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# Detection of *Francisella tularensis* in Blood by Polymerase Chain Reaction

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**We developed a polymerase chain reaction-based assay for *Francisella tularensis* which we evaluated by using spiked blood samples and experimentally infected mice. The assay detected both type A and type B *F. tularensis* at levels equivalent to one CFU/μl of spiked blood. Results from polymerase chain reaction-based assay of limiting dilutions of blood from mice infected with the live vaccine strain agreed closely with results from blood culture.**

Tularemia is a clinical syndrome caused by the facultative intracellular bacterium *Francisella tularensis*. There are two recognized biovars of the bacterium distributed over most of the temperate regions of the Northern Hemisphere. *F. tularensis* bv. tularensis (Jellison type A) is found in North America, primarily in the south central United States, and is the more pathogenic of the two biovars. *F. tularensis* type A causes several different disease syndromes depending on the method of exposure. Ulceroglandular infections are the most common type (ca. 80%) and are characterized by fever, swollen lymph nodes, and ulceration at the site of skin penetration, usually through the bite of an infected tick, mosquito, or deer fly; ingestion of *F. tularensis*-contaminated food or water produces oropharyngeal and/or gastrointestinal tularemia; and inhalation of aerosolized bacteria results in the pleuropulmonary form. Regardless of the route of inoculation, bacteria usually invade the regional lymph nodes and are disseminated via the systemic circulation to other organs.

*F. tularensis* bv. palaearctica (Jellison type B) is more widely distributed in nature and is found in Europe, Asia, and North America. It is considered to be less pathogenic than *F. tularensis* bv. tularensis, although studies have identified this biovar as a cause of disease in Finland (14), Japan (7), and the United States (6).

Although *F. tularensis* can be isolated from blood (8, 13), diagnosis by this method is notoriously unreliable. In a recent study in the southwest central United States, bacteria were isolated from only 13.2% of laboratory-confirmed cases (13). Laboratory confirmation is typically made by serodiagnosis (enzyme-linked immunosorbent assay or agglutination) (9, 11). Routine isolation of the organism from whole blood of infected mice is equally difficult, hampering studies of systemic dissemination in the live vaccine strain (LVS) in a mouse model of tularemia (4). Polymerase chain reaction (PCR) has been shown to be an extremely sensitive technique for the detection of infectious agents in blood. We developed a PCR assay specific for *F. tularensis* and report here its use in detecting *F. tularensis* septicemia with experimental models in vitro (LVS-spiked human blood) and in vivo (LVS infection of BALB/c mice).

The LVS of *F. tularensis* bv. palaearctica (ATCC 29684; American Type Culture Collection, Rockville, Md.) was used in these studies and grown in modified Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with ferric $PP_i$ and IsoVitaleX (Becton Dickinson, Cockeysville, Md.) (2). Bacteria were quantified by plating on supplemented Mueller-Hinton agar.

Specific-pathogen-free BALB/c mice (Harlan Sprague Dawley, Frederick, Md.) were inoculated intravenously with $2 \times 10^3$ *F. tularensis* cells (LVS). Blood was collected in EDTA by cardiac puncture 48 h later and pooled.

Animals used in this study were handled in accordance with the *Guide for Laboratory Animal Facilities and Care* (2a).

Primers for PCR are based on a previously published sequence of a T-cell epitope of a *F. tularensis* membrane protein (TUL4) (10). Primer sequences are as follows:

FT393: 5′-ATGGCGAGTGATACTGCTTG
FT642: 5′-GCATCATCAGAGCCACCTAA

PCR product was detected by using an oligonucleotide probe:

FT443: 5′-TCGTAATGTTAGCTGTATCATCATT

The PCR reaction mix contained 70 mM Tris (pH 8.8); 20 mM $NH_4SO_4$; 2.0 mM $MgCl_2$; 1 mM dithiothreitol; 0.1% Triton X-100; 10 μg of gelatin; 200 μM each dGTP, dCTP, and dATP; 400 μm dUTP; 200 pM each primer; and 5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). Amplification was carried out in a Perkin-Elmer DNA Thermal Cycler in 100-μl volumes for 39 cycles under the following conditions: two cycles of denaturation for 1 min at 95°C, annealing for 1 min at 56°C, and extension for 90 s at 72°C. Denaturation temperature was 90°C for the remaining 37 cycles. PCR product was detected on ethidium bromide-stained 1.5%

