SENTINEL LEVEL CLINICAL LABORATORY GUIDELINES

FOR

SUSPECTED AGENTS OF BIOTERRORISM

AND

EMERGING INFECTIOUS DISEASES

Francisella tularensis

American Society for Microbiology (ASM)

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PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A. Introduction

Francisella tularensis is a fastidious, tiny, Gram-negative coccobacillus and the etiological agent of tularemia. Due to its low infectious dose (<10 organisms) and ability to be acquired via inhalation, *F. tularensis* presents a risk to public health and safety and as such is classified as a Tier 1 select agent and toxin by the US Department of Health and Human Services.

This procedure describes the steps to rule out, recognize, and presumptively identify this organism in clinical specimens in Sentinel Clinical Laboratories. Such laboratories are defined as those certified to perform high complexity testing under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) by the Centers for Medicare and Medicaid Services for the applicable Microbiology specialty. Laboratory in-house testing includes Gram stains, and at least one of the following: lower respiratory tract, wound or blood cultures.

Sentinel Clinical Laboratories are not required to register with the Select Agent Program to conduct diagnostic testing for Select Agents, both Tier I and non-Tier 1. Testing for Select Agents may be performed by laboratories as long as the laboratory follows the policies listed in the reporting section of this document when a Select Agent cannot be ruled out. Consult with your designated LRN Reference Laboratory or refer to the CDC Division of Select Agents and Toxins website at http://www.selectagents.gov for questions.

NOTE: *F. tularensis* is a known risk for laboratory acquired infection (15, 17). As of October 2012, *F. tularensis* is considered Tier 1 select agents because it presents the greatest risk of deliberate misuse with most significant potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence. (www.gpo.gov/fdsys/pkg/FR-2012-10-05/html/2012-24389.htm) At a minimum, suspected isolates should be manipulated at BSL-2 using BSL-3 practices. The identification of *F. tularensis* should not be attempted with commercial identification systems because of the potential of generating aerosols and the high probability of misidentification. The most common misidentification of *F. tularensis* is *Haemophilus influenzae* (satellite or XV positive) and *Aggregatibacter* spp. (includes previous *Actinobacillus* spp.) (β-lactamase negative).

B. Geographic distribution

F. tularensis is widespread throughout the Northern Hemisphere and is responsible for recent tularemia outbreaks in several countries including

Norway and Turkey (5, 9). Within the United States, tularemia has been reported in every state with the exception of Hawaii and approximately 120 cases are reported a year http://www.cdc.gov/tularemia/statistics/map.html. Most patients have a recent history of animal contact (bite, scratch) or arthropod bite.

C. Disease and clinical presentation

Francisella tularensis is a zoonotic pathogen with an extremely wide host range that includes mammals, birds and amphibians (11). The primary hosts for *F. tularensis* are believed to be small rodents (hares, voles, muskrats). Humans usually acquire infection by direct contact with infected animals or by animal associated biting arthropods. Ticks, mosquitoes, and biting flies have all been implicated as capable vectors. Although less common, contaminated soil, water, infected carcasses, and aerosolized particles have also been documented as sources of infection (4). The most likely exposure in a bioterrorism attack is from an aerosol and the pneumonic form would likely be the primary clinical presentation. *Francisella tularensis* was actively pursued as a biological warfare agent by both the United States and the former Soviet Union (2,10). The bacterium's low infective dose and ability to infect via aerosol not only make it a potential bioterrorism agent but also a significant occupational hazard in the clinical microbiology laboratory.

The clinical symptoms of tularemia depend upon the infecting strain and the mode of transmission. Approximately 80% of tularemia cases present as ulceroglandular disease. The bacteria replicate at the localized site of penetration, where an ulcer usually forms. From the penetration site(s), bacteria are transported by the lymphatic system to regional lymph nodes which serve as a secondary site for continued replication. In severe cases, the bacteria may then disseminate through the bloodstream to other sites including the lungs, liver, and spleen. Other, less common presentations are glandular without lesion, oculoglandular, oropharyngeal, typhoidal (febrile) without lymphadenopathy, and pneumonic (most severe form).

