

Molecular Method for Discrimination between *Francisella tularensis* and *Francisella*-Like Endosymbionts[∇]

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Environmental studies on the distribution of *Francisella* spp. are hampered by the frequency of *Francisella*-like endosymbionts that can produce a misleading positive result. A new, efficient molecular method for detection of *Francisella tularensis* and its discrimination from *Francisella*-like endosymbionts, as well as two variants associated with human disease (unusual *F. tularensis* strain FnSp1 and *F. tularensis* subsp. *novicida*-like strain 3523), is described. The method is highly specific and sensitive, detecting up to one plasmid copy or 10 genome equivalents.

Francisella spp., which are gram-negative coccobacilli, are the etiological agents of tularemia. Currently, there are four subspecies recognized within the *F. tularensis* species (*F. tularensis* subsp. *tularensis*, *holarctica*, *mediasiatica*, and *novicida*), which present differences in virulence and geographical distribution (16). Recently, there have also been descriptions of strains associated with human diseases that presented atypical features with uncertain taxonomic position (6, 11, 19). Based on 16S rRNA gene sequences, several other organisms have been classified as probable members of the *Francisellaceae* family, including *Wolbachia persica* and “*Francisella*-like endosymbionts” (FLEs) (13, 15, 17). Although FLEs are believed to be nonpathogenic to humans, their pathogenicity to guinea pigs and hamsters has been demonstrated (4), and they have been detected recently in free-living small mammals (A. Toledo, R. Escudero, A. S. Olmeda, J. F. Barandika, M. A. Casado-Nistal, I. Jado, H. Gil, A. L. García-Pérez, and P. Anda, submitted for publication). The homology found in the 16S rRNA between *Francisella* spp. and FLEs poses a potential problem for laboratories that screen environmental samples such as ticks, due to PCR cross-reactivity (12). The significance of this problem has been heightened by the classification of *F. tularensis* as a category A agent of bioterrorism (5). The homology between the sequence of the lipoprotein-coding *lpnA* gene of *Francisella* pathogens and that of FLEs is high, although there is variability, and FLEs conform to a phylogenetic clade separate from *F. tularensis* species (15). We selected a 233-bp fragment on a variable region of *lpnA* that is able to differentiate between *F. tularensis* pathogens and FLEs by amplification and further hybridization with specific probes by reverse line blotting (RLB).

Primers and probes were designed with Oligo6 software

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(Molecular Biology Insights, Inc., West Cascade, CO), and the Basic Local Alignment Search Tool (BLASTn) was used for a preliminary assessment of the oligonucleotides' specificity (1). Available sequences from GenBank were aligned and analyzed by ClustalX and Mega4 software (8, 18). Figure 1 shows the alignment of representative sequences.

The region covering nucleotide positions 593 to 825 of *lpnA* (as in *F. tularensis* subsp. *holarctica* strain LVS; GenBank accession no. M32059) was amplified from each sample by using degenerated primers (Table 1) that allowed the amplification of DNA from all of the known subspecies and variants of *F. tularensis* and all of the described FLEs (2). Also, an internal amplification control was added to evaluate the presence of PCR inhibitors as described previously (10). PCR was performed in a 50- μ l reaction volume with 10 mM Tris-HCl, 50 mM KCl, 3 mM MgCl₂, 200 μ M of each deoxynucleoside triphosphate (dNTP) (Promega, Madison, WI), and 1.5 U of *Taq* gold DNA polymerase (Applied Biosystems, Branchburg, NJ). Primers were used at a final concentration of 0.5 μ M. PCR cycling included an initial denaturing step of 9 min at 94°C, followed by 40 cycles of 15 s at 94°C, 1 min at 60°C, and 4 min at 65°C and a final elongation step of 7 min at 65°C. The overall time required for PCR was 3 h.

For the hybridization, a Biometra OV3 mini hybridization oven (Cultek, S.L., Madrid, Spain) was used, and the RLB was performed as previously described (10), with few modifications as follows: 12.8 pmol/ μ l of each probe (Table 1) was attached to the membrane, the selected temperature for hybridization was 48°C, and the subsequent incubation with streptavidin-peroxidase conjugate (Roche Farma, S.S., Madrid, Spain) and the washing steps were performed at 40°C. Super Signal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) was used for the development of the membrane. The overall time required for the RLB was 3.5 h.