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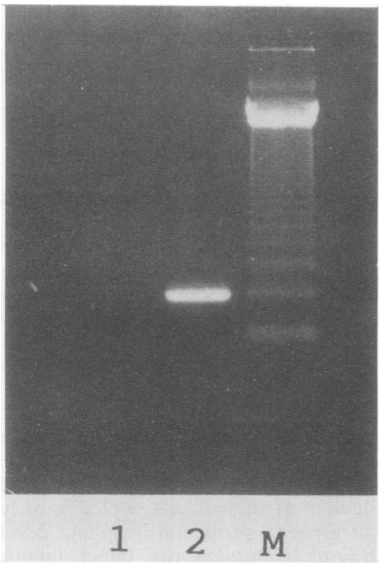


FIG. 1. Results of PCR of *F. tularensis*. Ten microliters of sample from a PCR reaction was electrophoresed on a 1.5% agarose gel. Lane 1, negative control (no DNA); lane 2, sample from PCR of *F. tularensis* (LVS) DNA; M, 123-bp marker.

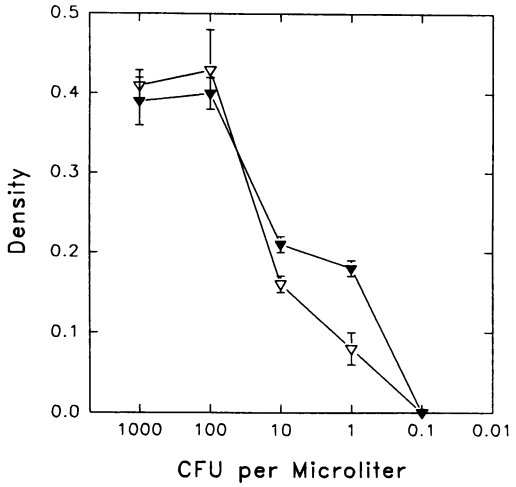


FIG. 2. PCR of a limiting dilution of LVS (▽) and spiked human blood (▼). Tenfold dilutions were tested by PCR in triplicate, and the reactions were quantitated by densitometer. The results are plotted as density ± standard error of the mean.

agarose gels (NuSieve 3:1 Agarose; FMC BioProducts). An oligonucleotide probe (FT443) was tailed with digoxigenin-11-dUTP by using terminal transferase (Boehringer Mannheim, Indianapolis, Ind.). The digoxigenin-labelled probe was used for dot blot analysis of PCR products following the manufacturer's recommendations (Genius system; Boehringer Mannheim). The reaction was quantitated by using a hand-held white light densitometer (model IQ-200; Tobias Associates, Ivyland, Pa.).

PCR of purified DNA resulted in a product whose apparent molecular weight on agarose gel electrophoresis was consistent with the 250-bp predicted size (Fig. 1). Restriction analysis of the product was carried out with the endonucleases *Dra*I, *Hin*dIII, and *Ssp*I, which produced fragments consistent with the sizes predicted from the published sequence (data not shown). *F. tularensis* bv. tularensis (type A; ATCC 6443) shares the TUL4 antigen, and PCR of this strain gave identical results.

Twelve different types of bacteria genetically unrelated to *F. tularensis* were grown on solid media. The colonies were suspended in phosphate-buffered saline (PBS), and 1 μl was added to the PCR reaction mix to test for cross-reactivity. All of the following organisms were negative by PCR (data not shown): *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella* sp. (non-typhi; C1, D), *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Vibrio cholerae*, and *Yersinia enterocolitica*. The PCR was also negative when tested against mouse, human, and *Plasmodium falciparum* DNA.

PCR (5 μl of sample per tube) was performed on *F. tularensis* (LVS) diluted 10-fold in PBS and assayed by dot blot analysis. In addition, aliquots of LVS at each dilution were added to human blood (10-μl bacterial suspension to 90 μl of blood), and total nucleic acid was extracted from each sample. Whole blood was digested at 60°C by the addition of proteinase K and 10× digestion buffer (final concentration: 10 mM Tris, pH 8.0; 0.45% Triton X-100; 0.45% Tween 20; 100 μg of proteinase K per ml). Total nucleic acid was extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol. DNA was dissolved to its original volume in distilled water. PCR of a 5-μl aliquot of total nucleic acid extracted from these spiked blood samples (equivalent to DNA from 5 μl of blood) was tested in PCR, and the product was analyzed by dot blot. PCR product could be detected in tubes containing 1 CFU/μl of the LVS (5 CFU per reaction mix) (Fig. 2) and from blood samples equivalent to 1 CFU/μl of blood (5 CFU per reaction mix).

