



Laboratory Procedures for the identification of *Bacillus anthracis*

General

The procedures described below function to rule out presumptively identified *Bacillus 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.**

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.

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 minutes 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 hour 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.

Collection of clinical specimens

Materials required:

- Sporocidal disinfectant
- Sputum cup
- Sterile cotton swabs
- Blood culture collection kit
- Stool collection cup

Cutaneous anthrax

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

Gastrointestinal anthrax

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

Inhalational anthrax

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

Materials and equipment needed for processing clinical specimens

- Class II Biological Safety Cabinet
- 5% Sheep blood agar plates [SBA] (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)
- Motility media
- Microscope
- 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)
- 37 °C incubator

Isolation from clinical specimens

Sputum specimens

- Inoculate 3 routine media for sputum specimens (i.e. SBA or broth enrichment).

Blood specimens

- Routine blood culture methods are sufficient.
- 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 in which clear zones around the bacilli may be evident. The presence of large encapsulated gram-positive rods in the blood is strongly presumptive for *B. anthracis* identification.
- If blood culture bottle is positive, perform a Gram stain directly and observe for rods. These blood cultures should also be subcultured to SBA plates.

Swab specimens

- Use one swab to inoculate 3 standard media for surface wounds (e.g., SBA or broth enrichment).
- Prepare a smear for Gram staining with the second swab.

Stool specimens

- Routine stool culture methods are sufficient (e.g., SBA or PEA plates).
- Do not use CVA or hectone agar plates.

CSF specimens

- If a clinical centrifuge with appropriate biocontainment tube holders is available, centrifuge the CSF specimen at 1500 X g for 15 minutes.
- Collect the sediment and prepare a smear for Gram staining.
- Inoculate the remainder of the sediment onto SBA and broth enrichment

Nasal swab specimens (asymptomatic persons with known inhalation exposure to *B. anthracis* or to credible anthrax threats)

- Streak a nasal swab from the patient on SBA and incubate at 37°C overnight. The next day, check for Gamma hemolysis around the colonies and perform Gram stain.
- Non-hemolytic colonies that show gram positive rods upon microscopic examination and are non-motile on the motility media (or by wet mount), are suspected to be *Bacillus* species. Although *B.*

anthracis is a spore-forming bacterium, the bacilli may or may not contain spores.

- In addition, the presence of capsules can be checked microscopically after staining with India ink.

Incubation and examination of cultures

- Cultures should be incubated at 35-37° C under ambient conditions.
- Cultures should be examined within 18-24 hours of incubation.
- Growth of *B. anthracis* may be observed as early as 8 hours after inoculation.

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

Colony characteristics of *B. anthracis*

- After incubation of SBA plates for 15-24 hours 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.
- 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 beta-hemolytic. However, weak hemolysis may be observed under areas of confluent growth in aging cultures and should not be confused with beta-hemolysis.

B. anthracis grows rapidly; heavily inoculated areas may show growth within 6-8 h and individual colonies may be detected within 12-15 hours. This trait can be used to isolate *B. anthracis* from mixed cultures containing slower-growing organisms.

Gram stain (perform by usual procedures)

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 may not always be observed from a 24-hour SBA plate. Absence of spores alone should not be used to rule out *B. anthracis*. Spores are not present in clinical samples unless exposed to atmospheric levels of CO₂; CO₂ 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% CO₂ 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.

Confirmation of *B. anthracis* at the state lab. GPHL has the reagents necessary for direct fluorescent antibody (DFA) test for the capsules and the cell wall polysaccharides. If both stainings are positive, *B. anthracis* is confirmed. In addition, phage lysis can be performed at GPHL as another confirmatory test. The DFA test is a rapid test and only takes 4 hours to complete.

Patient testing should be handled by the local hospital labs and clinics, as a part of their routine procedures described above. In case a local hospital laboratory is unable to perform the presumptive tests, the specimens should be sent to a contract lab and/or to GPHL for testing.

If bioterrorism is suspected, local hospitals and clinics should contact local law enforcement officers/FBI for referring the specimens to GPHL. In addition, any suspected *B. anthracis* culture should be submitted to GPHL for confirmatory testing.

Reference:

<http://www.bt.cdc.gov/Agent/Anthrax/Anthraxis20010417.pdf>

Important Contact information for the GPHL

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