ENVIRONMENTAL MICROBIOLOGY

Diversity of *Francisella* Species in Environmental Samples from Martha's Vineyard, Massachusetts

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Abstract We determined whether *Francisella* spp. are present in water, sediment, and soil from an active tularemia natural focus on Martha's Vineyard, Massachusetts, during a multiyear outbreak of pneumonic tularemia. Environmental samples were tested by polymerase chain reaction (PCR) targeting *Francisella* species 16S rRNA gene and *succinate dehydrogenase A* (*sdhA*) sequences; evidence of the agent of tularemia was sought by amplification of *Francisella tularensis*-specific sequences for the insertion element IS*FTu*2, 17-kDa protein gene *tul4*, and the 43-kDa outer membrane protein gene *fopA*. Evidence of *F. tularensis* subsp. *tularensis*, the causative agent of the human infections in this outbreak, was not detected from environmental samples despite its active transmission among ticks and animals in the sampling site. *Francisella philomiragia* was frequently detected from a brackish-water pond using *Francisella* species PCR targets, and subsequently *F. philomiragia* was isolated from an individual brackish-water sample. Distinct *Francisella* sp. sequences that are closely related to *F. tularensis* and *Francisella novicida* were detected from samples collected from the brackish-water pond. We conclude that diverse *Francisella* spp. are present in the environment where human cases of pneumonic tularemia occur.

Introduction

An outbreak of pneumonic tularemia was reported from the island of Martha's Vineyard, MA, USA in 2000 [6]; this

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outbreak has since continued, with cases from the island reported annually. In the 8 years of this outbreak, more than 90 tularemia cases have been reported from Martha's Vineyard, of which two thirds presented with pneumonic disease (personal communication, Dr. Bela Matyas, Massachusetts Department of Public Health). This unusual outbreak was preceded by a cluster of pneumonic tularemia cases in 1978 in seven inhabitants of a vacation cottage on the island, as well as five others that were not linked to the cottage [25]. The two pneumonic tularemia outbreaks on Martha's Vineyard emphasize the unique situation on the island as the proportion of pneumonic cases greatly exceeds that generally seen in other endemic regions of the USA. Tularemia in the USA is most commonly associated with an infected tick bite or hunting and skinning of infected mammals (e.g., rabbits, muskrats) and usually manifests as the ulceroglandular or glandular form of the disease [1].

A case–control study prepared by the Centers for Disease Control and Prevention and the Massachusetts Department of Public Health subsequently linked an increased risk of pneumonic tularemia with landscaping activities (OR=6.7; *P*=0.04) [6]. These findings suggested aerosolization of environmental material contaminated with *Francisella tularensis* subsp. *tularensis* (type A), the bacterial pathogen that is responsible for the tularemia cases on Martha's Vineyard based on identification of clinical and animal isolates. Field investigations prompted by the 2000 outbreak, however, failed to detect environmental evidence of this pathogen [6]. Whereas *F. tularensis* subsp. *holarctica* (type B) has been isolated or detected from multiple environmental sources, including water and mud, as well as fecal and urine material excreted by infected mammals, it is unclear whether type A has the same capacity for prolonged environmental survivability [3, 4, 14–16, 19, 22]. The nature of the fomites that serve as

the basis for pneumonic exposures on Martha's Vineyard remains undescribed.

To determine whether *F. tularensis* type A may accumulate in the environment in a Martha's Vineyard field site where it is intensely enzootic [5, 10, 12], we collected samples from or near permanent fresh and brackish-water body sources and analyzed these samples by polymerase chain reaction (PCR) for evidence of *Francisella* species. In addition, we described the diversity of *Francisella* spp. within environmental samples collected from this coastal site.

