

Level A Laboratory Procedures for Identification of *Bacillus anthracis*

- I. General:** The procedures described below function to rule out or presumptively identify *B. anthracis* from clinical specimens or isolates. These procedures should be performed in microbiology laboratories that use Biological Safety Level-2 (BSL-2) practices.
- II. Precautions:** Refer to Procedure for Laboratory Safety and Decontamination.
- III. Specimen**
- A. Acceptable specimens:** Collect other specimens if/as clinically indicated (e.g., cerebrospinal fluid [CSF], lymph node biopsy). Refer to Appendix for information on nasal swabs.
1. Cutaneous anthrax
 - a. Vesicular stage: Aseptically collect vesicular fluid on sterile swabs from previously unopened vesicles. Note: The anthrax bacilli are most likely to be seen by Gram stain in the vesicular stage.
 - b. Eschar stage: Collect eschar material by carefully lifting the eschar's outer edge; insert a sterile swab, then slowly rotate for 2-3 sec beneath the edge of the eschar without removing it.
 2. Gastrointestinal anthrax
 - a. Blood cultures: Collect appropriate blood volume and number of sets per laboratory protocol. In later stages of disease (2-8 days post-exposure) blood cultures may yield the organism, especially if obtained before antibiotic treatment.
 - b. Stool: Transfer ≥ 5 g of stool directly into a clean, dry, sterile, wide-mouth, leak-proof container.
 - c. Rectal swab: For patients unable to pass a specimen, obtain a rectal swab by carefully inserting a swab 1 inch beyond the anal sphincter.
 3. Inhalational anthrax
 - a. Blood cultures: Collect appropriate blood volume and number of sets per laboratory protocol.
 - b. Sputum: Collect >1 ml of a lower respiratory specimen into a sterile container. Inhalational anthrax usually does not result in sputum formation.
- B. Rejection criteria:** Use standard laboratory criteria.
- C. Specimen transport and storage:** Refer to Shipping Procedure
1. Swabs: Transport directly to laboratory at room temperature. For transport time >1 h, transport at 2-8°C.
 2. Stool: Transport unpreserved stool to laboratory within 1 h. For transport time >1 h, transport at 2-8°C; Cary-Blair or equivalent transport media is acceptable.
 3. Sputum: Transport in sterile, screw-capped container at room temperature when transport time is <1 h. For transport time >1 h, transport at 2-8°C.
 4. Blood culture: Transport directly to laboratory at room temperature.

IV. Materials

A. Reagents

1. Gram stain reagents
2. Catalase reagent (3% hydrogen peroxide)
3. Motility media (or slide, coverslips, saline for wet mount)
4. India ink (an optional test)
5. Sterile saline

B. Media

1. 5% sheep blood agar (SBA) or equivalent
2. Chocolate agar (CA)
3. MacConkey agar (MAC)
4. Phenyl ethyl alcohol agar (PEA)
5. Blood culture bottles
6. Tubed motility media
7. Tryptic soy broth (TSB), or equivalent
8. Thioglycolate broth or equivalent

C. Equipment/miscellaneous

1. Blood culture instrument (optional)
2. Light microscope with 10X, 40X and 100X objectives and 10X eyepiece
3. Microscope slides and coverslips
4. Disposable bacteriologic inoculating loops
5. Incubator, 35-37°C, ambient preferred (CO₂ enriched is acceptable)

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V. Quality control: Document all quality control results for the following tests per standard laboratory procedure/protocol.

VI. Procedure: Refer to Fig. A1a and A1b.

A. Stains and smears

1. Gram stain
 - a. Procedure: Perform Gram stain procedure/QC per standard laboratory protocol.
 - b. Interpretation
 - (1) *B. anthracis* is a large gram-positive rod (1-1.5 X 3-5 µm).
 - (2) Blood and impression smears: Vegetative cells seen on Gram stain of blood and impression smears are in short chains of 2-4 cells that are encapsulated, which may be seen on the Gram stain as clear zones around the bacilli. Spores are not present in clinical samples unless exposed to low CO₂ levels, such as those found in the atmosphere; higher CO₂ levels within the body inhibit sporulation. The presence of large encapsulated gram-positive rods in the blood is strongly presumptive for *B. anthracis* identification. Refer to Fig. A2.

