# BASIC
# LABORATORY PROTOCOLS

## FOR THE PRESUMPTIVE
## IDENTIFICATION OF

## *Yersinia pestis*

## CDC

### Centers for Disease Control
### and Prevention

This protocol is designed to provide laboratories
with techniques to identify microorganisms, in order to
support clinicians in their diagnosis of potential diseases.

## Credits

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# I. Introduction

## 1. Disease history and perspective

*Yersinia pestis*, the causative agent of plague, has a remarkable place in history. For centuries, plague represented disaster for those living in Asia, Africa and Europe. Populations were so affected that sometimes there were not enough people left alive to bury the dead (Gross, 1995). The cause of plague was unknown, and plague outbreaks contributed to massive panic in cities and countries where it appeared. It was believed that the disease was delivered upon the people by displeasure of the gods, other supernatural powers, or heavenly disturbance. Innocent people were blamed for spreading plague and were persecuted by the panicked masses. Numerous references in art, literature, and monuments attest to the horrors and devastation of plague epidemics. Even now, entering into the 21st century, a suspected plague outbreak can incite mass panic. The number of human plague infections is low when compared to diseases caused by other agents. Plague invokes an intense, irrational fear, disproportionate to its transmission potential in the post-antibiotic and vaccination era.

## 2. Epidemiology

A total of 18,739 human cases were reported from 20 countries to the World Health Organization between 1980-1994. Plague is most likely under-reported because of inadequate surveillance and laboratory capabilities in some countries. In the United States, plague is endemic in the western states (Figure 1). From 1980-1998, human cases in the United States have averaged 13 per year, with a high of 40 cases in 1983. The case fatality was about 15%. Thirty percent of cases have occurred among Native Americans living in the southwestern states, where plague is enzootic. Plague pneumonia, potentially transmissible by the person-to-person route via respiratory droplets, has occurred in 11% of cases in the United States between 1979-1997. Five of seven persons who were infected by inhalation were known to be exposed to infected domestic cats. The presence of plague in co-existing suburban and rural rodent populations on the outskirts of some southwestern and Pacific Coast cities underscores the risk of epidemic plague in the United States in the absence of effective surveillance and prevention. Most plague cases in the United States occur in the summer months, when the risk for exposure to infected fleas is greatest. The majority of cases are acquired in or near the patient's residence. Risks for acquiring the disease are associated with conditions that provide food and shelter for plague-susceptible rodents near human dwellings.

