

Phylogenetic Analysis of *Francisella tularensis* Group A.II Isolates from 5 Patients with Tularemia, Arizona, USA, 2015–2017

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We examined 5 tularemia cases in Arizona, USA, during 2015–2017. All were caused by *Francisella tularensis* group A.II. Genetically similar isolates were found across large spatial and temporal distances, suggesting that group A.II strains are dispersed across long distances by wind and exhibit low replication rates in the environment.

Francisella tularensis, a Tier 1 select agent (1), has 3 subspecies: *tularensis* (type A), *holarctica* (type B), and *mediasiatica* (Appendix 1 Figure, <https://wwwnc.cdc.gov/EID/article/25/5/18-0363-App1.pdf>). In humans, disease is caused by type A and type B. Type B is found throughout the Northern Hemisphere, type A only in North America, and *mediasiatica* only in central Asia (2). Type A is divided into 2 distinct subgroups, A.I and A.II (Appendix 1 Figure), that have little geographic overlap (3,4). A.II is found primarily in the mountainous region of western North America (3,4) and A.I throughout the central eastern regions and along the West Coast (3–5). Observational human data and limited experimental mouse data suggest A.II is less virulent than A.I but potentially more virulent

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than type B (6,7). Here, we describe 5 patients in Arizona, USA, during 2015–2017 with cases of tularemia (1 fatal), all caused by A.II (Appendix 2, <https://wwwnc.cdc.gov/EID/article/25/5/18-0363-App2.xlsx>).

The Study

Case-patient 1 was a 57-year-old previously healthy man who sought treatment July 12, 2015, for chills and an acute onset of fever >40°C. Five days before symptom onset, while camping at the northern rim of Grand Canyon National Park, he noted a small wound at the lateral aspect of his left elbow consistent with an insect bite. Cellulitis with regional lymphadenopathy developed on his left forearm, extending to the left axillary region. After surgical irrigation, debridement of the wound (August 8), and oral doxycycline treatment upon discharge, the patient fully recovered.

Case-patient 2 was a 55-year-old previously healthy woman who sought treatment on July 20, 2015, for sore throat and an acute onset of fever >40°C. She reported no outdoor activity except being in a Coconino County park 4 days before symptom onset. Despite receiving treatment with amoxicillin, her fever persisted; she returned 4 days later with myalgia, fatigue, headaches, and emesis. Her therapy was switched to sulfamethoxazole/trimethoprim, amoxicillin/clavulanate, and ceftriaxone. A 2-day hospitalization revealed left axillary lymphadenopathy with associated cellulitis in her left chest wall and breast. Her fever resolved with intravenous ceftriaxone and gentamicin. She received oral doxycycline upon discharge and fully recovered.

Case-patient 3 was a 73-year-old Coconino County woman with previous health conditions. She sought treatment in the summer of 2016 and died several days later (Appendix 2 Table). Details about this case-patient are presented elsewhere (8).

Case-patient 4 was a 24-year-old previously healthy woman from Navajo County who sought treatment in November 2016. A cat bite was the suspected source of infection, but the cat was euthanized without testing. Severe

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swelling and lymphadenopathy developed at the site of the bite; the patient was treated with antimicrobial drugs and recovered.

Case-patient 5 was a 52-year-old man who resided and traveled between both Coconino County and Pinal County. He sought treatment for dizziness, nausea, chills, headache, and body aches in June 2017. He was initially treated with antipyretics but returned to the hospital 2 days later. At this visit, he received treatment with several antimicrobial drugs and recovered. The source of his infection is unknown.

All illnesses were classified as ulceroglandular tularemia except the one in case-patient 3, which was classified as respiratory tularemia. Recovered isolates from all 5 patients tested positive for *F. tularensis* group A.II by PCR (Appendix 2 Table).

Comparisons of whole-genome sequencing and geographic data (Appendix 1) for these 5 isolates and 9 other A.II isolates (Appendix 2 Table) revealed 2 patterns. First, the 5 cases in humans during 2015–2017 were caused by isolates from distinct clades (Figure, panel A). The isolates in case-patients 2 and 3, who probably acquired the infection in the same city, were assigned to 2 different major phylogenetic clades (A.II.2 and A.II.8), suggesting distinct clades co-occur locally in the environment, a finding similar to that observed with type B and group A.I (9,10). Second, some closely related isolates were distant in geographic and temporal space (Figure, panels A, B). Isolates from case-patients 3 and 4 are highly similar, differing by

just 1 single-nucleotide polymorphism (SNP) across the core genome, despite being isolated >150 km and 5 months apart; they also differ by just 3–4 SNPs from a case that occurred in another location 9 years previous in 2007 (no. 8; Figure). Likewise, isolates from case-patients 2 and 5 differ by just 4 SNPs, despite being isolated from distant locations 2 years apart.

