbapenem inactivation method

The modified carbapenem inactivation method (mCIM) tests for the presence of a metallo-beta-lactamase (MBL). The zones of inhibition of the controls and *F. novicida* were examined as shown in **Table 6** below. *K. pneumoniae* BAA-1705 showed that it was positive for carbapenemase production, as the zones of inhibition were between 6 and 15mm. There was less than 5mm between the mCIM and the mCIM with EDTA, so the positive control did not produce a MBL. *K. pneumoniae* BAA-1706 showed that it was negative for carbapenemase production, as the zones of inhibition were greater than 19mm. There was less than 5mm between the mCIM and the mCIM with EDTA, so the negative control did not produce a MBL. *F. novicida* showed that it was negative for carbapenemase production, as the zones of inhibition were about 19mm. There was less than 5mm between the mCIM and the mCIM with EDTA, so *F. novicida* did not produce a MBL.

Table 6. Results of the mCIM test. The zones of inhibition were measured in millimeters.

	mCIM	mCIM + EDTA	Difference
***K. pneumoniae* BAA-1705 (+)**	8.4 ± 0.5	8.83 ± 0.6	0.43
***K. pneumoniae* BAA-1706 (-)**	19.6 ± 0.6	20.5 ± 0.6	0.9
***F. novicida* U112**	18.6 ± 0.4	19.4 ± 1.2	0.8

Chapter 4: Discussion

The results of the MIC, Kirby-Bauer antibiotic susceptibility tests, MHT, and CarbaNP tests all show that *F. novicida* does not produce a functional Class A carbapenemase. This might be due to the FTN_1072 gene having S-D-S at position 122 in its amino acid sequence instead of S-D-N, which is included in all Class A carbapenemases.

However, the MIC and Kirby-Bauer assays show that the gene FTN_1072 produces a beta-lactamase that targets penicillin and aztreonam antibiotics. FTN_1072 might also be involved in a decrease in susceptibility for meropenem, as shown in the Kirby-Bauer assays.

Previous research has shown that the *F. holarctica* LVS homologues to *F. tularensis* Schu S4 do not have any ESBL activity [21]. Therefore, the *F. novicida* U112 strain not producing an ESBL was within expectations.

Previous research has showed that there are no metallo-β-lactamase genes that have been discovered in *Francisella tularensis*, even though FTT_0783c has been annotated as a MBL-like gene [9,39]. The *F. novicida* gene FTN_1227 is homologous with FTT_0783c, so it was expected to not produce a MBL.

Appendix A

Supplementary Table A. Minimum Inhibitory Concentrations (MICs) of *F. novicida* U112 and mutants with different antibiotics. This data was previously collected in the van Hoek lab.

Bacterium		Antibiotic				
Gene/ Locus	**Fn mutant tested**	**Aztreonam**	**Imipenem**	**Biapenem**	**Doripenem**	**Meropenem**
***F. novicida* U112**		>256	>256	>256	>256	16
FTN_1002 blaA (*bla1*) FTT_0681c homologue	tnfn1_pw060419p02q192 tnfn1_pw060328p04q109 tnfn1_pw060328p04q112	>256 >256 >256	>256 >256 N/A	256 32 32	32 64 256	128 64 256
FTN_1072 ESBL (*bla2*) FTT_0611c homologue	tnfn1_pw060323p06q181 tnfn1_pw060418p01q102	>256 >256	>256 128	8 ≤1	8 ≤1	2 ≤1
FTN_1227 MBL FTT_0783 homologue	tnfn1_pw060329p01q182 tnfn1_pw060420p02q133	>256 >256	>256 >256	128 32	128 32	256 16

Bibliography

1. Biot, Fabrice V., Beth A. Bachert, Kevin D. Mlynek, Ronald G. Toothman, Galina I. Koroleva, Sean P. Lovett, Christopher P. Klimko, et al. 2020. "Evolution of Antibiotic Resistance in Surrogates of *Francisella tularensis* (LVS and *Francisella novicida*): Effects on Biofilm Formation and Fitness." *frontiers in Microbiology* 11: 593542. doi:10.3389/fmicb.2020.593542.

2. McLendon, Molly K., Michael A. Apicella, and Lee-Ann H. Allen. 2007. "*Francisella tularensis*: Taxonomy, Genetics, and Immunopathogenesis of a Potential Agent of Biowarfare." *Annual review of microbiology* 60: 167-185. doi:10.1146/annurev.micro.60.080805.142126.

3. Jones, Bradley D., Matthew Faron, Jed A. Rasmussen, and Joshua R. Fletcher. 2014. "Uncovering the components of the *Francisella tularensis* virulence stealth strategy." *frontiers in Cellular and Infection Microbiology* 4: 32. doi:10.3389/fcimb.2014.00032.

