15 August 2005

SENTINEL LABORATORY GUIDELINES FOR SUSPECTED AGENTS OF BIOTERRORISM

Yersinia pestis

American Society for Microbiology

Credits: *Yersinia pestis*

ASM Subject Matter Experts

Susan E. Sharp, Ph.D.
Kaiser Permanente
Portland, OR

Michael A. Saubolle Ph.D. D(ABMM)
Banner Good Samaritan Medical Center
Laboratory Sciences of Arizona
Banner Health System
Phoenix, AZ

ASM Laboratory Protocol Working Group

Vickie Baselski, Ph.D.
University of Tennessee at Memphis
Memphis, TN

Roberta B. Carey, Ph.D.

Peter H. Gilligan, Ph.D.
University of North Carolina
Hospitals/Clinical Microbiology & Immunology Labs
Chapel Hill, NC

Karen Krisher, Ph.D., D(ABMM)
Clinical Microbiology Institute
Wilsonville, OR

Judith Lovchik, Ph.D.
Public Health Laboratories, NYCDOH
New York, NY

Larry Gray, Ph.D.
TriHealth Laboratories and
University of Cincinnati College of Medicine
Cincinnati, OH

Rosemary Humes, MS, MT (ASCP) SM
Association of Public Health Laboratories
Washington, DC

Chris N. Mangal, MPH
Association of Public Health Laboratories
Washington, DC

Daniel S. Shapiro, M.D.
Lahey Clinic
Burlington, MA

Alice Weissfeld, Ph.D.
Microbiology Specialists, Inc.
Houston, TX

David Welch, Ph.D.
Medical Microbiology Consulting
Dallas, TX

Mary K. York, Ph.D.
MKY Microbiology Consultants
Walnut Creek, CA

Coordinating Editor

James W. Snyder, Ph.D.
University of Louisville
Louisville, KY

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I. GENERAL INFORMATION

A. Description of Organism

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's or Giemsa stains. This appearance has been referred to as "safety pin-like."

B. History

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 (Perry, 1997). 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 (Butler, 1983). Numerous references in art, literature, and monuments attest to the horrors and devastation associated with the plague bacillus. During 1998-2002, a total of 112 human cases of plague were reported from 11 western states (CDC, 2003).

C. Geographic Distribution

Plague is a zoonotic disease transmitted ordinarily from animals and their infected fleas. To date, plague has not been transmitted east of the Rocky Mountains. However, cases acquired in the western United States have presented on the east coast (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5231a1.htm). Most cases occur in the summer months and are associated with flea contact.

D. Clinical Presentation

Humans can acquire plague through the bite of infected fleas, direct contact with contaminated tissue, or inhalation (Gage, 1998). Clinically, plague may present in bubonic, septicemic, and pneumonic forms (Perry, 1997). 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 (Campbell, 1998). 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; however the communicability of this form of the disease would make control of this particular agent even more problematic.

II. PROCEDURES

A. General

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

B. Precautions (BSL recommendations, etc)

These procedures should be performed in microbiology laboratories that use Biological Safety Level-2 (BSL-2) practices which at a minimum have a biological safety cabinet. The CDC recommends that all manipulations be performed within a biological safety cabinet. Because of the infectious nature of this organism, the state public health laboratory/department should be consulted immediately if *Y. pestis* is suspected.

C. Specimens

1. Acceptable Specimens

   Specimens of choice will be determined by the clinical presentation:

   a. Lower respiratory tract (pneumonic): Bronchial wash or transtracheal aspirate ($\geq$1 ml). Sputum may be examined but this is not advised because of contamination by normal throat flora.

   b. Blood (septicemic): Collect appropriate blood volume and number of sets per established laboratory protocol. Note: In suspected cases of plague, an additional blood or broth culture (general nutrient broth) should be incubated at room temperature (22–28°C), temperature at which *Y. pestis* grows faster. Do not shake or rock the additional broth culture so that the characteristic growth formation of *Y. pestis* can be clearly visualized (see section G.3.b for description of growth characteristics).

   c. Aspirate of involved tissue (bubonic) or biopsied specimen: Tissue or aspirates that can be obtained for culture include liver, spleen, bone marrow, lymph node, and/or lung. Note: Aspirates may yield little material; therefore, a sterile saline flush may be needed to obtain an adequate amount of specimen. Note: Aspirated specimens should not be submitted in a syringe with the needle attached. The needle should first be removed and the syringe capped prior to transport to the laboratory.