Materials and Methods

Study Site

Martha's Vineyard is a 227-km^2 island off the coast of Massachusetts, USA. Our field site in the Town of Chilmark has been intensively studied since 2000, when pneumonic tularemia cases were first reported from the island. This site comprises a known natural focus for the agent of tularemia [10–12]. The site abuts Squibnocket Pond (brackish water) and also contains a permanent freshwater pond. A small freshwater marsh, which often loses standing water during the summer, is also located on the property between the two ponds. The site is an undeveloped and privately owned area of glacial outwash plain (elevation <5 m) with coastal grassland and beach scrub (herbaceous and grassy vegetation). The mean temperature and relative humidity (RH) from May through July are 24.4°C and 76.1% RH based upon data loggers placed at ground level within the actual sampling site in 2008.

Environmental Samples

The sampling design was similar to that previously reported during a followed up investigation of possible *F. tularensis*-positive air filter samples [2]. Water (100–300 mL) and soil samples were collected in sterile Whirlpak sampling bags (Nasco, Ft. Atkinson, WI, USA), and in some instances also in 50-mL conical tubes (Corning, Corning, NY, USA), from a marsh, a freshwater pond, and a brackish-water pond on our field site. Sediments were also collected with many of the water samples. Sampling was undertaken at approximate monthly intervals from April to September during 2005–2007.

Water samples were prepared for analysis by centrifugation at 600×*g* for 10 min; the supernatants were removed from the pelleted sediment. Supernatants were filtered using 0.22-μm cellulose nitrate filters (Whatman, Maidstone, England) and placed in a sampling bag with 10–15 mL of

the filtrate. Filter "washes" were obtained by massaging the filter for several minutes with the filtrate in order to dislodge particulates. Particulates were collected by centrifuging 0.25–1.0 mL of filter wash material, depending on the amount of particulates, in an Eppendorf 5415C microcentrifuge (Eppendorf, Westbury, NY, USA) at 8,000×*g* for 5 min and processed as described for other particulate matter (e.g., soil, sediment). All samples and filters were stored at 4°C until processed, usually within the month.

DNA Extraction

DNA was extracted from 0.25 to 0.50 g of sediments, mud, and soil samples (a lesser amount of material with high organic content (e.g., sediments, mud) was processed than those samples with less organic matter (e.g., sand)) using the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol with the exception of the lysis step; the bead-beating time was reduced to 5 min, and a 5-min incubation at 70°C was added to reduce DNA shearing. Extracted DNA was stored at −20°C until analyzed by PCR.

PCR

Samples were analyzed using previously published *Francisella* species 16S rRNA gene primers Fr153F.1/Fr1281R.1 [2]. The majority of samples were concurrently tested using *F. tularensis*-specific nested primer sets targeting the 43-kDa *Francisella* outer membrane protein (*fopA*) [9]. To ensure that the extracted sample content was not inhibitory to PCR, a primer set that broadly amplifies bacterial 16S rRNA gene was used in parallel. The set was designated 16SN and had been designed using Primer Express v2.0 (Applied Biosystems, Foster City, CA, USA; 16SN-forward (5′-CCAAAGAATAAGCACCGGCTA) and 16SN-reverse (5′- AATCCTGTTGATCCCCACG)). Samples that displayed inhibition were tested at a 1:10 dilution in PCR-grade water; if inhibition persisted, template was diluted to 1:100 in PCR-grade water and retested. Multiple extractions were performed on many of the collected water samples (e.g., on postcentrifuged sediments and/or multiple filters); thus, a sample was considered positive if one or more of such DNA extractions were PCR positive.

Samples that produced a *Francisella* spp. 16S rRNA amplicon (primers Fr153F.1/Fr1281R) were also tested using succinate dehydrogenase (*sdhA*) and 17-kDa lipoprotein (*tul4*) genes, an insertion element (IS*Ftu2*), and the *fopA* target if not tested initially with this set [2, 9, 23, 26]. Thermocycling conditions for the primary and secondary nested *fopA* reactions were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and a final extension time of 5 min at 72°C. The

thermocycling conditions for the *sdhA* PCR assay were 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 60 s with the final extension of 72°C for 5 min; conditions were the same for the *tul4*, IS*Ftu2*, 16S rRNA, and 16SN PCR assays with the exception of a 60°C annealing temperature.

