SENTINEL LEVEL CLINICAL LABORATORY GUIDELINES

FOR

SUSPECTED AGENTS OF BIOTERRORISM

AND

EMERGING INFECTIOUS DISEASES

Yersinia pestis

American Society for Microbiology (ASM)

Revised March 2016

For latest revision, see web site below:

https://www.asm.org/Articles/Policy/Laboratory-Response-Network-LRN-Sentinel-Level-C

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PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A. Introduction

Yersinia pestis is a nonmotile, slow-growing, facultative organism classified in the family *Enterobacteriaceae*. It appears as plump, gram-negative coccobacilli that are seen mostly as single cells or pairs, which may exhibit bipolar staining from a direct specimen if stained with Wright stains. This appearance has been referred to as "safety pin-like."

Y. pestis, the causative agent of plague, has a protracted history, being described in epidemics and pandemics since biblical times. In the Middle Ages, it was estimated to have killed up to 40% of the European population. In more recent history, pandemic plague began in China in the 1860s. It spread to Hong Kong by the 1890s and subsequently was spread by ship rats to the Americas, Africa, and other parts of Asia (8). As recently as the beginning of the 20th Century, India suffered more than 10 million deaths from plague, and in the 1960s and 1970s, Vietnam was engrossed in a plague epidemic (1). Numerous references in art, literature, and monuments attest to the horrors and devastation associated with the plague bacillus.

B. Geographic distribution

More recently, during 2007-2011, a total of 23 human cases of plague from the U.S. were reported to the CDC (3).

Plague is a zoonotic disease transmitted ordinarily from animals and their infected fleas. Most cases occur in the late winter to summer months and are associated with flea contact (7).

C. Diseases and Clinical Presentation

Humans can acquire plague through the bite of infected fleas, direct contact with contaminated tissue, or inhalation (4). The incubation period from flea bite to symptomatic disease is 2-10 days (5).

Clinically, plague may present in bubonic, septicemic, and pneumonic forms (8). Bubonic plague is characterized by sepsis that is accompanied by the sudden onset of fever, chills, weakness, headache, and the formation of painful buboes (swelling of regional lymph nodes of the groin, axilla, or neck). Septicemic plague is similar to bubonic plague, but lacks the swelling of the lymph nodes. Pneumonic plague, the most deadly form of the disease and the form that can be transmitted rapidly, presents as fever and lymphadenopathy with cough, chest pain, and often hemoptysis. Secondary pneumonia from hematogenous spread of the organisms can occur (secondary pneumonic plague). The organism can also occasionally be passed from human to human by close contact as in primary pneumonic plague (2). Primary pneumonic

plague is most likely the form that would be seen if *Y. pestis* were used in a bioterrorism event. This is due to the high likelihood of aerosol delivery; the communicability of this form of the disease would make control of this particular agent even more problematic (6).

The procedures described below are intended to **rule out** *Yersinia pestis* from human specimens when examining isolates from cultures.

D. CDC Case Definition

A confirmed case is the isolation of *Y. pestis* from a clinical specimen, or a fourfold or greater change in serum antibody titer to *Y. pestis* F1 antigen (http://wwwn.cdc.gov/NNDSS/script/casedef.aspx?CondYrID=800&DatePub=1/1/1996).

E. Presumptive Diagnosis

A presumptive case of plague is an elevated serum antibody titer(s) to *Yersinia pestis* fraction 1 (F1) antigen (without documented fourfold or greater change) in a patient with no history of plague vaccination, or detection of F1 antigen in a clinical specimen by fluorescent assay.

Note: sentinel clinical laboratories do not require registration with the Select Agent Program to conduct diagnostic testing for Select Agents, both Tier I and non-Tier
Testing for Select Agents may be performed by laboratories as long as the laboratory destroys any residual specimen and destroys or transfers the confirmed select agent with 7 days of receipt of a confirmed identification. Reporting of all identified Select Agents is still required; laboratories will need to complete Form 4. If the organism is transferred following identification, then the laboratory must also complete Form 2. 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.
As of October 2012, *Yersinia pestis* is considered a Tier 1 select agent 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)

II. SAFETY CONSIDERATIONS

A. These procedures should be performed in microbiology laboratories that use Biological Safety Level-2 precautions. All patient specimens should be handled as BSL-2 while wearing gloves and gowns and working in a biosafety cabinet (BSC). Subcultures should be performed in a Class II BSC. Plates should be

taped shut, and incubated. All further testing should be performed only in the BSC. Because of the infectious nature of this organism, the appropriate LRN reference level laboratory should be consulted immediately if *Y. pestis* is suspected. See following reference pages 159-160 for additional information on safety; http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf

