

Cutaneous Infection Caused by a Novel *Francisella* sp.

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A 69-year-old patient presented with a tender, thickly crusted skin lesion of 1 week's duration. A bacterial culture swab taken from the underlying granular tissue yielded a pure isolate of a Gram-negative coccobacillus, presumptively identified as a novel *Francisella* species via 16S rRNA and multilocus gene sequence analysis.

CASE REPORT

On 5 January 2011, a 69-year-old white male presented to a dermatology clinic with a tender lesion on his left ankle. The patient had been well until the previous week when he cut down an Arizona ash tree near his home in southern Louisiana, close to the Mississippi River. Branches from the tree scratched his legs and abraded his left ankle. He applied mupirocin ointment to the ankle lesion, but it became increasingly tender and erythematous. His underlying medical conditions included non-insulin-dependent diabetes, hypertension, hyperlipidemia, and a history of a squamous cell carcinoma excised from his right arm in March 2010. The patient denied fever, chills, or other systemic symptoms.

On initial examination, the patient was afebrile with a 4- by 6-cm thickly crusted erythematous nodule on the medial aspect of the left ankle, with surrounding erythema and tenderness to palpation (Fig. 1A). Incision and drainage of the nodule was attempted; however, no pus or purulent drainage was noted. Tissue underlying the eschar was pink and granular. A biopsy specimen of the underlying granular tissue was submitted for histopathology, deep fungal culture, and atypical mycobacterial culture; a swab was also taken for routine bacterial culture.

Initial histopathology of the biopsied tissue was interpreted as squamous cell carcinoma. The patient was referred to a general surgeon for excision of the lesion but, because of the surrounding erythema, was also prescribed 500 mg ciprofloxacin twice daily (BID) for 7 days. The patient noted improvement in the lesion after 24 h. Deep fungal and atypical mycobacterial cultures were both negative. A Gram-negative coccobacillus grew from the bacterial swab taken from the underlying granular tissue and was presumptively identified as *Francisella tularensis* (described below). Based on the isolation of a *Francisella* species from the lesion, an additional 7 days of ciprofloxacin was prescribed, and the pathologist who performed histopathology was contacted to review the initial biopsy specimen. The pathologist's amended report noted probable pseudoepitheliomatous hyperplasia overlying a dense suppurative granulomatous dermatitis, rather than squamous cell carcinoma. However, significant atypia was noted, and the remaining lesion was excised in the operating room on 4 March 2011. The area healed well without further sequelae. Subsequent independent histopathology of the biopsy tissue similarly indicated no evidence of squamous cell carcinoma but rather marked inflammation and pseudoepitheliomatous epidermal hy-

perplasia with a dense, mixed dermal infiltrate including scattered small neutrophilic microabscesses, histiocytes, lymphocytes, and plasma cells (Fig. 1B). Intraepidermal neutrophilic microabscesses were also seen (Fig. 1B).

Testing of the bacterial culture swab was performed at a commercial laboratory, where a pure culture of a small Gram-negative coccobacillus was recovered on both blood and chocolate agars but not MacConkey agar. The organism was forwarded to a second commercial laboratory, where it was presumptively identified via growth characteristics, colony morphology, Gram stain, and biochemical testing (catalase and oxidase) as *F. tularensis* and subsequently submitted to the nearby Orange County Public Health Laboratory (OCPHL) in Santa Ana, CA, on 19 January 2011. Characterization performed at OCPHL indicated the organism grew well on chocolate, cysteine heart, and buffered charcoal yeast extract agars after 24 h incubation at 35°C in 5% CO₂, whereas only minimal growth was observed on blood agar after 24 h. Biochemical testing revealed the bacterium was *o*-nitrophenyl- β -D-galactopyranoside (ONPG) positive and oxidase, catalase, indole, urease, gelatinase, and nitrate negative. It fermented maltose and glucose but did not utilize xylose, mannitol, lactose, sucrose, or fructose when grown on 1% cystine tryptic agar in the presence of the individual carbohydrates. Similarly, there was no fermentation of glycerol after 5 days in phenol red nitrate broth. The organism grew better in nutrient broth in the presence of 3% and 6% NaCl than in the presence of 0% NaCl. When the NaCl concentration was increased to 10%, there was no growth.

