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Isolation of *Francisella tularensis* from Infected Frozen Human Blood

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Francisella tularensis was isolated from human blood that was frozen for 3 months before it was examined. Before he became ill, the patient operated a "bush-hog" in an area thickly populated with rabbits. His illness was undiagnosed and untreated before his death. Portions of blood and tissue homogenates from necropsy were injected intraperitoneally into mice and inoculated onto glucose-cysteine-blood agar plates. *F. tularensis* did not grow from the culture plates, but mice inoculated with the blood died in 48 to 72 h. Fluorescent-antibody stains of mouse liver and spleen impression smears showed clumps of cells or amorphous masses of brightly staining envelope material around the cells. Tissue impressions of liver, spleen, and heart blood inoculated onto glucose-cysteine-blood agar plates yielded pure cultures of *F. tularensis*.

In the United States, over 90% of the human cases of tularemia, a disease acquired directly or indirectly from animals, are contracted from rabbits (5, 9), and the other cases are from other mammals, game birds, and blood-sucking arthropods (9). Laboratory diagnosis is based on isolating *Francisella tularensis*, detecting specific antibody, or demonstrating the organisms by fluorescent-antibody (FA) staining of exudate, tissue impression smears, or histological sections. At the present time, only one serological type of *F. tularensis* is known (8).

F. tularensis is rarely isolated from blood except during acute stages of the infection and before treatment. In the case reported here, a forensic pathologist collected blood and tissues at necropsy for toxicological studies, and *F. tularensis* was isolated from the blood, which was stored in a chest freezer for 86 days before it was examined. Histological organ examination suggested primary pneumonic tularemia as a possible diagnosis. Establishing an unequivocal diagnosis was very important both medically and epidemiologically, because compensation for a possible job-related death was involved.

MATERIALS AND METHODS

Case history. Thirty days before he died, a 34-year-old, previously healthy Negro male construction worker used a "bush-hog" machine to clear a lot in a rabbit-populous area. The next week he developed a frequent, mild, dry cough and deteriorated to the point that he remained in bed with fever and sweats for 3 days before he died. He died without being seen by a physician. Investigators did not find any evidence of out-of-town travel or of any other contact with wild game within the past 3 months. A routine necropsy, including removal of clotted blood and organ sections, was performed at the Medical University of South Carolina. Specimens were stored in a chest freezer at −25°C. Necropsy findings (FA-73-718) revealed left-lower-lobe lung consolidation with a 2-cm central abscess. Hilar and mediastinal nodes were enlarged with central gray, firm necrosis.

Clinical specimens received. Approximately 0.5 pint (ca. 0.2 liter) of blood, as well as brain, kidney, and liver sections, was stored frozen (−25°C) for 86 days before being shipped in dry ice to the Analytical Bacteriology Branch, Bacteriology Division, Center for Disease Control (CDC), with a request that they be examined for *F. tularensis*.

Processing of specimens. (i) Cultures. The best medium for isolating *F. tularensis* is glucose-cysteine-blood agar (GCBA) (BBL) supplemented with 5% rabbit blood (10), with rare isolations made on blood agar or on heart infusion agar (HIA). Several drops of blood from the thawed, macerated clot were streaked onto all three media. Tissue impression inoculations were made by touching the surface of the media with freshly cut pieces of the three organs. The plates were then streaked with an inoculating loop. Portions of homogenized tissue were also inoculated onto these media. Cultures were incubated in a candle jar at 35°C and examined daily for growth.

Biochemical identification is not usually necessary because cultural, morphological, and serological characteristics of *F. tularensis* are definitive (3).

(ii) Animal inoculations. Three mice were inoculated intraperitoneally with 0.2 ml of blood and three were inoculated with tissue homogenate. GCBA plates were inoculated from the cut surfaces

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of the liver and spleen and from the heart blood of mice succumbing to infection. Tissue impression and blood smears were also prepared for FA staining.

