Water as Source of Francisella tularensis Infection in Humans, Turkey

Selcuk Kilic,1 Dawn N. Birdsell,1 Alper Karagöz, Bekir Çelebi, Zekiye Bakkaloglu, Muzaffer Arikan, Jason W. Sahil, Cedar Mitchell, Andrew Rivera, Sara Maltinsky, Paul Keim, Duran Üstek, Rıza Durmaz, David M. Wagner

Francisella tularensis DNA extractions and isolates from the environment and humans were genetically characterized to elucidate environmental sources that cause human tularemia in Turkey. Extensive genetic diversity consistent with genotypes from human outbreaks was identified in environmental samples and confirmed water as a source of human tularemia in Turkey.

Tularemia is a disease caused primarily by 2 subspecies of Francisella tularensis: F. tularensis subsp. tularensis, which is restricted to North America; and F. tularensis subsp. holarctica, which is found widely throughout the northern hemisphere but is the only subspecies in most of Eurasia (1). Through whole-genome sequencing and canonical single-nucleotide polymorphism (canSNP) genotyping, F. tularensis subsp. holarctica has been divided into 4 major genetic groups (B.4, B.6, B.12, and B.16) consisting of multiple subgroups (Figure 1) (1–3). Geographic distribution of these subgroups in Europe, Japan, and the USA are well described (1–3).

The phylogeography of F. tularensis in Asia is poorly understood because of undersampling in many regions, but recent studies have revealed new insights. A report has described rich phylogenetic diversity of the bacterium in China (4), including the rare B.16 group (biovar japonica). Previously, B.16 was known only in Japan (1) and Turkey (6). Sweden reportedly has the highest overall phylogenetic diversity among regions worldwide (2).

In Turkey, tularemia cases in humans have increased since 2009 (7), but little is known about environmental sources. Tularemia was first reported in Turkey in 1936 and then was sporadically reported for several decades (7). After improved surveillance, the number of tularemia cases increased in the 1980s and led to registration of tularemia as a reportable disease in 2004 (7,8). Incidence has continued to increase since then (7), and tularemia is now considered a reemerging zoonotic disease in Turkey.

Patients with oropharyngeal signs and symptoms account for >90% of tularemia cases in Turkey (8), and cases emerge seasonally from August–March (7). Seasonality of incidence of cases is presumably associated with consumption of contaminated water (9), but confirming sources is difficult. Reports of confirmation of F. tularensis from water samples by PCR (10) or culture (6) are rare, and definitive studies that link water to tularemia in humans are lacking. How water sources become seasonally contaminated is also unknown, but contamination could be caused by rodents. Recently, F. tularensis was confirmed by PCR from 2 mice captured in Thrace (11), but in Turkey, confirmation has not been obtained from ticks or mosquitoes, which are known vectors of F. tularensis (1,4).

Genetic characterization of clinical samples from tularemia outbreaks in Turkey in 2011 showed that multiple phylogenetic groups cause disease in multiple regions across Turkey (5); however, no environmental samples were assessed in that study. We report our findings from genetically characterized samples positive for F. tularensis from environmental and human sources located in multiple active tularemia areas in Turkey. Our results provide new insights into F. tularensis transmission from environmental sources to humans.

The Study
To examine environmental reservoirs that could be possible sources for human infections, during 2010–2013, we sampled water sources and rodent populations from suspected sites where transmission of F. tularensis infection could occur in Turkey. To survey and compare phylogenetic diversity of environmental samples and clinical samples, we examined 33 clinical samples of mostly oropharyngeal tularemia cases from approximately the same sites where environmental samples were collected. DNA was extracted (DNeasy Blood & Tissue Kit, QIAGEN GmbH, Hilden, Germany) from 6 water, 1 rodent spleen, and 33 human samples (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0634-Techapp.pdf).