The geographic pattern suggests *F. tularensis* group A.II might be capable of long-distance dispersal, perhaps by wind, as has been suggested for type B (9,11). The temporal pattern, which also has been observed for type B (9,11), is consistent with a low evolutionary rate for A.II strains in the environment. This pattern suggests group A.II strains may persist in the environment in a dormant state, such as the viable but nonculturable state that has been described for type B (12).

Consistent with a low evolutionary rate in the environment, groups A.I and A.II appear to be highly monomorphic and have much less genetic variation than type B (Appendix 1 Figure). Type B was previously described as being less genetically diverse than type A as a whole when groups A.I and A.II were considered together (2). However, A.I and A.II are separated by large SNP distances with no intermediate lineages (Appendix 1 Figure), verifying these groups as highly distinct and warranting their analysis as separate groups.

In this study, just 309 SNPs were discovered among 14 A.II isolates separated by considerable geographic

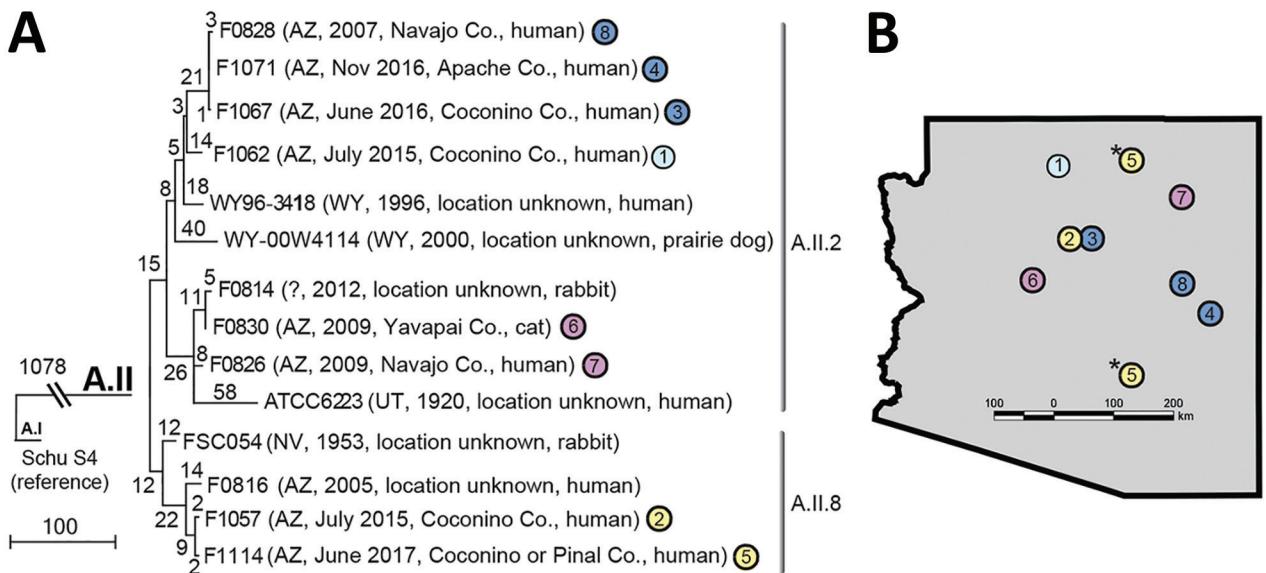


Figure. Phylogeny and geographic distribution of *Francisella tularensis* isolates, Arizona, 2005–2017. A) Maximum-parsimony tree of 14 *F. tularensis* subsp. *tularensis* A.II isolates from humans and other mammals constructed by using single-nucleotide polymorphisms (SNPs) discovered by whole-genome sequencing. The tree is rooted on A.I strain Schu S4. Scale bar indicates number of SNPs. Numbers along branches also indicate the number of SNPs the branches represent. Closely related isolates are indicated with circles of the same color (also indicated in panel B). Numbers within circles correspond to the identification numbers in Appendix 2 Table (<https://wwwnc.cdc.gov/EID/article/25/5/18-0363-App2.xlsx>). B) Known or suspected geographic origins of tularemia cases in Arizona. *Case 5 is represented twice to reflect the 2 possible geographic sources of this infection. Co., county.

(maximum >1,000 km) and temporal (maximum 96 years) distances (Figure, panel A). In a previous study (13), just 295 SNPs were discovered among 14 A.I isolates separated by similar temporal distances (maximum 65 years) and an even greater geographic distance (maximum >2,800 km). In contrast, type B exhibits much more diversity across smaller geographic and temporal scales. For example, 735 SNP differences were found in an analysis of 10 isolates from a respiratory tularemia outbreak in Sweden (9), even though the temporal (maximum 1 year) and geographic (maximum ≈201 km) distances among these isolates were much smaller. The patterns observed with group A.II isolates suggest that, as has been suggested for type B (9,11), both group A.I and A.II strains might also persist long term in the environment in a dormant state where replication is nonexistent or greatly arrested.