- (3) Growth on SBA or equivalent medium: *B. anthracis* forms oval, central-to-subterminal spores (1 X 1.5 µm) on SBA that do not cause significant swelling of the cell; frequently occur as long chains of bacilli. However, cells from growth on SBA regardless of the incubation conditions (ambient atmosphere or CO₂ enriched) are not encapsulated. Refer to Fig. A3a and Fig. A3b.
2. India Ink (optional procedure)
 - a. Purpose. Used to improve visualization of encapsulated *B. anthracis* in clinical samples such as blood, blood culture bottles, or cerebrospinal fluid (CSF).
 - b. Quality control
 - (1) Positive control strain: *Klebsiella pneumoniae* (or laboratory validated equivalent) will demonstrate a well-defined clear zone on SBA.
 - (2) Negative control strain: *E. coli* ATCC 25922 (or laboratory validated equivalent) will demonstrate no clear zone.
 - (3) Method controls: Perform the test with suspensions of fresh cultures of the control strains. Control strains should be assayed on each day of testing.
 - (4) Resolving out-of-control results: Check media, reagents, controls and equipment; replace or correct as appropriate. Document corrective actions and repeat test.
 - c. Procedure
 - (1) For the controls, transfer a small amount of growth (1 mm diameter) from each control SBA plate (positive control = *Klebsiella pneumoniae*; negative control = *Escherichia coli* ATCC 25922) into 0.5 ml saline and mix.
 - (2) For the unknowns, take 100 µl of sample (blood, CSF). Transfer 5-10 µl of unknown sample or control to a slide. Place a coverslip on the drop, and then add 5-10 µl of India ink to the edge of the coverslip. After the ink diffuses across the slide, view the cells using 100X oil immersion objective with oil on top of the coverslip.
 - d. Interpretation
 - (1) Positive result: The capsule will appear as a well-defined clear zone around the cells.
 - (2) Negative result: No zone will be present.
 - e. Reporting/actions
 - (1) Clinical specimens with encapsulated (visualized with India ink), gram-positive rods provide a presumptive identification of *B. anthracis*.
 - (2) Every effort should be made to obtain an isolate for continued testing and referral to state public health laboratory.
 - f. Limitations
 - (1) Interpretation of results requires trained/experienced staff.
 - (2) A negative test result should not be used to rule out *B. anthracis*.

B. Cultures

1. Inoculation and plating procedure: Inoculate and streak the following media for isolation of the respective specimen types. Note: Standard media should be used according to normal laboratory procedures.
 - a. Blood cultures: Process following routine laboratory protocol.
 - b. Cutaneous swab specimens: Plate directly on media used routinely for surface wounds such as SBA, MAC, and broth enrichment, and prepare smears for staining. Note: *B. anthracis* does not grow on MAC.

- c. Stool: Plate directly on appropriate media, such as PEA, SBA, and MAC.
Note: *B. anthracis* does not grow on PEA.
 - d. Sputum specimens: Plate directly on media used routinely, such as SBA, MAC, and CA, and prepare smears for staining.
2. Incubation
 - a. Temperature: 35-37°C
 - b. Atmosphere: Ambient preferred
 - c. Length of incubation: Hold primary plates for at least 3 days; read daily. Examine plates within 18-24 h of incubation. Growth of *B. anthracis* may be observed as early as 8 h after incubation.
 3. Colony characteristics of *B. anthracis*
 - a. 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 may be often comma-shaped projections from the colony edge, producing the "Medusa-head" colony. Refer to Fig. A4.
 - b. *B. anthracis* colonies on SBA usually have a tenacious consistency. When teased with a loop, the growth will stand up like beaten egg white; refer to Fig. A5. In contrast to colonies of *B. cereus* and *B. thuringiensis*, colonies of *B. anthracis* are not β -hemolytic; refer to Fig. A6. However, weak hemolysis may be observed under areas of confluent growth in aging cultures and should not be confused with β -hemolysis.
 - c. When examining primary growth media, it is important to compare the extent of growth on SBA plates with that on MAC. *B. anthracis* grows well on SBA but does not grow on MAC or PEA.
 - d. *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.
 4. Extent of identification: For the Level A laboratory, identification is limited to 'presumptive' only (see section VII, below, for specific criteria/key characteristics).

C. Motility test: Wet mount or motility medium

1. Purpose: Used to determine motility of suspected isolates; *B. anthracis* is nonmotile. Two methods are given, the wet mount and the motility medium test.
2. Wet mount procedure
 - a. Deliver 2 drops (approximately 0.1 ml) of TSB, or equivalent, into a sterile glass tube. Using an inoculating loop, transfer a portion of the suspect colony from a 12-20 h culture and suspend the growth in the broth medium.
 - b. Alternatively, a loopful of medium from a fresh broth culture can be used.
 - c. Transfer 10 μ l of the suspension to a microscope slide and overlay with a coverslip.
 - d. Examine slide under a microscope using the 40X objective (total magnification 400X; may also be viewed at 1000X with oil objective).
 - e. Discard slide(s) following standard laboratory procedures, such as into 0.5% hypochlorite solution.

