



# *Francisella philomiragia* Infection and Lethality in Mammalian Tissue Culture Cell Models, *Galleria mellonella*, and BALB/c Mice

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*Francisella (F.) philomiragia* is a Gram-negative bacterium with a preference for brackish environments that has been implicated in causing bacterial infections in near-drowning victims. The purpose of this study was to characterize the ability of *F. philomiragia* to infect cultured mammalian cells, a commonly used invertebrate model, and, finally, to characterize the ability of *F. philomiragia* to infect BALB/c mice via the pulmonary (intranasal) route of infection. This study shows that *F. philomiragia* infects J774A.1 murine macrophage cells, HepG2 cells and A549 human Type II alveolar epithelial cells. However, replication rates vary depending on strain at 24 h. *F. philomiragia* infection after 24 h was found to be cytotoxic in human U937 macrophage-like cells and J774A.1 cells. This is in contrast to the findings that *F. philomiragia* was non-cytotoxic to human hepatocellular carcinoma cells, HepG2 cells and A549 cells. Differential cytotoxicity is a point for further study. Here, it was demonstrated that *F. philomiragia* grown in host-adapted conditions (BHI, pH 6.8) is sensitive to levofloxacin but shows increased resistance to the human cathelicidin LL-37 and murine cathelicidin mCRAMP when compared to related the *Francisella* species, *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. *LVS*. Previous findings that LL-37 is strongly upregulated in A549 cells following *F. tularensis* subsp. *novicida* infection suggest that the level of antimicrobial peptide expression is not sufficient in cells to eradicate the intracellular bacteria. Finally, this study demonstrates that *F. philomiragia* is lethal in two *in vivo* models; *Galleria mellonella* via hemocoel injection, with a LD<sub>50</sub> of  $1.8 \times 10^3$ , and BALB/c mice by intranasal infection, with a LD<sub>50</sub> of  $3.45 \times 10^3$ . In conclusion, *F. philomiragia* may be a useful model organism to study the genus *Francisella*, particularly for those researchers with interest in studying microbial ecology or environmental strains of *Francisella*. Additionally, the Biosafety level 2 status of *F. philomiragia* makes it an attractive model for virulence and pathogenesis studies.

**Keywords:** *Francisella philomiragia*, murine model, mammalian cells, *Galleria mellonella*, pulmonary infection, intranasal

## INTRODUCTION

Members of the *Francisella* genus are small, non-motile, Gram-negative coccobacilli of the gamma-proteobacteria class (Sjostedt, 2007). *Francisella philomiragia* was first identified in an ailing muskrat located in Utah approximately 50 years ago, following the discovery of its related species, *F. tularensis* (Hollis et al., 1989). Mistakenly characterized as *Yersinia philomiragia* due to its 24% genomic homology with *Y. pestis* and similarities in morphology, it took 30 years to re-categorize the species as a member of the *Francisella* genus (Hollis et al., 1989).

*Francisella philomiragia* has an affinity for aquatic environments which may increase its host species potential (Anda et al., 2001; Tarnvik et al., 2004). The natural range of *F. philomiragia* reflects its preference for aquatic environments as it is found near bodies of water, particularly brackish or salt water in the mainland United States (Hollis et al., 1989; Whipp et al., 2003; Berrada and Telford, 2010; Siddaramappa et al., 2012; Whitehouse et al., 2012). *F. philomiragia* may exist naturally by forming biofilms on exposed surfaces of the environment and infecting the aquatic amoeba, *Acanthamoeba castellanii* (Verhoeven et al., 2010).

Virulence factors in *F. philomiragia* have not been well studied in this species, but likely include proteins encoded by the Francisella Pathogenicity Island (FPI) and phospholipase C (Zeytun et al., 2012), similar to other members of the *Francisella* genus (Nano and Schmerk, 2007; Dai et al., 2010).

A related species, *F. noatunensis* (formerly named *F. philomiragia noatunensis*), is pathogenic to many fish and mollusk species, which inflicts negative economic and health effects on fisheries (Kay et al., 2006; Ostland et al., 2006; Mauel et al., 2007; Mikalsen and Colquhoun, 2009). Mikalsen et al. (2009) previously asserted that *F. philomiragia* subsp. *noatunensis* is a fish pathogen that is not lethal to mice and does not pose a threat to human health (Mikalsen et al., 2009). However, in the time since that publication, *F. noatunensis* has been elevated to species level, which leaves the ability of *F. philomiragia* to infect mice in question and untested (Mikalsen and Colquhoun, 2009; Copley and Elkins, 2011).

Near-drowning victims are susceptible to numerous bacterial infections due to the direct inoculation of the bacteria into the lungs (Ender and Dolan, 1997; Relich et al., 2015). *F. philomiragia* infections have been reported in otherwise healthy individuals via direct lung exposure resulting from near-drowning experiences in brackish or salty water or immunocompromised individuals with contact to contaminated water or fish (Wenger et al., 1989; Ender and Dolan, 1997; Cora et al., 2010; Kreitmann et al., 2015). Despite differences in genomic sequences (88% homologous to *F. tularensis* subsp. *LVS* and 84% to *F. tularensis* subsp. *novicida* and *SchuS4*; Zeytun et al., 2012; Davenport et al., 2014; Johnson et al., 2015), slightly different plasmids (Le Pihive et al., 2009), and reports that *F. philomiragia* does not cause disease in mice (Mikalsen et al., 2009), some of these near drowning victims infected by *F. philomiragia* develop a severe pneumonic infection. This prompted further investigation on the similarity of *F. philomiragia* to *F. tularensis* subsp. *novicida* and subsp. *LVS*, strains related to virulent *F. tularensis* subsp.

