

Association of Different Genetic Types of *Francisella*-Like Organisms with the Rocky Mountain Wood Tick (*Dermacentor andersoni*) and the American Dog Tick (*Dermacentor variabilis*) in Localities Near Their Northern Distributional Limits

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Dermacentor andersoni and *Dermacentor variabilis* from allopatric and sympatric populations near their northern distributional limits were examined for the presence of *Francisella* species using molecular techniques that targeted 373 bp of the 16S rRNA gene. Although there was no evidence for the presence of *Francisella tularensis* in any tick, *Francisella*-like endosymbionts (FLEs) were common in *D. andersoni* and *D. variabilis* adults and immatures. A significantly greater proportion of female ticks contained FLEs compared to male ticks. In addition, significantly more *D. variabilis* adult individuals contained multiple FLE sequence types than did *D. andersoni* adults. Ten different types of FLEs were identified based on the sequence data, which has implications for diagnostic tests and epidemiological studies of *F. tularensis* in tick populations in Canada. The three most prevalent types of FLEs have been detected previously in *D. andersoni* or *D. variabilis* from other parts of their distributional ranges, whereas the other seven FLE types have not been reported previously. A comparison of the FLEs from both allopatric and sympatric populations of these two tick species provided insight into the relative host-specificity and the modes of transmission of these tick-borne bacteria. In general, each FLE type was specific for one tick species, suggesting vertical transmission of each bacterium. However, there were a few instances of potential cross-transfer of two FLE types to the other tick species at locations where *D. andersoni* and *D. variabilis* occurred in sympatry, suggesting that there may be occasional horizontal transmission of some FLEs.

There are four recognized species within the genus *Francisella* (31), three of which can cause disease in humans (53). *Francisella tularensis*, the causative agent of tularemia in the Northern Hemisphere (16), varies considerably in its transmission patterns, virulence and disease presentation in different geographical areas (13, 38, 45, 50, 57). In North America, there are two common subspecies of *F. tularensis*: *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* (16). Human infections with *F. tularensis* subsp. *holarctica* are mainly acquired through direct contact with infected beavers, muskrats, or lagomorphs, whereas in the United States, *F. tularensis* subsp. *tularensis* is often acquired by tick bites (8, 12). The American dog tick, *Dermacentor variabilis* Say, 1821, and the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles, 1908, are important for the transmission of *F. tularensis* in the eastern and western United States, respectively (16, 23). The distributional ranges of *D. andersoni* and *D. variabilis* are largely allopatric (i.e., separate), except in some parts of Montana, Wyoming, Colorado, North Dakota, and South Dakota (2, 35, 44, 54, 61) and in Saskatchewan, Canada (S. J. Dergousoff and N. B. Chilton, unpublished data), where they occur in sympatry. These two tick species, as well as *Dermacentor albipictus*, *Dermacentor occidentalis*, *Dermacentor hunteri*, and *Dermacentor nitens*, are also hosts of a number of bacteria that are closely related to *F. tularensis* (24, 46, 51). These so-called *Francisella*-like endosymbionts (FLEs) are generally of undetermined pathogenicity but sometimes assumed to be nonpathogenic (15, 51). However, infection studies with the “*Dermacentor andersoni* symbiont” (DAS) showed it to be pathogenic for chicken embryos and guinea pigs (6).

Francisella tularensis is also endemic in Canada (62). However, compared to the United States, relatively few human cases of tu-

laremia have been documented (5, 34), some of which have occurred in Saskatchewan and Alberta in western Canada (5, 7, 30, 42, 49). Most human cases of tularemia in western Canada have been associated with contact with infected wildlife (3, 36, 43, 52, 60) or livestock (4, 28). Sporadic occurrences of tularemia have been reported in beavers (40), muskrats (18, 40), jackrabbits (4), snowshoe hares (62), ground squirrels (4), and sheep (28). The most recent outbreak occurred in deer mice (*Peromyscus maniculatus*), following a population explosion in 2005 near Madison, Saskatchewan. The causative agent was identified as *F. tularensis* subsp. *holarctica*, but the source of infection was not determined (63).

Although the transmission cycle of *F. tularensis* in Canada is not well defined, ticks (*Dermacentor* spp.) have been implicated as potential vectors in western Canada. For example, adult *D. andersoni* was important in some of the first recognized cases of human and animal tularemia in southern Alberta (4, 28). *Francisella tularensis* has been recovered from *D. andersoni* in British Columbia, southern Alberta, and southern Saskatchewan during surveys conducted between 1938 and 1946 (20, 32, 33). In 1982, *F. tularensis* was detected in adult *D. andersoni* from Saskatchewan Land-

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TABLE 1 Localities and coordinates of the collection sites of adult *D. andersoni* and *D. variabilis*, and the number of ticks positive for the presence of the *Francisella* 16S rRNA gene at each locality