B. 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

1. Media
   a. Sheep blood agar (SBA) or equivalent
   b. Selective agar: MacConkey (MAC) or Eosin methylene blue (EMB) agar

2. Reagents
   a. Gram stain reagents
   b. Oxidase
   d. Catalase (3% hydrogen peroxide)
   e. Indole
   f. Urease test
   g. Motility media

NOTE: **Separate procedures for the biochemical tests listed above are located in the last section of the General Recommendations and Biochemical Procedures Section.**

IV. QUALITY CONTROL

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 *Yersinia pestis* 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.

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 4a within 90 days of receipt of the sample

(http://www.selectagents.gov).

V. SPECIMEN COLLECTION

A. Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing	
Lower respiratory tract	• Transport specimens in sterile, screw-capped containers at room temperature. • If it is known that material will be transported from 2-24 h after collection, then store and transport at 2-8°C.
Blood	• Transport samples directly to the laboratory at ambient temperature and place onto the blood culture instrument • Do not refrigerate • Follow established laboratory protocols for processing blood cultures
Aspirate, tissue or biopsy specimen	• Submit tissue or aspirate in a sterile container. • For small samples, add 1–2 drops of sterile normal saline to keep the tissue moist. • Transport the sample at room temperature for immediate processing. Keep the specimen chilled if processing will be delayed (> 2 h).
Swabs	• A swab of tissue is not recommended. • However, if a swab specimen is collected, the swab should be reinserted into an appropriate transport package and sent to the laboratory for immediate processing. Keep the specimen chilled if processing will be delayed (> 2 h).

B. Rejection of specimens
   1. Use established laboratory criteria for rejection of cultures
   2. Environmental or non-clinical samples are not processed by Sentinel laboratories; contact your designated LRN Reference Laboratory state public health laboratory directly.

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VI. SPECIMEN PROCESSING

A. Blood
   1. Aseptically inoculate liquid blood culture bottles with maximum amount of blood or body fluid per manufacturers' instructions. Incubate at 35-37°C.
   2. Alternatively, follow the manufacturer's instructions for the lysis-centrifugations method and inoculate pellet to BAP, CHOC and MAC. Incubate plates at 35-37°C in a humidified incubator with 5 to 10% CO_2.

B. For tissues, inoculate BAP, CHOC and MAC and incubate at 35-37°C in a humidified incubator. Humidity may be maintained by placing a pan of water in the bottom of the incubator or by wrapping the plates with gas permeable tape.

VII. INCUBATION AND EXAMINATION OF CULTURES

Use established inoculation and plating procedures for all specimen types. Tape plates shut to prevent inadvertent opening. Incubate at 25-28°C (optimal but optional) and/or 35–37°C (growth will be slower), in ambient air or 5% CO_2 for 5 days. Plates should be held for up to 7 days if the patient has been treated with antibiotics prior to culture collections.

VIII. CULTURE 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*.

A. Staining

1. Gram stain characteristics
   Gram stained specimens containing *Y. pestis* often reveal Gram-negative rods, 1–2 µm x 0.5 µm, that are seen mostly in single cells or pairs. The organism may demonstrate short chains in liquid media.

2. Wright or Giemsa stain characteristics
   Although not normally performed in the microbiology laboratory, Wright or Giemsa stains (Figure 1) performed on peripheral blood or tissue by hematology or histopathology may reveal the bipolar staining characteristics of *Y. pestis*, whereas the Gram stain may not. In patients with overwhelming sepsis, bipolar staining rods may be detected in peripheral blood smears. It is useful to note these characteristics in the event that hematology or histopathology asks for a microbiology consult.

Figure 1. Giemsa stained blood smear taken from a septicemic patient containing *Y.pestis* (1000x).