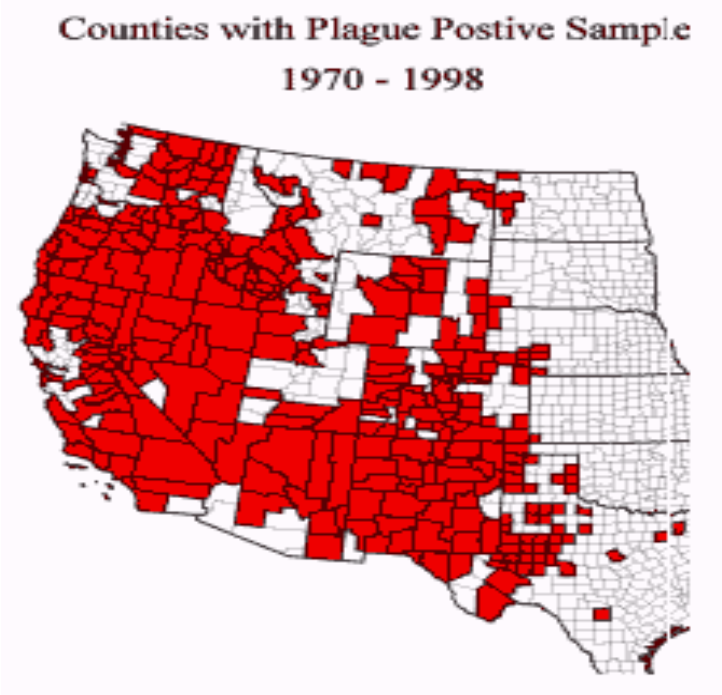


**Figure 1.** Epidemiological map of plague in the United States

### 3. Infection

Humans acquire plague from the bite of infected fleas, from direct contact with contaminated tissue, and by inhalation of bacteria-laden droplets (Gage, 1998). Bubonic plague is the most common form of infection, resulting from the bacteria being taken up by the host macrophages in the lymph nodes closest to the flea bite site. The affected lymph node becomes inflamed (bubo), enlarged, and painful as the bacteria replicate. From the infected lymph node, bacteria sometimes multiply and become blood-borne (bacteremic or septicemic) and occasionally lodge in the lungs (pneumonic). When plague infection becomes pneumonic, direct person-to-person transmission via bacterial aerosolization becomes possible (Campbell and Dennis, 1998). Progression of pneumonic plague is rapid, and, if untreated, may lead to death in a few days. However, pneumonic plague is rare and requires close contact for transmission to occur. Early diagnosis with prompt antibiotic treatment is effective against all forms of plague infection, and antibiotic-resistance to plague is rare (Dennis and Hughes, 1997).

### 4. Bacteriologic characteristics

The morphologic and physiologic description of *Y. pestis* is reviewed in Brubaker, 1972, and Bibel and Chen, 1976. *Y. pestis* is a facultative anaerobe that is non-motile and does not form spores. It stains easily with aniline dyes and appears as single or short-chained pleomorphic, gram-negative, fat rods (0.5 µm X 1.0 µm, coccobacillus). Under certain growth and staining conditions, the coccobacillus appears as a bipolar ("closed safety-pin") rod. In the laboratory, the optimum temperature for propagating *Y. pestis* is between 28°C-30°C at physiologic pH (7.2-7.6). On both solid media and in broth, *Y. pestis* has a characteristic appearance that may be used to aid in its identification.

Cultures grow slower (1.25 hours/generation time) than other bacteria and thus require longer incubation times for optimal growth. *Y. pestis* lacks many enzymatic functions such as adenine deaminase, aspartase, ornithine decarboxylase, glucose-6-phosphate dehydrogenase, and urease activities. It also lacks the ability to ferment most carbohydrates; primarily it utilizes glucose and mannitol for energy.

## 5. Treatment

Vaccination is recommended for laboratory personnel who are routinely exposed to live *Y. pestis*. Vaccination should also be considered for persons who have regular contact with wild rodents (hosts of plague) or their fleas in areas where plague is enzootic or epizootic (CDC, 1996). Currently, the killed plague vaccine is not produced in the United States. The efficacy of the killed vaccine in protecting humans has not been studied in randomized, controlled trials. Pneumonic plague has been documented in two vaccinated persons, suggesting that the vaccine may be ineffective against aerosolized plague. Newly developed vaccines targeting aerosolized plague are currently under investigation.

Antibiotic treatment is known to be effective. Recommended antibiotics for treatment of a plague case include streptomycin, gentamicin, tetracyclines, and chloroamphenicol. Tetracycline, doxycycline, or trimethroprim/sulfamethoxazole, which can be taken orally, are recommended for prophylaxis (CDC, 1996).

## 6. Precautions and environmental decontamination

Decontamination procedures should include autoclaving of all non-noxious materials. Where autoclaving is not possible, exposure to 10% diluted household bleach (0.5% hypochlorite), followed by 70% alcohol wash, is adequate to reduce surface contamination risks.

## II. Laboratory Procedures for the identification of *Yersinia pestis*

### 1. General

The intent of providing laboratory procedures in this manual is to assist the clinical microbiologist in the examination of a specimen or culture that may contain *Y. pestis*. Guidelines are provided to perform simple laboratory tests that will assist the microbiologist to presumptively rule out the presence of *Y. pestis*. These protocols are abstracted from the Centers for Disease Control and Prevention's (CDC's) laboratory manual for plague diagnostic tests (Chu, 1999).

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources and inclusion does not imply endorsement by the CDC, the U. S. Public Health Service, Department of Health and Human Services, or the other governmental agencies.

1.a. Handling of specimens

Working with *Y. pestis* requires Biological Safety Level II (BSL-2) facilities and practices. Specimens should only be handled in a BSL-2 laminar flow hood with eyes protected (safety glasses, eye shields); closed-front laboratory coats with cuffed sleeves and gloves stretched over the cuffed sleeves should be worn. Laboratory personnel must avoid any activity in the laboratory that puts individuals at risk for infectious exposure, including activities that might create aerosols or droplet dispersal. Laboratory benches should be decontaminated after each use and contaminated supplies disposed of into proper receptacles. Laboratory works should avoid touching mucosal surfaces with their hands (gloved or ungloved) and should not eat or drink in the laboratory. Each time before leaving the laboratory, laboratory workers should take off and reverse their gloves before disposing them in a biohazard container, wash their hands and remove their laboratory coat.

