Development of a Macrophage Cell Culture Method To Isolate and Enrich Francisella tularensis from Food Matrices for Subsequent Detection by Real-Time PCR

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ABSTRACT

Francisella tularensis is a gram-negative bacterium that can cause gastrointestinal or oropharyngeal tularemia in humans from ingestion of contaminated food or water. Despite the potential for accidental or intentional contamination of foods with F. tularensis, there are no techniques currently available to detect this organism in specific food matrices. In this study, a macrophage cell culture system is combined with real-time PCR to identify F. tularensis in food matrices. The method utilizes a mouse macrophage cell line (RAW 264.7) as host for the isolation and intracellular replication of F. tularensis. Exposure of macrophages to F. tularensis–contaminated food matrices results in uptake and intracellular replication of the bacteria, which can be subsequently detected by real-time PCR analysis of the DNA released from infected macrophage cell lysates. Macrophage monolayers were exposed to infant formula, liquid egg whites, and lettuce contaminated with varying quantities of F. tularensis. As few as 10 CFU/ml (or CFU per gram) F. tularensis was detected in infant formula and lettuce after 5 h postinfection. As few as 10 CFU/ml F. tularensis was detected in liquid egg whites after 18 h postinfection. Intracellular F. tularensis could also be isolated on Mueller-Hinton medium from lysates of macrophages infected with the bacteria in infant formula, liquid egg whites, and lettuce for subsequent confirmatory identification. This method is the first to successfully identify F. tularensis from select food matrices.

Francisella tularensis, the causative agent of tularemia, is a highly infectious organism that can cause severe, life-threatening illness in humans. As few as 10 bacteria are sufficient to cause infection via the respiratory system (12, 21, 51). In addition, tularemia can be contracted via the bite of an arthropod vector, handling of contaminated animal products, and ingestion of contaminated food or water. The severity of infection can vary, and it is based on the infecting strain, the dose, and the route of inoculation. This bacterial pathogen is most often associated with rabbits, rodents, and beavers, but has been isolated in over 250 vertebrate and invertebrate species (44). F. tularensis is considered a potential biological weapon due to its extreme infectivity and potential for airborne transmission. Subspecies holarctica (also known as type B) is a less virulent subspecies found mostly in Europe and Asia and is rarely fatal in humans (44, 55). Much of the knowledge of the biology of F. tularensis has been obtained by using an attenuated form of type B, referred to as the live vaccine strain (LVS). Despite its attenuation in humans, LVS remains fully virulent in mice, making this strain a safe and popular alternative model for the study of the pathogenesis of F. tularensis. Subspecies mediastatica causes a less severe form of tularemia, while F. novicida is not considered pathogenic in immunocompetent humans (14, 59).

Infection by F. tularensis is promoted by its ability to survive and multiply within macrophages. In addition to macrophages, F. tularensis has also been shown to multiply within a wide range of nonphagocytic cells including hepatocytes, fibroblasts, and endothelial cells, although macrophages are believed to be the preferred location for intracellular replication (9, 14, 34, 55, 59). F. tularensis entry into macrophages occurs by a unique method involving asymmetric, spacious pseudopod loops (9). Once inside the macrophage phagosome, the bacterium circumvents the phagosome-lysosome fusion pathway and escapes into the cytoplasm, where replication occurs (8). Various genes on a recently discovered Francisella pathogenicity island are

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required for intracellular survival, including pdpA, pdpD, iglA, and iglC (11, 19, 20, 41). These genes show no similarity to other known bacterial genes and appear to be unique to F. tularensis (55).

Although F. tularensis is not considered an enteric pathogen, ingestion of contaminated food or water can result in bacterial colonization of the throat or intestine, leading to oropharyngeal or gastrointestinal tularemia, respectively. Oropharyngeal tularemia is not typically associated with a high fatality rate; however, rapid progression to the more fulminating secondary pneumonic tularemia can occur from bacteremic spread to the lungs, resulting in a high rate of mortality (14, 18). Gastrointestinal tularemia symptoms can range from a mild diarrhea to development of extensive bowel ulceration, with a fatal outcome (14). Outbreaks of oropharyngeal or gastrointestinal tularemia throughout parts of the northern hemisphere have been well documented (5, 7, 23, 25, 27, 36, 37, 39, 47, 58).