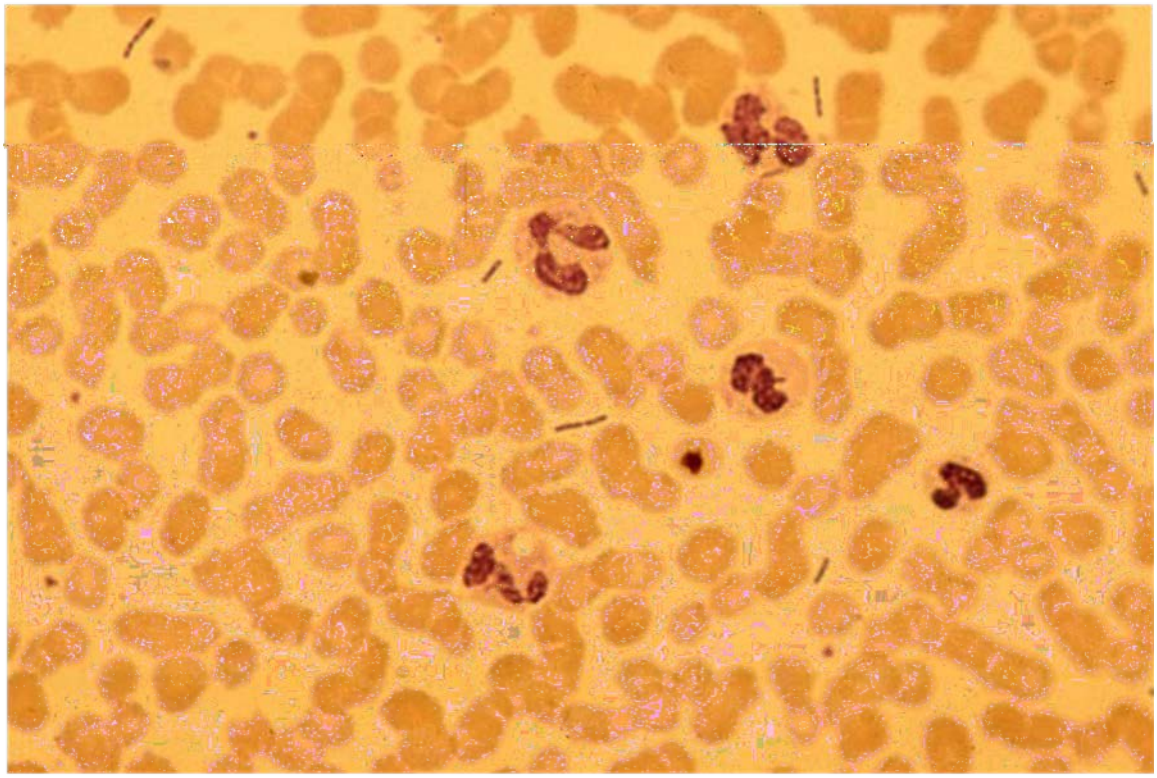

Photo courtesy of Dr. Michael Saubolle, Phoenix, AZ

B. Colony Morphology:
Agar plates: *Y. pestis* grows as grey-white, translucent colonies, usually too small to be seen as individual colonies at 24 h. After incubation for 48 h, colonies growing on SBA are approximately 1-2 mm in diameter, gray-white to slightly yellow and opaque. Older cultures may have "Fried Egg" appearance. There is little or no hemolysis of the sheep red blood cells. At 48 h, *Y. pestis* will grow as small, non-lactose fermenting colonies on MAC or EMB agar.

Figure 2: Blood plate at 48 hours growing *Y. pestis*.

Photo courtesy of APHL

Figure 3: Blood plate at 48-72 hours showing 'fried egg' appearance.

Photo courtesy of APHL

C. Biochemical Reactions/Tests

1. Use established laboratory procedures for catalase, oxidase, indole and urease tests (see also General Introduction, Recommendations and Biochemical Procedures).
2. Commercial biochemical identification systems may misidentify the organism and should not be used.

E. Presumptive Identification
Any isolate, from the respiratory tract, blood or lymph node, possessing the major characteristics noted below should be suspected as *Y. pestis.* Also, refer to Table 1 and Flow Chart for rule-out protocols.

a. Pinpoint colony at 24 h on SBA
b. Non-lactose fermenter, may not be visible on MAC or EMB at 24h
c. Oxidase, indole, urease negative
d. Catalase positive

NOTE: Full identification and susceptibility testing should not be performed. Confirmatory identification is made by an LRN Reference Level Laboratory; refer to https://emergency.cdc.gov/lrn/biological.asp.

Table 1: Differentiation of other important *Yersinia* species

***Yersina* species**	**Oxidase**	**Catalase**	**Urea (35°C)***	**Indole**
Y. pseudotuberculosis	negative	positive	positive	negative
Y. enterocolitica	negative	positive	positive	variable
Y. frederiksenii	negative	positive	positive	positive
Y. kristensenii	negative	positive	positive	variable
Y. ruckeri	negative	positive	negative	negative
Y. pestis	**negative**	**positive**	**negative**	**negative**

**Y. pseudotuberculosis* and *Y. enterocolitica* give stronger reactions in urea agar or broth when incubated at 25-28°C, but incubation at this temperature is not necessary to demonstrate urease production.