### 2. Processing of clinical specimens

Specimens are opened inside the BSL-2 safety cabinet. The BSL-2 cabinet interior is kept clean and free of unnecessary equipment to avoid compromising airflow. A covered disposal pan is set inside the cabinet for holding used materials. All inoculated plates are taped so that they will not open inadvertently. Respiratory masks (National Institute Occupational Safety and Health-approved particulate respirators) should be available for use if working with large volumes or during potential aerosolization situations. If *Y. pestis* cannot be ruled out or if a biological infectious agent is identified, modifications in the handling of the specimen should be considered.

### 3. Presumptive identification of *Y. pestis*

3.a. Gram Stain

3.a.1. Perspective

Direct microscopic examination of specimens and cultures by Gram stain can provide a rapid presumptive diagnosis. Gram stain results, the shape of cell (cocci, bacilli), and the type of cell arrangement (single, chained, clustered) visualized under light microscopy can provide an indication of what the etiologic agent may be.

Crystal violet (gentian violet) is the primary stain that will bind to the peptidoglycan present in the cell walls of some bacterial cells. Iodine is added as a mordant to fix the bound dye. If the cell wall does not contain peptidoglycan, crystal violet is easily washed off with acid or alcohol (decolorizer). A secondary dye (counterstain), safranin, is added after the decolorization step. If the primary stain does not bind, the unstained cells will easily adsorb safranin. Thus, gram-positive cells are purple (peptidoglycan-containing), while gram-negative cells are pink (little or no peptidoglycan).

3.a.2. Application

Smears may be prepared, in order of likely positive results, from cultures, bubo aspirates, spleen, liver, blood and sputum specimens for staining.

3.a.3. Reagents

Gram stain kit (Fisher Scientific or equivalent supplier)
Control slide (Fisher Scientific or equivalent supplier)

3.a.4. Materials

Microscope slides
Gas or alcohol burner
Staining rack for slides
Microscope with high power and oil immersion objectives

3.a.5. Procedure

Prepare a thin smear from the specimen on the slide. Let the specimen dry. Heat-fix the smear by passing the slide quickly through a flame; this process kills the bacterial cells and binds the bacterial proteins to the slide.

Proceed with staining following kit instructions, or individual laboratory standard protocol. Air dry; examine the smear by using the oil immersion objective on the microscope (800-1000X magnification). The slide may be stored at ambient room temperature after removing the immersion oil, or discarded into a disposal receptacle for glass.

3.a.6. Interpretation

The presence of 1.0-µm X 0.5-µm, fat, gram-negative (pink), and sometimes bipolar-staining bacilli, seen mostly as single cells or short-chains (Figure 2), along with clinical symptoms compatible with plague, suggests that the specimen may contain plague. The presence of a gram-negative, bipolar-staining bacilli alone is not enough to identify the specimen as containing plague organisms. Efforts should be made to isolate *Y. pestis* by inoculating the specimen in sheep blood agar (SBA) plates and brain heart infusion broth (BHI). If growth on SBA and BHI yields culture with characteristics suggestive of *Y. pestis*, the culture should be forwarded to the state health laboratory for definitive identification.