Many barriers presently exist for the detection of F. tularensis in foods. For example, detection using traditional enrichment techniques is ineffective due to the extreme fastidious and slow-growing nature of the bacterium, which is readily outcompeted in growth by the resident bacteria normally found in foods. In addition, recent advances in the detection of bacterial pathogens from clinical specimens, including molecular methods such as PCR, can be ineffective for the detection of F. tularensis in foods, due to the presence of PCR-inhibitory components. Numerous food products contain compounds that are among the most common PCR inhibitors such as organic and phenolic compounds, glycogen, fats, and calcium (64). These methods often do not discriminate between the presence of living and dead cells.

In this study, we developed a novel detection system to allow for the specific identification of viable F. tularensis in various food matrices. This detection system is based on the ability of F. tularensis to invade and replicate within a macrophage, as part of its life cycle within a host. In theory, cultured eukaryotic cell lines exposed to F. tularensis–contaminated foods will allow the penetration and replication of F. tularensis while confining food particles and noninvasive bacteria to the extracellular environment, thus allowing the isolation and enrichment of intracellular F. tularensis for subsequent detection by commercially available techniques such as real-time PCR. In practice, a suitable mammalian cell monolayer is exposed to a particular food matrix suspected of harboring F. tularensis. The exposure is promoted for sufficient time to allow cell contact and engulfment of F. tularensis. The mammalian cell monolayer is then washed sufficiently to remove the food matrix and extracellular microorganisms. The infected cell monolayer is reconstituted with fresh medium and incubated further to allow for intracellular multiplication of F. tularensis (postinfection). After the infection is terminated, the culture medium is discarded, the infected cells are disrupted, and the DNA present in the resultant lysate is analyzed by real-time PCR using primers and a probe specific for a unique F. tularensis DNA sequence. We used this technique to investigate the efficiency of both presumptive and confirmatory identification of F. tularensis in infant formula, liquid egg whites, and lettuce.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** F. tularensis LVS was obtained from Karen Elkins (U.S. Food and Drug Administration, Rockville, MD) and was used in all experiments. LVS was routinely grown on modified Mueller-Hinton (MH) broth or agar supplemented with 0.1% glucose, 0.025% ferric pyrophosphate, and 2% IsoVitaleX (Becton Dickinson, Cockeysville, MD). MH agar also included 2.5% fetal calf serum (American Type Culture Collection, Manassas, VA). For confirmatory identification of LVS in lettuce, MH agar was supplemented with colicin (7.5 μg/ml), lincomycin (0.5 μg/ml), trimethoprim (4 μg/ml), ampicillin (10 μg/ml), and amphotericin B (2.5 μg/ml). LVS was routinely grown at 37°C in a 5% CO2 incubator. LVS working cultures were preserved in MH broth at −80°C.

**Food products.** Liquid infant formula, iceberg lettuce, and liquid egg whites (99% real eggs with yolks removed) were purchased from local supermarkets and kept at 4°C until use. Iceberg lettuce was cut and used unwashed for all experiments.

