

Characterization of Two Unusual Clinically Significant *Francisella* Strains

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We have isolated two phenotypically distinct nonfastidious *Francisella* strains (Fx1 and Fx2) from the blood of compromised patients with pneumonia and compared them with eight other *Francisella* strains, including *Francisella tularensis* biovar *tularensis*, *F. tularensis* biovar *novicida*, and *F. philomiragia*. Our isolates grew well on sheep blood agar, chocolate agar, modified Thayer-Martin agar, and Trypticase soy agar. Fx1 and Fx2 were determined to be within the *Francisella* genus by cellular fatty acid analysis and by the utilization of glucose, production of H₂S and catalase, and lack of motility, oxidase, nitrate reductase, and gelatinase. They were additionally shown to belong to the species *F. tularensis* by sequencing of two variable regions comprising approximately 500 nucleotides of the 16S rRNA gene. Also, RNA probe hybridization confirmed their belonging to the species *F. tularensis*. However, the new strains, which are not identical, are distinguished from other *F. tularensis* strains by growth characteristics, repetitive extragenic palindromic PCR fragment pattern, and some biochemical tests. Key biochemical differences included the findings that Fx1 was positive for β-galactosidase and arabinose hydrolysis and that both strains were citrulline ureidase positive and glycerol negative. Commercial *F. tularensis* antiserum agglutinated stock *F. tularensis* strains but not Fx1, Fx2, *F. tularensis* biovar *novicida*, or *F. philomiragia*; serum from either patient failed to agglutinate or only weakly agglutinated commercial antigen but showed agglutination when tested against each patient's respective isolate. Fx1 and Fx2 produced β-lactamase. Because of their good growth, negative serology, and biochemical profile, the organisms could be misidentified in the clinical laboratory if standard strategies or commercial identification systems are used.

The taxonomy of the genus *Francisella* has been in transition. The four recognized groups of *Francisella*, some of which have at times been given the status of species, are *Francisella tularensis* biovar *tularensis* (also called *F. tularensis* group A), *F. tularensis* biovar *palaeartica* (also called *F. tularensis* group B), *F. tularensis* biovar *novicida* (formerly *F. novicida*), and *F. philomiragia* (formerly *Yersinia philomiragia*). The first three groups have been combined into one species on the basis of their high degree of relatedness determined by sequencing of genes coding for 16S rRNA (16S rDNA) (>97%) (5, 6, 22) or DNA hybridization (9).

Historically, *F. tularensis* has been described as fastidious since it grows slowly, forms small colonies, and may require cysteine-glucose blood agar for isolation (21). In fact, because of the difficulty in cultivating *F. tularensis*, most cases of tularemia have been diagnosed by the clinical picture and serology only (3, 13). However, recent reports document isolation of *F. tularensis* by using enriched (i.e., now standard) chocolate agar, Thayer-Martin agar, Mueller-Hinton agar, and cysteine yeast extract agar and by using blood culture bottles (17, 19). The isolation of cysteine-independent strains has also been reported (1). PCR has recently been developed to overcome the perceived lack of sensitivity of culture (4, 11). An exception is that both *F. tularensis* biovar *novicida* and *F. philomiragia* grow well on routine media (9).

Growth and biochemical characterization (9, 16), cellular

fatty acid composition analysis (1, 10), antigen agglutination (21), and probes based on the 16S rDNA (5, 6, 20) have been used to identify *F. tularensis*. Some (21) consider that biochemical differentiation within the genus is neither necessary nor worth the potential risk to laboratory personnel. Repetitive extragenic palindromic sequences-PCR (REP-PCR) pattern analysis has been used to distinguish strains of the family *Enterobacteriaceae* and species of *Bartonella* and *Mycobacteria* (2, 18, 22) but has not previously been used for *Francisella* strains. In clinical practice, we encountered two unusual clinically significant *Francisella* strains which might have been misidentified by routine strategies. We used the above methods in combination with serological testing to characterize the strains.

