Detection of *Francisella tularensis* in Ulcers of Patients with Tularemia by PCR

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The diagnosis of human cases of tularemia is usually confirmed by the demonstration of an antibody response to *Francisella tularensis*, which occurs about 2 weeks after the onset of disease. Due to a high risk of infection in the laboratory, cultivation of the causative agent tends to be avoided. During an outbreak in Sweden, the use of PCR for diagnosing the ulceroglandular form of tularemia was evaluated. Extraction and preparation of *F. tularensis* DNA from swab samples from the wounds of patients with tularemia involved the use of the nuclease inhibitor guanidine thiocyanate. The DNA was detected by multiplex PCR targeting the 16S rRNA gene and a 17-kDa lipoprotein gene of *F. tularensis*. In 29 of 40 (73%) patients with serologically confirmed tularemia, *F. tularensis* DNA was successfully amplified. Considering the limitations of current diagnostic procedures, PCR may become useful for the early diagnosis of tularemia.

Francisella tularensis, the causative agent of tularemia, is a facultative, intracellular, gram-negative bacterium. There are two main biovars of the species. *F. tularensis* biovar tularensis (Jellison type A) is found in North America and is associated with rabbits and ticks (3). It usually causes fulminant disease, and before the introduction of antibiotics, the mortality rate was ~10%. *F. tularensis* biovar palaeartica (type B) occurs in Asia and Europe and to some extent also in North America. It is associated with rodents and hares, and in Europe, mosquitoes are the predominant vector (12). An association with water voles and other water-living mammals is also indicated. Compared to *F. tularensis* type A, type B causes less severe disease, with a mortality rate of <1% in the preantibiotic era.

Depending on the route of entry, tularemia occurs in two major clinical forms (16). Inhalation of contaminated dust results in respiratory tularemia, whereas arthropod transmission or direct contact with an infected animal leads to the ulceroglandular form. Ulceroglandular tularemia presents with a vesicular or papulovesicular lesion at the site of bacterial entry. The skin lesion is often slight and is usually not a reason to seek medical attention. Predominant symptoms are regional lymph node enlargement, malaise, and fever.

The laboratory diagnosis of tularemia relies mainly on serology. Irrespective of the use of agglutination techniques or enzyme immunoassay (EIA), antibodies are usually not detected until the end of the second week of disease (10). Culture techniques tend to be avoided, primarily because of a high risk of infection in the laboratory. Besides, *F. tularensis* is fastidious and relatively slow growing. Rapid techniques for the specific demonstration of the organism, such as immunofluorescence staining, RNA hybridization, and assay of *F. tularensis* antigen in urine (6, 9, 17), have been tried but are not generally included as tests for routine diagnostics.

PCR has so far not been applied to the diagnosis of human tularemia. In experimental work, it has been used for the sensitive and specific detection of *F. tularensis* in blood from infected mice (7, 11). In the study described here we tested by

PCR specimens obtained from the wounds of patients in the acute phase of tularemia. According to the results, the technique is a promising alternative for the diagnosis of ulceroglandular tularemia.

MATERIALS AND METHODS

Bacteria. *F. tularensis* LVS was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. Ten different species of other bacterial genera were obtained from the American Type Culture Collection (ATCC): *Bacillus subtilis* ATCC 6633, *Coxiella burnetii* ATCC VR-616, *Escherichia coli* ATCC e23716, *Legionella pneumophila* ATCC 33152, *Listeria monocytogenes* ATCC 19111, *Pseudomonas aeruginosa* ATCC 14502, *Salmonella typhimurium* ATCC 19585, *Staphylococcus aureus* ATCC 6538, *Yersinia pestis* ATCC 23053, and *Yersinia enterocolitica* ATCC 9610. Moreover, one strain of each of the following species was collected in a clinical laboratory: *Clostridium sporogenes*, *Enterococcus faecalis*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and streptococci belonging to Lancefield's serological groups B and C.

Patient samples. In August and September 1995, tularemia occurred in two areas of central Sweden, along the Västerdalälven River and the Ljusnan River, respectively. Through general practitioners, wound and blood samples were obtained from patients seeking advice for suspected ulceroglandular tularemia. Fifty patients (age range, 2 to 81 years; mean age, 44 years; 28 females; 22 males) were included; none of the patients had received antibiotic treatment before the day of the visit. Informed consent was obtained from all patients. The time interval from the onset of disease to the visit was 1 to 14 days (mostly 3 to 7 days). Material was collected from the ulcer by use of a cotton swab, which was transported for 1 to 4 days at ambient temperature in a sterile tube containing 0.3 ml of saline. From each patient, blood samples were drawn during the acute phase and 3 to 5 weeks after the onset of disease for assay for *F. tularensis*-specific antibodies.