*SchuS4*, and whether it may be an opportunistic pathogens in humans. This comparison was achieved through the use of *in vitro* experiments using cell models involved in tularemia infections (macrophages, lungs, and liver) and *in vivo* animal infection models (*G. mellonella* and BALB/c mice).

## MATERIALS AND METHODS

### Bacterial Strains

*Francisella tularensis* Live Vaccine Strain (*LVS*; ATCC 29684), *F. tularensis* subsp. *novicida* (ATCC 15482), and *F. philomiragia* (ATCC 25015) were obtained from the American Type Culture Collection (Manassas, VA, USA). All bacterial strains were streaked onto Chocolate II Agar (GC II Agar with Hemoglobin and IsoVitaleX™, BD 221267) and single colonies were inoculated into Brain Heart Infusion (BHI pH 6.8) broth (TekNova, Hollister, CA, USA).

### Tissue Culture Cells

Murine macrophages, J774A.1 (ATCC TIB-67), human hepatocellular carcinoma cells, HepG2 (ATCC HB-8065), and human Type II alveolar epithelial cells, A549 (ATCC CCL-185), were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen #10566-016) supplemented with 10% fetal bovine serum (FBS) as per the manufacturer's recommendations. Human U937 macrophage-like cells (ATCC CRL-1593.2), were cultured in RPMI 1640 media with 2 mM L-glutamine and 10% FBS as per the manufacturer's recommendations (Lonza # 3163826). U937 cells were differentiated from monocytes to macrophages as instructed by manufacturer.

### Infection Protocol for A549, J774A.1, HepG2, and U937 Cells

Cells were infected at a multiplicity of infection (MOI) of approximately 500, as previously described (Han et al., 2008; Hegedus et al., 2008; Ahmad et al., 2010), with a 2-h preinfection and 1-h gentamicin pulse. Briefly, cells were seeded ( $10^5$ /well) in a 48-well plate and allowed to attach overnight. After verifying successful cell attachment, culture media was gently removed and rinsed twice with culture media. *Francisella* strains were grown to mid-logarithmic phase, collected by centrifugation (10 min at  $4000 \times g$ , 4°C), washed three times with 1x phosphate buffered saline (PBS), and diluted in serum-free DMEM to a verified bacterial concentration (CFU/mL). Dilutions of bacteria were used to infect each cell line at MOI = 500. Sets of three wells were prepared for each condition ( $n = 3$ ). Characteristically, *Francisella* infects host cells inefficiently, despite its infectivity via multiple routes in animals and humans. Therefore, the standard MOI of 500 CFU was used to infect cells with the *Francisella* strains in order to achieve infection of most of the cells (Lai et al., 2001; Lai and Sjostedt, 2003). Cells were then incubated with bacteria at 37°C, 5% CO<sub>2</sub>. After a 2-h incubation, well media was gently aspirated, washed twice with PBS, and treated with 50 µg/mL gentamicin in serum-free DMEM for 1 h to kill extracellular bacteria. Following the gentamicin pulse, cell media

was gently aspirated, replaced with DMEM supplemented with 10% FBS and 5  $\mu\text{g}/\text{mL}$  gentamicin, and allowed to incubate for 24 h at 37°C, 5% CO<sub>2</sub>. (Lai et al., 2001; Han et al., 2008; Ahmad et al., 2010) Cells were lysed and plated on Chocolate agar for CFU determination.

### Cytotoxicity Assay of Mammalian Tissue Cultured Cells Infected with *Francisella*

PrestoBlue Cell Viability Reagent (A-13261, Life Technologies, Carlsbad, CA, USA) was used according to the manufacturer's protocol. This reagent functions by using the reducing environment of the cell's cytosol to determine cell viability. The reagent contains a cell-permeable compound, which is blue in color. When added to viable cells, it encounters the reducing environment and modifies the reagent to become a red fluorescent, which can be detected by fluorescence or absorbance measurements. Briefly, reagent was added to infected cells 24 h post gentamicin-pulse at a 1:10 ratio. The reagent was incubated with cells at 37°C for 2 h. Fluorescence was measured at excitation and emission spectra of 560 and 590 nm, respectively. Three wells were used per condition ( $n = 3$ ). Data was averaged and a no-cell well was subtracted as background. Data was then plotted using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) to reflect the cytotoxic effect of *Francisella* species on eukaryotic cell lines.

### EC<sub>50</sub> Antimicrobial Assays

Peptides used in this study were custom synthesized by ChinaPeptides Company (Shanghai, China) and had purities of  $\geq 95\%$  based on chromatographic analysis of the purified peptides. Antimicrobial activity (EC<sub>50</sub>) assays of the antibiotic control levofloxacin, human cathelicidin LL-37, and murine cathelicidin mCRAMP were performed against *F. philomiragia*, *F. tularensis* subsp. *novicida*, and *F. tularensis* subsp. *LVS* as previously described (Amer et al., 2010). Briefly,  $1 \times 10^5$  CFU/well of *Francisella* species were grown in BHI (pH 6.8), added to a sterile 96-well plate and incubated with serial dilutions of peptide or antibiotic in 10 mM phosphate buffer for 3 h at 37°C. Dilutions were plated in triplicate on tryptic soy agar with 1% cysteine for 24 h; colonies were counted to determine survival ( $n = 3$ ). This experiment was performed three independent times. Bacterial survival was calculated by a ratio of the number of colonies on each experimental plate to the average number of colonies on the control plates lacking peptide or antibiotic application. The EC<sub>50</sub> was determined using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) to plot the percent survival versus log of peptide or antibiotic concentration (log  $\mu\text{g}/\text{mL}$ ) and fitting data to a standard sigmoidal dose-response curve as previously described (Blower et al., 2015).

