DETECTION OF FRANCISIELLA TULARENSIS IN INFECTED MAMMALS AND VECTORS USING A PROBE-BASED POLYMERASE CHAIN REACTION

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Abstract. We investigated the use of a TaqMan 5’ nuclease assay (5NA) directed against the Francisella tularensis outer membrane protein (Fop) gene and a polymerase chain reaction–enzyme immunoassay (PCR-EIA) directed against the tul 4 gene for detection of this organism in experimentally infected mice and in field-collected tick vectors. We also evaluated the use of specially formulated filter paper (FTA©) for rapid sample preparation. The 5NA had a detection limit of 1 pg of genomic DNA (<100 colony-forming units) and could be completed within several hours. The PCR-EIA could detect 1 pg of genomic DNA and 10 attograms (ag) (22 copies) of cloned insert, but takes longer to perform. Both assays were genus-specific, and successfully detected F. tularensis in mouse tissues (5NA) and in tick extracts (PCR-EIA). The FTA paper provided inexpensive, rapid, template preparation for the tick extracts, mouse tissues, and DNA obtained from clinical specimens. These probe-based assays have the potential to provide rapid, real-time/high-throughput molecular diagnostics in field situations.

Tularemia is a zoonosis caused by the gram-negative, pleomorphic, nonmotile bacterium Francisella tularensis. Rodents and lagomorphs are principle natural hosts. In the United States, infection is usually acquired from handling animal skins or carcasses, and much less frequently from tick (Dermacentor spp.) or deer fly (Chrysops spp.) bites. It is also possible to acquire the disease from drinking water that has been contaminated with animal feces and urine, or by eating inadequately cooked meat. Francisella tularensis can be infectious via aerosol route, and laboratory-acquired cases are not uncommon. Because of the relative ease with which this agent can be cultivated, and its highly infectious nature, there is some concern that it may be used for biological warfare or bioterrorism. Accordingly, rapid detection of F. tularensis in any situation where its use as a threat agent is suspected would be valuable.

Prior to the development of the polymerase chain reaction (PCR), diagnosis of tularemia was dependent on clinical parameters, immunologic assays, and/or inoculation of fluid or tissue specimens into culture media or laboratory mice. The latter assays obviously require a minimum time period (commonly 24–48 hr) for adequate growth of the organism. In addition, culturing F. tularensis requires appropriate Biosafety 3 level facilities. Historically, laboratory-based outbreaks of tularemia are not uncommon when this agent is handled using inadequate safety protocols. Immunologic assays (agglutination and ELISA) can be confounded by serum cross-reactivity with antigens of other genera of bacteria (Brucella, Yersinia, and Proteus).

The first PCR assay for F. tularensis was reported by Long and others, who used primers specific for the tul 4 membrane protein/T cell epitope to detect Jellison type A and B biovars from the blood of experimentally infected mice. A dot-blot of the PCR products, using an oligonucleotide probe, allowed detection of one colony-forming unit (cfu) in 1 μl of blood. Subsequently, Fupol and others used primers for the Fop A gene, an outer membrane protein, to detect F. tularensis in a murine model. Chinese researchers used a PCR to detect F. tularensis in experimentally infected mice and in aerosols generated via a nebulizer in a military medical sciences 500-liter steel chamber. Sjostedt and others, investigating a large outbreak of tularemia in Sweden, used a PCR to detect F. tularensis in DNA extracted from swabs taken from skin ulcers of patients. An example of the use of a PCR for detection of F. tularensis in clinical specimens from the United States is provided by Dolan and others.

We are interested in developing novel, second-generation, nucleic acid–based technologies for the accurate and timely detection of various pathogens. One technique being evaluated is the 5’ nuclease assay (5NA). This has been used to detect Listeria, human papillomaviruses, Salmonella, hepatitis C virus, Aspergillus, tuberculosis, plague, and orthopoxviruses. The TaqMan® System (Perkin Elmer/Applied Biosystems, Foster City, CA) represents a 5NA in which a reporter dye (6-carboxy-fluorescein [FAM], tetrachloro-6-carboxyfluorescein [TET], 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein [JOE], or VIC, proprietary name; Perkin-Elmer) is covalently linked to the 5’ end of a selected oligonucleotide probe. A quenching dye (6-carboxy-tetramethyl-rhodamine [TAMRA]) is covalently linked to the 3’ end of the same oligonucleotide. The probe can be added to a conventional PCR master mixture.