Recently, a study published by Nübel et al. (14) showed different *lpnA* sequences within *F. tularensis* subspecies. This difference consists of a deletion of 36 bp in the region between nucleotide positions 662 and 697 (as in *F. tularensis* subsp. *holarctica* strain LVS; GenBank accession no. M32059). Consequently, the following probes were designed (Table 1): a

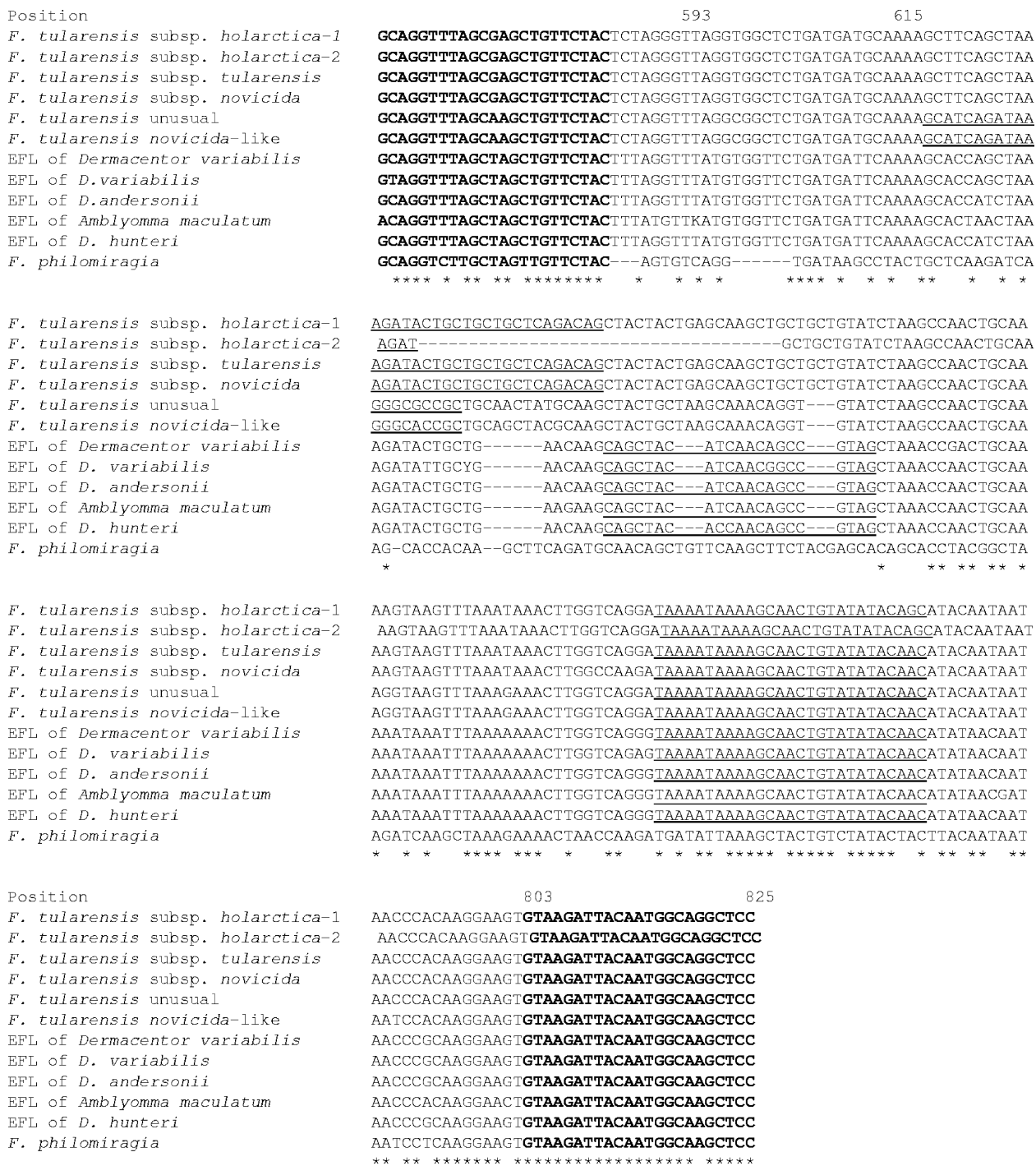


FIG. 1. Sequence alignment of a 233-nucleotide fragment of *lpnA* of representative subspecies of *F. tularensis* and FLE, as well as *F. philomiragia* (nucleotide positions 593 to 825, as in *F. tularensis* subsp. *holarctica* strain LVS [GenBank accession no. M32059]). Boldfaced nucleotides represent primers, and underlined nucleotides represent probes, as in Table 1. The accession numbers are as follows: *F. tularensis* subsp. *holarctica*-1 strain LVS, GenBank no. M32059; *F. tularensis* subsp. *holarctica*-2 strain LVS, GenBank no. AM261157; *F. tularensis* subsp. *tularensis* strain Schu S4, AJ749949; *F. tularensis* subsp. *novicida* strain Utah 112, CP000439; unusual *F. tularensis* strain FnSp1, EU363482; *F. tularensis novicida*-like strain, AY243029; FLE of *Dermacentor variabilis*, AY375420; FLE of *D. variabilis*, AY375421; FLE of *Dermacentor andersonii*, AY375413; FLE of *Amblyomma maculatum*, AY375422; FLE of *Dermacentor hunteri*, AY375417; *F. philomiragia* strain 2669, AY243030.

generic probe (P-FRAG) that hybridized with the different subspecies of *F. tularensis*, FLEs and other variants described (FnSp1 and 3523); a probe for all the *F. tularensis* subspecies which do not present that deletion (P-TUL); a generic probe

