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Multiple Locus Variable Number Tandem Repeat Analysis of *Francisella tularensis*

C.W. Stratilo and D.E. Bader
DRDC Suffield

Defence R&D Canada

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Abstract

Francisella tularensis, the causative agent of tularemia, is highly virulent with an extremely low infective dose. Although *F. tularensis* comprises four subspecies (*tularensis, holarctica, mediasiatica,* and *novicida)*, the two most clinically important subspecies are the highly virulent *F. tularensis tularensis* (nearctica, biovar type A) and the moderately virulent *F. tularensis holarctica* (palaearctica, biovar type B). *F. tularensis* is considered a potential biological weapon and listed as a Category A select agent by the Centers for Disease Control (CDC). A PCR-based typing system that targets variable number tandem repeats (VNTR) at multiple loci (MLVA) can distinguish very closely related bacterial strains. In this report we describe the MLVA typing of 24 strains of *F. tularensis* in the DRDC Suffield collection using 25 VNTR loci. The DRDC Suffield strains, although quite heavily weighted towards *F. tularensis holarctica* (18 strains), do have representation from other subspecies including *F. tularensis tularensis* A.I, *F. tularensis tularensis* A.II, *F. tularensis holarctica* B.V Japan; and *F. tularensis mediasiatica*.

Résumé

Francisella tularensis, agent étiologique de la tularémie, présente une forte virulence et une dose infectieuse extrêmement faible. Bien que *F. tularensis* se divise quatre sous-espèces (*tularensis, holarctica, mediasiatica* et *novicida)*, les deux plus importantes sur le plan clinique sont *F. tularensis tularensis* (biovar Nearctica, type A), qui est de forte virulence, et *F. tularensis holarctica* (biovar palaearctica, type B), qui est de virulence modérée. Considéré comme une arme biologique potentielle, *F. tularensis* est classé comme une substance désignée de catégorie A par les Centers for Disease Control (CDC). Il existe un système de typage par PCR ciblant des répétitions en tandem en nombre variable (VNTR) dans plusieurs locus (MLVA) qui permet de distinguer des souches bactériennes étroitement apparentées. Le présent rapport décrit le typage par MLVA de 24 souches de *F. tularensis* de la collection de RDDC Suffield dans 25 locus de VNTR. Bien que les souches de RDDC Suffield appartiennent majoritairement à la sous-espèce *F. tularensis holarctica* (18 souches), d'autres sous-espèces sont représentées, notamment *F. tularensis tularensis* A.I, *F. tularensis tularensis* A.II, *F. tularensis holarctica* B.V Japan et *F. tularensis mediasiatica*.

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Executive summary

Multiple Locus Variable Number Tandem Repeat Analysis of *Francisella tularensis*

Chad Stratilo; Doug Bader; DRDC Suffield TM 2009-144; Defence R&D Canada – Suffield; October 2009.

Introduction: *Francisella tularensis*, the causative agent of tularemia, is considered a potential biological warfare agent and is listed as a Category A select agent by the Centers for Disease Control (CDC). It exists as four subspecies. Of these, *F. tularensis tularensis* is highly virulent with an extremely low infective dose and appears to be ubiquitous throughout the United States and Canada, while the less pathogenic *F. tularensis holarctica* is endemic throughout the Northern Hemisphere. High resolution genetic subtyping of *F. tularensis* would facilitate better understanding of it geographical distribution and host species; such knowledge could also possibly assist forensic investigations where intentional use is suspected.

Results: DRDC Suffield in partnership with the Public Health Agency of Canada National Microbiology Laboratory (PHAC–NML) previously established a molecular typing method for *B. anthracis* (the causative agent of anthrax) based on different numbers of short repetitive regions between strains at multiple locations within the organism's genomic DNA (MLVA) which allows very closely related bacterial strains to be distinguished. This technique has now been applied to the 24 strains of *F. tularensis* held by DRDC Suffield. Although the DRDC Suffield collection is quite heavily weighted towards *F. tularensis holarctica* (18 strains), genetic relationships between the various subspecies were revealed through MLVA using 25 locations in the genome. The strains present in the DRDC Suffield collection, do have representation from other subspecies including *F. tularensis tularensis* A.I, *F. tularensis tularensis* A.II, *F. tularensis holarctica* B.V Japan; and *F. tularensis mediasiatica*.

Significance: Although a discriminatory MLVA typing scheme has been developed, there is no information as to the origin of fourteen of these strains. With so few strains of known pedigree, further characterization of the genetic diversity of *F. tularensis* in Canada will not be possible until a larger repository of isolates with known pedigrees has been established.

Future Plans: Although this project is complete, it is hoped that the methods and results established in this paper can be used as a structure when typing additional samples of *F. tularensis*. DRDC Suffield does not currently have any plans to collect further isolates.

Sommaire

Multiple Locus Variable Number Tandem Repeat Analysis of *Francisella tularensis*

Chad Stratilo; Doug Bader; DRDC Suffield TM 2009-144; R & D pour la défense Canada – Suffield; Octobre 2009.

