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Discrimination between *Francisella tularensis* and *Francisella*-Like Endosymbionts when Screening Ticks by PCR

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The presence of *Francisella*-like endosymbionts in tick species known to transmit tularemia poses a potential diagnostic problem for laboratories that screen tick samples by PCR for *Francisella tularensis*. Tick samples initially considered positive for *F. tularensis* based on standard 16S rRNA gene PCR were found to be positive only for *Francisella*-like endosymbionts using a multitarget *F. tularensis* TaqMan assay (IS*Ftu2*, *tul4*, and *iglC*) and 16S rRNA gene sequencing. Specificity of PCR-based diagnostics for *F. tularensis* should be carefully evaluated with appropriate specimen types prior to diagnostic use.

The *Francisellaceae* family is composed of a group of closely related organisms that are widespread in nature. *Francisella philomiragia* and *Francisella tularensis* are the two recognized species of the *Francisella* genus (2, 15), with the latter being the etiologic agent of the zoonotic disease tularemia. Humans can acquire tularemia through consumption of contaminated food or water, inhalation of infectious aerosols, contact with infected animals, or the bite of an infected vector (1, 15). In North America three subspecies of *F. tularensis* have been described: *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *novicida* (2, 15).

Based on a high degree of similarity in 16S rRNA gene sequences, several other organisms have been classified as probable members of the *Francisellaceae* family, though their genera have not been determined (15). These include the intracellular bacterium *Wolbachia persica*, isolated from the tick *Argas arboreus*, and endosymbionts of the tick species *Amblyomma maculatum*, *Ornithodoros porcinus*, and *Ornithodoros moubata* (8, 14, 16, 17). *Francisella*-like endosymbionts have also been identified in multiple *Dermacentor* tick species, including *D. variabilis*, *D. andersoni*, *D. hunteri*, *D. nitens*, *D. occidentalis*, and *D. albipictus* (7, 14, 17). Characterization of these organisms has largely been limited to PCR-based methods, since they are not readily culturable on microbiological agar.

The presence of closely related *Francisella*-like organisms in tick species capable of transmitting tularemia (*D. variabilis*, *D. andersoni*, and *D. occidentalis*) (5) poses a challenge for accurate identification of *F. tularensis* by PCR. The significance of this problem has recently been heightened by the classification of *F. tularensis* as a category A agent of bioterrorism (3) and the increased use of PCR-based surveillance for *F. tularensis* in tick and environmental samples. Here we show that tick pools initially positive for *F. tularensis* based on a 16S rRNA gene PCR assay (9, 10, 11) were determined to be positive only for *Francisella*-like endosymbionts. This report highlights the need for careful evaluation of PCR-based diagnostics in laboratories that screen ticks for *F. tularensis.*

In 2002 and 2003, tularemia was identified in two dead rabbits (*Sylvilagus audoboni*) found in an urban area of San Diego County (10). To assess potential public health risk, San Diego County Vector Control dragged for ticks on vegetation in proximity to where the rabbits had been found. A small number of ticks were also collected from animals submitted for necropsy and from owners who had pulled ticks off of their pets. A total of 3,202 ticks were identified and pooled at the San Diego County Animal Disease Diagnostic Laboratory (332 pools of *D. occidentalis*, 32 pools of *D. variabilis*, and 25 pools of ticks whose species was not determined). Each pool consisted of 1 to 12 ticks. DNA was extracted and purified from all tick pools using the DNeasy tissue extraction protocol (QIAGEN, Valencia, CA).