(P-ENDO2) for all known FLEs; and an additional probe (P-FnSp1) for two atypical human isolates (FnSp1 and 3523). It is remarkable that the probe P-TUL will catch all the *F. tularensis* subspecies that show a complete *lpnA* sequence.

TABLE 1. Probes and primers used in the study

Organism(s)	Primer	Probe	Sequence ^a	Source or reference
<i>Francisella</i> spp.	FT593		5'-bio-GYAGGTTTAGCKAGCTGTTCTAC	This study
	FT825		5'-bio-GGAGCYTGCCATTGTAATCTTAC	This study
<i>F. tularensis</i> ^b		P-FRAG	5'-a-TAAAATAAAAGCAACTGTATATACARC	This study
<i>F. tularensis</i> subsp. <i>tularensis</i> , <i>holarctica</i> , and <i>novicida</i> ^c		P-TUL	5'-a-AGATACTGCTGCTGCTCAGACAG	This study
<i>Francisella</i> -like endosymbiont		P-ENDO2	5'-a-CAGCTACATCAACRGCCGTAG	This study
Unusual <i>F. tularensis</i>		P-FnSp1	5'-a-GCATCAGATAAGGGCGCCGC	This study
<i>Cannabis sativa</i> (internal control)	IC-F		5'-bio-ATGATGCTGAGGGTATGTCCTAC	10
	IC-R		5'-bio-GTTTTCTCCTCCACCACCACG	10
		P-IC	5'-a-GTGGACACTTTAGTGAGGAGG	10

^a bio, biotin modification; a, aminolink modification.

^b For all subspecies of *F. tularensis*, *Francisella*-like endosymbiont and unusual *F. tularensis*.

^c *F. tularensis* subsp. *tularensis*, *holarctica*, and *novicida* with no deletion in *lpnA*.

Given this, the difference in reactivities observed with P-FRAG and P-TUL will differentiate among those two sequence types.

To be used as positive controls, since the strains were not available, synthetic DNA fragments were constructed follow-

ing the sequences of *lpnA* from an FLE from *Dermacentor variabilis* (GenBank accession no. AY375420) (15) and *F. tularensis* subsp. *novicida*-like isolate 3523 (GenBank accession no. AY243029) (19) and by using overlapping primers up to 75 bp long in consecutive PCRs (Table 2). The fragments were

