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Early Recognition of Atypical *Francisella tularensis* Strains Lacking a Cysteine Requirement

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Seven cultures referred to in our laboratories as unidentified gram-negative bacilli or *Haemophilus* species were identified as atypical strains of *Francisella tularensis* lacking a requirement for cysteine or enriched medium for growth. The use of cellular fatty acid composition analysis facilitated early recognition of this pathogen and prompt implementation of appropriate biosafety measures.

Francisella tularensis is the causative agent of tularemia, an acute, infectious, febrile granulomatous zoonosis (6). It is among the leading agents associated with laboratory-acquired infections, and owing to its highly infectious nature, laboratory personnel are cautioned to handle cultures in a level 3 biosafety containment facility (7, 13, 14). Primary isolation of *F. tularensis* is facilitated by using enriched medium supplemented with cysteine; on such medium, growth typically develops in 2 to 4 days as small (1 mm), smooth, gray opaque colonies. However, on conventional media such as blood and chocolate agar, the organisms either fail to grow or take several days (7 to 10 days) to grow (4, 6, 13). Thus, the requirement for cysteine and enriched medium for stimulated growth has traditionally served as a useful characteristic for presumptive identification of this important human pathogen (6, 13).

We describe here seven strains of *F. tularensis* which, on primary isolation, failed to demonstrate a requirement for cysteine and enriched medium but grew well on conventional laboratory medium. The isolates were originally referred to in our laboratories as unidentified gram-negative bacilli or as a *Haemophilus* species. The seven strains recovered from six patients from four Canadian provinces (Ontario, Manitoba, Saskatchewan, and British Columbia) were as follows (Laboratory Centre for Disease Control [LCDC] reference number, source): 88-552, thoracentesis fluid; 91-258, blood; 91-259, blood; 91-305, right eye; 91-391, mesenteric lymph node; 91-693, left thumb abscess; 92-569, blood. Samples 91-258 and 91-259 originated from the same patient. There were no reported mortalities associated with these strains. The clinical history of the strain from one patient (91-693) has recently been published (2); the investigators did not provide a detailed microbiological description of their isolate except to say that it grew well on chocolate agar in 72 h.

Cellular fatty acid (CFA) composition analysis of these strains, which was carried out at LCDC, provided strong presumptive evidence that these bacteria could be classified as *Francisella* species, since the CFAs of members of this genus are sufficiently distinctive that all other bacteria can be excluded (4, 9). Identification to the species level was confirmed by agglutination with commercially acquired specific antiserum and other phenotypic traits (13).

Upon initial subculture, all bacteria appeared as small, white or gray-white colonies on Columbia agar base (Oxoid, Basingstoke, England) supplemented with 5% defibrinated sheep erythrocytes (Qualicum Laboratories, Ottawa, Ontario, Canada) within 24 to 48 h after incubation at 35°C in 5% CO_2. Single colony isolates were Gram stained and appeared as weakly staining, tiny gram-negative coccobacilli. Bipolar staining was an inconsistent feature among these strains.

For CFA composition analysis, the growth conditions, fatty acid derivatization and gas-liquid chromatography of CFAs, and data integration and reporting by using the Microbial Identification System and Library Generation System (LGS) software (MIDI, Newark, Del.) have been described previously (1). A similarity index value was generated for each CFA profile and was compared with those in a commercial data library (CLIN, version 3.7; MIDI) and an in-house library (LCDC1), both of which were rooted to the method called AEROBE, version 3.7 (MIDI). Each strain was found to have a CFA composition characteristic for the genus *Francisella* (Table 1), being distinguishable by the presence of a large volume of the CFA 3-hydroxy-octadecanoate (3OH-18:0 [9]). That fatty acid has not been found in significant amounts in any other genus of bacteria to date (4, 9) and therefore can be considered a reliable marker for this genus. It was found in the present study, however, that in the peak naming table associated with AEROBE, version 3.7, CFA 3OH-18:0 was inadvertently misnamed CFA 20:3,ω6,9,12c (equivalent chain length [ECL], 19.545). We understand that a correction will appear in the next version of MIDI's software (10). Each strain demonstrated a significant degree of matching against the library entry contained in CLIN for *F. tularensis* and did not match any other entry (Table 1). Similarity indices to the CLIN entry ranged from 0.24 to 0.76 and generally improved when the strains were incubated for 48 to 72 h or if two platefuls of bacteria grown on blood agar were used (Table 1). The similarity indices to the LCDC1 library ranged from 0.780 to

