

## *Francisella tularensis* Strain Typing Using Multiple-Locus, Variable-Number Tandem Repeat Analysis

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***Francisella tularensis*, the etiological agent of tularemia, is found throughout the Northern hemisphere. After analyzing the *F. tularensis* genomic sequence for potential variable-number tandem repeats (VNTRs), we developed a multilocus VNTR analysis (MLVA) typing system for this pathogen. Variation was detected at six VNTR loci in a set of 56 isolates from California, Oklahoma, Arizona, and Oregon and the *F. tularensis* live vaccine strain. PCR assays revealed diversity at these loci with total allele numbers ranging from 2 to 20, and Nei's diversity index values ranging from 0.36 to 0.93. Cluster analysis identified two genetically distinct groups consistent with the current biovar classification system of *F. tularensis*. These findings suggest that these VNTR markers are useful for identifying *F. tularensis* isolates at this taxonomic level. In this study, biovar B isolates were less diverse than those in biovar A, possibly reflecting the history of tularemia in North America. Seven isolates from a recent epizootic in Maricopa County, Ariz., were identical at all VNTR marker loci. Their identity, even at a hypervariable VNTR locus, indicates a common source of infection. This demonstrates the applicability of MLVA for rapid characterization and identification of outbreak isolates. Future construction of reference databases will allow faster outbreak tracking as well as providing a foundation for deciphering global genetic relationships.**

Tularemia is a disease with extensive geographic occurrence throughout North America, Asia, and Europe and is caused by the bacterium *Francisella tularensis*. *F. tularensis* is a small (0.2 to 0.7  $\mu$ m), highly virulent, gram-negative intracellular pathogen. Tularemia occurs in over 250 mammalian species, including humans (13). Although *Francisella* is found in arthropod vectors and infected mammal reservoirs, the bacterium can also be isolated from water, animal feces, and mud (10). The disease has been associated with outbreaks in Spain (1997), the Smolensk Province of Russia (1997), and South Dakota (1984), among others. Clinically, tularemia presents in two major forms: ulceroglandular and respiratory. Ulceroglandular tularemia is primarily contracted from arthropod vectors and direct contact with contaminated animals (10). Respiratory tularemia is associated with the inhalation of contaminated aerosols or dust (10).

The genus *Francisella* has two species, *F. tularensis* and *F. philomiragia*. Of these, *F. philomiragia* is relatively rare, less virulent, and most often associated with water (5). The more common, *F. tularensis*, has four subspecies, two of which are sometimes referred to as biovars. *F. tularensis* subsp. *tularensis* (*nearctica*, biovar type A) is found primarily in mammalian hosts and arthropod vectors of North America and has also recently been isolated in Europe (4). This subspecies exhibits the highest virulence of the four subspecies. The more moderately pathogenic *F. tularensis* subsp. *palaeartica* (*holoartica*, biovar type B) is mainly waterborne in Europe and Asia and to

a lesser degree in North America (12). *F. tularensis* subsp. *mediaasiatica* has only been isolated from locations in the post-Soviet republics of Central Asia (11). Finally, the *F. tularensis* subsp. *novicida* type strain was isolated from a Utah water sample in 1950.

The identification of *F. tularensis* and its subspecies differentiation has traditionally been accomplished by growth characteristics and biochemical analysis (6). *F. tularensis* subsp. *tularensis* (biovar A) ferments glycerol and glucose and produces citrulline ureidase (4). *F. tularensis* subsp. *palaeartica* (biovar B) ferments only glucose and does not produce citrulline ureidase (4). Recently the capture enzyme-linked immunosorbent assay has been applied in the detection of human *F. tularensis* using lipopolysaccharide-specific monoclonal antibodies (3). Studies of the 16S rRNA gene have demonstrated a sequence similarity of at least 98% between the two observed species of *Francisella*, *F. tularensis* and *F. philomiragia* (2), revealing their close phylogenetic relationship and allowing their discrimination. Repetitive extragenic palindromic, enterobacterial repetitive intergenic, and random amplified polymorphic DNA analyses have all proven useful for subspecies discrimination (12). Although these methods provide rapid species and subspecies differentiation, they do not appear applicable to individual strain discrimination (6). A strain-typing tool with greater resolving power would enhance our abilities to differentiate individual strains, detect transmission parameters, and assist in the control of tularemia outbreaks.