3. Motility medium test procedure
 - a. Using a sterile inoculating needle, remove a portion of growth from an isolated, suspect colony after 18-24 h incubation.
 - b. Inoculate the motility medium 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.
 - c. Incubate the tube at 35-37°C in ambient atmosphere for 18-24 h.
4. Interpretation of motility results: Lack of motility is unusual among *Bacillus* species and is therefore useful in the preliminary identification of *B. anthracis* isolates.
 - a. Wet mount
 - (1) Positive result: Motile organisms will be observed moving throughout the suspension. Observe that the movement may be sluggish/slower than that of the positive controls .
 - (2) Negative result: Nonmotile organisms either do not move or move with Brownian motion.
 - b. Motility test
 - (1) Positive result: Motile organisms will form a diffuse growth zone around the inoculum stab.
 - (2) Negative result: Nonmotile organisms, such as *B. anthracis*, will form a single line of growth that does not deviate from the original inoculum stab.
5. Quality control
 - a. Positive control strain: *Pseudomonas aeruginosa* ATCC 35032 or laboratory-validated equivalent will demonstrate motility.
 - b. Negative control strain: *Acinetobacter* spp. ATCC 49139 or laboratory-validated equivalent will show no motility
 - c. 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.
6. Resolving out-of-control results
 - a. Check media, reagents, controls and equipment; replace or correct as appropriate. Document corrective actions and repeat test.
 - b. Check purity and identity of control strains and repeat testing.

VII. Interpretation and reporting

A. Presumptive identification criteria: Refer to Table A1.

1. Direct smears from clinical samples, such as blood, CSF, or skin lesion (eschar) material: Encapsulated gram-positive rods
2. From growth on SBA or equivalent media: Large gram-positive rods (may stain gram-variable after 72 h of culture). Spores may be found in culture, under non-CO₂ atmosphere (but not on direct examination). Spores are nonswelling and oval-shaped.
3. Rapid, aerobic growth, and tenacious colonies on sheep blood agar.
4. Catalase positive
5. Nonmotile: In addition to *B. anthracis*, *B. cereus* var. *mycoides* is nonmotile.
6. Nonhemolytic on SBA, ground-glass appearance of colonies

B. Rule out: While hemolysis, gram stain morphology, or motility can be used for rule out when the result provides clear evidence that the isolate is not *B. anthracis* (e.g., a clearly visible zone of beta hemolysis), a combination of two Level A tests is recommended for rule out.

C. Reporting/action

1. Consult with state public health laboratory director (or designate) if *B. anthracis* is suspected.
2. General instruction and information
 - a. Preserve original specimens pursuant to a potential criminal investigation and possible transfer to an appropriate LRN laboratory.
 - b. Environmental/nonclinical samples and samples from announced events are not processed by Level A Laboratory; submitter should contact the state public health laboratory directly.
 - c. The state public health laboratory/state public health department will coordinate notification of local FBI agents as appropriate.
 - d. Assist local law enforcement efforts in conjunction with guidance received from the state public health laboratory.
 - e. The state public health laboratory/state public health department may request transfer of suspicious specimens prior to presumptive testing.
 - f. 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; refer to Shipping Procedure.
3. Immediately notify state public health laboratory director (or designate) and state public health department epidemiologist/health officer if *B. anthracis* cannot be ruled out and a bioterrorist event is suspected.
4. Immediately notify physician/infection control according to internal policies if *B. anthracis* cannot be ruled out.
5. If *B. anthracis* is ruled out, proceed with efforts to identify using established procedures.