**Sample preparation.** Working cultures of LVS were grown overnight on MH agar plates at 37°C in a 5% CO2 incubator. LVS from MH plates were inoculated into 50 ml of MH broth and grown overnight at 37°C, in a shaker bath. Cultures were harvested by centrifugation at 16,000 × g for 10 min at room temperature and washed once with phosphate-buffered saline (PBS, pH 7.4; American Type Culture Collection). For the preparation of artificially contaminated infant formula and liquid egg whites, the cell suspension of the pure culture was resuspended to a final population of 108 CFU/ml in infant formula or liquid egg whites. These were then 10-fold serially diluted in the same food matrix to obtain a range of samples containing approximately 106 to 108 CFU/ml. Uninoculated samples of infant formula and liquid egg whites were also prepared as negative controls. A final volume of 1 ml of the corresponding sample dilutions was used immediately for infection of RAW 264.7 cell monolayers. Appropriate dilutions of LVS directly from each food matrix were spread plated onto MH agar and grown for at least 3 days in a 37°C, 5% CO2 incubator to determine accurate numbers of the initial inocula. For the preparation of artificially contaminated iceberg lettuce, LVS was grown as described above and 10-fold serially diluted in PBS. A series of Whirl-Pak filter bags (Seward, London, UK) were packed with 25 g of cut iceberg lettuce and inoculated with the corresponding bacterial dilution to obtain a range of samples containing from 108 to 101 CFU/g of iceberg lettuce. An uninoculated Whirl-Pak filter bag containing 25 g of lettuce was also prepared as a negative control. After 15 min at room temperature, 25 ml of macrophage growth medium (Dulbecco’s modified Eagle’s medium [American Type Culture Collection] containing 10% [vol/vol] fetal calf serum) was added to each filter bag and stomached for 60 s at normal speed in a Stomacher 400 (Seward). A final volume of 1 ml of the corresponding sample lettuce homogenates was used immediately for infection of RAW 264.7 cell monolayers. Appropriate dilutions of LVS in PBS were spread plated onto MH agar and grown for at least 3 days in a 37°C, 5% CO2 incubator to determine accurate numbers of the initial inocula.

**Tissue culture infections.** RAW 264.7 macrophages (American Type Culture Collection) were routinely grown at 37°C in a 5% CO2 atmosphere in macrophage growth medium supplemented with 100 μg/ml penicillin and streptomycin. RAW 264.7 macrophages were seeded into six-well tissue culture plates containing...
2.0 ml of macrophage growth medium per well at a density of 1.5 × 10⁶ cells per well and cultured to confluency for 24 h prior to use. Prior to infection, cultured cells were washed once with PBS. In two replicate experiments, cell monolayers were infected in duplicate with LVS in liquid egg whites, infant formula, iceberg lettuce homogenate, and macrophage growth medium at population densities of 10⁸ to 10⁹ CFU/ml. Uninoculated liquid egg whites, infant formula, lettuce homogenate, and macrophage growth medium were also added as negative controls. Plates were centrifuged at 600 × g for 5 min to promote bacteria-macrophage cell contact and incubated for 2 h at 37°C in 5% CO₂. Macrophage monolayers were then washed three times with PBS and reconstituted with either macrophage growth medium alone or with macrophage growth medium containing 10 μg/ml gentamicin and 2.5 μg/ml amphotericin B and placed in a 37°C, 5% CO₂ incubator for 5 h or 18 h, respectively. All macrophage monolayers were then removed from plates by using cell scrapers, and the cells were transferred to microfuge tubes and centrifuged at 600 × g for 10 min. Supernatants were removed and cells were washed once with PBS. Infected macrophages were then resuspended in 30 μl of distilled H₂O. Cells were then boiled for 10 min, cooled for 5 min, and centrifuged at 16,000 × g for 10 min. The resultant supernatants were used for real-time PCR analysis.

Real-time PCR. All reactions were run in a final volume of 25 μl using the Smart Cycler II apparatus (Cepheid, Sunnyvale, CA). Primers (23kDaF and 23kDaR) and probe (23kDaP) were previously designed and shown to target specifically the unique iglC gene, which encodes a 23-kDa protein required for intracellular survival of F. tularensis (62). The fluorogenic probe contains a 5'-carboxy-fluorescein fluorophore and a 3' black hole quencher. The reaction components and final concentrations used were 6 mM MgCl₂, 100 μM each primer and probe, 200 μM each deoxy nucleoside triphosphates, 1 unit of TaKaRa Ex Taq HS polymerase (Takara Bio Inc., Shiga, Japan), 1X Ex Taq buffer, and 3 μl of each template DNA. Thermal cycling parameters were set for an initial 90 s denaturation step at 94°C, which was followed by 45 cycles at 94°C for 10 s for DNA denaturation, with subsequent annealing and extension at 60°C for 15 s. Positive reactions were recorded as the measurement of at least 30 fluorescent units above baseline occurring before the completion of 45 cycles.