TABLE 2. Strains and organisms included in this study

Species (no. of strains) or organism and origin	Strain or isolate	GenBank or ATCC accession no.	Reactivity with probe ^a :			
			P-TUL	P-FRAG	P-ENDO2	P-FnSp1
<i>Francisella</i> -like endosymbiont						
Synthetic DNA		GenBank no. AY375420	-	+	+	-
<i>F. tularensis</i> subsp. <i>holarctica</i>						
Live vaccine strain, Russia	LVS	ATCC 29684	+	+	-	-
Human and hare, outbreak, 1997, Spain (2)	FT7 and FT13		+	+	-	-
Human, outbreak, 2007, Spain (3)	BZO20, -21, and -22		+	+	-	-
Tick, 1949, Moscow area, Russia	FSC 257	GenBank no. EF208975	+	+	-	-
Tick, 1941, Montana	FSC 012	GenBank no. EF208972	+	+	-	-
Human, Sweden	FSC200	GenBank no. EF208977	+	+	-	-
<i>F. tularensis</i> subsp. <i>tularensis</i>						
Human ulcer, 1941, Ohio	B-38	ATCC 6223	+	+	-	-
<i>F. tularensis</i> subsp. <i>novicida</i>						
Water, 1950, Utah	Utah 112	ATCC 15482	+	+	-	-
Human blood, 1991, Texas	Fx1	GenBank no. EF208976	+	+	-	-
Human blood, 1991, Texas	Fx2	GenBank no. EF208978	-	+	-	-
Unusual <i>F. tularensis</i> , Spain	FnSp1	GenBank no. EU363482	-	+	-	+
<i>F. tularensis</i> subsp. <i>novicida</i> -like						
Synthetic DNA	3523	GenBank no. AY243029	-	+	-	+
<i>F. philomiragia</i>						
Human, Sweden	CCUG 12603		-	-	-	-
Water, U.S.	CCUG 19701		-	-	-	-
<i>Anaplasma phagocytophilum</i>	108		-	-	-	-
<i>Borrelia burgdorferi</i> sensu stricto	B31		-	-	-	-
<i>Coxiella burnetii</i>	Nine Mile		-	-	-	-
<i>Legionella pneumophila</i>	S5/S		-	-	-	-
<i>Rickettsia conorii</i>	VR-613		-	-	-	-
<i>Orientia tsutsugamushi</i>	Kato		-	-	-	-
<i>Mycoplasma pneumoniae</i> ^b			-	-	-	-
<i>Chlamydia pneumoniae</i> ^b			-	-	-	-
<i>Ixodes ricinus</i>			-	-	-	-
<i>Rhipicephalus sanguineus</i>			-	-	-	-
C3H mouse			-	-	-	-
Human			-	-	-	-

^a As shown in Fig. 2. +, positive; -, negative.

^b DNA was extracted from scraped slides for indirect immunofluorescence (Vircell, S.L., Granada, Spain).