Swab samples were similarly obtained from the wounds of out- and inpatients of the Department of Infectious Diseases, University Hospital of Umeå, Umeå, Sweden. Tularemia did not occur in the region of the hospital at the time of sampling, and the patients had no signs of tularemia. By routine culture at the Clinical Hospital Laboratory, 49 samples yielded 75 bacterial strains representing 16 species of gram-positive cocci and gram-negative rods.

Culture diagnostics of tularemia. Cotton swabs were inoculated onto modified Thayer-Martin agar plates (13), and the plates were incubated at 37°C in 5% CO₂ for 6 days. Growth of *F. tularensis* was confirmed by the slide agglutination test with a commercial antiserum (Difco Laboratories, Augsburg, Germany), biochemical analysis, and DNA amplification by PCR.

EIA. For EIA, microplates were coated with a carbohydrate-protein complex of *F. tularensis* (13). Serum samples diluted twofold from 1/16 were assayed for immunoglobulin G (IgG) and IgM antibodies. The upper limit of the reference range for IgG and IgM titers was 64. A fourfold increase in titer or a titer of ≥256 was considered to confirm the diagnosis.

Preparation of DNA in patient samples. The transport tube containing the cotton swab was subjected to freezing-thawing, a method previously shown to efficiently lyse *F. tularensis* (4). After thawing, the tube was vigorously vortexed and a 30-μl aliquot was used for each assay. Four different protocols for treatment of the aliquots prior to the PCR analysis were assessed. Method A involved

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no treatment at all. Method B was based on a commercially available kit for preparation of DNA from whole blood and other body fluids (QIAamp blood kit; Qiagen, Hilden, Germany); for method B we followed the instructions of the manufacturer. By method C, the 30- μ l aliquot was mixed with 0.5 ml of a buffer containing 1% (wt/vol) sodium dodecyl sulfate and 1 mg of proteinase K per ml. After phenol-chloroform-isoamyl alcohol (12:12:1) extraction and subsequent ethanol precipitation of DNA, ether treatment was performed to remove residual phenol. Method D was a modification (8) of a previously described method (1) based on the binding of DNA to uniform glass beads (Glass milk; Bio 101, La Jolla, Calif.) in the presence of the chaotropic nuclease inhibitor guanidine isothiocyanate (Sigma, St. Louis, Mo.). When the samples were prepared by method D, they could be stored for at least 1 month without the loss of amplifiable DNA. For methods B, C, and D, the prepared DNA was resuspended in 20 μ l of Tris-EDTA.

PCR. Samples of 5 μ l prepared by method A, B, C, or D or 10^6 bacteria of a given strain suspended in 5 μ l of saline were subjected to PCR with primers specific for the sequences of the 16S rRNA gene and a gene encoding a 17-kDa lipoprotein of *F. tularensis* LVS (5, 13). The 16S rRNA gene primers, designated FT5 and FT11 and yielding a 1.1-kb DNA fragment, are specific to strains belonging to the genus *Francisella* (5). The 17-kDa lipoprotein gene primers, denoted TUL4-435 (5'-GCT GTA TCA TCA TTT AAT AAA CTG CTG) and TUL4-863 (5'-TTG GGA AGC TTG TAT CAT GGC ACT), yielded a 0.4-kb fragment. Amplification with the two primer pairs was performed simultaneously. Samples were added to a PCR mixture containing (at a final concentration) 200 μ M (each) deoxynucleoside triphosphate mixture, *Taq* reaction buffer (Advanced Biotechnologies, London, United Kingdom), 0.6 μ M (each) primer, and 3 mM $MgCl_2$ in a total reaction volume of 25 μ l. The tubes were heated to 80°C before the addition of 1 U of thermostable *Taq* polymerase (Advanced Biotechnologies). The reaction mixtures were subjected to 30 cycles of amplification in a DNA thermal cycler (model 4800; Perkin-Elmer, Norwalk, Conn.). An amplification cycle consisted of denaturation for 30 s at 94°C, primer annealing to the template at 65°C for 1 min, and primer extension at 72°C for 1 min. When the amplification was extended to 35 cycles, no additional product was visible. Separately, samples were also subjected to amplification with primers specific to introns of the human β -actin gene. The primer sequences were 5'-TGG GCC TCG TCG CCC ACA TA and 5'-ATG GAT GAT GAT ATC GCG GCG.

