

**BASIC
LABORATORY PROTOCOLS**

**FOR THE PRESUMPTIVE
IDENTIFICATION OF**

Bacillus anthracis

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.

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I. Introduction

1. Overview

Anthrax is a zoonotic disease that is transmissible to humans through handling or consumption of contaminated animal products. The etiologic agent of anthrax, *Bacillus anthracis*, is a spore forming gram-positive bacillus. Although anthrax can be found globally in temperate zones, it is more often a risk in countries with less standardized and less effective public health programs. Areas currently listed as high risk are South and Central America, Southern and Eastern Europe, Asia, Africa, the Caribbean, and the Middle East. In these regions, herbivorous wildlife mammals, such as deer, wildebeest, elephants, and domesticated livestock, such as goats, sheep, cattle, horses, and swine, are at highest risk for disease. These animals usually become infected while grazing on contaminated land, eating contaminated feed or drinking from contaminated water holes. *B. anthracis* spores can remain viable in soil for many years. Anthrax infrequently occurs in livestock in North America; however, anthrax outbreaks have been reported among deer from Louisiana and Texas up through the Midwest and among wood buffalo in the Northwest Territory in Canada. Animal infections in the United States are reported most often in Texas, Louisiana, Mississippi, Oklahoma, and South Dakota. Birds, amphibians, reptiles, and fish are not directly susceptible to anthrax infection. However, some carnivorous mammals, such as dogs, lions, and omnivorous mammals such as swine, may be susceptible to anthrax infection through consumption of meat from infected animals.

2. Human infection

Humans can become infected with *B. anthracis* by handling products or consuming undercooked meat from infected animals. Infection may also result from inhalation of *B. anthracis* spores from contaminated animal products such as wool or the intentional release of spores during a bioterrorist attack. Human-to-human transmission has not been reported. Three forms of anthrax occur in humans: cutaneous, gastro-intestinal, and inhalational.

2.a. Cutaneous anthrax

Cutaneous infections occur when the bacterium or spore enters a cut or abrasion on the skin, such as when handling contaminated wool, hides, leather or hair products (especially goat hair) from infected animals. Skin infection begins as a raised itchy bump or papule that resembles an insect bite. Within 1-2 days, the bump develops into a fluid-filled vesicle, which ruptures to form a painless ulcer (called an eschar), usually 1-3 cm in diameter, with a characteristic black necrotic (dying) area in the center. Pronounced edema is often associated with the lesions because of the release of edema toxin by *B. anthracis*. Lymph glands in the adjacent area may also swell. Approximately 20% of untreated cases of cutaneous anthrax result in death either because the infection becomes systemic or because of respiratory distress caused by edema in the cervical and upper thoracic regions. Deaths are rare following appropriate antibiotic therapy, with lesions becoming sterile within 24 h and resolving within several weeks.

2.b. Gastrointestinal anthrax

The gastrointestinal form of anthrax may follow the consumption of contaminated meat from infected animals and is characterized by an acute inflammation of the intestinal tract. Initial signs of nausea, loss of appetite, vomiting, and fever are followed by abdominal pain, vomiting of blood, and severe diarrhea. The mortality rate is difficult to determine for gastrointestinal anthrax but is estimated to be 25%-60%.

2.c. Inhalation anthrax

This form of anthrax results from inhaling *B. anthracis* spores, and is most likely following an intentional aerosol release of *B. anthracis*. After an incubation period of 1-6 days (depending on the number of inhaled spores), disease onset is gradual and nonspecific. Fever, malaise, and fatigue may be present initially, sometimes in association with a nonproductive cough and mild chest discomfort. These initial symptoms are often followed by a short period of improvement (ranging from several hours to days), followed by the abrupt development of severe respiratory distress with dyspnea (labored breathing), diaphoresis (perspiration), stridor (high-pitched whistling respiration), and cyanosis (bluish skin color). Shock and death usually occur within 24-36 h after the onset of respiratory distress, and in later stages, mortality approaches 100% despite aggressive treatment. Physical findings are usually nonspecific. The chest X-ray is often pathognomonic (disease-specific) revealing a widened mediastinum with pleural effusions, but typically without infiltrates.