Locality	Coordinates (decimal degrees)		<i>D. andersoni</i>		<i>D. variabilis</i>	
	Latitude (north)	Longitude (west)	No. tested	No. <i>Francisella</i> positive (%)	No. tested	No. <i>Francisella</i> positive (%)
Lethbridge, Alberta	49.73721	112.84751	100	73 (73)	— ^a	
Saskatchewan Landing Provincial Park, Saskatchewan	50.64528	107.96310	82	79 (96)	96	92 (96)
Grasslands National Park, Saskatchewan	49.21666	107.70000	17	15 (88)	1	1 (100)
Buffalo Pound Provincial Park, Saskatchewan	50.57582	105.31356	33	28 (85)	79	70 (89)
Douglas Provincial Park, Saskatchewan	51.02966	106.46590	14	14 (100)	40	40 (100)
Danielson Provincial Park, Saskatchewan	51.25933	106.89580	61	59 (97)	99	98 (99)
Outlook, Saskatchewan	51.48807	107.05817	18	16 (89)	12	11 (92)
Harris, Saskatchewan	51.73448	107.58370	100	83 (83)	12	9 (75)
Blackstrap Provincial Park, Saskatchewan	51.79760	106.45833	— ^a		105	95 (91)
Wakaw, Saskatchewan	52.60297	105.85426	— ^a		44	44 (100)
Minnedosa, Manitoba	50.24715	99.83870	— ^a		99	85 (86)
Kenora, Ontario	49.90153	94.49324	— ^a		30	28 (93)
Total			425	367 (86)	617	573 (93)

^a Tick species does not occur at this locality.

ing Provincial Park based on the results of transmission experiments in rabbits (26). Despite these reports, the prevalence of *F. tularensis* and FLEs in ticks in western Canada is unknown. The aim of the present study was to use PCR-based methods to determine the prevalence of *Francisella* and FLEs in sympatric and allopatric populations of *D. andersoni* and *D. variabilis* from 12 localities near their northern distributional limits, which includes Saskatchewan Landing Provincial Park, a location where *F. tularensis* in adult ticks has been detected previously. In addition, we examined if the different types of FLEs were specific for either tick species in sympatric populations of *D. andersoni* and *D. variabilis*.

MATERIALS AND METHODS

Collection of ticks. A total of 1,042 adult male and female ticks (425 *D. andersoni* and 617 *D. variabilis*) were collected by flagging grass and shrubs or were removed from vertebrate hosts at 12 localities in southwestern Canada (Table 1). Questing ticks obtained by flagging were collected in May and June of 2005 and from April to June in 2006. Some adult ticks were also collected from humans, horses, dogs, skunks, and raccoons between May and June in 2005 and 2007. Immature ticks (nine *D. andersoni* nymphs, six *D. variabilis* nymphs and 71 *D. variabilis* larvae) were collected from 13 deer mice (*Peromyscus maniculatus*), seven meadow voles (*Microtus pennsylvanicus*), and eight western jumping mice (*Zapus princeps*) trapped in Saskatchewan Landing Provincial Park in June and July of 2008, and in April of 2009. An additional seven *D. variabilis* nymphs and 143 *D. variabilis* larvae were collected from one deer mouse, one western jumping mouse, five meadow voles and 10 southern red-backed voles (*Myodes gapperi*) from Blackstrap Provincial Park (Saskatchewan) in May through July of 2009. All adult ticks were identified based on morphological examination. Adults of *D. andersoni* and *D. variabilis* are easily distinguished from one another, and from those of *D. albipictus* (a species that occurs in sympatry with the other two species [61]), based on differences in the morphology of their spiracular plates (27). The species identity of representative individuals was also verified using a PCR-based assay (11). Immature ticks were examined by microscopy to confirm they belonged to the Metastriata. Their species identity was determined using the same PCR assay as for the adults; however, amplicons were subjected to a restriction fragment length polymorphism (RFLP) analysis using *AluI* (Fermentas) according to the manufacturer's instructions. The RFLP analysis was used to confirm that the internal transcribed spacer 2 (ITS-2) amplicons of *D. variabilis* individuals were not from *D. albipictus*, which

has an ITS-2 amplicon of the same size. The ITS-2 sequence of *D. variabilis* lacks the restriction site for *AluI* present in the ITS-2 sequence of *D. albipictus* (11).

DNA preparation. Total genomic DNA (gDNA) was extracted and purified from each tick using a modification of the tissue protocol for the DNeasy tissue kit (Qiagen, Valencia, CA). Individual ticks were placed in 1.5-ml micropestle tubes (Kontes), to which 180 μ l of buffer ATL (Qiagen) was added. Ticks were homogenized by grinding with micropestles (Kontes) attached to a cordless drill. Proteinase K (20 μ l of 15 μ g/ μ l) was added to the homogenate. Samples were incubated for 16 h at 55°C. The gDNA was purified according to the DNeasy tissue kit protocol, except that gDNA was eluted twice from the spin columns using 50 μ l of buffer AE. The two elutions derived from the same tick were combined in a single tube and stored at -20°C.

PCR and single-strand conformation polymorphism (SSCP) of 16S rRNA gene. The presence of *Francisella* DNA in adult ticks was tested using a PCR targeting 373 bp of the bacterial 16S rRNA gene with the primers NC-Fran16S-F (5'-CAACATCTGGACCGAT-3') and NC-Fran16S-R (5'-TGCGGGACTTAACCCAACAT-3'), which were designed to be specific for *F. tularensis* and FLEs. PCRs were carried out in 25- μ l volumes containing 200 μ M each deoxynucleoside triphosphate (dNTP) (Fermentas, Burlington, Canada), 3 mM MgCl₂, 25 pmol (1 μ M) of each primer, 0.5 U of recombinant *Taq* DNA polymerase (Fermentas), 2.5 μ l 10 \times PCR buffer with (NH₄)₂SO₄ (Fermentas), and 2 μ l of template gDNA. A negative control (i.e., without gDNA) and positive control were included in each set of reactions. PCRs were performed in a thermocycler (iCycler; Bio-Rad, Hercules, CA) using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. The presence of *Francisella* DNA in immature ticks was tested using a nested PCR (nPCR) because of the possibility that the relative numbers of bacteria within an individual larva or nymph may be significantly lower than in an adult tick. The first phase of the nPCR targeted 1,141 bp of the bacterial 16S rRNA gene with primers F11 (5'-TACCAGTTGGAAACGACTGT-3') and F5 (5'-CCTTTTGGAGTTTCGCTCC-3') (17). Each reaction mixture contained 200 μ M each dNTP (Fermentas), 2.5 mM MgCl₂, 25 pmol (1 μ M) of each primer, 0.5 U of recombinant *Taq* DNA polymerase (i*Taq*; Bio-Rad), 2.5 μ l 10 \times PCR buffer (Bio-Rad), and 2 μ l of template gDNA. The PCR conditions used were 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 65°C for 60 s, and 72°C for 60 s and a final extension at 72°C for 5 min. PCR products, including those of the negative controls, were then purified by adding shrimp alkaline phosphatase (0.014 U/ μ l) (New England BioLabs, Pick-