Introduction ou contexte : *Francisella tularensis*, agent étiologique de la tularémie, est considéré comme une arme biologique potentielle et est classé comme une substance désignée de catégorie A par les Centers for Disease Control (CDC). Il se divise en quatre sous-espèces. Parmi celles-ci, *F. tularensis tularensis*, qui présente une forte virulence et une dose infectieuse extrêmement faible, semble être répandu partout aux États-Unis et au Canada, et *F. tularensis holarctica*, moins pathogène, est endémique partout dans l'hémisphère Nord. Le sous-typage génétique à haute résolution de *F. tularensis* aiderait à éclaircir sa répartition géographique et ses espèces hôtes; ces connaissances pourraient également faciliter les enquêtes judiciaires dans les cas où une utilisation intentionnelle est soupçonnée.

Résultats : RDDC Suffield a déjà établi, en partenariat avec le Laboratoire national de microbiologie (LNM) de l'Agence de la santé publique du Canada (ASPC), une méthode de typage moléculaire de *B. anthracis* (agent étiologique de la maladie du charbon) fondée sur de courtes régions répétitives en nombre variable entre des souches dans plusieurs locus d'ADN génomique (MLVA), ce qui permet de distinguer des souches bactériennes étroitement apparentées. Cette méthode a été appliquée aux 24 souches de *F. tularensis* détenues par RDDC Suffield. Bien que les souches de RDDC Suffield appartiennent majoritairement à la sous-espèce *F. tularensis holarctica* (18 souches), les relations génétiques entre différentes sous-espèces ont été examinées par MLVA dans 25 locus du génome. Parmi les souches faisant partie de la collection de RDDC Suffield, on compte également *F. tularensis tularensis* A.I, *F. tularensis tularensis* A.II, *F tularensis holarctica* B.V Japan et *F. tularensis mediasiatica*.

Importance : Bien qu'une méthode de typage discriminatoire par MLVA ait été mise au point, on ne dispose d'aucune information concernant l'origine de 14 de ces souches. Par conséquent, il ne sera pas possible de caractériser davantage la diversité génétique de *F. tularensis* au Canada tant qu'on n'aura pas établi un référentiel plus vaste d'isolats dont l'origine généalogique est connue.

Perspectives : Bien que le projet soit terminé, nous espérons que les méthodes et les résultats présentés pourront servir de base au typage d'autres échantillons de *F. tularensis*. À l'heure actuelle, RDDC Suffield ne prévoit pas prélever d'autres isolats.

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Introduction

Francisella tularensis, the causative agent of tularemia, is a small, gram-negative, intracellular pathogen. *F. tularensis* is highly virulent with an extremely low infective dose [1]. Tularemia infections have been documented in more than two hundred animal species. The transmission cycle of *F. tularensis* is not well characterized. The factors supporting tularemia foci, both environmentally and ecologically, remain largely unknown. *F. tularensis* is one of the most pathogenic microorganisms known due to its highly infectious nature and mortality rate. It is considered a potential biological weapon and listed as a Category A select agent by the Centers for Disease Control (CDC) [2].

F. tularensis comprises the four subspecies *tularensis, holarctica, mediasiatica,* and *novicida*. The two most clinically important subspecies of tularemia are the highly virulent *F. tularensis tularensis* (nearctica, biovar type A) and the moderately virulent *F. tularensis holarctica* (palaearctica, biovar type B) [3].

F. tularensis tularensis is found primarily in mammalian hosts and arthropod vectors of North America [4]. This subspecies exhibits the highest virulence of the four subspecies. *F. tularensis tularensis* has two subpopulations that are geographically distinct (A.I. and A.II) [4].

F. tularensis holarctica is found in much of the Northern Hemisphere [5] and has been grouped into five distinct groups (*F. tularensis holarctica* I to V). *F. tularensis holarctica* Group V contains isolates from Japan that are phenotypically similar to *F. tularensis holarctica* but genetic evidence would suggest they represent a fifth *F. tularensis* subspecies.

F. tularensis mediasiatica has only been isolated from locations in the post-Soviet republics of Central Asia and little is known concerning its pathogenicity to humans [5]. *F. tularensis novicida* is rarely isolated and found mostly in North America [6].

F. tularensis subspecies show highly conserved genomic sequences between strains of diverse origin. All four subspecies are genetically similar with 99.8 % identity among 16S rRNA genes [7]. Numerous typing methods that show concordant results (repetitive element PCR [8], PFGE [9], insertion sequence-element probe restriction fragment length polymorphism analysis [10] and whole genome microarray [11]) have been described. Variable number tandem repeats (VNTRs) have been used in numerous bacteria species (e.g. *B. anthracis* and *Y. pestis)* for strain specific discrimination, including *F. tularensis* [12 – 14]. Using several VNTR loci in combination, a robust PCR-based typing system allows bacteria of the same species to be differentiated from each other even if they are genetically very homogeneous.

MLVA typing (multiple-locus variable-number tandem repeat analysis) of *F. tularensis* has been successfully carried out using 192 isolates revealing 120 genotypes [15]. The MLVA typing system for *F. tularensis* uses twenty five VNTR loci for strain discrimination. These loci are amplified by PCR using fluorescently labelled primers. The PCR products can be sized by fragment size analysis or they can be sequenced directly using capillary electrophoresis.

In this report we describe the MLVA typing of 24 strains of *F. tularensis* from the DRDC Suffield collection using 25 VNTR loci. Strains were typed using singleplex PCR reactions that were later pooled for analysis. The amplicons were distinguished from one another using different fluorescent dyes or expected amplicon sizes, prior to capillary electrophoresis.

Materials and Methods

Bacterial Strains and DNA Isolation

The strains typed in this study are part of the DRDC Suffield strain collection (Table 1).