VIII. References

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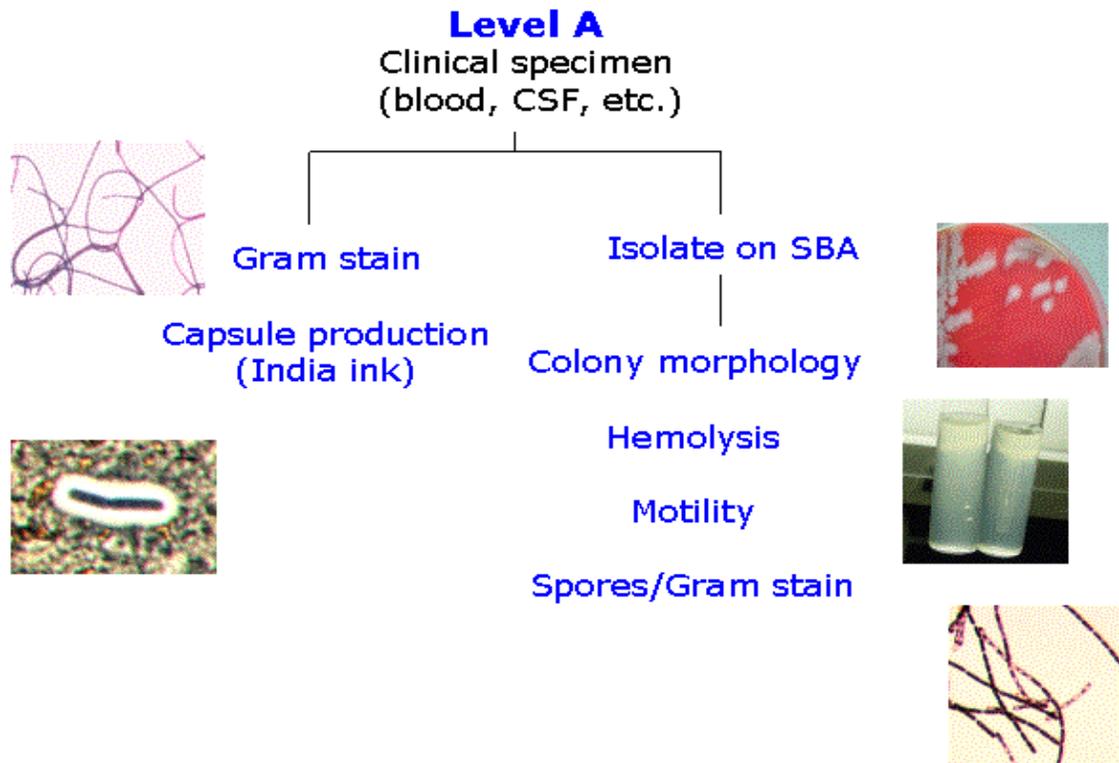
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Figure A1a. Flowchart of *B. anthracis* Level A procedures.