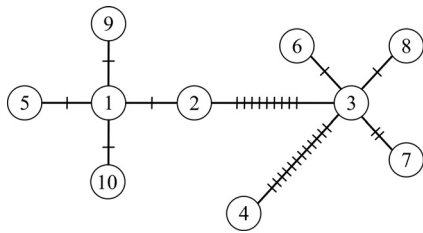


FIG 1 Minimum spanning network tree depicting the relationships of the 10 FLE sequence types detected in *D. andersoni* and *D. variabilis* from western Canada, based on comparisons of the partial sequence of the 16S rRNA gene. FLE types 5, 9, and 10 were found exclusively in *D. andersoni*, while types 3, 4, 6, 7, and 8 were found only in *D. variabilis*. Types 1 and 2 were detected in both *D. andersoni* and *D. variabilis*. Hatch marks indicate the number of nucleotide differences between sequences of adjacent types.

ering, Canada) and exonuclease I (0.27 U/ μ l) (Fermentas) and incubating the mixture at 37°C for 15 min and then at 80°C for 15 min. The second phase of the nPCR was conducted with 2 μ l of purified PCR products (including the negative-control samples) using primers NC-Fran16S-F and NC-Fran16S-R and the same PCR conditions used for the adult ticks. Additional negative-control samples were also included. Amplicons were subjected to electrophoresis on SYBR safe-stained 1.5% agarose-Trisborate-EDTA (TBE) gels, and their banding patterns were visualized by UV transillumination. Some of the initial amplicons produced from the adult ticks were subjected to DNA sequencing to verify the specificity of the PCR assay.

SSCP analyses (11) were performed on all samples that were PCR positive. This mutation scanning technique can be used to differentially display DNA sequences that differ by one or more nucleotides (19). In the present study, SSCP was used to prescreen all amplicons for genetic variation before selecting representative samples of each different SSCP profile for DNA sequencing. Samples that were sequenced previously were used as mobility controls in SSCP gels. Where possible, multiple amplicons of each SSCP profile were prepared for DNA sequencing.

DNA sequence analyses. Amplicons from 76 adult ticks and 21 immature ticks were column purified (MinElute DNA purification kit; Qiagen) and then sequenced using primers NC-Fran16S-F and NC-Fran16S-R in separate reactions. The 16S rRNA gene sequences, excluding primer sites, were manually aligned, and a BLAST search was performed to determine sequence similarity with those of other bacterial 16S rRNA gene sequences deposited in GenBank. Included in the analyses were the 16S rRNA gene sequences of different subspecies of *F. tularensis*. A minimum spanning network tree depicting the relationships of the different genetic types of FLEs was constructed using the TCS program (Fig. 1) (9).

Nucleotide sequence accession numbers. The sequences of representative samples obtained in the present study have been deposited in GenBank under accession numbers [FR872824](https://doi.org/10.1093/nucleic-acids/gab012) to [FR872833](https://doi.org/10.1093/nucleic-acids/gab012).

RESULTS

A total of 1,042 adult and 236 immature (214 larvae and 22 nymphs) ticks were tested individually for the presence of *Francis-*

sella DNA by PCR. All samples that were positive by PCR produced a single band of the expected size (approximately 370 bp) on an agarose gel. The proportion of adult ticks at each locality that were PCR positive for *Francisella* DNA ranged from 73% to 100%; however, significantly more *D. variabilis* (93%) were positive than *D. andersoni* adults (86%) ($\chi^2_1 = 12.09$, $P < 0.05$, $n = 1,042$) (Table 1). There were also significant differences in the proportion of male and female *D. andersoni* (82% and 90%, respectively) ($\chi^2_1 = 378.29$, $P < 0.001$, $n = 425$) and *D. variabilis* (88% and 97%, respectively) ($\chi^2_1 = 150.10$, $P < 0.001$, $n = 617$) that were PCR positive for *Francisella* DNA. For the immature ticks, 89% of the *D. andersoni* nymphs, 69% of the *D. variabilis* nymphs, and 52% of the *D. variabilis* larvae were PCR positive for *Francisella* DNA (Table 2). A significantly lower proportion of *D. variabilis* larvae from Saskatchewan Landing Provincial Park were PCR positive for *Francisella* DNA than those from Blackstrap Provincial Park ($\chi^2_1 = 26.75$, $P < 0.001$, $n = 214$).