Case reports. (i) First case. A 55-year-old diabetic man was hospitalized on 11 June 1991 with a 3-week history of progressive weakness, dyspnea, chest pain, and restlessness. He had been maintained on prednisone (20 mg daily by mouth) for inflammatory nodular skin lesions of unknown etiology. He lived in an isolated beachfront cottage in Galveston, Tex., which he shared with a tick-infested dog. On admission, the patient appeared restless but was oriented. He had a temperature of 99°F (ca. 37°C), a pulse of 100/min, a respiratory rate of 20/min, and blood pressure of 120/70 mm Hg. Pertinent abnormal findings included diffuse lung wheezing and moderate hepatosplenomegaly. On the third hospital day, he developed chills, fever (103.6°F [ca. 39.8°C]), headache without neck stiffness, and leukocytosis (leukocyte count, $14.3 \times 10^3/\text{mm}^3$). Magnetic resonance imaging of the brain revealed on T2-weighted images multiple areas of increased signal measuring up to 12 mm in diameter. Analysis of cerebrospinal fluid revealed 27 erythrocytes per mm^3 , 25 leukocytes per mm^3 (92%

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TABLE 1. Strains and sources

| Strain or species | Our designation | Previous designation | Source |
|------------------------|-----------------|----------------------|--------------------------------------|
| Fx1 | Fx1 | BCUL 3664 | Blood, present case |
| Fx2 | Fx2 | BCUL 110 | Blood, present case |
| <i>F. tularensis</i> | Ft 31 | AZ91-1624, type A | Blood, CDC ^a (T. Quan) |
| | Ft 32 | AL91-1623, type B | Blood, CDC (T. Quan) |
| | Ft 78 | T6755 | Rabbit (D. Shoemaker) |
| | Ft 79 | AL91-1623 | Blood, CDC (D. Shoemaker) |
| | Ft 80 | 88R-144 | Rabbit, Indiana (D. Shoemaker) |
| | Ft 77 | 88R-675 | Rabbit, Indiana (D. Shoemaker) |
| <i>F. novicida</i> | Fn | ATCC 15482 | Water, Utah |
| <i>F. tularensis</i> | Ft 6223 | ATCC 6223 | Human, ATCC ^b type strain |
| <i>F. philomiragia</i> | Fph | ATCC 25015 | Muskrat, Utah |

^a CDC, Centers for Disease Control and Prevention.

^b ATCC, American Type Culture Collection.

mononuclear cells), a glucose concentration of 84 mg/dl, and a protein concentration of 107 mg/dl. Gram, fluorochrome, and India ink stains and agglutination tests for cryptococcal and bacterial antigens were all negative. A plain roentgenogram of the chest revealed new pleura-based infiltrates in the right lower lung, but the patient was not producing sputum and he refused evaluation by a percutaneous lung biopsy. A blood screen for vasculitis, an electroencephalogram, an echocardiogram, and a four-vessel cerebral angiogram were all noncontributory.

Both blood cultures that had been obtained at the onset of fever grew a *Francisella* species (Fx1). Cerebrospinal fluid cultures and serologic testing for *Francisella* of serum obtained 2 weeks after the positive blood culture were negative. The patient was thought to have disseminated infection due to Fx1 manifesting with bacteremia, pneumonia, and brain abscesses. He defervesced while receiving parenteral therapy with expanded-spectrum cephalosporins, and a follow-up chest roentgenogram demonstrated substantial resolution of the right lower lung infiltrates.

(ii) **Second case.** A 43-year-old man was admitted in January 1995 because of a 1-week history of fever, chills, cough productive of yellow sputum, shortness of breath, nausea, occasional vomiting and diarrhea, and 45-lb (ca. 20-kg) weight loss over the preceding 6 months. He denied chest pain or hemoptysis. The patient had a history of pustular dermatitis for 3 years. Physical examination on admission revealed a cachectic, dehydrated man who was highly febrile (105°F [ca. 40.6°C]), tachypneic (24/min), and tachycardiac (98/min) but with preserved blood pressure (105/60 mm Hg). He had decreased breath sounds with rales in the lower half of the left lung, consistent with the findings of lingular infiltrates by plain roentgenogram of the chest. Pertinent abnormal laboratory test findings included pancytopenia and elevated alkaline phosphatase (378 U/liter) and serum glutamic oxalacetic transferase (94 U/liter).

No pathogens were recovered from sputum samples, which were of low quality. Blood cultures drawn at the time of admission and processed for acid-fast bacilli, fungi, and routine bacteria grew only a *Francisella* species (Fx2). The patient became afebrile, and his pulmonary symptoms improved while he was receiving a 2-week course of ampicillin-sulbactam for bacteremia associated with pneumonia. However, because of worsening pancytopenia, a bone marrow aspirate and biopsy were taken, revealing slightly hypocellular marrow and absent iron stores but no evidence of malignancy or granulomas. Ultrasound and computerized axial tomographic examinations of the abdomen demonstrated only moderate hepatosplenomeg-

aly. Serum protein electrophoresis indicated the presence of polyclonal hyperglobulinemia, suggestive of liver and/or renal disease. A percutaneous liver biopsy demonstrated lymphohistiocytic infiltration with granuloma formation and an abundance of plasma cells. Bacterial and special stains and cultures of bone marrow and liver specimens were all negative. Serologic testing performed at the Veterans Affairs (VA) Reference Laboratory for *F. tularensis* on serum samples obtained 17 days and 6 weeks after the first blood culture was negative. The patient refused further workup and was discharged against medical advice.