F. tularensis-specific testing indicated that the isolate was negative by the direct fluorescent-antibody assay (1) and by the Laboratory Response Network (LRN) real-time PCR for *F. tularensis* (3 of 3 PCR assays were negative). For 16S rRNA gene sequencing, the MicroSEQ microbial identification system (Life Technologies, Grand Island, NY) and associated protocols were used. A 475-bp region of the 16S rRNA gene sequence showed the highest similarity to *Francisella* spp., specifically 95% to *Francisella philomira*-

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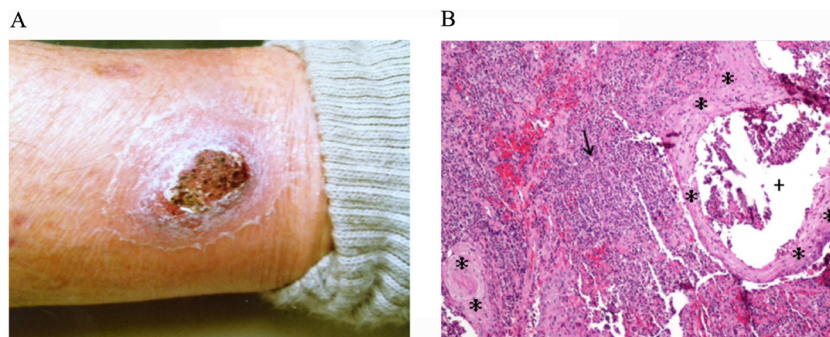


FIG 1 (A) The cutaneous lesion from which *Francisella* sp. LA11-2445 was isolated. (B) Histopathology (100 \times magnification) of a fragmented punch biopsy specimen. The arrow marks a dense, mixed dermal infiltrate of neutrophils, histiocytes, and lymphocytes. An intraepidermal neutrophilic microabscess is shown (+). The asterisks (*) indicate pseudoepitheliomatous epidermal hyperplasia.

gia and 93% to *F. tularensis* when using the MicroSEQ ID 16S rDNA 500 Library and 96% to *F. noatunensis* subsp. *noatunensis* GM2212 (DQ309246) when using GenBank for comparison.

For identification of the *Francisella* species, the isolate was sent to the Centers for Disease Control and Prevention (CDC), Fort Collins, CO. Amplification and sequencing of a \sim 1,300-bp segment of the 16S rRNA gene from the isolate (designated LA11-2445) was performed using primers and PCR conditions described previously (2). Segments of the *pgm*, *rpoA*, *rpoB*, *tpiA*, *groEL*, *sdhA*, and *atpA* genes were amplified and sequenced using PCR primers and conditions described in references 2, 3, and 4. Previous studies have shown that concatenation and analysis of the housekeeping genes chosen for this study yield phylogenies congruent with those based on whole-genome sequences of *Francisella* species (3, 5, 6). Available homologous sequences from *Francisella* species and other gammaproteobacteria were obtained from GenBank for nucleotide comparison. All sequences were trimmed in Lasergene v9.0 (DNASTAR, Madison, WI) and then exported into MEGA v5 (7) to generate alignments, distance comparisons, average nucleotide identity (ANI), and phylogenetic trees using maximum likelihood (ML). The generalized time-reversible nucleotide substitution model with gamma distribution (4 gamma categories) and invariant sites (GTR+I+G) was used for ML analyses. Statistical reliability of nodes was evaluated through bootstrap analysis with 1,000 replicates.

Distance comparison of a 1,205-bp fragment of the 16S rRNA gene sequence revealed that isolate LA11-2445 shared \geq 95.5% sequence identity with all other *Francisella* spp. analyzed, compared to only 89.2% and 88.1% with *Fangia hongkongensis* and *Caedibacter taeniopiralis*, respectively. Maximum 16S rRNA sequence identity (99.56%) was observed between isolate LA11-2445 and *Francisella* sp. GP-2009. Phylogenetic analysis of 16S rRNA gene sequences revealed that LA11-2445 fell into a clade (statistical support of 81%) consisting of *Francisella* organisms associated with marine environments, including (i) GP-2009, an uncultured endosymbiont of a marine ciliate from the Adriatic Sea, (ii) TX077310 and TX077308, two isolates cultured from Gulf of Mexico seawater samples, (iii) Shimane-1 and Miyagi-1, two *F. halioticida* strains isolated from moribund giant abalone in Japan, and (iv) SSW64Au, SSW58Ap, and 46 T0h-oil, three uncultured bacteria from either the Salton Sea or the Berre lagoon, France (Fig. 2).