2. Rejection Criteria

   a. Use established laboratory criteria.

   b. Dried specimens, i.e.; powders, etc., should be referred to the state public health laboratory for analysis. Environmental and nonclinical samples should not be processed by Sentinel laboratories; submitter should contact the state public health laboratory directly.

3. Specimen transport and storage
   a. Respiratory/sputum: 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 container and transport at 2–8°C.

   b. Blood: Transport samples directly to the laboratory at ambient temperature. Hold them at ambient temperature until they are placed onto the blood culture instrument or incubator. Do not refrigerate. Follow established laboratory protocol for processing blood cultures.

   c. Tissue aspirate/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 of the specimen will be delayed.

   d. Swabs: A swab of tissue is not recommended. However, if a swab specimen is taken, the swab should be reinserted into the transport package for transport.

D. Materials

1. Media
   a. General nutrient rich media: Sheep blood agar (SBA) or equivalent

   b. General nutrient rich broth: Brain heart infusion (BHI) or equivalent

   c. Selective agar: MacConkey (MAC) or Eosin methylene blue (EMB) agar

   d. Blood culture, standard blood culture system

2. Reagents
   a. Gram stain reagents

   b. Wright-Giemsa or Wayson stain

   c. Oxidase reagents

   d. Catalase reagent (3% hydrogen peroxide)

   e. Urease test (e.g., Christensen agar, biochemical kit)

3. Equipment/supplies
   a. Microscope slides

   b. Heat source for fixing slides: Burner (gas, alcohol), heat block

   c. Staining rack for slides

d. Microscope with high power and oil immersion objectives

e. Bacteriologic loops, sterile

f. Incubator: Ambient atmosphere, 28°C and 35–37°C

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources; inclusion does not imply endorsement by the Centers for Disease Control and Prevention, the Department of Health and Human Services, the Federal Bureau of Investigation, the American Society for Microbiology, the Association of Public Health Laboratories, or any other contributor.

E. Quality Control

Perform quality control of media and reagents according to package inserts, CLSI/NCCLS document M22-A3, and CLIA standards, using positive and negative controls appropriate for each media and reagent. Document all quality control results according to standard laboratory practices.

F. Stains and smears: Gram stain

1. Gram stain

a. Procedure: Perform Gram stain procedure/quality control per standard laboratory protocol. Smears for staining may be prepared in order of likely positive results (i.e., cultures, bubo aspirates, tissue, blood, and sputum specimens).

b. Characteristics: Direct microscopic examination of specimens and cultures by Gram stain can provide a rapid presumptive identification. Stained specimens containing *Y. pestis* often reveal plump, gram-negative rods, 1–2 µm X 0.5 µm, that are seen mostly as single cells or pairs and short chains in liquid media (Fig. A1). Note: Patients with pneumonic plague may be secondarily infected with *Streptococcus pneumoniae*. Both of these organisms may be visualized in the sputum smears. It is imperative to evaluate such smears for the presence of gram-negative rods around the leukocytes (not necessarily intracellularly).

2. Other stains

a. Presence of bipolar-staining bacterial cells in these smears should trigger the suspicion of plague. The Wright stain often reveals the bipolar staining characteristics of *Y. pestis*, whereas the Gram stain may not. The Wright-Giemsa stains are the most reliable for accurately highlighting the bipolar staining characteristics of these gram-negative rods (Fig. A2). Note that in patients with overwhelming sepsis, bipolar staining rods may be detected in peripheral blood smears.

b. Wayson stain, another polychromatic stain, can be used instead of Wright-Giemsa.

3. Additional work:

Another smear may be prepared for referral to the state public health laboratory.