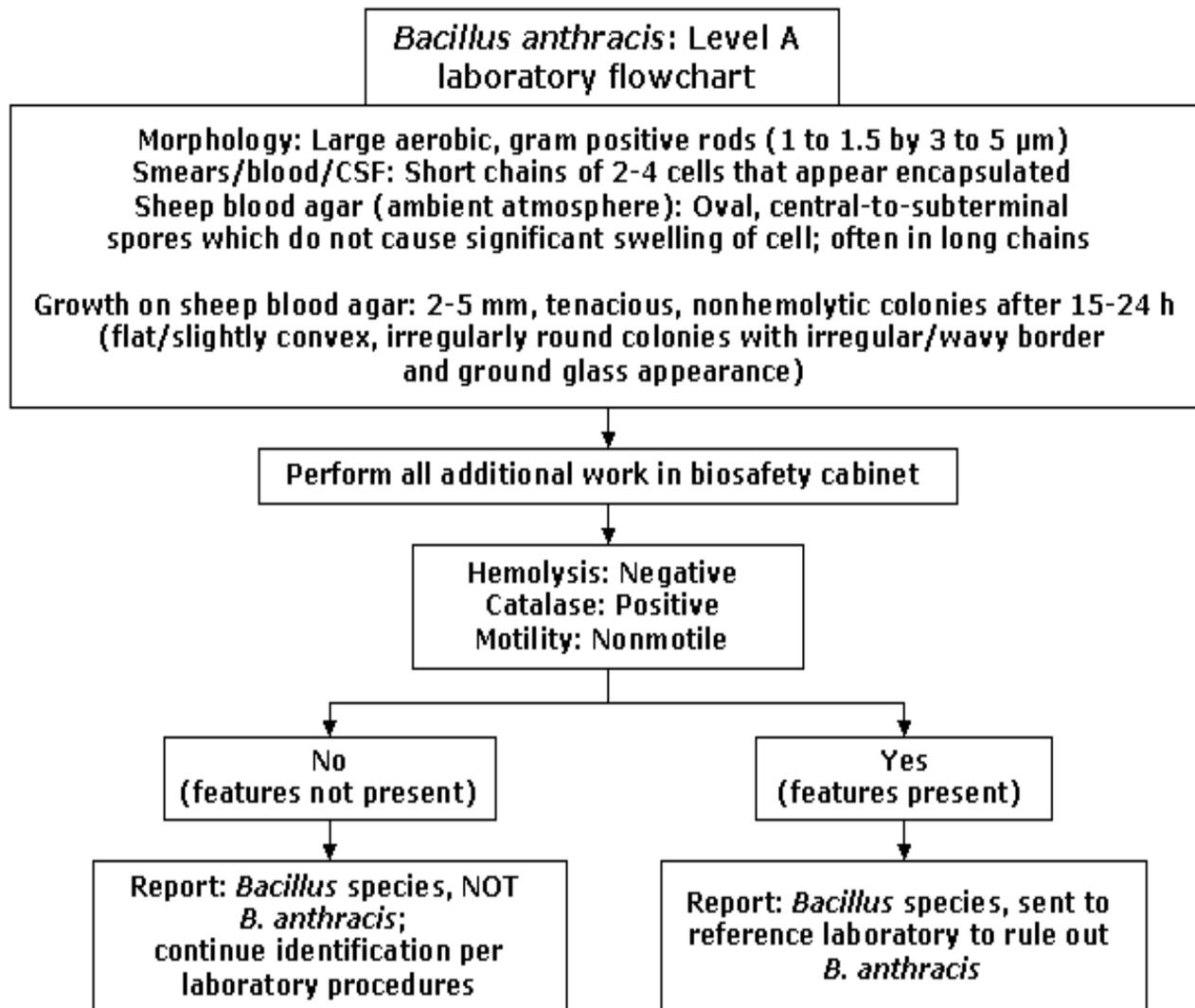


Figure A1b: Level A flowchart for *Bacillus anthracis*

Figure A2. Gram stain of *B. anthracis* in rhesus monkey blood, magnification 1000X