Simple sequence repeats or variable-number tandem repeats (VNTRs) have been shown to provide high-level discriminatory power for strain identification (14). This stems from the high mutability of repeat copy number in tandem arrays. Most genomes examined contain numerous VNTRs and, in combi-

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TABLE 1. VNTR PCR primer sequences

Primer name	Forward sequence	Reverse sequence
FT-V1	GATTTTTGGGGTTTTCTCTAAACATTTCTAAAATCTGCTTATC	GCAAACATTATTACCTTATACAGGTTATGGTAGTGGAATC
FT-V2	GTCATACCTTCTCATGTTTATTAATTACGATGTCTC	GTTTCAGGTTAAAACCTTTTCTATTGCCATAGC
FT-V3	GCGGTTTAGCTATTTTCAATATAATTTAAGTTTTTTGTGC	GTTGGTAAGGTTTTTTTATTCTAGTGCTTTTTGATTATCC
FT-V4	GAAATTCACCTACTCAACTGGGAGAATATAGCAAGGC	GCAAATTTATTTTAGGAGTCCACGTTGGATCATC
FT-V5	GTGGTCTTTTAAGCGTCTTAGCAAGCTCGAC	GGTACCCATCCCATATGTAAGTACAAAATGTAGC
FT-V6	GGACCAGAATACTACACAAAAGTCTATATAATGGTGAAG	GCTCCATATTACTCAAATAAAATACCCTGAATATAAAAAATATCATC
C1-C4	TCCGGTTGGATAGGTGTTGGATT	CGCGGATAATTTAAATTTCTATA

nation, can be used to develop a robust PCR-based marker typing system. When multiple-locus VNTR analysis (MLVA) is used, great discriminatory capacity and accurate estimation of genetic relationships can be obtained (1, 8, 9). We report here the successful application of MLVA for strain discrimination among a group of 55 North American *F. tularensis* isolates from locations including California, Oklahoma, Arizona, and Oregon. We have also included the *F. tularensis* live vaccine strain (LVS) as a reference strain in this analysis. Six novel VNTR loci were identified from genome sequences in this study. In addition, we have used a seventh locus described previously by Johansson et al. (7). Polymorphisms at these loci were then used to resolve the 56 isolates into 39 unique types, which demonstrated higher-level relationships consistent with the current biovar classification.

#### MATERIALS AND METHODS

**Genomic analysis.** The *F. tularensis* strain SHU S4 (biovar A) partial genomic sequence was downloaded from the *Francisella tularensis* website (<http://www.medmicro.mds.qmw.ac.uk/ft>) and used to identify potential VNTR loci. We screened approximately 120 contigs of available sequences for the presence of tandem repeats (9) by using the DNASTar software program Genequest (Lasergene, Inc., Madison, Wis.). This program locates and displays tandem and non-tandemly repeated arrays. Confirmation of the repeated sequence structure was performed using dot plot similarity analysis with the software program Megalign (Lasergene, Inc.).

**PCR amplification of VNTR loci.** MLVA primers were developed around 33 potential VNTR loci using the DNASTar program PrimerSelect. However, only six primer sets ultimately amplified polymorphic VNTR loci (Table 1). Reagents used in the PCRs were obtained from Life Technologies. Primers were designed with annealing temperatures from 65 to 61°C, though they were used under annealing conditions of 4°C lower. While shorter primers would work, these high temperatures were chosen for more rapid thermocycling and because of constraints of the AT-rich sequence. The sequence of the seventh primer set was obtained from Johansson et al. (7). An annealing temperature of 64°C was used for the C1-C4 primer set (Table 1).

PCR amplification of the seven variable loci from 55 *F. tularensis* isolates was carried out in the following mixture: 2 mM MgCl<sub>2</sub>, 1× PCR buffer, 0.1 mM concentrations of deoxynucleoside triphosphates, 1 μM concentrations of R110, R6G, or Tamra phosphoramidite fluorescence-labeled dUTPs (Perkin-Elmer Biosystems), 0.5 U of *Taq* polymerase, 1.0 μl of template DNA, 0.5 μM forward primer, 0.5 μM reverse primer, and filtered sterile water to a volume of 12.5 μl. The reaction mixtures were incubated at 94°C for 5 min and then cycled at 94°C for 30 s, 61 or 56°C for 30 s, 72°C for 30 s, and 94°C for 30 s for 35 cycles, with a final incubation at 72°C for 5 min.

**Isolate DNA.** DNA isolated by heat lysis (8) from a total of 55 *F. tularensis* strains was obtained from the California Department of Health (32 of the 55 strains), the Oklahoma Department of Health (10 of the 55 strains), and the Arizona Department of Health (7 of the 55 strains) (Table 2). The *F. tularensis* LVS culture strain was obtained as a gift from John Wright, U.S. Army, Dugway, Utah.

**Automated genotyping.** Fluorescently labeled amplicons were sized by denaturing polyacrylamide gel electrophoresis on an ABI 377 DNA Sequencer. Analysis was accomplished using the Genescan and Genotyper software (9). The PCR product was diluted threefold and mixed 1:1 with equal parts of a 5:1 formamide-

dextran blue dye mixture and a size standard prior to electrophoresis. Bioventures ROX 1000 size standards were used for estimating amplicon sizes. Because amplicon sizes determined by migration relative to standards do not always agree with the sizes predicted by direct nucleotide sequence determination, at least one allele for each locus was completely sequenced. All gels were analyzed using ABI filter set A.

