JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2003, p. 5492–5499 Vol. 41, No. 12
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.12.5492–5499.2003

Development of a Multitarget Real-Time TaqMan PCR Assay for Enhanced Detection of *Francisella tularensis* in Complex Specimens

Jessica L. Versage,† Darlena D. M. Severin,‡ May C. Chu, and Jeannine M. Petersen*

Bacterial Zoonoses Branch, Division of Vector-Borne Infectious Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention, Ft. Collins, Colorado 80522

Received 4 June 2003/Returned for modification 4 August 2003/Accepted 26 August 2003

Tularemia is the zoonotic disease caused by the gram-negative coccobacillus *Francisella tularensis*. Its wide distribution in the environment poses a challenge for understanding the transmission, ecology, and epidemiology of the disease. *F. tularensis* is also considered a potential biological weapon due to its extreme infectivity. We have developed a multitarget real-time TaqMan PCR assay capable of rapidly and accurately detecting *F. tularensis* in complex specimens. Targeted regions included the IS*Ftu2* element and the *23kDa*, *fopA*, and *tul4* genes. Analysis of the four TaqMan assays demonstrated that three (IS*Ftu2*, *23kDa*, and *tul4*) performed within our established criterion of a detection limit of one organism. The combined use of the three assays was highly specific, displaying no cross-reactivity with the non-*Francisella* bacteria tested and capable of differentially diagnosing both *F. tularensis* and *Francisella philomiragia*. When the multitarget TaqMan assay (IS*Ftu2*, *23kDa*, and *tul4*) was compared to culturing, using environmentally contaminated specimens, the TaqMan PCR assay was significantly more sensitive than culturing ($P \leq 0.05$). The sensitive and specific nature of this rapid multitarget TaqMan assay provides a valuable new tool that with future evaluations can be used for analyzing clinical specimens, field samples during bioterrorism threat assessment, and samples from outbreaks and for improving our understanding of the ecology and environmental prevalence of *F. tularensis*.

Francisella tularensis is a gram-negative coccobacillus that is the etiologic agent of the disease tularemia (5, 6, 9, 31). It was first described in 1911 in Tulare County, Calif., and since has been reported throughout North America and Eurasia. *F. tularensis* naturally occurs in ~150 species of vertebrates, including lagomorphs and rodents, in ~100 species of invertebrates, and in contaminated soil, water, and vegetation (18, 25). It has a low level of natural transmission to humans, with an average of 124 reported cases per year in the United States from 1990 to 2000 (5). Humans become infected through arthropod bites (ticks and deerflies), contact with infected tissues, ingestion of contaminated water or food, and inhalation of infective aerosols. The clinical presentation of tularemia varies depending upon the route of infection. While the most common manifestation is ulceroglandular tularemia, pneumonic tularemia is the most severe form of the disease. *F. tularensis* is considered a biological weapon due to its extreme infectivity, as <10 organisms can cause severe disease (7).

The genus *Francisella* consists of two species, *F. tularensis* and *F. philomiragia* (6, 31). Species and subspecies of *Francisella* differ with regards to their geographic distribution and virulence in humans. Of the four *F. tularensis* subspecies, *F. tularensis* subsp. *tularensis*, also known as type A, has the highest mortality rate in humans (31). Human cases have been limited to North America, though type A organisms have been isolated from arthropods in Europe (16). *F. tularensis* subsp. *holarctica*, also known as type B, is widely distributed throughout the Northern Hemisphere and rarely causes fatal disease. *F. tularensis* subsp. *mediaasiatica* is isolated in focal regions of Central Asia and has not been associated with human disease. *F. tularensis* subsp. *novicida* is infrequently associated with human disease in North America and more recently in Australia (33). *F. philomiragia* appears to be an opportunistic pathogen, primarily causing serious disease in immunocompromised patients or near-drowning victims. *F. philomiragia* has been isolated from patients in Europe, North America, and Australia (33).

Human outbreaks of tularemia caused by both *F. tularensis* subsp. *tularensis* and *holarctica* have been reported in the United States, Spain, Sweden, Norway, Russia, and Kosovo (1, 2, 3, 8, 11, 27, 28). Infections were associated with contaminated food and water, aerosolized bacteria produced during landscaping and farming, handling or skinning of lagomorphs, arthropod bites, and crayfish fishing. Since *F. tularensis* is present in a diverse number of animals and environmental habitats, a large variety of field and environmental samples, from carcasses, fish, vectors, water, soil, lawn clippings, hay, feces, and urine, need to be tested. Thus, for many of these outbreaks, transmission sources are not accurately understood due to laboratory challenges associated with testing, detecting, and recovering *F. tularensis* from complex and varied specimens.

Diagnostic methods for presumptive and confirmatory identification of *F. tularensis* include a direct fluorescently labeled antibody (DFA) test, PCR, and culturing. The DFA test is a rapid, specific test (6, 20), but it has a sensitivity limitation of ~10^6 cells/ml. Gel-based PCR assays targeting the *tul4* and *fopA* genes have been developed, but they lack sensitivity and

* Corresponding author. Mailing address: Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Foothills Campus, P.O. Box 2087, Ft. Collins, CO 80522. Phone: (970) 266-3524. Fax: (970) 221-6476. E-mail: nzp0@cdc.gov.

† Present address: College of Medicine, University of Florida, Gainesville, FL 32611.

‡ Present address: Epidemiology and Biostatistics, School of Public Health, Loma Linda University, Loma Linda, CA 92350.

TABLE 1. *F. tularensis* TaqMan primers and probes

Gene target	Primer or probe[a]	Sequence (5′ → 3′)	Amplicon size (bp)
IS*Ftu2*	ISFtu2F	TTGGTAGATCAGTTGGTGGGATAAC	97
	ISFtu2R	TGAGTTTTACCTTCTGACAACAATATTTC	
	ISFtu2P	AAAATCCATGCTATGACTGATGCTTTAGGTAATCCA	
23kDa	23kDaF	TGAGATGATAACAAGACAACAGGTAACA	84
	23kDaR	GGATGAGATCCTATACATGCAGTAGG	
	23kDaP	TCAGTTCTCACATGAATGGTCTCGCCA	
tul4	Tul4F	ATTACAATGGCAGGCTCCAGA	91
	Tul4R	TGCCCAAGTTTTATCGTTCTTCT	
	Tul4P	TTCTAAGTGCCATGATACAAGCTTCCCAATTACTAAG	
fopA	FopAF	ATCTAGCAGGTCAAGCAACAGGT	87
	FopAR	GTCAACACTTGCTTGAACATTTCTAGATA	
	FopAP	CAAACTTAAGACCACCACCCACATCCCAA	

[a] F, forward primer; R, reverse primer; P, probe.

rapidity in comparison to real-time PCR assays (13, 23, 29). Culturing is the Centers for Disease Control and Prevention (CDC) "gold standard" for confirmatory diagnosis of infection. However, *F. tularensis* is a slow-growing, fastidious organism that requires 24 to 72 h for growth on cysteine-enriched agar (6, 31) and its growth is often out-competed by contaminating bacteria, particularly when environmental samples are being tested. *F. tularensis* is also notorious for causing laboratory-acquired infections and must be handled under biosafety level 3 (BSL-3) conditions (6).