Figure A3a. Gram stain of *B. anthracis* from SBA, magnification 1000X

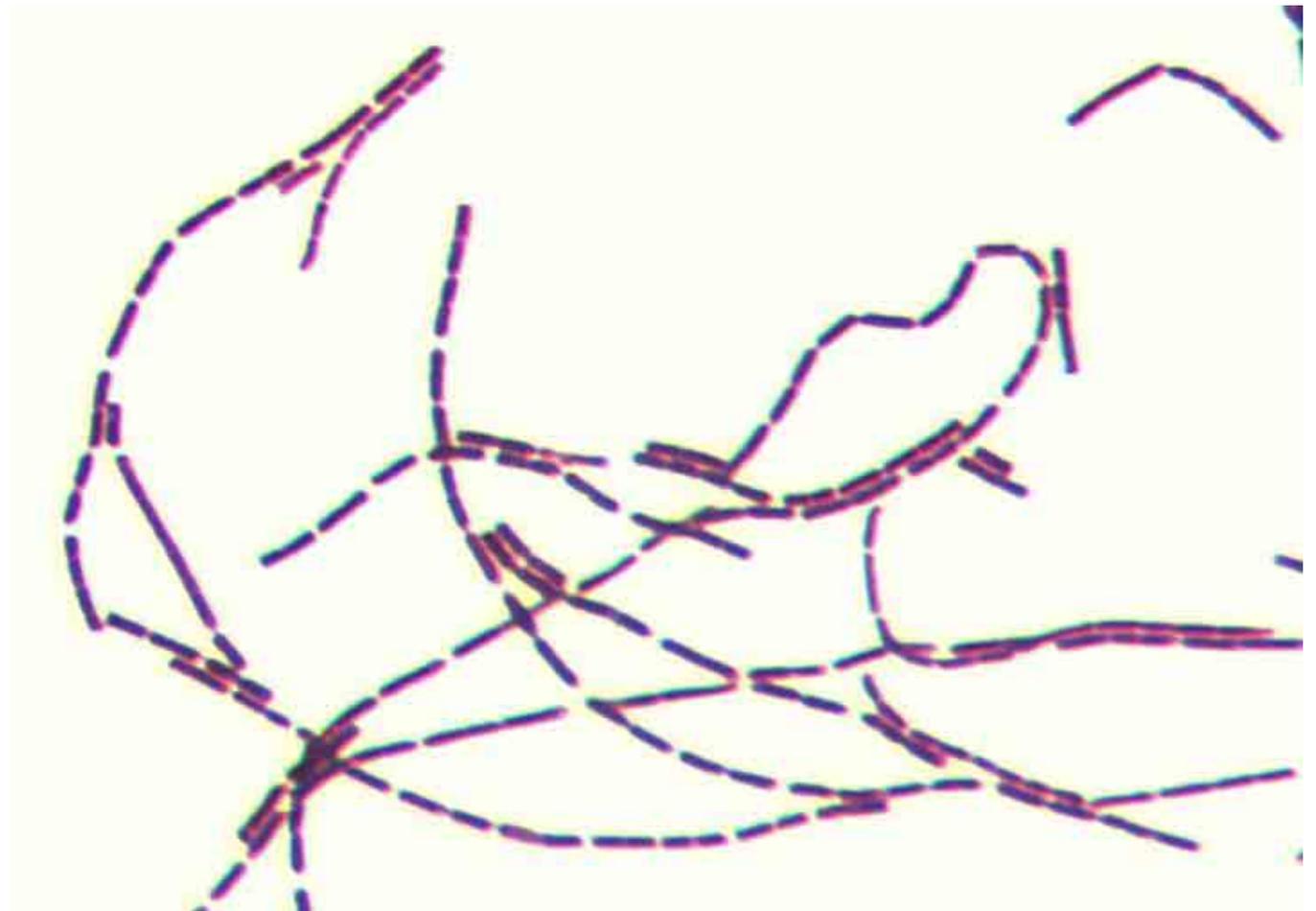


Figure A3b. Gram stain of *B. anthracis* with spores, magnification 1000X



Figure A4. *B. anthracis* colony morphology; overnight cultures on SBA.