B. anthracis can be detected by Gram stain of the blood and by blood culture with routine media, but often not until late in the course of the illness. Only vegetative encapsulated bacilli are present during infection. Spores are not found within the blood, partially because CO₂ levels in the body inhibit sporulation. Studies of inhalation anthrax in non-human primates (i.e., rhesus monkeys) showed that bacilli and toxins appear in the blood within 2-3 days of exposure. The appearance of toxins coincides with the appearance of bacilli in the blood, and tests are available to rapidly detect the toxins.

3. Antibiotic therapy

Most *B. anthracis* strains are sensitive to a broad range of antibiotics. Penicillin, ciprofloxacin, or doxycycline are usually recommended for the treatment of anthrax. To be effective, treatment should be initiated early. If left untreated, the disease is highly fatal.

4. Anthrax vaccine

An anthrax vaccine for humans is licensed for use in the United States. The vaccine is a cell-free filtrate that contains protective antigen and alum. The vaccine is reported to be 93% effective in protecting against cutaneous anthrax. Animal studies have suggested that the vaccine may also be protective against aerosol challenge. The anthrax vaccine is distributed by BioPort Corporation, Lansing, Mich.

B. anthracis is considered a potential biological warfare threat agent. The U.S. Department of Defense recommends anthrax vaccination of all U.S. active duty military personnel. According to the Advisory Committee for Immunization Practices (ACIP) civilians who should receive anthrax vaccine include persons who come in contact in the workplace with imported animal hides, furs, bonemeal, wool, animal hair (especially goat hair), and bristles and persons engaged in diagnostic or investigational activities which may put them in contact with anthrax spores. The vaccine should be administered only to healthy men and women aged 18-65 years since all studies to date have been conducted exclusively in that population. Pregnant women should not be vaccinated, because it is not known whether the anthrax vaccine can cause fetal harm.

The anthrax vaccination protocol consists of 3 subcutaneous injections given 2 weeks apart followed by 3 additional subcutaneous injections given at 6, 12, and 18 months. Annual booster injections of the vaccine are required to maintain immunity. Mild local reactions consisting of slight tenderness and redness at the injection site of the skin occur in approximately 30% of recipients. A moderate local reaction can occur if the vaccine is given

to anyone with a past history of anthrax. Severe local reactions occur infrequently and consist of extensive swelling of the forearm in addition to the local reaction. Systemic reactions characterized by flu-like symptoms occur in fewer than 0.2% of vaccinees.

II. Laboratory Procedures for the identification of *Bacillus anthracis*

1. General

The procedures described below function to rule out presumptively identified *B. anthracis* in clinical specimens or isolates. These procedures should be performed in microbiology laboratories that utilize Biological Safety Level 2 (BSL 2) practices. Laboratory coats and gloves shall be worn when processing specimens and performing tests. Safety glasses or eye shields are recommended. Any activities that bring hands in contact with mucosal surfaces (for example, eating, drinking, smoking, or applying make-up) are prohibited. Hands should be washed before leaving the laboratory. Anthrax vaccination is not required.

Disclaimer. Names of vendors or manufacturers are provided as examples of suitable product sources and inclusion does not imply endorsement by the Centers for Disease Control and Prevention, the United States Public Health Service, Department of Health and Human Services, the United States Army, or the Federal Bureau of Investigation.

1.a. Handling of samples

For safety considerations, analysis of samples for biological threat agents is performed within a certified Class II biological safety cabinet (BSC). Procedures requiring removal of items from a BSC, such as slides for microscopy, should follow published microbiological practices and precautions. When using a BSC, assure that the cabinet does not contain unnecessary items that will interfere with proper airflow and function. As for any procedure involving infectious materials, standard personal protective gear should be used, such as latex gloves and laboratory coats, or disposable over garments. Additional respiratory protection should also be considered with materials or analytical procedures determined to be potentially hazardous outside the BSC. Once a biological agent has been identified, modifications in handling of samples can then be considered.

1.b. Decontamination

Commercially available household bleach solutions contain 5.25% hypochlorite and, when diluted 1:10, are effective in routine decontamination of surfaces and instruments after working with *B. anthracis*. Contaminated items such as pipettes, needles, loops, and microscope slides should be immersed in decontamination solution until autoclaving. Work surfaces, such as a biological safety cabinet (BSC), should be wiped down before and after use with decontamination solution. The method of decontamination of a spill depends upon the nature of the spill. Spills involving fresh cultures or samples known to have low concentrations of spores should be flooded with decontamination solution and soaked for 5 min before cleanup. Spills that involve samples with high concentrations of spores, involve organic matter, or occur in areas of lower than room temperature (refrigerators, freezers) should be exposed to decontamination solution for at least 1 h before cleanup. Personnel involved in the cleanup of any spill should wear gloves, safety glasses, and a laboratory coat or gown during the cleanup process. Respiratory protection should be considered for spills in which a substantial aerosolization is suspected.