**Statistical analysis.** Pairwise genetic differences among isolates were estimated using a simple matching coefficient. The clustering method used to evaluate genetic relationships was the unweighted pair group method with arithmetic mean (UPGMA) of the software package PAUP4a (D. Swofford, Sinauer Associates, Inc., Sunderland, Mass.). The diversity (*D*) for each marker was calculated as  $D = [1 - \Sigma(\text{allele frequencies})^2]$  (15).

#### RESULTS AND DISCUSSION

**Identification and diversity of VNTR markers.** We identified 33 repeated sequence motifs as potential VNTRs from the 1.84 Mb of the available *F. tularensis* genomic sequence. Five primer pairs failed to support PCR amplification and 22 were amplified but no variation was detected. These failures may be due to the preliminary nature of the available *F. tularensis* genome sequence. Ultimately, we observed six polymorphic VNTR loci (Table 3) among 55 *F. tularensis* isolates from California, Oklahoma, Arizona, and Oregon (Table 2) and the LVS.

The allele number in these six loci ranged from two alleles for Ft-V5 and Ft-V6 to 20 alleles for the hypervariable marker Ft-V4 (Table 3). This variation may be due to genomic restraints on the production of large repeat arrays, which could confer more flexibility in the variation of small repeats. Previous studies in *Yersinia pestis* (9) indicate higher copy number repeats exhibit higher allelic variability than lower copy repeats. Likewise, in our study we observed greater variability in loci with higher repeat copy numbers. For example, marker Ft-V2 (Table 3) has a repeat copy number of 18 in the SHU S4 strain and exhibits 10 alleles, while marker Ft-V5 with a copy number of 5 exhibits only 4 alleles (Table 3). In general, we found small repeat motifs were less variable than larger repeat motifs. Marker Ft-V6, with a 2-bp repeat motif, displayed only 2 alleles, while 10 alleles were observed for the 16-bp repeat motif of marker Ft-V2 (Table 3). The repeat motifs displayed a range from 2 to 21 bp in length (Table 3). The smallest array size ranged from 2 bp for marker Ft-V2 to 5 bp for Ft-V3 (Table 3). The largest array size ranged from 6 bp for markers Ft-V5 and Ft-V6 to 27 bp for the hypervariable marker Ft-V4 (Table 3). Whether these observations are generalizable is difficult to discern, given that only six loci have been characterized.

Marker utility is partially determined by observed diversity. Diversity values ranged from 0.36 to 0.96, with an overall