At least 20 different SSCP banding patterns (i.e., profiles) were detected among the 1,068 PCR products. Many amplicons had SSCP patterns that were comprised of at least two different patterns, suggesting that some ticks contained more than one sequence type that differed from one another by one or more nucleotides. DNA sequencing analyses of representative amplicons of each SSCP banding pattern type revealed that the bacteria present in ticks were not *F. tularensis* but *Francisella*-like endosymbionts. A total of 10 different sequence types of FLEs were identified among the tick samples (Tables 3 and 4), some of which were represented by more than one SSCP banding pattern. Multiple FLE sequence types were detected in 24% of the adult ticks, as evident by the presence of two nucleotides at one or more positions in the DNA sequence. The proportion of PCR positive adult ticks that contained more than one sequence type was significantly different between *D. andersoni* (3%) and *D. variabilis* (38%) ($\chi^2_1 = 150.18$, $P < 0.001$, $n = 940$) (Table 3). None of the 128 *Dermacentor* immatures contained multiple FLE types (Table 4).

There were 29 variable nucleotide positions in the alignment of the partial 16S rRNA gene sequences (321 to 336 bp) of the 10 FLEs and *F. tularensis* (Table 5). This included a 12-bp deletion (positions 172 to 183) in the sequence of FLE type 4, which was otherwise identical in sequence to FLE type 3. Pairwise comparisons of all 10 sequences revealed differences at one to 21 nucleotide positions between each type of FLE. A comparison of the partial 16S rRNA gene sequences of the 10 FLEs with sequence data available on GenBank revealed that FLE types 4 to 10 had unique sequences, whereas the sequences of FLE types 1 to 3 were reported previously. For instance, the 336-bp sequence of FLE type 1 was identical to the 16S rRNA gene sequence of FLEs detected in *D. andersoni* (GenBank accession no. [AY375397](https://doi.org/10.1093/nucleic-acids/gab012) and [AY375398](https://doi.org/10.1093/nucleic-acids/gab012) [51]). FLE type 2 was identical in sequence to an FLE in

TABLE 2 Localities of the collection sites of *D. andersoni* nymphs and *D. variabilis* larvae and nymphs, and the number of ticks positive for the presence of the *Francisella* 16S rRNA gene at both localities

Locality	<i>D. andersoni</i> nymphs		<i>D. variabilis</i> larvae		<i>D. variabilis</i> nymphs	
	No. tested	No. <i>Francisella</i> positive (%)	No. tested	No. <i>Francisella</i> positive (%)	No. tested	No. <i>Francisella</i> positive (%)
Blackstrap Provincial Park	— ^a	— ^a	143	92 (64)	7	7 (100)
Saskatchewan Landing Provincial Park	9	8 (89)	71	19 (27)	6	2 (33)

^a This species does not occur at this locality.

TABLE 3 Genetic types of FLEs detected in adult *D. andersoni* and *D. variabilis* from each locality

Locality ^a	FLE types in <i>D. andersoni</i>					FLE types in <i>D. variabilis</i>										
	1	5	1 and 2	1 and 9	1 and 10	1	2	3	4	8	2 and 3	2, 3, and 4	2 and 7	3 and 4	3 and 7	3 and 8
Allopatric tick populations																
Lethbridge	73	0	0	0	0	0	33	14	0	0	47	0	0	0	1	0
Blackstrap Provincial Park						0	34	0	0	0	10	0	0	0	0	0
Wakaw						0	44	4	1	0	34	0	0	0	1	1
Minnedosa						0	19	0	0	0	8	0	0	0	0	0
Kenora																
Sympatric tick populations																
Saskatchewan Landing Provincial Park	76	0	2	1	0	0	55	2	0	0	35	0	0	0	0	0
Grasslands National Park	15	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Buffalo Pound Provincial Park	27	1	0	0	0	0	34	8	0	0	24	2	1	0	0	0
Douglas Provincial Park	13	0	0	0	1	0	24	0	0	0	16	0	0	0	0	0
Danielson Provincial Park	59	0	0	0	0	1	64	3	0	1	29	0	0	0	0	0
Outlook	16	0	0	0	0	0	5	0	0	0	6	0	0	0	0	0
Harris	76	0	3	4	0	0	7	0	0	0	2	0	0	0	0	0
Total	355	1	5	5	1	1	320	31	1	1	211	2	1	2	2	1

^a Coordinates given in Table 1.

TABLE 4 Genetic types of FLEs detected in *D. andersoni* nymphs and *D. variabilis* larvae and nymphs from each locality

Locality	FLE types in <i>D. andersoni</i>		FLE types in <i>D. variabilis</i>			
	1	5	2	3	6	7
Saskatchewan Landing Provincial Park	7	1	93	4	1	1
Blackstrap Provincial Park	0	0	20	0	0	1

D. variabilis (accession no. AY795979 [24] and AY375406 [51]), and FLE type 3 was identical to that of a *Francisella*-like *Derma-centor variabilis* endosymbiont (“*dermacentor variabilis francisella*”) in *D. variabilis* (accession no. AY795976 to AY795978 [24]). The sequences of all FLE types differed ≥ 1 bp compared to the 16S rRNA gene sequences of the different subspecies of *F. tularensis*.