Real-time TaqMan PCR is a rapid assay with high sensitivity (21, 22). The utilization of a fluorogenically labeled probe enhances sensitivity by at least 7 orders of magnitude compared to conventional PCR (21). In addition, the fluorogenic probe allows for real-time data collection due to the production of a fluorescent signal during amplification, eliminating the need for gel electrophoresis.

For this study, four individual real-time TaqMan PCR assays were developed for combined use for the detection of *F. tularensis* in a LightCycler instrument (Roche Applied Science, Indianapolis, Ind.). We chose to develop TaqMan PCR assays, as opposed to other real-time assays, since TaqMan assays can provide high specificity due to binding of two primers as well as a probe. Currently, a real-time TaqMan PCR assay for *F. tularensis* is available through the Laboratory Response Network (CDC, Atlanta, Ga.). However, due to national security concerns regarding *F. tularensis* as a potential biological weapon, these reagents have limited distribution and use. Therefore, we developed a real-time TaqMan PCR assay that would be available for public use for research, clinical, or surveillance purposes. Our assay development included two goals: (i) the identification of multiple *F. tularensis*-specific genomic targets that could be utilized in combination (multitarget assay) and (ii) the reliable detection of one organism of *F. tularensis*. Multiple targets would enhance specificity by reducing the likelihood of obtaining false positives due to reliance on a single target. Additionally, a detection limit of one organism increases the likelihood of detecting *F. tularensis* in environmental samples (e.g., water, hay, and grass) in which the number of organisms is expected to be quite low.

Regions targeted for TaqMan assay development were specific for *Francisella* and included the IS*Ftu2* element, a newly described insertion element-like sequence present in multiple copies (32; Y. Zhou, S. W. Bearden, Z. L. Berrada, L. G. Carter, B. M. Yockey, S. K. Urich, and M. C. Chu, unpublished data), the *23kDa* gene, which encodes a protein that is expressed upon macrophage infection (14), and the *tul4* and *fopA* genes, which encode outer membrane proteins (26, 30). The IS*Ftu2*, *23kDa*, and *tul4* assays were each capable of detecting one organism and therefore were combined into a single multitarget assay for further analysis. This multitarget assay was species specific, able to differentially diagnose both *F. tularensis* and *F. philomiragia*. Comparison of the *F. tularensis* TaqMan assay with culturing, using contaminated specimens, demonstrated that the combined TaqMan PCR assay was significantly more sensitive than culturing ($P \leq 0.05$). Thus, the *F. tularensis* multitarget TaqMan PCR assay described here should be of valuable use for detecting *F. tularensis* during future outbreaks and field studies.

MATERIALS AND METHODS

TaqMan primers and probes. The GenBank sequences for *F. tularensis* IS*Ftu2* (accession no. AY06), *23kDa* (accession no. Y08861), *tul4* (accession no. M32059), and *fopA* (accession no. AF097542) were used to design four sets of TaqMan primers and probes (Table 1). For primer and probe design, ABI Primer Express software was used (Applied Biosystems, Foster City, Calif.). Prior to synthesis of primer-probe sets by the CDC Biotechnology Core Facility (Atlanta, Ga.), the sequences were subjected to a BLAST search to verify their specificity for *Francisella*. All fluorogenic probes were synthesized with a 6-carboxy-fluorescein reporter molecule attached to the 5′ end and a Black Hole Quencher (BioSearch Technologies, Inc., Novato, Calif.) attached to the 3′ end.

PCR optimization. TaqMan PCR conditions were optimized with 10 ng of DNA from *F. tularensis* subsp. *tularensis* (strain SchuS4). All reactions were performed in a final volume of 20 μl and contained LightCycler Fast Start DNA master hybridization probe mix (Roche) at a 1× final concentration, primers (500 nM), probes (100 nM), $MgCl_2$, and 0.5 U of uracil-DNA glycosylase per reaction. For the *23kDa*, *tul4*, and *fopA* TaqMan assays, the final $MgCl_2$ concentration was 4 mM, and for the IS*Ftu2* assay, the final concentration was 5 mM. The optimum annealing temperature for all four TaqMan assays was 60°C. Thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 10 s and 60°C for 30 s, and then 45°C for 5 min.

Bacterial isolates. The specificities of the four TaqMan assays were evaluated with DNA from 87 non-*Francisella* species isolated from both animals and humans (Table 2). Bacterial cultures were obtained from the Poudre Valley Hospital Clinical Laboratory (Ft. Collins, Colo.), Denver Children's Hospital (John James), and Colorado State University Veterinary Diagnostic Laboratory (Doreene Hyatt). All isolates were stored in brain heart infusion broth with 10% glycerol at −70°C. DNA from *Brucella* species was kindly provided by R. Martin Roop II (East Carolina University, Greenville, N.C.). Barry Fields (CDC) kindly provided DNA from *Legionella* species.

DNAs were also analyzed from a total of 55 wild-type *Francisella* isolates which originated from a variety of sources, including humans, animals, and water (Table 3). The 55 isolates included *F. tularensis* subsp. *tularensis* ($n = 18$), *F.*

TABLE 2. Evaluation of cross-reactivity of IS*Ftu2, 23kDa, tul4*, and *fopA* TaqMan PCR assays using non-*Francisella* bacterial isolates ($n = 87$)[a]

Genus	Species	No. tested
Acinetobacter	*A. calcoaceticus*	2
Bacillus	*Bacillus* spp.	3
Brucella	*B. abortus* (2308, RB51), *B. melitensis* 16M, *B. suis* biovar II	4
Corynebacterium	*C. diphtheriae*	3
Enterobacter	*E. cloacae*	4
Escherichia	*E. coli*	9
Hemophilus	*H. influenzae*	3
Klebsiella	*K. oxytoca, K. pneumoniae*	11
Legionella	*L. anisa, L. bozemani, L. feeleii, L. longbeachae, L. pneumoniae*	5
Moraxella	*M. catarrhalis*	1
Proteus	*P. mirabilis*	4
Pseudomonas	*P. aeruginosa*	8
Serratia	*S. marcesans*	2
Staphylococcus	*S. aureus*, coagulase-negative staphylococci	9
Streptococcus	*S. faecalis, S. pneumoniae*	7
Yersinia	*Y. enterocolitica, Y. pestis, Y. pseudotuberculosis*	12

[a] Results for all isolates for all four assays were negative, with a negative result being no DNA amplification after 45 cycles.

tularensis subsp. *holarctica* ($n = 18$), *F. tularensis* subsp. *novicida* ($n = 4$), and *F. philomiragia* ($n = 15$) and were selected from the CDC reference strain collection (Ft. Collins, Colo.). All *Francisella* isolates were confirmed by characteristic growth on cysteine heart agar with 9% chocolatized blood (CHAB) at 37°C, by DFA test, and by biochemical profile (Biolog GN2 identification system; Biolog Inc., Hayward, Calif.).