Ten microliters of each tick pool was screened using a proprietary 16S rRNA gene PCR assay (Engene Biotechnology, Rancho Santa Fe, CA) (Table 1). PCR amplification conditions were 94°C for 2 min 30 s followed by 40 cycles at 94°C for 30 seconds, 56°C for 1 min 30 s, and 72°C for 1 min. DNA extracted from tissues of the two *F. tularensis*-infected rabbits served as positive controls (Table 1). Of the 389 pools tested, positive ticks were identified in 2 pools (10 ticks) of *D. occidentalis* (2,948 total ticks), 14 pools (56 ticks) of *D. variabilis* (94 total ticks), and 5 pools (5 ticks) of ticks whose species was not determined (160 total ticks) (Table 1). 16S rRNA gene PCR positive results were also obtained using a published *Francisella* 16S rRNA gene primer set (F11-F5) (Tables 1 and 2) (14). PCR amplification conditions for this assay were 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

Since the 16S rRNA gene sequence is highly conserved among *Francisellaceae* members, further testing was performed to determine whether the 16S PCR positive results were due to

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TABLE 1. PCR results for San Diego samples and controls

Identifier[a]	Species[b] (no. of ticks)	PCR assay results[e]		
		San Diego 16S rRNA gene standard PCR	F11-F5 16S rRNA gene standard PCR	Multitarget TaqMan assay (IS*Ftu2*, *tul4*, and *iglC*)
2022859[c]	*Sylvilagus audoboni*	+	ND	+
2032026[c]	*Sylvilagus audoboni*	+	ND	+
2030223	*D. variabilis* (1)	+	ND	−
2030224	*D. variabilis* (1)	+	+	−
2030362	*D. variabilis* (7)	+	ND	−
2030363	*D. variabilis* (1)	+	ND	−
2030997	*D. variabilis* (1)	+	+	−
2040278	*D. variabilis* (2)	+	ND	−
2040369	*D. variabilis* (11)	+	+	−
2040460[d]	*D. variabilis* (1)	+	+	−
2040445	*D. variabilis* (4)	+	+	−
2040409	*D. variabilis* (1)	+	+	−
2040384[d]	*D. variabilis* (1)	+	+	−
2040372	*D. variabilis* (12)	+	+	−
2040370	*D. variabilis* (3)	+	+	−
2040371	*D. variabilis* (10)	+	+	−
2032437	*D. occidentalis* (9)	+	+	−
2031093[d]	*D. occidentalis* (1)	+	ND	−
2032666	Canine (1)	+	+	−
2040006	Canine (1)	+	+	−
2040431	Cat (1)	+	+	−
2040386	Mountain lion (1)	+	+	−
2032922	Coyote (1)	+	+	−
MV1[d]	*D. variabilis* (1)	ND	+	−
MV2	*D. variabilis* (1)	ND	ND	−
MV3	*D. variabilis* (1)	ND	ND	−
MV1 (spiked)		ND	ND	+

[a] The identifier for the first 23 samples corresponds to the accession number assigned at the San Diego Animal Disease Diagnostic Laboratory.
[b] Ticks whose species was not identified are referred to by source.
[c] *F. tularensis*-positive rabbits.
[d] Samples subjected to 16S rRNA gene sequencing.
[e] ND, not determined.

F. tularensis or *Francisella*-like endosymbionts. The *F. tularensis* multitarget TaqMan assay is a real-time PCR assay directed against an insertion sequence-like element (IS*Ftu2*), a gene that encodes an outer membrane protein (*tul4*), and a gene expressed upon macrophage infection (*iglC*) (18). Identification of *F. tularensis* requires all three targets to be positive (18). Previous evaluation of this assay with a panel of organisms showed no cross-reactivity with non-*Francisella* species and the ability to differentiate between the two species *F. tularensis* and *F. philomiragia* (18).

TABLE 2. Primers used for 16S rRNA gene amplification and sequencing

Primer use and name	Primer sequence (5′–3′)	Reference or source
Amplification		
F11 (F)	TACCAGTTGGAAACGACTGT	4
F5 (R)	CCTTTTTGAGTTTCGCTCC	4
63F	CAGGCCTAACACATGCAAGTC	6
1387R	GGGCGGWGTGTACAAGGC[a]	6
Sequencing		
101F	ACTGGCGGACGGGTGAGTAA	12
537R	CGTATTACCGCGGCTGCTGG	12
519F	CAGCAGCCGCGGTAATAC	13
926R	CCGTCAATTCCTTTGAGTTT	13
327F	CTACGGGAGGCAGCAGTGGGGAATA	This study
727F	ACCGATACTGACACTGAGGGACGAA	This study
949F	CGATGCAACGCGAAGAACCT	This study

[a] W, bases T, U, or A.