The incubation period for tularemia averages 3 to 5 days but may range from 1 to 21 days (13). Onset of disease is sudden with fever accompanied by chills, headache, malaise, anorexia, and fatigue. Depending on the route of infection, other prominent symptoms may include cough, myalgias, chest discomfort, vomiting, sore throat, abdominal pain, and diarrhea. Without treatment, fever lasts an average of 32 days, and chronic debility, weight loss, and adenopathy may persist for many months longer. Tularemia associated mortality has decreased significantly since the introduction of effective antimicrobial therapy and the overall case fatality has dropped to approximately 2% (12).

D. Case definition

The CDC case definition of tularemia (http://wwwn.cdc.gov/nndss/document/2012_Case%20Definitions.pdf) is a febrile illness which presents in several distinct forms including:

- Ulceroglandular: cutaneous ulcer with regional lymphadenopathy
- Glandular: regional lymphadenopathy with no ulcer
- Oculoglandular: conjunctivitis with preauricular lymphadenopathy
- Oropharyngeal: stomatitis or pharyngitis or tonsillitis and cervical lymphadenopathy
- Intestinal: intestinal pain, vomiting, and diarrhea
- Pneumonic: primary pleuropulmonary disease
- Typhoidal: febrile illness without early localizing signs and symptoms

A confirmatory diagnosis requires culture isolation of *F. tularensis* from clinical specimens or a fourfold or greater change in serum antibody titer to *F. tularensis* antigen.

E. Presumptive diagnosis

A presumptive diagnosis can be made by demonstrating elevated titers of specific antibodies to *F. tularensis* antigen in the serum from a patient with no prior vaccination history. Although not widely available, presumptive diagnosis can also be made by detection of *F. tularensis* in clinical specimens by direct fluorescent antibody assay.

II. SAFETY CONSIDERATIONS

A. *Francisella tularensis* is a highly infectious pathogen. Human clinical specimens (not cultures/isolates) suspected of containing *F. tularensis* can be processed with BSL-2 practices. If the initial Gram stain and/or culture is suggestive of *F. tularensis*, all manipulation should be performed in a biological safety cabinet (BSL-2) with BSL-3 practices (20). Nonclinical (environmental or animal) samples or specimens should not be processed in hospital laboratories. Veterinary laboratories are equipped to handle animal specimens. Nonclinical specimens should be directed to the designated LRN Reference Laboratory.

B. All patient specimens should be handled and processed in BSL-3 or BSL-2 with BSL-3 precautions, wearing gloves and lab coats or gowns and working in a Class II biosafety cabinet (BSC). Subcultures should be performed in a Class II BSC. Plates should be taped shut and incubated in 5 to 10% CO_2. All further testing should be performed only in the Class II BSC while wearing gloves to protect from infections through the skin.

C. Decontamination of laboratory surfaces is easily accomplished using a fresh

solution of 10% bleach. Plates and specimens should be destroyed as directed by the LRN Reference Laboratory when the identification is confirmed.

III. MATERIALS (see glossary for official abbreviations that do not require definitions)

A. Media

1. Blood culture
    a. One of the commercially available automated blood culture systems should be used for body fluids, bone marrow aspirates and blood specimens
    b. Biphasic system with both agar and liquid in the bottle, such as Septi-Chek (BD BBL Sparks, MD) or Hemoline (bioMérieux Durham, NC)
    c. Lysis-centrifugation system, ISOLATOR (Wampole Laboratories, Cranbury, NJ)
2. Media used for primary culture or inoculation of pellet from lysis-centrifugation system and subculturing of positive blood culture bottles
    a. BAP
    b. CHOC
    c. MAC or EMB
    d. Cysteine-supplemented agar: Thayer-Martin (TM), Buffered Charcoal Yeast Extract (BCYE), Cysteine Heart Agar (CHA)
    e. Cysteine-supplemented broth – Thioglycollate with 1% IsoVitalex.