The patient lives in rural Texas (Liberty County) and owns a dog but had not been in the woods or hunting in the past 6 years. Although he drank bottled water, he used well water for food preparation and bathing. Because his skin lesions may have provided a portal of entry for *Francisella* organisms, we cultured the well water. One liter of the well water was collected and filtered, and the retentate was plated on modified Thayer-Martin medium. This medium grew an almost pure culture of a gram-negative rod identified by cellular fatty analysis as a *Comamonas* sp., but no *Francisella* species was isolated from the well water.

MATERIALS AND METHODS

Strains and biochemical identification. The designations and sources of strains examined are listed in Table 1. Biochemical tests were performed by conventional methods (9, 20) and by use of RapID NH and RapID ANA (Innovative Diagnostic Systems, Atlanta, Ga.) commercial identification kits according to the manufacturer's directions. Organisms used as inocula were grown on chocolate agar in an 8 to 10% CO₂ atmosphere for 24 to 72 h (the strains that formed smaller colonies were held longer). The RapID NH panel tests for preformed enzymes which catalyze the hydrolysis of proline, *o*-nitrophenyl-β-galactoside (ONPG), glucose, fatty acid esters, phosphate, and urea; the utilization of glucose, sucrose, and ornithine; the formation of indole; and the reduction of nitrate and resazurin. The RapID ANA panel tests for the hydrolysis of urea, the hydrolysis of various aryl-substituted glycosides (e.g., L-arabinoside, D-galactoside, D-glucoside, and N-acetyl-β-D-glucosaminide), the hydrolysis of arylamides (e.g., leucyl-glycine-, glycine-, proline-, phenylalanine-, arginine-, serine-, and pyrrolidonyl-β-naphthylamide), and formation of indole. Growth on various media was compared by uniformly inoculating plates with 1 drop of a turbid suspension of organisms, streaking for isolation, and measuring the largest isolated colonies. Media were incubated at 35°C in an elevated (6 to 8%) CO₂ atmosphere. Testing for the presence of β-lactamase was performed by using a cefinase disk (BBL Microbiology Systems, Cockeysville, Md.).

Cellular fatty acid analysis. Whole-cell fatty acids were extracted and analyzed as previously reported (2) except that the organisms were grown on chocolate agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) for 24 to 48 h. Analysis was performed with an automated Hewlett-Packard HP 5890 II Microbial Identification System (MIDI Systems, Newark, Del.) using an automatic sampler, an integrator, and a computer. Fatty acid peaks were analyzed via software containing a library of cell wall lipids of clinically relevant bacteria. Although each isolate was analyzed more than once, only results from a single run were used for comparison (2, 2a).

Serological agglutination tests. Serum samples were obtained at 12 days for patient 1 and at 17 days and 6 weeks for patient 2. The tube agglutination test was performed here and at the VA Reference Laboratory (Lexington, Ky.) according to the manufacturers' instructions. The standard slide test was performed by dispensing 50 μ l of serum onto a slide and adding a drop of the antigen (BBL, Becton Dickinson Microbiology Systems). The antigen and serum were mixed together, and the slide was placed on a shaker for 4 min at 125 rpm. The slides were examined macroscopically and microscopically for agglutination.

Each patient's serum samples were also tested in a slide agglutination assay against his own isolate and other isolates. The isolates were cultured for 48 h on chocolate agar in an elevated CO₂ atmosphere, harvested in 0.85% NaCl with 0.5% phenol to a turbidity of a 3.0 MacFarland standard, and left overnight at 4°C. Slide agglutination was performed as described above, using the killed organisms as the antigen.