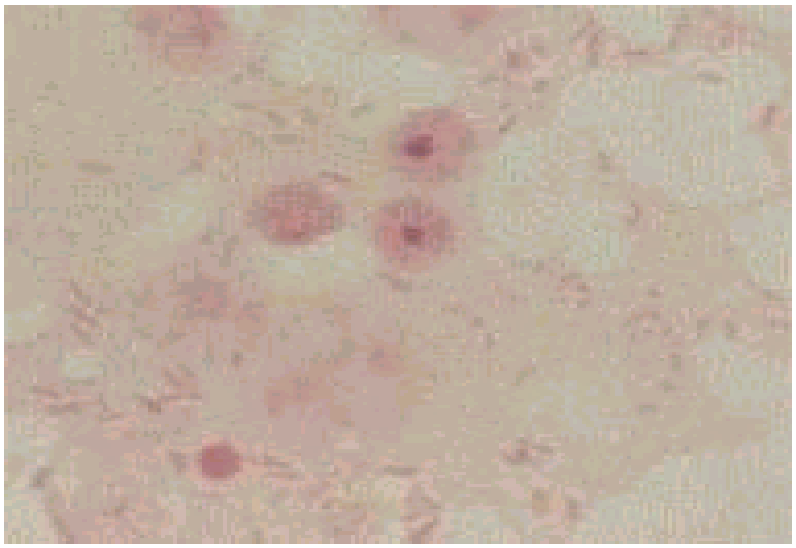

**Figure 2.** Gram stain of mouse liver tissue infected with *Y. pestis*, 800X

3.a.7. Quality control

Gram staining of test specimens should be carried out with parallel staining of known gram-positive (e.g., *Staphylococcus aureus* or *Bacillus subtilis*) and gram-negative (e.g., *Escherichia coli* or *Klebsiella pneumoniae*) organisms to ensure proper staining results. If this is a frequently performed procedure in the laboratory, parallel staining once a week would be adequate. Reagents, such as iodine and alcohol, should be fresh and the crystal violet stain should be kept free of yeast contamination by filtration. The microscope lens and objectives should be kept dust-free and oil-free. The microscope light source should be aligned to deliver the fullest light. The microscope mirrors should be free of dust and fungus growth.

## 3.b. Differential Stains (Wayson, Wright/Giemsa)

### 3.b.1. Perspective

Polychromatic differential stains such as Wayson and Wright-Giemsa provide contrast staining so that bacteria, tissue, and blood cell components are easily seen. Basic fuchsin and methylene blue in the Wayson stain bind to the bacterial cells which, upon staining, appear as pink-blue cells with granules at end of the cell, thus making the cell look like a closed safety pin. Wright-Giemsa stain, typically used in clinical laboratories for differentiating blood cell types, binds to *Y. pestis* cells (Figure 3) in much the same way as methylene blue, yielding bipolar-staining *Y. pestis* cells. This differential polychromatic appearance can be seen with different types of organisms; therefore, Wayson stain alone is not diagnostic for *Y. pestis*.

### 3.b.2. Application

Wayson stain is used for a rapid assessment of a specimen. The most suitable materials for Wayson staining include a bubo aspirate, sputum, blood smears and tissues (lung, spleen, liver).

### 3.b.3. Reagents

a) Wayson stain
b) Solution A
Basic fuchsin..... 0.2 g
Methylene blue..... 0.75 g
Ethanol (95%)..... 20.0 ml
Mix together and filter through Whatman no.1 paper or equivalent.
c) Solution B
Phenol (aqueous)..... 5.0 ml
Distilled water..... 195.0 ml
Mix solution A with solution B. Store at ambient room temperature in a dark glass bottle for 5 years.
d) Methanol
e) Wright/Giemsa stain kit (Fisher Scientific or equivalent vendor)

### 3.b.4. Materials needed

Microscope slides
Coplin jar for methanol
Gas or alcohol burner
Staining rack for slides
Microscope with high power and oil immersion objectives

### 3.b.5. Procedure

Prepare a thin smear or impression touch-preparation on a slide. Let the specimen dry on the slide. Fix the smear in absolute methanol for a minimum of 3 minutes. Then let the methanol on the slide evaporate completely (methanol fixation yields more contrasting staining than heat fixation). Alternatively, heat-fix the smear, which permits acceptable staining results.

Flood the smear with stain. For Wayson stain, leave on for only 5-10 seconds. For Wright/Giemsa, follow manufacturer's directions. Prolonged staining of smears results in darker backgrounds, which may mask the cells.

Wash the slide in tap water. Let the stained smear air dry. Examine the slide under light microscope by using a high-power or oil immersion objective for adequate magnification. The slide may be saved by removing immersion oil, then storing it at ambient room temperature; or, discard slide into glass disposal receptacle.

3.b.6. Interpretation

Consistent, striking bipolar safety-pin morphology of plump bacilli is characteristic of *Yersinia*, *Pasteurella spp.* and other organisms (Figure 3a, Giemsa; Figure 3b, Wayson). Bipolar appearance is not unique to *Y. pestis*; therefore, specimens taken from areas with a wide variety of normal flora (nasal, pharyngeal, and throat) may lead to mistaken interpretation. All *Y. pestis* may stain as bipolar cells, but all bipolar-staining cells are not *Y. pestis*. Bacterial cells picked from freshly passaged agar/broth growth tend to exhibit very little bipolarity, because the cells are too small; however, upon prolonged incubation, the cells would be more likely to exhibit the characteristic bipolar safety-pin shapes. When stained smear reveals the cells with the characteristic safety-pin morphology, the specimen should be forwarded to the state health department for isolation, identification and evaluation.