### Waxworm Infection

*Galleria mellonella* larvae (Vander horst Wholesale, St. Mary's, OH, USA, 16 per group) were infected following previous reports (Aperis et al., 2007; Dean et al., 2011; McKenney et al., 2012). *G. mellonella* were infected by injecting 10  $\mu\text{L}$  of bacteria into the hemocoel via a right proleg and incubated at 37°C. Each larva

received bacterial concentrations of  $1 \times 10^6$ ,  $5 \times 10^5$ ,  $1 \times 10^5$ ,  $5 \times 10^4$ ,  $1 \times 10^4$ ,  $5 \times 10^3$ ,  $1 \times 10^3$ ,  $5 \times 10^2$ , or  $1 \times 10^2$  CFU/mL with 16 larvae per group. Waxworms were examined once a day for death. Bacterial concentrations were verified via retrospective plating and counting of CFUs.

### Murine Infection

BALB/c mice (Harlan, Frederick, MD, USA, five per group) were infected intranasally with 20  $\mu\text{L}$  of the following concentrations of bacteria:  $1 \times 10^6$ ,  $5 \times 10^5$ ,  $1 \times 10^5$ ,  $5 \times 10^4$ ,  $1 \times 10^4$ ,  $5 \times 10^3$ , or  $1 \times 10^3$  CFU/20  $\mu\text{L}$ . Mice were examined twice a day for signs of illness or death. Bacterial concentrations were verified via retrospective plating and counting of CFUs. Animal experiments were approved by and conducted in compliance with regulations of the Institutional Animal Care and Use Committee (Protocol # 0236) of George Mason University. All experiments were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (2011) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002).

### Statistical Analysis

Antimicrobial EC<sub>50</sub> assays were performed in triplicate with  $n = 3$  for each experiment, and representative experiments are shown. Standard deviations of the mean of each set are represented on each graph as error bars. Additionally the confidence interval (95%) is provided for EC<sub>50</sub> determinations to demonstrate statistical overlap of data. Student's *t*-test was performed and *p* values of  $p < 0.05$  was considered statistically different.

The survival curves were performed with an  $n = 16$  for *G. mellonella* and an  $n = 5$  for BALB/c mice and were analyzed using the Mantel-Cox test, which is used to test the null hypothesis that survival curves are not different between groups. This test does not assume a normal distribution, allows for censored data, and is based off of the chi-squared test, which allows for a minimum of five samples.

## RESULTS

During pulmonary tularemia infections, bacteria colonize the alveolar macrophages, the lungs, and the liver (Hall et al., 2007; Faron et al., 2015). *F. philomiragia* was evaluated to see if it infected cell lines representative of these systems *in vitro*: murine macrophage cells, J774A.1, human Type II alveolar epithelial cells, A549, and human hepatocyte-like cells, HepG2. These cell lines have been previously shown by us and others to be susceptible to infection by *F. tularensis* subsp. *novicida* and *LVS* (Qin and Mann, 2006; Han et al., 2008; Amer et al., 2010; Bradburne et al., 2013).

*Francisella tularensis* readily infects macrophages and proliferates within these cells (Anthony et al., 1991; Golovliov et al., 1997; Lai et al., 2001; Bolger et al., 2005). It is characteristic for *Francisella* replication to occur with little cytotoxicity until the cell becomes overburdened (at about 48 h post infection) and

will experience cell death. *F. philomiragia* was found to be able to infect and proliferate in murine macrophages at 24 h to higher levels than what was seen for *F. tularensis* subsp. *novicida* and subsp. *LVS* (Figure 1A,  $p < 0.05$ ). These results with the *in vitro*