B. Reagents

1. Appropriate disinfectant such as 10% bleach
2. Gram stain reagents
3. Catalase (3% hydrogen peroxide)
4. Oxidase (0.5 tetramethyl-p-phenylenediamine)
5. Culture of *Staphylococcus aureus* ATCC 25923 for satellite test
6. β-lactamase disk (Thermo-Scientific or BD BBL)

C. Equipment and supplies

1. Class II Biosafety cabinet (BSC)
2. PPE (gloves, solid front gown)
3. 35-37°C incubator (5-10% CO_2)
4. Light microscope with 100x objective and 10x eyepiece
5. Microscope slides and cover slips
6. Pipettes, inoculating loops and swabs
7. Cytospin centrifuge (optional)
8. Automated blood culture instrument (optional)

IV. QUALITY CONTROL

A. Perform quality control of media and reagents according to package inserts, most recent CLSI document M22, and CLIA standards, using positive and negative controls. Do not use *Francisella tularensis* as a control organism, due to its infectious nature. Examine culture plates for contamination, poor hemolysis, cracks, and drying. Confirm the ability of CHOC to support growth of fastidious organisms. Quality control of biochemical tests using a positive and negative reacting organism should generally be performed on each lot of reagent. For frequency of quality control, refer to manufacturer guidelines and state and federal regulations. Refer to the biochemical test section for procedures and quality control organisms for each test.

B. It is desirable for Sentinel laboratories to prescribe to a proficiency program designed to test the competency of Sentinel Laboratories in detection of agents of bioterrorism. Should the laboratory identify a select agent, the laboratory is required to fill out and submit Form 4b within 90 days of receipt of the sample (http://www.selectagents.gov).

V. SPECIMEN COLLECTION AND TRANSPORT

A. Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing	
Tissue Biopsy, scraping of an ulcer, or conjunctival swab	• For small tissue samples, add several drops of sterile normal saline into a sterile container to keep the tissue moist • Transport at room temperature for immediate processing • If processing of specimen is delayed beyond 2 hours, keep specimen chilled (2-8°C) • Amies transport media is an appropriate transport medium
Aspirate Lymph node or lesion	• Submit in a sterile container • Transport at room temperature for immediate processing. • If processing of specimen is delayed beyond 2 hours, keep specimen chilled (2-8°C)
Bone Marrow	• Submit in a sterile container • Transport at room temperature for immediate processing • If processing of specimen is delayed beyond 2 hours, keep specimen chilled (2-8°C)
Blood	• Transport inoculated bottles directly to laboratory at room temperature • Hold at room temperature until placed into automated, continuous monitoring blood culture incubators • Do not refrigerate
Respiratory Secretions	• Submit in a sterile container • Transport at room temperature for immediate processing • If processing of specimen is delayed beyond 2 hours, keep specimen chilled (2-8°C)

Serum	• Collect at least 1 ml without anticoagulant for serologic diagnosis • Store at 4°C until testing is performed. • Acute specimen is collected as soon as possible after onset of disease • Convalescent-phase should be collected >14 days after the acute specimen.

B. Rejection of specimens
1. Use established laboratory criteria.
2. Environmental or non-clinical samples are not processed by Sentinel Laboratories, contact designated LRN Reference Laboratory directly.

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VI. SPECIMEN PROCESSING

A. Blood and Bone Marrow
1. Aseptically inoculate liquid blood culture bottles with maximum amount of blood or body fluid per manufacturers' instructions. Incubate at 35°C.
2. Alternatively, follow the manufacturer's instructions for the lysis-centrifugation method and inoculate pellet to BAP, CHOC, MAC, Cysteine-supplemented agar, Thioglycollate broth. Incubate plates at 35°C in a humidified incubator with 5 to 10% CO_2.
3. Hold bottles at room temperature until placed into automated, continuous monitoring blood culture incubators.

B. Tissues
1. Inoculate BAP, CHOC, MAC, Cysteine-supplemented agar, Thioglycollate broth and incubate at 35°C in a humidified incubator with 5 to 10% CO_2.
2. Humidity may be maintained by placing a pan of water in the bottom of the incubator, sealing the plates with gas permeable tape also increases humid conditions.
3. MAC need not be incubated in CO_2.
4. Prepare smear for Gram stain.