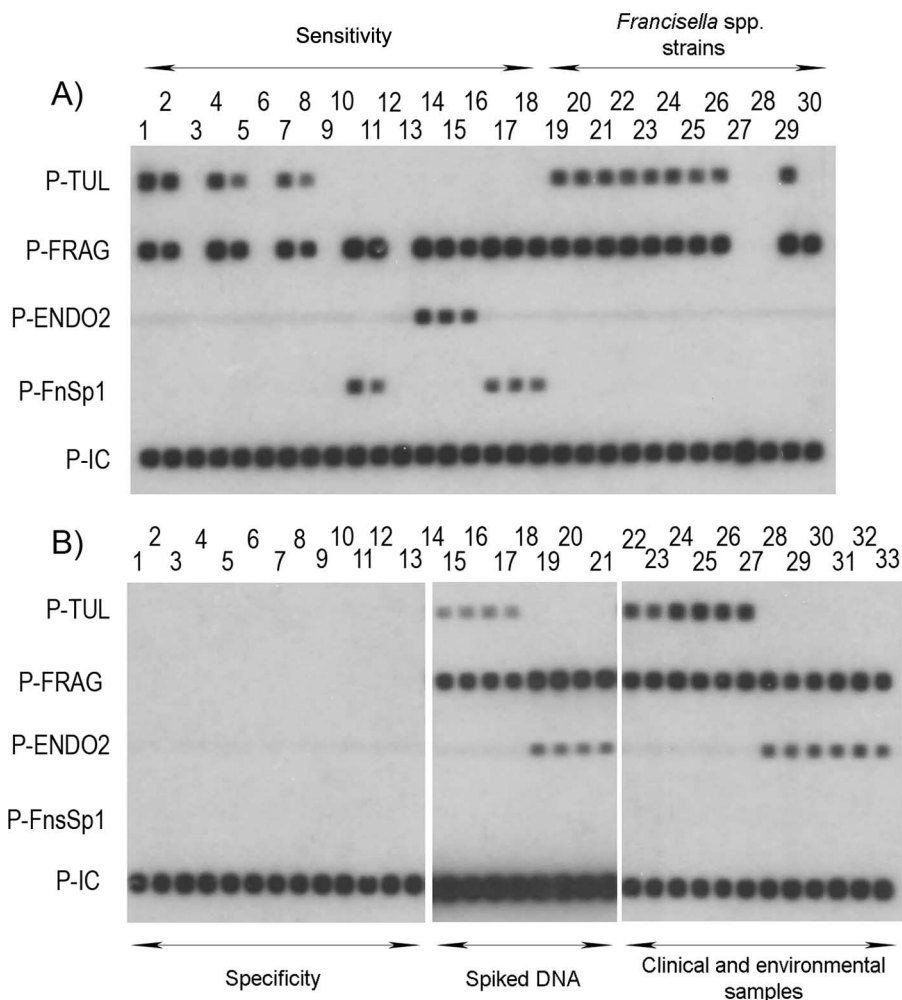


FIG. 2. RLB results of different samples, as in Table 2. (A) Lanes 1 to 3, 10^2 , 10^1 , and 1 GE, respectively, *F. tularensis* subsp. *holarctica* strain LVS; lanes 4 to 6, 10^2 , 10^1 , and 1 GE, respectively, *F. tularensis* subsp. *tularensis* strain B-38; lanes 7 to 9, 10^2 , 10^1 , and 1 GE, respectively, *F. tularensis* subsp. *novicida* strain Utah 112; lanes 10 to 12, 10^2 , 10^1 , and 1 GE, respectively, unusual *F. tularensis* isolate FnSp1; lanes 13 to 15, 10^2 , 10^1 , and 1 copy, respectively, of plasmid FLE; lanes 16 to 18, 10^2 , 10^1 , and 1 copy, respectively, of synthetic DNA of *F. tularensis* subsp. *novicida*-like isolate 3523; lane 19, strain FSC 257; lane 20, strain FSC 012; lane 21, strain FSC200; lane 22, strain FT7; lane 23, strain FT13; lane 24, strain BZO20; lane 25, strain BZO21; lane 26, strain BZO22; lanes 27 and 28, *F. philomiragia* strains CCUG 12603 and CCUG 19701, respectively; lanes 29 and 30, *F. tularensis* subsp. *novicida* strains Fx1 and Fx2, respectively. (B) Lane 1, negative control (water). Lanes 2 to 13 show the results of specificity assays and reactivities of different *Francisella* strains: lane 2, *Anaplasma phagocytophilum*; lane 3, *Borrelia burgdorferi* sensu stricto; lane 4, *Coxiella burnetii*; lane 5, *Legionella pneumophila*; lane 6, *Rickettsia conorii*; lane 7, *Orientia tsutsugamushi*; lane 8, *Mycoplasma pneumoniae*; lane 9, *Chlamydia pneumoniae*; lane 10, questing *Ixodes ricinus* ticks from the Basque Country (3); lane 11, *Rhipicephalus sanguineus*; lane 12, C3H mouse DNA; and lane 13, human DNA. Lanes 14 to 17 contain DNAs of different origins spiked with 10 GE of *F. tularensis* subsp. *holarctica* strain LVS: lane 14, *I. ricinus*; lane 15, *R. sanguineus*; lane 16, C3H mouse DNA; and lane 17, human DNA. Lanes 18 to 21 contain DNAs of different origins spiked with 1 copy of plasmid FLE: lane 18, *I. ricinus*; lane 19, *R. sanguineus*; lane 20, C3H mouse DNA; and lane 21, human DNA. Lanes 22 to 27 contain clinical samples, including four lymph node aspirates (lanes 22 to 25), one skin exudate (lane 26), and one skin biopsy specimen (lane 27) from patients with tularemia. Lanes 28 and 29 contain samples from the small mammal *Apodemus sylvaticus*. Lanes 30 to 33 contain samples from ticks.

cloned in pGEM-T Easy vectors (Promega Biotech Ibérica, S.L., Madrid, Spain) by following the manufacturer's instructions and sequenced to check their fidelity. The plasmid copy numbers were quantified by spectrophotometry with a Nano-Drop ND-1000 spectrophotometer (Nucliber, Madrid, Spain). Genomic DNAs from *F. tularensis* subsp. *holarctica* strain LVS, *F. tularensis* subsp. *tularensis* strain B-38, *F. tularensis* subsp. *novicida* strain Utah 112, and the atypical isolate FnSp1 (6), all obtained after purification with a QIAamp DNA minikit (IZASA S.A., Barcelona, Spain), were used as positive controls as well (Table 2). To determine the sensitivity of the assay, 1,

10, and 10^2 copies/genomic equivalent (GE) of each plasmid/strain were tested per reaction.