Confirmatory identification. LVS-contaminated liquid egg whites, infant formula, lettuce, and macrophage growth medium were prepared, and RAW 264.7 cell monolayers were infected as described above. Plates were centrifuged at 600 × g for 5 min to promote bacteria-macrophage cell contact and incubated for 2 h at 37°C in 5% CO₂. Infected cell monolayers were then washed three times with PBS and reconstituted with macrophage growth medium and placed in a 37°C, 5% CO₂ incubator for 5 h. Cell monolayers were then washed once with PBS and transferred to microfuge tubes for centrifugation at 600 × g for 10 min. Infected RAW 264.7 cells were lysed by the addition of 80 μl of 0.1% sodium deoxycholate in PBS for approximately 2 min. The released bacteria were serial diluted 10-fold in PBS, plated onto MH agar plates, and incubated at 37°C in 5% CO₂ for at least 3 days or until the appearance of suspect F. tularensis colonies. Suspect F. tularensis colonies were streaked onto fresh MH plates and incubated for at least 3 days at 37°C in 5% CO₂. Colonies from the pure culture were suspended in 30 μl of dH₂O in microfuge tubes, boiled for 10 min, centrifuged for 10 min, and 3 μl of the supernatant was used for real-time PCR identification using the iglC-specific primers and probe.

### RESULTS

#### Real-time PCR primer and probe set specificity. The primer and probe set used in this study were previously developed and shown to be specific for a segment of the F. tularensis iglC gene and displayed no cross-reactivity to a host of other bacterial pathogens (62). In order to determine whether the primer and probe set cross-reacted with DNA from various common foodborne pathogens such as *Listeria*, *Salmonella*, *Vibrio*, *Shigella*, *Clostridium botulinum*, and *Enterobacter sakazakii*, we conducted real-time PCR analysis of the supernatants from boiled bacterial isolates. No fluorescent signal above background levels were observed after 45 cycles in any of the non-Francisella bacteria tested, whereas both *F. tularensis* subsp. *holarctica* and *F. novicida* showed fluorescent signals above background levels before the completion of 45 cycles (Table 1).

### Macrophage cell culture and real-time PCR identification of LVS from artificially contaminated macrophage growth medium. Murine macrophages have previously been shown to sustain intracellular growth of *F. tularensis*, whereas macrophage growth medium alone fails to support extracellular bacterial replication (2). In order to test the macrophage cell culture method, we used RAW 264.7 murine macrophages as the eukaryotic cell host. RAW 264.7 macrophages were infected with LVS population densities ranging from 10⁸ to 10⁹ CFU/ml in macrophage growth medium alone. Analysis of the DNA from the RAW 264.7 cell lysates by real-time PCR using the iglC-specific primer and probe set revealed a detection limit of 10 CFU/ml for both 5- and 18-h postinfections (Table 2). Lysates from RAW 264.7 cells exposed to uninoculated macrophage growth medium (negative control) failed to illicit a fluorescent signal before the completion of 45 cycles, indicating that the real-time PCR reaction was specific for LVS DNA and not to any nonspecific DNA sequence from RAW 264.7 cells. Standard curve analysis (relationship be-
**TABLE 2.** Mean cycle threshold values from macrophage cell culture and real-time PCR detection of LVS from artificially contaminated medium, infant formula, and liquid egg whites at 5 and 18 h postinfection

<table>
<thead>
<tr>
<th>LVS population (CFU/ml)</th>
<th>5 h</th>
<th>18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Infant formula</td>
</tr>
<tr>
<td>10^8</td>
<td>14.27</td>
<td>19.60</td>
</tr>
<tr>
<td>10^7</td>
<td>17.17</td>
<td>22.79</td>
</tr>
<tr>
<td>10^5</td>
<td>24.25</td>
<td>29.56</td>
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<td>10^4</td>
<td>27.99</td>
<td>33.64</td>
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<td>10^3</td>
<td>31.35</td>
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<td>39.20</td>
<td>39.79</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

^a Cycle threshold values are the average of two experiments performed in duplicate.