A.II appears to be the main and perhaps only group of *F. tularensis* present in the environment in Arizona, although group A.I and type B are known to be present in neighboring states (14). However, all available archival isolates from human and wildlife sources in Arizona (Appendix 2 Table) were assigned to the A.II group (Appendix 1), consistent with other reports, indicating the presence of only group A.II from animal and human sources from Arizona (3,4). In 2000, type B isolates were obtained from captive animals in an Arizona zoo, but these infections were suspected to be imported rather than locally acquired (2).

Conclusions

In summary, we report 5 cases of tularemia in humans (including 1 fatality) that occurred in Arizona during 2015–2017, and all were caused by A.II isolates. Phylogeographic patterns suggest *F. tularensis* A.II strains might persist in the environment in a dormant state and be dispersed long distances, perhaps by wind.

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About the Author

Dr. Birdsell is an associate director at the Pathogen and Microbiome Institute, Northern Arizona University, in Flagstaff, Arizona. Her primary research interests are the evolution and phylogeography of *F. tularensis*.

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Appendix

***Francisella tularensis* Culture, DNA Extraction, and Assignment to Major Genetic Groups**

For case 4 (Northern Arizona University identification no. F1071), *Francisella tularensis* was obtained from the wound site 10 days after the initial onset of symptoms and isolated by culture at a commercial laboratory. The sample was confirmed positive at the Arizona State Public Health Laboratory (Phoenix, Arizona, USA), which provided Northern Arizona University (Flagstaff, Arizona, USA) staff with DNA extracts of the cultured isolate for analysis. Information on the procedures used to confirm *F. tularensis* in each case is presented in Appendix 2 Table (<https://wwwnc.cdc.gov/EID/article/25/5/18-0363-App2.xlsx>).

Isolates were first cultured in the hospitals where patients received medical attention; isolates were sent to Northern Arizona University for additional analysis. We cultured on Cysteine Heart Agar (Remel, <https://www.thermofisher.com>) for 72 hours at 37°C. DNA was extracted by using QIAGEN kits (<https://www.qiagen.com>). DNA extracts of *F. tularensis* isolates were genetically typed into phylogenetic groups by using published assays (1).

DNA Sequencing

We sequenced the DNA extracts of 10 isolates (the 5 isolates we obtained from humans in Arizona and 5 archival isolates from humans, environmental sources, or sources of unknown origin; Appendix 2 Table). DNA library preparations for whole-genome sequencing were performed by using the KAPA Low Throughput Library Preparation Kit with SPRI Solution and

Standard PCR Library Amplification/Illumina series (KK8232; KAPA Biosystems, <https://www.kapabiosystems.com>) with modifications. The adapters and 8-bp index oligos were based on Kozarewa and Turner (2) and purchased from Integrated DNA Technologies (<https://www.idtdna.com>). The quality and quantity of genomic DNA was evaluated by 0.7% agarose gel analysis. We fragmented ≈ 1 μg of DNA per sample with a SonicMan (Matrical Technologies, <https://www.matrical.in>) using the following parameters: 75.0-s prechill, 16 cycles, 10.0-s sonication, 100% power, 75.0-s lid chill, 10.0-s plate chill, and 75.0-s postchill. The sonicated DNA was size selected to target 600–650 bp by performing fragment separation with Agencourt AMPure XP beads (A63882; Beckman Coulter, <https://www.beckmancoulter.com>); the fragmented DNA was eluted into 42.5 μL of elution buffer.

During the enzymatic steps, half the enzyme volume was used with the full volume of buffer as described in the KAPA's library preparation protocol. For library preparation, 3 separate reactions were carried out for each sample. In the first reaction (end repair), 2.5 μL of enzyme and 5 μL of buffer were used, followed by a 1.6 \times AMPure XP bead cleanup and elution in 43.5 μL of elution buffer. In the second reaction (A-tailing), 1.5 μL of enzyme and 5 μL of buffer were used, followed by a 1.6 \times AMPure XP bead cleanup and elution in 36.5 μL of elution buffer. In the third reaction (quick ligation), we used 2.5 μL of enzyme, 10 μL of buffer, and 1 μL of 10 μM adaptor oligo mix (2), followed by a 0.9 \times AMPure XP bead cleanup and elution in 30 μL of elution buffer. The PCR was optimized to improve yield and genome coverage.