The extractions were confirmed F. tularensis–positive by using PCR and targeting the tul4 gene (12). Analysis

Author affiliations: Public Health Institution of Turkey, Ankara, Turkey (S. Kilic, A. Karagöz, B. Çelebi, Z. Bakkaloglu, R. Durmaz); Northern Arizona University, Flagstaff, Arizona, USA (D.N. Birdsell, J.W. Sahil, C. Mitchell, A. Rivera, S. Maltinsky, P. Keim, D.M. Wagner); Istanbul University, Istanbul, Turkey (M. Arikan); Medipol University, Istanbul (D. Üstek)

DOI: http://dx.doi.org/10.3201/eid2112.150634

1These authors contributed equally to this article.
by using 21 published canSNP assays, as previously described (5), assigned these samples to 3 major phylogenetic groups and distinct subgroups: B.16 (n = 11); B.6 (2 subgroups: B.6/7/10, n = 1; and B.10/11, n = 6); and B.13 (2 subgroups: B.27, n = 5; and B.20/21/33, n = 17) (Figure 1; online Technical Appendix Table 1). Of the subgroups, 3 were previously unknown in Turkey: B.6/7/10, B.10/11, and B.16. The 7 environmental samples collected included most of the known phylogenetic diversity in Turkey and represented the 3 major groups: B16, B6 (B.6/7/10 and B.10/11), and B.13 (the group previously known to be in Turkey). Of the subgroups identified, all but B.6/7/10 were also found in the human samples.

To determine detailed associations between environmental and human clinical samples, we examined the genetic diversity among these samples by using multilocus variable number of tandem-repeats analysis (MLVA) (13). All samples contained a single MLVA genotype (online Technical Appendix Figure, panels A–C); no mixed allele calls were observed at any of the examined variable number of tandem-repeats loci. Three different environmental samples (F0922, F0910, and F0916) had canSNP and MLVA genotypes that were identical to those of clinical samples (online Technical Appendix Table 1). In 2 instances (F0910 and F0916), the environmental sample and its respective genetically identical clinical sample(s) were recovered from different geographic regions, resulting in identical genotypes being found in different localities and suggesting that close genotypes are dispersed widely in Turkey. One environmental sample (F0922) had genetic, geographic, and temporal data (online Technical Appendix Figure, panel A) concordant with data from human samples. This water sample shared identical canSNP and MLVA genotypes with 5 clinical samples recovered 2 weeks previously at the same locality, strongly suggesting that the human cases are linked with this infected water source.

The genetic characterization of *F. tularensis* from environmental sources provides insights into transmission of tularemia from the environment to humans, but little is known about how water is contaminated. The seasonal

---

**Figure 1.** Phylogeography of *Francisella tularensis* subsp. *holarctica*. A) Global distribution of known phylogenetic groups determined on the basis of previous studies (2–4); enlarged map of Turkey shows locations of phylogenetic groups identified among the 40 samples positive for *F. tularensis* examined in this and previous studies (5). Circle size indicates number of samples (small circles, 1–3; medium circles, 4–6; large circles, 7–9). Colors of circles (human samples) and triangles (environmental samples) represent the phylogenetic subgroups to which these samples were assigned (see panel B). Subgroup B.16 (biovar *japonica*) is represented by the dot inside the brown circles and triangles. B) Phylogenetic tree for *F. tularensis* subsp. *holarctica* constructed on the basis of current canonical single-nucleotide polymorphism genotyping. Red numbers indicate nomenclature of canonical single-nucleotide polymorphism groups. Terminal subgroups representing sequenced strains are shown as stars, and intervening nodes representing collapsed branches are indicated by circles. Countries of origin for samples assigned to relevant phylogenetic groups are as follows: AUT, Austria; CE, central Europe, unknown country; CHN, China; CZE, Czech Republic; DEU, Germany; FIN, Finland; GEO, Georgia; HUN, Hungary; ITA, Italy; NOR Norway; ROU, Romania; RUS, Russia; SWE, Sweden; TUR, Turkey; UKR, Ukraine; USA, United States. CHN* indicates approximate phylogenetic placement because of a lack of resolved information on single-nucleotide polymorphisms (4). TUR** indicates identification from a previous study (5).
Francisella tularensis Infection in Humans, Turkey

nature of human outbreaks suggests that water sources are not constant reservoirs but rather are contaminated by another source. Rodents were identified as reservoirs (21% tularemia positive) in Bulgaria, where mainly oropharyngeal tularemia is endemic (14). We found a rodent sample (F0910) with canSNP and MLVA genotypes identical to an oropharyngeal clinical sample (F0898) (online Technical Appendix Table 1), a finding consistent with water contamination that originates from animal sources. However, the converse is also possible: animals could become infected by contaminated water.