Fluorescence emissions from the reporter dye moiety are normally quenched by fluorescence resonance energy transfer to the quencher (TAMRA) moiety. However, upon cleavage from the 5’ end of the hybridized probe by the activity of Taq polymerase, the reporter dye fluorescence is no longer quenched. As the amplification reactions proceed, more amplicons become available for probe binding, and consequently the fluorescence signal intensity per cycle increases. The increased signal intensity can be detected and interpreted by the ABI 7700 Model Sequence Detector (Perkin Elmer/Applied Biosystems), a combination thermal cycler, laser, and detection/software system. It uses a computer al-
algorithm to calculate $\Delta R_n$, a parameter measuring fluorescence intensity.\(^6\) Data for individual reactions are graphically displayed as $\Delta R_n$ on the y axis and cycle number on the x axis. A threshold, defined as 10 SD above the mean baseline fluorescence observed in no template controls, from cycles 3 to 15 is also displayed on the graph. A given reaction is considered positive if its $\Delta R_n$ curve exceeds the threshold at the completion of the assay (usually 30–40 cycles).

The value $C_t$ refers to the cycle number at which a given amplification plot crosses the threshold; the lower the $C_t$ value, the more template is present in the sample. Using a series of known standards, it is possible to construct a standard curve by plotting quantity versus the $C_t$ value. A given reaction is considered positive if its $\Delta R_n$ curve exceeds the threshold at the completion of the assay (usually 30–40 cycles).

When a series of known standards are used, it is possible to quantify the amount of template present in a given sample.

Here, we report on the development and use of a 5NA and a PCR-EIA for the detection of \textit{F. tularensis}.

**Materials and Methods**

**Bacterial species and strains.** The bacterial strains and species used, chosen because they represent either possible threat agents, or other intracellular parasites with life histories similar to \textit{Francisella} are shown in Table 1. Some were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and others were obtained from strains and stocks maintained at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) (Fort Detrick, Frederick, MD). Some were obtained from clinical isolates made at the Frederick Memorial Hospital (Frederick, MD) and identified by the MicroScan® (Dade MicroScan, Inc., West Sacramento, CA) protocol. Four cultures of \textit{F. tularensis} were gifts from Dr. David Shoemaker (USAMRIID) and Dr. Ted Hadfield (Walter Reed Army Institute for Research, Washington, DC). These are 88-R-675 and T6755, isolated by Shoemaker from Eastern cottontail rabbits in Illinois; AL91-1623, originally isolated from a human case in Alabama by Dr. Thomas Quan (Centers for Disease Control and Prevention, Fort Collins, CO); and AZ91-1624, also isolated by Dr. Quan, from a human case in Arizona. Genomic DNA for the \textit{F. tularensis} Schu strain was provided by one of the authors (AS).

The \textit{Bartonella henselae} culture was provided by Dr. Russ Regnery (Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, GA), while the \textit{Ehrlichia} spp. ( provisionally designated the human granulocytic ehrlichiosis agent) was provided by Dr. Robert F. Massung (same location) and forwarded by Ellen Stromdahl (Division of Vectorborne Infectious Diseases, Fort Collins, CO) protocol. For the virulent \textit{F. tularensis} strains, aliquots of cultures were treated with reagents from the Invitrogen (Carlsbad, CA) Easy-DNA® kit and DNA was extracted using phenol-chloroform-isoamyl alcohol/ethanol precipitation. For detection limit assays, \textit{F. tularensis} LVS strain genomic DNA was used as template.

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>$C_t$</th>
<th>Species</th>
<th>$C_t$</th>
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<td>Liver from mice (n = 2) infected with \textit{F. tularensis} LVS (mean ± SD)</td>
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<tr>
<td>Spleen from mice (n = 2) infected with \textit{F. tularensis} LVS (mean ± SD)</td>
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<td>Uninfected controls (n = 2) (mean ± SD)</td>
<td>28.0 ± 0</td>
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</tbody>
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* $C_t$ = threshold cycle values as calculated by SDS version 1.8 software (Applied Biosystems). Values <30 indicate a positive reaction.

† HGE = human granulocytic ehrlichiosis.

‡ These samples were analyzed by a 28-cycle, nested PCR. Mean ± SD are shown.
Clinical samples. Clinical samples included DNA extracted from swabs taken from skin ulcers of 8 Swedish patients suspected of being infected with tularemia.2 The DNA had previously been assayed using a PCR that amplified a segment of the F. tularensis 17-kD lipoprotein tul 4 gene.17 The DNA was spotted as 3–5-μl aliquots onto FTA® (Fitz Co., Maple Plain, MN) filter paper along with DNA extracted from the F. tularensis Schu strain. Upon receipt at USAMR-RIID, the samples were processed as described below.

