Comparative Analysis of PCR versus Culture for Diagnosis of Ulceroglandular Tularemia

ANDERS JOHANSSON,1 LENNART BERGLUND,2 ULLA ERIKSSON,3 INGELA GÖRANSSON,3 RALFH WOLLIN,4 MATS FORSMAN,3 ARNE TÄRNVIK,1 AND ANDERS SJÖSTEDT3,5*

Department of Clinical Microbiology, Division of Infectious Diseases,1 and Department of Clinical Microbiology, Division of Bacteriology,5 Umeå University, S-901 85 Umeå, Primary Health Care Center, S-827 25 Ljusdal, Department of Microbiology, Defence Research Establishment, S-901 82 Umeå,3 and Department of Bacteriology, Swedish Institute of Infectious Disease Control, S-105 21 Stockholm,4 Sweden

Received 4 May 1999/Accepted 30 September 1999

PCR and culture were comparatively evaluated for their abilities to demonstrate Francisella tularensis in wound specimens from tularemia patients during an outbreak in Sweden in 1998. For transport of the specimens used for PCR, a buffer solution containing a nuclease inhibitor was used, and for transport of the specimens used for culture, a commercial transport system was selected after experimental comparison of various systems. Of 40 patients with culture- and/or serology-verified ulceroglandular tularemia, PCR detected F. tularensis DNA in 30 (75%) patients, whereas culture detected bacterial growth in 25 (62%) patients. Compared to data from a previous study, the present inclusion of a nuclease inhibitor in the transport medium did not improve the sensitivity of the PCR, whereas the sensitivity of the culture procedure was significantly increased by selection of the system used for transport. Among eight patients with clinically suspected tularemia but with negative serology and culture, specimens from four patients showed detectable DNA. In three of these patients the diagnosis was verified by the demonstration of an F. tularensis-specific T-cell response in vitro. In conclusion, PCR was more sensitive than culture for demonstration of F. tularensis in wound specimens. Besides, we showed that tularemia may proceed without development of serum antibodies, and in these patients, PCR may be of special importance for verification of the diagnosis.

Francisella tularensis is endemic throughout the Northern Hemisphere and causes outbreaks of tularemia in various mammals including rodents, lagomorphs, and humans. In humans, the clinical presentation depends on the route of entrance of the bacteria. The ulceroglandular form of the disease is acquired either by direct contact with an infected animal or by vector transmission. Patients typically present with fever, enlarged and tender lymph nodes, and an ulcer at the place of entry (4, 5). The skin lesion is usually slight, and the appearance of an infected insect bite need not actually differ from that of a noninfected bite.

F. tularensis is highly virulent, and in diagnostic work involving culture procedures, nonvaccinated staff are at high risk of acquiring clinical disease (3). In most clinical laboratories, serology is the only diagnostic test used. Exceptions are patients with septicemia in whom tularemia may be diagnosed more or less accidentally by growth of F. tularensis in blood cultures. Consequently, some work is performed to optimize the blood culture procedure for F. tularensis (11, 12). There is, however, little experience with the use of culture of wound specimens in clinical diagnostic work. The optimal way of sampling and the optimal handling of wound specimens during transport are unknown, and therefore, the potential efficacy of the procedure is also unknown.

Rapid methods for the identification of F. tularensis such as the immunofluorescence assay and the enzyme-linked immunosorbent assay for the detection of antigen and the RNA hybridization assay have been tried but have so far not been included in routine diagnostics (9; M. Forsman, K. Kuoppa, A. Sjöstedt, and A. Tärnvik, Letter, Eur. J. Clin. Microbiol. Infect. Dis. 9:784–785, 1990; A. Tärnvik, S. Löfgren, L. Öhlund, and G. Sandström, Letter, Eur. J. Clin. Microbiol. 6:318–319, 1987). We recently introduced PCR for the demonstration of F. tularensis in wound specimens (16). The method showed a high degree of specificity, and by use of spiked samples, a sensitivity of 105 bacteria was demonstrated. In an outbreak of ulceroglandular tularemia in Sweden in 1995, F. tularensis DNA was successfully amplified from wound specimens from 29 of 40 patients. In that study, specimens were sent in saline. When various methods for treatment of the specimens prior to the PCR analysis were compared, the best success was achieved by use of a protocol that included the nuclease inhibitor guanidine thiocyanate as the lysis agent.

The use of PCR for the direct diagnosis of ulceroglandular tularemia is thus highly promising, and more work on the conditions that might influence the assay seems to be warranted. By inclusion of a nuclease inhibitor in the transport medium, we addressed in the present study the question of whether degradation during transport might adversely affect the outcome of PCR. As regards culture, we compared various transport systems by experimental inoculation and storage. When in 1998 a new outbreak of ulceroglandular tularemia occurred in the same geographic region as the 1995 outbreak (16), we compared the sensitivity of PCR with that of culture.