TABLE 2. *F. tularensis* strains

Strain name	State <sup>a</sup> -county	Sample information	Year
CA-ALA-1	CA-Alameda	Isolated by animal passage from live ticks	1981
CA-ALA-2	CA-Alameda	Human, cutaneous ulcer	1985
CA-ALP	CA-Alpine	<i>Spermophilus lateralis</i>	1985
CA-BUT-1	CA-Butte	Human, type B, blood isolate	1983
CA-BUT-2	CA-Butte	Human, node aspirate	1982
CA-COC-1	CA-Contra Costa	Human, infected insect bite	1995
CA-COC-2	CA-Contra Costa	Human, type B, cervical abscess, Pinote	1999
CA-COC-3	CA-Contra Costa	Human, type B, empyema fluid, San Pablo	1999
CA-ELD	CA-El Dorado	Cat, V940395	1994
OR-BND	OR-Bend	Human, sputum	1991
CA-INY-1	CA-Inyo	Human, neck node, Bishop	1984
CA-INY-2	CA-Inyo	Human, infected insect bite, Bishop	1985
CA-KRN-1	CA-Kern	<i>Spermophilus beecheyi</i> , Frazier Park, Camp Tecuya	1996
CA-KRN-2	CA-Kern	Gopher, V988	1999
CA-LAS	CA-Lassen	Human, epitrochlear node, hunted rabbits	1984
CA-MAR-1	CA-Marin	Human, axillary mass	1998
CA-MAR-2	CA-Marin	Human, pleural fluid, San Rafael	1999
CA-PLU	CA-Plumas	<i>Eutamias quadrimaculatus</i> , V96-2017	1986
CA-SDI	CA-San Diego	Ground squirrel, V940370	1994
CA-SFR	CA-San Francisco	Human, type B, neck mass	1999
CA-SLO-1	CA-San Luis Obispo	<i>Microtus californicus</i> , V98-1492	1998
CA-SLO-2	CA-San Luis Obispo	<i>Microtus californicus</i>	1998
CA-SLO-3	CA-San Luis Obispo	Human, lymph node aspirate	1982
CA-SC/SM	CA-Santa Clara or San Mateo	Squirrel, San Mateo or Santa Clara county	1997
CA-SCL-1	CA-Santa Clara	Squirrel monkey, Stanford	1993
CA-SCL-2	CA-Santa Clara	Human, sputum	1994
CA-SCR-1	CA-Santa Cruz	Human, neck lymph node, Watsonville	1984
CA-SCR-2	CA-Santa Cruz	Human, blood, has roaming house cat	1991
CA-SCR-3	CA-Santa Cruz	Human, cat scratch on finger	1983
CA-TUL	CA-Tulare	<i>Spermophilus lateralis</i> , V84-1197	1984
CA-3245	CA-Unknown	Unknown	1997
CA-3603	CA-Unknown	Cat, V92-451	1992
CA-VEN	CA-Ventura	Human, pleural fluid, handled dead rabbit	1993
CA-YOL-1	CA-Yolo	Capuchin monkey, type B, UC Davis	1999
CA-YOL-2	CA-Yolo	Squirrel monkey, Sacramento Zoo, type B	1989
CA-YOL-3	CA-Yolo	Rhesus monkey subman, UC Davis, type B	1989
CA-YOL-4	CA-Yolo	Rhesus monkey isolate, UC Davis, type B	1990
CA-YOL-5	CA-Yolo	Monkey, Sacramento City Zoo	1988
AZ-MAR-1	AZ-Maricopa	<i>Sylvilagus arizonae</i> (cotton rat), spleen	2000
AZ-MAR-2	AZ-Maricopa	<i>Sylvilagus auduboni</i> (cottontail rabbit), liver	2000
AZ-MAR-3	AZ-Maricopa	<i>Spermophilus variegatus</i> (rock squirrel)	2000
AZ-MAR-4	AZ-Maricopa	Tamarin monkey, spleen	2000
AZ-MAR-5	AZ-Maricopa	<i>Spermophilus beecheyi</i> , Frazier Park, Camp Tecuya	2000
AZ-MAR-6	AZ-Maricopa	<i>Sigmodon arizonae</i> (cotton rat)	2000
AZ-MAR-7	AZ-Maricopa	Tamarin monkey, liver	2000
OK-ADA	OK-Adair	Human	2000
OK-CAN	OK-Canadian	Human, ulceroglandular, wound culture	1993
OK-CHK	OK-Cherokee	Human, blood culture, deceased	2000
OK-HUG	OK-Hughes	Human, blood culture, hunting exposure	1993
OK-OKL-1	OK-Oklahoma	Human	1994
OK-OKL-2	OK-Oklahoma	Human, blood culture, deceased	2000
OK-OTW	OK-Ottawa	Human	1992
OK-TUL-1	OK-Tulsa	Human, wound culture	1994
OK-TUL-2	OK-Tulsa	Human, ulceroglandular, wound culture	1996
OK-TUL-3	OK-Tulsa	Human, wound culture	1993
LVS		LVS, type B, ATCC 29629	

<sup>a</sup> CA, California; OR, Oregon; AZ, Arizona; OK, Oklahoma.

average diversity index of 0.53 (Table 3). Markers with high diversity values, such as Ft-V4 with a *D* of 0.96 (Table 3), may have high mutation rates or be under environmental pressure to diversify. Diverse markers have the greatest discriminatory power for the identification of genetically similar strains, but their capacity would be compromised if selection were impor-

tant. VNTR marker loci that exhibit relatively low diversity values, such as Ft-V3 with a *D* of 0.36 (Table 3), may have utility for species, subspecies, and biovar identification.

The nucleotide sequence structure of the VNTR loci is well illustrated by dot plot analysis (Fig. 1). The center diagonal line in each panel of Fig. 1 represents the identity of the sequence

TABLE 3. VNTR marker attributes

Marker locus	Repeat motif	GenBank accession no.	Repeat size (nt) <sup>b</sup>	SHU S4 strain array size (nt)	Largest array size (nt)	Smallest array size (nt)	No. of alleles	Diversity <sup>a</sup>
Ft-V1	TTGGTGAACCTTTCTTGCTCTT	AY037288	21	3	7	4	4	0.7
Ft-V2	TTTCTACAAATATCTT	AY037290	16	18	14	2	10	0.53
Ft-V3	TTAATG	AY037289	6	4	7	5	2	0.36
Ft-V4	AATAAGGAT	AY037287	9	25	27	3	20	0.93
Ft-V5	GT	AY037291	2	5	6	3	4	0.53
Ft-V6	TA	AY037286	2	5	6	4	2	0.51
Avg value								0.53

<sup>a</sup> Diversity was determined by the equation  $D = 1 - \sum(\text{allele frequency})^2$ .  
<sup>b</sup> nt, nucleotides.