4. Ellis, Jill, Petra C. F. Oyston, Michael Green, and Richard W. Titball. 2002. "Tularemia." *Clinical Microbiology Review* 15(4): 631-646. doi:10.1128/CMR.15.4.631-646.2002.

5. Celli, Jean, and Thomas C. Zahrt. 2013. "Mechanisms of *Francisella tularensis* Intracellular Pathogenesis." *Cold Spring Harbor Perspectives in Medicine* 3(4): a010314. doi:10.1101/cshperspect.a010314.

6. Roberts, Lydia M., Shraddha Tuladhar, Shaun P. Steele, Kristina J. Riebe, Ching-ju Chen, R. Ian Cumming, Sarah Seay, et al. 2014. "Identification of Early Interactions between Francisella and the Host." *Infection and Immunity* 82(6): 2504-2510. doi:10.1128/IAI.01654-13.

7. Oyston, Petra C. F., Anders Sjostedt, and Richard W. Titball. 2004. "Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*." *Nature Review Microbiology* 2(12): 967-78. doi:10.1038/nrmicro1045.

8. Tärnvik, Arne, and May C. Chu. 2007. "New approaches to diagnosis and therapy of tularemia." *Annals of the New York Academy of Sciences* 1105:378-404. doi:10.1196/annals.1409.017.

9. Kassinger, Stephen J., and Monique L. van Hoek. 2021. "Genetic Determinants of Antibiotic Resistance in Francisella." *frontiers in Microbiology* 12: 644855. doi:10.3389/fmicb.2021.644855.

10. Klis, S., Stienstra, Y., Phillips, R. O., Abass, K. M., Tuah, W., & van der Werf, T. S. (2014). Long Term Streptomycin Toxicity in the Treatment of Buruli Ulcer: Follow-up of Participants in the BURULICO Drug Trial. *PLoS Neglected Tropical Diseases*, 8(3): e2739. doi:10.1371/journal.pntd.0002739

11. Su, F.-Y., Chen, J., Son, H.-N., Kelly, A. M., Convertine, A. J., Ratner, D. M., & Stayton, P. S. (2018). Polymer-augmented liposomes enhancing antibiotic delivery against intracellular infections. *Biomaterials Science*, 6(7):1976-1985. doi:10.1039/c8bm00282g

12. Ikäheimo, I., H. Syrjälä, J. Karhukorpi, R. Schildt, and M. Koskela. 2000. "In vitro antibiotic susceptibility of *Francisella tularensis* isolated from humans and animals." *Journal of Antimicrobial Chemotherapy* 46(2): 287-90. doi:10.1093/jac/46.2.287.

13. Rojas-Moreno, C., Bhartee, H., Vasudevan, A., Adiga, R., & Salzer, W. (2018). Tetracyclines for Treatment of Tularemia: A Case Series. *Open Forum Infectious Diseases*, 5(9): ofy176. doi:10.1093/ofid/ofy176

14. Sutera, Vivien, Yvan Caspar, Sandrine Boisset, and Max Maurin. 2014. "A new dye uptake assay to test the activity of antibiotics against intracellular *Francisella tularensis*." *frontiers in Cellular and Infection Microbiology* 4:36. doi:10.3389/fcimb.2014.00036.

15. Caspar, Y., Hennebique, A., & Maurin, M. (2018). Antibiotic susceptibility of Francisella tularensis subsp. holarctica strains isolated from tularaemia patients in France between 2006 and 2016. *The Journal of Antimicrobial Chemotherapy*, 73(3):687-691. doi:10.1093/jac/dkx460

16. Georgi, E., Schacht, E., Scholz, H. C., & Splettstoesser, W. D. (2012). Standardized broth microdilution antimicrobial susceptibility testing of Francisella tularensis subsp. holarctica strains from Europe and rare Francisella species. *The Journal of Antimicrobial Chemotherapy*, 67(10):2429-33. doi:10.1093/jac/dks238

17. Martínez-Martínez, S., Rodríguez-Ferri, E.-F., Rodríguez-Lázaro, D., Hernández, M., Gómez-Campillo, J.-I., Del Carmen Martínez-Nistal, M., . . . Gutiérrez-Martín, C.-B. (2021). In Vitro Antimicrobial Susceptibilities of Francisella tularensis subsp. holarctica Isolates from Tularemia Outbreaks That Occurred from the End of the 20th Century to the 2020s in Spain. *Antibiotics (Basel, Switzerland)*, 10(8):938. doi:10.3390/antibiotics10080938

18. Caspar, Y., & Maurin, M. (2017). Francisella tularensis Susceptibility to Antibiotics: A Comprehensive Review of the Data Obtained In vitro and in Animal Models. *frontiers in Cellular and Infection Microbiology*, 7:122. doi:10.3389/fcimb.2017.00122