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TABLE 1. CFA composition and similarity index values[a]

LCDC no. (age[b])	% of total fatty acid							SI (CLIN)[d]
	10:0	2OH-10:0	14:0	16:0	3OH-16:0	18:1, ω9c	3OH-18:0[c]	
88-552 (48 h)	18	3	13	16	6	10	25	0.60
91-258 (72 h)	19	5	12	16	5	11	21	0.57
91-259 (72 h)	18	6	12	16	5	11	21	0.44
91-305 (48 h)	18	4	13	18	5	11	22	0.70
91-391 (24 h)	22	2	12	18	4	11	17	0.24
91-693 (24 h; 2 p[e])	29	1	10	16	3	9	18	0.62
92-569 (24 h)	23	2	12	16	4	9	23	0.76

[a] For fatty acid composition, the number to the left of the colon is the number of carbon atoms and the number to the right of the colon is the number of double bonds. Values are percentages of total fatty acids and are rounded off to the nearest whole percent. OH, hydroxy group present at carbon atom. All strains also contained trace (0.3 to 0.7%) to small (0.8 to 3%) amounts of C12:0, C13:1$^{12-13}$, C16:1,ω9c, C17:0, C18:2 (Summed Feature 6), C18:0, C3OH-17:0, C20:2,ω6,9c (ECL 19.744), and C20:0.

[b] Age of bacteria when harvested from one Columbia blood agar plate with 5% sheep erythrocytes after incubation at 35°C in 5% CO_2.

[c] As discussed in the text, in the peak naming table of AEROBE, version 3.7, this peak was inadvertently named C20:3,ω6,9,12c.

[d] SI, similarity index values, scored from negative (no match) to 1.0, as generated in comparison with the CLIN data library.

[e] Two plates of blood agar.

0.940 and also did not match any other entry, with the higher degree of matching in comparison with CLIN being attributed to the differences in the media that were used (data not shown).

Once the strains were presumptively identified by CFA composition analysis as *F. tularensis*, they were tested against *Francisella* agglutinating serum (Difco Laboratories, Detroit, Mich.), and all strains reacted strongly in the serum. All strains were found to be unreactive to all tests except catalase (3); therefore, routine phenotypic testing did not contribute significantly toward strain identification (6, 13).

Human infections of *F. tularensis* in Canada are thought to occur rarely (11). This observation was corroborated in the present study, because the strains described here represent the total number of *F. tularensis* strains referred to the LCDC over a 4-year period (1988 to 1992). The sources of isolation of our strains are typical for this species (13); however, isolation from blood, as for two of the strains, is generally considered to be a rare occurrence because of difficulties in recovering the organism from patients with bacteremia (8).

In view of the fact that there were no reported case mortalities or laboratory-acquired infections associated with these strains, it is interesting to speculate that strains that lack a requirement for cysteine and enriched media for growth may be less virulent than classical *F. tularensis* strains. It has been suggested that there may be an association between geographic location (13), passage on artificial media (12), specific genotypes (5, 12), phenotypic properties (3, 4), and virulence among *F. tularensis* strains. Regardless, it is recommended that examination of isolates for characteristics associated with less virulent biovars of *F. tularensis* also require biosafety level 3 facilities to conform with risk guidelines (7, 14).

In summary, bacteriologists in diagnostic and reference centers should be aware that strains of *F. tularensis* which lack an overt requirement for cysteine or enriched medium for growth may be encountered in their facilities. At least one such atypical strain of *F. tularensis* has been described previously as part of a case study of oculoglandular tularemia (15). The isolation of a strictly aerobic, tiny, weakly staining, gram-negative coccobacillus which lacks the aforementioned growth requirements should lead one to suspect *Francisella* species. The use of CFA composition analysis coupled with agglutination in specific antiserum can facilitate early recognition of these atypical strains of *F. tularensis* and prompt implementation of appropriate biosafety containment of this hazardous pathogen.

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