with itself, while parallel diagonals indicate directly repeated sequences. For example, the nucleotide structure in Ft-V1 has four 21-bp repeats represented by the one diagonal and three parallel lines (Fig. 1). The Ft-V4 marker locus shows a compound repeat structure, where the 9-nucleotide repeat sequence differs within the array. The allele presented in Fig. 1 has eight repeats of AACAAAGAC and 12 repeats of AATA-

AGGAT. Although three nucleotide differences separate these repeats now, they doubtlessly are derived from a common ancestor. Mutations must have arisen and spread to adjacent repeats until differentiation occurred to create the current mixed-sequence array. Repeat copy number variation among our strain collection is present in both sides of this array, which has been documented by direct DNA sequence analysis (data

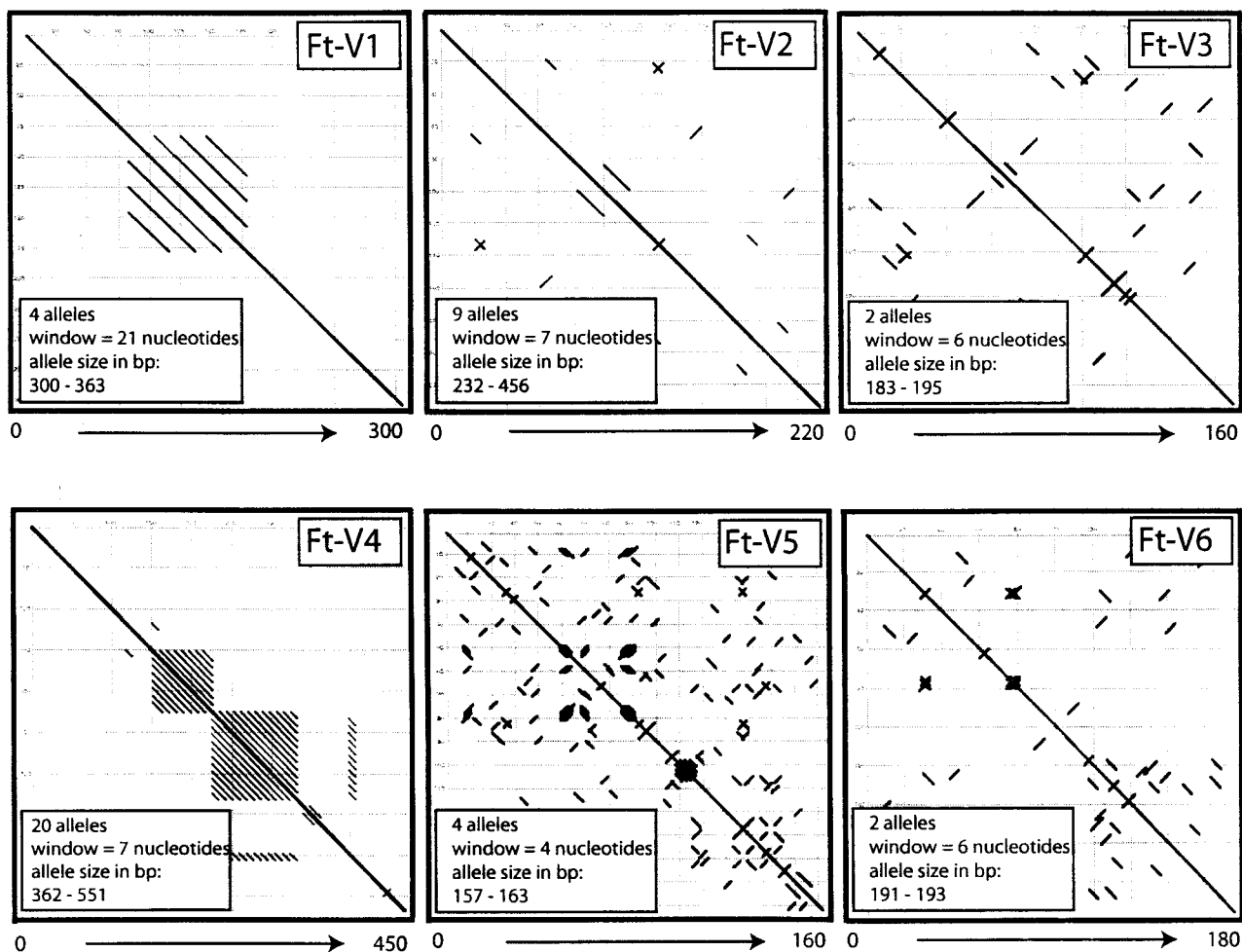


FIG. 1. Dot plot homology at individual marker loci. Dot plot homology analysis was performed using self comparison of each VNTR locus's nucleotide sequence. The panels represent the entire amplicon generated with primers from Table 1. All analyses used a 100% similarity requirement as indicated in each panel. The allele sizes presented in this figure are 321, 248, 183, 479, 159, and 191 bp (Ft-V1 through Ft-V6, respectively).