The presence of DNA in samples containing bacteria of defined species was confirmed by amplification of a fragment of the 16S rRNA gene by using two previously described universal primers targeting positions 9 to 27 and 984 to 962, respectively (5).

After amplification, 5 μ l of each reaction mixture was subjected to electrophoresis in a 2% agarose gel, and the amplified gene products were visualized with UV light after ethidium bromide staining. Samples containing saline were run together with each set of patient samples. None of these control samples was PCR positive.

Southern blot analysis. To confirm the identity of the 17-kDa lipoprotein gene product, Southern blotting was performed. A fragment encompassing nucleotides 614 to 813 of the gene (14) was used as the probe. The probe was labeled by random priming by incorporating digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany), according to the instructions of the manufacturer. To prepare the blots, PCR products were transferred from agarose gels onto nylon filters (Boehringer Mannheim). Prehybridization and hybridization were performed as described previously (15) in an aqueous solution at 68°C. For visualization of the reaction, antidigoxigenin alkaline phosphatase-conjugated Fab fragments (Boehringer Mannheim) were used. To detect alkaline phosphatase activity, the luminescent substrate CSPD (Boehringer Mannheim) was added, and the filters were exposed to X-ray film for 10 to 15 min.

RESULTS

Comparison of methods for preparation of DNA. Samples from 10 randomly selected patients were used to compare four different methods of extraction and preparation of DNA. Methods A, B, C, and D yielded successful amplifications from none, one, three, and eight of these samples, respectively. A

FIG. 1. Analysis by PCR of samples containing various numbers of *F. tularensis* LVS organisms. Lanes 1 to 6, amplicons from DNA extracted from 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , and 10^0 bacterial cells, respectively. The 1.1-kb fragment represents the amplification product of the 16S rRNA gene and the 0.4-kb fragment of the 17-kDa lipoprotein gene. The 1.0- and 0.5-kb fragments of the molecular size markers are indicated on the left.

reason for the more successful preservation of DNA by the guanidine isothiocyanate extraction of method D may be that the buffer inhibited the nuclease activity present in the samples. The method allowed for the detection of 10^2 *F. tularensis* LVS organisms, as determined by visualization of the two amplicons on an agarose gel (Fig. 1). No DNA was amplified from samples containing 10^6 organisms of any of 16 control strains representing various clinically relevant species. The presence of DNA in the culture lysates was confirmed by amplification with universal primers of a 970-bp fragment of the 16S ribosomal DNA. In the subsequent analyses, method D was used.

Patient analysis. Of 50 patients seeking medical attention during the tularemia outbreak, serology was confirmatory for 40 patients and negative for 10 patients. By culture, 10 of 50 wound swab samples showed growth of *F. tularensis*. These 10 samples all originated from patients who were serologically confirmed to have tularemia.

PCR was performed with wound swab samples from all 50 patients. Of those 40 subjects who were serologically confirmed to have tularemia, successful amplification of the 17-kDa lipoprotein gene of *F. tularensis* was found for 29 (73%) of the subjects and successful amplification of the 16S rRNA gene was found for 20 (50%) of the subjects (Table 1). All the latter 20 patients were among those in whose samples the 17-kDa lipoprotein gene was identified. Results for six samples with and six samples without demonstrable amplicons are presented in Fig. 2. The identity of the amplified 17-kDa lipoprotein gene was consistently confirmed by Southern blot hybridization (Fig. 2). Among those 10 subjects who were negative by serology, *F.*

TABLE 1. Detection of *F. tularensis*-specific DNA in wound samples by PCR

Clinical condition	No. of samples yielding amplified DNA/total no. of samples (%) after amplification of gene of:		
	β -Actin	17-kDa lipoprotein	16S rRNA
Tularemia, serologically confirmed	32/40 (80)	29/40 (73)	20/40 (50)
Tularemia suspected, serologically negative ^a	8/10 (80)	1/10 (10)	1/10 (10)
Diagnosis other than tularemia	43/49 (88)	1/49 (2)	0/49 (0)

^a Sera of all patients lacked antibodies to *F. tularensis* when the patients' sera were tested in the acute phase of disease and 3 to 5 weeks after the onset of disease.