We combined 2 μL of DNA, 2 μL of each 10 μM indexing primer, 25 μL of KAPA 2 \times HIFI PCR Master Mix (KAPA Biosystems), and 19 μL of molecular grade water and used the following PCR parameters: 2 min at 98 $^{\circ}\text{C}$ (initial denaturation); 8 cycles of 30 s at 98 $^{\circ}\text{C}$, 20 s at 65 $^{\circ}\text{C}$, and 30 s at 72 $^{\circ}\text{C}$ (amplification); and 5 min at 72 $^{\circ}\text{C}$ (final extension). The libraries were purified with a 0.9 \times AMPure XP bead cleanup and eluted into 50.0 μL of elution buffer. The final libraries were quantified by using the KAPA ABI Prism Library Quantification Kit (KK4835; KAPA Biosystems) and pooled together at equimolar concentrations.

The pool was quantified by using the KAPA ABI Prism Library Quantification Kit, and the quality of the pool was assessed with the Agilent DNA 1000 Kit (5067-1504; Agilent

Technologies, <https://www.agilent.com>) on the Bioanalyzer DNA 1000 Chip (Agilent Technologies). The samples, spiked with 1% PhiX, were sequenced on an Illumina MiSeq instrument by using the 600-cycle MiSeq Reagent Kit v3 (MS-102-3003; Illumina, <https://www.illumina.com>) with the standard Illumina procedure. The appropriate sequencing primers were added to the MiSeq kit as described (3). Sequence data was submitted to NCBI under BioProject PRJNA398413 (Sequence Read Archive accession nos. SRR5937764–SRR5937772, SRR6205344; Appendix 2 Table).

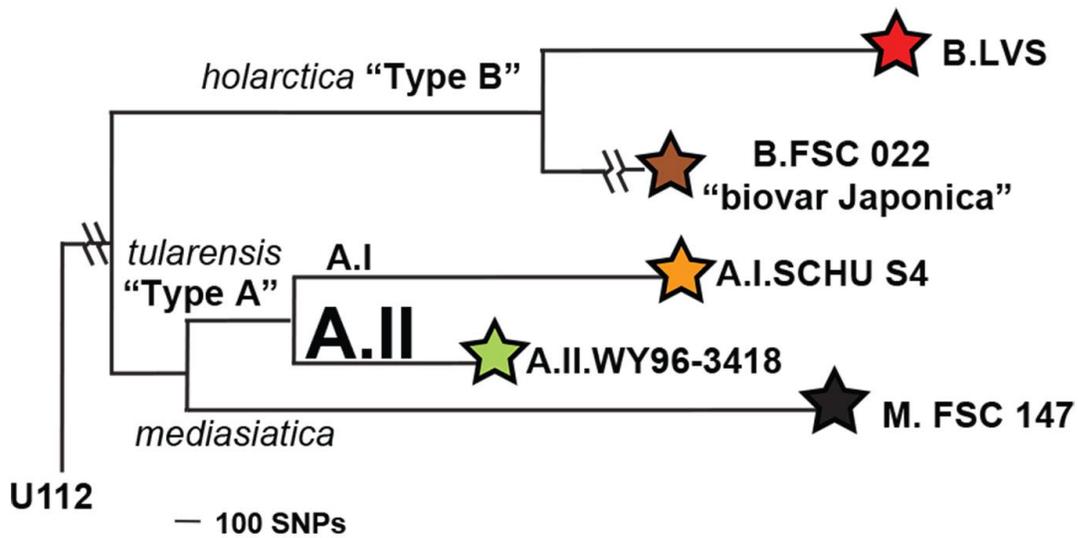
Single-Nucleotide Polymorphism Discovery and Phylogeny

Core genome single-nucleotide polymorphisms (SNPs) were identified among *F. tularensis* genomes with NASP (4). Reads were aligned to the reference assembly (GenBank accession no. NC_006570.2) with Bowtie 2 (used for SNP identification) (5). If reads were not available for a genome, paired Illumina reads were simulated for the genome with ART (6). SNPs were identified with the UnifiedGenotyper method in Genome Analysis Toolkit (7,8). SNPs were removed from the matrix if the read depth was $<10\times$ or the allele frequency proportion was $<90\%$. SNPs identified in duplicated regions of the reference assembly with NUCmer self-alignments (9,10) were filtered from the SNP matrix. A total of 1,308 SNPs were called from a 1,140,352-bp core genome alignment (NASP quality_breadth). Two SNPs were phylogenetically conflicting. A maximum parsimony tree was inferred on high-quality, core genome SNPs with MEGA 6.0 (11). SCHU S4 (GenBank accession no. NC_006570.2) was the outgroup for the phylogenetic analyses.

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Appendix Figure. Phylogenetic tree indicating the major genetic groups and subgroups within *Francisella tularensis*. Colored stars indicate representative whole-genome sequences from these different groups, which were used to construct this phylogeny. SNP, single-nucleotide polymorphism.