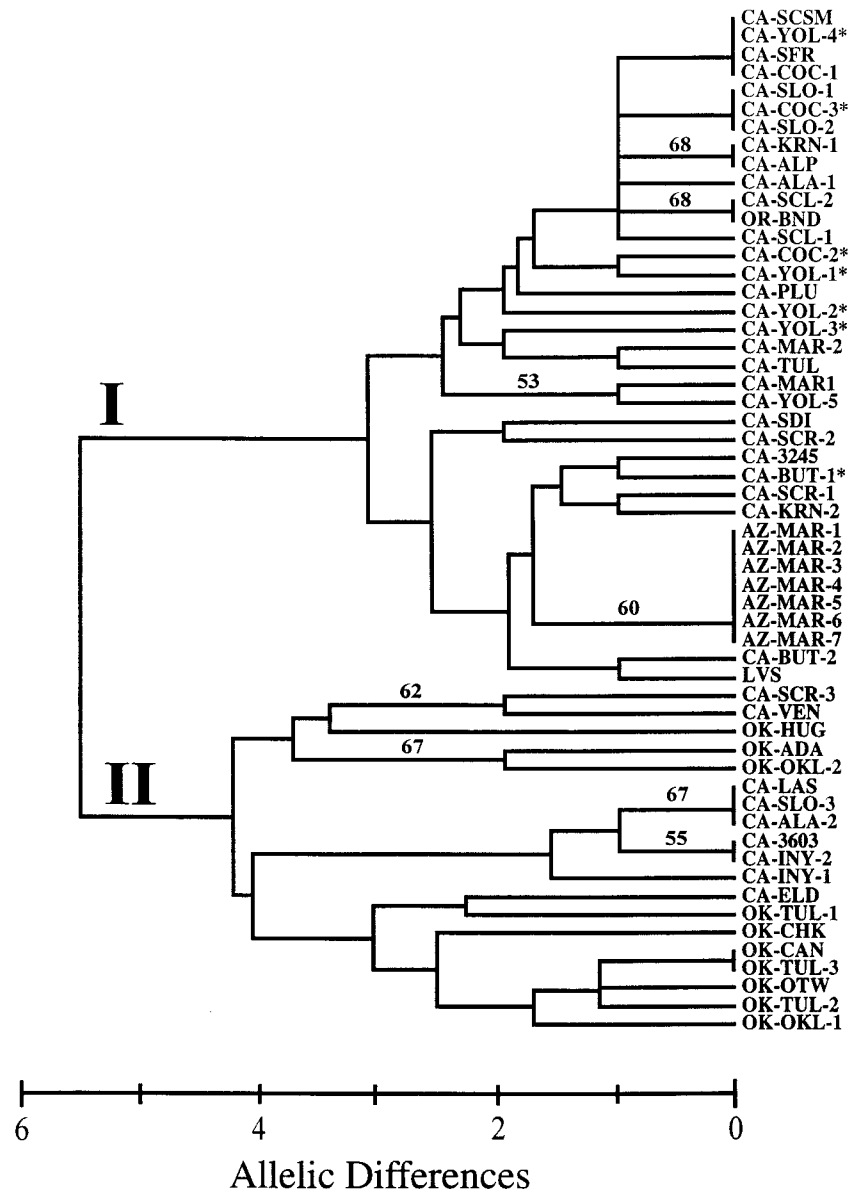


FIG. 2. Dendrogram based upon six MLVA markers. This dendrogram was generated using UPGMA analysis based upon allele differences among isolates. Letters to the right of each branch correspond to geographical origin: California isolates are identified by a county-specific three letter code, Arizona county isolates are designated by AZ, and Oklahoma isolates are listed as OK. Numbers associated with branch lengths represent bootstrap values using 1,000 simulations. An asterisk designates strains of known *F. tularensis* biovar B classification.

not presented). Because the repeat size on both sides of the array is the same, such variation is detectable only by nucleotide sequence determination.

**Genetic relationships among isolates.** In order to understand the genetic relationships among samples, genetic distances among the *F. tularensis* isolates were calculated using the seven marker loci and then subjected to UPGMA cluster analysis.

Thirty-nine unique marker allele-size combinations (genotypes) were observed among the 56 isolates. Two major clusters were apparent and subdivisions also occurred within these groups (Fig. 2). These genetic clusters are primarily due to distinct allele frequencies, since no absolute fixed allelic dif-

ference exists between cluster I and cluster II (Table 4). The average genetic distance is approximately 5.8 allelic differences (out of 7 possible) between cluster I and cluster II (Fig. 2).