REP-PCR. Previously described methods using conserved primers corresponding to REP sequences in a PCR were used (2, 18). For our usual protocol, organisms were grown on chocolate media and the plates were incubated at 35°C with 8% CO₂. The organisms were harvested with a sterile swab and resuspended in 0.9% sterile saline to a 3.0 MacFarland standard turbidity. The PCR primers REP1R and REP2-1 have been previously described (22). The reaction mixture consisted of 5.0 μ l of 5 \times polymerase buffer, 2.5 μ l of dimethyl sulfoxide, 0.5 μ l of deoxynucleoside triphosphates (25 mM), 1 μ l (50 pmol) of each primer, 4 μ l of sample organism suspension, 0.4 μ l of *Taq* polymerase (Perkin-Elmer Cetus), and 10 μ l of water, overlaid with 50 μ l of light mineral oil. Amplifications were performed on a Coy thermocycler as follows: 1 cycle of 95°C for 6 min; 35 cycles of 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min; 1 cycle of 65°C for 16 min; and final holding at 4°C until analysis. The products were separated on a 1.2% agarose (Sigma, St. Louis, Mo.) gel stained with ethidium bromide. For analysis of the band patterns, bands were compared with DNA molecular weight marker III (λ DNA digested with *Eco*RI and *Hind*III) (Sigma). Restriction fragment length polymorphism molecular weight software (DNA ProScan, Nashville, Tenn.) was used to assess the pattern similarity; lanes within 10% similarity were given the same letter designation.

rDNA hybridization and 16S rRNA gene sequence homology. The oligonucleotides FT1 (specific to *F. tularensis* biovar palaeartica), FT2 (specific to *F. tularensis* biovar tularensis), and FT3 (specific to any *F. tularensis* strain) were end labeled at the 5' end with ³²P and T4 polynucleotide kinase as previously described (5). Bacterial isolates were heated at 65°C for 60 min before the direct filter hybridization experiments, which were performed essentially as described by Forsman et al. (5). Briefly, the bacterial suspensions were directly applied in 10-fold dilution series onto the filters with a dot blot manifold without prior extraction of nucleic acids. The filters were then treated with sodium dodecyl sulfate to make rDNA accessible for hybridization (5), which was performed according to standard procedures.

Two variable regions, comprising a total of 475 to 520 nucleotides (approximate location according to *E. coli* nomenclature, 340 to 590 and 1100 to 1350), of the 16S rRNA gene of both Fx1 and Fx2 were sequenced as previously described (6) and compared with known sequences.

Susceptibility testing. Disk diffusion susceptibility testing was performed by a standard procedure (12a) using *Haemophilus* test medium (HTM) agar designed for susceptibility testing of *Haemophilus* species. Zone diameters and interpretive standards for *Haemophilus* spp. were used to determine susceptibility.

Nucleotide sequence accession number. The determined 16S rRNA gene sequences of Fx1 and Fx2 have been deposited in the EMBL data library under accession no. X98070 and X98071 for Fx1 and X98068 and X98069 for Fx2.

RESULTS

Clinical culture results. For patient 1, both blood cultures that had been obtained at the onset of fever grew a small, aerobic, gram-negative coccoid organism (Fx1) in the aerobic bottle of the BACTEC blood culture system after 3 days. The organism was susceptible to chloramphenicol, ciprofloxacin, imipenem, and ceftriaxone and resistant to ampicillin, penicillin, amoxicillin-clavulanate, aztreonam, cephalothin, and trimethoprim-sulfamethoxazole. The Vitek NHI and RapID NH panels suggested an identification of *Haemophilus actinomycescomitans* or *Neisseria meningitidis* (no. 44104) and no identification (no. 7710), respectively. There was no growth in the Vitek GNI panel.

For patient 2, at 6 days the blood culture became positive for a small, aerobic, gram-negative coccoid organism (Fx2) in the aerobic bottle of the Bac-t-Alert system. The organism was susceptible to amikacin, gentamicin, and ciprofloxacin and resistant to ampicillin, piperacillin, ampicillin-sulbactam, ceftriaxone, aztreonam, and erythromycin. The Vitek GNI, Vitek NHI, and RapID NH panels suggested identifications of *Acin-*

TABLE 2. Sizes of isolated colonies at 2 and 7 days on two media

| Strain and day | Colony diam (mm) on: | |
|----------------|----------------------|-----------------|
| | Chocolate agar | SB-TSA |
| Ft 6223 | | |
| 2 | <0.1 | NG ^a |
| 7 | 1.0 | NG |
| Ft 80 | | |
| 2 | 0.3 | <0.1 |
| 7 | 1.8 | 0.4 |
| Ft 77 | | |
| 2 | 0.4 | <0.1 |
| 7 | 1.5 | 0.3 |
| Ft 78 | | |
| 2 | 0.3 | <0.1 |
| 7 | 1.5 | 0.3 |
| Ft 79 | | |
| 2 | <0.1 | <0.1 |
| 7 | 1.0 | ND ^b |
| Ft 31 | | |
| 2 | 0.8 | <0.1 |
| 7 | 1.2 | 0.5 |
| Ft 32 | | |
| 2 | 0.8 | <0.1 |
| 7 | 1.2 | 0.3 |
| Fx2 | | |
| 2 | 1.5 | 1 |
| 7 | 1.8 | 1.3 |
| Fx1 | | |
| 2 | 1.6 | 0.8 |
| 7 | 2.3 | 1.6 |
| Fn | | |
| 2 | 2.5 | 1.5 |
| 7 | 4.0 | 2.0 |
| Fph | | |
| 2 | 3.4 | 1.0 |
| 7 | 5.0 | 3.0 |