DNA from clinical and environmental sources was obtained using a QIAamp DNA blood extraction kit (Qiagen). Clinical samples from patients consisted of lymph node aspirates, skin exudates, and skin biopsy specimens from the outbreak of tularemia that occurred in Spain in 1997 (9). Questing ticks and small mammals from Madrid (Toledo et al., submitted) as well as questing ticks from the Basque Country (3) were also included in this study.

The sensitivity of the method was excellent. One plasmid copy

and 10 GE were detected when synthetic or genomic DNAs of the positive controls were tested, respectively (Fig. 2A, lanes 1 to 18). As expected, the strains of *F. tularensis* subsp. *holarctica* (Fig. 2A, lanes 19 to 26) reacted with P-FRAG and P-TUL, and *F. tularensis* subsp. *novicida* strain Fx1 reacted with P-FRAG and P-TUL, whereas Fx2 only reacted with P-FRAG, since this strain presents the deletion mentioned above (Fig. 2A, lanes 29 and 30). Finally *F. philomiragia* did not react with any of the designed probes (Fig. 2A, lanes 27 and 28). In addition, the test was performed again with 10 GE of *F. tularensis* subsp. *holarctica* strain or 1 plasmid copy of synthetic DNA of FLEs in the presence of foreign DNA free of pathogens (300 ng of human DNA, 300 ng of DNA from *Ixodes ricinus* and *Rhipicephalus sanguineus* specimens, and 300 ng of a C3H mouse DNA), showing no loss of sensitivity (Fig. 2B, lanes 14 to 21). Sources for the DNA as well as a summary of the results are shown in Table 2.

No cross-reaction was observed either with any of the microorganisms tested for specificity (10^2 GE of different related pathogens or an estimated similar amount of DNA from intracellular pathogens, such as *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Coxiella burnetii*, *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Rickettsia conorii*, and *Orientia tsutsugamushi*) (Fig. 2B, lanes 2 to 9) or with 300 ng of host DNA or vector DNA free of pathogens (Fig. 2B, lanes 10 to 13).

All of the tularemia samples from patients (23 samples) were positive by PCR-RLB, yielding a sensitivity of 100% (data not shown). A representative sample of this result is shown in Fig. 2B, lanes 22 to 27. FLEs were detected in ticks collected from vegetation as well as from small mammals (Fig. 2B, lanes 28 to 33).

These results demonstrated that this method has shown excellent sensitivity and specificity values and also has the ability to differentiate among FLEs and known human pathogens, including atypical human isolates (FnSp1 and 3523) not included in routine practice for identification due to rare occurrence. Consequently, it can be used in epidemiological surveys, especially in environmental studies where FLEs are expected to be present. The primers used in this method are generic for any known *F. tularensis* strain and FLEs, and sequencing of new variants that could arise in the future will provide us with the possibility of designing new probes for their detection.

Kugeler et al. have recently described a method that differentiates between *F. tularensis* and FLEs (12). Their approach, using a multitarget TaqMan assay, has been shown to be both sensitive and specific, but sequencing of 16S rRNA was necessary for the identification of FLEs in the samples tested. The methodology proposed here is easy to set up and robust, as has been shown with other pathogens (7, 10). Moreover, this method has been tested under field conditions and is able to detect FLEs in both ticks and small mammals, as well as *F. tularensis* in ticks and humans, all without sequencing. However, considering the available information regarding FLEs, samples reactive to P-ENDO2, specific for FLEs, should be subjected to sequencing of additional genes to further assess the specificity of the probe.

As a result of increased concern over the terrorist use of agents such as *F. tularensis*, an extensive understanding of the diversity and distribution of this organism is required. Here, we have described a simple method for the detection and differ-

entiation among *F. tularensis* subspecies, FLEs, and new atypical *Francisella* variants recently described as associated with human disease that has high sensitivity and specificity. The generic approach used allows the detection of all known members of these groups. Moreover, this method represents an excellent and versatile tool for performing clinical and environmental studies.

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