(iii) **FA staining.** Duplicate smears made from tissue impressions, blood, and growth from GCBA plates were allowed to dry in air and were heat fixed before half were stained with *F. tularensis* conjugate and half with normal rabbit conjugate that had been absorbed with *F. tularensis* cells to serve as a negative control. Smears were stained for 30 min, rinsed for 10 min in phosphate-buffered saline, pH 7.6, dried, and mounted in buffered glycerol. Slides were examined with a Leitz microscope equipped with the HBO-200 mercury arc lamp (Osram Co.) for excitation and with a 5840 primary filter and a Wratten 2A secondary filter.

(iv) **Histological sections.** Sections of human and mouse tissue were fixed in 10% neutral formalin, paraffin embedded, and cut into thin sections (5 μm or less). The paraffin was removed and the sections were stained by the usual histological procedures (8).

(v) **Agglutination tests.** The patient's blood was centrifuged to obtained serum for agglutination tests. Antigens were prepared from both known strains and from the culture isolated from the patient's blood. The turbidity of all antigens was adjusted to a McFarland no. 4 standard (10). Doubling dilutions of sera were made, beginning with 1:10 through 1:5,120. In the test, 0.2 ml of each serum dilution was combined with antigen and mixed. The tubes were incubated in a 37°C water bath for 2 h. The results were read at that time and again after the tubes were refrigerated overnight.

RESULTS

Cultures of specimens. No *F. tularensis* grew on plates from the patient's tissue imprints, homogenates, or blood. However, all cultures from the specimens did have many, very large mucoid colonies. Subcultures of these colonies were sent to the Enterobacteriology Branch, CDC, where they were subsequently identified as *Klebsiella pneumoniae* type 5 from brain and spleen and *K. pneumoniae* type 51 from blood.

Animal inoculations. Mice injected with tissue homogenates from the patient showed no signs of illness, although two mice inoculated with the patient's blood died within 3 days, and the third died 1 day later. The mice were necropsied, and the livers and spleens were removed. Fluid from these organs and heart blood were inoculated onto fresh GCBA. In 24 h there was light confluent growth and a few pinpoint-sized colonies; after 48 to 72 h of incubation, there was heavy confluent growth typical of *F. tularensis*. The medium had a greenish color, which turned brown with continued incubation. The Gram stain showed very small gram-negative rods and coccobacillary forms. Tissue impression smears were made for FA staining, and pieces of tissue were placed in 10% neutral formalin for histological study. *K. pneumoniae* was not isolated from the mouse tissues, nor were organisms resembling this species seen in the smears.

FA staining. Organisms stained by the *F. tularensis* conjugate were not found in the patient's tissues; organisms in tissue impression smears from the mice and from the growth on GCBA plates were brilliantly fluorescent. In tissues, the organisms were often clumped or appeared as an amorphous mass of brightly staining envelope material surrounding the cells. From the culture, cells were more easily distinguishable as brilliantly stained, individual, very small rods.

Histological sections. Histopathological examination of liver sections from the patient showed scattered foci of coagulative necrosis containing nuclear debris and surrounded by minimal mononuclear inflammatory infiltrate. No granulomas were seen. The autolyzed kidney had one isolated focus of coagulative necrosis in the outer cortex. These pathological changes are compatible with, but not diagnostic for, early tularemia. The brain was distorted by freezing, but no necrotic lesions were identified. The only organisms seen in the Brown-Breen-stained sections were large gram-negative rods (*Klebsiella*?) within a single arachnoid venule. No others were seen in the Gomori or Giemsa stains from any tissues (Pathology Division, CDC). Bacterial stains to demonstrate *Mycobacterium tuberculosis* were negative, but rods were demonstrated by a special zinc-free methylene blue stain (performed by Betty Hall, Medical University of South Carolina, Charleston, S.C.).