DNA extraction. For isolation of genomic DNA from non-*Francisella* species, bacteria were freshly cultured on 5% sheep blood agar at 37°C. All *Francisella* isolates were freshly cultured on CHAB at 37°C in a BSL-3 laboratory, with use of appropriate protective gear, including closed front gown, N95 mask, and gloves. Template DNA was isolated from gram-negative bacteria by use of the QiaAmp DNA mini kit (Qiagen Inc., Valencia, Calif.) and from gram-positive bacteria by use of the MasterPure DNA purification kit (Epicentre, Madison, Wis.). For purification of DNA from *Francisella* isolates, lysis was performed under BSL-3 conditions. Once DNA was lysed, purification reactions were performed on the benchtop in a BSL-2 laboratory. Concentrations of DNA were approximated by electrophoresis on a 1% agarose gel and visual comparison to known concentrations of DNA (1-kb EZ Load molecular ruler; Bio-Rad Laboratories, Hercules, Calif.). All samples were stored at −20°C until use. For preservation of the integrity of the genomic DNAs, aliquots were prepared and freeze-thawing was minimized.

Real-time TaqMan PCR assays. PCR amplification and product detection were performed in a LightCycler instrument (Roche). For each TaqMan run, both negative (no template) and positive (1 ng of purified *F. tularensis* strain SchuS4 DNA) controls were included (one of each for every five samples tested). If any of the controls failed, the run was repeated. For evaluation of genomic DNA purified from bacterial cultures, 1 ng from both *Francisella* and non-*Francisella* species was tested; 1 ng of *F. tularensis* DNA is ≈500,000 genome equivalents (GEs) (genome size, 2 MB) and provides a strong fluorescent TaqMan signal (cycle threshold [C_t] value, ≤18). The C_t values were determined by the LightCycler instrument for each reaction by setting the *y* axis to F1/F3 and performing automatic quantification using the second derivative maximum method. To verify that amplified products were the correct size, amplicons were electrophoresed on 3.5% agarose gels and visualized by staining with ethidium bromide.

Sensitivity determinations. TaqMan assays were evaluated by use of *F. tularensis* subsp. *tularensis* (strain SchuS4) and subsp. *holarctica* (strain LVS) and two independent methods for determining sensitivity (CFU and GEs). For determination of CFU, single colonies were picked from a fresh culture and suspended in phosphate-buffered saline (pH 7.2) containing 5 mM $MgCl_2$ to a final density of 56% transmittance at 590 nm. Tenfold serial dilutions, to 10^{-9}, were made and 50 μl of each dilution was spread evenly in duplicate on CHAB. Plates were incubated for 48 h at 37°C, at which time colonies were counted and the results from the two plates were averaged. For standard curves based on CFU, 50 μl was removed from the 10^{-1} to 10^{-9} dilutions and added to 150 μl of Tris-EDTA buffer (pH 8.0). DNA was extracted by boiling lysis at 95°C for 10 min, and 1 μl from each dilution was tested. For standard curves based on GEs, DNA was extracted from the 10^{-1} dilutions by use of the MasterPure DNA purification kit (Epicentre). The DNA concentration was approximated by electrophoresis in a

TABLE 3. Panel of wild-type *Francisella* strains used for evaluation of TaqMan assays

Francisella species (*n*)	Origin	Host or Source	CDC no.
F. tularensis subsp. *tularensis* (18)	Ohio	Human	SchuS4
	Massachusetts	Human	MA002987
	Colorado	Cat	CO012364
	Colorado	Rabbit	CO013713
	North Dakota	Human	ND000952
	Kansas	Cat	KS000948
	Oklahoma	Human	OK002731
	North Carolina	Rabbit	NC993990
	Arkansas	Human	AR000028
	Utah	Human	UT983134
	New Mexico	Rabbit	NM990295
	North Carolina	Rabbit	NC973057
	North Carolina	Cat	NC015379
	Arkansas	Rabbit	AR982147
	Oklahoma	Human	OK004337
	Arkansas	Human	AR011117
	Missouri	Human	MO011907
	California	Human	CA020099
F. tularensis subsp. *holarctica* (18)	Russia	Rat	LVS
	Oregon	Monkey	OR960246
	Canada	Human	CN985979
	Arizona	Rat	AZ001325
	Illinois	Human	IL003633
	Missouri	Human	MO011673
	Kentucky	Human	KY001708
	Ohio	Prairie dog	OH013209
	Utah	Human	UT002098
	Spain	Rabbit	SP986120
	Indiana	Rat	IN983055
	Colorado	Vole	CO961243
	California	Human	CA990837
	Indiana	Human	IN002758
	Arizona	Squirrel	AZ001324
	California	Monkey	CA993992
	Spain	Human	SP982108
	New Mexico	Human	NM002642
F. tularensis subsp. *novicida* (4)	Louisiana	Human	GA993548
	California	Human	GA993549
	Utah	Water	GA993550
	Utah	Human	UT014992
F. philomiragia (15)	California	Human	GA012793
	Colorado	Human	GA012794
	New York	Human	GA012795
	California	Human	GA012796
	Pennsylvania	Human	GA012797
	Connecticut	Human	GA012799
	Connecticut	Human	GA012800
	New York	Human	GA012801
	California	Human	GA012802
	New Mexico	Human	GA012803
	Virginia	Human	GA012804
	Massachusetts	Human	GA012806
	Unknown	Human	GA012807
	Utah	Water	GA012810
	Utah	Water	GA012811

TABLE 4. Evaluation of *F. tularensis* multitarget TaqMan PCR assay using mouse tissues[a]

Subjects (*n*)	CDC strain no.	Result by multitarget TaqMan assay[b] (IS*Ftu2*, *23kDa*, and *tul4*)
Mice infected with *F. tularensis* subsp. *tularensis* (5)	Schu4	+
	ND000952	+
	CO013713	+
	MA002987	+
	CO012364	+
Mice infected with *F. tularensis* subsp. *holarctica* (5)	LVS	+
	OR960246	+
	AZ001325	+
	IL003633	+
	CN985979	+
Healthy uninfected mice (5)		−

[a] For each mouse (15), the liver and spleen were tested.
[b] +, positive result, $9 \leq C_t \leq 25$; −, negative result, no DNA amplification after 45 cycles.