MATERIALS AND METHODS

Bacteria. F. tularensis live vaccine strain (LVS) (ATCC 29684) was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. A virulent strain of F. tularensis (strain FSC200) was isolated from a patient during the 1998 outbreak of ulceroglandular tularemia in central Sweden. F. tularensis strains were handled under biosafety level 3 laboratory conditions. Pseudomonas sp. strain CF600 was kindly provided by Victoria Shingler, Umeå University, Umeå, Sweden.
TABLE 1. Survival of F. tularensis in transport media

| Strain          | Transport medium | Survival (no. of bacteria) at the following times postinoculation:
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>FSC200&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Saline</td>
<td>9.4 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Modified Stuart</td>
<td>4.1 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Copan</td>
<td>6.2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATCC 29684&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Saline</td>
<td>8.6 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Modified Stuart</td>
<td>4.5 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Copan</td>
<td>3.8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>The bacteria were inoculated in transport medium and were stored at room temperature for the indicated period of time. Each value represents the mean number of bacteria in duplicate samples.

<sup>b</sup>The inoculum was ~5.5 x 10<sup>7</sup>. The strain was isolated from a wound sample of a patient during the 1998 outbreak along the Ljusnarn River in Sweden.

<sup>c</sup>The inoculum was ~1.5 x 10<sup>8</sup>. The strain was the live vaccine strain F. tularensis LVS.

Bacterial transport systems. Four different transport systems were compared. First, a modified variant of the nonnutrient Stuart medium intended for transport of gonococci (13) has been used for several decades for transport of bacterial specimens and was used during the 1995 outbreak of ulceroglandular tularemia (16). Second, a modified Thayer-Martin medium (14) routinely used in the laboratory for culture of F. tularensis was evaluated. It was supplemented with 7.5 mg of colistin (Sigma, St. Louis, Mo.) per liter, 2.5 mg of amphotericin B (Bristol-Myers Squibb, New Brunswick, N.J.) per liter, 0.5 mg of lincomycin (Upjohn, Kalamazoo, Mich.) per liter, 4 mg of trimethoprim (Duchefa, Haarlem, The Netherlands) per liter, and 10 mg of ampicillin (Duchefa) per liter as described previously (2). Sterile saline (3-ml volumes) was also evaluated as a transport medium. Sterile cotton-tipped wood applicators (Selseftrate AB, Spånga, Sweden) were used for inoculation of F. tularensis in all these media. Finally, we assessed a commercial transport system designed for transport of common anaerobic and aerobic pathogens (7, 10); J. L. Perry, D. R. Ballou, and Spånga, Sweden) were used for inoculation of F. tularensis LVS and to the respective target was amplified separately. Aliquots were removed after cycles 10, 15, 20, 25, and 30, and the quantity of each amplicon was plotted in relation to the number of cycles. In three experiments, no significant differences in the slope coefficients for the linear portions of two curves were found. The amount of 16S rDNA on ethidium bromide-stained agarose gels was estimated by identifying the dilution of the competitor fragment that after amplification showed the same intensity as the ampiclon of the sample DNA after correction for the different lengths of the two fragments.

Assay of proliferative T lymphocyte response. Peripheral blood mononuclear cells were prepared from heparinized blood by centrifugation on a Ficoll-Metrizoate gradient (Lymphoprep; NYCOMED AS, Oslo, Norway), and cultures were established. Each culture (200 µl) contained 3 x 10<sup>5</sup> mononuclear cells. The culture medium consisted of RPMI-HEPES (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 15% pooled human serum, 100 µg of gentamicin per ml, and 2 mM L-glutamine. Concanavalin A (10 µg/ml) or heat-killed F. tularensis (10<sup>5</sup> cells/ml) was used as a stimulating agent. In preliminary experiments, these concentrations were found to be optimal. To estimate the proliferative response, five replicate cultures were incubated at 37°C for 4 days, pulsed for 6 h with 1 µCi of [³H]thymidine, and harvested. The proliferative indices were calculated by dividing the mean for cultures with and without stimulating agent. Concanavalin A-stimulated cell cultures all showed proliferative indices of >.