Francisella-like endosymbiont control DNA (MV1, MV2, and MV3) isolated from *D. variabilis* ticks on Martha's Vineyard (Heidi Goethert, Harvard University) was utilized to test whether the F11-F5 16S primer set and the *F. tularensis* multitarget TaqMan assay could distinguish between *F. tularensis* and *Francisella*-like endosymbionts. Multitarget real-time PCRs were performed with 1 μl of purified DNA and previously described reaction conditions (18). Whereas *D. variabilis* endosymbiont DNA was positive with the F11-F5 16S rRNA gene primer set, the endosymbiont samples tested negative by the *F. tularensis* multitarget assay (Table 1). The negative result was not due to inhibition, as amplification of all three targets occurred when endosymbiont DNA was spiked with *F. tularensis* DNA (Table 1). Thus, the multitarget assay does not cross-react with *D. variabilis Francisella*-like endosymbionts.

All 16S rRNA gene PCR positive organisms (ticks and rabbits) from San Diego County were then tested with the multitarget TaqMan assay. Whereas both rabbit samples were positive for *F. tularensis*, all tick pools were negative. This suggests that the 16S PCR positive tick pools were negative for *F. tularensis* (Table 1).

To determine whether *Francisella*-like endosymbionts were

TABLE 3. 16S rRNA gene sequence comparison

Strain	% Sequence identity[a]														
	Study samples				*Francisella*-like endosymbiont sequences (GenBank)							*Francisella* sequences (GenBank)			
	D. occidentalis 2031093	*D. variabilis* 2040384	*D. variabilis* 2040460	*D. variabilis* symbiont MV1	*D. variabilis* symbiont AF166256	*D. occidentalis* symbiont (CA) AY375402	*D. occidentalis* symbiont (WA) AY375403	*D. variabilis* symbiont (WA) AY375404	*D. andersoni* symbiont AF001077	*O. moubata* symbiont (B) AB00152	*O. porcinus* symbiont AF166257	*F. tularensis* subsp. *tularensis* Z21932	*F. tularensis* subsp. *holarctica* Z21931	*F. tularensis* subsp. *novicida* L26084	*F. philomiragia* Z21933
D. occidentalis 2031093		99.8	99.8	99.8	99.6	100	99.8	99.8	99.4	99.4	99.1	97.4	97.3	97.6	95.9
D. variabilis 2040384			100	99.8	99.6	99.8	100	100	99.4	99.4	99.1	97.4	97.3	97.6	95.9
D. variabilis 2040460				99.8	99.6	99.8	100	100	99.4	99.4	99.1	97.4	97.3	97.6	95.9
D. variabilis symbiont MV1					99.6	99.8	99.8	99.8	99.4	99.4	99.1	97.4	97.3	97.6	95.9

[a] Sequence identity was calculated using the MegAlign program of DNASTAR, using bases 141 to 1152 of the complete *F. tularensis* subsp. *tularensis* 16S rRNA gene sequence in GenBank (Z21932).

present in the tick pools, a portion of 16S rRNA gene was sequenced from three tick samples: two *D. variabilis* samples (2040384 and 2040460) and a *D. occidentalis* sample (2031093). One *Francisella*-like endosymbiont DNA sample from *D. variabilis* collected on Martha's Vineyard was sequenced as a control (MV1). 16S rRNA gene was amplified using either eubacterial 16S rRNA gene primers (63F and 1387R) or *Francisella*-specific primers (F11-F5) when the eubacterial primers provided mixed sequence data (10 μM final concentration) (Table 2), Pure*Taq* Ready-to-go PCR beads (Amersham Biosciences, Piscataway, NJ), and 1 μl of template DNA. The amplification program consisted of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. Products were purified using a QIAquick PCR purification kit (QIAGEN) and sequenced using a CEQ 8000 DNA capillary sequencer (Beckman Coulter, Fullerton, CA) and its associated protocol. All nucleotide positions were sequenced at least twice, and the final sequence was analyzed to ensure that there was no mixed sequence.