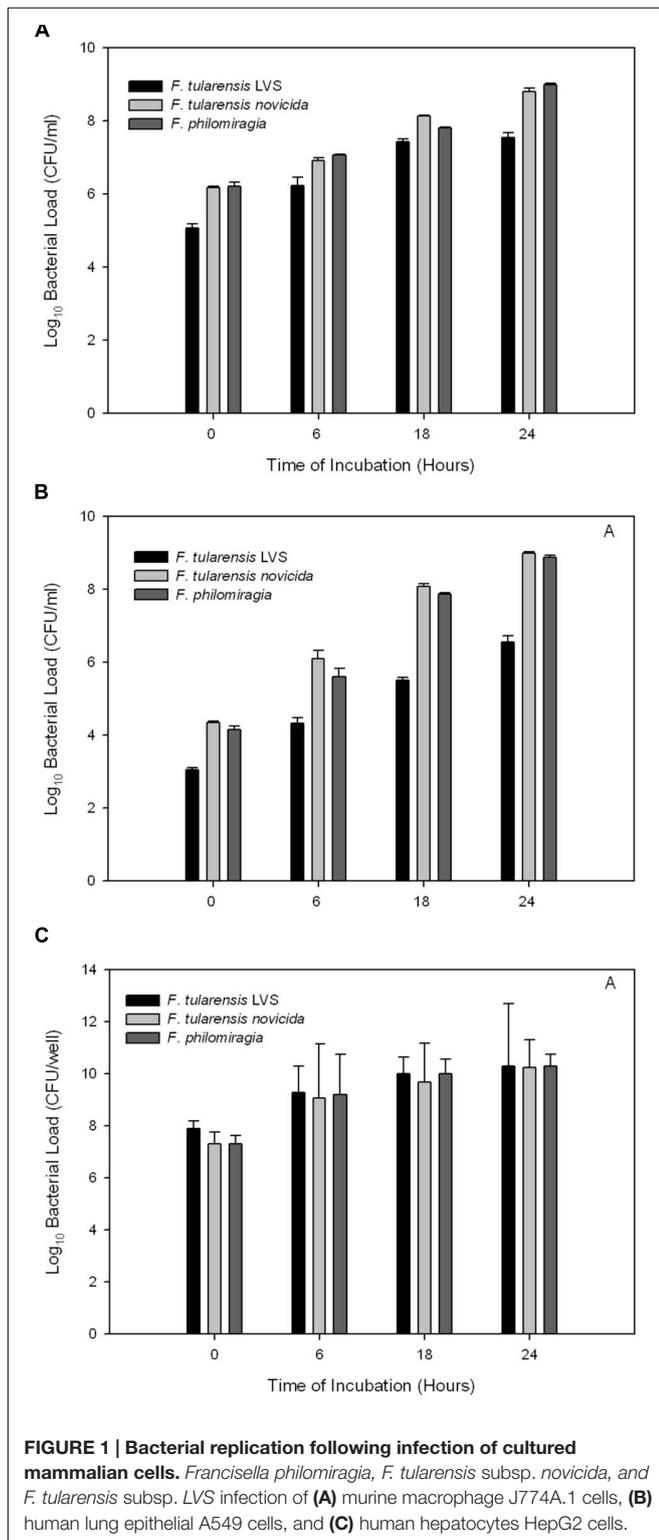
macrophage model suggest that *F. philomiragia* may be capable of infecting mammalian macrophages *in vivo*. Furthermore, these results suggest that *F. philomiragia* may be able to infect the alveolar macrophages in the lungs of near-drowning victims, which results in the clinical disease resembling tularemia that can afflict these patients.

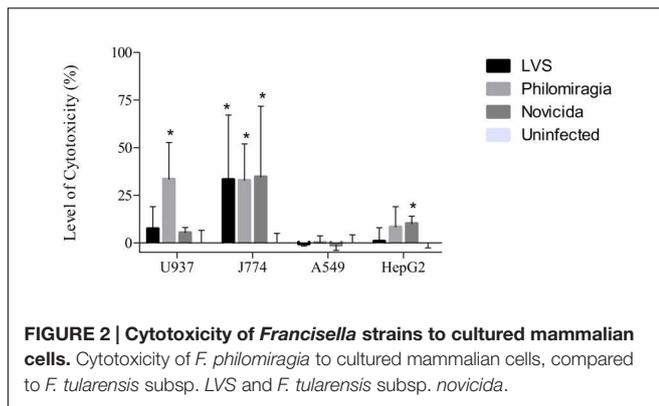
Human alveolar epithelial cells are known to be infected by *Francisella* both *in vitro* and *in vivo* (Hall et al., 2007; Faron et al., 2015). Here, experiments utilizing A549 cells showed that *F. philomiragia* infects this cell type to a lesser extent than *F. tularensis* subsp. *novicida* but more than subsp. *LVS* (Figure 1B,  $p < 0.05$ ). The infection of this cell type suggests another potential mechanism by which the near-drowning infections in human could occur by this organism due to the direct inoculation of the lung.

*Francisella* infection of and proliferation in hepatocytes has been observed in human tularemia patients and animal models (Conlan and North, 1992; Lamps et al., 2004; Rasmussen et al., 2006; Ray et al., 2010). The fully virulent *F. tularensis* subsp. *SchuS4* replicates well in cultured HepG2 cells (Qin and Mann, 2006). In these experiments, *F. tularensis* subsp. *LVS* infected human hepatocyte-like cells well, and replicated faster than *F. tularensis* subsp. *novicida* and *F. philomiragia*. However, by 24 h post infection, there were no differences between the bacterial burdens of the three *Francisella* species in HepG2 cells (Figure 1C,  $p > 0.05$ ). This suggests that the *F. philomiragia* infections could potentially lead to liver damage, consistent with a tularemia infection.

*Francisella tularensis* is said to be a “stealth” pathogen, promoting its intracellular survival by not causing high cytotoxicity, among other mechanisms (Sjostedt, 2006; Jones et al., 2014). It was previously shown that infections of A549 cells by *F. tularensis* subsp. *LVS* (at 500 MOI) for 24 h did not cause significant cytotoxicity, although CFU increased significantly (Han et al., 2008; Bradburne et al., 2013). This high MOI of 500 is standard for *Francisella* infection protocols, as it is not taken up into non-phagocytic cells readily (Lai et al., 2001; Lai and Sjostedt, 2003; Telepnev et al., 2003). These studies were expanded to all three strains of *Francisella* investigated here (*F. tularensis* subsp. *LVS*, *F. tularensis* subsp. *novicida*, and *F. philomiragia*) and J774A.1, A549, and HepG2 cells.