2. Collection of clinical specimens

2.a. Materials

Sporocidal disinfectant
Sputum cup

Sterile cotton swabs
Blood culture collection kit
Stool collection cup

2.b. Cutaneous anthrax

- 2.b.1. Vesicular stage: The organism is best demonstrated in this stage. Soak two dry sterile swabs in vesicular fluid from a previously unopened vesicle.
2.b.2. Eschar stage: Rotate two swabs beneath the edge of the eschar without removing the eschar.

2.c. Gastrointestinal anthrax

- 2.c.1. If the patient is able to produce a stool specimen, stool cultures should be performed. Obtain a stool culture.
2.c.2. In later stages of disease, blood cultures will yield the organism, especially if specimens are obtained prior to antibiotic treatment.

2.d. Inhalation anthrax

- 2.d.1. If respiratory symptoms are present and sputum is being produced, obtain a specimen for culture and smear.
2.d.2. In later stages of disease (2-8 days post exposure) blood cultures may yield the organism, especially if specimens are drawn before antibiotic treatment.

3. Materials needed for processing of clinical specimens

5% Sheep blood agar plates [SBA] (BD Bioscience or Remel, Inc. or equivalent)
MacConkey agar plates (BD Bioscience or Remel, Inc. or equivalent)
Phenyl ethyl alcohol agar (PEA) plates (for stool specimens)(BD Bioscience or Remel, Inc. or equivalent)
Trypticase soy broth (BD Bioscience or Remel, Inc. or equivalent)
Clean glass microscope slides
Sterile cotton swabs (commercially available specimen transport swabs for aerobic culture are preferred)
Disposable bacteriologic inoculation loops
Clinical centrifuge with appropriate biocontainment tube holders
Sporicidal disinfectant (0.5% sodium hypochlorite or 0.5% calcium hypochlorite)

4. Isolation from clinical specimens

4.a. Sputum specimens

Inoculate 3 routine media for sputum specimens (i.e. SBA, MacConkey agar, broth enrichment).

4.b. Blood specimens

- 4.b.1. Routine blood culture methods are sufficient.
4.b.2. There may be enough organisms in the blood to see them on direct smears by Gram stain. *B. anthracis* appears as short chains of 2-4 cells which are encapsulated as evidenced by clear zones around the bacilli. The presence of large encapsulated gram-positive rods in the blood is strongly presumptive for *B. anthracis* identification.

4.b.3. If blood culture bottle is positive, perform a Gram stain directly and observe for encapsulated rods. These blood cultures should also be subcultured to SBA and MacConkey agar plates.

4.c. Swab specimens

4.c.1. Use one swab to inoculate 3 standard media for surface wounds (e.g., SBA, MacConkey agar, or broth enrichment).

4.c.2. Prepare a smear for Gram staining with the second swab.

4.d. Stool specimens

4.d.1. Routine stool culture methods are sufficient (e.g., SBA, MacConkey agar, or PEA plates).

4.d.2. Do not use CVA or hectone agar plates.

4.e. CSF specimens

4.e.1. If a clinical centrifuge with appropriate biocontainment tube holders is available, centrifuge the CSF specimen at 1500 X g for 15 minutes.

4.e.2. Collect the sediment and prepare a smear for Gram staining.

4.e.3. Inoculate the remainder of the sediment onto SBA and broth enrichment media (tryptic soy broth or thioglycollate).

5. Incubation and examination of cultures

5.a. Cultures should be incubated at 35-37° C under ambient conditions.

5.b. Cultures should be examined within 18-24 h of incubation. Growth of *B. anthracis* may be observed as early as 8 h after inoculation.

6. Differential tests for the presumptive identification of *B. anthracis*

6.a. Colony characteristics of *B. anthracis*

6.a.1. After incubation of SBA plates for 15-24 h at 35-37° C, well isolated colonies of *B. anthracis* are 2-5 mm in diameter. The flat or slightly convex colonies are irregularly round, with edges that are slightly undulate (irregular, wavy border), and have a ground-glass appearance. There are often comma-shaped projections from the colony edge, producing the "Medusa head" colony.