^a NG, no growth.

^b ND, not determined.

etobacter lwoffii (presumptive, 97%, no. 10020000000); unidentified but possibly *Cardiobacterium hominis*, *H. actinomycescomitans*, or *Neisseria subflava* (no. 17100); and *N. meningitidis* or *Neisseria sicca* (no. 3510 and 3710), respectively.

Growth and biochemical results. By gram stain, Fx1 and Fx2 isolates were gram negative and were initially coccoid, becoming a more typical bipolar coccobacillus (0.2 by 0.2 to 0.5 μ m) on subculture. They grew on blood, enriched chocolate, modified Thayer-Martin, Mueller-Hinton, and charcoal-yeast extract agars but not on MacConkey agar with bile. The colonies were round, moist, and gray. Representative comparative cultural characteristics of the clinical isolates and stock strains are shown in Table 2 as the diameters of the largest colonies at 2 and 7 days on different media. The most fastidious strain was the type strain, *F. tularensis* biovar tularensis ATCC 6223, which grew on chocolate agar but did not grow on either Trypticase soy agar with 5% sheep blood (SB-TSA) or Trypticase soy agar without blood (TSA). Growth differences between the rest of the stock *F. tularensis* strains and Fx1, Fx2, *F. philomiragia*, and *F. tularensis* biovar novicida are notable, as by 2 days on SB-TSA the colonies from the first group can barely be seen, whereas the colonies from the latter group are at least 1 mm in diameter and show less growth enhancement on chocolate agar. In addition, at 14 days, the first group formed much smaller colonies on TSA than on SB-TSA (0.1- to 0.5-mm diameter on TSA compared with 1- to 2-mm diameter on SB-TSA), whereas *F. philomiragia*, *F. tularensis* biovar

TABLE 3. Summary of differential characteristics of tested strains

| Test system | Test category or substrate | Result for strain ^a : | | | | |
|----------------------|--|----------------------------------|------------|--|---------|---------|
| | | Fx1 | Fx2 | Ft (6 strains) | Fn | Fph |
| RapID NH | ID no. | 7710 | 3510, 3710 | 2510, 2500 | 3710 | 7730 |
| | Hydrolysis of amides (proline) | + | + | - | + | + |
| | Hydrolysis of glucosides (ONPG) | + | - | - | - | + |
| | Utilization of carbohydrates (sucrose) | + | wk/+ | - | + | + |
| | Reduction of resazurin | + | + | -/+ | + | + |
| RapID ANA | ID no. | 410773 414773 | 000773 | 00016/73 (2 strains) 00076/73 (4 strains) | 000773 | 014773 |
| | Hydrolysis of L- arabinoside and ONPG | +, + | -, - | -, - | -, - | -, + |
| | Hydrolysis of amides (leucine, glycine, and proline) | +, +, + | +, +, + | -, -, wk or +, +, + | +, +, + | +, +, + |
| | | + | | wk | + | + |
| Miscellaneous tests | Growth on SB-TSA at 2 days, 1 mm | + | + | - | + | + |
| | Growth in 6% NaCl | - | - | - | + | + |
| | Citrulline ureidase/glycerol ^b | +/- | +/- | +/+ or -/- | +/+ | ND |
| | Oxidase (tetramethyl- <i>p</i> -PDD) | - | - | | - | + |
| Serological tests | Agglutination of patient 1 serum diluted 1/10 | ++ | - | - | - | - |
| | Agglutination of patient 2 serum diluted 1/10 | + | + | - | - | + |
| Nucleic acid testing | Hybridization with probes FT1/FT2 ^b | -/+ | -/+ | -/+ or +/- | -/+ | -/- |
| | REP-PCR pattern | B | B | A | C | D |

^a As only one or a few strains are represented in each column, the data should be understood to be valid for the strain(s) and not to necessarily define the species or subspecies. wk, weak; wk/+, weakly positive; ND, not determined.