This study confirmed that *F. tularensis* subsp. *LVS* is not significantly cytotoxic toward A549 cells and, furthermore, it was found that *F. tularensis* subsp. *novicida* and *F. philomiragia* also displayed little cytotoxicity in this cell line at 24 h (Figure 2). HepG2s were also minimally affected by cytotoxic effects of *F. tularensis* subsp. *LVS* and displayed only 10 and 8% cytotoxicity from *F. tularensis* subsp. *novicida* and *F. philomiragia* infections at 24 h, respectively. The murine macrophage cell line, J774A.1, demonstrated greater susceptibility to the cytotoxic effects of *Francisella*, with all strains demonstrating about 33% cytotoxicity at 24 h ( $p < 0.05$ ) consistent with previous reports (Lai et al., 2001; Lai and Sjostedt, 2003). However, additional cytotoxicity studies showed that *F. philomiragia* is highly cytotoxic to the human macrophage-like cell line, U937, (33%,  $p < 0.05$ ) while *F. tularensis* subsp. *LVS* and subsp. *novicida* showed only 7 and 5% cytotoxicity, respectively. The differences between the





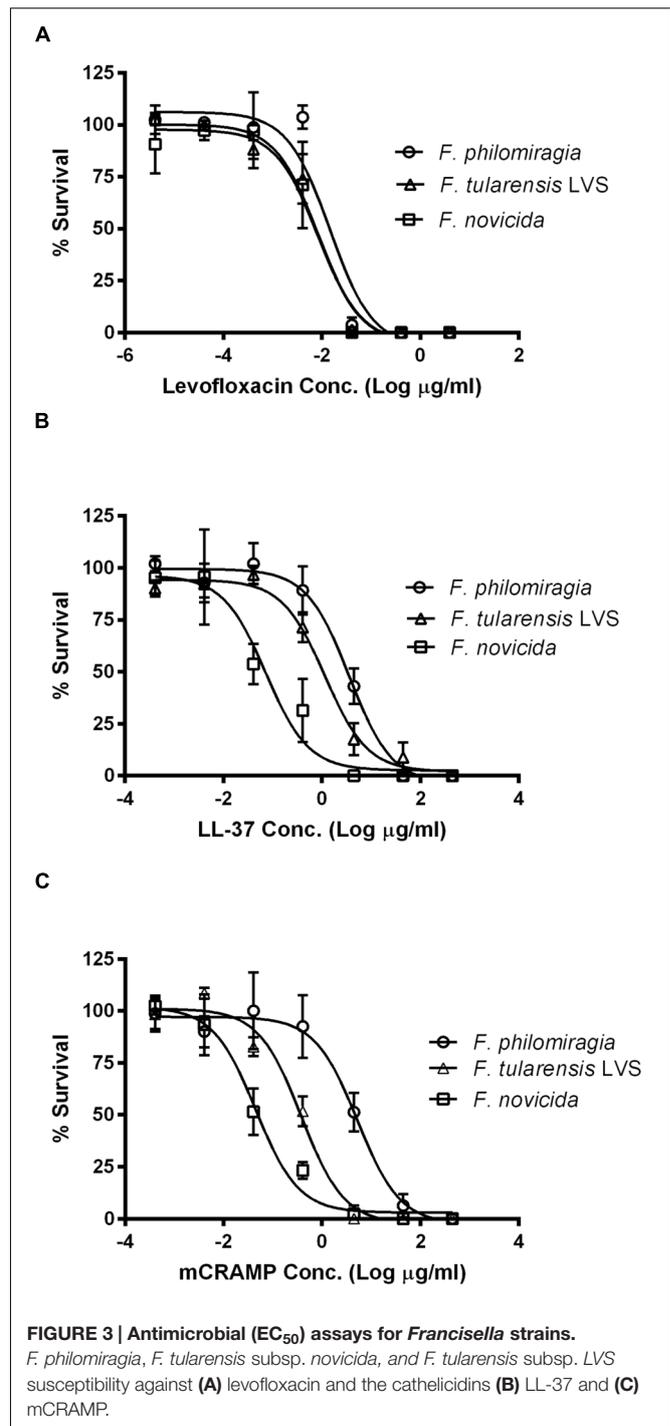
human and murine macrophage cell lines are not yet understood in regard to *Francisella* infections. Previously, differences in *Francisella* intracellular replication have been noted between rat and murine macrophages (Anthony et al., 1991), however, other causes for the cytotoxicity differences other than species of origin are possible. These findings are consistent with the intracellular replication lifestyle of other *Francisella* species (Sjostedt, 2006).

Here, *F. philomiragia* was found to infect and replicate in the same cell types and have the same general level of cytotoxicity in those cell types as *F. tularensis* subsp. *LVS* and subsp. *novicida*, with the exception of the U937 cells ( $p < 0.05$ ). The susceptibility of *F. philomiragia* to two antimicrobial peptides, LL-37, a human cathelicidin, and mCRAMP, a murine cathelicidin, known to be expressed by host cells and have killing activity against *F. tularensis* subsp. *novicida* and subsp. *LVS* was examined (Amer et al., 2010). This is important because host defense against *Francisella* infection relies not only on antibody production, but also on the response of the innate immune system (Allen, 1962; Metzger et al., 2007; Kirimanjeswara et al., 2008).

*Francisella* species, including *F. philomiragia*, are known to be highly susceptible to levofloxacin under MIC conditions (Nelson et al., 2010; Georgi et al., 2012), thus it was used as a control for the EC<sub>50</sub> antimicrobial assays. The EC<sub>50</sub> for levofloxacin of *F. philomiragia* is 0.0146 μg/mL (14.6 ng/mL; **Figure 3A**) while the *F. tularensis* subsp. *LVS* EC<sub>50</sub> is 0.00827 μg/mL (8.27 ng/mL) and the *F. tularensis* subsp. *novicida* EC<sub>50</sub> is 0.00843 μg/mL (8.43 ng/mL; **Table 1**). These values are statistically the same within the 95% confidence intervals ( $p > 0.05$ ) and are consistent with the MICs previously reported (Georgi et al., 2012).