^b ND, not detected.

between the real-time PCR cycle threshold values and the initial LVS CFU per milliliter used for RAW 264.7 cell infection revealed that detection of the *iglC* gene from RAW 264.7 cell infections by real-time PCR was linear ($R^2 \geq 0.99$) over the 8 orders of magnitude ($10^8$ to $10^1$ LVS CFU/ml) (Fig. 1). Gel electrophoresis of the real-time PCR amplifications revealed DNA fragments with a size corresponding to the *iglC* segment, indicating that the fluorescent signal was derived specifically from LVS DNA (data not shown). These data suggest that LVS was internalized into RAW 264.7 cells and can subsequently be detected by real-time PCR from infected macrophage cell lysates.

**Macrophage cell culture and real-time PCR detection of LVS from artificially contaminated infant formula and liquid egg whites.** To evaluate the effectiveness of LVS detection in infant formula and liquid egg whites by using the macrophage cell culture technique and real-time PCR, RAW 264.7 macrophages were infected with LVS in either liquid infant formula or liquid egg whites. Analysis of RAW 264.7 cell lysates from 5- and 18-h postinfections by real-time PCR using the *iglC*-specific primer and probe set revealed a detection limit of 10 CFU/ml for infant formula at 5 and 18 h postinfection and liquid egg whites at 18 h postinfection (Table 2). A detection limit of 10 CFU/ml was established by using real-time PCR of lysates from macrophages infected with 10-fold dilution series of LVS in macrophage growth medium (diamonds), infant formula (triangles), or liquid egg whites (squares). Both 5-h (A) and 18-h (B) postinfected macrophages were boiled, and the released DNA was analyzed by real-time PCR using the *iglC*-specific primers and probe. Real-time PCR cycle threshold values were plotted against the initial LVS inocula used to infect RAW 264.7 macrophages.

**FIGURE 1.** Standard curves generated from cycle threshold values obtained by real-time PCR of lysates from macrophages infected with a 10-fold dilution series of LVS in macrophage growth medium (diamonds), infant formula (triangles), or liquid egg whites (squares). Both 5-h (A) and 18-h (B) postinfected macrophages were boiled, and the released DNA was analyzed by real-time PCR using the *iglC*-specific primers and probe. Real-time PCR cycle threshold values were plotted against the initial LVS inocula used to infect RAW 264.7 macrophages.
TABLE 3. Mean cycle threshold values from macrophage cell culture and real-time PCR detection of LVS from artificially contaminated iceberg lettuce at 5 and 18 h postinfection

<table>
<thead>
<tr>
<th>LVS population (CFU/g)</th>
<th>Cycle threshold*</th>
<th>5 h</th>
<th>18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁸</td>
<td>15.38</td>
<td>15.64</td>
<td></td>
</tr>
<tr>
<td>10⁷</td>
<td>18.95</td>
<td>17.56</td>
<td></td>
</tr>
<tr>
<td>10⁶</td>
<td>22.81</td>
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<td>10⁵</td>
<td>26.01</td>
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<td>10⁴</td>
<td>29.48</td>
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<td>10³</td>
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<td>10²</td>
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<td>39.51</td>
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</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* Cycle threshold values are the average of two experiments performed in duplicate.

** ND, not detected.

10² CFU/ml was achieved for liquid egg whites at 5 h post-infection. Lysates from RAW 264.7 cells exposed to un inoculated infant formula and liquid egg whites (negative controls) failed to illicit a fluorescent signal before the completion of 45 cycles, indicating that the real-time PCR reactions were specific for LVS DNA and not to any nonspecific reactions with RAW 264.7 cell DNA or residual food particles. Standard curve analysis revealed that the detection of the iglC gene from 5- and 18-h–postinfected RAW 264.7 cell lysates were linear for LVS isolated from samples infected with 10⁸ to 10¹ CFU/ml in liquid infant formula and liquid egg whites (Fig. 1). These data demonstrate that the macrophage cell culture method and real-time PCR combination can correctly identify LVS in infant formula from 5- and 18-h postinfections and in liquid egg whites from 18-h postinfections, without loss in specificity or sensitivity, whereas a loss of 1 order of magnitude was observed for detection of LVS in liquid egg whites from 5-h postinfections.