VII. INCUBATION AND EXAMINATION OF CULTURES

NOTE: Isolation of *F. tularensis* from blood cultures is often delayed compared to other bloodstream pathogens. Increasing incubation time to 10 days may aid in detection.

A. Blood culture bottles
   1. Incubate non-automated broth blood cultures for 14 days, with direct observations for turbidity daily and blind subculture at 7 days, followed by terminal subculturing of negative blood cultures.
   2. Incubate automated systems for 10 days and perform terminal subcultures.
   3. Daily observation for growth in the bottles is either automated or visual, depending on the system.
   4. For blind subculturing, inoculate to BAP, CHOC, MAC, Cysteine-supplemented agar, Thioglycollate broth and incubate at 35°C in a humidified incubator with 5 to 10% CO_2.
   5. For positive broth cultures, inoculate to BAP, CHOC, MAC, Cysteine-supplemented agar. Place a dot or streak of *Staphylococcus aureus* ATCC 25923 culture on the BAP in the first quadrant of the plate. Incubate plates at 35°C in a humidified incubator with 5 to 10% CO_2 until growth appears.

B. Plate culture incubation times
   1. Recommended incubation of BAP, CHOC, MAC, Cysteine-supplemented agar, Thioglycollate broth is at 35°C in a humidified incubator with 5 to 10% CO_2.
      a. 7 days for tissue specimens
      b. A minimum of 3 days for blind blood culture subculture plates, but incubation can be extended up to 7 days.
   2. MAC need only be incubated for 3 days at 35°C in ambient air or 5 to 10% CO_2.
   3. All plates either from direct inoculation of specimens or from subculture of broths should be examined daily for growth of tiny colonies.

VIII. IDENTIFICATION

If your laboratory uses Mass Spectrometry (MALDI-TOF or bioMérieux) for bacterial identification and if the manufacturer provides your facility with an alternate tube extraction method, it is recommended that the resulting extract be filtered using a 0.2 µ (or less) filter. This additional step is recommended to reduce the risk of laboratory contamination with viable bacteria and spores.

Using automated systems, including Mass Spectrometry (MALDI-TOF, bioMerieux) technology may result in exposure to dangerous pathogens, and could result in erroneous identification, Ex: *Bacillus anthracis* misidentified as *B. cereus*; *Yersinia pestis* misidentified as *Y. pseudotuberculosis*.

NOTE: The rule out or referral of *F. tularensis* is accomplished by utilizing the *F. tularensis* identification flow chart (see below) and the following tests.

A. Gram stain

1. Stain suspicious colonies from agar plates and positive blood culture bottles, prepare and fix smears in a Class II BSC.
2. *F. tularensis* is a tiny, poorly counterstaining (by safranin) Gram-negative coccobacillus (0.2 to 0.5 µm by 0.7 to 1.0 µm). The Gram stain interpretation may be difficult because the cells are minute and faintly staining (Fig. 1). Counterstaining with basic fuchsin instead of safranin may increase resolution of the stained cells. *F. tularensis* are smaller than *Haemophilus influenzae* and bipolar staining is not a distinctive feature of *F. tularensis*.
3. Another smear may be prepared and Gram-stained, substituting basic fuchsins for safranin as the counterstain. Smear resolution may also be enhanced by staining with acridine orange.