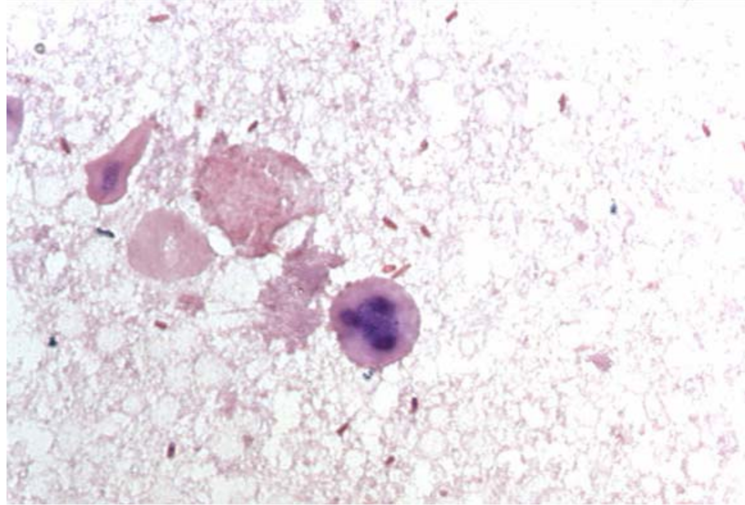

Figure A1. Gram stained touch-prep of liver from Y. *pestis* infected mouse (1000x)

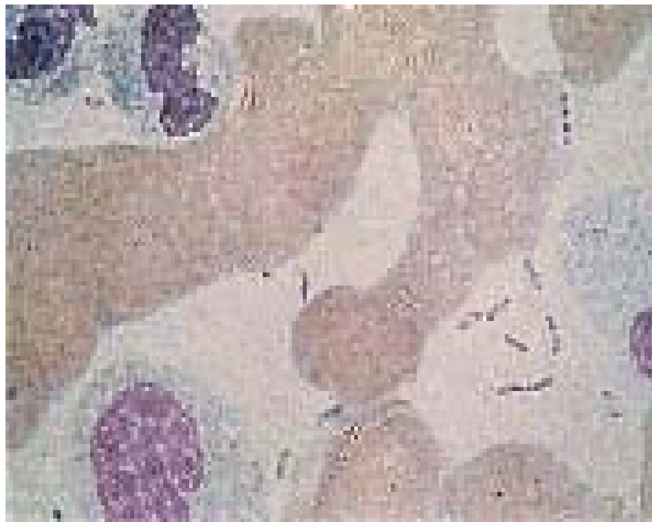

Figure A2. Giemsa stain of blood smear taken from septicemic patient containing Y. *pestis* (800X). Note: bipolar–staining “closed safety pin”-shaped cells

G. Cultures

1. Inoculation and plating procedure

   Use established inoculation and plating procedure. For tissues, use established laboratory procedure to inoculate media (e.g., grind, touch-preparation, or by using a sterile wood stick). Then, tape plates shut in 2 places (or use alternative method) to prevent inadvertent opening.

2. Incubation
   a. Temperature: 28-30°C (optimal); 35–37°C (grow more slowly).

   b. Atmosphere: Ambient, use of 5% CO_2 is acceptable.

   c. Length of incubation: Hold primary plates for 5 days. Plates should be held for up to 7 days if the patient has been treated with bacteriostatic antibiotic.

3. Colony characteristics
   a. Agar plates: *Y. pestis* grows as gray-white, translucent colonies, usually too small to be seen as individual colonies at 24 h. After incubation for 48 h, colonies are about 1–2 mm in diameter, gray-white to slightly yellow, and opaque. Under 4X enlargement, after 48–72 h of incubation, colonies have a raised, irregular "fried egg" appearance, which becomes more prominent as the culture ages (Fig. A3a). Colonies also can be described as having a “hammered copper,” shiny surface (Fig. A3b). There is little or no hemolysis of the sheep red blood cells. *Y. pestis* will grow as small, non-lactose fermenting colonies on MAC or EMB agar.

   b. Broth tubes: *Y. pestis* grows in clumps that are typically described as flocculant” or “stalactite” in appearance when the broth culture is not shaken or mixed. At 24 h, the growth is seen as clumps that hang along the side of the tube. After 24 h the growth settles to the bottom of the tube described as “cotton fluff.”