All PCR assays were performed using MJ Research PTC-200 thermocyclers (Bio-Rad Laboratories, Richmond, CA, USA). Amplicons were generated using 12.5 μL of Promega GoGreen Taq 2× Master Mix, 2.0 μL DNA (or PCR-grade water for no-template controls (NTC)), 0.5 μL molecular-grade bovine serum albumin (10 mg/ml), 0.4 μM of forward and reverse primers, and PCR-grade water to a total reaction volume of 25 μL. Amplification products were separated on a 1.5% Tris–acetate–EDTA agarose gel and visualized by ethidium bromide. Amplicons submitted for sequencing were excised from the gel and purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA).

In order to minimize the risk of contamination, PCR reactions were prepared in a PCR workstation (Cleanspot, Coy Lab Products, Grass Lake, MI, USA) separated from areas used for DNA extraction, amplification, and PCR product handling. Dedicated equipment was used. Negative (no template) and positive controls using DNA from *F. tularensis* Live Vaccine Strain (BEIR NR-14) were included with each run.

Sequence and Phylogenetic Analysis

Gel-purified PCR products were sent to the University of Maine DNA Sequencing Facility for sequencing using the amplicon target PCR primers. Sequences were compared to those deposited in GenBank using BLAST analysis. Consensus and single-strand sequences were aligned using GeneDoc Multi-Sequence Alignment Editor and ClustalW2 (EMBL-EBI, Cambridge, UK). Chimera analysis on the 16S rRNA gene products were performed using the Bellerophon server with parameters set for Huber-Hugenholtz correction and a window size of 300 [13]. Phylogenetic trees of representative sequences were constructed using the neighbor-joining Jukes-Cantor algorithm in MEGA (v 4.0) with 500 bootstrap replicates; the trees were condensed with a consensus value cutoff set to 60%.

Culture

A subset of samples that tested PCR-positive using the *fopA* primers were subsequently cultured on cysteine heart agar (CHA; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 9% sheep blood (Lampire, Coopersburg, PA, USA). Samples that were cultured from 2007 were inoculated onto selective CHAB as described by Petersen et al. [21] but without the addition of 2.5 mg/mL of amphotericin. The inoculated CHAB plates were incubated at 37°C overnight or until growth was observed, and colonies that were morphologically consistent with *Francisella* spp. [18, 24] were picked and subcultured onto a new CHAB plate. Afterwards, the loop was placed in 50-μL PCR-grade water to wash off residual bacterial cells and boiled at 100°C for 5–10 min.

Nucleotide Sequence Accession Numbers

Sequences analyzed in this study were deposited into GenBank. GenBank accession numbers for 16S rDNA sequences are EU503153 to EU503168 and EU492359. Accession numbers for *sdhA* sequences are EU492352 to EU492358. Accession numbers for *tul4* sequences are FJ360762 to FJ360765, and accession numbers for *fopA* sequences are FJ394026 to FJ394028.

Results

Francisella Species Detected in Environmental Samples by PCR

To determine whether *Francisella* spp. were present in the environment in a site where *F. tularensis* type A was intensely enzootic, we analyzed water and soil samples by PCR (Table 1). All samples collected from and around the freshwater pond (*n*=19) and the marsh from our field site (*n*=54) tested negative for the *Francisella* spp. 16S rRNA gene target. Of 83 samples from the brackish-water pond, 23 were positive for the *Francisella* spp. 16S rRNA gene target. Of the 23 samples that contained *Francisella* spp. 16S rRNA target, 16 were also positive for *sdhA* and 15 were *fopA*-positive, primarily from the water filter samples. All *fopA*-positive samples, as well as three additional *fopA*-negative samples, were also IS*FTu2* PCR positive (*n*=23). Of the 23 16S rRNA gene-positive samples, 14 were *tul4* positive.