Amplicons for the tul 4 gene fragment from one patient were cycle sequenced using the dye-terminator method and the ABI model 377 fluorescent automated sequencer (Perkin Elmer/Applied Biosystems). Identification of sequenced amplicon was performed by submission to the National Center for Biotechnology Information (Bethesda, MD) BLAST (Basic Local Alignment Search Tool) program.

Mouse tissues. Infection of mice with the F. tularensis LVS strain followed established protocols.19 Mice (BALB/cByJ) were intradermally inoculated with 105 cfu, a dose that results in morbidity (splenomegaly, but no bacteremia or ulceration at the inoculation site), and rarely, mortality. Mice were killed 5 days post-inoculation. Sample processing using the FTA paper followed the manufacturer’s instructions. Briefly, small portions (1 cm x 1 cm) of liver and spleen were either firmly pressed, or the cut surface of the liver was gently blotted, onto the paper. Samples (up to 20 μl) of tail blood were deposited onto the paper with a pipettor. Uninfected mouse tissues and/or uninfected human blood were used as controls. After allowing the paper to dry, disks 3.0 mm in diameter were punched out of the paper and washed three times (5 min per wash) with FTA reagent, followed by three washes for 5 min with TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) and a final soak in 100% cold ethanol for 5 min. The disks were then dried in a 37°C oven and used as template for the PCR.

The DNA extracted from an aliquot of the same bacterial stock (F. tularensis LVS strain ATCC 29684 maintained at the Center for Biologics Evaluation and Research) used to infect the mice was used for dynamic range assays. Genomic DNA was isolated from these bacteria using phenol-chloroform-isooamyl alcohol and ethanol precipitation.14

Tick specimens. Dermacentor reticulatus, Isodes ricinus, and Haemaphysalis concinna adult male and female ticks were collected as part of an ongoing prevalence study conducted in South Moravia and Lower Austria.19 Protocols for tick collection, processing, and evaluation of infection status were performed as previously described.19–21 Briefly, adult tick cohorts, comprised mostly of pooled (range = 5–29, but usually about 10) ticks, sorted by study site, collection date (1985, 1988, 1996–1998), and sex, were homogenized in 2 ml of sterile phosphate-buffered saline (PBS). The extracts were then centrifuged at low speed (70 x g), and the supernatant fraction was inoculated (subcutaneously, 0.25 ml) into the left inguinal region of 4-week old outbred mice. Mice were observed for 10 days following inoculation, and spleens were removed from dead animals, homogenized in sterile PBS, and a portion of the homogenate was spread onto thioglycollate-glucose-blood agar plates. Samples of the bacterial colonies were examined by Gram and Giemsa staining, and assayed for agglutination with rabbit anti-F. tularensis serum. Light microscopy was also performed on smears of spleens removed from dead mice. Accordingly, if tularemia bacteria were cultured from a dead mouse, and observed in spleen smears, then the ticks contributing the inoculum were considered to harbor viable F. tularensis.

The number of F. tularensis infectious units present in the tick extracts was estimated by titrating in laboratory mice. Briefly, tick homogenates were diluted serially up to 106 and each dilution was inoculated into several mice. The 50% lethal dose (LD50) value was calculated for each extract, and its antilogarithm was multiplied by 0.693. On the basis of the Poisson distribution, LD50 infectious titers per tick extract were converted to infectious particles per tick extract and found to range from 6 to 175,300.

For the PCR-EIA, 5-μl aliquots from the tick extracts were deposited onto 3.0 mm FTA TM filter paper disks. After drying, the disks were transported and stored at ambient temperature until processing as described above.

5’ nuclease PCR. We used a 707-basepair (bp) segment of the F. tularensis Fop A gene, Genbank accession number M32059,23 as our target for primers and probes. The outer primer set was forward primer MS1 5’-CAG CTA CAC AAA GCA GTG G-3’ (nucleotides 490–511) and the reverse primer is MAI 5’-CAC CAT TTA CTT AGC ACG C-3’ (nucleotides 1176–1197). The inner primer set is forward primer IS1 5’-CCT CAA GAT AGA ACT GGC 3’ (nucleotides 588–605) and the reverse primer is IAI 5’-GCA CCA ATC ATG TTA GCA CCC G-3’ (nucleotides 970–991). The Taqman® (Perkin Elmer) probe sequence is 5’TET AGG TAG AGG ATT TGC TGG TTT AGG TAMRA-p3’, (nucleotides 731–757), where TET refers to the reporter dye and TAMRA to the quencher dye 6-carboxytetramethylrhodamine attached to the 3’-guanidine residue. A phosphate is also attached to the 3’-thymine residue to prevent extension of bound probe during amplification.