6.a.2. Colonies on SBA usually have a tenacious consistency. When teased with a loop, the growth will stand up like beaten egg white. In contrast to colonies of *B. cereus* and *B. thuringiensis*, colonies of *B. anthracis* are not β -hemolytic. However, weak hemolysis may be observed under areas of confluent growth in aging cultures and should not be confused with β -hemolysis.

6.a.3. When examining primary growth media, it is important to compare the extent of growth on SBA plates with that on MacConkey agar plates. *B. anthracis* grows well on SBA, but does not grow on MacConkey agar. *B. anthracis* grows rapidly; heavily inoculated areas may show growth within 6-8 h and individual colonies may be detected within 12-15 h. This trait can be used to isolate *B. anthracis* from mixed cultures containing slower-growing organisms.



Figure 1. *B. anthracis* and *B. cereus* colony morphology. Overnight cultures of *B. cereus* (left side of plate) and *B. anthracis* (right side) on SBA.

6.b. Gram stain morphology of *B. anthracis*

6.b.1. Procedure

Perform Gram stain by usual procedures.

6.b.2. Interpretation of results

B. anthracis is a large gram-positive rod (1-1.5 X 3-5 μm) that forms oval, central-to-subterminal spores (1 X 1.5 μm) on SBA that do not cause significant swelling of the cell. Spores are not present in clinical samples unless exposed to atmospheric levels of CO_2 ; CO_2 levels within the body inhibit sporulation. Vegetative cells seen on Gram stain of blood and impression smears are in short chains of 2-4 cells that are encapsulated. However, cells from growth on SBA under ambient conditions, are not encapsulated and occur as long chains of bacilli. When grown on nutrient agar in the presence of 5% CO_2 or on other basal media supplemented with 0.8% sodium bicarbonate, virulent strains will yield heavily encapsulated bacilli. The capsule can be visualized microscopically using India Ink.

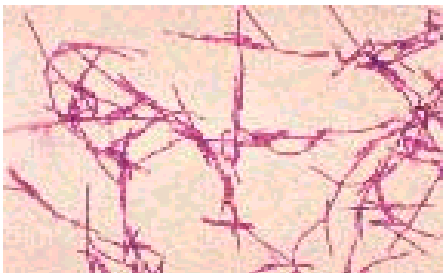


Figure 2. Gram stain of *B. anthracis* from SBA
Magnification 1,000X.

6.c. India ink staining of clinical samples (blood and CSF) for capsule

6.c.1. Purpose

India ink is useful for improving visualization of encapsulated *B. anthracis* in clinical samples such as blood, blood culture bottles, or cerebrospinal fluid (CSF).

6.c.2. Materials

Microscope slides

Cover glasses

India ink (Bactidrop, Remel, Inc., Catalog # 21-518 or equivalent).

Microscope with 100X oil immersion objective

6.c.3. Controls

a) Control strains

(1) Positive control: *Klebsiella pneumoniae* on SBA or equivalent

(2) Negative control: *E. coli* ATCC 25922 or equivalent

b) Method controls: Perform the test with suspensions of fresh cultures of the control strains. Control strains should be assayed on each day of testing.

c) Resolving an out-of-control result: check the purity and identity of the control strains and repeat the test.

6.c.4. Procedure

a) For the controls, transfer a small amount of growth (1 mm diameter) from each control SBA plate into 0.5 ml whole EDTA-treated blood or serum. Mix.

b) For the unknowns, take 100 μ l of sample (blood, CSF)

c) Transfer 5-10 μ l of unknown or control to a slide, place a cover glass on the drop, and then add 5-10 μ l of India ink to the edge of the cover glass.

d) After the ink diffuses across, view the cells using 100X oil immersion objective with oil on top of the cover glass.

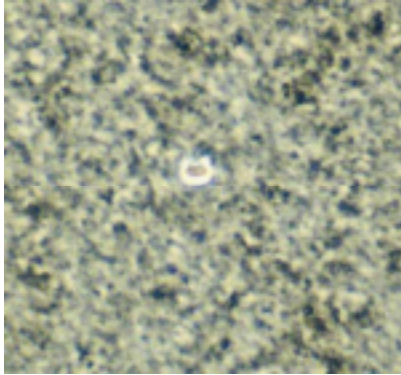


Figure 3. India ink staining of *B. anthracis*

6.c.5. Interpretation of results

The capsule will appear as a well-defined clear zone around the cells for the positive control. No zone should be present in the negative control.