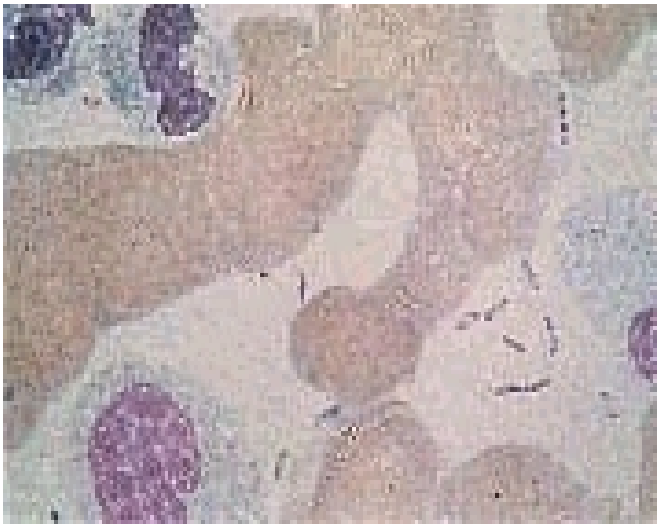

**Figure 3a.** Giemsa stain of blood smear taken from septicemic patient containing *Y. pestis* (800X). Note: bipolar–staining "closed safety pin"-shaped cells

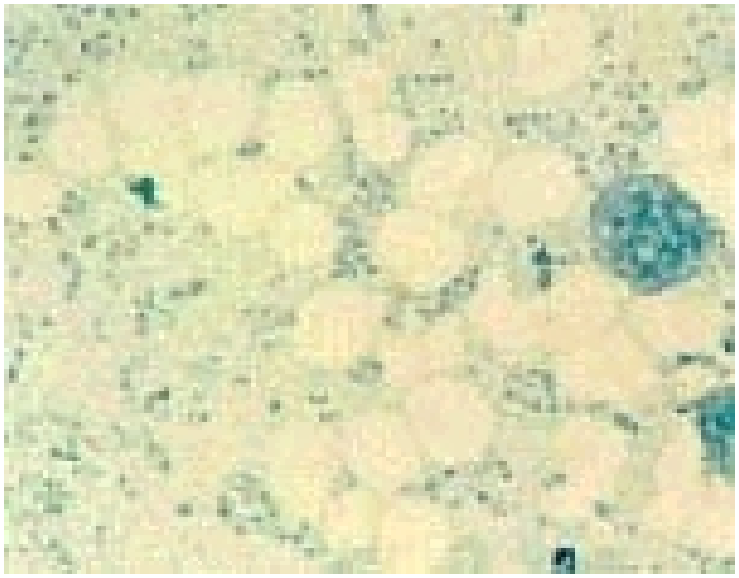

**Figure 3b.** Wayson staining of *Y. pestis* –infected mouse spleen, 1000X

3.b.7. Quality control

Control slides of specimens containing bipolar-staining *Y. pestis* cells and uninfected tissue smears should be prepared, fixed and stored dry at -20° C for use as reference. Each batch of Wayson stain should be tested with both bipolar (e.g., *Yersinia/Pasteurella spp.*, and *E. coli*) and non-bipolar-staining cells (e.g., *B. subtilis* and *Francisella tularensis*).

## 3.c. Growth Characteristics in Brain Heart Infusion Broth

### 3.c.1. Perspective

*Y. pestis* grows well in nutrient-rich broth such as brain heart infusion (BHI), trypticase soy or nutrient broth. The organisms exhibit a characteristic growth formation in clear broth, whose appearance may be used as an aid to its identification. Because of its slower growth, *Y. pestis* may be quickly overwhelmed by contaminants but the characteristic clumped growth may still be seen in the broth tube growth. Inoculation in clear, enriched media such as BHI may assist in the recovery of *Y. pestis* but is not critical to isolation.