Sections of liver and spleen from mice infected with the patient's blood were Brown-Breen stained, but no organisms were seen. Sections stained with FA typically were tiny, rod-shaped, coccobacillary forms or clumps without clearly distinguishable morphology. The intensity of fluorescence was considerably less than that seen in the fresh impression or culture smears, because histopathological procedures alter the surface antigens to reduce fluorescence as much as from 4+ to 2+.

Agglutination tests. The patient's serum had an agglutination titer of 1:1,280 with known *F. tularensis* antigen when tested in the Bacterial Immunology Branch, CDC. The culture isolated from the patient was confirmed as *F. tularensis* by agglutination with known high-titered rabbit anti-*F. tularensis* serum.

DISCUSSION

Because humans accidentally become infected with a disease which in the United

States usually involves wild animals (primarily rabbits) and their arthropods, it is useful to establish animal or vector contact (2). The history, the necropsy, and the pathological findings in this case indicate pulmonary tularemia. The patient operated a bush-hog in areas of known, dense rabbit populations. This piece of equipment can shred anything in its path, including small animals such as rabbits and most underbrush. Rabbit burrows were no doubt disturbed by the machine, and litter from the burrows formed part of a dust cloud that the deceased inhaled as he worked. This is the probable source of infection in the absence of other known animal contact. Also, the size of *F. tularensis* cells (0.2 by 0.2 to 0.7 μm) would allow them to easily penetrate the alveoli of the lungs. The incubation period for *F. tularensis* is generally considered 3 to 10 days (3). That time interval fits well with that probable for exposure of the patient and with the time he became ill.

An airborne epidemic of tularemia with many pneumonic-type cases has been described (11). Most cases were among farmers who became infected from breathing dust from hay in barns and in stables occupied by infected voles. *F. tularensis* was isolated from dead voles and vole feces in the barns and from hay in the infested areas (11).

Generally, *F. tularensis* can be easily isolated from livers and spleens of rodents and lagomorphs who have died of the disease. We were unable to culture it from the liver, brain, or kidney available from this human case. The significance of finding *K. pneumoniae* in the blood, brain, kidney, and liver is not clear. It is possible that this organism interfered with the isolation of the tularemia agent.

This case was unusual in that the organism was recovered postmortem under much less than optimal circumstances, reemphasizing the value of using mice to isolate *F. tularensis* (8). Since these clinical specimens had been stored frozen for nearly 3 months before they were examined, a significant number of organisms may have died. This could account for the lack of growth in direct cultures of the organs and blood. In a susceptible animal, however, the presence of only one highly virulent *F. tularensis* cell can, theoretically, establish infection (1, 3, 9). The presence of *F. tularensis* is confirmed by isolating it in cultures from tissue impressions on GCBA and FA stains of organisms in tissue impression smears. The mice resisted infection with the *Klebsiella* and other bacteria, whereas infection was established by small numbers of *F. tularensis* present in the patient's blood. The data presented here establish the diagnosis of primary pulmonary tularemia. The evidence strongly suggest that the mode of infection was the inhalation of contaminated dust while performing on-the-job duties of operating a bush-hog. This case was without legal precedent and was finally settled out of court (South Carolina Industrial Commission, docket no. 46552).

The efficacy of detecting *F. tularensis* by FA staining has been established (3, 4, 6, 12). Although White and McGavran (12) successfully stained *F. tularensis* in formalin-fixed tissues processed by the usual histopathological procedures, our experience has been that FA staining of the organisms in smears from fresh tissue is superior. The fact is commonly accepted that *F. tularensis* cannot be demonstrated by such common histological stains as Giemsa, Gomori, and Brown-Breen (7).

Reasonable safety precautions should be exercised in processing specimens and isolating *F. tularensis* or any other potential pathogen. Specimens shipped to other laboratories for identification must be packaged and labeled to conform with Section 72.25, Part 72, Title 42, *Code of Federal Regulations*, which governs the interstate shipment of etiological agents and diagnostic specimens. Detailed instructions are presented in the manual *Collection, Handling, and Shipment of Microbiological Specimens* (4).

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