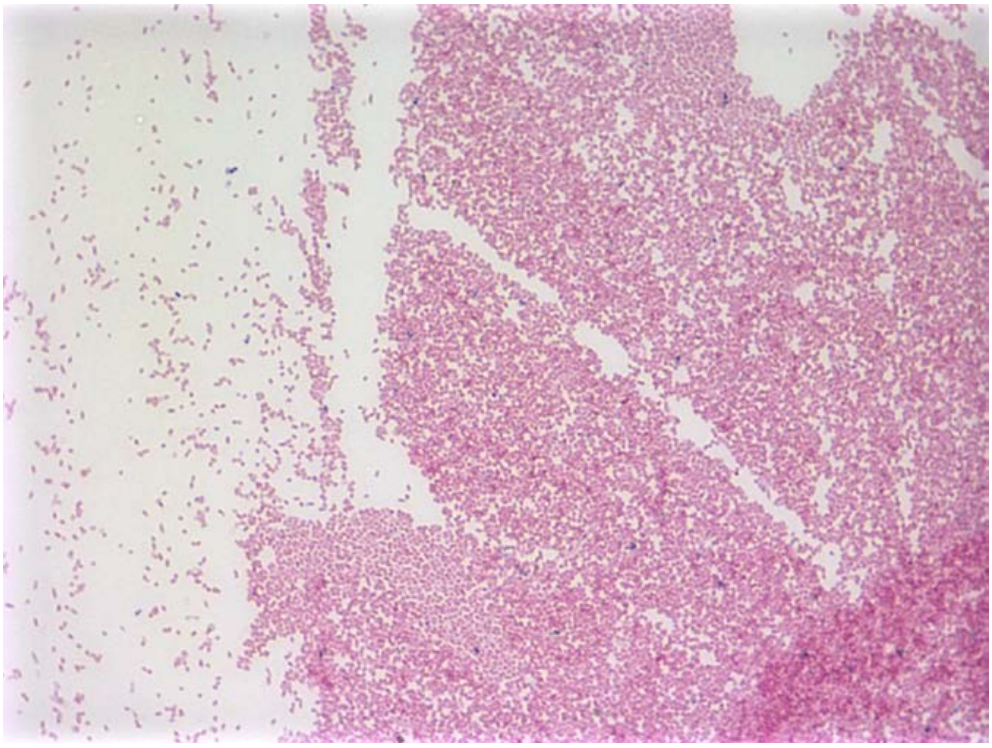


Figure 1. Gram stain of *F. tularensis* (X 1000)
Photo courtesy of Cheryl Gauthier, MA Dept. of Public Health

B. Cultures

1. Because these organisms require cysteine supplementation, *F. tularensis* may at first grow on BAP, but upon subsequent passage fail to grow on standard BAP. On cysteine supplemented agar plates (such as CHOC), colonies are gray-white, opaque and usually too small to be seen at 24 hours on most media. After incubation for 48 hours or more, colonies are about 1-2 mm in diameter, white to grey to bluish-grey, opaque, flat, with an entire edge, smooth, and have a shiny surface (Fig. 2). *F. tularensis* will not grow on MAC or EMB plates.

2. *F. tularensis* is a known risk for laboratory-acquired infections (17, 18). If characteristic Gram stain and culture yields oxidase-negative and catalase-negative colonies, it may be *F. tularensis*. Avoid aerosols and perform all subcultures in a Class II BSC. Plates should be taped shut and all further testing should be performed only in the BSC, using Biosafety level III practices. Further identification should not be attempted with commercial automated or kit identification systems, because of the danger of aerosol production due to preparing high concentrations of organisms.