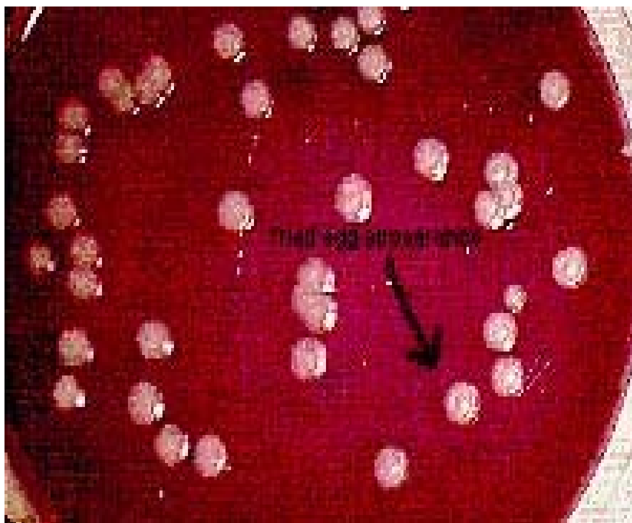

Figure 2. 72 h *Y. pestis* culture exhibiting a “fried egg” appearance.

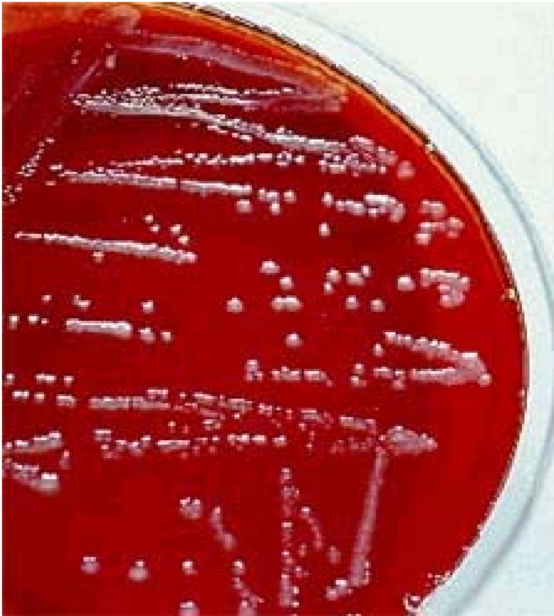

Figure 3. 48 h *Y. pestis* culture with characteristic "hammered copper" morphology.

H. Biochemical Reactions/Tests

1. Procedure

   Use established laboratory procedures for catalase, oxidase, and urease tests. Refer to Flow Chart.

2. Interpretation

   Follow established laboratory practice.

3. Additional notes

   Commercial biochemical identification systems may misidentify the organism. Refer to Flow Chart.

I. Interpretation

1. Presumptive Identification

   Any isolate, from the respiratory tract, blood or lymph node, containing the major characteristics noted below should be suspected as *Y. pestis.* Also, refer to Flow Chart.

   a. Bipolar staining rod (Wright-Giemsa) on direct smear

   b. Pinpoint colony at 24 h on SBA

   c. Non-lactose fermenter, may not be visible on MAC or EMB at 24h

   d. Oxidase and urease negative

   e. Catalase positive

f. Growth often better at 28°C

J. Miscellaneous testing

In an era of possible release of microorganisms as biological agents of terror, concern must be raised for sole reliance on diagnostic reagents targeting specific *Y. pestis* antigens or antibodies. *Yersinia pestis* has a genetic diversity that can potentially be manipulated to alter parameters for specific DNA sequences, thereby abrogating the use of available sensors. Thus, older methods relying on culture and overall phenotypic organism characteristics still play a major role in the recognition and diagnosis of *Y. pestis.* On the other hand, in non-bioterrorism situations, normal diagnosis of plague has been enhanced by more novel and rapid techniques (Anisimov, et. al., 2004).

1. Rapid diagnostic tests (RDTs)

   Several methods targeting the F1 antigen released by *Y. pestis in situ* (in the bubo and blood) have been described in the diagnosis of acute phase plague. These include enzyme-linked immunosorbent assay (ELISA), the immunogold chromatography dipstick assay and the direct immunofluorescent assay (DFA). These tests are not normally readily available, especially in the U.S. (Rahalison, et. al., 2000; Stevenson, et. al., 2003).

   A recent RDT dipstick method was described which had capability of detecting 0.5 ng/ml of *Y. pestis* F1 antigen, was rapid (15 minutes to complete), and had 100% sensitivity and specificity when tested against laboratory strains of *Yersinia spp*. (Chanteau, et. al., 2003). In clinical field trials the RDT detected 41.6% and 31% more positive clinical specimens than did traditional microbiologic and ELISA methods, respectively. The shelf-life, unfortunately, was only 21 days. This RDT holds promise for rapid diagnosis for plague where larger volume testing is predicated and can be used in medically resource-poor areas lacking technical or adequately trained staff support.