Recovery of F. tularensis in various transport systems. To compare the various transport media, a virulent strain (strain FSC200) of F. tularensis, which was isolated from a Swedish patient in 1998 and passed only once, and live vaccine strain F. tularensis LVS (ATCC 29684) were used. Bacteria were grown on modified Thayer-Martin medium and were suspended in saline at a density of 10<sup>7</sup> cells/ml. Portions of 0.1 ml were collected from the suspension by inserting a tipped applicator for 5 s. The applicator was stored in a transport medium at room temperature for a given period of time, removed, and rotated vigorously for 60 s in 200 µl of saline for determination of viable counts. For PCR analysis, a 50-µl portion was transferred to a tube containing 1.0 ml of the guanidine isothiocyanate-containing buffer. The DNA of the lysed bacteria was prepared as described previously (8) and was subjected to PCR with TUL4-435 and TUL4-863 as primers. Applicators inoculated in saline were used as negative controls. After amplification, a 5-µl portion of each reaction mixture was subjected to electrophoresis in a 2% agarose gel. The amplified gene products were visualized with UV light after ethidium bromide staining.
-actin DNA was not detected, indicating that these specimens days after inoculation of 1.5
a significant reduction in bacterial numbers within 4 h, and 2
the strain used, storage in saline or Stuart medium resulted in
plifiable from all 30 (75%) of these 40 patients (Table 2).
F. tularensis
after 7 days of storage showed the presence of
36/40 (90) 30/40 (75) 25/40 (62) 30/31 (97)
F. tularensis
tients the diagnosis was confirmed by serology and/or culture.
were sent in Amies agar with charcoal for culture and in gua-
were more preservative. When the
Survival of F. tularensis in various transport media. The survival of F. tularensis after storage in modified Stuart me-
medium, saline, or Amies agar with charcoal was tested for two
F. tularensis, a recent patient isolate, strain FSC200, and the live vaccine strain, strain LVS. Irrespective of the strain used, storage in saline or Stuart medium resulted in
F. tularensis
DNA was amplified.

**RESULTS**

Survival of F. tularensis in various transport media. The survival of F. tularensis after storage in modified Stuart me-
dium, saline, or Amies agar with charcoal was tested for two
different strains of F. tularensis, a recent patient isolate, strain FSC200, and the live vaccine strain, strain LVS. Irrespective of the strain used, storage in saline or Stuart medium resulted in
a significant reduction in bacterial numbers within 4 h, and 2
days after inoculation of 1.5 × 10^6 or 5.5 × 10^6 organisms, no
viable bacteria were demonstrated (Table 1). In contrast,
Amies agar with charcoal (Copan) preserved bacterial viability
for 1 week, and after 7 days bacterial numbers had decreased
>1 log_{10}. Irrespective of the medium, PCR analysis performed
after 7 days of storage showed the presence of F. tularensis
DNA. Control samples containing saline showed no visible
amplicons. Thayer-Martin medium modified by the addition of
antibiotics was repeatedly found to preserve bacterial viability
as effectively as Amies agar with charcoal (data not shown).

Culture and PCR analysis of wound specimens. Wound specimens from 48 patients with clinically suspected tularemia
were sent in Amies agar with charcoal for culture and in gua-
indine isothiocyanate-containing buffer for PCR. For 40 pa-
tients the diagnosis was confirmed by serology and/or culture.
F. tularensis was isolated from 25 (62%) of these 40 patients,
and F. tularensis DNA was successfully amplified from 30
(75%) of these 40 patients (Table 2). β-Actin DNA was am-
plifiable from all 30 F. tularensis DNA-containing specimens.
For 4 of 10 F. tularensis DNA-negative specimens, however,
β-actin DNA was not detected, indicating that these specimens
may not have contained significant amounts of biological ma-
terial. Thus, F. tularensis DNA was detected in 30 (83%) of 36
patients from whose samples β-actin DNA was amplified.

**DISCUSSION**

Although the nutritional requirements of F. tularensis have been known for decades, there has been little experience with
the use of culture for the routine diagnosis of tularemia. Due
to the high degree of virulence of the organism, culture of
wound specimens is normally avoided (5). A more general use
of culture for the diagnosis of tularemia has been reported only
from Scandinavia, where the less virulent subspecies F. tula-
rensisa subsp. holarctica is endemic (4, 16). In one of the latter
studies (16), the sensitivity of the culture procedure was found
to be as low as 25%. In that study a modified Stuart medium
was used for transport, but it was found to be suboptimal in the
present study. According to the present experiments with
spiked samples, a commercial transport medium and a medium
recommended for culture were more preservative. When the