Yersinia pestis Identification Flowchart

Major Characteristics of *Yersinia pestis*

Gram Stain Morphology: Gram-negative, plump rods, 0.5 x 1-2 μm.

Colony Morphology: Slow growing at 35°C with either pinpoint colonies or no growth on BAP after 24 h; colonies after 48 h are 1-2mm, gray-white to slight yellow and opaque; nonlactose fermenter on MAC/EMB.

Specimen is blood, sputum, or lymph node aspirate

↓

Oxidase: Negative
Catalase: Positive
Indole: Negative
Urease: Negative

→ No

Not *Yersinia pestis*. Continue identification per routine laboratory procedures. May be other *Yersinia* spp

↓ Yes

Y. pestis not ruled out. Send to LRN Reference Level Laboratory.
Report: *Y. pestis* cannot be ruled out; isolate referred to LRN Reference Level Laboratory.

WARNING: Some of the automated identification systems do not identify *Y. pestis* adequately. *Y. pestis* has been falsely identified as *Y. pseudotuberculosis*, *Shigella,* H_2S negative *Salmonella*, *Acinetobacter* and *Pseudomonas* species.

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. *Y. pestis* is suspected and cannot be ruled out if the isolate fulfills the following characteristics:

- Plump Gram negative bacilli
- Slow growing, at 35°with either pinpoint colonies or no growth on SBA after 24 h; colonies after 48 h are 1-2mm, gray-white to slight yellow and opaque; non-lactose fermenter on MAC/EMB
- Positive for catalase and negative for oxidase, indole, and urease.

B. Notifications and submission of cultures if *Y. pestis* cannot be ruled out by above characteristics.

1. Generate a report to the physician that *Y. pestis* 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 confirmative 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. state public health department epidemiologist/health officer) as required by local and state communicable disease reporting requirements. The state 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 the infection preventionists and/or infectious disease service so that the patient can be treated appropriately, infectious precautions can be taken, 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, which will need to be destroyed or transferred once identification has been completed.

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

D. If *Y. pestis* 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 *Y. pestis* is not appropriate for Sentinel Laboratories to perform. Table 2 lists the appropriate antibiotics for use against *Y. pestis.*

Table 2: Antibiotics for use against *Y. pestis*

Antibiotic	Dosage	Duration
Streptomycin	15 mg/kg IM daily	10 days
Doxycycline (starting)	200 mg IV	One dose
Doxycycline (repeat dose)	100 mg IV twice daily	10 days
Gentamicin	5 mg/kg IM/IV daily	10 days
Ciprofloxacin	500 mg oral BID	10 days
Chloramphenicol (starting)	25mg/kg QID	One day
Chloramphenicol (repeat dose)	15mg/kg QID	10 days

Reference: http://www.freemd.com/yersinia-pestis/treatment.htm (accessed 3/25/13).

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.

D. 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. Alternatively, contaminated items should be soaked in 10% bleach or 10% formalin for 24 hours.

E. 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

Limitations

1. *Y. pestis* will grow on general nutrient-rich media but its growth rate is slower than that of most other bacteria; therefore, its presence may be masked by other organisms that replicate faster.
2. The bipolar appearance of cells following Wright or Giemsa staining is not unique to *Y. pestis*. Other *Yersinia* spp., enteric bacteria, and other Gram-negative organisms, particularly *Pasteurella* spp., can exhibit the same staining characteristic.
3. Characteristic clumped growth in unshaken broth culture is not an exclusive feature of *Y. pestis*. Some *Y. pseudotuberculosis* and *Streptococcus*

pneumoniae can exhibit the same growth features.

4. Some of the automated identification systems do not identify *Y. pestis* adequately. *Y. pestis* have been falsely identified as *Y. pseudotuberculosis*, *Shigella*, H_2S-negative *Salmonella*, or *Acinetobacter* (9).
5. Because of the limitations in identification of *Y. pestis* using methods typically found in microbiology laboratories, a high level of suspicion is essential. For blood isolates in particular, isolation of any *Yersina* spp or H_2S-negative *Salmonella* may be an indication to evaluate the clinical condition of the patient to determine if plague is a possibility.
6. Additionally, isolation of *Shigella* from blood is highly unlikely as is isolation of *Acinetobacter* from a case of severe community-acquired pneumonia or sepsis, and should immediately raise suspicion.

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