Analysis of the 7 environmental F. tularensis subsp. holarctica samples from Turkey revealed extensive phylogenetic diversity that represents most known major groups in the world. Three of the 4 major F. tularensis subsp. holarctica phylogenetic groups (B.4, B.6, B.12, and B.16) are found in Turkey, including the highly basal B.16 group (biovar japonica) (Figure 1). This finding indicates that no single phylogenetic type is dominant in Turkey, unlike in Western Europe (3). Diversity was also represented in the clinical samples, suggesting that all major groups have similar capacities to cause disease, as other studies have suggested (15).

To gain insights into the evolutionary origin of the B.16 group, we examined the phylogenetic relationships among 3 published B.16 strains: 1 from Turkey (PHIT_FT049) (6) and 2 from Japan (FSC021 and FSC022) (GenBank accession nos. CP007148.1, SRX147922, and DS264138.1, respectively; Figure 2). We generated a global core-genome SNP phylogeny (online Technical Appendix) for these 3 B.16 strains and 5 strains from other groups (online Technical Appendix Table 2). As expected, PHIT-FT049 clusters with the Japanese B.16 strains from Japan and shares 448 putative SNPs; however, it is also distinct from the 2 strains from Japan, which together share 640 putative SNPs (Figure 2). The distinctiveness of the B.16 strain from Turkey strongly suggests that it has an evolutionary history different from that of the Japanese strains. The MLVA phylogeny of B.16 strains (online Technical Appendix Table 1) reveals greater diversity among the 8 strains from Japan than among the 8 strains from Turkey. These data show that the B.16 strains from Turkey and Japan are highly distinct, and the greater diversity in strains from Japan supports the possibility that the place of ancestral origin of the B.16 group is Asia.

Conclusions

Phylogenetically diverse strains of F. tularensis subsp. holarctica are environmentally established in Turkey and cause human disease. The strains in Turkey now include many phylogenetic groups previously found only in Scandinavia or Asia.

Acknowledgments

We thank Charles Williamson, Katy Califf, Bridget Barker, and Heidie Hornstra-O’Neill for assistance with the manuscript.

This study was funded by the US Department of Homeland Security, Science and Technology Directorate, Award NBCH2070001, and by the Cowden Endowment in Microbiology at Northern Arizona University.

Dr. Kilic is a professor and a principal investigator of F. tularensis at the Public Health Institution of Turkey, National Tularemia Reference Laboratory, Ankara, Turkey. His research interests include the evolution, epidemiology, and control of bacterial zoonoses.

References

1. Vogler AJ, Birdsell D, Price LB, Bowers JR, Beckstrom-Sternberg SM, Auerbach RK, et al. Phylogeography of Francisella tularensis: global expansion of a highly fit clone. J Bacteriol. 2009;191:2474–84. http://dx.doi.org/10.1128/JB.01786-08
2. Karlsson E, Svensson K, Lindgren P, Byström M, Sjödin A, Forsman M, et al. The phylogeographic pattern of Francisella tularensis in Sweden indicates a Scandinavian origin of EuroSiberian tularemia. Environ Microbiol. 2013;15:634–45. http://dx.doi.org/10.1111/1462-2920.12052

3. Gyurahnecz M, Birdsell DN, Splęttstoesser W, Seibold E, Beckstrom-Stenbreg SM, Makrai L, et al. Phyllogeography of Francisella tularensis ssp. holarctica, Europe. Emerg Infect Dis. 2012;18:290–3. http://dx.doi.org/10.3201/eid1802.111305

4. Wang Y, Peng Y, Hai R, Xia L, Li H, Zhang Z, et al. Diversity of Francisella tularensis subsp. holarctica lineages, China. Emerg Infect Dis. 2014;20:1191–4. http://dx.doi.org/10.3201/eid2007.130931

5. Öz Sürekci Y, Birdsell DN, Çelik M, Karadağ-Oncel E, Johansson A, Forsman M, et al. Phylogenetically diverse Francisella tularensis strains cause human tularemia in Turkey. Emerg Infect Dis. 2015;21:173–5. http://dx.doi.org/10.3201/eid2101.141087

6. Kiliç S, Celebi B, Acar B, Atas M. In vitro susceptibility of isolates of Francisella tularensis from Turkey. Scand J Infect Dis. 2013;45:337–41. http://dx.doi.org/10.3109/00365548.2012.751125

7. Kiliç S. Tularemia: the pathogen and epidemiology [in Turkish]. Turkiye Klinikleri J.E.N.T–Special Topics. 2014;7:52–61.