The sensitivity of *F. philomiragia* to cationic antimicrobial peptides has not been well studied. This organism is highly resistant to colistin and polymyxin B, which are cationic cyclic peptide antibiotics (Petersen et al., 2009; Stephens et al., 2016). It was previously demonstrated that expression of the human cathelicidin LL-37 in A549 cells is strongly induced by *F. tularensis* subsp. *novicida* infection (Amer et al., 2010). This is of interest as *Francisella* bacteria replicate directly in the cytosol of the infected cells (Wehrly et al., 2009), and thus the bacteria may be able to be killed by expression of these innate immunity peptides by the afflicted cell.

The antimicrobial peptides, LL-37 and mCRAMP, were tested for their killing activity against *F. philomiragia* in



10 mM phosphate buffer, pH 7.2. The EC<sub>50</sub> of LL-37 against *F. philomiragia* was determined to be 3.61 μg/mL (**Figure 3B**). In contrast to *Francisella* sensitivity to LL-37 in other species (EC<sub>50</sub> of 1.15 and 0.0724 μg/mL in *F. tularensis* subsp. *LVS* and *novicida*; Amer et al., 2010; Flick-Smith et al., 2013), *F. philomiragia* is more resistant to this human cathelicidin peptide ( $p < 0.05$ ). The EC<sub>50</sub> of LL-37 against *F. tularensis* subsp. *novicida* found here is statistically similar to previously reported values due to

**TABLE 1 | Summary of Antimicrobial (EC<sub>50</sub>) assays for *Francisella* strains.**

		<i>Francisella tularensis</i> subsp. <i>LVS</i>	<i>Francisella tularensis</i> subsp. <i>novicida</i>	<i>Francisella philomiragia</i>
Levofloxacin	EC <sub>50</sub> (μg/ml)	0.00827	0.00843	0.0146
	95% CI	(0.00524–0.0131)	(0.00413–0.0172)	(0.00696–0.0305)
mCRAMP	EC <sub>50</sub> (μg/ml)	0.381	0.0453	5.27
	95% CI	(0.239–0.607)	(0.0284–0.0723)	(2.93–9.46)
LL-37	EC <sub>50</sub> (μg/ml)	1.15	0.0724	3.61
	95% CI	(0.604–2.18)	(0.0331–0.158)	(2.36–5.53)

EC<sub>50</sub> values plus 95% confidence intervals for *F. philomiragia*, *F. tularensis* subsp. *novicida*, and *F. tularensis* subsp. *LVS* susceptibility against levofloxacin and the cathelicidins LL-37 and mCRAMP.

overlapping 95% confidence interval values (Amer et al., 2010). Some small difference could also be due to the “host-adapted phenotype” growth conditions used to grow the bacteria for this study (BHI pH 6.8) compared to growth in Tryptic Soy Broth with Cysteine (TSB-C) media that was used previously (Amer et al., 2010). Growth in BHI (pH 6.8) is known to alter the surface carbohydrate and gene expression in a way that mimics the “host-adapted” phenotype of *Francisella* (Zarrella et al., 2011). In conclusion, *F. philomiragia* is more resistant to LL-37 than other *Francisella* species that were verified here (*F. tularensis* subsp. *LVS* EC<sub>50</sub> = 1.15 (threefold),  $p = 0.011$ , and *F. tularensis* subsp. *novicida* EC<sub>50</sub> = 0.0724 μg/mL (50-fold),  $p = 0.0011$ ; Amer et al., 2010; Flick-Smith et al., 2013).

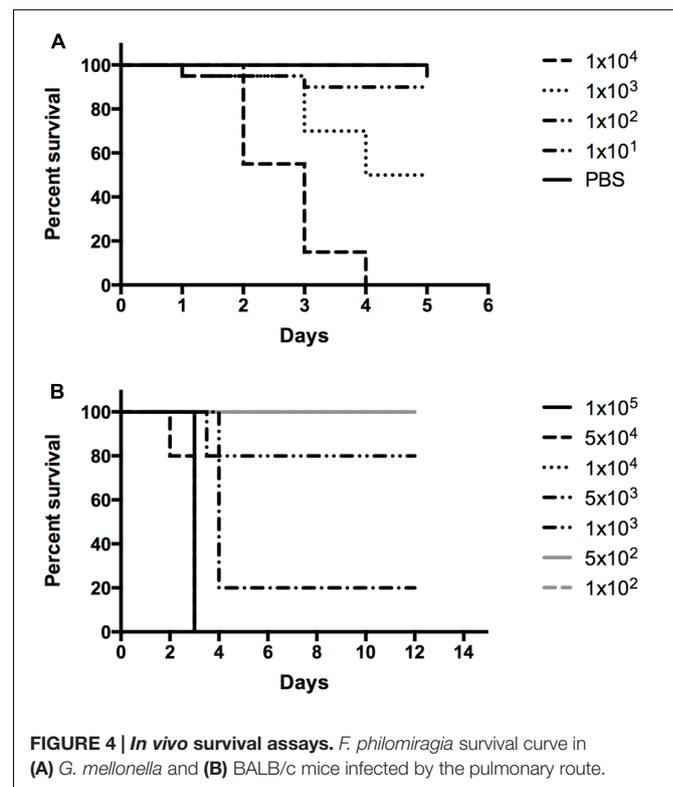
*Francisella* susceptibility to mCRAMP has not been previously reported. The EC<sub>50</sub> of mCRAMP against *F. philomiragia* was determined to be 5.27 μg/mL (Figure 3C). No previous reports of *Francisella* susceptibility to mCRAMP were found; however, for this murine cathelicidin peptide, *F. philomiragia* is significantly more resistant ( $p < 0.05$ ) than *F. tularensis* subsp. *novicida* (EC<sub>50</sub> = 0.0453 μg/mL) or *F. tularensis* subsp. *LVS* (EC<sub>50</sub> = 0.381 μg/mL). Here it was found that *F. philomiragia* is 14–116-fold more resistant to mCRAMP (EC<sub>50</sub> = 5.27 μg/mL) than *F. tularensis* subsp. *LVS* (0.381 μg/mL,  $p = 0.007$ ) and subsp. *novicida* (0.0453 μg/mL,  $p = 0.0051$ ).