Incongruent phylogenetic topologies for *Francisellaceae* mem-

bers have previously been shown for 16S rRNA compared to whole-genome sequence data (5). Therefore, in-frame gene sequences amplified from the housekeeping genes, *pgm* (567 bp), *rpoA* (264 bp), *rpoB* (255 bp), *tpiA* (321 bp), *groEL* (837 bp), *sdhA* (444 bp), and *atpA* (318 bp), were analyzed as concatenated sequences. Analysis of both 4- and 7-locus concatenated sequences was performed, as sequence data for all 7 genes of the most closely related *Francisellaceae* members, including *F. halioticida*, were not available in GenBank. Uncultured *Francisella* spp. also could not be included due to the absence of sequence information in GenBank for genes other than 16S rRNA genes. Concatenation of the 4 loci, in the order *pgm*, *rpoA*, *rpoB*, and *tpiA*, generated a 1,596-bp sequence, which was compared to homologous sequences from 17 other *Francisella* strains. Phylogenetic analysis indicated strong statistical support (100%) for the resulting topology, which consisted of two previously recognized clades, clade 1 (*F. philomiragia*, *F. noatunensis*, and *Francisella* sp. TX077308) and clade 2 (*F. tularensis*, *F. novicida*, and *F. hispaniensis*), and *F. halioticida* and LA11-2445 formed a separate clade (Fig. 3A). The ANI between LA11-2445 and *F. halioticida* was 89.3% across the four genes, compared to an ANI of 81 to 82% with all other *Francisella* spp. in clade 1 and clade 2.

All 7 loci linked in the order *pgm*, *rpoA*, *rpoB*, *tpiA*, *groEL*, *sdhA*, and *atpA* yielded a 3,014-bp sequence. The same overall tree topology was observed for the 7-locus data set compared with the 4-locus data set, with 93 to 100% bootstrap support for clades 1 and 2 and with LA11-2445 separate from both clades 1 and 2 (Fig. 3B). The topologies observed for both the 4-locus and 7-locus phylogenetic trees (clade 1 and clade 2), with the exception of *F. noatunensis* subsp. *orientalis*, are also consistent with topologies generated using whole-genome sequence data (8), suggesting accurate placement of LA11-2445 into the *Francisella* genus via these analyses. These results indicate that *Francisella* sp. LA11-2445 falls into a clade with *F. halioticida* but represents a unique species, as the ANI observed between LA11-2445 and *F. halioticida* was 89.9% across 4 loci, below the 95% cutoff for bacterial species. Comparative analyses have shown that an ANI of 95% corresponds to a 70% reassociation value via DNA-DNA hybridization, the cutoff and method historically used to delineate bacterial species (9).

To look at the susceptibility of LA11-2445 to antibiotics used for treatment of *Francisella* infections, broth microdilution was performed with ciprofloxacin, chloramphenicol, doxycycline,