Sequences were manually adjusted in CEQ 8000 (version 5.0) software and exported for assembly and analysis in Lasergene (DNASTAR, Madison, WI). A segment of 16S rRNA gene corresponding to bases 141 to 1152 of the complete *F. tularensis* subsp. *tularensis* 16S rRNA gene sequence in GenBank (Z21932) was compared against the following GenBank sequences: Z21931 (*F. tularensis* subsp. *holarctica*), Z21933 (*F. philomiragia*), L26084 (*F. tularensis* subsp. *novicida*), AB001522 (*O. moubata* symbiont B), AF166257 (*O. porcinus* symbiont), AF166256 (*D. variabilis* symbiont), AY375404 (*D. variabilis* symbiont, WA), AY375402 (*D. occidentalis* symbiont, CA), AY375403 (*D. occidentalis* symbiont, WA), and AF001077 (*D. andersoni* symbiont). Sequence identity was calculated using the ClustalW multiple alignment program (DNASTAR, Madison, WI).

The 16S rRNA gene sequences from the San Diego tick samples showed >99% identity to published *Francisella*-like endosymbiont 16S rRNA gene sequences compared to >97% identity to all subspecies of *F. tularensis* and 95.9% identity to *F. philomiragia*, indicating the presence of *Francisella*-like endosymbionts in these samples (Table 3). All sequenced samples shared nucleotide deletions and substitutions with published 16S sequences for the *Francisella*-like endosymbionts. The F11-F5 16S rRNA gene primer sequences were entirely conserved in all sequenced samples, indicating that the 16S rRNA gene PCR false positives were due to cross-reactivity with *Francisella*-like endosymbionts.

This report highlights the need for careful evaluation of PCR-based diagnostics when testing ticks for *F. tularensis*. Laboratories must be aware of the issue of PCR cross-reactivity due to the presence of *Francisella*-like endosymbionts in ticks known to transmit *F. tularensis*. The *Francisella*-like endosymbiont of *D. variabilis* has been identified in *D. variabilis* ticks collected in Idaho, Washington, Connecticut, Massachusetts, and in this report, California. The distribution and prevalence of *Francisella*-like endosymbionts in tick species that transmit tularemia is largely unknown, as only a few studies have been performed (14, 17).

Here we show that *F. tularensis* 16S rRNA gene PCR assays

cross-react with *Francisella*-like endosymbionts of two *Dermacentor* species (Table 1). Due to the high level of conservation in 16S rRNA gene of *Francisellaceae* members, 16S rRNA gene-based PCR should not be used to identify *F. tularensis* when other *Francisellaceae* members might be present. In addition, two different standard *tul4* PCR assays have also been shown to cross-react with the *Francisella*-like endosymbionts of *Dermacentor* species (7, 14). The specificity of PCR-based tests must be considered when testing other environmental specimens, such as soil and water, for *F. tularensis*. Undescribed members of the *Francisellaceae* family are possibly widespread in nature.

In conclusion, the multitarget TaqMan assay (18) (IS*Ftu2*, *tul4*, and *iglC*) can discriminate *F. tularensis* from *Francisella*-like tick endosymbionts of *D. variabilis* and *D. occidentalis* and may be useful in laboratories that screen these species for *F. tularensis*. Using multiple targets as criteria for a positive sample has the added advantage of minimizing the likelihood of cross-reactivity. Further evaluations are required for *Francisella*-like endosymbionts of other tick species as well as for environmental specimens.

Nucleotide sequence accession numbers. Sequences were submitted to GenBank and possess the following accession numbers: AY805304 (*D. occidentalis* 2031093), AY805305 (*D. variabilis* 2040384), AY805306 (*D. variabilis* 2040460), and AY805307 (*D. variabilis* MV).

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