### 3.c.2. Application

Specimens taken from clinical materials or pure cultures should be inoculated into two broth tubes and incubated at 28° C (for faster growth) and at 37° C (for expression of the diagnostic F1 antigen) as part of the isolation or the confirmation procedure.

### 3.c.3. Reagents

Brain heart infusion broth (BHI) (Difco Laboratories or equivalent supplier). The height of broth in the tube must reach at least 5 cm to visualize the characteristic growth of *Y. pestis*.

### 3.c.4. Materials

Sterile bacteriologic loops or sterile wooden sticks/toothpicks
Incubators: 28° C and 35-37° C
Pipets
Test tube racks

### 3.c.5. Procedure

Clinical specimens and pure culture: Two broth tubes should be inoculated with a small sample and incubated at 28° C and at 35-37° C, respectively without agitation. After incubation for 24-48 hours, carefully remove tubes, without agitation, from the incubator, and examine for the characteristic growth pattern. After completion of work, the inoculated broth tubes should be decontaminated by autoclaving before discarding the fluids and washing.

### 3.c.6. Interpretation

After 24-48 hours of incubation, the cultures in broth can be described as suspended flocculent or crumbly clumps ("stalactites"). These clumps are visible at the side and bottom of the tube with the rest of the medium remains clear (Figure 4). Longer incubation will result in the clumps of cells falling to the bottom of the tube and loss of the characteristic formation, but the medium above will still remain clear. The characteristic formation of *Y. pestis* cells can be seen in broth culture even if the culture is contaminated; the broth will be cloudy but the clumps will be visible.

The presence of clumped growth on the side of the broth tube and an appropriate clinical history, suggests this may be *Y. pestis*. Also, *Y. pseudotuberculosis* and *Streptococcus pneumoniae* may exhibit the same type of clumping; therefore, this characteristic growth formation in broth is not a confirmatory indicator of *Y. pestis*. Highly passaged *Y. pestis* strains adapted to laboratory media may lose the capacity to form clumps in broth. Additional culture isolation on sheep blood agar and further evaluation by the state health laboratory for identification should be undertaken to validate the observation.

**Figure 4.** BHI broth tubes inoculated with *Y. pestis* (left), and *Y. pseudotuberculosis* (right), at 48 hours

3.c.7. Quality control

Each lot of broth should be tested for its ability to support both control gram-positive/negative strains as well as a known *Y. pestis* strain and to show the characteristic growth pattern.

3.d. Growth characteristics on Sheep Blood Agar

3.d.1. Perspective

Sheep blood agar (SBA) is a general bacteriologic medium used for the isolation and examination of colonial morphology of bacterial organisms. *Y. pestis* organisms are not fastidious and will grow well on any nutrient medium including SBA. Plague bacilli grow slower than most bacteria at 37° C, but at 28° C they will grow faster than most. Enrichment of medium with 6% sheep red blood cells instead of the standard 5% provides more nutrition and shortens the incubation period. Even though *Y. pestis* may grow faster at 28° C, a plate should also be incubated at 37° C since diagnostic tests for plague depend primarily on expression of the temperature-regulated F1 antigen.

SBA plates are used as the standard solid medium for the isolation and culture of *Y. pestis*. If, however, SBA plates are not available, other general solid medium such as BHI agar, nutrient agar or trypticase soy agar may be used, though growth of the organism will be slower and colonies will be smaller.

3.d.2. Application

For isolation and propagation of *Y. pestis*, clinical specimens or pure cultures are streaked onto two SBA plates for incubation at both 28° C and at 37° C.

3.d.3. Reagents

Sheep blood agar plates (SBA, 4%-6% sheep blood) (Difco Laboratories, Fisher Scientific or equivalent supplier)

3.d.4. Materials

Sterile bacteriologic loops or wood sticks/toothpicks
Incubators: 28° C and 37° C
Microscope, stereoscope 4X magnification
Tape, paper

3.d.5. Procedure

For cultures, use the sterile loop/stick to inoculate SBA plates. For tissues, the samples are obtained by using the sterile wood stick to punch into the tissue several times, especially in visibly necrotic areas, and then transferring the materials on the wood stick to the agar surface. Inoculate two SBA plates and streak to obtain isolated colonies.