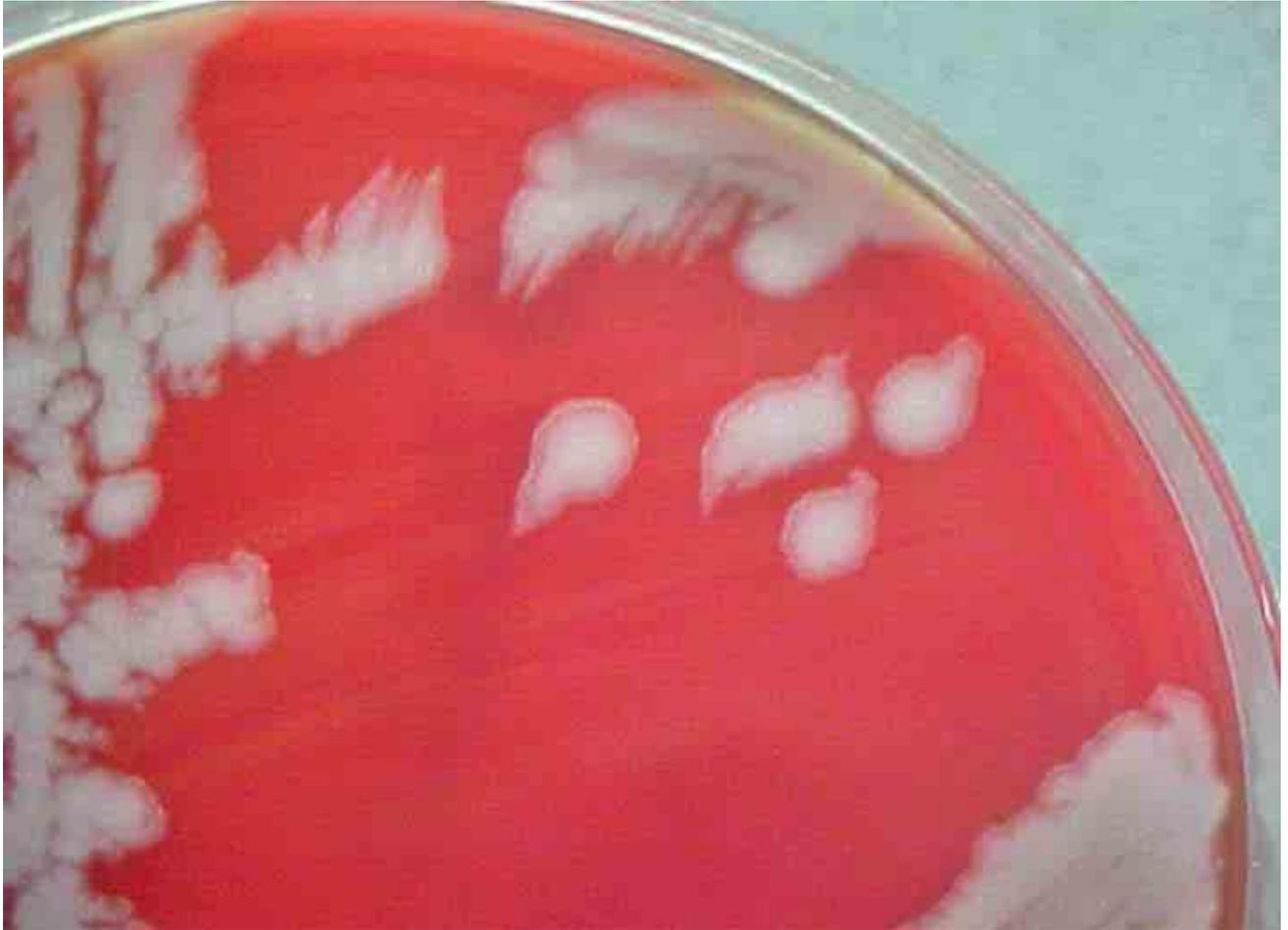


Figure A5. Tenacious colonies of *B. anthracis* on SBA



Figure A6. *B. anthracis* and *B. cereus* colony morphology; overnight cultures of *B. cereus* (left side of plate) and *B. anthracis* (right side) on SBA.