8. Erdem H, Ozturk-Engin D, Yesilyurt M, Karabay O, Elaldi N, Celebi G, et al. Evaluation of tularemia courses: a multicentre study from Turkey. Clin Microbiol Infect. 2014;20:O1042–51. http://dx.doi.org/10.1111/1469-0691.12741

9. Willeke A, Meric M, Grunow R, Sayan M, Finke EJ, Splęttstoesser W, et al. An outbreak of oropharyngeal tularemia linked to natural spring water. J Med Microbiol. 2009;58:112–6. http://dx.doi.org/10.1099/jmm.0.002279-0

10. Ulu Kiliç A, Kiliç S, Sencan I, Cieek Sentürk G, Gürbüz Y, Tütüncü EE, et al. A water-borne tularemia outbreak caused by Francisella tularensis subsp. holarctica in Central Anatolia region [in Turkish]. Mikrobiyol Bul. 2011;45:234–47.

11. Unal Yılmaz G, Gurcan S, Özkan B, Karadenizli A. Investigation of the presence of Francisella tularensis by culture, serology and molecular methods in mice of Thrace Region, Turkey [in Turkish]. Mikrobiyol Bul. 2014;48:213–22. http://dx.doi.org/10.5578/mb.7028

12. Sjöstedt A, Knoppa K, Johannson T, Sandström G. The 17 kDa lipoprotein and encoding gene of Francisella tularensis LVS are conserved in strains of Francisella tularensis. Microb Pathog.

13. Vogler AJ, Birdsell D, Wagner DM, Keim P. An optimized, multiplexed multi-locus variable-number tandem repeat analysis system for genotyping Francisella tularensis. Lett Appl Microbiol. 2009;49:140–4. http://dx.doi.org/10.1111/j.1472-765X.2008.02484.x

14. Christova I, Gladnishka T. Prevalence of infection with Francisella tularensis, Borrelia burgdorferi sensu lato and Anaplasma phagocytophilum in rodents from an endemic focus of tularemia in Bulgaria. Ann Agric Environ Med. 2005;12:149–52.

15. Johansson A, Lärkeryd A, Widerström M, Mörtberg S, Myrtännäs K, Öhrman C, et al. An outbreak of respiratory tularemia caused by diverse clones of Francisella tularensis. Clin Infect Dis. 2014;59:1546–53. http://dx.doi.org/10.1093/cid/ciu621

Address for correspondence: David M. Wagner, Northern Arizona University, PO Box 4073, Flagstaff, AZ 86011, USA; email: Dave.Wagner@nau.edu
Water as Source of *Francisella tularensis* Infection in Humans, Turkey

Technical Appendix

Details of Samples and Reference Strains in This Study

Detailed Methods for Constructing the Phylogeny in Figure 2

Published reference genome assemblies (Technical Appendix Table 2) were downloaded from GenBank (1). Assemblies were aligned against the reference genome, *F. tularensis* subsp. *holarctica* OSU18, by using MUMer (2). The reference genome was also aligned against itself; regions that aligned >1 time represent duplication events and were filtered from downstream analyses. Single-nucleotide polymorphisms compared with the reference were concatenated, and a maximum-parsimony phylogeny (Figure 2) was inferred on a concatenation of ≈15,000 single-nucleotide polymorphisms by using Phangorn (3).