To evaluate the use of FTA paper for assaying clinical specimens, a conventional PCR was performed using primers specific for the F. tularensis tul 4 gene (Genbank accession number M32059), which codes for a 17-kD membrane protein. The primer sequences were forward primer MS2 5’-CCT CAG CTA AAG ATG CTT C-3’ (nucleotides 648–666) and reverse primer MA2 5’-GCA CTG AGA ACC TTC TGG AGC C-3’ (nucleotides 820–841). The same gene was used, albeit with different primers, for the PCR of the clinical specimens in the original study conducted in Sweden.3

Polymerase chain reaction. The TaqMan PCR used 10 pmol of probe, 0.5–1.0 μM (50–100 pmol) of each primer, 6.0 mM MgCl2, 1 unit of AmpliTaq® (Perkin Elmer) polymerase, 1 μl of nucleotide mixture (200 μM of each dNTP), and 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl).

Cycling conditions were 10 min at 95°C (initial denaturation), followed by 15 sec at 95°C and 1 min at 60°C for 40 cycles. For the nested PCR, the cycle number for each round of the PCR was reduced to 28. The amplifications were performed using the Applied Biosystems (Foster City, CA) 7700 Model Sequence Detector in optical tubes and caps. Following the assay, data were analyzed using the Sequence Detection System (Applied Biosystems) software, version 1.0.5, and later, upgrade version 1.6.

For presentation of TaqMan assay graphic data, images of amplification plots were obtained using the SimpleText®
screen capture format. To convert these images into more flexible jpeg and tiff formats, we used a program for Macintosh (Cupertino, CA) computers called GraphicConverter (shareware; Lemke Software, Peine, Germany, http://www.lemkesoft.de/).

When necessary, 10–15 μl of product was subjected to electrophoresis on 1% agarose gels, visualized by staining with ethidium bromide, and viewed using the Stratagene (La Jolla, CA) EagleEye® II still video camera system.

The PCR-EIA's amplifying the tul 4 gene were performed using the MS2/MA2 primer pair with 0.5 μM (50 pmol) of each primer, 1.5 mM MgCl2, 2.5 units of Amplitaq® polymerase, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), and digoxigenin-11 dUTP labeling mix (Boehringer Mannheim, Indianapolis, IN). Following amplification, 10 μl of product was denatured with 20 μl of alkaline lysis solution, then hybridized to a biotin-labeled probe derived from the F. tularensis tul 4 gene (5'-CAA TTG TAA TCT TAC ACT TCC TTG TGG-3', nucleotides 791–817, accession no. M32059) at a concentration of 250 ng/ml in 200 μl of hybridization solution. The probe/amplonc solution was then transferred to a streptavidin-coated microliter plate in duplicate. Following incubation for 60 min at 37°C, the plates were washed 3 times with 100 mM NaCl, pH 7.5, 3% Tween, and 200 μl of horseradish peroxidase–conjugated antibody to digoxigenin (10 mM/m) was added and incubated for 30 min at 37°C. The plates were then washed as before and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution was added. Optical density readings on the plate were recorded at 15, 30, and 60 min at a wavelength of 405 nm (reference filter = 492 nm). A threshold value for positivity was calculated by multiplying the standard deviation of the mean from the 3 no template controls by the T statistic value at 2 degrees of freedom and a 99% confidence interval. Sample values (minus the mean of the no template controls) which exceeded the threshold, were considered positive.

For use as a positive control in the PCR-EIA, an F. tularensis AL91-1623 strain tul 4 amplicon was cloned into the pCR®2.1 vector (Invitrogen). The resultant plasmid preparation used in the assays as standards of 100 pg and 1 fg.

Clinical specimens used in this research were obtained from patients who provided informed consent under the legal and ethical guidelines established by Umea University (Umea, Sweden). The care and use of mice in this study were in accordance with United States Department of Agriculture regulations on the humane use of laboratory animals for research purposes.

RESULTS

Detection limit and specificity. A logarithmic dilution series (100–0.001 ng) of F. tularensis LVS strain genomic DNA was used to determine the detection limit of our Fop probe. When used with primary PCRs, the Fop gene probe detected at least 0.001 ng/ml (Figure 1). This is equivalent to <100 cfu of the original bacterial stock from which the DNA was extracted. When cloned amplicon was used as a template, the detection limit was 10 pg/ml, equivalent to 2 × 106 copies. However, we observed that use of diluted probe and/or genomic DNA target older than several weeks
FIGURE 2. Dynamic range of the Francisella tularensis tul 4 polymerase chain reaction–enzyme immunoassay (PCR-EIA). The bar graph depicts the average optical density (OD) (y axis) at 30 min plotted against serial dilutions of plasmid DNA containing cloned amplicon (x axis). Shown above the bars is an agarose gel image of the corresponding PCR products. NTC = no template controls. ag = attograms.