1% agarose gel and visual comparison to known concentrations of DNA (1-kb EZ Load molecular ruler; Bio-Rad). Tenfold serial dilutions (to 10^{-9}) of DNA were then made and 1 μl from each dilution was tested. GE calculations were based on assuming a 2-MB genome size for *F. tularensis*. Standard curves based on both CFU and GEs were plotted as C_t versus log input. Standard deviations were calculated based on the averages of three independent experiments performed by the same operator. To assess the log-linear relationship of the assays, the linear regression and square regression coefficients (R^2) were calculated.

Animal tissues. A total of 10 different *F. tularensis* strains, 5 *F. tularensis* subsp. *tularensis* and 5 *F. tularensis* subsp. *holarctica* (Table 4), were inoculated into 10 pathogen-free Swiss Webster mice (IACUC protocol 00-06-018-MUS). In all cases, 100 CFU were administered by subcutaneous injection. All inoculations were performed in a BSL-3 animal facility, and appropriate biosafety measures were followed. Animals were sacrificed when signs and symptoms of tularemia were evident (3 to 5 days after inoculation). Liver and spleen tissues were excised from each mouse (total of 20 tissue samples) and DNA was extracted from 25 mg of liver and 10 mg of spleen by the QiaAmp DNA mini kit (Qiagen Inc.) tissue protocol. DNA samples were divided into aliquots and stored frozen (−20°C) until use. For real-time PCR analysis of total DNA (animal plus bacterial) from tissues, 1 μl of purified DNA was used.

Comparison of the multitarget TaqMan PCR assay to culturing. A total of 86 tissues from a naturally occurring tularemia prairie dog outbreak were evaluated to compare multitarget TagMan PCR with culturing: 46 tissues were from animals succumbing to tularemia and 40 tissues were from healthy uninfected animals (4). Tissues were inoculated onto CHAB or CHAB containing antimicrobials (19), incubated for 7 days at 37°C, and checked daily for growth. For DNA extraction, liver and spleen tissues were processed and stored by the animal tissue protocol described above. For real-time PCR analysis, 1 μl of purified DNA was tested under blind coding.

Statistical analysis. The differences between test sensitivities were evaluated by McNemar's test.

RESULTS

Initial evaluation. To demonstrate that the four TaqMan assays amplified their respective genomic targets from *F. tularensis*, we performed an initial assessment of the assays using *F. tularensis* subsp. *tularensis* and *holarctica*. Logarithmic amplification was observed for all four genomic targets ($12 \leq C_t \leq 19$) and DNA fragments of the expected sizes were produced (Table 1; data not shown).

Specificity. Specificity of the TaqMan assays was determined by use of DNAs from 87 bacterial strains originating from both animal and human sources (Table 2). These bacterial species were chosen for comparison because they represent environmental bacteria, organisms found in the same infection sites as *F. tularensis* (respiratory tract and wounds), vector-borne organisms, and potentially cross-reactive bacteria (*Brucella* and *Legionella*). No evidence of cross-reactivity was detected with the four TaqMan assays, as evidenced by the absence of DNA amplification after 45 cycles (Table 2).

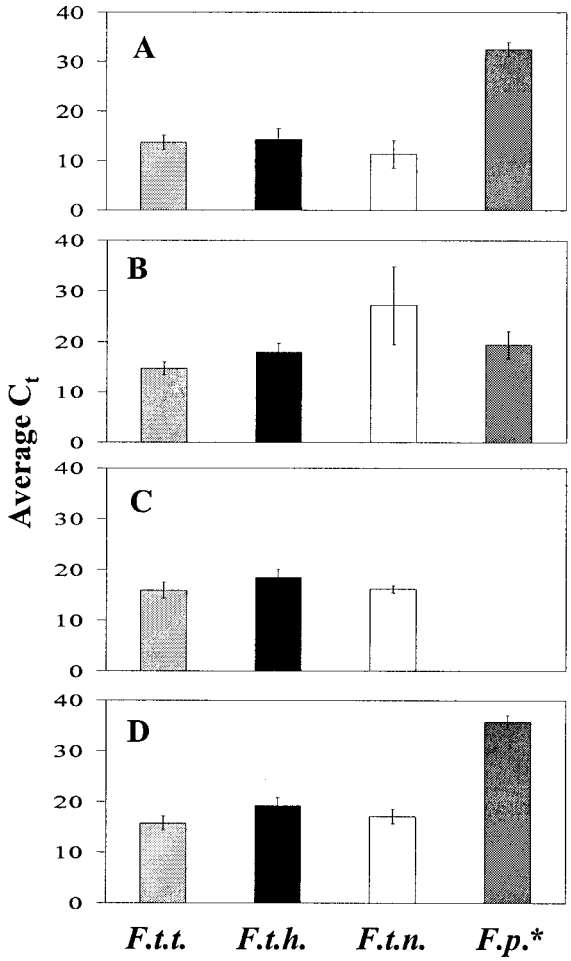

FIG. 1. Average C_t values for the IS*Ftu2* (A), *23kDa* (B), *tul4* (C), and *fopA* (D) assays. C_t values for the 18 *F. tularensis* subsp. *tularensis* strains (*F.t.t.*), 18 *F. tularensis* subsp. *holarctica* strains (*F.t.h.*), 4 *F. tularensis* subsp. *novicida* strains (*F.t.n.*), and 15 *F. philomiragia* stains (*F.p.*) and their respective standard deviations are shown. ∗, for *F. philomiragia*, the *23kDa* C_t value represents the average for four isolates in which *23kDa* was detected.