Table 1. F. tularensis strains typed in this study

DRDC Designation	Source[a]	Origin (trivial name)[b]	*Francisella* subspecies[c]
Swed1	SDRA	Squirrel, Georgia (SnMF) Group A	*F. tularensis tularensis* A.I
Swed2	SDRA	Human, Ohio (SCHU) Group A	*F. tularensis tularensis* A.I
Swed3	SDRA	Water supply (Helena)	*F. tularensis holarctica*
Swed4	SDRA	Eigelsbach strain	*F. tularensis holarctica*
Swed5	SDRA	Human, Japan (Jap4)	*F. tularensis holarctica* B.V
Swed6	SDRA	Human, Sweden (SBL R45)	*F. tularensis holarctica*
Swed7	SDRA	Human, Utah Group A	*F. tularensis tularensis* A.II
Swed8	SDRA	Gerbil, Middle Asia CIS (120)	*F. tularensis mediasiatica*
Swed9	SDRA	Water, Russia (P-13863)	*F. tularensis holarctica*
Swed10[d]	SDRA	Live vaccine strain (LVS)	*F. tularensis holarctica*
FT58	PHAC-NML	Unknown	*F. tularensis tularensis* A.I
FT65	PHAC-NML	Unknown	*F. tularensis holarctica*
FT67	PHAC-NML	Unknown	*F. tularensis holarctica*
FT68	PHAC-NML	Unknown	*F. tularensis holarctica*
FT69	PHAC-NML	Unknown	*F. tularensis holarctica*
FT70	PHAC-NML	Unknown	*F. tularensis holarctica*
FT71	PHAC-NML	Unknown	*F. tularensis holarctica*
FT72	PHAC-NML	Unknown	*F. tularensis holarctica*
FT73	PHAC-NML	Unknown	*F. tularensis holarctica*
FT76	PHAC-NML	Unknown	*F. tularensis holarctica*
FT77	PHAC-NML	Unknown	*F. tularensis holarctica*
FT105	PHAC-NML	Unknown	*F. tularensis holarctica*
FT106	PHAC-NML	Unknown	*F. tularensis holarctica*
FT109	PHAC-NML	Unknown	*F. tularensis holarctica*

[a] SDRA = Swedish Defense Research Agency, PHAC-NML = Public Health Agency of Canada - National Microbiology Laboratory.
[b] Origin and trivial names were provided by the Agency supplying the strains.
[c] *Francisella* subspecies designations are based on the analysis presented in this paper .
[d] Also referred to as FTLVS or FTLVS-3 in this paper.

Strain DNA was isolated in the BSL3 bacterial suite using MasterPure™ DNA & RNA Purification Kits (Epicentre Biotechnologies, Madison, WI, USA), Phase lock gels® (Eppendorf, Westbury, NY, USA) or GNOME® DNA Isolation Kits (QBiogene, Irvine, CA, USA) according to the manufacturer's instructions. Concentrations of approximately 10 ng/μL were obtained from each strain tested. DNA from *Francisella tularensis* live vaccine strain (LVS) or an LVS equivalent was used as a positive control. Sterile 18 megaohm double distilled water (DDW) was used as a negative control.

MLVA

PCR amplification of the 25 variable loci was carried out in a total volume of 25 μL. The final concentration of the PCR reagents were 2 mM $MgCl_2$, 1× PCR buffer, 2 mM dNTPs, 0.5 U Taq polymerase (Life Technologies, Inc., Rockville, Md.), 1.0 μL of template DNA, 0.5 μM forward primers, 0.5 μM reverse primer. The primers that were used, as well as the 5' phosphoramidite fluorescent dyes used with each primer, are listed in Table 2 and were prepared by IDT (Integrated DNA Technologies Inc., Coralville, IA, USA).

Table 2. Primers used for amplification of VNTR loci

Locus Name	Primer name and 5' modification	Primer Sequence 5' – 3'
Ft-M1	Ft-M1F- 6-FAM Ft-M1R	GAGCTGGTCAAGTTTATTTAAGTA CAGGATTGCTTGAACATGATA
Ft-M2	Ft-M2F -TAMRA Ft-M2R	TTTATGATAAGGATGATTTAAAACAAAATA GCTTAAATCTCGCAATACCATGTAAT
Ft-M3	Ft-M3F-TAMRA Ft-M3R	GTTTTCACGCTTGTCTCCTATCA CAAAAGCAACAGCAAAATTCACAAA
Ft-M4	Ft-M4F-TAMRA Ft-M4R	AAAAGGGCGGGTTACTGAGG GTATCAAATAGCGCAAAAATAACTGC
Ft-M5	Ft-M5F-HEX Ft-M5R	TAGGCATGACAAACCCTGCTAT CAGCTCGAACTCCGTCATAC[e]
Ft-M6	Ft-M6F-6-FAM Ft-M6R	TTTTGGGTTTTCTCTAAACATTTCTA CAATTCAGCGAAACCCTATCTTA
Ft-M7	Ft-M7F-HEX Ft-M7R	ATTGGGTGATTTGGATGGTTG CAGCTCGAACTCCGTCATAC[e]
Ft-M8	Ft-M8F-HEX Ft-M8R	AGTAATCTAGCCAAGGTAATA CAGCTCGAACTCCGTCATAC[e]

[e] This reverse primer targets the VNTR locus within the multicopy *F. tularensis* insertion sequence element, ISFtu1. Forward primers at these loci target alternate genomic locations to include the 16 bp VNTR locus