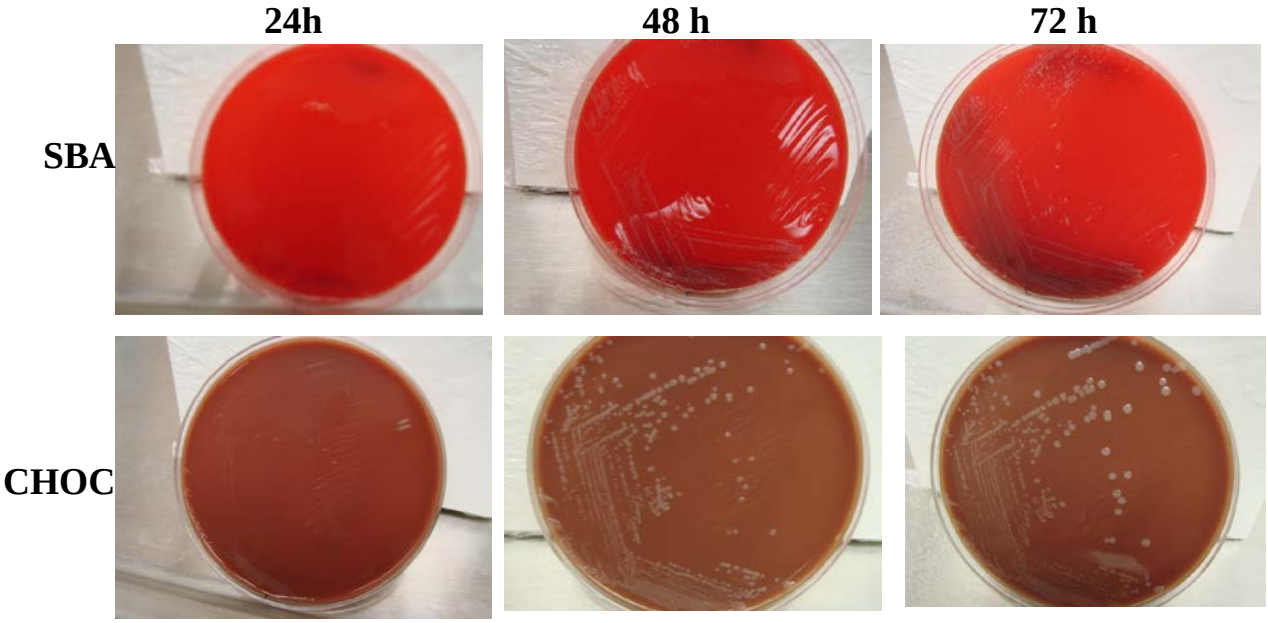


Figure 2. *F. tularensis* on Chocolate and Sheep Blood Agar at 24, 48 and 72 hours. Photo courtesy of: MAJ Todd Kijek, USAMRIID

C. If the above criteria are met, perform the following biochemical tests in a Class II BSC.

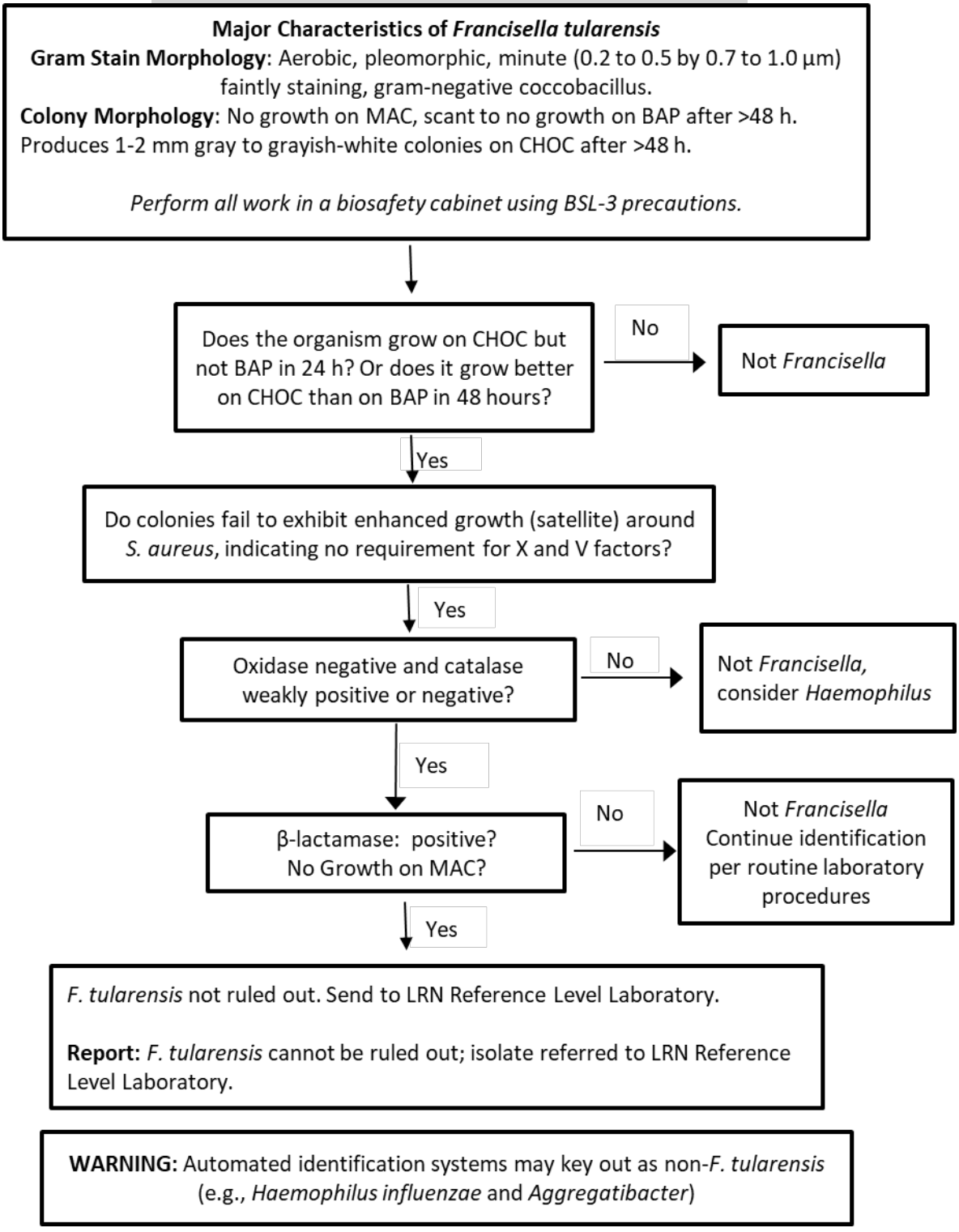
NOTE: Refer to General Introduction and Recommendations section for a listing of biochemical test and associated procedures.