Most of the 10 FLE types were found in either *D. andersoni* or *D. variabilis*. However, two of the FLE sequence types occurred in adult ticks of both species. FLE type 1 was the most prevalent sequence variant in adult *D. andersoni*, found in 99.7% of the PCR positive ticks; however, it also occurred in a single *D. variabilis* adult (<1%) (Table 3). This was also the most prevalent FLE type in the *D. andersoni* nymphs (Table 4). The FLE type 2 sequence variant was the most prevalent type in *D. variabilis*, occurring in 93% of PCR positive adult ticks and 94% of the PCR positive immature ticks (Tables 3 and 4). This type also occurred in five (1%) *D. andersoni* females: three from Harris and two from Saskatchewan Landing Provincial Park. All five of these ticks also contained FLE type 1 (Table 3). Infections of FLE type 1 in *D. variabilis* and FLE type 2 in *D. andersoni* appeared to be rare and only occurred at localities where these tick species were sympatric. In contrast, seven of the FLE types, which occurred at relatively low prevalences in the present study, were found exclusively in a single tick species. The DNA sequences of the least common FLE types in *D. andersoni* (types 5, 9, and 10) differed by a single nucleotide compared to the sequence of type 1, the most prevalent type in *D. andersoni*. Similarly, the FLE types which were relatively rare in *D. variabilis* (i.e., types 4, 6, 7, and 8) had 16S rRNA gene sequences most similar to that of FLE type 3 (Fig. 1), the second most common type of FLE in *D. variabilis* (Table 3). FLE type 3 was detected in 44% of the *D. variabilis* adults found positive by PCR. Most (85%) of *D. variabilis* adults infected with FLE type 3 also contained FLEs of type 2 (Table 3). FLE type 6 was only detected in a single *D. variabilis* larva (Table 4).

DISCUSSION

Francisella tularensis is endemic in western Canada (42, 62). Despite this, little is known of the relative prevalence of this pathogen in different geographical areas, or of its natural transmission cycle, in western Canada. In the present study, we did not detect, using PCR-SSCP and DNA sequencing, the presence of *F. tularensis* in any of the 1,278 ticks collected from 12 localities in western Canada. This includes Saskatchewan Landing Provincial Park, a locality where, in 1982, questing *D. andersoni* adults were found to be infected with *F. tularensis* (26). However, this was not unexpected since *F. tularensis* infections in ticks are often sporadic (25). This

TABLE 5 Multiple sequence alignment of the 29 variable nucleotide positions of the 16S rRNA gene fragment (336 bp) obtained from the 10 FLE types found in *D. andersoni* and *D. variabilis* adults from 12 localities in western Canada and for the different subspecies of *Francisella tularensis*

Taxon	Alignment position ^a																													
	61	82	172	173	174	175	176	177	178	179	180	181	182	183	188	189	196	198	206	208	215	217	227	260	287	288	289	306	326	
Type 1	G	C	G	A	A	T	T	G	A	C	G	G	G	G	C	G	T	G	A	T	C	C	T	G	G	G	C	C		
Type 5	G	.
Type 9	A
Type 10	C
Type 2	G
Type 4	A	T	-	-	-	-	-	-	-	-	-	-	-	.	.	T	G	C	G	.	T	T	.	A	
Type 3	A	T	A	T	G	C	G	.	T	T	.	A	
Type 6	A	T	A	T	G	C	G	.	T	T	.	A	.	.	.	G	
Type 8	A	T	A	T	T	G	C	G	.	T	T	.	A	
Type 7	A	T	A	T	G	C	G	.	T	T	.	A	A	T	.	.	
<i>F. tularensis</i> ^b	G	.	.	.	A	

^a A dot (.) represents the identical nucleotide, and a dash (-) indicates a deletion with respect to the sequence of the type 1 FLE. The alignment position indicates the nucleotide position relative to the 3' end of the forward primer.

^b Sequence of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida* (GenBank accession no. AJ749949, AY968229, AY968235, and AY968237).

appears to be the case for ticks in Saskatchewan Landing Provincial Park because *F. tularensis* was also not detected in any *D. andersoni* collected in 1971 and between 1974 and 1981 (26). Given that *F. tularensis* is often maintained in endemic foci at a low prevalence (21, 25, 29), estimations of the prevalences of *F. tularensis* in northern populations of *D. andersoni* and *D. variabilis* in western Canada will require surveys to be conducted involving a large number of ticks from numerous locations over multiple consecutive years.

Although *F. tularensis* was not detected in *D. andersoni* or *D. variabilis* in our study, we did detect the DNA of FLEs in a large proportion of adult ticks at each locality. This was not surprising given that FLEs have been reported from many genera of ticks (10, 41, 46, 47, 51, 58, 59). The combined results of our SSCP and DNA sequencing analyses revealed the existence of 10 types of FLEs. The partial 16S rRNA gene sequences of seven FLEs (types 4 to 10) represented new sequence types based on comparisons with sequence data available in GenBank, whereas the three most prevalent types of FLEs (types 1, 2, and 3) were identical in sequence to FLEs reported in *D. andersoni* or *D. variabilis* from a number of locations in the United States and Canada (24, 39, 51). The partial 16S sequences of FLE types 1 and 2 were also identical in sequence to those previously found in other species of *Dermacentor* in North America (39, 51). Therefore, a larger fragment of the 16S rRNA gene or a second genetic marker would be needed to distinguish FLE types 1 to 3 in *D. andersoni* and *D. variabilis* from those FLEs in the other species of *Dermacentor*. Nonetheless, we were able to identify different genotypes of FLEs in *D. andersoni* and *D. variabilis* based on a relatively small part of the 16S rRNA gene sequence.