Multiple *Francisella* Species Identified by Sequence Analysis

16S rRNA gene sequences from 13 samples, including that from an isolate derived from the brackish-water pond, were identified as *Francisella philomiragia*. These sequences were 99–100% identical to *F. philomiragia* sequences available in GenBank, with the exception of one amplicon (extraction MB33) that aligned more closely to *F. philomiragia* subsp. *noatunensis* (previously referred to as *F. piscicida* (AM403242), a fish pathogen). In one sample (extraction MB 89), the 16S rRNA gene sequence obtained

Table 1 PCR results

| Sample location | No. of samples tested | PCR results | | | | |
| | | 16s rRNA | sdhA | F. tularensis specific | | |
				fopA	ISFTu2	tul4
Field site marsh	**54**	**0**	**nt**	**0**	**nt**	**nt**
Water (filter or pellet)	25	0		0		
Soil/sediment/sand	29	0		0		
Squibnocket pond (brackish water)	**83**	**23**	**16**	**15**	**19**	**14**
Water (filter or pellet)	42	19	14	12	16	12
Soil/sediment/sand	41	4	2	3	3	2
Middle pond (freshwater)	**19**	**0**	**nt**	**0**	**nt**	**nt**
Water (filter or pellet)	10	0		0		
Soil/sediment/sand	9	0		0		

nt not tested

clustered with *F. tularensis* species (99% similarity, 1,098/1,100 nucleotides) and groups with sequence from a *Francisella novicida* clone, but there were not enough differences for a definitive identification based on the phylogenetic analysis (Fig. 1). There were no chimeras detected from the 16S rRNA gene sequences using the Bellerophon analysis [13].

SdhA consensus sequences were obtained from seven environmental samples; phylogenetic analysis of sequences revealed that these samples contain DNA that is similar to that of *Francisella* spp. previously identified from environmental samples, with 96–99% similarity to *Francisella* spp. in GenBank. These environmental samples cluster together and are distinct from other *Francisella* species in the phylogenetic analysis (Fig. 2).

BLAST results of consensus sequences the IS*FTu2* amplicons (*n*=5) were between 97% and 100% similar to *F. tularensis* sequences. Sequences obtained for the *tul4* gene target (*n*=4) were 95–97% identical to *F. tularensis* sequences in GenBank while results for *fopA* (*n*=3) sequences were more closely identified with *F. tularensis* subsp. *holarctica* and *F. novicida* (99–100%). Even with sequences from these three targets, we cannot assign a more specific identity to the agent or agents that are present in our environmental samples other than to genus *Francisella*.

Isolation of *Francisella philomiragia* Species by Culture

Three initial *fopA*-positive samples were cultured on CHAB (2005), and five were inoculated onto selective CHAB (2007). Cultures were checked at 24 and 48 h; any cultures that were not overgrown with indigenous bacteria or fungi were checked daily thereafter. *F. philomiragia* was isolated from a brackish-water sample, sample number 080107-14, by directly culturing filter wash material onto selective

CHAB. No other *Francisella* sp. was recovered from direct cultures of environmental material that tested positive by PCR, indicating that either nonculturable bacteria were present or other indigenous bacteria overgrew potentially culturable bacteria from these samples.

Discussion

We sought to detect evidence of environmental *F. tularensis* and other *Francisella* spp. from a site on the island with known intense enzootic *F. tularensis* type A activity in dog tick and mammal populations [5, 10, 12]. *F. tularensis*-infected ticks have been found each year from 2001 to 2007 within 50 m of the brackish-water pond and marsh that were sampled. The pond and marsh receive water runoff via sloughs from a large portion of our study site, including the 290-m-diameter tularemia microfocus [12] and thus would be likely to have *F. tularensis* from carcasses (or other fomites) deposited within. Our failure to detect *F. tularensis* in the pond or marsh could be explained by the bacteria failing to be washed into such bodies of water from surrounding habitat, instability of the bacterium in water or soil, an artifact of our sampling, or a combination of these factors.