1. MAC—No Growth
2. BAP—Scant growth
3. Oxidase – negative
4. Catalase – negative (or weakly positive)
5. Satellite growth – negative
6. β-lactamase – positive

D. Presumptive identification and referral to LRN Reference Laboratory (*Francisella tularensis* Identification Flowchart)

E. Confirmatory identification is made by an LRN Reference Level Laboratory; refer to http://www.bt.cdc.gov/lrn/biological.asp

Francisella tularensis Identification Flowchart



Note: Biochemical test procedures and quality control instructions can be found at the end of the *General Recommendation and Biochemical Testing Procedures* document.

POST ANALYTICAL CONSIDERATIONS

IX. REPORTING, NOTIFICATION, AND TRANSFER

A. *F. tularensis* is suspected and cannot be ruled out if the isolate fulfills the following characteristics:
- Faintly staining tiny Gram negative coccobacillus
- Scant to no growth on BAP, grayish white colonies on CHOC after 48 hrs
- Not growing on MAC in 48 h, no satellite growth around *S. aureus*
- Oxidase-negative, catalase- weak positive or negative, and β-lactamase positive.

B. Notifications and submission of cultures if *F. tularensis* cannot be ruled out by above characteristics.
1. Generate a report to the physician that *F. tularensis* species cannot be ruled out.
2. Do not attempt full identification and susceptibility testing in the Sentinel Clinical Laboratory.
3. Immediately notify your designated LRN Reference Laboratory, which will provide the referring laboratory with guidance and recommendations for retaining the specimen or isolate and submission for confirmatory identification.
4. Preserve original specimens pursuant to a potential criminal investigation and transfer to your designated LRN Reference Laboratory in accordance with state and local requirements. In particular, the appropriate material, including blood culture bottles, tubes and plates, and actual clinical specimens (aspirates, biopsies, sputum specimens) should be documented, and either submitted to the LRN Reference Laboratory or saved until the Reference Laboratory confirms the identification.
5. Do not ship specimens or cultures to LRN Reference Laboratories without prior arrangements.
6. Notify other public health authorities (e.g. local public health department epidemiologist/health officer) as required by local and state communicable disease reporting requirements. The public health laboratory/state public health department will notify law enforcement officials (state and federal), such as local FBI agents, as appropriate**.**
7. Within the hospital setting, immediately notify infection preventionists and/or infectious disease service so that the patient can be treated appropriately, infectious precautions can be implemented, and a further investigation of the patient's history can be made.
8. Consult with the LRN Reference Level Lab about additional clinical specimens that may be submitted for testing.
9. Initiate documentation, showing the specimen identification control, notification and transfer to the designated LRN Reference Laboratory, and documentation of all plates and tube cultures. Cultures will need to be destroyed or transferred once identification has been completed.