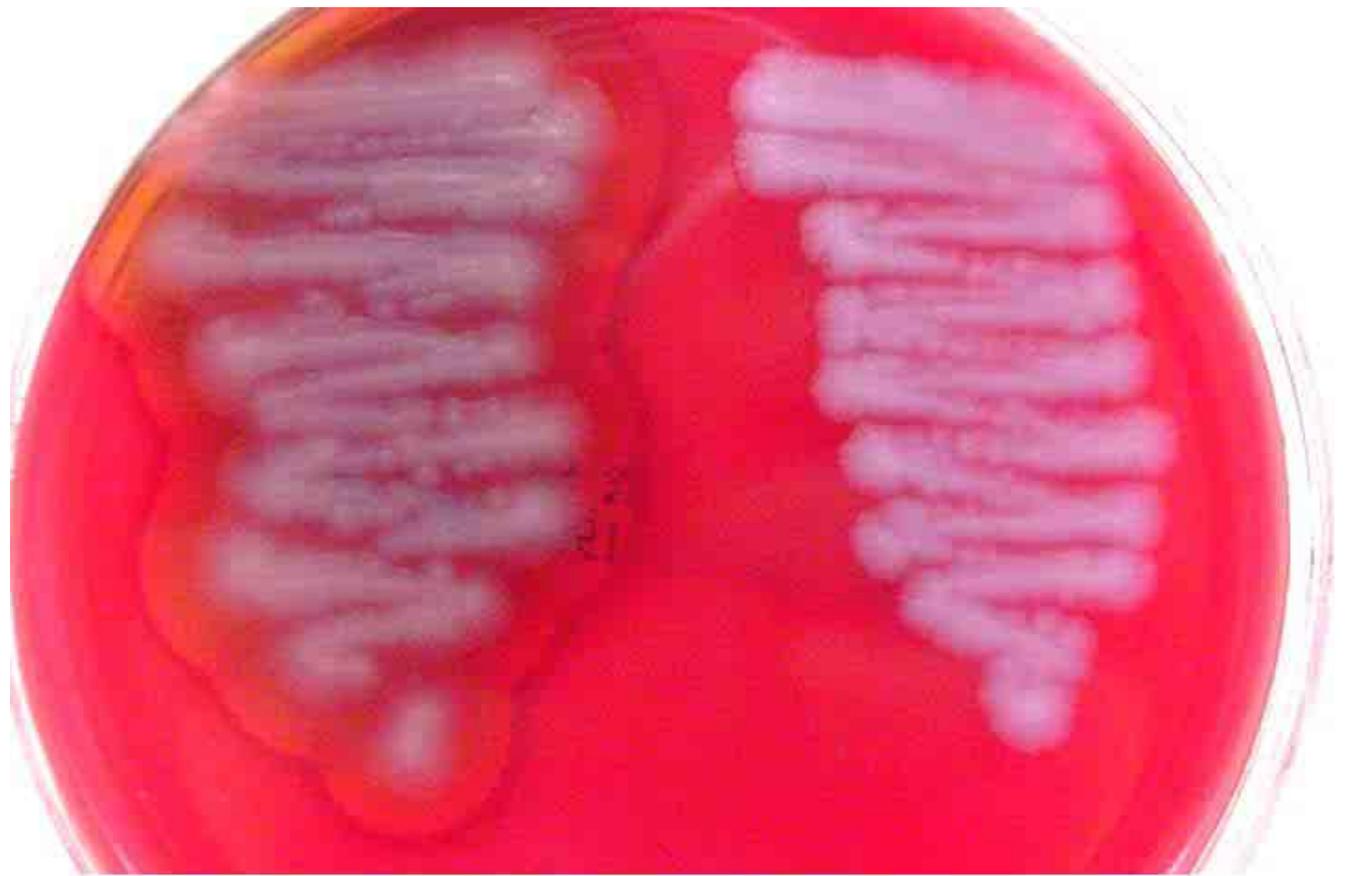


Table A1. Presumptive identification of *B. anthracis*

Lab Level	Type of sample	Presumptive identification	
		Characteristic	Method
A	Clinical sample	1. Gram-positive rods AND 2. Capsule	Gram stain India ink stain
A	Isolate	1 Spore-former AND 2. Colony morphology AND 3. Nonhemolytic AND 4. Nonmotile	Gram stain Observation on SBA Observation on SBA Motility medium or Wet mount

IX. Appendix: Nasal specimens for *Bacillus anthracis* screening

A. General: Nasal specimens (nares culture) should ONLY be used to support a confirmed exposure to *B. anthracis* or during an ongoing epidemiologic investigation. Gram stain of nasal specimens for *B. anthracis* spores is not recommended. Refer to limitations section below.

B. Materials: Swab (Dacron, rayon or other synthetic swabs are preferred over cotton) and transport medium for culture.

C. Procedure

1. Selection
 - a. The specimen of choice is a swab specimen taken at least 1 cm inside the nares.
 - b. Lesions in the nose require samples from the advancing margin of the lesions.
2. Method
 - a. Carefully insert the moistened swab (saline, sterile water) at least 1 cm into the nares.
 - b. Firmly sample the inside of the nares by rotating the swab and leaving it in place for 10 to 15 sec.
 - c. Withdraw the swab, insert it into its transport container, and submit the sampling unit to the laboratory for culture.
3. Labeling
 - a. Label the swab container with patient information.
 - b. Indicate, if possible, the degree or likelihood of exposure.
4. Transport
 - a. Transport the specimen to the laboratory as soon as possible.
 - b. Do not refrigerate specimens for culture.
5. Culture: Heat Shock
 - a. Remove the swab from transport container and place it into 1.5 ml of sterile saline or a nutrient broth such as trypticase soy broth, brain heart infusion broth, or equivalent. Vigorously twist the swab, and recap the tube.
 - b. Leave the swab in the tube. Place the broth suspension into a 65°C water bath for 30 min.
 - c. Plate 100-200 µl of broth on 5% sheep blood agar plate and incubate at 35-37°C for 18-24 h. Many *B. anthracis* will have visible growth in 12-18 h; observe for characteristics of *B. anthracis*.

D. Interpretation: Observe colony morphology for typical *Bacillus* colonies, look for lack of hemolysis, perform Gram stain, and evaluate for *B. anthracis* characteristics as described in the Level A laboratory protocol.

E. Reporting: If *B. anthracis* cannot be ruled out, submit the isolate to the state public health laboratory/department for confirmation. Refer to Level A reporting.

F. Limitations: Nasal cultures taken to evaluate for the presence of anthrax spore have not been evaluated for sensitivity or specificity. Nasopharyngeal and throat specimens are not recommended for anthrax screens and should not be submitted. Nasal cultures are NOT recommended for screening those who are asymptomatic and without known exposure.

G. Procedure Notes

1. Anterior nares cultures, without an indication of the presence of a lesion, are routinely examined only for presence of *Staphylococcus aureus* and β -hemolytic streptococci. Because of the unknown sensitivity of this method for detecting *B. anthracis* spores, interpret negative results with caution.
2. Anterior nares cultures cannot be used to predict a subsequent infection with *B. anthracis*, and should not be submitted in lieu of blood and other appropriate specimens from symptomatic patients.
3. Anaerobic cultures are not done on nasal specimens. *B. anthracis* produces spores in culture only when grown in air.
4. Nasal swabs may also be plated directly onto sheep blood agar prior to or without heat shocking, however normal nasal flora may overgrow very low numbers of *Bacillus* colonies.
5. Pediatric needs: Use the same procedure substituting a small fine-wire or nasopharyngeal swab to sample the anterior nares.