   Several polymerase chain reaction (PCR) detection methods have been described for detection of *Y. pestis* genetic material, but most have not been well studied against human clinical specimens (Hinnebusch and Schwan, 1993; Stevenson, et. al., 2003). However, recently a PCR method targeting a 501bp fragment of the *Y. pestis caf1* gene was evaluated with human specimens (Rahalison, et. al., 2000). Its sensitivity reached 89% in culture-proven patients with plague and 80.7% in patients diagnosed using F1 antigen detection methods. At this time, PCR methods are not recommended for routine diagnosis of plague.

2. Serology

   As with any tests requiring initiation of antibody production, serologies may be useful for epidemiologic studies or for retrospective confirmation of *Y. pestis* infection. They are, however, of little value for routine diagnosis of acute phase disease. Confirmation requires at least a four-fold rise in antibody titer to the F1 antigen (CDC, 1994), whereas a single titer rise to >1:16 is only suggestive of the diagnosis of plague in the right clinical setting (Butler, 1994).

K. 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 organisms that replicate faster.

2. Bipolar staining of cells is not an exclusive feature limited to *Y. pestis*. *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. pseudtuberculosis* 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* (Wilmoth, et. al., 1996). *Y. pestis* is alkaline slant/acid butt in triple sugar iron. In most conventional biochemical or commercial identification systems, the organism appears relatively inert, making further biochemical testing of little value. A list of manual and automated identification tests with *Y. pestis* in their databases can be found in a review article on the commercial identification of gram negative bacilli (O'Hara, 2005).

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. Isolation of *Shigella* from blood is highly unlikely and should immediately raise suspicion. Similarly, isolation of *Acinetobacter* from a case of severe community-acquired pneumonia or sepsis is also unlikely.

L. Reporting/Action

1. Sentinel laboratories should consult with state public health laboratory director (or designate) prior to or concurrent with testing if *Y. pestis* is suspected by the physician (Fig. A4).

2. Immediately notify state public health laboratory director (or designate) and state public health department epidemiologist/health officer if *Y. pestis* cannot be ruled out. The state public health laboratory/state public health department will notify law enforcement officials (state and federal).

3. Immediately notify physician/infection control according to internal policies if *Y. pestis* cannot be ruled out.

4. Preserve original specimens pursuant to a potential criminal investigation and possible transfer to an appropriate LRN laboratory. The 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. Start chain of custody documentation if appropriate.

5. Obtain guidance from the state public health laboratory as appropriate (e.g., requests from local law enforcement or other local government officials).

6. If *Y. pestis* is ruled out, proceed with efforts to identify using established procedures.

M. Therapy

Streptomycin has historically been the preferred treatment for plague and is FDA-approved for this purpose. .Streptomycin may not be widely and immediately available. The aminoglycosides, tetracyclines, and chloramphenicol are antimicrobials routinely used to treat plague. Among newer classes of antimicrobials, *Y. pestis* is susceptible to fluoroquinolones *in vitro*, and fluoroquinolones are efficacious in treating experimental plague in animals (http://www.bt.cdc.gov/agent/plague/trainingmodule/5/06.asp).

Antimicrobial therapy for specific patient populations are shown below (Inglesby, et. al., 2000):

Perferred Choice	**Alternative Choice**
ADULT: Streptomycin, 1g IM twice daily Gentamicin, 5 mg/kg IM or IV once daily or 2 mg/kg loading dose followed by 1.7 mg/kg IM or IV three times daily	**ADULT:** Doxycycline, 100 mg IV twice daily or 200 mg IV once daily Ciprofloxacin, 400 mg IV twice daily Chloramphenicol, 25 mg/kg IV 4 times daily
CHILDREN: Streptomycin, 15 mg/kg IM twice daily (maximum daily dose 2 g) Gentamicin, 2.5 mg/kg IM or IV 3 times daily	**CHILDREN:** Doxycycline, If >= 45 kg, give adult dosage If < 45 kg, give 2.2 mg/kg IV twice daily (maximum 200 mg/dl) Ciprofloxacin, 15 mg/kg IV twice daily Chloramphenicol, 25 mg/kg IV 4 times daily
PREGNANT WOMEN: Gentamicin, 5 mg/kg IM or IV once daily or 2 mg/kg loading dose followed by 1.7 mg/kg IM or IV three times daily	**PREGNANT WOMEN:** Doxycycline, 100 mg IV twice daily or 200 mg IV once daily Ciprofloxacin, 400 mg IV twice daily