C. If *F. tularensis* culture is requested by the physician or a bioterrorist event is suspected, Sentinel Laboratories should consult with the designated LRN Reference Laboratory prior to or concurrent with testing. Obtain guidance from the state public health laboratory as appropriate (e.g., requests from local law enforcement or other local government officials). FBI and public health laboratory/public health department will coordinate the transfer of isolates/specimens to a higher-level LRN laboratory as appropriate**.**

D. If *F. tularensis* species is ruled out, proceed with efforts to identify using established procedures.

E. If other cases are suspected or there is a laboratory exposure, collect samples to submit to the designated LRN Reference Laboratory for serological testing.

X. SUMMARY/SPECIAL CONSIDERATIONS

A. Antimicrobial susceptibility

1. Antimicrobial susceptibility testing of *F. tularensis* is neither needed nor appropriate for Sentinel Laboratories to perform.
2. *Francisella tularensis* infections are treatable with narrow-spectrum antibiotics. All *Francisella* isolates examined to date are β-lactamase positive, so penicillins and cephalosporins are not effective and should not be used to treat tularemia (14).
3. Resistance rarely occurs and should be confirmed in a reference laboratory equipped to test agents of bioterrorism in a BSL level 3 facility.
4. For laboratories that perform susceptibility testing, CLSI M45-A2 lists "susceptible only" breakpoints for gentamicin, streptomycin, tetracycline, doxycycline, ciprofloxacin, levofloxacin and chloramphenicol.
5. Post-exposure prophylaxis recommendations for laboratory workers should be referred to the infectious disease service and exposure reported to occupational health.

B. Select Agent reporting and compliance

1. Reporting of all identified Select Agents is still required, even though Sentinel laboratories are not required to register under the Select Agent Rule.
2. The laboratory must complete Form 2 within one week (7 days) following notification of the confirmed identification. For further guidance and access to the necessary forms, consult with your designated LRN Reference Laboratory or refer to the CDC Division of Select Agents and Toxins website at www.selectagents.gov.
3. Reporting all identified Select Agents is required by completing Form 4 A within 7 days of confirmed identification. If the isolate is from a Proficiency test sample, Form 4 B is to be completed within 90 days of receipt of the sample.
4. Your designated LRN Reference Laboratory will advise you with

completion of required forms (e.g., Forms 2, 3, and 4). Always refer to www.selectagents.gov for the latest guidance and versions of these forms.

C. Destruction

1. Once the identification of the isolate is confirmed, the Sentinel Laboratory Select Agent regulations require that the residual specimen and cultures of the isolate be destroyed or transferred to an approved Select Agent entity within 7 days of confirmed identification. Your designated LRN Reference Laboratory must advise you on destruction or transfer of isolates.
2. Generally all plates and clinical material that contain the organism should be autoclaved, incinerated on-site or submitted to the designated LRN Reference Laboratory for disposal.
3. If no autoclave is available, contaminated items should be soaked in 10% bleach or 10% formalin for 24 h.

D. Packing and shipping

1. Refer to the ASM Packing and Shipping Sentinel Guidelines.
2. All materials sent to your designated LRN Reference Laboratory must be shipped in compliance with IATA and DOT regulations.

XI. LIMITATIONS

1. Environmental or non-clinical samples should not be processed by Sentinel laboratories, contact designated LRN Reference Laboratory directly.
2. *F. tularensis* is a known risk for laboratory-acquired infections (17,18).
3. Avoid aerosols and perform all subcultures in a Class II BSC.
4. Plates should be taped shut and all further testing should be performed only in the BSC, using Biosafety level III practices.
5. Further identification should not be attempted with commercial automated or kit identification systems, because of the danger of aerosol production due to preparing high concentrations of organisms.

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