**Macrophage cell culture and real-time PCR detection of LVS from artificially inoculated iceberg lettuce.**

To determine the effectiveness of the macrophage cell culture and real-time PCR method for detection of F. tularensis in solid foods as well as in foods more heavily contaminated with resident microbial populations, macrophages were exposed to unwashed iceberg lettuce artificially inoculated with various population densities of LVS. Analysis of RAW 264.7 cell lysates from 5- and 18-h postinfections by real-time PCR using the iglC-specific primer and probe set revealed a detection limit of 10 CFU/g in lettuce from both 5- and 18-h postinfections (Table 3). No fluorescent signal was detected from the uninoculated lettuce, indicating that the real-time PCR reaction was specific for LVS DNA and not to any nonspecific reactions with RAW264.7 cell DNA, residual lettuce particles, or DNA from the background resident microbial population. Standard curve analysis revealed that detection of the iglC DNA from 5- and 18-h–postinfected RAW 264.7 cell lysates was linear for LVS isolated from samples infected with 10⁸ to 10¹ CFU/g in iceberg lettuce (data not shown). In order to enumerate the indigenous microflora population in lettuce, uninoculated lettuce homogenates were serially diluted in PBS, plated onto MH agar plates, and incubated for at least 24 h at 37°C in a 5% CO₂ atmosphere. The average microflora count for two separate experiments was approximately 5 × 10⁶ CFU/g of iceberg lettuce. These data suggest that the eukaryotic cell culture method can be adapted for detection of F. tularensis in solid foods such as lettuce, without loss of sensitivity by implementation of 1:1 dilutions in macrophage growth medium. In addition, the high background level of resident food microorganisms does not reduce the specificity or sensitivity of LVS detection in lettuce.

**Confirmatory identification of LVS in foods by the macrophage cell culture technique.** A presumptive-positive result by the macrophage cell culture and real-time PCR method must be followed up by confirming the identity of F. tularensis in pure culture. Confirmatory detection for traditional foodborne bacterial pathogens usually consists of isolating target colonies on selective medium by using traditional culture methods. Unfortunately, F. tularensis grows poorly on most microbiological medium and is easily outcompeted in growth by resident microflora normally found on foods. To evaluate the macrophage cell culture method for confirmatory identification of LVS in food matrices, RAW 264.7 macrophages were infected with LVS population densities ranging from 10⁸ to 10⁶ CFU/ml (or CFU per gram) in macrophage growth medium, infant formula, liquid egg whites, and lettuce. At 5 h postinfection, macrophages were lysed with 0.1% sodium deoxycholate in PBS, and the released LVS were plated on MH agar plates, and incubated for at least 72 h at 37°C in a 5% CO₂ atmosphere. Suspect colonies were streaked for isolation on MH agar and incubated for at least 72 h. Colonies were chosen and boiled as previously described under “Materials and Methods,” and the released DNA was analyzed by real-time PCR using the iglC-specific primer and probe set. Confirmatory identification revealed a detection limit of 10 CFU/ml (or CFU per gram) in macrophage growth medium, infant formula, and lettuce at 5 h postinfection (Table 4). However, the detection of LVS in liquid egg whites at 5 h postinfection was limited to 10³ CFU/ml. Lysates from RAW 264.7 cells exposed to uninoculated macrophage growth medium, infant formula, liquid egg whites, and lettuce (negative controls) failed to produce LVS colonies on MH agar plates.

**DISCUSSION**

Methods used previously to detect F. tularensis from clinical specimens include serology (enzyme-linked immunosorbent assay, direct fluorescence antibody), molecular biology (PCR, Western blotting, gene probes), and classic microbiology (cultivation, biochemical profiling and susceptibility testing) techniques (14, 22, 52, 56, 62). However, the application of these methods for the detection of F. tularensis in food matrices has not been reported. Real-time PCR is a recent advance in molecular detection, used successfully for the identification of a wide array of bac-
TABLE 4. Confirmatory detection of LVS in medium, infant formula, liquid egg whites, and lettuce by lysis of 5-h-postinfected macrophages, plating on MH agar, and real-time PCR analysis of resultant colonies