19. Chalhoub, H., Harding, S. V., Tulkens, P. M., & Bambeke, F. V. (2020). Influence of pH on the activity of finafloxacin against extracellular and intracellular Burkholderia thailandensis, Yersinia pseudotuberculosis and Francisella philomiragia and on its cellular pharmacokinetics in THP-1 monocytes. *Clinical Microbiology and Infection*, 26(9):1254.e1-1254.e8. doi:10.1016/j.cmi.2019.07.028

20. Mullen, N., Raposo, H., Gudis, P., Barker, L., Humphries, R. M., Schmitt, B. H., . . . May, M. (2017). Induction of β-Lactamase Activity and Decreased β-Lactam Susceptibility by CO2 in Clinical Bacterial Isolates. *mSphere*, 2(4):e00266-17. doi:10.1128/mSphere.00266-17

21. Antunes, Nuno T., Hilary Frase, Marta Toth, and Sergei B Vakulenko. 2012. "The Class A β-Lactamase FTU-1 Is Native to *Francisella tularensis*." *Antimicrobial Agents and Chemotherapy* 56(2):666-671. doi:10.1128/AAC.05305-11.

22. Toth, Marta, Viktoria Vakulenko, Nuno T. Antunes, Hilary Frase, and Sergei B. Vakulenko. 2012. "Class A Carbapenemase FPH-1 from Francisella philomiragia." *Antimicrobial Agents and Chemotherapy* 56(6):2852-2857. doi:10.1128/AAC.00223-12.

23. Heine, Henry S., Lynda Miller, Stephanie Halasohoris, and Bret K. Purcell. 2017. "In Vitro Antibiotic Susceptibilities of *Francisella tularensis* Determined by Broth Microdilution following CLSI Methods." *Antimicrobial Agents and Chemotherapy* 61(9): e00612-17. doi:10.1128/AAC.00612-17.

24. Vollmer, Waldemar, Didier Blanot, and Miguel A. de Pedro. 2008. "Peptidoglycan structure and architecture." *FEMS Microbiology Reviews* 32(2):149-67. doi:10.1111/j.1574-6976.2007.00094.x.

25. Spidlova, Petra, Pavla Stojkova, Vera Dankova, Iva Senitkova, Marina Santic, Dominik Pinkas, Vlada Philimonenko, and Jiri Stulik. 2018. "*Francisella tularensis*

D-Ala D-Ala Carboxypeptidase DacD Is Involved in Intracellular Replication and It Is Necessary for Bacterial Cell Wall Integrity." *frontiers in Cellular and Infection Microbiology* 8: 111. doi:10.3389/fcimb.2018.00111.

26. Fisher, Jed F., and Shahriar Mobashery. 2020. "Constructing and deconstructing the bacterial cell wall." *Protein Science* 29(3): 629-646. doi:10.1002/pro.3737.

27. Papp-Wallace, Krisztina M., Andrea Endimiani, Magdalena A. Taracila, and Robert A. Bonomo. 2011. "Carbapenems: past, present, and future." *Antimicrobial Agents and Chemotherapy* 55(11):4943-60. doi:10.1128/AAC.00296-11.

28. Bush, Karen. 2018. "Past and Present Perspectives on β-Lactamases." *Antimicrobial Agents and Chemotherapy* 62(10):e01076-18. doi:10.1128/AAC.01076-18.

29. Lee, Chang-Ro, Jung Hun Lee, Moonhee Park, Kwang Seung Park, Il Kwon Bae, Young Bae Kim, Chang-Jun Cha, Byeong Chul Jeong, and Sang Hee Lee. 2017. "Biology of Acinetobacter baumannii: Pathogenesis, Antibiotic Resistance Mechanisms, and Prospective Treatment Options." *frontiers in Cellular and Infection Microbiology* 7:55. doi:10.3389/fcimb.2017.00055.

30. Bradford, Patricia A. 2001. "Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat." *Clinical Microbiology Reviews* 14(4):933-51. doi:10.1128/CMR.14.4.933-951.2001.

31. Singh, R., Saxena, A., & Singh, H. (2009). Identification of group specific motifs in Beta-lactamase family of proteins. Journal of Biomedical Science, 16(1): 109. doi:10.1186/1423-0127-16-109

32. Queenan, A. M., & Bush, K. (2007). Carbapenemases: the Versatile β-Lactamases. *Clinicak Microbiology Reviews*, 20(3): 440–458. doi:10.1128/CMR.00001-07

33. Bina, Xiaowen R, Chunmei Wang, Mark A Miller, and James E Bina. 2006. "The Bla2 beta-lactamase from the live-vaccine strain of *Francisella tularensis* encodes a functional protein that is only active against penicillin-class beta-lactam antibiotics." *Archives of Microbiology* 186(6):219-28. doi:10.1007/s00203-006-0140-6.