A large proportion (>85%) of *D. andersoni* and *D. variabilis* at localities near their northern distributional limits contained FLEs, which are similar to rates of infection for FLEs in these two species in more southern parts of their geographical ranges (i.e., 55 to 97%) (24, 46). These bacteria occur in the Malpighian tubules and/or the ovaries of female ticks (6, 46, 47), and transovarial transmission of FLEs has been shown to be very efficient (96 to 100%) in *D. andersoni* (46) and *D. variabilis* (24). The infection rate of FLEs in *D. variabilis* larvae in the present study also suggests

that FLEs are vertically transmitted. Transovarial transmission is an important mechanism by which FLEs are maintained in a large proportion of individuals within the tick populations. However, many *D. andersoni* and *D. variabilis* males in western Canada were also found to contain FLEs, but it is not known if these individuals would contribute to the maintenance of FLEs in tick populations. Few studies have examined *D. andersoni* or *D. variabilis* males for the presence of FLEs. However, the salivary glands and reproductive tissues of *D. andersoni* males from Bitterroot Mountains (Montana) were found not to contain the DAS FLE found in *D. andersoni* females from the same locality (46). If vertical transmission is the only means by which FLEs are maintained in a tick population, then *Dermacentor* males would represent a dead-end host for FLEs.

In the present study, 24% of the ticks containing FLEs were infected with multiple types. This is consistent with the results of previous studies where coinfections of multiple types of FLEs in *Dermacentor* adults were relatively common (24, 51). However, we found a significant difference between the two tick species in the frequency of multiple infections. Very few *D. andersoni* (3%) were infected with multiple types of FLEs, whereas significantly more infected *D. variabilis* (38%) were coinfecting with two or three types of FLE.

Previous studies have determined the types of FLEs in *D. andersoni* and *D. variabilis* adults from allopatric populations (24, 39, 46, 51). However, the present study differed in that the FLE types present in *D. andersoni* and *D. variabilis* adults and immatures were compared from both allopatric and sympatric populations of ticks. Such a comparison provides insights into the modes of transmission (i.e., the occurrence of horizontal and/or vertical transmission) and host specificity of the different FLE types. Our results revealed a significant difference in the types of FLEs present in *D. andersoni* and *D. variabilis*, even in sympatric populations of these two species. Most FLE types were specific for a single tick species; however, the two most prevalent types (i.e., types 1 and 2) were found in both species of tick but in different relative frequencies. This infection pattern may be explained by horizontal transmission from one species of tick to the other through a vertebrate host. There is a recent report of the detection of FLEs (e.g.,

GenBank accession no. [EU315913](#)) in wood mice (*Apodemus sylvaticus*) in Europe (15), which suggests that horizontal transmission of some FLE types from ticks to small mammals can occur. However, horizontal transmission of FLEs has not been demonstrated in experimental infections (1, 6, 46). At localities where *D. andersoni* and *D. variabilis* occur in sympatry, immature stages of both species do parasitize the same small mammal hosts (e.g., mice and voles) (Dergousoff and Chilton, unpublished data), thus there is the potential for horizontal transmission of FLEs from one tick species to the other via a vertebrate host. However, at Saskatchewan Landing Provincial Park, there was no evidence of FLEs of type 1 in *D. variabilis* immatures or of type 2 or 3 in *D. andersoni* nymphs.

It is not known if there is an epidemiological significance of FLEs in ticks. Studies have not indicated that these organisms are pathogenic to ticks or affect their fecundity (1, 6, 24, 46). Furthermore, multiple FLE types can be cotransmitted transovarially (24), or with *Rickettsia* spp. (46) or *Anaplasma phagocytophilum* (1). Thus, the presence of FLEs does not appear to inhibit the vertical transmission of other FLE strains or distantly related organisms. Additional studies are needed to determine if there is a negative correlation between the presence of FLEs and the occurrence of *F. tularensis* in ticks and if they affect the vectorial capacity of their tick hosts (24).

In conclusion, multiple types of FLEs were found in northern populations of *D. andersoni* and *D. variabilis*. However, each FLE type was primarily found in a single tick species, and this host specificity of FLEs was generally maintained at locations where both tick species occurred in sympatry because there were very few examples of potential transfer of FLEs from *D. andersoni* to *D. variabilis* and vice versa. Although the three most common FLE types have been found previously in *D. andersoni* or *D. variabilis* in other parts of their geographical range, seven of the FLE types detected in the present study represented new sequence types. This finding expands our knowledge on the genetic diversity of FLEs in ticks. The continual discovery of new FLEs in ticks (41, 56) and species of *Francisella* in fish and mammals (37, 48, 55) and humans (14, 31) shows that the family *Francisellaceae* is much more diverse than previously realized (38). Furthermore, the presence of multiple FLEs in *D. andersoni* and *D. variabilis* that are genetically similar to *F. tularensis* has important implications for diagnosis and epidemiological studies of tularemia in Canada. These require molecular techniques that can reliably distinguish among the different subspecies and subtypes of *Francisella* and the different types of FLEs (15, 22, 39). Our study has demonstrated that PCR-SSCP, combined with DNA sequencing, is an effective approach to examine a large number of ticks for the presence of different bacteria within the family *Francisellaceae*.