In comparison, the IS*Ftu2* element and *23kDa*, *tul4*, and *fopA* genes were all detected when template DNAs from 18 *F. tularensis* subsp. *tularensis*, 18 *F. tularensis* subsp. *holarctica*, and 4 *F. tularensis* subsp. *novicida* isolates were tested (Table 3; Fig. 1). When the genomic targets were amplified from *F. tularensis* subsp. *tularensis*, C_t values averaged 14, 15, 16, and 16 for IS*Ftu2*, *23kDa*, *tul4*, and *fopA*, respectively (Fig. 1). For the amplification of IS*Ftu2*, *23kDa*, *tul4*, and *fopA* from *F. tularensis* subsp. *holarctica*, C_t values averaged 14, 18, 19, and 19 (Fig. 1). The average C_t value for *F. tularensis* subsp. *novicida* for the *23kDa* assay was 27, while it was 11, 16, and 17, respectively, for the IS*Ftu2*, *tul4*, and *fopA* assays (Fig. 1). Appropriately sized

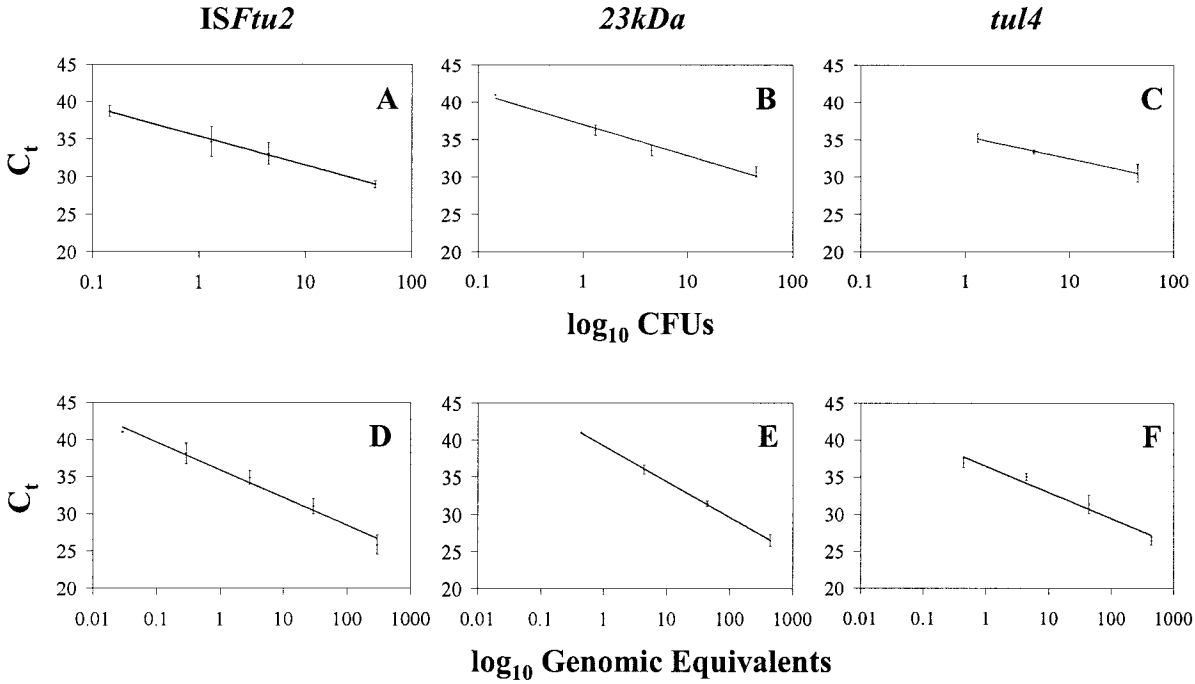

FIG. 2. Cell (top) and DNA (bottom) standard curves for *F. tularensis* subsp. *tularensis* (strain SchuS4). The relationship between 10-fold serial dilutions of CFU versus C_t for the IS*Ftu2* (A), *23kDa* (B), and *tul4* (C) TaqMan assays is shown in the top panels; the calculated square regression coefficients were 0.98, 0.9971, and 0.9967, respectively. The relationship between 10-fold serial dilutions of GEs versus C_t for the IS*Ftu2* (D), *23kDa* (E), and *tul4* (F) TaqMan assays is shown in the bottom panels; the calculated square regression coefficients were 0.9853, 0.9997, and 0.9632, respectively. The log-linear regression and standard deviations are indicated.

DNA products were produced from all *F. tularensis* subspecies tested.

To determine if the TaqMan assays were species specific for *F. tularensis*, DNAs from 15 *F. philomiragia* isolates were tested (Table 3). The IS*Ftu2* and *fopA* TaqMan assays amplified products of the appropriate sizes from the 15 *F. philomiragia* DNAs; however, the average C_t values, 33 and 36, respectively, were consistently higher than those observed for *F. tularensis* (Fig. 1). The region of the *23kDa* gene targeted by the TaqMan assay was only amplified from 4 of the 15 *F. philomiragia* isolates tested. The region of *tul4* targeted in our assay could not be amplified from any of the *F. philomiragia* DNAs, demonstrating that the *tul4* TaqMan assay is specific for *F. tularensis.* Thus, the combined use of the *tul4* assay with either the IS*Ftu2* or *fopA* TaqMan assay is discriminatory for the two *Francisella* species, *F. tularensis* and *F. philomiragia.*

Sensitivity. We next generated standard curves to determine the detection limits of the four TaqMan assays. For cell standard curves, DNA was amplified from 10-fold dilutions of *F. tularensis* suspensions (subsp. *tularensis* [strain SchuS4]) and *holarctica* [strain LVS]) with known CFU. Analysis of amplification curves for all four TaqMan assays showed that each assay displayed similarly shaped curves over the 10-fold dilution series. For both *F. tularensis* subsp. *tularensis* and subsp. *holarctica*, the detection limit for the *tul4* TaqMan assay was ~1 CFU, while it was <1 CFU for the IS*Ftu2* and *23kDa* assays (Fig. 2A to C; data not shown). All three assays showed a linear log correlation. In contrast, the *fopA* TaqMan assay had a detection limit of ~45 CFU (data not shown).

Since growth of live bacteria on culture plates (CFU) may not be an accurate estimate of the total number of colonies present (both live and dead), especially for a fastidious organism like *F. tularensis*, the detection limits were also calculated based on DNA concentrations. For the *23kDa*, *tul4*, and *fopA* assays, we assumed that 1 CFU was equal to 1 GE, and for the IS*Ftu2* assay, we assumed that 1 CFU was equal to ~15 and 28 GEs for *F. tularensis* subsp. *tularensis* and *holarctica*, respectively. When logarithmic dilutions of both *F. tularensis* subsp. *tularensis* and *holarctica* were tested, the detection limit for the *23kDa* and *tul4* assays was ~1 GE and for the IS*Ftu2* assay was <1 GE (Fig. 2D to F; data not shown). The IS*Ftu2*, *23kDa*, and *tul4* TaqMan assays had a linear log correlation. As observed for cell standard curves, the *fopA* assay had the highest limit of detection (~44 GEs) based on DNA concentrations (data not shown). Therefore, further analysis was only performed with the IS*Ftu2*, *23kDa*, and *tul4* TaqMan assays, all of which met the established criterion of detection of 1 CFU or GE.