**TABLE 4. T-cell response to F. tularensis in wound samples but
no evidence of tularemia by culture or serology**

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Sex</th>
<th>Serum antibodies to F. tularensis/a</th>
<th>F. tularensis DNAb</th>
<th>Time of institution of antibiotics (days after onset)</th>
<th>T-cell response to F. tularensisc</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>Male</td>
<td>−</td>
<td>+</td>
<td>2</td>
<td>8.8</td>
</tr>
<tr>
<td>67</td>
<td>Female</td>
<td>−</td>
<td>+</td>
<td>2</td>
<td>9.8</td>
</tr>
<tr>
<td>42</td>
<td>Female</td>
<td>−</td>
<td>+</td>
<td>1</td>
<td>3.1</td>
</tr>
</tbody>
</table>
| 59      | Female| −                               | Not insti-
tuted      | 1                             | 1.0              |

a Not detected in any of at least four serum samples collected over a period of ≥3 months.
b Detectable DNA in wound specimen.
c After 4 days of incubation with heat-killed F. tularensis, T cells were pulsed with [3H]thymidine. An index representing the ratio of the mean incorporation of five cultures with and without antigen is shown. Reference subjects without any previous history of exposure to F. tularensis (n = 7) all showed indices of <2.3.
commercial medium was used as the transport medium for specimens from the present outbreak, a sensitivity of 62% was recorded. The transport medium thus seemed to be important for the success of culture of F. tularensis from wound specimens.

Due to hazards associated with culture, ulceroglandular tularemia seems to be a perfect target for gene-based pathogen identification. PCR for identification of the gene encoding a 17-kDa outer membrane lipoprotein allows the sensitive identification of F. tularensis. The gene is conserved among various strains of F. tularensis and shows no significant similarity to other prokaryotic or eukaryotic gene sequences in current gene banks. F. tularensis is not closely related to organisms known to be associated with human infection or colonization (6), thus further minimizing problems with interpretation of the findings.

According to the present results and those of a previous study (16), the sensitivity of PCR applied to wound specimens from patients with ulceroglandular tularemia is ~75%. In principle, a failure to detect DNA may be due either to inefficient sampling or to degradation of DNA during transport. The present and previous results favor the first explanation. If degradation was the main problem, the present inclusion of a nuclease inhibitor in the transport medium would have been expected to result in an increased sensitivity. The sensitivity was, however, not higher than that found when samples were sent in saline (16). Moreover, some samples would have been expected to contain relatively small amounts of amplifiable DNA. When six samples were subjected to quantitative PCR, all were found to have large amounts of DNA (10^6 to 10^7 genomic equivalents). In line with this, we previously showed that the success of PCR amplification was not decisively affected by the time of transport (16).

If the transport is not the weak link of the present PCR application, difficulties associated with sampling may be the more important. Some of those specimens that lacked amplifiable F. tularensis DNA also lacked amounts of β-actin DNA sufficient for PCR detection, indicating that in those samples, no or very little biological material was present. By contrast, β-actin DNA was amplified from all 30 samples in which F. tularensis-specific DNA was detectable. Moreover, only 1 of the 10 PCR-negative samples was culture positive. It should be recalled that in some patients with tularemia, the ulcer is very slight, and it may even be difficult to distinguish between an infected and a noninfected mosquito bite (4). Thus, the sensitivity of the PCR may well be restricted by difficulty with obtaining representative material. To improve sensitivity, intense rubbing of the wound surface may be tried, and sampling from more than one lesion may also be attempted. When lesions are minute or dry, a skin biopsy might be useful.

In the present study we identified four patients with clinically suspected tularemia but with repeated negative serology results when their sera were tested up to 12 weeks after the onset of symptoms and with negative culture results. When subjected to the T-cell stimulation test, three of them showed a strong cell-mediated immune response to F. tularensis. In previous vaccination trials, such a lack of correlation between serological and cell-mediated responses to the organism has been demonstrated, and similar to the patients in the present study, some of the vaccinees showed a strong T-cell response but no antibodies (18, 19). In clinical studies of tularemia, there are virtually no reports of the absence of antibodies in patients who were monitored by serologic testing for several weeks. A problem, however, is that the presence of serum antibodies is often used as an inclusion criterion for a study, and seronegative patients with tularemia may thereby be excluded a priori. In the present study those three patients who failed to respond with detectable antibodies all received treatment within 2 days of the onset of disease. An inhibitory effect of the early institution of antibiotics on the serological response in tularemia has been suggested previously (15). In essence, PCR may verify some cases of tularemia that would otherwise escape detection.

Thus, under transport conditions believed to preserve the viability of F. tularensis, in the present study PCR showed a sensitivity of ~75% and culture showed a sensitivity of 62%. Moreover, PCR allowed detection of some cases of tularemia that failed to be detected by culture or serology. The sensitivity of PCR may possibly be restricted by difficulty with obtaining representative material from all patients.

ACKNOWLEDGMENTS

We thank Stig Granström for expert advice on transport systems for culture specimens and Michal Kroca and Thorsten Johansson for help with lymphocyte stimulation assays.

Financial support was obtained from the Swedish Medical Research Council (grant 9485), Västerbottens Landssting, and the Medical Faculty, Umeå University, Umeå, Sweden.

REFERENCES