34. Rohmer, Laurence, Christine Fong, Simone Abmayr, Michael Wasnick, Theodore J Larson Freeman, Matthew Radey, Tina Guina, et al. 2007. "Comparison of *Francisella tularensis* genomes reveals evolutionary events associated with the

emergence of human pathogenic strains." *Genome Biology* 8(6): R102. doi:10.1186/gb-2007-8-6-r102.

35. Siddaramappa, Shivakumara, Jean F Challacombe, Jeannine M Petersen, Segaran Pillai, Geoff Hogg, and Cheryl R Kuske. 2011. "Common Ancestry and Novel Genetic Traits of *Francisella novicida*-Like Isolates from North America and Australia as Revealed by Comparative Genomic Analyses." *Applied and Environmental Microbiology* 77(15): 5110-5122. doi:10.1128/AEM.00337-11.

36. Hollis, D G, R E Weaver, A G Steigerwalt, J D Wenger, C W Moss, and D J Brenner. 1989. "*Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup novicida (formerly *Francisella novicida*) associated with human disease." *Journal of Clinical Microbiology* 27(7):1601-8. doi:10.1128/jcm.27.7.1601-1608.1989.

37. Clarridge, J E 3rd, T J Raich, A Sjösted, G Sandström, R O Darouiche, R M Shawar, P R Georghiou, C Osting, and L Vo. 1996. "Characterization of two unusual clinically significant Francisella strains." *Jornal of Clinical Microbiology* 34(8):1995-2000. doi:10.1128/jcm.34.8.1995-2000.1996.

38. Bröms, Jeanette E., Lena Meyer, Kun Sun, Moa Lavander, and Anders Sjöstedt. 2012. "Unique Substrates Secreted by the Type VI Secretion System of *Francisella tularensis* during Intramacrophage Infection." *PLoS One* 7(11): e50473. doi:10.1371/journal.pone.0050473.

39. Uniprot.org

40. Urich, Sandra K., and Jeannine M. Petersen. 2008. "In Vitro Susceptibility of Isolates of *Francisella tularensis* Types A and B from North America." *Antimicrobial Agents and Chemotherapy* 52(6): 2276-2278. doi:10.1128/AAC.01584-07.

41. beiresources.org

42. 2019. "Microbiology guide to interpreting minimum inhibitory concentration (MIC)." *INDEXX.*

43. Smith, Kenneth. 2019. "Opening the Black Box of Phenotypic Carbapenemase Detection." *American Society for Microbiology.*

44. Tankeshwar, Acharya. 2021. "Modified Hodge Test (MHT) for Carbapenemase Detection." *Microbe Online.*

45. Pasteran, Fernando, Nathalie Tijet, Roberto G Melano, and Alejandra Corso. 2015. "Simplified Protocol for Carba NP Test for Enhanced Detection of Carbapenemase Producers Directly from Bacterial Cultures." *Journal or Clinical Microbiology* 53(12): 3908-11. doi:10.1128/JCM.02032-15.

46. Rudresh, Shoorashetty Monohara, Giriyapur Siddappa Ravi, Lakshminarayanappa Sunitha, Sadiya Noor Hajira, Ellappan Kalaiarasan, and Belgode Narasimha Harish. 2017. "Simple, rapid, and cost-effective modified Carba NP test for carbapenemase detection among Gram-negative bacteria." *Journal of Laboratory Physicians* 9(4): 303-307. doi:10.4103/JLP.JLP_138_16.

47. Rawat, Deepti, and Deepthi Nair. 2010. "Extended-spectrum β-lactamases in Gram Negative Bacteria." *Journal of Global Infectious Diseases* 2(3): 263-274. doi:10.4103/0974-777X.68531.

48. Watt, Christine, Marie Louie, and Andrew E. Simor. 2000. "Evaluation of Stability of Cefotaxime (30-µg) and Ceftazidime (30-µg) Disks Impregnated with Clavulanic Acid (10 µg) for Detection of Extended-Spectrum β-Lactamases." *Journal of Clinical Microbiology* 38(7): 2796-2797. doi:10.1128/JCM.38.7.2796-2797.2000.

49. Tsai, Ya-Min, Shining Wang, Hui-Chuan Chiu, Cheng-Yen Kao, and Li-Li Wen. 2020. "Combination of modified carbapenem inactivation method (mCIM) and EDTA-CIM (eCIM) for phenotypic detection of carbapenemase-producing Enterobacteriaceae." *BMC Microbiology* 20: 315. doi:10.1186/s12866-020-02010-3.

Biography

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