Locus Name	Primer name and 5' modification	Primer Sequence 5' – 3'
Ft-M9	Ft-M9F-6-FAM Ft-M9R	AAGGACCTATTTTTACATCAGT CAGCTCGAACTCCGTCATAC[e]
Ft-M10	Ft-M10F-HEX Ft-M10R	GTTGGCGAACCTAAAATAATAGC CAGCTCGAACTCCGTCATAC[e]
Ft-M11	Ft-M11F-TAMRA Ft-M11R	AAACCTACAATCAACATCTGACAAT TTGTTATATTAACCTCATCAGTTCAATTTA
Ft-M12	Ft-M12F-6-FAM Ft-M12R	CGCTAGATGGTGCTGATACTATCTT CCTGCTAGAAAACCCATATTTACAT
Ft-M13	Ft-M13F-HEX Ft-M13R	TTTGCAACTACTAGGTGTGGAGAT TTGATATTCCAAATGATCAAGTTTT
Ft-M14	Ft-M14F-TAMRA Ft-M14R	ACCGCCATCTTTTCTATCATAAT AACCTTAAGTGATAAATATAACCCAAAA
Ft-M15	Ft-M15F-HEX Ft-M15R	GCATGGACATGAGTGTCTATGGCGTAGATC GATAAAGGAATGTTTTAAATAATGTGATGTTTTGCATC
Ft-M16	Ft-M16F- HEX Ft-M16R	AGGAAAGCATACCCAACATTATT CCAAAGATCGCCGTGATT
Ft-M17	Ft-M17F-6-FAM Ft-M17R	GCTATAGCAGTAAATGTAGGCTCAA ACATATCGGTGGATCACTATCAA
Ft-M18	Ft-M18F-6-FAM Ft-M18R	AACAGCCTTCAAACCACCTT CATAAAATACAGCTTCAATAAACAATCTT
Ft-M19	Ft-M19F-6-FAM Ft-M19R	TCCGGTTGGATAGGTGTTGGATT AGGCGGAGATCTAGGAACCTTT
Ft-M20	Ft-M20F-6-FAM Ft-M20R	GCATAACTTTTGAGACAATTGGTGCAGATGATC GACCGCCAGTATATGCTTGACCTTGACTCC
Ft-M21	Ft-M21F-TAMRA Ft-M21R	CCACAGCTAGCCAGACCAAAT AGTTTGGCGCGAGCTAAT
Ft-M22	Ft-M22F-HEX Ft-M22R	GTCAAAATCTCAAGATGAGCAAATATTTGAATGGT GGAGTTTTTTCTCGTCCGCTGTTAGTGATTT
Ft-M23	Ft-M23F-TAMRA Ft-M23R	TGAGATGTGGAACTTTATAGGTTCAA TGTAAACTAAAAGATAACTAATGGCAATTT
Ft-M24	Ft-M24F-TAMRA Ft-M24R	ATACGGTCCTAATAATATTCCTGTCA ATTCATTTATAGATGCCTTTGTTACC
Ft-M25	Ft-M25F-HEX Ft-M25R	GTGGTCTTTTAAGCGTCTTAGCAAGCTCGAC GGGTACCCATCCCATATGTAAGTACAAATGTAGC

The PCR reaction mixtures were incubated at 94 ºC for 5 min and then cycled at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s for 35 cycles with a final incubation of 72 °C for 5 min using GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA, USA). PCR reagents

were obtained from Life Technologies, Inc. or Applied Biosystems Inc. PCR products (2 µL) were then pooled as described in Table 3 and diluted with DDW to a final volume of 80 µL.

Table 3. Product pools for fragment analysis on ABI 3100

Primer	Amplicon Size	5' Fluorescent Tag	Pool #
Ft-M10	617-857	HEX	1
Ft-M20	255-435	6-FAM	1
Ft-M24	459-522	TAMRA	1
Ft-M25	157-161	HEX	1
Ft-M1	310-316	6-FAM	2
Ft-M22	242-272	HEX	2
Ft-M23	435-504	TAMRA	2
Ft-M16	221-241	HEX	3
Ft-M19	316-342	6-FAM	3
Ft-M21	403-445	TAMRA	3
Ft-M14	442-454	TAMRA	4
Ft-M15	222-234	HEX	4
Ft-M9	248-360	6-FAM	4
Ft-M17	351-363	6-FAM	5
Ft-M4	434-459	TAMRA	5
Ft-M7	207-303	HEX	5
Ft-M12	345-365	6-FAM	6
Ft-M3	432-711	TAMRA	6
Ft-M5	208-304	HEX	6
Ft-M2	463-583	TAMRA	7
Ft-M6	311-437	6-FAM	7
Ft-M8	286-382	HEX	7
Ft-M11	392-422	TAMRA	8
Ft-M13	325-361	HEX	8
Ft-M18	372-390	6-FAM	8

One µL of the diluted, pooled PCR products was combined with eight µL of HiDi Formamide and one µL of the combined size standard (0.5 µL Rhodamine-X MapMarker (70 – 400 bp) and 0.5 µL CST ROX (420 – 800 bp)) from BioVentures Inc., Murfreesboro, TN.

The PCR amplification products were sized using the Applied Biosystems 3100 Genetic Analyzer with a 36 cm capillary and filter set D for fragment analysis. Allele names were established by direct sequencing of PCR amplicons by PHAC–NML and DRDC Suffield. The bin sets for *F. tularensis* alleles are found in Table 4. Phylogenetic analysis was carried out using UPGMA options from Bionumerics software (Applied Maths, Inc., Austin, TX, USA).