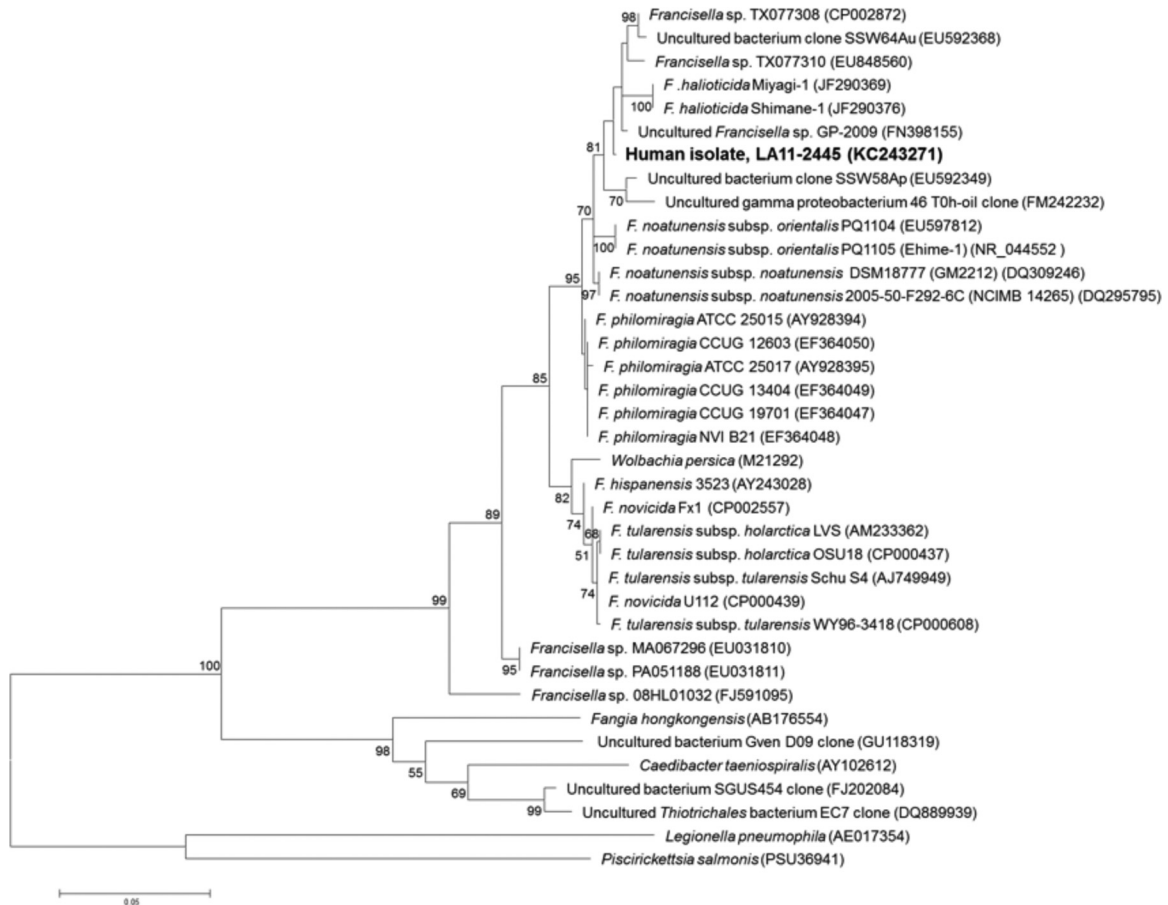


FIG 2 Maximum-likelihood (ML) analysis showing the relationship of the clinical isolate, LA11-2445, to other *Francisellaceae* members based on a 1,205-bp region of the 16S rRNA gene. Bootstrap support values >50% are indicated. The scale bar corresponds to 0.05 substitutions per nucleotide position. GenBank accession numbers are indicated following strain designations.

gentamicin, levofloxacin, streptomycin, and tetracycline, utilizing the Clinical and Laboratory Standards Institute approved susceptibility method and breakpoints for *F. tularensis* (10, 11). MICs to the aforementioned antibiotics were as follows: 0.004 $\mu\text{g/ml}$ for ciprofloxacin, 1.0 $\mu\text{g/ml}$ for chloramphenicol, 0.25 $\mu\text{g/ml}$ for doxycycline, 0.06 $\mu\text{g/ml}$ for gentamicin, 0.008 $\mu\text{g/ml}$ for levofloxacin, 0.5 $\mu\text{g/ml}$ for streptomycin, and 1.0 $\mu\text{g/ml}$ for tetracycline. All fell within the susceptible range, consistent with the patient's positive response to treatment with ciprofloxacin.

Members of the *Francisella* genus range from intracellular pathogens with different host preferences (*F. tularensis*, *F. noatunensis*, and *F. haliotidica*) to specialized endosymbionts (*Francisella*-like endosymbionts) to generalists believed capable of a free-living existence (*F. philomiragia* and *F. novicida*) (8), with only a subset of *Francisella* spp. linked to human illness. The intracellular pathogen *F. tularensis* is the causative agent of tularemia, an acute and fatal illness in animals and humans, whereas *F. noatunensis* and *F. haliotidica* infect and cause death in fish and abalone, respectively (3, 6, 12). *F. novicida* and *F. philomiragia* are infrequent causes of opportunistic infections in compromised individuals or saltwater near-drowning victims and are associated with saltwater or brackish water (13, 14). In recent years, several

unclassified *Francisella* spp., also linked to marine and brackish environments, have been identified based on 16S rRNA gene analysis of uncultured samples or by isolation and characterization of the organism (15–18). To date, none of these *Francisella* species have been associated with human infections.

Here, we identify a *Francisella* species recovered from a human cutaneous lesion. Results of phylogenetic analyses, including an ANI of only 89.8% across 4 housekeeping genes to *F. haliotidica*, the closest *Francisellaceae* member for which sequence is available, are consistent with this organism representing a novel *Francisella* species. Further description of this *Francisella* sp. is limited by the lack of similar strains and the availability of gene sequences, other than 16S rRNA gene sequences, for closely related uncultured *Francisella* species. *F. haliotidica* and the uncultured *Francisella* spp. are all associated with marine environments. Consistent with this, *Francisella* sp. LA11-2445 was determined to be slightly halophilic, growing better in the presence of 6% NaCl than in the absence of NaCl.