FIG. 2. Analysis by PCR of wound swab samples from tularemia patients. Lanes 1 to 12, samples from 12 individuals with serologically confirmed ulceroglandular tularemia, respectively. The samples analyzed in lanes 1 to 6 were considered positive. (A) Ethidium bromide-stained gels after electrophoresis of the PCR products. The 1.1-kb fragment represents the amplification product of the *F. tularensis* 16S rRNA gene and the 0.4-kb fragment of its 17-kDa lipoprotein gene. (B) Blotting of the same PCR products onto nylon filters and hybridization with a probe specific to the 17-kDa lipoprotein gene. The 1.0- and 0.5-kb fragments of the molecular size markers are indicated on the left.

tularensis DNA was detectable in 1 subject. In this patient, both *F. tularensis* genes were detected and the patient showed a clinical picture typical of tularemia.

When the time interval from the onset of disease to sampling was less than 4 days, 5 to 8 days, and 9 to 14 days, *F. tularensis* DNA was demonstrable in 13 of 18, 10 of 13, and 4 of 5 patients serologically confirmed to have tularemia, respectively. When samples arrived after 1, 2, 3, and 4 days of transportation, the DNA was successfully amplified from samples from 12 of 13, 7 of 12, 8 of 10, and 2 of 4 subjects, respectively. Thus, within the time intervals studied, the duration of the disease and the time of transportation did not seem to decisively affect the outcome of the analysis.

To check for the preservation of DNA in the wound swab samples, amplification of the human β -actin gene was performed. The β -actin gene was detected in samples from 40 of 50 patients with confirmed or suspected tularemia (Table 1). Of 11 patients diagnosed with tularemia by serology but lacking demonstrable *F. tularensis* DNA, seven subjects were also negative for the β -actin gene. The failure to detect *F. tularensis* DNA and β -actin DNA in these samples may have been due to degradation during transport or inhibition of DNA amplification.

When 49 specimens from patients without any clinical evidence of tularemia and living outside the region of the outbreak were subjected to PCR, the 16S rRNA gene of *F. tularensis* was not at all demonstrable (Table 1). In one specimen, the 17-kDa lipoprotein gene was detected. No bacteria were isolated from the ulcer of this patient. The β -actin gene was amplified from 43 of the 49 specimens.

DISCUSSION

In the present study, wound swab samples were taken without regard to the duration of disease and were sent to the laboratory at ambient temperature, with a transport time of 1 to 4 days. Under these circumstances, PCR confirmed the diagnosis for 73% of patients with serologically confirmed tularemia.

The two primer pairs used in the PCR allowed for a highly specific DNA amplification. The 16S rRNA gene primers have previously been found to enable specific amplification of *Francisella* DNA, despite the conserved nature of the gene (5). The 17-kDa lipoprotein, a major membrane protein of *F. tularensis* LVS, and the encoding gene (14) show no significant similarity to prokaryotic or eukaryotic sequences deposited in current data banks. Importantly, though, the encoding gene is con-

served among various strains of the species, including highly virulent type A strains isolated in North America (15).

Primers based on the 17-kDa lipoprotein gene allowed for a more effective amplification of DNA than did primers based on the 16S rRNA gene (Table 1). This might be explained by a difference in the melting temperature (T_m) between the two primer pairs. Due to the conserved nature of the 16S rRNA gene sequences, annealing had to be performed at a temperature close to the T_m of the primers in order to ensure sufficient specificity (5). The annealing temperature used (65°C) was several degrees lower than the T_m of the 17-kDa lipoprotein gene primers, resulting in a more efficient amplification of this gene.

In most clinical laboratories the diagnosis of tularemia is usually confirmed by the demonstration of an antibody response to *F. tularensis*. Normally, this does not allow for a definite diagnosis within the first 2 weeks of disease. Therefore, techniques for the early diagnosis of tularemia are desirable. However, culture techniques are afflicted with safety problems, and unvaccinated staff working with the organism usually acquire tularemia (2). Handling of clinical specimens for PCR is probably less risky, as indicated by the lack of spread of tularemia among humans. We showed here that PCR can be successfully applied for the detection of *F. tularensis* in samples from skin lesions of *F. tularensis*-infected humans. Previously, the technique has been used to detect the organism in blood and organs from experimentally infected mice (7, 11). The results of those studies and experience from work with other organisms (8) indicate that the PCR can also be useful for the identification of *F. tularensis* in other types of clinical specimens from humans.

In conclusion, the results of this study indicate that *F. tularensis* DNA can be detected by PCR in a majority of patients with ulceroglandular tularemia and, possibly, that the sensitivity may be further increased by trying to reduce the degradation of DNA during the transport of specimens. Previously applied techniques for the early diagnosis of tularemia have not become included in routine work. Compared to those techniques, PCR is more versatile and should be easily maintained in laboratories in regions where tularemia is endemic.

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