Table 4. F. tularensis bin set panels[f]

Marker	Bin Parameters	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8
Ft-M1	Sequence (bp)	310[g]	313						
	Location	309[h]	312						
	Offset(left/right)	1.0/1.0	1.0/1.0						
Ft-M2	Sequence (bp)	451	463	511	559	577	595	613	
	Location	453	465	512	560	578	596	615	
	Offset(left/right)	1.5/ 1.5	1.5/1.5	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	
Ft-M4	Sequence (bp)	434	439	444					
	Location	439	444	449					
	Offset(left/right)	1.5/1.0	1.5/1.0	1.0/1.0					
Ft-M5	Sequence (bp)	192	208	224	240	256			
	Location	189	205	221	237	253			
	Offset(left/right)	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0			
Ft-M6	Sequence (bp)	311	332	353	374	395			
	Location	312	334	354	376	398			
	Offset(left/right)	1.0/1.0	1.0/1.0	1.0/1.25	1.0/1.0	1.0/1.0			
Ft-M7	Sequence (bp)	175	191	207					
	Location	173.5	189	206					
	Offset(left/right)	1.5/1.5	1.0/1.0	1.0/1.0					
Ft-M8	Sequence (bp)	236	253	270	286	302			
	Location	235	250	267	283	300			
	Offset(left/right)	1.0/1.0	1.0/1.0	1.5/1.0	1.0/1.0	1.0/1.0			
Ft-M9	Sequence (bp)	216	232	248	344				
	Location	214	230	246	342				

[f] Bin set panel data for the Ft-M3 marker is found at the end of this table.
[g] Allele size was determined by sequence analysis and accepted as the allele name for a given locus.
[h] Allele size was determined by fragment sizing analysis but was not used for the allele name for a given locus .

Marker	Bin Parameters	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8
	Offset(left/right)	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0				
Ft-M10	Sequence (bp)	345	361	370	425	489	505	553	617
	Location	343	361	369	424	490.91	506	555	619
	Offset(left/right)	1.0/1.0	2.0/1.5	1.0/1.0	1.0/1.0	1.0/1.0	1.5/1.5	1.0/1.0	1.0/1.0
Ft-M11	Sequence (bp)	392							
	Location	393							
	Offset(left/right)	1.0/1.0							
Ft-M12	Sequence (bp)	335	345						
	Location	334	344						
	Offset(left/right)	1.0/1.0	1.0/1.0						
Ft-M13	Sequence (bp)	313	325						
	Location	311	323						
	Offset(left/right)	1.0/1.0	1.0/1.0						
Ft-M14	Sequence (bp)	436	442	448					
	Location	438	443	449					
	Offset(left/right)	1.0/1.0	1.0/1.0	1.0/1.0					
Ft-M15	Sequence (bp)	222	228						
	Location	220	226						
	Offset(left/right)	1.0/1.0	1.0/1.0						
Ft-M16	Sequence (bp)	211	221						
	Location	208	218						
	Offset(left/right)	1.0/1.0	1.0/1.0						
Ft-M17	Sequence (bp)	345	351						
	Location	344	350						
	Offset(left/right)	1.0/1.0	1.0/1.0						
Ft-M18	Sequence (bp)	360	372						
	Location	359	371						
	Offset(left/right)	1.0/1.5	1.0/1.5						
Ft-M19	Sequence (bp)	286	316						
	Location	285	315						
	Offset(left/right)	1.0/1.0	1.0/1.5						
Ft-M20	Sequence (bp)	255	267	303	345	351	393	435	519
	Location	255	266	301	345	351	392.5	434	518

Marker	Bin Parameters	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8
	Offset(left/right)	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0
Ft-M21	Sequence (bp)	396	403	410	417				
	Location	398.5	405.5	412.5	419.5				
	Offset(left/right)	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0				
Ft-M22	Sequence (bp)	242	248	254	272				
	Location	238	244	250	268				
	Offset(left/right)	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0				
Ft-M23	Sequence (bp)	412	435						
	Location	412	435						
	Offset(left/right)	1.0/1.0	1.0/1.0						
Ft-M24	Sequence (bp)	459	480						
	Location	462	484						
	Offset(left/right)	1.0/1.0	1.0/1.0						
Ft-M25	Sequence (bp)	157	159						
	Location	154	156						
	Offset(left/right)	1.0/0.5	1.0/1.0						

Marker	Bin Parameters	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7
Ft-M3	Sequence (bp)	234	279	297	306	315	324	333
	Location	234	282	300	309	318	327	337
	Offset (left=right)	1.0	1.0	1.0	1.5	1.5	1.5	1.5
Marker	**Bin Parameters**	**Allele 8**	**Allele 9**	**Allele 10**	**Allele 11**	**Allele12**	**Allele 13**	**Allele 14**
Ft-M3	Sequence (bp)	342	351	360	369	378	387	396
	Location	345	354.5	364	371	381	390	402
	Offset (left=right)	1.5	1.5	1.5	1.0	1.0	1.0	1.0
Marker	**Bin Parameters**	**Allele 15**	**Allele 16**	**Allele 17**	**Allele 18**	**Allele 19**		
Ft-M3	Sequence (bp)	405	432	441	459	477		
	Location	407	436	445	457	484		
	Offset (left=right)	1.0	1.0	1.0	1.5	1.5		

Results and Discussion

The amplicon sizes of the 24 *Francisella* strains typed in this study for all 25 loci are found in Annex A. The amplicon sizes are based on sequencing data. Amplicon sizes were found to be within the expected range based on other published reports [15]. Identification of our strains to a specific subspecies and group (Table 1) was made possible based on evaluating amplicons for those loci that comprise a distinct allele or set of alleles compared to data in other published reports [15].