Analysis of tissue specimens. To test the ability of the IS*Ftu2*, *tul4*, and *23kDa* TaqMan assays to identify *F. tularensis* in biological specimens, infected mouse tissues were analyzed. Whereas DNAs isolated from spleens and livers of control mice were negative in the assays, DNAs isolated from tissues of *F. tularensis*-infected mice were consistently positive by all three assays (Table 4). In addition, tissue samples spiked with known concentrations of *F. tularensis* DNA showed no evidence of inhibition (data not shown). Thus, the IS*Ftu2*, *23kDa*, and *tul4* assays can be used in combination to accurately detect *F. tularensis* in sterile tissue specimens.

Comparison of the *F. tularensis* multitarget TaqMan PCR assay to culturing. We next compared the sensitivity of the

TABLE 5. Comparison of multitarget TaqMan assay with culturing for detection of *F. tularensis* in complex specimens

Samples (*n*)[a]	No. (%) of positive results by:	
	Culture	Multitarget TaqMan assay (IS*Ftu2*, *23kDa*, and *tul4*)
Infected animal carcasses (46)	40 (86.9)	46 (100)
Uninfected animals (40)	0 (0)	0 (0)

[a] The tissues tested were spleens and livers. In all cases, the same tissue was tested by both culturing and real-time PCR.

combined IS*Ftu2*, *23kDa*, and *tul4* TaqMan assay to culturing, since culturing is the CDC gold standard for the diagnosis of *F. tularensis*. Because our goal was to develop an enhanced assay capable of accurately identifying *F. tularensis* in the presence of contaminating bacteria, our comparison focused on *F. tularensis*-infected animal carcasses. Culturing of these tissues on CHAB medium showed them to be highly contaminated with environmental bacteria, including *Proteus*, *Pseudomonas*, and *Staphylococcus* (J. M. Petersen, unpublished data).

F. tularensis was isolated from 40 of the 46 carcasses by culturing, yielding a recovery rate of 86.9% (Table 5). In comparison, the combined IS*Ftu2*, *23kDa*, and *tul4* TaqMan assay accurately detected *F. tularensis* in 46 of the 46 carcasses (100%), with average C_t values of 20, 22, and 22, respectively. None of the 40 (0%) *F. tularensis*-negative tissues were positive by the TaqMan assays. The difference in sensitivity between culturing and the multitarget TaqMan is statistically significant ($P \leq 0.05$), with the combined TaqMan assay being more sensitive than culturing for testing of complex specimens from naturally occurring outbreaks.

DISCUSSION

For the present study, we developed a highly sensitive and specific multitarget real-time TaqMan PCR assay that exceeds the detection limit of existing real-time PCR assays for *F. tularensis* (10, 17). This multitarget assay is directed against three different genomic loci, the IS*Ftu2* element and the *23kDa* and *tul4* genes, and is capable of detecting 1 CFU or GE of *F. tularensis*. We are confident in the detection limit of this assay, as two independent methods (CFU and GE) were utilized and produced similar results. This multitarget TaqMan assay is also species specific and can differentially diagnose both *F. tularensis* and *F. philomiragia*. Identification of *F. tularensis* occurs when all three targets (IS*Ftu2*, *23kDa*, and *tul4*) are positive, whereas identification of *F. philomiragia* occurs when the IS*Ftu2* assay is positive and the *tul4* assay is negative.

Multiple genomic loci (multitarget) were targeted in the real-time TaqMan PCR assay in order to enhance the specificity of the test. While the *tul4* gene has been targeted in previous standard and real-time PCR assays (10, 23, 29), this is the first time that either the *23kDa* gene or the IS*Ftu2* element have been utilized. The *23kDa* gene is unique to *Francisella*, and research has focused on the role the gene product plays in macrophage infection and pathogenesis (14, 15). The IS*Ftu2* element, a recently described insertion sequence-like element, was chosen for development since it is present in multiple copy numbers (12 to 17 copies in *F. tularensis* subsp. *tularensis*, 26 to 30 copies in subsp. *holarctica*, 6 to 18 copies in subsp. *novicida*, and 1 to 2 copies in *F. philomiragia*), thus ensuring maximum sensitivity for the assay (32; Y. Zhou et al., unpublished data). Indeed, the IS*Ftu2* assay was the most sensitive of the three and had a detection limit of <1 organism for both *F. tularensis* subsp. *tularensis* and *holarctica*.

Enhanced specificity of the multitarget TaqMan assay (IS*Ftu2*, *23kDa*, and *tul4*) was shown upon evaluating a large panel of *Francisella* and non-*Francisella* isolates. First, the assay showed no evidence of cross-reactivity with non-*Francisella* bacteria (environmental bacteria, common respiratory tract and wound bacteria, and vector-borne organisms), as judged by the absence of DNA amplification after 45 cycles. In addition, the assay displayed no evidence of cross-reactivity when tissues known to be contaminated with environmental bacteria were tested. Second, the multitarget assay detected all *Francisella* species from a variety of sources, demonstrating its ability to reliably identify wild-type isolates. Third, the multitarget assay was species specific. Due to the specificity of the assay, at this point in the evaluation, we have chosen not to define a cutoff value for a positive result. Ideally, we would like to set as high a cutoff as possible to increase the likelihood of detecting positive samples in which numbers of bacteria are expected to be quite low. Defining this cutoff will require further evaluation with a variety of clinical and environmental specimens.

Because we tested a large panel of *Francisella* isolates for this study, we were able to make several interesting observations regarding the taxonomy of the genus. Our results are consistent with the recent classification of subsp. *novicida* as a subspecies of *F. tularensis* (31), since all subsp. *novicida* isolates, including a recent one from Australia, tested positive for all three genomic targets (33; data not shown) (Fig. 1). DNA from *F. tularensis* subsp. *novicida* did show a small difference from *F. tularensis* subsp. *tularensis* and *holarctica*. On average, higher C_t values were observed for the *23kDa* assay with subsp. *novicida* (average C_t, 27) than with subsp. *tularensis* and *holarctica* (average C_t, 16). Since the concentrations of template DNA were the same, it is likely that the *23kDa* gene sequence is somewhat divergent between subsp. *novicida* and subsp. *tularensis* and *holarctica*.