This increased resistance of *F. philomiragia* to cationic antimicrobial peptides could be due to differences in the LPS (Siddaramappa et al., 2012) or other surface properties of *F. philomiragia* compared to *F. tularensis* subsp. *novicida* or *LVS* perhaps due to differential expression of high-molecular weight carbohydrates in the “host-adapted” phenotype (Zarrella et al., 2011).

*Galleria mellonella* has been demonstrated to be a useful model for *Francisella* infection (Aperis et al., 2007; Ahmad et al., 2010; McKenney et al., 2012; Sprynski et al., 2014), thus a survival curve evaluating the mortality of *G. mellonella* during *F. philomiragia* infection was examined. As shown in Figure 4A, *F. philomiragia* injection is lethal to *G. mellonella* in a manner similar to *F. tularensis* subsp. *LVS* (Aperis et al., 2007; Ahmad et al., 2010). The LD<sub>50</sub> of *F. philomiragia* in *G. mellonella* is  $\sim 1.8 \times 10^3$  CFU/mL or  $\sim 18$  CFU with an inoculation volume of 10 μL. For comparison, the LD<sub>50</sub> of *F. tularensis* subsp. *novicida* in *G. mellonella* is  $\sim 1.2 \times 10^2$  CFU/mL or  $\sim 1$  CFU due to an inoculation volume of 10 μL (McKenney et al., 2012). The mean and median times to death for *F. philomiragia* are 2.79 and 3 days,

respectively. Since *F. philomiragia* is able to infect *G. mellonella* similarly to other laboratory strains of *Francisella*, infection of BALB/c mice by the intranasal route of infection was tested for this organism.

BALB/c mice are a common experimental model for *Francisella* infections and they are susceptible to *Francisella* infection by the pulmonary route (intranasal or aerosol), among other routes; no studies have been reported for *F. philomiragia* infections of insect models, mice, rats, or marmosets (Chen et al., 2003; Conlan et al., 2003; Cowley and Elkins, 2011). To conform to these standards and expand the *in vivo* results obtained with *G. mellonella*, a survival curve evaluating the lethality of *F. philomiragia* in BALB/c mice when delivered via intranasal administration (mimicking near-drowning experiences) was examined. As shown in Figure 4B, intranasal *F. philomiragia* is lethal to BALB/c mice with an approximate LD<sub>50</sub> of  $3.45 \times 10^3$



CFU. This is very comparable to the intranasal *F. tularensis* subsp. *LVS* LD<sub>50</sub> ( $1 \times 10^3$  CFU) in the same species of mice but is higher than the 100 CFU intranasal LD<sub>50</sub> of *F. tularensis* subsp. *novicida* (Aperis et al., 2007).

## DISCUSSION

Multiple reports of severe pneumonic infections of humans following near-drowning experiences (Hollis et al., 1989; Wenger et al., 1989) suggested that direct or large inoculation of *F. philomiragia* into the lung by this method is sufficient to allow for infection of normal, healthy human lungs, potentially via infection of the alveolar macrophages and/or lung epithelial cells. However, this organism is not generally regarded as a human pathogen and its ability to infect mammalian cells is generally uncharacterized. In addition, the highly related organism, *F. noatunensis*, was found to be unable to infect laboratory mice (Mikalsen et al., 2009). Thus, *F. philomiragia* was compared to *F. tularensis* subsp. *novicida* and subsp. *LVS* regarding its ability to infect human and murine cells was further studied.

*Francisella philomiragia* was shown to be capable of infecting a murine macrophage cell line, J774A.1, which are commonly used for *Francisella* studies, with statistically higher levels ( $p < 0.05$ ) than more commonly studied strains of *Francisella* (Hegedus et al., 2008; Pechous et al., 2008; Ahmad et al., 2010). Similarly, *F. philomiragia* was also found to infect a human Type II alveolar epithelial cell line, A549, at statistically higher levels than *F. tularensis* subsp. *LVS*. This is the first demonstration of *F. philomiragia* infecting Type II alveolar epithelial cells and is a significant contribution to the understanding of the potential interactions of *F. philomiragia* within the human lung. These findings suggest a potential mechanism by which near-drowning in brackish water known to contain *F. philomiragia* (Ottem et al., 2007) could potentially lead to infection through interaction of the bacteria with Type II alveolar epithelial cells of the lung and/or alveolar macrophages (Gentry et al., 2007; Hall et al., 2007; Craven et al., 2008; Faron et al., 2015). Furthermore, these results suggest that aerosol exposure to *F. philomiragia* could potentially lead to pulmonary infections in humans if inhaled via an aerosol. Given the wide distribution of *F. philomiragia*, in particular its known presence in various bodies of water within the United States, this potential route of infection should be further investigated.