<table>
<thead>
<tr>
<th>LVS population (CFU/ml or g)</th>
<th>RT-PCR detection</th>
<th>Medium</th>
<th>Infant formula</th>
<th>Liquid egg whites</th>
<th>Lettuce</th>
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<tr>
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<tr>
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<td>+</td>
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<td>+</td>
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<tr>
<td>10^2</td>
<td>+</td>
<td>+</td>
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<td>ND</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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<td>ND</td>
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</tr>
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</table>

a +, positive PCR result from suspect LVS colonies. 

b ND, no LVS colonies detected.

terial pathogens in foods (13, 17, 28, 48, 53, 63). Various food matrices have been shown to interfere with PCR, thus limiting the detection of foodborne bacterial pathogens (1, 28, 45, 46, 49). Dairy products such as milk and eggs have previously been shown to promote PCR inhibition (64, 65). Nontarget bacterial cell debris and DNA could behave as PCR inhibitors (64). As a consequence of the PCR inhibitory nature of specific foods, a sample processing step is often required to isolate the target organism from potential PCR inhibitory food particles and competing microorganisms prior to initiating the real-time PCR assay. Previous methods used to separate food particles from a target organism prior to PCR include immunomagnetic separation (31, 61), Flinders Technology Associates filtration (32), activated charcoal systems (1), and selective culture (35, 50). Unfortunately, these methods result in low PCR detection sensitivity, do not distinguish between live and dead cells, give rise to false-positive results, and/or are extremely time-consum ing and labor-intensive.

The technique presented here uses a macrophage cell monolayer that acts as host for the physical separation of F. tularensis from both food particles and competing resident microflora. Once F. tularensis is separated from inhibitory food ingredients and concentrated via intracellular multiplication to detectable levels, the bacteria can be identified via real-time PCR. We demonstrated that this system is capable of identifying as few as 10 CFU/ml (or CFU per gram) of LVS in infant formula, liquid egg whites, and lettuce and displays a broad range of detection of 10^8 CFU/ml (or CFU per gram) to 10^1 CFU/ml (or CFU per gram). The overall analysis time for presumptive detection of LVS in each food tested was 9 h for 5-h postinfections and 22 h for 18-h postinfections, with no false positives recorded. Another advantage of the macrophage cell culture technique is that only living F. tularensis cells are able to reside and multiply within macrophages, thus restricting detection to viable F. tularensis. In addition, despite its fastidious and slow-growing nature, F. tularensis can be detected in foods heavily contaminated with indigenous background microflora, without loss of sensitivity. Moreover, macrophage cell lines can be maintained indefinitely in the laboratory so that the cell culture technique can be administered promptly on receipt of foods suspected of F. tularensis contamination. However, the requirement for macrophage cell lines to perform the detection method may restrict the application of this technique to laboratories that have access to tissue culture facilities and personnel skilled in cell culture techniques.

One of the main advantages of the eukaryotic cell culture system is that it not only provides a means for rapid presumptive detection of F. tularensis in foods when combined with real-time PCR, but also can separately be used for confirmatory identification by lysis of infected macrophages and plating released bacteria on appropriate solid medium as well. Similar to the presumptive detection of LVS, a detection limit of 10 CFU/ml (or CFU per gram) was observed at 5 h postinfection in medium, infant formula, and lettuce. However, the confirmatory detection limit was only 10^3 CFU/ml from 5-h postinfections in liquid egg whites compared with 10^2 CFU/ml from presumptive identification. An 18-h postinfection assay was performed in liquid egg whites but did not improve the confirmatory detection limit of 10^3 CFU/ml (data not shown). It has been reported previously that F. tularensis can exist in a viable but nonculturable state under conditions of stress such as nutrient deprivation (16). The viable but nonculturable phenomenon is not limited to F. tularensis but has been shown to occur in a large number of bacterial pathogens (26, 40, 43, 57). It is conceivable that F. tularensis is exposed to stress-inducing conditions in the presence of liquid egg whites during the initial infection such that minute populations of the organisms that are taken up by macrophages are no longer able to grow on MH medium but nevertheless remain viable and can be subsequently detected by real-time PCR during presumptive identification. Viable but nonculturable F. tularensis does not appear to remain virulent (16) and hence may no longer pose a human threat if ingested.