FIGURE 3. Results of the Francisella tularensis tul 4 gene polymerase chain reaction (PCR) performed on DNA extracted from ulcer swabs taken from Swedish patients with tularemia. The DNA was spotted onto FTA for transport to the United States, where disks punched from the paper were used directly as template for the PCR. Numbers below each lane refer to patients. NTC = no template controls; + = F. tularensis-positive controls. The 100-, 200-, and 300-basepair rungs of the 100-basepair ladder are indicated.

tended to give at least one log less sensitivity with decreased ΔR values.

The specificity of the probe was assayed by using a panel of 21 species and strains of bacteria (Table 1) as blinded templates the for 5′ nuclease PCR. The assay was 100% genus-specific; only F. tularensis spp., and F. philomiragia gave positive results (i.e., they had C values below that of the no template controls and other bacterial genera at the completion of n cycles).

When genomic DNAs obtained from 4 virulent isolates of F. tularensis (88-R-675, T6755, AL91-1623, and AZ91-1624) were assayed, all samples gave satisfactory threshold values (Table 1).

When genomic DNA isolated from the LVS strain of F. tularensis used for the 5NA was assayed by the PCR-EIA, we detected 1 pg/μl. Using the cloned tul 4 amplicon as template, we detected 10 attograms (ag), equivalent to 22 copies (Figure 2). Assuming that each bacterium harbors one copy of the tul 4 gene, then the detection limit is equivalent to 22 bacteria; however, this involves use of ideal template, and in actual clinical or environmental samples the detection limit may be several magnitudes larger. Using a blind panel of 24 bacterial DNAs similar to those listed in Table 1, the PCR-EIA was 100% specific, with probe hybridizing only to Francisella spp. templates.

Clinical specimens. Overseas air-shipment of aliquoted DNA obtained from the ulcers of Swedish tularemia patients presented difficulties, insofar as keeping the samples refrigerated during their period of transport. Samples arrived at room temperature, and degradation of the DNA template could not be ruled out. When assayed with the Fop gene 5′ nuclease assay, none of the 11 samples were positive, although 5 of the samples were reactive with a human β-globin housekeeping gene probe (Perkin Elmer, Foster City, CA). To investigate alternative means of transoceanic shipment of samples, DNA extracted from another 8 clinical specimens, along with purified DNA from the F. tularensis Schu strain, was deposited onto FTA paper in Sweden. The paper was then mailed to our laboratory. Initial analysis with our 5′ nuclease assay indicated that only Schu strain DNA was positive by primary PCR (Table 1): with the nested PCR, one sample was faintly positive. However, when a conventional PCR assay was performed, using primers for the tul 4 gene, all 8 samples were positive (Figure 3). When amplicons from sample number 27 were sequenced, they were found to have >99% homology with the published tul 4 sequence.17

Mouse tissues. The use of FTA paper to prepare templates for PCR assays was also evaluated using organs and blood collected from mice experimentally infected with the F. tularensis LVS (vaccine) strain. The best results were obtained when FTA paper disks, upon which small pieces of liver and spleen had been firmly pressed immediately after dissection, were assayed within 24 hr using a nested PCR for the Fop gene. The nested portion of the assay used the TaqMan probe, and both liver and spleen from infected mice were positive by this assay (Figure 4).