F. tularensis tularensis represent the most virulent of the *F. tularensis* subspecies. Four strains analyzed in this study (Swed1, Swed2, Swed7, and FT58) were identified as *F. tularensis tularensis* A type strains but were also further differentiated as being A.I or A.II type strains (Table 1). Our analysis indicated that the Swed7 strain is an *F. tularensis tularensis* A.II strain while the other three strains, Swed1, Swed2, and FT-58 are *F. tularensis tularensis* A.I strains. *F. tularensis tularensis* A.I strains have a subspecies specific VNTR at locus 17 repeated three times resulting in a total amplicon size of 351 bp while in all other subspecies and groups, the VNTR is repeated two times resulting in a total amplicon size of 345 bp. Similarly for locus 22, *F. tularensis tularensis* A.I strains have a specific VNTR of two repeat units (242 bp) while all other subspecies and groups have VNTRs of 3 to 14 repeats (248-326 bp) [15]. Locus 20 comprises a 12 bp VNTR that is repeated between 3 and 39 times [15]. In strain SCHU, it is repeated just three times resulting in a 255 bp fragment [15]. The Swed2 strain in our collection is also designated as strain SCHU and it generated a 255 bp amplicon at locus 20, which supports this designation. The other two type A.I strains (Swed1 and FT-58) generated a 255 bp amplicon as well indicative of A.I strains. By contrast, *F. tularensis tularensis* A.II strains have a VNTR repeated between 22-25 times at locus 20. Strain Swed7 had an allele of 519 bp or a VNTR of 25 repeats at locus 20. Together the data showed that Swed7 does not share the group specific VNTRs of the other three A type strains and, therefore, it appears to be an *F. tularensis tularensis* A.II strain.

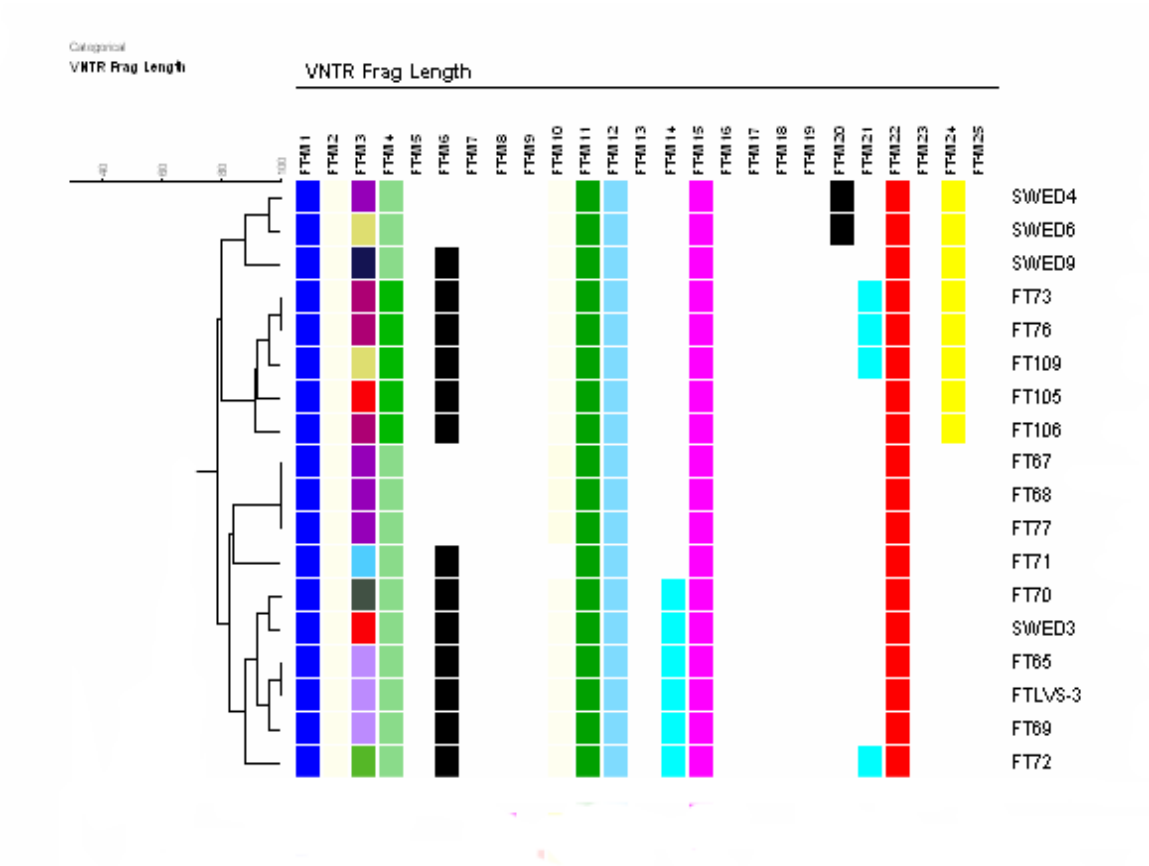
One strain, Swed8, was identified as *F. tularensis mediasiatica* due to the unique allele at locus 1. With all other subspecies and their groups, the 3 bp VNTR is repeated three times for a total amplicon size of 310 bp but for *F. tularensis mediasiatica*, the 3 bp VNTR is repeated four times which generated an amplicon of 313 bp [15]. This designation is further supported by locus 11 where *F. tularensis mediasiatica* has a specific VNTR of three repeats while all other subspecies have a VNTR of four repeats or greater [15]. This was observed in our analysis of Swed8 as well.