Our sampling focused around water sources because *F. tularensis* type B has previously been recovered from streams and ponds during tularemia epizootics in wildlife populations [14–16, 19]; much of the suggested environmental stability attributes (survival within soil or water) of type A are inferred from studies of type B as opposed to having been directly demonstrated. In order to increase the cumulative volume of material tested, numerous samples were collected at the various collection points around the water bodies. The sample volume varied based on the

Figure 1 Phylogenetic tree of 16S rDNA sequences. Neighbor-joining analysis was done using Jukes-Cantor parameters and 500 bootstrap replicates; tree was condensed to show consensus values >60%. The *boxed* groups represent those clades which include sequences from the brackish-water pond. DNA extraction designation and GenBank accession numbers follow the sample name. Reference sequences are designated as follows: *Ftt*, *F. tularensis* subsp. *tularensis*; *Fth*, *F. tularensis* subsp. *holarctica*; *Ftmed*, *F. tularensis* subsp. *mediasiatica*; *Fnov*, *F. novicida*; *Fpisc*, *F. piscicida*; *UPBclone*, uncultured proteobacteria clone; *Fphil*, *F. philomiragia*; *FrancSp*, *Francisella* species clinical isolates; and *Franc* endosymbiont, *Francisella* endosymbiont from the tick, *Dermacentor variabilis*
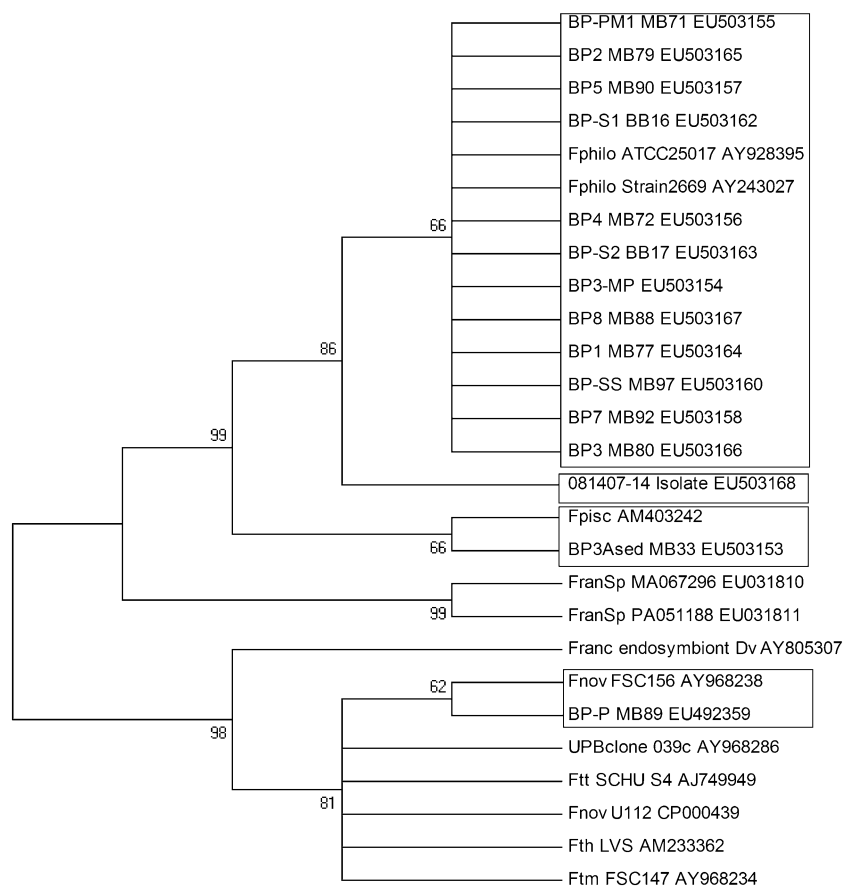

condition of the water body at the time of collection (e.g., water level, heavy plant/organic content) as this affected downstream processing (e.g., filtration, DNA extraction).