When the primary PCR alone was performed on 15 mice
were both significant at P relation statistic. The Spearman correlation coefficient (r_s) Spearman’s rank correlation statistic and Kendall’s rank correlation statistic. The square of this statistic (d_j^2) between the two ranking scores for each sample was calculated. From 86 different pools of ticks (plus a replicate of n_1005), cohorts of F. tularensis 0.0028. The majority of positive ticks were I. ricinus 1985–1998. **Table 2** Results of the polymerase chain reaction–enzyme immunoassay (PCR-EIA) for the Francisella tularensis tul 4 gene performed on extracts from ticks collected in South Moravia and Lower Austria, 1985–1998. Year Species Mouse bioassay* PCR-EIA† 1998 Dermacentor reticulatus 2/17 4/17 1997 Ixodes ricinus 1/1 1/1 Haemaphysalis concinna 0/1 0/1 1996 D. reticulatus 7/9 4/9 1. ricinus 0/9 29 1995 D. reticulatus 20/20 18/20 1. ricinus 1/1 1/1 1988 D. reticulatus 4/1 1/1 1985 I. ricinus 1/1 1/1 * Number of tick pools lethal to mice/number of tick pools assayed. † Number of tick pools positive by PCR-EIA/number of pools assayed. using FTA papers either spotted with tail blood or simply touched with the cut surface of the dissected liver, only a very faint band was visible for one of the 10 infected mice assayed, and no probe activity was observed for these samples. When liver samples from these mice were subjected to DNA extraction using phenol-chloroform-isooamyl alcohol/ethanol precipitation, and 3-μg aliquots used as template for 5’ nuclease assay, 3 of the 10 mice were positive (i.e., C_t < 40), but their amplification plots were below an ΔRn value of 0.5. The DNA extracted from livers of 5 mice infected with Listeria monocytogenes housed in the same room as the LVS mice were negative (i.e., C_t = 40). When 0.3 μg of DNA was used as template, no positive results were observed. **Tick specimens.** The FTA disks spotted with extracts from 86 different pools of ticks (plus a replicate of n = 10 ticks) were assayed using the tul 4 PCR-EIA because of its superior sensitivity. The majority of positive ticks were D. reticulatus (56 of 73 cohorts, 76%), with 3 (25%) of 12 cohorts of I. ricinus positive, and none of the cohorts of H. concinna positive (Table 2). The background-corrected readings for the positive samples ranged from 0.03 to 2.4 optical density units, with the 100 pg cloned insert positive control set at 2.4; negative samples ranged from 0 to 0.0033, with no template controls at 0 and substrate/conjugate controls at 0.0028. Was there a correlation between the PCR-EIA optical density reading and the concentration of F. tularensis infectious units present for each tick pool extract? A nonparametric statistical test was performed. Briefly, samples were ranked in ascending order, according to each parameter (optical density units and infectious units), and the difference (d_j) between the two ranking scores for each sample was calculated. The square of this statistic (d_j^2) was used to calculate Spearman’s rank correlation statistic and Kendall’s rank correlation statistic. The Spearman correlation coefficient (r_s = 0.709) and the Kendall correlation coefficient (τ = 0.475) were both significant at P < 0.05. These values indicate a positive, but moderate, correlation between optical density units and infectious units. If the original mouse inoculation/lethality bioassay is used as a gold standard, and replicated data for n = 10 ticks is included, then for 86 pools of ticks plus 10 replicates of these pools (a total of 96 samples), 47 true positives, 23 true negatives, 10 false negatives, and 6 false positives were observed. Therefore, the epidemiologic sensitivity of the PCR-EIA was 82% and the specificity was 79%; the positive and negative predictive values were 88% and 79%, respectively. **Discussion** There were two main objectives in designing the assays and protocols. First, we were interested in developing high-throughput, probe-based assays for the detection of F. tularensis nucleic acids in different types of specimens. The 5’ nuclease PCR assay uses real-time detection of fluorogenic probe emissions, while the PCR-EIA involves hybridization of a biotinylated probe with digoxigenin-labeled PCR amplicons. Our ultimate goal is to use these assays in field situations. Second, we were interested in using protocols that would allow safe, inexpensive, and efficacious collection and processing of samples for the PCR. Because of the highly infectious nature of F. tularensis preparations, it was necessary to devise a technique that satisfied safety and security concerns, but allowed us to obtain PCR-quality material without maintaining an expensive, cumbersome cold chain. Accordingly, we investigated the use of a filter paper-based sample preparation method. Published articles on the use of the PCR to detect F. tularensis have varying sensitivities according to the template used: from one bacterium in 5 μl of blood from experimentally infected mice to the genomic DNA equivalent of 100 bacteria. Junhui and others were able to detect 1 cfu in 1 μl of diluent used as template, and found their assay allowed detection of F. tularensis in aerosolized formulations. Using the TaqMan Fop probe, we observed a detection limit of 1 pg/μl for F. tularensis LVS strain genomic DNA. When the number of cfu in the starting bacterial suspension is converted into nanograms of extracted DNA, this quantity is equivalent to <100 cfu. For the tul 4 PCR-EIA, if we assume one copy of the tul 4 gene per bacterium, then the estimated sensitivity is approximately 22 bacteria (when optimum template is used). While use of the reduced-virulence LVS strain of F. tularensis is permissible in Biosafety level 2-equipped laboratories, it would probably not be encountered in the wild, or in bioterrorism situations. Accordingly, we investigated the 5’ nuclease assay for detection of 4 virulent strains of F. tularensis (Table 1). The ability of the probe to recognize these strains indicates it may also have utility in a clinical or veterinary setting, where these organisms would most likely be encountered domestically. Both our 5NA and PCR-EIA showed positive results for F. philomiragia. This organism was formerly classified as Yersinia philomiragia and exhibits a high degree (>98%) of 16S rRNA sequence homology with F. tularensis. While a documented human pathogen, F. philomiragia is isolated mainly from immunocompromised patients, and encountering it in a biological warfare/terrorism scenario is unlikely. In comparing the two assays, it is apparent that the PCR-EIA is more sensitive than the 5’ nuclease assay, and the tul
4 target offers a greater detection limit than the Fop gene. Theoretically, the use of a nested 5' nuclease assay could improve its detection limit, and we found that with mouse tissue–impregnated FTA paper, a nested assay was necessary to detect target DNA. However, the TaqMan assay was designed to be a one-tube, closed system, and the plasticware is not amenable to the sequential opening and closing steps a nested PCR assay demands. Consequently, cross-contamination and false-positive results are difficult to avoid, even with the best of handling procedures.