The 38 California isolates were found clustered in both of the two major subdivisions: clusters I and II (Fig. 2). California strains CA-YOL-5, CA-YOL-2, CA-YOL-3, CA-YOL-1, CA-YOL-4, CA-SFR, and CA-BUT-1 were previously identified as *F. tularensis* subsp. *tularensis* biovar B (Table 1) and are all found in cluster I (Fig. 2). The *F. tularensis* LVS also grouped within cluster I (Fig. 2) and is of known biovar B classification. The 37 strains within cluster I assembled into three discernible though weakly supported groups (Fig. 2). The average distance among these cluster I subgroups is approximately three mark-

TABLE 4. *F. tularensis* allele size

Cluster and strain	Allele size (bp)						
	Ft-V1	Ft-V2	Ft-V3	Ft-V4	Ft-V5	Ft-V6	C1-C4 <sup>a</sup>
Cluster I							
CA-SC/SM	303	230	183	434	157	191	300
CA-YOL-4	303	230	183	434	157	191	300
CA-SFR	303	230	183	434	157	191	300
CA-COC-1	303	230	183	434	157	191	300
CA-SLO-1	303	230	183	425	157	191	300
CA-COC-3	303	230	183	425	157	191	300
CA-SLO-2	303	230	183	425	157	191	300
CA-KRN-1	303	230	183	389	157	191	300
CA-ALP	303	230	183	389	157	191	300
CA-ALA-1	303	230	183	407	157	191	300
CA-SCL-2	303	230	183	416	157	191	300
OR-BND	303	230	183	416	157	191	300
CA-SCL-1	303	230	183	443	157	191	300
CA-COC-2	303	230	183	425	159	191	300
CA-YOL-1	303	230	183	434	159	191	300
CA-PLU	345	230	183	425	157	191	300
CA-YOL-2	303	230	183	407	155	191	300
CA-YOL-3	282	230	183	407	159	191	300
CA-MAR-2	282	230	183	452	157	191	300
CA-TUL	282	230	183	470	157	191	300
CA-MAR-1	324	230	183	461	159	191	300
CA-YOL-5	324	230	183	461	157	191	300
CA-SDI	324	230	183	551	159	193	300
CA-SCR-2	345	230	183	488	159	193	300
CA-3245	324	230	183	443	157	193	300
CA-BUT-1	324	230	183	479	157	193	300
CA-SCR-1	345	230	183	479	157	193	300
CA-KRN-2	303	230	183	479	157	193	300
AZ-MAR-1	345	230	183	470	157	193	300
AZ-MAR-2	345	230	183	470	157	193	300
AZ-MAR-3	345	230	183	470	157	193	300
AZ-MAR-4	345	230	183	470	157	193	300
AZ-MAR-5	345	230	183	470	157	193	300
AZ-MAR-6	345	230	183	470	157	193	300
AZ-MAR-7	345	230	183	470	157	193	300
CA-BUT-2	282	230	183	371	157	193	300
LVS	282	230	183	443	157	193	300
Cluster II							
CA-SCR-3	282	342	195	497	161	193	330
CA-VEN	282	327	195	506	161	193	330
OK-HUG	282	342	195	434	157	193	?
OK-ADA	282	358	195	443	157	191	330
OK-OKL-2	282	230	195	461	157	191	330
CA-LAS	282	214	183	362	159	193	330
CA-SLO-3	282	214	183	362	159	193	330
CA-ALA-2	282	214	183	362	159	193	330
CA-3603	282	214	183	371	159	193	330
CA-INY-2	282	214	183	371	159	193	330
CA-INY-1	282	214	183	371	163	195	330
CA-ELD	282	423	195	524	159	193	330
OK-TUL-1	282	408	195	533	159	193	?
OK-CHK	324	374	195	452	159	193	330
OK-CAN	303	374	195	398	159	193	?
OK-TUL-3	303	358/342 <sup>b</sup>	195	434/452 <sup>b</sup>	159	193	?
OK-OTW	303	358	195	515	159	193	?
OK-TUL-2	303	358	195	452	159	193	?
OK-OKL-1	303	390	195	461	159	193	330

<sup>a</sup> As reported in Johansson et al. (7). ?, amplicon size data absent due to PCR amplification failure.

<sup>b</sup> Markers displayed two allele sizes in this strain.

ers. California isolates CA-SC/SM, CA-YOL-4, CA-SFR, and CA-COC-1 showed 100% identity, as did CA-SLO-1, CA-COC-3, and CA-SLO-2 (Fig. 2). Within cluster I, the Oregon isolate (OR-BND) showed 100% identity (Fig. 2) with the

isolate from Santa Clara County (CA-SCL-2); both strains were isolated from sputum collections (Table 1).

Cluster II includes 9 California strains of unknown biovar type and 10 Oklahoma strains (biovar A), which assembled into three apparent groups (Fig. 2). Examination of the California spatial distribution within both major clusters reveals completely overlapping geographic locations (Fig. 2). For example, isolates from San Luis Obispo County are found in both major clusters (CA-SLO-1 and CA-SLO-3), as are samples from Alameda County (CA-ALA-1 and CA-ALA-2).