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REFERENCES

- Baldrige GD, et al. 2009. Transovarial transmission of *Francisella*-like endosymbionts and *Anaplasma phagocytophilum* variants in *Dermacentor albipictus* (Acari: Ixodidae). *J. Med. Entomol.* **46**:625–632.
- Bishopp FC, Trembley HL. 1945. Distribution and hosts of certain North American ticks. *J. Parasitol.* **31**:1–54.
- Black DM, Thomson JA. 1958. Tularemia in British Columbia. *Can. Med. Assoc. J.* **78**:16–18.
- Bow MR, Brown JH. 1943. Tularemia in the "Seven Persons Coulee," Alberta. *Can. J. Public Health* **34**:415–418.
- Bow MR, Brown JH. 1946. Tularemia. A report on 40 cases in Alberta, Canada, 1931–1944. *Am. J. Public Health Nations Health* **36**:494–500.
- Burgdorfer W, Brinton LP, Hughes LE. 1973. Isolation and characterization of symbionts from Rocky Mountain wood tick, *Dermacentor andersoni*. *J. Invertebr. Pathol.* **22**:424–434.
- Canadian Cooperative Wildlife Health Center. 1995. Tularemia in muskrats and beaver. *Can. Coop. Wildl. Health Centre Newsl.* **3-2**:1–12.
- Choi E. 2002. Tularemia and Q fever. *Med. Clin. North Am.* **86**:393–416.
- Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* **9**:1657–1659.
- de Carvalho IL, Santos N, Soares T, Zé-Zé L, Nuncio MS. 2011. *Francisella*-like endosymbiont in *Dermacentor reticulatus* collected in Portugal. *Vector Borne Zoonotic Dis.* **11**:185–188.
- Dergousoff SJ, Chilton NB. 2007. Differentiation of three species of ixodid tick, *Dermacentor andersoni*, *D. variabilis* and *D. albipictus*, by PCR-based approaches using markers in ribosomal DNA. *Mol. Cell Probes* **21**:343–348.
- Eisen L. 2007. A call for renewed research on tick-borne *Francisella tularensis* in the Arkansas-Missouri primary national focus of tularemia in humans. *J. Med. Entomol.* **44**:389–397.
- Eisen RJ, et al. 2009. Time course of hematogenous dissemination of *Francisella tularensis* A1, A2, and type B in laboratory mice. *Am. J. Trop. Med. Hyg.* **80**:259–262.
- Escudero R, et al. 2010. A possible novel *Francisella* genomic species isolated from blood and urine of a patient with severe illness. *Clin. Microbiol. Infect.* **16**:1026–1030.
- Escudero R, et al. 2008. Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts. *J. Clin. Microbiol.* **46**:3139–3143.
- Foley JE, Nieto NC. 2010. Tularemia. *Vet. Microbiol.* **140**:332–338.
- Forsman M, Sandstrom G, Sjostedt A. 1994. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int. J. Syst. Bacteriol.* **44**:38–46.
- Fyvie A, Ross WG, Labzoffsky NA. 1959. Tularemia among muskrats on Walpole Island, Lake St. Clair, Ontario. *Can. J. Comp. Med. Vet. Sci.* **23**:153–156.
- Gasser RB, et al. 2006. Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. *Nat. Protoc.* **1**:3121–3128.
- Gibbons RJ. 1939. Survey of Rocky Mountain spotted fever and sylvatic plague in western Canada during 1938. *Can. J. Public Health* **30**:184–187.
- Goethert HK, Saviet B, Telford SR, III. 2009. Metapopulation structure for perpetuation of *Francisella tularensis tularensis*. *BMC Microbiol.* **9**:147.
- Goethert HK, Shani I, Telford SR, III. 2004. Genotypic diversity of *Francisella tularensis* infecting *Dermacentor variabilis* ticks on Martha's Vineyard, Massachusetts. *J. Clin. Microbiol.* **42**:4968–4973.
- Goethert HK, Telford SR, III. 2010. Quantum of infection of *Francisella tularensis tularensis* in host-seeking *Dermacentor variabilis*. *Ticks Tick-borne Dis.* **1**:66–68.
- Goethert HK, Telford SR, III. 2005. A new *Francisella* (Beggiatiales: Francisellaceae) inquiline within *Dermacentor variabilis* say (Acari: Ixodidae). *J. Med. Entomol.* **42**:502–505.
- Goethert HK, Telford SR, III. 2009. Nonrandom distribution of vector ticks (*Dermacentor variabilis*) infected by *Francisella tularensis*. *PLoS Pathog.* **5**:e1000319.
- Gordon JR, McLaughlin BG, Nitiuthai S. 1983. Tularemia transmitted by ticks (*Dermacentor andersoni*) in Saskatchewan. *Can. J. Comp. Med.* **47**:408–411.
- Gregson JD. 1956. The Ixodoidea of Canada. Publication 930. Canada Department of Agriculture, Ottawa, Canada.
- Gwatkin R, Painter RH, Moynihan IW. 1942. Tularemia in sheep. *Can. J. Comp. Med. Vet. Sci.* **6**:163–168.