Because of the relatively sparse numbers of cases of tularemia in the United States, we used experimentally infected mouse tissues, DNA extracted from human cases in Sweden, and tick extracts from the Czech Republic to validate our assay. We initially tested our Fop probe on 11 samples of soluble DNA extracted from clinical specimens obtained from patients with tularemia in Sweden. These samples originated from a study using a PCR of the 16S rRNA and 17-kD lipoprotein to detect F. tularensis in 40 serologically diagnosed patients. The original study observed a positivity rate of 73% for the 40 samples; the investigators noted that the positivity rate might be higher, if not affected by degradation of some DNA samples. Unfortunately, the 11 samples mailed to us spent at least 48 hr at a warehouse before we received them. Therefore, we attempted to improve the stability of template and the ease of intercontinental transport by using another cohort of 8 samples, as well as F. tularensis Schu strain DNA, deposited onto FTA filter paper. For the Fop probe, F. tularensis Schu strain DNA (280–840 ng) yielded exceptionally high ΔRn values (>4.0), but the 8 clinical specimens did not give detectable signals. When tul 4 primers were used in the conventional PCR, faint but definite amplicons were generated for all 8 specimens (Figure 3) and, upon sequencing, were found to be 99% homologous to F. tularensis. The negative results observed with the Fop TaqMan assay, compared with the positive findings with the conventional tul 4 PCR, indicate that the latter gene may be a more amplifiable target for the PCR.

When we used the FTA paper to process samples of infected mouse tissues, we obtained positive 5' nuclease PCR results (Figure 4), but these were dependent on prompt use of paper forcibly impregnated with liver and spleen material, in a nested PCR format. Gently pressing the cut surface of a dissected organ or spotting aliquots of tail blood onto the paper was not sufficient to impregnate the paper with enough bacterial DNA for detection by the 5NA. It should be noted that F. tularensis LVS as a cellular pathogen has a more transient bacteremia, and multiplies to a lesser concentration in the tissues of the host, compared with more virulent strains.

These results, and those of the human specimens, indicate that the 5NA requires a level of template higher than that required for nested PCR, to yield positive results. This may be due to the interaction of fluorogenic probe emissions, or laser illumination of the tube, with the enclosed paper disk. We are currently investigating the use of other filter paper–based systems (Isocode; Schleicher and Schuell, Keene, NH) in an effort to address this issue. It is interesting to note that Junhui and others used a PCR assay to detect tul 4 gene fragments in blood samples from 38 (82.6%) of 46 mice receiving an inoculation of 15 cfu. Fulop and others also investigated the use of mouse tissues as targets for an F. tularensis Fop gene PCR. These investigators evaluated a variety of sample preparation methods for use on blood and spleen specimens, and reported best results with a guanidine thiocyanate/DNA-binding silica protocol, in conjunction with a nested PCR.

We were interested in assaying infected tick specimens for two reasons. First, ticks are vectors of tularemia and in many instances troops may be exposed to them in field situations; accordingly, a rapid and high-throughput test for the presence of the bacteria may be useful in disease surveil-

**Figure 4.** Results of the Francisella tularensis Fop gene 5' nuclease assay performed on FTA paper-prepared organ smears obtained from 2 LVS strain infected mice. Samples from liver and spleen were amplified by a nested polymerase chain reaction with TaqMan probe during the nested portion of the assay. The ΔRn value is plotted on the y axis and the cycle number is plotted on the x axis. Amplification plots A1 and A2 are liver samples and A3 and A4 are spleen samples. Plots A5, A6, and A7 are FTA blanks/no template controls (the positive control was omitted for clarity).
lance of bivouacked personnel. Second, because tularemia is a comparatively rare disease in the United States, and many laboratories are not equipped to handle this agent, obtaining enough naturally infected clinical or veterinary specimens to adequately evaluate our assays was problematic. Ticks provided a reasonably inexpensive and readily processed alternative, and we were able to assay 86 pools of ticks using the PCR-EIA.