The use of molecular methods such as PCR to analyze natural water sources and soil samples for the presence of *F. tularensis* has been previously documented [2, 8, 29]. Consistent with the approach of these published reports, we utilized broad-range primers targeting the *Francisella* species 16S rRNA gene and *sdhA* gene and a battery of additional previously published *F. tularensis*-specific primers (*tul4*, IS*Ftu2*, and *fopA*) to analyze environmental samples for the presence of *Francisella* spp. We expected all 23 of the *Francisella* sp. 16S rRNA gene-positive samples to also be positive for the *sdhA* gene target; however, we found that only 16 of these samples were positive. Interestingly, concordance was better when comparing the *sdhA* gene target and the presumptively *F. tularensis*-specific PCR assays; of the *sdhA*-positive samples, 14 were *fopA* positive (nested PCR) and *tul4* positive. There were more IS*Ftu2* positives than *sdhA* positives (*n*= 19); however, the former primer set is more sensitive than the latter due to the presence of multiple insertion sequence elements in characterized *F. tularensis* genomes [27]. Potential factors, such as low concentration of target DNA and/or complex environmental media background in which

DNA was extracted, may have affected the overall concordance between the *Francisella* sp.-specific PCR assays. We also cannot discount the possibility that previously uncharacterized *Francisella* spp. in the environment may not be amplified by the primers used in our study because of differences at the priming sites.

Sequencing and phylogenetic analysis of the 16S rRNA gene PCR products identified *F. philomiragia* as the predominant *Francisella* sp. in PCR-positive environmental samples taken from the brackish-water pond. This is not an unexpected finding; many human infections with *F. philomiragia* are seawater-derived [28], and *F. philomiragia* subsp. *noatunensis* (proposed *F. noatunensis* comb. nov) and other closely related *Francisella* sp. (i.e. *F. piscicida*, proposed *F. noatunensis* comb. nov.) are fish pathogens that included several oceanic species [18]. We obtained one 16S rRNA gene product (extract MB 89) that was not identified as *F. philomiragia*; this sequence was 99% identical to known *F. tularensis* subspecies and phylogenetically grouped more closely with *F. novicida* sequences in GenBank (Fig. 1). *F. novicida* is commonly associated with brackish-water environments and, while a definitive identification could not be made from this sequence, it is likely that the organism from which this sequence derived is a *F. novicida*-like bacterium. Although

Figure 2 Phylogenetic tree of *sdhA* sequences. Neighbor-joining analysis was done using Jukes-Cantor parameters and 500 bootstrap replicates; tree was condensed to show consensus values >60%. The *boxed* groups represent those clades which include sequences from the brackish-water pond. DNA extraction designation and gene GenBank accession numbers follow the sample name. Reference sequences are designated as follows: *Ftt*, *F. tularensis* subsp. *tularensis*; *Fth*, *F. tularensis* subsp. *holarctica*; *Ftm*, *F. tularensis* subsp. *mediasiatica*; *Fnov*, *F. novicida*; *UPBclone*, uncultured proteobacteria clone; *Fphil*, *F. philomiragia*; and *FrancSp*, *Francisella* species from clinical isolates
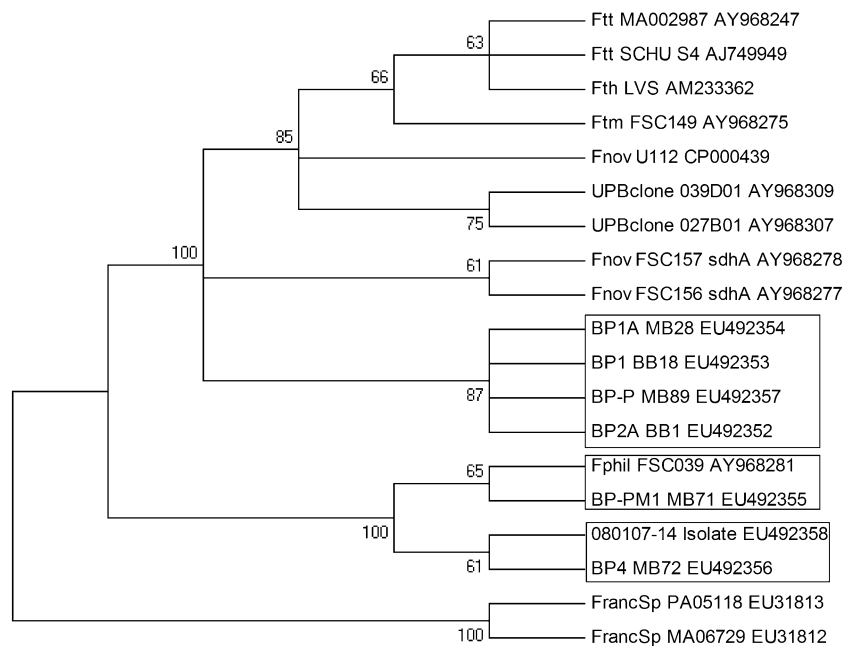
F. philomiragia sequences were frequently obtained from 16S rRNA gene-positive samples, BLAST results and phylogenetic analysis for most of the *sdhA* sequences obtained from the same samples were not identified as *F. philomiragia*, suggesting that multiple *Francisella* spp. may be simultaneously present in the samples. The *sdhA* sequences are clearly that of previously uncharacterized *Francisella* sp. and cluster together in their own distinct clade (Fig. 2), and the presence of identical sequences in multiple samples suggests that the sequences are not the result of random chimeric formation during the PCR process. As with the *sdhA* gene target sequences, phylogenetic analyses of sequences obtained from a subset of positive samples for the nested *fopA* and *tul4* gene targets also show that these environmental samples contain DNA from a species that is closely related to known *F. tularensis* subspecies but again cluster together in a separate clade (data not shown).