^b Except for new strains, data are from the literature (14).

novicida, and Fx1 did not need the blood supplement and grew about the same on SB-TSA and TSA. Fx2 grew significantly less well than Fx1 on TSA. *F. philomiragia* had mucoid colonies and the most copious growth.

Distinguishing characteristics of the strains are shown in Table 3. All strains were strict aerobes, weakly catalase positive, H₂S positive, nitrate reductase negative, nonmotile, and negative for the tested biochemicals of the two panels not listed in Table 3 (except *F. philomiragia* for phosphatase). All 11 strains were positive for β-lactamase, as are almost all *Francisella* isolates (15). Fx1 grew poorly and *F. philomiragia* and *F. tularensis* biovar novicida grew well in 6% NaCl; no other strains grew. All strains with strong growth were positive for proline and sucrose, but only Fx1 and *F. philomiragia* were positive for ONPG. Only *F. philomiragia* gave a strongly positive oxidase reaction; the occasional very weak positive reaction given by other strains is notable primarily because it allows confusion with other oxidase-positive organisms. The RapID NH number indicated that the organism could be a *Neisseria* species (3710 and 7710), *N. meningitidis* (3510), a *Gardnerella* sp. (3710), or an *Actinobacillus* sp. (2500), all of which could be confused with *Francisella* spp. by Gram stain and/or colony morphology. Although growth-based systems were not suitable for comparing strains as they did not support the *F. tularensis* biovar tularensis strains, the Vitek NHI panel gave a 99% confidence level for the identification of the Fx1 isolate as *H. actinomycetemcomitans*.

Both Fx1 and Fx2 were citrulline ureidase positive and glycerol negative. These results are surprising, as other strains which are recognized by the FT2 probe (below) are citrulline ureidase and glycerol positive.

Cellular fatty acid analysis. The cellular fatty acid profile for *Francisella* spp. is distinctive, with the predominant fatty acids being C_{10:0} (22%), C_{14:0} (15%), C_{16:0} (18%), 3 OH C_{18:0} (17%), and the unusual C_{22:0}, C_{24:0}, and C_{24:10} (10). All strains analyzed in this study were named *F. tularensis* by the MIDI

clinical database. However, when data from within a single run are compared, the organisms segregate into three closely related groups; *F. tularensis* biovar tularensis ATCC 6223 and *F. philomiragia* were the most distinct, with all other *Francisella* strains more closely clustered (Fig. 1). Our observations confirm the atypical nature of the type strain (16).

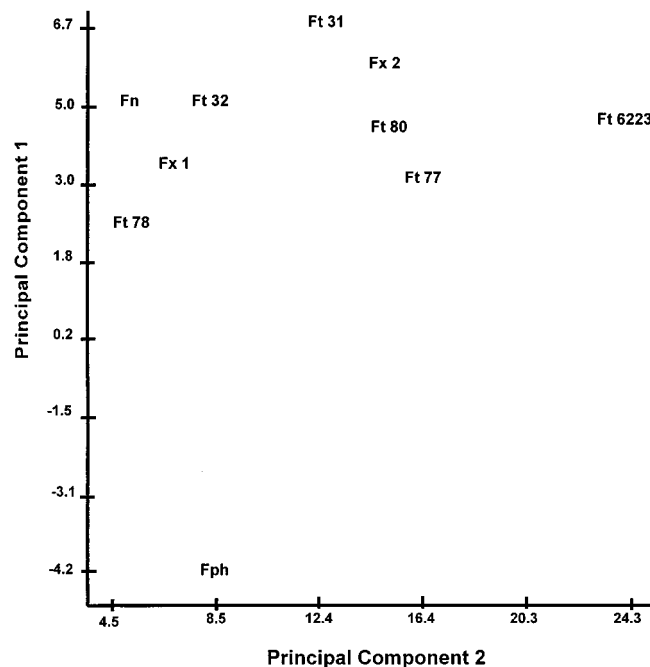


FIG. 1. Cellular fatty acid analysis plotted as a function of the two most discrepant fatty acids (the principal components).