We chose to assay the ticks with the more sensitive of our probe-based assays, the *tul* 4 PCR-EIA. The results showed satisfactory sensitivity and specificity (82% and 79%, respectively). Using non-parametric statistics, we calculated a moderate, but significant correlation between the PCR-EIA optical density reading and the infectious units of *F. tularensis* originally present in the tick extracts. We cannot be sure whether some of the PCR-EIA false-positive results were due to cross-reactivity of our primers and probe to the as yet unnamed tick symbiont assigned to the genus *Francisella*; this nonpathogenic bacterium is present in *Derma-
centor* in the United States and is transovarially transmit-
ted.2,23 Because lethal infection in inoculated mice was the criterion for declaring a cohort of ticks as infected, it may be that some of the PCR-EIA false-positive samples contained *F. tularensis*, but these bacteria were not able to cause lethal infections in these immunocompetent mice.

Our PCR-EIA did show false-negative results for 10 disks (9 samples and 1 replicate). We and other investigators (Goldosbrough M, Life Technologies, Gaithersburg, MD, unpublished data) have occasionally observed FTA disks that fail to amplify; this may be one reason why there were false-negative results. Alternatively, the low numbers of infectious units present in the majority of these 10 samples (of the 10, 9 had infectious units from 6 to 10, and 1 sample had a value of 55), as well as an inadvertent freeze-thawing that occurred during freezer failure (Hubalek Z, unpublished data), may explain these results. Obviously, a concurrent PCR for a tick housekeeping gene would be an indicator of the quality of an FTA sample as template; while same-tube multiplexing with differently labeled probes is currently not possible with the PCR-EIA, it is feasible with fluorogenic *TaqMan* probes, although this would add expense to the assay.

In conclusion, we have demonstrated that a 5NA using fluorogenic probes for the *Fop* gene can successfully detect *F. tularensis* in experimentally infected mice. Using the PCR-EIA with a biotinylated probe for the *tul* 4 gene, we can detect *F. tularensis* in naturally infected vectors. And, using a simple, inexpensive, and rapidly performed sample preparation method, FTA paper, we were able to detect *F. tularensis* in DNA from clinical specimens using a conventional PCR and in vectors using a PCR-EIA. The PCR-EIA provides a high level of sensitivity, while the *TaqMan* 5NA allows investigators to perform the assays in a real-time, high-throughput format that obviates the need for agarose gel electrophoresis analysis of PCR products. Accordingly, 5NA assays can be completed more rapidly than conventional PCR assays. Since we first designed and obtained our *Fop* probe, newer recommendations for designing *TaqMan* probes have been released by Perkin Elmer; these include shorter amplicon length (100–150 basepairs), higher numbers of cytosine nucleotides, and other parameters that can serve to improve probe performance. It is therefore reasonable to expect the improvement of the sensitivity of our *TaqMan* assay in the near future. Interestingly, Pusterla and others27 used a *TaqMan* probe specific for bacteria of the *Ehrlichia phagocytophila* genogroup to screen both tick specimens (n = 2,320), and bovine leukocytes (21 samples each from 2 cows) from Switzerland. The assay used a 106-bp fragment of the 16S rRNA gene as a target, and the investigators observed equal sensitivity and specificity compared with a conventional, nested PCR originally performed on these specimens.

The time savings afforded by the use of fluorogenic probe-based PCR assays would obviously be of importance in those situations where timely identification of a possible threat agent is necessary. While the platforms currently used for real-time PCR, such as the ABI Prism® 7700 (Applied Biosystems) are rather bulky and not currently suitable for field use, a newer generation of miniature, analytical thermal cyclers are being developed.29 The utility of such devices for performing a 5NA for orthopoxviruses has already been demonstrated.30 We anticipate that in the near future, such platforms will be increasingly available for use with existing fluorogenic probe-based PCR assays to provide field-oriented capabilities in rapid diagnostics.

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REFERENCES

1. Evans ME, Friedlander AM, 1997. Tularemia. Sidell FR, Tak-
ations, 303–512.