In addition to detection of F. tularensis subsp. holarctica (LVS), preliminary results show that F. novicida (strain U112) can also be detected using this method (data not shown). Previous studies have shown that the primer and probe set used here was specific for the three subspecies of F. tularensis (tularensis, holarctica, and mediiasiatica) and F. novicida and exhibited no cross-reactivity to DNA from 87 non-Francisella strains (62). We show that the primer-probe set does not cross-react with over 40 isolates of common foodborne bacterial pathogens. Therefore, it is likely that this method will be specific for the detection of other species and subspecies of F. tularensis as well.

In theory, the macrophage cell culture and real-time PCR technique, or variations of this technique, could be used to detect other foodborne pathogens that exhibit intracellular life cycles such as Listeria monocytogenes, Shigella spp., Yersinia spp., and Salmonella serovars. In fact, preliminary experiments have demonstrated that a modified version of the macrophage cell culture and real-time PCR
method can identify as few as 10 CFU/ml Salmonella Enteritidis in raw shell eggs (10). The method may also conceivably be employed for the detection of F. tularensis in water, clinical, and environmental samples, all of which may contain a low amount of F. tularensis, contain PCR inhibitory substances, and be highly contaminated with competing background microflora. Future work will seek to demonstrate the efficacy of the technique for the detection of F. tularensis in other complex matrices.

F. tularensis is currently listed as one of four category A bacterial agents that can potentially be used as a biological weapon. The extreme infectivity and high fatality rate associated with the pneumonic form of tularemia make the aerosol route the most likely mode of dissemination in the event of a biological terrorist attack (12). However, the distribution of F. tularensis via the food supply may present an alternative way to circulate the bacterium to large populations as an act of bioterrorism (3, 6). F. tularensis readily survives the high acid content of the stomach and the oral administration of 10^{10} CFU of type A F. tularensis to human volunteers resulted in infection that required antibiotic intervention to prevent further symptoms (24, 60). The 50% lethal dose in mice and monkey models after oral inoculation of F. tularensis is 10^9 and 10^7, respectively, which is in the range for other more common foodborne pathogens such as Yersinia enterocolitica, Salmonella Typhimurium, L. monocytogenes, and Escherichia coli O157: H7 (30, 60). Moreover, F. tularensis has been shown to persist in specific foods and water as well as survive cold or freezing conditions for extended periods of time (4, 21), thus increasing the possibility of the organism reaching its intended target in a viable form in the event that the food supply is intentionally contaminated.

Risk assessment studies to determine the probability of particular foods being used as a vehicle for the delivery of F. tularensis in the event of an intentional biological attack have not been reported. Infant formula is of particular concern, due to the potentially greater susceptibility of immunologically immature infants to F. tularensis infection. In fact, oropharyngeal tularemia has been shown to be more common in children than adults (21). The dose required for infection in infants is unknown and may be much lower than that estimated in healthy adults. Therefore, the ability to detect low levels of bacteria in infant formula is critical. The macrophage cell culture method detects as few as 10 CFU/ml.

Detection of as few as 10 CFU/ml (or CFU per gram) in liquid egg whites and lettuce demonstrates the applicability of the method for the detection of F. tularensis in solid and semisolid foods, respectively. However, the physical and/or chemical makeup of certain foods could potentially damage macrophage monolayers and may require a dilution step in order to homogenize solid foods and/or diminish damaging chemical components. A 1:1 dilution of lettuce in macrophage growth medium was required to obtain liquid homogenate. Although the dilution step reduces the number of bacteria that are initially exposed to macrophage monolayers, the sensitivity of detection of 10 CFU/g was not compromised. Alternatively, liquid egg whites (semisolid) did not require a dilution step and were added directly to the macrophage monolayer, and detection of 10 CFU/ml was achieved.

The endemic nature of F. tularensis throughout the northern hemisphere, the severity of infection from ingestion, and the potential use as a biological weapon have raised awareness of the importance of developing rapid and sensitive techniques to detect F. tularensis in food matrices. We report the first method for the identification of F. tularensis in specific food matrices. This method could be used as a rapid preliminary screen of foods for the presence of F. tularensis with subsequent confirmation in case of accidental or intentional contamination.

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REFERENCES