Further molecular characterization of the *Francisella* spp. detected in these environmental samples would be facilitated by isolation of the bacteria that DNA sequences were obtained; however, attempts to isolate these bacteria of interest were generally unsuccessful. Although methods have been proposed for enhancing the possibility of directly culturing *F. tularensis* from heavily contaminated tissues onto appropriate laboratory media such as CHAB-containing antibiotics [21], our personal observation is that direct culture of environmental samples for the purpose of isolating *F. tularensis* (or *F. tularensis*-like organisms) is challenging given the probability of nontarget overgrowth during the prolonged incubation which is sometimes required to culture *F. tularensis* on laboratory media [24].

New selective CHAB medium that effectively reduces the growth of environmental microflora, thereby allowing the isolation of *Francisella* spp. from seawater [20], will undoubtedly be useful for future environmental studies. Further challenges to the isolation of *Francisella* spp. from these complex samples are that bacteria may exist in a viable but nonculturable state [7] or that novel *Francisella* spp. may have special laboratory media requirements that preclude cultivation on CHAB. It is possible that isolation of *F. tularensis* or other *Francisella* spp. may have been more successful by utilizing animal inoculation in a susceptible animal model (e.g., mouse, guinea pig). Earlier studies by Jellison et al. relied on the use of guinea pigs for recovery of *F. tularensis* (subspecies not reported) from freshwater and mud samples from streams at which tularemia epizootics were occurring in beavers (*Castor canadensis*) [15, 19].

Although we did not detect *F. tularensis* type A in our environmental samples, we detected other *Francisella* spp. sequences from these samples, demonstrating the efficacy of our sampling protocol and analysis. Our data and that of others demonstrate that the "*F. tularensis*-specific" primer sets (*fopA*, *tul4*) amplify DNA from bacteria other than the agent of tularemia (*F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*) and should be used with caution with environmental samples taken for outbreak investigation. [2]. Diverse *Francisella* spp., including two distinct *Francisella* tick endosymbionts [11], *F. philomiragia*, *F. novicida*-like bacteria, and *F. tularensis*-like environmental bacteria all coexist in our study site with the agent of tularemia, *F. tularensis* type A. It is likely that other sites that are enzootic or epizootic for tularemia might

contain similar diversity. The recent isolation of two novel strains of *Francisella* sp. from clinical samples, in which at least one case was possibly associated with a coastal environment, suggests that diverse *Francisella* spp. may be human pathogens (particularly for immunocompromised individuals) [17] and highlights the utility of environmental sampling to provide information about the prevalence of known and yet uncharacterized *Francisella* and their potential interactions.

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