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United States Army Medical Research Institute of Infectious Diseases

6.d. Motility test: Wet mount or motility medium

6.d.1. Purpose

This test determines the motility of suspect isolates. *B. anthracis* is a nonmotile species. This characteristic is unusual among *Bacillus* species and is therefore useful in the preliminary identification of *B. anthracis* isolates. Two methods are given: the wet mount and the tubed motility test.

6.d.2. Materials

a) For wet mount procedure

Precleaned microscope slides

Cover glasses

Sterile distilled water

Disposable bacteriologic inoculating loop

Light microscope with 40X objective and 10X eyepiece

Sterile glass tube

b) For tubed motility test

Tubed motility media (Remel, Inc., Catalog # 06-1408 or equivalent), 5 ml per tube

Sterile disposable 1 µl inoculating loop or needle

6.d.3. Controls

a) Control strains

(1) Positive control: *Pseudomonas aeruginosa* ATCC 35032 or equivalent

(2) Negative control: *Acinetobacter* spp. ATCC 49139 or equivalent

b) Method controls: Perform the test with fresh cultures of the control strains using the same method as with unknowns. Control strains should be assayed on each day of testing.

c) Resolving an out-of-control result: Check the purity and identity of the control strains and repeat the test.

6.d.4. Procedures

Wet mount and motility

a) Wet mount procedure

(1) Deliver 2 drops (approximately 0.1 ml) of sterile distilled water into the sterile glass tube.

(2) Using the inoculating loop, sample a suspicious colony from a 12-20 h culture and suspend the growth in the water. (Alternatively, a loopful of medium from a fresh broth culture can be used).

(3) Transfer 1 drop of the suspension to the microscope slide and overlay with the cover glass.

(4) Examine the slide under the microscope using the 40X objective (total magnification = 400X).

(5) Discard slides in 0.5% hypochlorite solution.

b) Motility test medium procedure

(1) Using the sterile inoculating needle, remove some growth from an isolated, suspicious colony of an 18-24 h culture.

(2) Inoculate the motility tube by carefully stabbing the needle 3-4 cm into the medium and then drawing the needle directly back out so that a single line of inoculum can be

observed.

(3) Incubate the tube aerobically at 35-37° C for 18-24 h.

c) Interpretation of results

(1) For wet mount: Motile organisms can be observed moving randomly throughout the suspension. Nonmotile organisms either fail to move or move with Brownian motion.

(2) For motility test medium: Nonmotile organisms, such as *B. anthracis*, will form a single line of growth that does not deviate from the original inoculum stab. Motile organisms will form a diffuse growth zone around the inoculum stab.

7. Presumptive identification key for *B. anthracis*

7.a. From clinical samples, such as blood, CSF, or lesion material: encapsulated gram-positive rods.

7.b. Gram-positive, broad rod, spore-positive: *Bacillus* species.

7.c. Spores are nonswelling and oval shaped; ground glass appearance of colonies: *Bacillus* morphology group 1 (includes *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. cereus* var. *mycoides*)

7.d. Nonmotile: *B. anthracis* and *B. cereus* var. *mycoides*

7.e. Nonhemolytic: presumptive *B. anthracis*

8. Actions if a presumptive *B. anthracis* colony is identified and suspected as a bioterrorist threat agent

8.a. Preserve original specimens pursuant to a potential criminal investigation.

8.b. Contact local FBI, state public health laboratory, and state public health department.

8.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 *B. anthracis* is suspected as a bioterrorist threat agent.

9. Listed vendors

American Type Cell Culture [ATCC], 800-638-6597

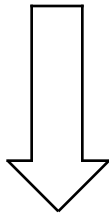
Remel, Inc., 800-255-6730

Becton-Dickinson Bioscience [BD], 800-675-0908

III. Appendix

***Bacillus anthracis* ID Overview Flowchart**

**Presumptive ID
of
*Bacillus anthracis***
(Gram stain,
Hemolysis, Motility)



**Basis for ID and
Actions**
Capsulated,
Spore-forming,
Gram- positive Rod
in Blood Culture
Notify Local FBI,
state public health
laboratory, and state
public health
department.