29. Gyurancz M, et al. 2011. Investigation of the ecology of *Francisella tularensis* during an inter-epizootic period. *Vector Borne Zoonotic Dis.* 11:1031–1035.
30. Harris TA. 1956. Tularaemia among farmer-trappers in northwestern Saskatchewan. *Can. Med. Assoc. J.* 74:60–61.
31. Huber B, et al. 2010. Description of *Francisella hispansiensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson et al. 1955) Olsufiev et al. 1959 as *Francisella tularensis* subsp. *novicida* comb. nov. and emended description of the genus *Francisella*. *Int. J. Syst. Evol. Microbiol.* 60:1887–1896.
32. Humphreys FA. 1947. Some observations regarding tick and insect-borne infections in western Canada. *Can. J. Comp. Med.* 11:187–192.
33. Humphreys FA, Campbell AG. 1947. Plague, Rocky Mountain spotted fever, and tularaemia surveys in Canada. *Can. J. Public Health* 38:124–130.
34. Isaac-Renton M, et al. 2010. Tularaemia in British Columbia: a case report and review. *BCM J.* 52:303–307.
35. James AM, et al. 2006. Distribution, seasonality, and hosts of the Rocky Mountain wood tick in the United States. *J. Med. Entomol.* 43:17–24.
36. Jellison WL. 1974. Tularaemia in North America 1930–1974. University of Montana Foundation, Missoula, MT.
37. Kamaishi T, et al. 2005. Identification and pathogenicity of intracellular *Francisella* bacterium in three-line grunt *Parapristipoma trilineatum*. *Fish Pathol.* 40:67–71.
38. Keim P, Johansson A, Wagner DM. 2007. Molecular epidemiology, evolution, and ecology of *Francisella*. *Ann. N. Y. Acad. Sci.* 1105:30–66.
39. Kugeler KJ, et al. 2005. Discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts when screening ticks by PCR. *Appl. Environ. Microbiol.* 71:7594–7597.
40. Langford EV. 1954. An outbreak of tularaemia in beaver and muskrat in Waterton Lakes National Park, Alberta. *Can. J. Comp. Med. Vet. Sci.* 18:28–30.
41. Machado-Ferreira E, Piesman J, Zeidner NS, Soares CA. 2009. *Francisella*-like endosymbiont DNA and *Francisella tularensis* virulence-related genes in Brazilian ticks (Acari: Ixodidae). *J. Med. Entomol.* 46:369–374.
42. Martin T, Holmes IH, Wobeser GA, Anthony RF, Greefkes I. 1982. Tularaemia in Canada with a focus on Saskatchewan. *Can. Med. Assoc. J.* 127:279–282.
43. McNabb A. 1930. Tularaemia: The first case reported in Canada. *Can. J. Public Health* 21:91–92.
44. Merten HA, Durden LA. 2000. A state-by-state survey of ticks recorded from humans in the United States. *J. Vector Ecol.* 25:102–113.
45. Molins CR, et al. 2010. Virulence differences among *Francisella tularensis* subsp. *tularensis* clades in mice. *PLoS One* 5:e10205.
46. Niebylski ML, Peacock MG, Fischer ER, Porcella SF, Schwan TG. 1997. Characterization of an endosymbiont infecting wood ticks, *Dermacentor andersoni*, as a member of the genus *Francisella*. *Appl. Environ. Microbiol.* 63:3933–3940.
47. Noda H, Munderloh UG, Kurtti TJ. 1997. Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. *Appl. Environ. Microbiol.* 63:3926–3932.
48. Nylund A, Ottem KF, Watanabe K, Karlsbakk E, Krossoy B. 2006. *Francisella* sp. (Family Francisellaceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. *Arch. Microbiol.* 185:383–392.
49. Office of the Medical Health Officer. 2007. Human tularaemia cases. *Public Health Matters MHO Newsl.* 13:2.
50. Reese SM, et al. 2010. Transmission dynamics of *Francisella tularensis* subspecies and clades by nymphal *Dermacentor variabilis* (Acari: Ixodidae). *Am. J. Trop. Med. Hyg.* 83:645–652.
51. Scoles GA. 2004. Phylogenetic analysis of the *Francisella*-like endosymbionts of *Dermacentor* ticks. *J. Med. Entomol.* 41:277–286.
52. Scott JW, Macbeth RAL. 1946. Tularaemia (with a report of nine cases). *Can. Med. Assoc. J.* 55:564–566.
53. Sjöstedt A. 2005. Family III. Francisellaceae fam. nov., p 199–209. In Brenner DJ, Krieg NR, and Staley JT (ed), *Bergey's manual of systematic bacteriology*, 2nd ed, vol 2. Springer, East Lansing, MI.
54. Sonenshine DE. 1979. Zoogeography of the American dog tick, *Dermacentor variabilis*, p 123–134. In Rodriguez JG (ed), *Recent advances in acarology*, vol 2. Academic Press, New York, NY.
55. Soto E. 2010. *In vivo* and *in vitro* pathogenesis of *Francisella asiatica* in *Tilapia nilotica* (*Oreochromis niloticus*). Ph.D. thesis. Louisiana State University, Baton Rouge, LA.
56. Sréter-Lancz Z, Széll Z, Sréter T, Márialigeti K. 2009. Detection of a novel *Francisella* in *Dermacentor reticulatus*: a need for careful evaluation of PCR-based identification of *Francisella tularensis* in Eurasian ticks. *Vector Borne Zoonotic Dis.* 9:123–126.
57. Staples JE, Kubota KA, Chalcraft LG, Mead PS, Petersen JM. 2006. Epidemiologic and molecular analysis of human tularaemia, United States, 1964–2004. *Emerg. Infect. Dis.* 12:1113–1118.
58. Suitor EC, Jr, Weiss E. 1961. Isolation of a Rickettsialike microorganism (*Wolbachia persica*, n. sp.) from *Argas persicus* (Oken). *J. Infect. Dis.* 108:95–106.
59. Sun LV, Scoles GA, Fish D, O'Neill SL. 2000. *Francisella*-like endosymbionts of ticks. *J. Invertebr. Pathol.* 76:301–303.
60. Walker WJ, Moore CA. 1971. Tularaemia: experience in the Hamilton area. *Can. Med. Assoc. J.* 105:390–396.
61. Wilkinson PR. 1967. The distribution of *Dermacentor* ticks in Canada in relation to bioclimatic zones. *Can. J. Zool.* 45:517–537.
62. Wobeser G, Campbell GD, Dallaire A, McBurney S. 2009. Tularaemia, plague, yersiniosis, and Tyzzer's disease in wild rodents and lagomorphs in Canada: a review. *Can. Vet. J.* 50:1251–1256.
63. Wobeser G, Ngeleka M, Appleyard G, Bryden L, Mulvey MR. 2007. Tularaemia in deer mice (*Peromyscus maniculatus*) during a population irruption in Saskatchewan, Canada. *J. Wildl. Dis.* 43:23–31.