In addition, it was demonstrated that *F. philomiragia* infects HepG2 cells, a human hepatocyte-like cell line. This finding suggests that *F. philomiragia* may be able to replicate in the liver in infected near-drowning victims. The liver is one of the main organs infected by *F. tularensis* strains and liver failure following overwhelming organ infection is thought to be the primary cause of death in mice suffering from tularemia (Conlan and North, 1992). Patients suffering from *F. philomiragia* pneumonia should be closely observed for sequelae similar to those found in tularemia infections caused by *F. tularensis* species.

Cytotoxicity data after 24 h of infection show that *F. philomiragia* is similar to *F. tularensis* subsp. *novicida*

and subsp. *LVS* in most of the studied cell lines. Little cytotoxicity was seen in A549 cells (~0%, similar to other species) and HepG2 cells (8%, more than subsp. *LVS* but similar to subsp. *novicida*), and moderate cytotoxicity in J774A.1 cells (32%, similar to other species). However, the U937 human macrophage-like cell line only showed high cytotoxicity (33%) from *F. philomiragia* and not the other *Francisella* strains studied. This observation will be the subject of future investigation to understand the difference in U937 susceptibility.

Susceptibility testing using the antimicrobial peptides LL-37, a human cathelicidin, and mCRAMP, a murine cathelicidin, showed that these peptides were highly active *in vitro* against *F. philomiragia*. Despite being active in killing the bacteria *in vitro*, this antimicrobial peptide host defense mechanism is clearly insufficient to control *F. philomiragia* infections in infected cells or *in vivo*.

*Francisella philomiragia* was found to be lethal for both *in vivo* models tested: *G. mellonella* and BALB/c mice. *G. mellonella* has been demonstrated to be a useful *in vivo* model for *Francisella* infection (Aperis et al., 2007; Ahmad et al., 2010; McKenney et al., 2012; Sprynski et al., 2014), thus the survival of *G. mellonella* during *F. philomiragia* infection was examined. In *G. mellonella*, *F. philomiragia* was shown to be fatal in concentrations similar to *F. tularensis* subsp. *LVS*, with an LD<sub>50</sub> of 18 CFU. This similarity supports the ability of *G. mellonella* to be used as an effective model for *Francisella* infection but also suggests that *F. philomiragia* is capable of infecting a range of hosts similar to other *Francisella* strains.

*Francisella philomiragia* is not generally regarded as a pathogen of humans or animals but is considered an environmental species of the genus (Anda et al., 2001; Tarnvik et al., 2004; Verhoeven et al., 2010). In some cases, *F. philomiragia* infections in near-drowning victims individuals are observed (Wenger et al., 1989; Ender and Dolan, 1997). An intranasal infection of mice by *F. philomiragia* was used to mimic lung exposure seen in drowning victims and test the susceptibility BALB/c mice to this organism. *F. philomiragia* was shown to be fatal in BALB/c mice by intranasal-delivered inoculum concentrations similar to *F. tularensis* subsp. *LVS*, with an LD<sub>50</sub> of  $3.45 \times 10^3$  CFU; however, this is significantly higher than the 10 CFU LD<sub>50</sub> seen with *F. tularensis* subsp. *novicida*. Thus, contrary to the result for *F. noatunensis* (Mikalsen et al., 2009), *F. philomiragia* is able to infect laboratory mice. These results call for further studies to determine the full host range of *F. philomiragia*.

## CONCLUSION

These studies show that *F. philomiragia* results in similar *in vitro* and *in vivo* infections to the *F. tularensis* subspecies *novicida* and *LVS* for the evaluated strains. It was demonstrated for the first time that there is potential for significant and robust *F. philomiragia* infection in macrophages, lung, and liver cells. *F. philomiragia* infection of human alveolar epithelial cells and macrophages suggests a mechanism for infection in the lungs of near-drowning patients. The high level of *F. philomiragia*

intracellular replication in all three cell types suggests that *F. philomiragia* follows an infection course similar to tularemia caused by *F. tularensis* subspecies. It was previously demonstrated that infections of *G. mellonella* and pulmonary infections of BALB/c mice were fatal with similar LD<sub>50</sub>s to *F. tularensis* subsp. *LVS*. The results of these *in vitro* and *in vivo* experiments confirm earlier suggestions that *F. philomiragia* may be an emerging opportunistic human pathogen (Mailman and Schmidt, 2005; Sjodin et al., 2012) and that cellular and animal models of *Francisella* infection could also be used to study *F. philomiragia*. It would be of interest to evaluate all the available *F. philomiragia* strains for their ability to infect the various tissue culture and murine models.

It was found that *F. philomiragia* is comparable to the other Biosafety level 2 strains of *Francisella* in many respects but unusual in its effect on human U937 cells. This finding will open some interesting new avenues of research regarding pathogenesis and virulence of *F. philomiragia*. In addition, this work also positions *F. philomiragia* as another important organism in the field of *Francisella* research, especially for researchers interested in questions of microbial ecology or environmental persistence of members of the genus *Francisella*.

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## AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication. MLV conceived the study; MLV and CNP wrote the manuscript; CNP, SLP, RJB, SA, and MM contributed experimental data and contributed to the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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