For safety, tape the top and bottom of the petri dish together in two places to keep them together; incubate plates, one at 28° C (for faster growth) and another at 37° C (for F1 antigen expression), for at least 24-48 hours. Examine plates for characteristic colonies. After completion of work, decontaminate the SBA by autoclaving before discarding the contents.

3.d.6. Interpretation

*Y. pestis* grows as grey-white, translucent colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours, colonies are about 1-2 mm in diameter, grey-white to slightly yellow color and opaque. Highly passaged and laboratory-adapted strains grow faster and colonies are larger. Under 4X enlargement, after 48-72 hours of incubation, colonies have a raised, irregular "fried egg" morphology, which becomes more prominent as the culture ages (Figure 5a). Colonies also can be described as having a "hammered copper," shiny surface (Figure 5b). There is little or no hemolysis of the sheep red blood cells. The characteristic growth on SBA should also be further examined with more specific diagnostic tests. SBA plates should be forwarded to the state health laboratory.

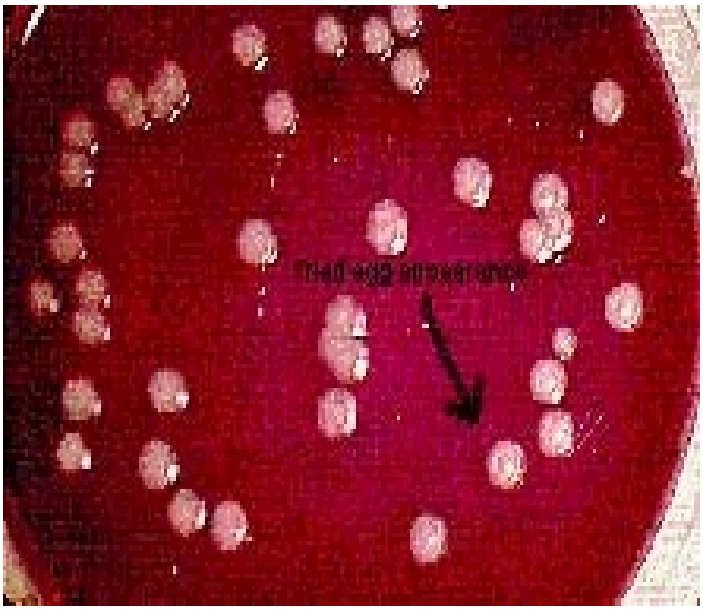

**Figure 5a.** 72-hour *Y. pestis* culture with characteristic "fried egg" morphology

**Figure 5b.** 48-hour *Y. pestis* culture with characteristic “hammered copper” morphology

3.d.7. Quality control

SBA can either be purchased commercially or made in the laboratory under aseptic conditions. Each batch of plates must be checked for sterility by incubating plates at 37° C for at least 24 hours. Test each batch of plates to ensure that they can support bacterial growth using a panel of control strains (e.g., *S. aureus*, *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*). Supplies of reliable sheep red blood cells may be difficult to obtain. In this case, nutrient-rich medium may be used instead of SBA, keeping in mind that the growth rate may be slower and the colony size may be smaller.

**4. Actions if a presumptive *Y. pestis* colony is identified and suspected as a bioterrorist threat agent**

4.a. Preserve original specimens pursuant to a potential criminal investigation.
4.b. Contact the local FBI, state public health laboratory, and the state public health department.
4.c. Local FBI agents will forward isolates to a state health department laboratory as is necessary. Consultation with a state health department laboratory is strongly encouraged as soon as *Y. pestis* is suspected as a bioterrorist threat agent.

**5. Listed vendors**

Difco Laboratories, 800 521-0851
Fisher Scientific, 800-766-7000
BD Bioscience [BBL], 800-675-0908

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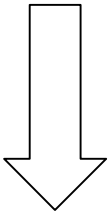
## IV. Appendix: General overview flowchart

### ***Yersinia pestis*** **Presumptive Identification Flowchart**

**Suspected Identification of *Yersinia pestis***

Clinical findings, Exposure history, and
Gram stain:
(gram-negative, bipolar staining bacteria)



**Basis for Presumptive Identification and Notification of Authorities**

Wayson staining showing “safety pin” coccobacilli
Distinctive “hammered copper” or “fried egg” colonial morphology
Clumped growth in broth culture that leaves medium clear

**Notify** Submitting Agency, Local FBI,
State Public Health Laboratory, and State Public Health Department