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Isolation of *Francisella tularensis* by Centrifugation of Shell Vial Cell Culture from an Inoculation Eschar

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A 52-year-old man was admitted to the hospital following the development of an inoculation eschar and fever six days after being bitten by a tick. He was clinically diagnosed as suffering from rickettsiosis. Eschar biopsy cultures on standard bacteriological media remained sterile. However, inoculation of cells with the homogenized specimen by the centrifugation-shell vial technique (M. Marrero and D. Raoult, Am. J. Trop. Med. Hyg. 40:197–199, 1989) resulted in the recovery of a bacterium. Determination of the sequence of the 16S rRNA gene amplified from the organism and comparison of the sequence to other sequences identified it as a strain of *Francisella tularensis*, whereas the specific serology was still negative. Our findings demonstrate that the centrifugation-cell culture, which is a tool for investigation of tick-transmitted diseases, may have the potential to serve as a method for the cultural isolation of *F. tularensis*.

Tularemia, caused by the facultative intracellular bacterium *Francisella tularensis*, is endemic in certain areas of the northern hemisphere. Diagnosis relies upon clinical, epidemiological, and microbiological evidence (6). In France it is a rare disease, being diagnosed mainly in the northeastern part of the country. Current laboratory diagnosis of tularemia relies upon serology, as cultivation of *Francisella tularensis* can be performed only on special media in high-level biosafety containment-equipped laboratories because of its highly infectious nature (6). In our laboratory we have developed a centrifugation-cell culture system, the shell vial assay (4), which has been adapted for bacterial isolation and which is used routinely in a biosafety level 3-equipped laboratory (1) for the isolation of rickettsiae and other strictly or facultatively intracellular bacteria from tissue biopsies, especially tick-bite eschars, and blood samples. In this report we describe the first application of this method to the clinical isolation of *F. tularensis*.

A 52-year-old, immunocompetent man was walking in a forest near Mulhouse, in Alsace, eastern France, on 1 May 1997, when he was bitten on his back by a tick. Four days later, he developed a painful interscapular inoculation eschar at the site of the bite. Within two additional days he developed an infectious syndrome with fatigue, high-grade fever (104°F [40°C]), chills, headache, myalgias, cutaneous hyperesthesia, and a right supraclavicular adenopathy. Despite oral antibiotic treatment combining 3,000 mg of amoxicillin with 750 mg of clavulanic acid per day, the febrile syndrome persisted and the patient was admitted to the hospital in Mulhouse. On physical examination, the physician observed an interscapular papule with centralized necrosis surrounded by an inflamed area and pustules. The patient also had a painless, mobile, right supraclavicular adenopathy.

The following biological results were identified: an inflammatory syndrome (C-reactive protein, 65.4 mg/liter; fibrinogen, 6.2 g/liter; erythrocyte sedimentation rate, 26 mm [first hour]),

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leukocyte count of 4,300/mm^3, thrombopenia (139,000 platelets/mm^3), and hepatic cytolysis (alanine-aminotransferase, 55 IU/liter; aspartate aminotransferase, 47 IU/liter).

F. tularensis microagglutination assay was negative on 13 May, and thus the physician suspected a rickettsiosis. The same serum sample and an eschar biopsy were therefore sent to our laboratory for rickettsia serology and culture. Tetracycline (200 mg per day) was then administered for two weeks, and the patient fully recovered within a few days of the start of this therapy.

The cutaneous biopsy was inoculated onto human embryonic lung (HEL) fibroblasts in shell vials on 14 May by methods described previously (4, 5). All of the following steps were performed under a class II biosafety hood in a biosafety level 3-equipped laboratory (1). Briefly, the eschar biopsy was homogenized in 1 ml of sterile brain heart infusion broth, and the homogenate was aspirated into a 1-ml syringe through a 27 gauge needle to filter out coarse material. Following Gram and Gimenez staining regimens, which were negative, the sample was inoculated into shell vials (3.7 ml; Sterilin, Feltham, England) containing a monolayer of confluent HEL fibroblasts grown on a 1-cm^2 coverslip. Three shell vials were inoculated and then centrifuged for 1 h at 700 × g and 22°C. The brain heart infusion was discarded and replaced with culture medium (Eagle's minimal essential medium with 4% fetal bovine serum and 2 mM L-glutamine). After incubation for three days at 32°C, a coverslip from one shell vial was stained by the Gimenez method. Small extracellular and apparently intracellular coccobacilli were observed, but they failed to react with anti-*Rickettsia conorii* or anti-*Coxiella burnetii* antiserum when incorporated into an indirect immunofluorescence assay. However, the patient's own serum reacted to these bacteria, with antibody titers of 1:32 for immunoglobulin G (IgG) and 1:32 for IgM. We could also grow the bacterium on L929 mouse fibroblasts. To determine whether it would grow in the cell culture medium alone, the isolated microorganism was inoculated and cultivated under the same conditions described above but without cells. No growth of the organism was obtained. At the same time, the same specimen was inoculated onto 5% sheep blood agar, chocolate agar, and Trypticase soy

agar (bioMérieux, Marcy l'Etoile, France) and incubated in a 5% CO_2 atmosphere for 24 h, but the culture remained sterile.