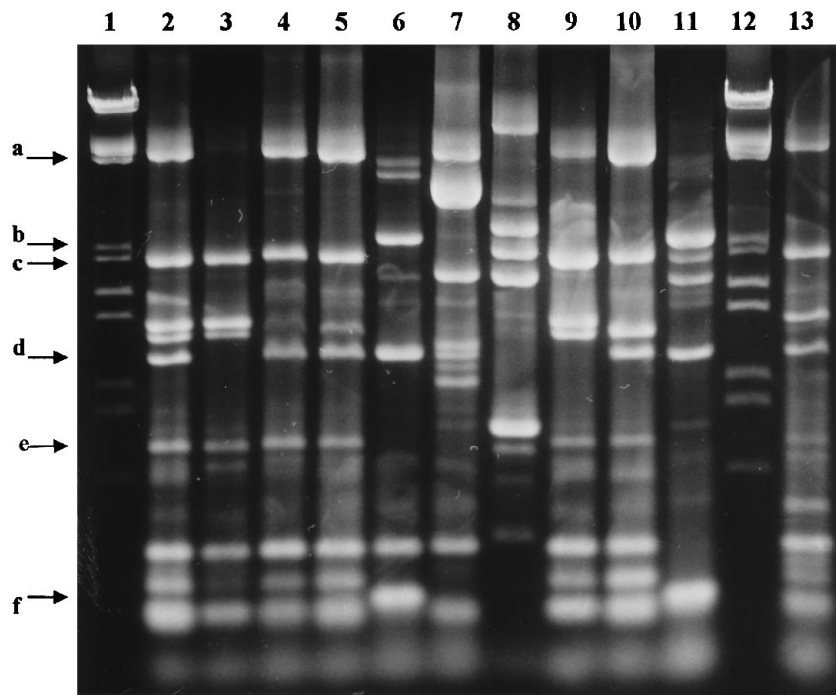


FIG. 2. REP-PCR patterns. Lanes 2, 3, 4, 5, 9, 10, and 13, *F. tularensis* strains Ft 31, 32, 77, 78, 79, 80, and 6223, respectively; lane 6, Fx1; lane 11, Fx2; lane 7, Fn; lane 8, Fph; lanes 1 and 12, markers (λ DNA digested with *EcoRI* and *HindIII* [Sigma]). Arrows a and b indicate molecular masses of 4,200 and 2,000 Da, respectively.

REP-PCR pattern analysis. In Fig. 2, the REP-PCR patterns for all strains are compared, with some predominant bands indicated. Although the patterns cannot measure the quantitative degree of relatedness, with the DNA ProScan software we can assess similarity and identify unlike strains; the taxonomic relationship then can be determined by other methods. Although there are bands that are not specific for a group (e.g., band d), the REP-PCR patterns for Fx1 and Fx2 are similar to each other and distinct from those of other strains in that they uniquely possess bands b and f. All other strains of *F. tularensis* (Fig. 2, lanes 2, 4, 5, 10, and 13) have bands c and e and the band below f in common. The REP-PCR patterns for *F. tularensis* biovar novicida and *F. philomiragia* are both unique. Since for many of the organisms we have studied (2, 18) the REP-PCR technique seems to define organism groups somewhere between a species and a strain (where and at how many places the REP primers will bind cannot be predicted beforehand without knowledge of the complete genome sequence), it is possible that if more strains of *F. tularensis* biovar novicida and *F. philomiragia* were examined, additional patterns for these organisms would be seen.

rDNA probe hybridization and 16S rRNA gene sequence homology. By probe hybridization, both Fx1 and Fx2 hybridized with probe FT2, indicating that they are in the same group as type A (*F. tularensis* biovar tularensis and *F. tularensis* biovar novicida). They were negative for probe FT1, which hybridizes with type B strains (*F. tularensis* biovar palaeartica). The new strains were also positive with probe FT3, which hybridizes with all *F. tularensis* biotypes but not with *F. philomiragia*.

The sequence of the two variable regions (475 to 520 nucleotides) demonstrated at most four nucleotide differences (>99% identity) when these sequences were compared to previously published *F. tularensis* sequences. The sequences showed a 93 to 95% identity compared to *F. philomiragia*. Sequences from all other species showed <90% homology.

Serology. By standard testing both in-house and at the VA Reference Laboratory, our patients' serum samples were negative against the commercially available *F. tularensis* antigens in tube agglutination assays. The purpose of the slide agglutination assay performed in our laboratory was both to test the patient sera (using commercial antigen) and to identify the isolates (using commercial antibody). A portion of the results are summarized in Table 3. The commercial *F. tularensis* control antiserum agglutinated the control antigen and all tested stock *F. tularensis* isolates (Ft 6223, Ft 32, and Ft 77) but not *F. tularensis* biovar novicida, *F. philomiragia*, Fx1, or Fx2. We found that there could be some nonspecific agglutination and weak agglutination when the serum was used full strength, so we tested both undiluted and 1/10-diluted samples. Serum from patient 1 was strongly positive (all agglutinations could be seen macroscopically) at both dilutions against his own isolate but negative against all other isolates (*F. tularensis* biovar novicida, *F. philomiragia*, Fx2, three strains of *F. tularensis* biovar tularensis, and the commercial antigen). However, patient 2 serum agglutinated all antigens to some extent when undiluted but only *F. philomiragia*, Fx1, and Fx2 antigens when diluted. The standard slide agglutination tests at the VA Reference Laboratory were negative.