The remaining 19 strains that were typed in this study, were found to be *F. tularensis holarctica* strains. As mentioned previously, *F. tularensis holarctica* is found in much of the Northern Hemisphere [5] and has been grouped into five distinct groups. *F. tularensis holarctica* Group V contains isolates from Japan that are phenotypically similar to *F. tularensis holarctica* but genetic evidence would suggest they represent a fifth *F. tularensis* subspecies. *F. tularensis holarctica* from Japan has a VNTR of between 11 and 18 repeats at locus 20 [15]. One of the 19 *F. tularensis holarctica* in this study, Swed5, had an allele of 435 bp or a VNTR of 18 repeats indicating that it is a member of the group *F. tularensis holarctica* from Japan. This is also supported by the source information about Swed5 (Table 1).

The remaining 18 *F. tularensis holarctica* strains were found to be part of the *F. tularensis holarctica* subspecies belonging to B groups I – IV. These strains were identified as such using

locus 25 which contains a VNTR of four repeats (157 bp) while all other subspecies have a VNTR of five repeats (159 bp). The phylogenetic relationships of the 18 *F. tularensis holarctica* B group I – IV strains are shown in Figure 1 (the other six *F. tularensis* strains were not included in the phylogenetic analysis due to very few isolates and a desire not to weight the loci nor to weight the measure of difference).

Figure 1. The phylogenetic relationship of 18 F. tularensis holarctica strains in the DRDC Suffield Collection



The *F. tularensis holarctica* B group breaks into four distinct groups which represent the four cohesive groups (B.I -IV) within this subspecies [15]. The phylogenetic analysis of the 18 DRDC Suffield *F. tularensis holarctica* strains appeared to break into 4 clades as well. Unfortunately, due to a lack of reference strains identified by Johansson *et al*. [15], the only sub group that could be assigned to our strains was *F. tularensis holarctica* B.III since this group includes the live vaccine strain (LVS) as does our collection (indicated as FTLVS-3 in Figure 1 but also referred to as SWED10 or FT-LVS in this paper). The clade that contains the LVS strain also includes FT70, Swed3, FT65, FT69, and FT72, which are thus identified as *F. tularensis holarctica* B.III

strains. Unfortunately, for the reasons stated above, no further designations could be assigned from our phylogenetic data.

It should be noted that while there was good correlation between our MLVA data and that of others, there were inconsistencies as well. For example, locus 19 should have a VNTR of one repeat for all B groups whereas all other groups should have a VNTR of two repeats [15] resulting in a difference between the two alleles of 13 bp. By contrast, our results indicated a uniform difference of 30 bp between the alleles of the B group and all other groups (286 bp compared to 316 bp). In addition, Swed5 (*F. tularensis holarctica* from Japan) was found to have a 222 bp allele at locus 15. Other published reports state that *F. tularensis holarctica* strains from Japan have a common allele of 228 bp present at this locus. Our results indicated a polymorphism at this locus for this group [15]. Furthermore, in this study, locus 20 has a repeat unit of 12 bp as indicated in the literature [15], but PHAC-NML has identified several alleles that appear to be separated by differences that were divisible by 6 but not 12. Therefore, it is possible that locus 20 may contain interrupted repeats or the VNTR repeat unit is 6 bp in length.

We also noticed differences in the size of the amplicons (number of bp) determined by fragment length sizing analysis versus direct sequence analysis, both of which were performed on the same sequencer (Applied Biosystems 3100, Applied Biosystems Inc.). The difference between length established by sequencing and the length determined by fragment analysis differs even though there may be no actual size differences. These discrepancies may be attributed to the type of phosphoramidite fluorescent dye used in 5' labeling for fragment analysis. The allele name representing the VNTRs at a given locus was designated according to the size of the fragment that was determined using sequence analysis as opposed to the size obtained by fragment analysis. The difference between amplicon length established by fragment length analysis and direct sequencing can be determined by comparing the allele name, established by direct sequencing, with the location of the allele established by fragment length analysis as depicted in Table 4.

In summary, while the DRDC Suffield *F. tularensis* collection is quite small, comprising just 24 isolates, MLVA analysis of these strains identified the subspecies to which they belong and established their genetic relationships. The DRDC Suffield strains, although quite heavily weighted towards *F. tularensis holarctica* (18 strains), do have representation from other subspecies including *F. tularensis tularensis* A.I (Swed1, Swed2, FT58), *F. tularensis tularensis* A.II (Swed7), *F. tularensis holarctica* B.V, Japan (Swed5); and *F. tularensis mediasiatica* (Swed8).

The MLVA typing method established in this project allows additional strains to be typed and placed within the established matrix at any time in the future. The molecular typing data obtained for the *F. tularensis* strains in our collection provides the capability to track these strains should their integrity come into question. High resolution subtyping could be used for forensic investigations where intentional use is suspected. Understanding the genetic diversity of *F. tularensis* in natural populations within Canada and worldwide would help to distinguish a natural outbreak from an intentional use situation. This would require a large scale typing effort comprising a larger number of medical and environmental *F. tularensis* isolates than is currently present in the DRDC Suffield collection.