Our findings are consistent with the bacterium being responsible for the patient's infection, as this *Francisella* sp. was the only organism isolated from the ankle lesion, the lesion showed marked inflammation, and the patient responded positively to treatment with ciprofloxacin. The mode of transmission is unclear. Given the relatedness of this organism with other *Francisella*

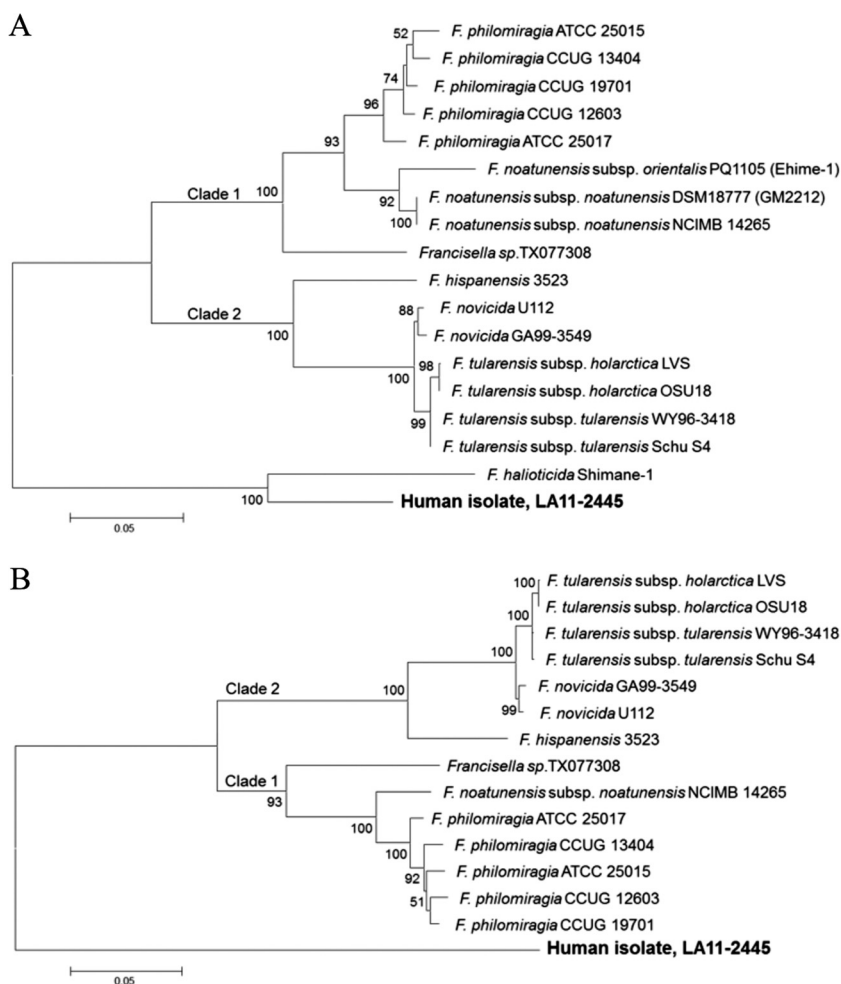


FIG 3 Maximum-likelihood (ML) analysis showing the relationship of the clinical isolate, LA11-2445, to other *Francisellaceae* members based on concatenated *pgm*, *rpoA*, *rpoB*, and *tpiA* gene sequences (A) and concatenated *pgm*, *rpoA*, *rpoB*, *tpiA*, *groEL*, *sdhA*, and *atpA* gene sequences (B). Bootstrap support values of >50% are shown. The scale bars correspond to 0.05 substitutions per nucleotide position. GenBank accession numbers for the *pgm*, *rpoA*, *rpoB*, *tpiA*, *groEL*, *sdhA*, and *atpA* gene sequences from LA11-2445 are KC243274, KC243275, KC243276, KC243278, KC243272, KC243273, and KC243277, respectively.

spp. associated with marine environments and the patient's residing near brackish waterways, we hypothesize that the bacterium was introduced from the environment into an open wound caused by the patient's tree-cutting activities.

The phylogenetic landscape of *Francisella* continues to expand with the availability of genomic sequencing and publicly available sequence repositories. Although *Francisella* was once thought to be a fairly small genus comprised of only three species (*F. tularensis*, *F. novicida*, and *F. philomiragia*), it has become increasingly clear in the last decade that additional *Francisella* spp. are widespread in the environment, with some of these species having the potential to cause infections in humans.

Nucleotide sequence accession numbers. The 16S rRNA, *pgm*, *rpoA*, *rpoB*, *tpiA*, *groEL*, *sdhA*, and *atpA* gene sequences from LA11-2445 were deposited in GenBank under accession numbers [KC243271](#), [KC243274](#), [KC243275](#), [KC243276](#), [KC243278](#), [KC243272](#), [KC243273](#), and [KC243277](#), respectively.

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The findings and conclusions in this report are those of the authors

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