The tularemia cases represented by the California isolates group into two separate clusters (I and II), suggesting the presence of a very subdivided reservoir of *F. tularensis* biovars in this region. Temporal overlap is evident as both major clusters contain samples obtained throughout the 1980s and 1990s, ruling out a separation in time (Table 1). Although the samples are well separated in collection date, there appear to be few genetic changes occurring over this period. These casual observations were combined with a formal statistical analysis using a Mantel test (significance level of  $P > 0.05$ ; data not shown) and indicated that geographic and temporal data are not correlated with the genetic type. The great diversity and nongeographic partitioning of this diversity suggest a complex disease cycle in California involving both biovars A and B. Either the pathogen is frequently transported into the regions or a highly diverse reservoir exists to generate distinctive outbreaks, biovars notwithstanding.

In contrast to the California strains, the year 2000 tularemia cases clustered in Maricopa County, Ariz., are related, indeed identical, to each other when analyzed using our methods. Seven isolates of *F. tularensis* subsp. *haloaretica* (biovar B) from this epizootic showed 100% identity and assembled within the third group of cluster I (Fig. 2). The identity is apparent even with marker Ft-V4, which is highly diverse. The lack of any Ft-V4 allelic difference is consistent with a recent common clonal ancestor. The absence of allelic variation among the Arizona strains strongly supports a point-source epidemiological model (where the infection spreads from a single origin) rather than a model with multiple sources. Host victims were a mixture of captive and wild animals, but these data do not indicate whether the disease spread from captive to wild animals or vice versa. However, further characterization of the resident animal reservoir could provide evidence to evaluate these two alternate hypotheses.

Historically, Oklahoma represents one of the three largest *F. tularensis* reservoirs in the United States. The 10 Oklahoma strains and 9 California strains clustered together into two minor groups within cluster II (Fig. 2). California strains CA-LAS, CA-SLO-3, and CA-ALA-2 appeared identical within the second minor group of cluster II, as did strains CA-3603 and CA-INY-2 (Fig. 2). Of the Oklahoma strains, only OK-CAN and OK-TUL-3 showed 100% identity (Fig. 2). It should be noted that markers Ft-V2 and Ft-V4 each displayed two allele sizes in the aforementioned strains (Table 4). It is possible that this result is due to strain contamination; this result is reported in Table 4 but was not used for the phylogenetic analysis shown in Fig. 2. All Oklahoma isolates appear loosely affiliated with the nine California strains found in cluster II (Fig. 2). While all 10 of the Oklahoma isolates are *F. tularensis* subsp. *tularensis* (biovar A), the VNTRs easily divided them

into nine unique genotypes. The observed marker differences among Oklahoma samples are most consistent with a model of multiple emergences from an animal reservoir. A somewhat diverse reservoir is likely to exist in Oklahoma, given these unique types.

Previous studies identified a marker (C1-C4) that allows discrimination between *F. tularensis* biovars A and B (7). Analysis of our strains at this locus revealed two alleles, which was consistent with the previous study and our knowledge of the biovar classification of *F. tularensis* (Table 4). The allelic variation that we observed in this marker clearly supports our cluster I and cluster II categories and validates that they represent biovar A (cluster II) and biovar B (cluster I).

In our study we found that cluster II isolates are much more diverse than cluster I isolates (Fig. 2). Because all of our isolates are from North America, this difference may reflect the history of tularemia on this continent rather than the inherent diversity of the two types. Biovar B may be a historically recent import to North America and, hence, its isolates are less diverse due to a colonization bottleneck. Future comparative studies of European and Asian *F. tularensis* isolates will be a test of this hypothesis.

The application of MLVA to the genetic characterization of *F. tularensis* isolates has provided significant strain discriminatory power. Using relatively few markers against these North American isolates, our data reflect the successful application of MLVA in discriminating between major *Francisella* groups consistent with the current biovar classifications (Fig. 2). Although subspecies classification appears possible with MLVA, this approach is most powerful when applied to the rapid discrimination between individual outbreak strains for epidemiological analysis. The contrast between the Arizona and the Oklahoma or California isolates illustrates this well. Because MLVA data can be standardized (Table 4), they are easily compared to data generated at dispersed laboratories, unlike other methods commonly employed. In this regard, these data are similar to nucleotide sequence data. Future studies across multiple laboratories will be able to directly compare MLVA data with the results reported here. A multilaboratory electronic database will allow for fast characterization and identification of *F. tularensis* isolates from outbreaks and provide the foundation for deciphering global genetic relationships.

It is known that VNTRs provide a potential mechanism for metabolic regulation as well as offering great potential for antigenic variation and environmental adaptation (14). While these studies do not address such issues, our identification and characterization of VNTR variation provides a starting point for such research.

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