The LVS, *F. tularensis* bv. palaearctica, while relatively avirulent in humans, produces disease in mice similar to that produced by type A in humans (4). Intravenous inoculation of mice with $10^3$ LVS bacteria produces a lethal infection terminating at approximately 72 h. We examined whole blood and plasma for the presence of bacteria. Blood was centrifuged at 1,500 × *g* for 5 min, and the number of bacteria in plasma was estimated by PCR and by culture. No organisms were detected in 10 μl of plasma by PCR, and inoculation of 1 ml of plasma onto supplemented Mueller-Hinton plates showed 20 CFU/ml of plasma. The negative PCR in this case is probably due to the small volume of plasma tested. The number of bacteria detected by culture is equivalent to 1 CFU/50 μl of plasma. Extraction and concentration of DNA from larger volumes of plasma would increase the chances of detection by PCR.

An estimate of the numbers of organisms per unit volume of whole blood was made by PCR with limiting dilution and compared to culture results. Blood samples were serially diluted 10-fold, and DNA was extracted from a 500-μl aliquot at each dilution. A 1-ml aliquot of blood at each dilution was also plated, and CFU were counted 4 days later. *F. tularensis* could be detected by PCR at a dilution equivalent to 0.1 μl of blood, corresponding to a concentration in the original sample of from $10^3$ to $10^4$ organisms per ml of blood. This is consistent with the culture estimate of $2 \times 10^3$ CFU/ml. A somewhat higher estimate was obtained by limiting dilution of a DNA extract prepared directly from an aliquot of whole blood. When twofold dilutions were made from these extracts, PCR detected *F. tularensis* at a dilution corresponding to 0.025 μl of blood ($2 \times 10^4$ to $4 \times 10^4$

organisms per ml of blood). The difference between these two methods of estimation may lie in the fact that *F. tularensis* is a facultative intracellular bacterium and is probably associated with leukocytes. While little work has been done on the relationship between *F. tularensis* and monocytes (12), it has been shown that macrophages of the peripheral lymph nodes of mice infected with *F. tularensis* (strain SCHU S4) contain numerous bacteria (5), and *F. tularensis* can be grown in rodent macrophages in vitro (1, 3). PCR and culture of diluted blood provide an estimate of the number of infected cells. An infected cell, while it contains numerous bacteria, is diluted and cultured and appears on PCR as a single CFU. The estimate derived from dilution of an extract of whole blood is a function of the number of organisms per unit volume of blood. Lysis and extraction of infected, circulating leukocytes release numerous bacteria, each of which is subsequently diluted and detected. Estimation of the number of *F. tularensis* cells by PCR of whole-blood DNA would necessarily result in a higher estimate than culture alone or PCR of dilutions of whole blood.

The PCR assay for *F. tularensis* is a sensitive method for the detection of this pathogen in blood. Tularemia is problematic to diagnose in the acute phase of the disease, since the organism is difficult and very slow to culture. Under ideal laboratory conditions in which special culture media were available and in which a rough concentration of *F. tularensis* was known, we were able to demonstrate, by culture, significant numbers of bacteria in whole blood and few pathogens in plasma. In clinical situations, however, the conditions for isolation of *F. tularensis* are far from ideal. Culture of this organism from blood samples of known cases of tularemia is less than 20%. The semiquantitative results we obtained by using limiting dilutions of blood show that DNA amplification is comparable to blood culture in its ability to detect the organism. Dilution from a whole-blood extract gave a higher estimated count of bacteria and appears to be more sensitive. The assay was able to detect a single bacterium in a 5-μl aliquot of spiked human blood (Fig. 2). Thus PCR was equally as sensitive as culture in detecting *F. tularensis* under ideal conditions. PCR can be expected to be considerably more reliable and specific in clinical situations.

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