**Technical Appendix Table 1.** Details of samples from study of *Francisella tularensis* infection, Turkey

| Original ID* | NAU ID† | County/Region | City | Source | Sample Type | Date       | SNP subgroup‡ |
|--------------|---------|----------------|------|--------|-------------|------------|--------------|
| PHIT-FT049, F283§ | F0915   | Central Anatolia | Ankara | Water | DNA extract from isolate cultured from water | 3/12/2012 | B.16         |
| F059         | F0892   | Aegean          | Afyonkarahisar | Human lymph node | DNA extract from clinical sample | 2/19/2010 | B.16         |
| F060         | F0893   | Aegean          | Afyonkarahisar | Human lymph node | DNA extract from clinical sample | 2/19/2010 | B.16         |
| F062         | F0894   | Aegean          | Afyonkarahisar | Human lymph node | DNA extract from clinical sample | 2/19/2010 | B.16         |
| F063         | F0895   | Aegean          | Afyonkarahisar | Human lymph node | DNA extract from clinical sample | 2/19/2010 | B.16         |
| F064         | F0896   | Aegean          | Afyonkarahisar | Human lymph node | DNA extract from clinical sample | 2/19/2010 | B.16         |
| F069         | F0899   | Central Anatolia | Kayseri | Human lymph node | DNA extract from clinical sample | 2/24/2010 | B.16         |
| F071         | F0900   | Aegean          | Afyonkarahisar | Human lymph node | DNA extract from clinical sample | 2/26/2010 | B.16         |
| F072         | F0901   | Aegean          | Afyonkarahisar | Human lymph node | DNA extract from clinical sample | 2/26/2010 | B.16         |
| F085         | F0902   | Aegean          | Afyonkarahisar | Human lymph node | DNA extract from clinical sample | 3/19/2010 | B.16         |
| F272         | F0912   | Central Anatolia | Çankırı | Human throat swab | DNA extract from clinical sample | 2/8/2012 | B.16         |
| F015         | F0884   | Central Anatolia | Çankırı | Human throat swab | DNA extract from isolate cultured from human | 1/8/2010 | B.28/29     |
| F026         | F0885   | Black Sea       | Amasya | Human throat swab | DNA extract from isolate cultured from human | 1/18/2010 | B.28/29     |
| F217         | F0907   | Eastern Anatolia | Sivas  | Human throat swab | DNA extract from isolate cultured from human | 4/12/2011 | B.28/29     |
| F043         | F0890   | Central Anatolia | Yozgat | Human lymph node | DNA extract from isolate cultured | 1/27/2010 | B.28/29     |
| Original ID* | NAU ID† | County/Region | City | Source | Sample Type | Date       | SNP subgroup‡ |
|-------------|---------|---------------|------|--------|-------------|------------|--------------|
| F039        | F0889   | Black Sea     | Tokat| Human lymph node | DNA extract from isolate cultured from human | 1/22/2010 | B.27/28      |
| F039        | F0923   | Aegean        | Denizli| Water | DNA extract from isolate cultured from water | 12/12/2013 | B.20/21/33   |
| F049        | F0891   | Central Anatolia | Kirsehir| Human throat swab | DNA extract from isolate cultured from water | 2/8/2010 | B.20/21/33   |
| F065        | F0897   | Black Sea     | Tokat| Human lymph node | DNA extract from isolate cultured from human | 2/19/2010 | B.20/21/33   |
| F067        | F0898   | Central Anatolia | Kirikkale| Human conjunctival swab | DNA extract from isolate cultured from human | 2/8/2010 | B.20/21/33   |
| F236        | F0908   | Black Sea     | Ordu | Human throat swab | DNA extract from isolate cultured from human | 4/12/2011 | B.20/21/33   |
| F282        | F0914   | Central Anatolia | Sivas| Water | DNA extract from isolate cultured from water | 3/6/2012 | B.20/21/33   |
| F244        | F0910   | Central Anatolia | Ankara| Rodent/spleen | DNA extract from isolate cultured from rodent | 11/14/2011 | B.20/21/33   |
| F037        | F0888   | Central Anatolia | Corum| Human lymph node | DNA extract from isolate cultured from human | 1/20/2010 | B.20/21/33   |
| F027        | F0886   | Black Sea     | Amasya| Human throat swab | DNA extract from isolate cultured from human | 1/18/2010 | B.20/21/33   |
| F033        | F0887   | Black Sea     | Amasya| Human throat swab | DNA extract from isolate cultured from human | 1/18/2010 | B.20/21/33   |
| F237        | F0909   | Eastern Anatolia | Elazig| Human lymph node | DNA extract from isolate cultured from human | 8/16/2011 | B.20/21/33   |
| F091        | F0903   | Central Anatolia | Yozgat| Human lymph node | DNA extract from isolate cultured from human | 4/13/2010 | B.20/21/33   |
| F159        | F0904   | Central Anatolia | Kayseri| Human blood | DNA extract from isolate cultured from human | 1/29/2011 | B.20/21/33   |
| F285        | F0916   | Eastern Anatolia | Malatya| Water | DNA extract from isolate cultured from water | 4/5/2012 | B.20/21/33   |
| F163        | F0905   | Central Anatolia | Kayseri| Blood | DNA extract from isolate cultured from water | 2/2/2011 | B.20/21/33   |
| F252        | F0911   | Eastern Anatolia | Mus | Human throat swab | DNA extract from isolate cultured from human | 12/15/2011 | B.20/21/33   |
| F176        | F0906   | Eastern Anatolia | Bingöl| Human throat swab | DNA extract from isolate cultured from human | 2/21/2011 | B.20/21/33   |
| F278        | F0913   | Eastern Anatolia | Malatya| Water | DNA extract from isolate cultured from water | 2/21/2012 | B.6/7/10     |
| F293        | F0917   | Eastern Anatolia | Agri | Human throat swab | DNA extract from isolate cultured from human | 2/13/2013 | B.10/11      |
| F294        | F0918   | Eastern Anatolia | Agri | Human throat swab | DNA extract from isolate cultured from human | 2/14/2013 | B.10/11      |
| F295        | F0919   | Eastern Anatolia | Agri | Human throat swab | DNA extract from isolate cultured from human | 2/14/2013 | B.10/11      |
| F297        | F0920   | Eastern Anatolia | Agri | Human throat swab | DNA extract from isolate cultured from human | 2/14/2013 | B.10/11      |
| F292        | F0921   | Eastern Anatolia | Agri | Human throat swab | DNA extract from isolate cultured from human | 2/14/2013 | B.10/11      |
**Technical Appendix Table 2. Reference strains used in study of *Francisella tularensis* infection, Turkey**