Concurrently, DNA was extracted from ground eschar biopsy with the QIAmp Tissue kit (QIAGEN GmbH, Hilden, Germany) according to the supplier's recommendations. Initially, these extracts were used as templates in a spotted fever group rickettsia-specific PCR (8). However, no amplification product was obtained. DNA was extracted from the cultivated bacteria (as described above) and then subjected to a PCR assay incorporating the versatile primers fD1 and rP2, derived from the 16S rRNA-encoding gene sequences (13). This amplification yielded a 1,400-bp fragment, which was sequenced as previously described (9). Sequencing reactions were resolved on 6% polyacrylamide gels (Ready Mix Gels, automated laser fluorescent grade; Pharmacia Biotech Europe, Brussels, Belgium), and electrophoresis was performed in the automated laser fluorescent DNA sequencer (Pharmacia Biotech Europe) in 1× TBE buffer, pH 8 (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA). The sequence obtained was compared with other sequences in the GenBank database with FASTA, in the PHYLIP software (7). A 99.9% sequence similarity was obtained with *F. tularensis* biogroup palearctica. Once the organism was identified, we subcultured the isolated bacterium on a special medium (cystine–glucose–5% sheep blood agar) and incubated it at 37°C in a 5% CO_2 atmosphere for four days. On the second day, blue-gray, round, smooth colonies that were moderately alpha-hemolytic appeared. Confirmation of the identification of *F. tularensis* was obtained by a slide agglutination test (Difco, Detroit, Mich.).

A second serum sample was obtained on 30 May, 25 days after the onset of symptoms. The *F. tularensis* microagglutination assay was positive with this sample, with a titer of 1:40. A third serum sample, taken on 4 July, yielded a titer of 1:160. With the indirect immunofluorescence assay, titers of antibody to the patient's strain of 1:64 for IgG and 1:32 for IgM were found for the second serum sample; antibody titers of 1:256 for IgG and 1:64 for IgM were found for the third.

In Europe the most commonly encountered human-pathogenic *F. tularensis* strain is *F. tularensis* biogroup palearctica, which is transmitted by mosquitos and ticks (2, 6). The most common portals of entry in humans are the skin and the respiratory tract (2). In areas of high endemicity, physicians are aware of the six classic forms of tularemia, namely, ulceroglandular, glandular, oculoglandular, pharyngeal, typhoidal, and pneumonic. Diagnosis is guided by recognition by clinical symptoms and confirmed by serological results or culture. Several serology methods are available, including tube agglutination, microagglutination, hemagglutination, and enzyme-linked immunosorbent assays (10, 12). Serological diagnosis requires a fourfold or greater rise in antibody titer between acute- and convalescent-phase sera, although samples may need to be repeated at 7- to 10-day intervals before a rise in antibody titer is demonstrated. Because of its highly biohazardous nature, only laboratories with specially equipped biohazard containment facilities and experienced personnel are able to work with *F. tularensis*. This bacterium has been recovered from blood, pleural fluid, lymph nodes, wounds, sputum, gastric aspirate, and inoculation eschar biopsies with specialized media. Colonies become apparent after 2 to 4 days of incubation (6). Other methods for rapid diagnosis include direct fluorescent-antibody staining of smears and tissues, antigen detection in urine, RNA hybridization with a 16S rRNA probe, and PCR (3, 11).

In France, tularemia is encountered only rarely, and since *F. tularensis* does not grow on standard bacteriological media, French physicians do not generally include it in the differential diagnosis of tick-bite infections. The national incidence of tularemia may therefore be underestimated.

The centrifugation-shell vial system (4) is a versatile method which can be applied to many viruses and most facultative or strictly intracellular bacteria. As a reference center for the diagnosis and study of rickettsioses, we have used this technique routinely and successfully to cultivate *Rickettsia* spp., *C. burnetii*, and *Bartonella* spp. from clinical specimens, including inoculation eschar biopsies. Because of the considerable virulence and contagiousness of *C. burnetii*, and to avoid any risk of aerosolization, in our laboratory the culture and DNA amplification procedures with any specimen are routinely performed in a biosafety level 3 facility under a class II biosafety hood. Identification of every isolated bacterium is obtained following PCR amplification and sequencing of genes coding for 16S rRNA. In the present study, although the suspected diagnosis was a rickettsiosis, use of the shell vial system permitted the isolation of *F. tularensis* from our patient at a stage when clinical symptoms and serology had not permitted a diagnosis. To our knowledge, this is the first diagnosis of tularemia made using the shell vial cell culture of a patient's specimens. Since shell vials remain closed during the centrifugation and incubation steps and cells are fixed with methanol before staining, we believe that this technique is less hazardous than culture on solid media for the isolation of *F. tularensis*.

The results of this study suggest that the centrifugation-shell vial system supports the cultural recovery of *F. tularensis* from a biopsy specimen. Presently, it is unclear whether the organism is capable of intracellular growth in the two cell lines tested but apparently the cells are a necessary prerequisite since the organism is incapable of growing in the cell-free shell vial medium. Although additional study is needed, we recommend the routine use of the shell vial cell culture system for the isolation of tick-transmitted pathogens in patients with an inoculation eschar. When performed as described in this study, the procedure is safe and time-saving.

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