DISCUSSION

The classical *F. tularensis* biovar tularensis strain is associated with tick-borne tularemia in rabbits, produces a well-described group of syndromes (ulceroglandular, glandular, typhoidal, and oculoglandular), is the most common *Francisella* sp. isolated in North America and is highly virulent. Most infections are diagnosed by serology. *F. tularensis* biovar palaeartica is associated with water-borne disease of rodents and has been isolated from humans, rodents, water, and agricultural products in Europe, Asia, and the Americas. In humans,

it causes a milder form of tularemia. The type strain of *F. novicida* was isolated from water in Utah in 1951. Although originally thought to produce tularemia in rodents but not to infect humans, it has been reported to cause glandular tularemia (isolate from lymph nodes) in an otherwise healthy individual and typhoidal tularemia (isolate from blood) in a compromised host and may be closely related to *F. tularensis* biovar tularensis (9). Of the 14 *F. philomiragia* strains isolated from sterile sites, 9 were from blood, 3 were from pleural fluid or lung biopsy, 1 was from cerebrospinal fluid, and 1 was from peritoneal fluid. The patients infected with these isolates had associated chronic granulomatous disease (five patients), history of near drowning (five patients), myeloproliferative disease (two patients), or no noted underlying disease event (two patients). Pneumonia was the most common infection noted; however, fever without distinct focus of infection, meningitis, and peritonitis were also reported (9, 23).

Bacteremia and pneumonia, as observed in both of our cases, are frequently seen in patients with infection due to *F. tularensis* biovar tularensis presenting as pneumonic or typhoidal tularemia. However, there are certain clinical aspects of these two cases, such as the presence of immunosuppressive underlying disease and the involvement of the central nervous system (observed with the first case), that are very rarely seen in patients infected with *F. tularensis* biovar tularensis (7). The clinical presentation and negative serologic studies of these two cases are somewhat similar to those of infections caused by the other nonfastidious *Francisella* organisms, *F. tularensis* biovar novicida and *F. philomiragia* (9, 14, 23).

As determined by both the DNA probe assay and 16S rRNA gene sequence homology, Fx1 and Fx2 are *F. tularensis* species. Because they hybridized with the FT2 probe, they may be more similar to *F. tularensis* biovar tularensis and *F. tularensis* biovar novicida than to *F. tularensis* biovar palaeartica. (Animal virulence studies performed by T. Quan at the Rickettsia Laboratory, Centers for Disease Control and Prevention, by standard methods showed no difference between *F. tularensis* biovar tularensis and Fx1 [17a]). However, in contrast to the former strains, which are citrulline ureidase and glycerol positive, Fx1 and Fx2 were citrulline ureidase positive and glycerol negative (20). *F. tularensis* biovar palaeartica is usually citrulline ureidase and glycerol negative (20). Although Fx1 and Fx2 are not biochemically identical, they are similar in growth, have the distinct citrulline ureidase and glycerol reactions, and have similar REP-PCR patterns. It has previously been shown that REP-PCR is useful for distinguishing species of both *Bartonella* and *Mycobacteria* (2, 18), and possibly REP-PCR is distinguishing a subspecies or biovar here. On the other hand, when more strains of the *F. tularensis* group are examined, it may be found that there will be similar strains in, for example, the *F. tularensis* biovar novicida group.

The epidemiology of our strains is unknown. Although both patients owned tick-carrying dogs, neither had engaged in activities that are associated with tularemia, such as rabbit hunting. Our investigation of the well water was negative for *Francisella* spp., but it has been well established by both culture and PCR that *Francisella* organisms are associated with water (4, 8, 12).

Reports of the isolation of relatively easy-to-grow *Francisella* spp. are not common. The reason for this may be that the strains are in fact rare (we isolated the two strains over a 4-year period) or that clinical laboratories do not recognize such organisms as *Francisella* spp. because they grow better than expected, can be negative in standard serological testing, and do not agglutinate in commercially available *F. tularensis* antisera or code correctly in most commercially available identification systems.

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