Our results are also consistent with the designation of *F. tularensis* and *F. philomiragia* as two distinct species within the *Francisella* genus (31). All *F. philomiragia* isolates tested positive by the IS*Ftu2* assay. The difference in the IS*Ftu2* C_t values for *F. philomiragia* (average C_t, 33) and *F. tularensis* (average C_t, 14) can be attributed to either sequence divergence or fewer copy numbers of the IS*Ftu2* element in *F. philomiragia* (Y. Zhou et al., unpublished data). The *tul4* gene was not detected in any of the *F. philomiragia* isolates used in this study or in a single isolate tested in another study (10). DNA hybridization studies with *F. tularensis* have shown that the *tul4* gene is present in *F. philomiragia* (30), suggesting that the region of the *tul4* gene targeted in our assay is divergent enough in *F. philomiragia* to not be recognized by the *tul4* assay. In addition, the majority of *F. philomiragia* isolates were negative by the *23kDa* TaqMan assay. The *23kDa* gene has never been studied in *F. philomiragia*, and therefore, it is unclear whether this gene is divergent or missing entirely from this species. Since the *23kDa* gene is known to be important for intracellular growth of *F. tularensis* in macrophages (14, 15), it

will be interesting to test whether *F. philomiragia* can also infect macrophages.

Here we show that the multitarget TaqMan assay (IS*Ftu2*, *23kDa*, and *tul4*) is significantly more sensitive ($P \leq 0.05$) than culturing for testing of complex specimens. Tissues from dead animals are usually highly contaminated with environmental bacteria, making culture recovery a challenge since *F. tularensis* is a slow-growing, fastidious organism. Past studies have had limited success in isolating *F. tularensis* from dead animals, with culture recovery rates from carcasses only around 30% (24). More recently, *F. tularensis* has also been reported to persist in a viable but noncultivable state (12). Despite the challenges associated with culturing *F. tularensis* from carcasses, the multitarget TaqMan assay detected *F. tularensis* in 100% of cases, demonstrating that the multitarget TaqMan assay is advantageous for the detection of *F. tularensis* in complex specimens.

Current studies are under way to evaluate the ability of the TaqMan assay for use in testing environmental and field specimens associated with outbreaks of tularemia, for bioterrorism assessment and response, and to further understanding of the ecology and transmission cycles of the organism. To date, the assay has proved to accurately detect *F. tularensis* in animal carcasses, urine, feces, and ticks (data not shown). Future evaluations using water, soil, and grass will also be important. For translation of this assay to the clinical diagnostic laboratory, evaluations between culturing and the multitarget TaqMan assay using clinical specimens such as bronchial fluid, blood, pleural fluid, and lymph node aspirates must be performed. In humans, antibiotic treatment can prevent the recovery of a culture, making PCR an important diagnostic tool. In the case of a bioterrorism event, for which results would be needed immediately, PCR can give rapid identification and guide further testing. While obtaining a culture is an irrefutable confirmation of the presence of live *F. tularensis*, PCR is an invaluable diagnostic when a sample is unculturable or contaminated or when results are needed immediately.

In conclusion, while other PCR assays exist for the detection of *F. tularensis*, our multitarget TaqMan PCR assay has many advantages because it consists of three genetic targets, is a real-time rapid assay, can differentially diagnose *F. tularensis* and *F. philomiragia*, and is sensitive to 1 CFU or 1 GE. In a bioterrorism event, when the testing of clinical and environmental samples would need to be done quickly and accurately for immediate assessment and response, the multitarget assay would provide an indispensable tool. It should also prove to be of great value for testing a wide variety of environmental and field samples. In the future, the IS*Ftu2*, *23kDa*, and *tul4* TaqMan assays can be combined into a single multiplex reaction by labeling the probes with emitters of different wavelengths, with the caveat that the multiplex assay will need to be carefully evaluated to ensure that there is no compromise to the sensitivity. While this TaqMan PCR assay was developed on a LightCycler instrument, it can be translated upon evaluation to any real-time platform, such as the I-Cycler, SmartCylcer, or ABI 7000 instrument.

ACKNOWLEDGMENTS

We thank Rob Weyant for kindly providing the *F. philomiragia* and *F. tularensis* subsp. *novicida* strains. We also thank the Poudre Valley Hospital Clinical Laboratory, Connie Ohlson, John James, R. Martin Roop II, Barry Fields, Doreene Hyatt, Nord Zeidner, Scott Bearden, and the Colorado State University Veterinary Diagnostic Laboratory for providing bacterial strains. We thank Brain Halloway at the CDC Biotechnology Core Facility for initial assessment and synthesis of the TaqMan primer-probe sets. We also acknowledge Tara Sealy and Craig Sampson for technical assistance.

This research was supported in part by an appointment to the Emerging Infectious Diseases (EID) Fellowship Program (to J.V.) administered by the Association of Public Health Laboratories (APHL) and funded by the CDC.