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Annex A - MLVA typing results at 25 loci for 24 *F. tularensis* strains[i]

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
FT58	310	463	333	434	224	332	191	270	344	505	392	345	325	442	222	221	351	372	316	255	417	242	435	459	159
FT65	310	451	360	439	192	395	175	253	216	361	392	345	313	448	228	211	345	360	286	255	396	248	412	459	157
FT67	310	451	351	439	192	311	175	253	216	370	392	345	313	442	228	211	345	360	286	255	396	248	412	459	157
FT68	310	451	351	439	192	311	175	253	216	370	392	345	313	442	228	211	345	360	286	255	396	248	412	459	157
FT69	310	451	360	439	192	374	175	253	216	361	392	345	313	448	228	211	345	360	286	255	396	248	412	459	157
FT70	310	451	324	439	192	353	175	253	216	361	392	345	313	448	228	211	345	360	286	255	396	248	412	459	157
FT71	310	451	396	439	192	332	175	253	216	345	392	345	313	442	228	211	345	360	286	255	396	248	412	459	157
FT72	310	451	315	439	192	332	175	253	216	361	392	345	313	448	228	211	345	360	286	255	403	248	412	459	157
FT73	310	451	333	444	192	332	175	253	216	361	392	345	313	442	228	211	345	360	286	255	403	248	412	480	157
FT76	310	451	333	444	192	332	175	253	216	361	392	345	313	442	228	211	345	360	286	255	403	248	412	480	157
FT77	310	451	351	439	192	311	175	253	216	370	392	345	313	442	228	211	345	360	286	255	396	248	412	459	157
FTLVS[j]	310	451	360	439	192	395	175	253	216	361	392	345	313	448	228	211	345	360	286	255	396	248	412	459	157
FT105	310	451	342	444	192	332	175	253	216	361	392	345	313	442	228	211	345	360	286	255	396	248	412	480	157
FT106	310	451	333	444	192	353	175	253	216	361	392	345	313	442	228	211	345	360	286	255	396	248	412	480	157
FT109	310	451	306	444	192	332	175	253	216	361	392	345	313	442	228	211	345	360	286	255	403	248	412	480	157
SWED1	310	577	360	434	192	311	207	253	248	425	392	345	325	442	222	221	351	372	316	255	396	242	412	459	159
SWED2	310	463	432	434	208	311	207	286	248	617	392	345	325	442	222	221	351	372	316	255	403	242	435	459	159
SWED3	310	451	342	439	192	353	175	253	216	361	392	345	313	448	228	211	345	360	286	255	396	248	412	459	157
SWED4	310	451	351	439	192	311	175	253	216	361	392	345	313	442	228	211	345	360	286	267	396	254	412	480	157
SWED5	310	559	234	439	192	311	175	270	216	393	392	345	313	442	222	211	345	360	286	435	403	248	412	459	159
SWED6	310	451	306	439	192	311	175	253	216	361	392	345	313	442	228	211	345	360	286	267	396	254	412	480	157
SWED7	310	595	279	434	192	311	175	253	216	345	392	345	313	442	228	211	345	360	316	519	396	248	412	459	159
SWED8	313	451	459	434	256	395	175	236	232	361	372	335	313	436	228	211	345	360	316	303	396	272	412	459	159
SWED9	310	451	297	439	192	353	175	253	216	361	392	345	313	442	228	211	345	360	286	255	396	254	412	480	157

[i] Amplicon sizes are based on sequence analysis.
[j] Also referred to as SWED10 or FTLVS-3 in this paper.

List of symbols/abbreviations/acronyms/initialisms

CDC	Centers for Disease Control
CRTI	Chemical, Biological, Radiological-Nuclear, and Explosives Research and Technology Initiative
DDW	double distilled water
DRDC	Defence Research & Development Canada
Ft or FT	*Francisella tularensis*
MLVA	multiple-locus variable-number tandem repeat analysis
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PHAC-NML	Public Health Agency of Canada-National Microbiology Laboratory
SDRA	Swedish Defense Research Agency
VNTR	variable-number tandem repeats

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Francisella tularensis, the causative agent of tularemia, is highly virulent with an extremely low infective dose. Although *F. tularensis* comprises four subspecies (*tularensis, holarctica, mediasiatica,* and *novicida)*, the two most clinically important subspecies are the highly virulent *F. tularensis tularensis* (nearctica, biovar type A) and the moderately virulent *F. tularensis holarctica* (palaearctica, biovar type B). *F. tularensis* is considered a potential biological weapon and listed as a Category A select agent by the Centers for Disease Control (CDC). A PCR-based typing system that targets variable number tandem repeats (VNTR) at multiple loci (MLVA) can distinguish very closely related bacterial strains. In this report we describe the MLVA typing of 24 strains of *F. tularensis* in the DRDC Suffield collection using 25 VNTR loci. The DRDC Suffield strains, although quite heavily weighted towards *F. tularensis holarctica* (18 strains), do have representation from other subspecies including *F. tularensis tularensis* A.I, *F. tularensis tularensis* A.II, *F. tularensis holarctica* B.V Japan; and *F. tularensis mediasiatica*.

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Francisella tularensis; MLVA; molecular typing

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