| Reference strain | WGS accession no.        |
|------------------|--------------------------|
| FSC022           | AAYD000000000.1           |
| FSC021           | SRX147922                 |
| PHIT_FT049       | CP007148.1                |
| FSC200           | NC_019551.1               |
| LVS              | NC_007880.1               |
| FTNF002-00       | NC_009749.1               |
| OSU18            | NC_008369.1               |
| Schu S4          | NC_006570.2               |

*WGS, whole genome shotgun sequencing data, National Center for Biotechnology Information, Bethesda, MD, USA.*
Technical Appendix Figure. Multilocus variable number of tandem repeats analysis (MLVA) trees constructed on the basis of distance matrix. Environmental samples (water and * rodent source) are indicated with bolded font. Scale bar indicates genetic distance. A) MLVA phylogeny for the B.10/11 group, which is rooted by using the SCHU S4 strain published in GenBank. B) MLVA phylogeny for the B.13 group, which is rooted by using the SCHU S4 strain. C) MLVA phylogeny for the B.16 group, which is rooted by using the SCHU S4 strain.
References

1. Benson DA, Karsch-Mizrachi I, Clark K, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Res. 2012;40:D48–53. http://dx.doi.org/10.1093/nar/gkr1202

2. Delcher AL, Salzberg SL, Phillippy AM. Using MUMmer to identify similar regions in large sequence sets. Curr Protoc Bioinformatics. 2003;Chapter 10:Unit 10.3. http://dx.doi.org/10.1002/0471250953.bi1003s00 PMID: 18428693

3. Schliep KP. phangorn: phylogenetic analysis in R. Bioinformatics. 2011;27:592–3. http://dx.doi.org/10.1093/bioinformatics/btq706

4. Gyuranecz M, Birdsell DN, Splettstoesser W, Seibold E, Beckstrom-Sternberg SM, Makrai L, et al. Phylogeography of Francisella tularensis subsp. holarctica, Europe. Emerg Infect Dis. 2012;18:290–3. http://dx.doi.org/10.3201/eid1802.111305