REFERENCES

1. **Anda, P., J. Segura del Pozo, J. M. Diaz Garcia, R. Escudero, F. J. Garcia Pena, M. C. Lopez Velasco, R. E. Sellek, M. R. Jimenez Chillaron, L. P. Sanchez Serrano, and J. F. Martinez Navarro.** 2001. Waterborne outbreak of tularemia associated with crayfish fishing. Emerg. Infect. Dis. **7:**575–582.
2. **Andres Puertas, C., M. L. Mateos Baruque, I. Buron Lobo, M. J. Gonzalez Megido, C. Rebollo Cuadrado, and L. A. Sangrador Arenas.** 1999. Epidemic outbreak of tularemia in Palencia. Rev. Clin. Esp. **199:**711–715.
3. **Berdal, B. P., R. Mehl, H. Haaheim, M. Loksa, R. Grunow, J. Burans, C. Morgan, and H. Meyer.** 2000. Field detection of *Francisella tularensis.* Scand. J. Infect. Dis. **32:**287–291.
4. **Centers for Disease Control and Prevention.** 2002. Public health dispatch: outbreak of tularemia among commercially distributed prairie dogs, 2002. Morb. Mortal. Wkly. Rep. **51:**688–689.
5. **Centers for Disease Control and Prevention.** 2002. Tularemia—United States, 1990–2000. Morb. Mortal. Wkly. Rep. **51**:182–184.
6. **Chu, M. C., and R. Weyant.** 2003. *Francisella* and *Brucella*, p. 789–797. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C.
7. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Barlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2001. Tularemia as a biological weapon—medical and public health management. JAMA **285:**2763–2773.
8. **Eliasson, H., J. Lindback, J. P. Nuorti, M. Arneborn, J. Giesecke, and A. Tegnell.** 2002. The 2000 tularemia outbreak: a case-control study of risk factors in disease-endemic and emergent areas, Sweden. Emerg. Infect. Dis. **8:**956–960.
9. **Ellis, J., P. C. F. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. J. Clin. Microbiol. **15:**631–646.
10. **Emanuel, P. A., R. Bell, J. L. Dang, R. McClanahan, J. C. David, R. J. Burgess, J. Thompson, L. Collins, and T. Hadfield.** 2003. Detection of *Francisella tularensis* within infected mouse tissues by using a handheld PCR thermocycler. J. Clin. Microbiol. **41:**689–693.
11. **Feldman, K. A., R. E. Enscore, S. L. Lathrop, B. T. Matyas, M. McGuill, M. E. Schriefer, D. Stiles-Enos, D. T. Dennis, L. R. Petersen, and E. B. Hayes.** 2001. An outbreak of primary pneumonic tularemia on Martha's Vineyard. N. Engl. J. Med. **345:**1601–1606.
12. **Forsman, M., E. W. Henningson, E. Larsson, T. Johansson, and G. Sandstrom.** 2000. *Francisella tularensis* does not manifest virulence in viable but non-culturable state. FEMS Microbiol. Ecol. **31:**217–224.
13. **Fulop, M., D. Leslie, and R. Titball.** 1996. A rapid, highly sensitive method for the detection of *Francisella tularensis* in clinical samples using the polymerase chain reaction. Am. J. Trop. Med. **54:**364–366.
14. **Golovliov, I., M. Ericsson, G. Sandström, A. Tärnvik, and A. Sjöstedt.** 1997. Identification of proteins of *Francisella tularensis* induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kilodalton protein. Infect. Immun. **65:**2183–2189.
15. **Gray, C. G., S. C. Cowly, K. K. Cheung, and F. E. Nano.** 2002. The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. FEMS Microbiol. Lett. **215:**53–56.
16. **Guryèová, D.** 1998. First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. Eur. J. Epidemiol. **14:**797–802.
17. **Higgens, J. A., Z. Hubalek, J. Halouzka, K. L. Elkins, A. Sjöstedt, M. Shipley, and M. Ibrahim.** 2000. Detection of *Francisella tularensis* in infected mammals and vectors using a probe-based polymerase chain reaction. Am. J. Trop. Med. **62:**310–318.
18. **Hopla, C. E., and A. K. Hopla.** 1994. Tularemia, p. 113–126. *In* G. W. Beran and J. H. Steele (ed.), Handbook of zoonoses, 2nd ed. CRC Press, Inc., Boca Raton, Fla.
19. **Johansson, A., L. Berglund, U. Eriksson, I. Göransson, R. Wollin, M. Forsman, A. Tärnvik, and A. Sjöstedt.** 2000. Comparative analysis of PCR versus culture for diagnosis of ulceroglandular tularemia. J. Clin. Microbiol. **38:**22–26.
20. **Karlsson, K. A., and O. Söderlund.** 1973. Studies of the diagnosis of tularemia. Contrib. Microbiol. Immunol. **2:**224–230.
21. **Leutenegger, C. M.** January 2001, posting date. The real-time TaqMan PCR

and applications in veterinary medicine. Vet. Sci. Tomorrow **1.** [Online.] http://www.vetscite.org.
22. **Lie, Y. S., and C. J. Petropoulos.** 1998. Advances in quantitative PCR technology: 5′ nuclease assays. Curr. Opin. Biotechnol. **9**:43–48.
23. **Long, G. W., J. J. Oprandy, R. B. Narayanan, A. H. Fortier, K. R. Porter, and C. A. Nacy.** 1993. Detection of *Francisella tularensis* in blood by polymerase chain reaction. J. Clin. Microbiol. **31:**152–154.
24. **Mörner, T., G. Sandstrom, R. Mattsson, and P. O. Nilsson.** 1988. Infections with *Francisella tularensis* biovar *palaearctica* in hares (*Lepus timidus*, *Lepus europaeus*) from Sweden. J. Wildl. Dis. **24:**422–433.
25. **Mörner, T.** 1992. The ecology of tularemia. Rev. Sci. Technol. **11:**1123–1130.
26. **Nano, F. E.** 1988. Identification of a heat-modifiable protein of *Francisella tularensis* and molecular cloning of the encoding gene. Microb. Pathog. **5:**109–119.
27. **Reintjes, R., I. Dedushaj, A. Gjini, T. R. Jorgensen, B. Cotter, A. Lieftucht, F. D'Ancona, D. T. Dennis, M. A. Kosoy, G. Mulliqi-Osmani, R. Grunow, A. Kalaveshi, L. Gashi, and I. Humolli.** 2002. Tularemia outbreak investigation in Kosovo: case control and environmental studies. Emerg. Infect. Dis. **8:**69–73.
28. **Rogutskii, S. V., M. M. Khramtsov, A. V. Avchinikov, O. I. Gar'kavaia, N. V. Larchenkova, A. M. Golovanov, T. A. Parfenova, and L. B. Rudkovskaia.** 1997. An epidemiological investigation of a tularemia outbreak in Smolensk province. Z. Mikrobiol. Epidemiol. Immunobiol. **2:**33–37.
29. **Sjöstedt, A., U. Eriksson, L. Berglund, and A. Tärnvik.** 1997. Detection of *Francisella tularensis* in ulcers of patients with tularemia by PCR. J. Clin. Microbiol. **35**:1045–1048.
30. **Sjöstedt, A., A. Tärnvik, and G. Sandström.** 1991. The T-cell-stimulating 17-kilodalton protein of *Francisella tularensis* LVS is a lipoprotein. Infect. Immun. **59:**3163–3168.
31. **Sjöstedt. A.** Family XVII. *Francisellaceae*, genus I. *Francisella. In* D. J. Brenner (ed.), Bergey's manual of systemic bacteriology, in press. Springer-Verlag, New York, N.Y.
32. **Thomas, R., A. Johansson, B. Neeson, D. Isherwood, A. Sjöstedt, J. Ellis, and R. W. Titball.** 2003. Discrimination of human pathogenic subspecies of *Francisella tularensis* by using restriction fragment length polymorphism. J. Clin. Microbiol. **41:**50–57.
33. **Whipp, M. J., J. M. Davis, G. Lum, J. de Boer, Y. Zhou, S. W. Bearden, J. M. Petersen, M. C. Chu, and G. Hogg.** 2003. Characterization of a *novicida*-like subspecies of *Francisella tularensis* isolated in Australia. J. Med. Microbiol. **52:**839–842.