Note: CLSI document M100-S15 outlines MIC interpretive standards for *Y. pestis* antimicrobial susceptibility testing (CLSI, 2005). This document also recommends

extreme caution when testing this organism and outlines biological safety practiced that must be adhered to. The document also warns that although *in vitro* studies have shown *Y. pestis* to be susceptible to β-lactam antibiotics, these antibiotics should not be reported as susceptible.

Yersinia pestis: Sentinel Laboratory Flowchart

Morphology: Facultative, bipolar, 0.5 by 1.0 to 2.0 um, gram negative rods.
Growth: Slow growing, pinpoint (1-2mm), gray-white to opaque, colonies on sheep blood agar after 24 h. Non-lactose fermenter, +/- growth on MAC/EMB at 24 h.

↓

Oxidase: Negative
Catalase: positive
Urea: Negative
Indole: Negative

WARNING: Automated identification systems often key out as non-*Y.pestis* (e.g., *Shigella,* H_2S-negative *Salmonella, Acinetobacter* and *Y.pseudotuberculosis*).

↓ No (features not present) → Report: *Y. pestis* is ruled out; continue identification per laboratory procedures.

↓ Yes (features present) → Report: Suspect, could not rule out; refer to state public health laboratory for confirmation.

CDC Contact Numbers:
English—888-246-2675
Español—888-246-2857
TTY—866-874-2646

Recommended Websites:
www.asm.org
www.cdc.gov
http://www.bt.cdc.gov/lrn
http://www.bt.cdc.gov/training/index.asp
http://www.bt.cdc.gov/agent/agentlist.asp

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Three references removed:

1. Bible and Chen, 1976.
2. Brubaker, 1972.
   Koneman, et al., 19

ASM acknowledges CDC for the use of its photos.

V. APPENDIX

A. Change Record

1. 04 August 2005
   a. Title Change to "Sentinel Laboratory Guidelines for Suspected Agents of Bioterrorism"
   b. Sentinel used instead of Level A throughout Guideline.
   c. On Credits page, new contact information for Lovchik, Saubolle, Shapiro and Welch.
   d. Under I. A. Description, added additional description of organism when stained with Wright's or Giemsa stains.
   e. Under I. B. History, information added on the number of Plague cases (112) in eleven western states between 1998-2002.
   f. Under I. C. Geographic Distribution, added MMWR article about cases acquired in the western U.S. that have presented on the east coast.
   g. Under I. D. Clinical Presentation, added information about aerosol delivery.
   h. Under II. B. Precautions, added recommendation that all manipulations be performed in a biological safety cabinet at a minimum.
   i. Under II. C. 1. c. Specimens, added additional note regarding aspirated specimens.
   j. Under II. C. 1. c. Specimens, added lymph nodes to list of biopsies/aspirates that could be sampled.
   k. Under II. C. 2. b. Rejection Criteria, added example (powder) to dried specimens.
   l. Under II. C. 3. Changed title of section to "Specimen Treatment and Storage."
   m. Under II. F. 2. Added: Note that in patients with overwhelming sepsis, bipolar staining rods may be detected in peripheral blood smears.
   n. Figure A. I, photo is 800x.
   o. Figure A. II., photo is 800x, and added additional note, "closed safety pin shaped cells."
   p. Under II. H. Biochemical Reactions/Tests, added, "refer to flow chart."
   q. Under II. I. Interpretation, added new subsection, "Presumptive Identification."
   r. Under II., new section J. added, "Miscellaneous Testing" including information on rapid diagnostic tests and serology.
   s. Under II. K. Limitations, new reference to O'Hara article sited.
   t. Under II. K. Limitations, added 5, emphasizing the need to have a high level of suspicion.
   u. Under II, new section M. added, "Therapy."
   v. Under III. References, 10 new references added (#1, 3, 5, 6, 7, 9, 10, 11, 13, 14) and three references removed.