Sentinel Level Clinical Laboratory Guidelines for Suspected Agents of Bioterrorism and Emerging Infectious Diseases

Bacillus anthracis and *Bacillus cereus* biovar *anthracis*

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Preanalytical Considerations

I. Principle

A. Introduction

Bacillus anthracis, the agent of anthrax, is a zoonotic disease that is transmissible to humans through consumption or handling of contaminated products. It is an aerobic, spore-forming, nonmotile, large gram-positive bacterium. Genetically similar to *B. anthracis*, *Bacillus cereus* biovar *anthracis* (*Bcbva*) was identified in the early 2000's in Cameroon (strains CA) and Côte d'Ivoire (strains CI) [1, 2]. These strains were recovered from gorillas and chimpanzees with anthrax-like disease. The organism has since been recovered from elephants, goats, and blow flies in other countries of Africa [1-3]. *Bcbva* strains produce all of the primary *B. anthracis* virulence factors; thus, they are now considered to be select agents in the United States (see Table 1). *B. anthracis* and *Bcbva* strains are classified as Tier 1 select agents because they present a great risk of deliberate misuse with significant potential for mass casualties or devastating effect to the economy, critical infrastructure, or public confidence, and pose a severe threat to the public health and safety as defined by the Centers for Disease Control and Prevention (CDC). At this time, no human infections caused by *Bcbva* have been described, although a seroprevalence study in Côte d'Ivoire showed a high rate of antibodies to *Bcbva*-specific antigens among residents living near the Tai National Park, a wildlife refuge [4].

This procedure describes steps to recognize and rule out these organisms from clinical specimens in sentinel clinical laboratories. Such laboratories are defined as those that are certified to perform high complexity testing under the Clinical Laboratory Improvement Amendments of 1988 (CLIA'88) by the Centers for Medicare and Medicaid Services (CMS) for the applicable microbiology specialty. Such laboratory testing includes Gram stains and processing of at least one of the following specimen types: lower respiratory tract, wound, or blood.

NOTE: Sentinel laboratory rule out of *B. anthracis* and *Bcbva* requires a combination of differentiation of morphological characteristics and performance of four simple, conventional tests (Gram stain, catalase, observation of hemolysis, and motility). Automated systems and manual multi-test kit identification methods must not be used for the identification of either organism. This is due to the danger of misidentification as another species within the *Bacillus* genus, and of potential exposure due to aerosolization.

Table 1. Comparison of *B. anthracis*, *B. cereus*, and *Bcbva* characteristics (adapted from Klee et al.). This table is informational, and these tests must only be performed by sentinel clinical laboratories or Laboratory Response Network (LRN) reference laboratories.

Characteristic	*B. anthracis*	*B. cereus*	*Bcbva* CI[1]	*Bcbva* CA[2]	Level[3]
Hemolysis[4]	-	+	-	-	St
Motility[5]	-	+	+/-	+/-	St
Gamma phage susceptibility[6]	+	-	-	-	Ref
Penicillin G[7]	S	R	S	R	Ref
Capsule[8]	+	Absent *in vitro*	+	+	Ref

[1] CI = Côte d'Ivoire strains, from chimpanzees
[2] CA = Cameroon strains, from gorillas/chimpanzees
[3] St = Sentinel Laboratory Procedure, Ref = LRN Reference Laboratory Procedure
[4] Hemolysis: + = beta-hemolytic on sheep blood agar; - = non-hemolytic
[5] Motility: + = motile; - = non-motile. +/- = *B. cereus* biovar *anthracis* strains are usually motile, including those recovered from gorillas, chimpanzees, and elephants; *B. cereus* biovar *anthracis* goat strains from Democratic Republic of the Congo were non-motile [1].
[6] Gamma phage susceptibility: + = susceptible; - = resistant.
[7] S= susceptible; R = resistant (measured by minimal inhibitory concentration, S= ≤0.12 µg/ml)
[8] Requires specialized media. Very rarely expressed on routine test media such as blood agar or chocolate agar

B. Geographic Distribution

Anthrax is endemic in southern Europe, parts of Africa, Australia, Asia, and North and South America. It persists in arid deserts with the majority of cases reported from Iran, Turkey, Pakistan, and Sudan [6]. In the United States, human cases of anthrax are rare, but infections in wildlife and livestock continue to be reported in various states including North and South Dakota, Nevada, Texas and California [7].

Currently, *Bcbva* strains have only been found in certain African countries, including Cameroon, Côte d'Ivoire, and Central African Republic [2, 3]. These strains are known to cause an anthrax-like disease in gorillas and chimpanzees, and have been isolated from other animals, including elephants and goats.

The potential use of *B. anthracis* as a bioterrorism agent to inflict disease and death following contact with or inhalation of spores has been a concern for centuries [8]. Although developed by several countries, including the United States, for possible military purposes, anthrax weapons were banned by the Biological Weapons Convention of 1972. Nonetheless, biocrimes with the use of agents other than *B. anthracis* have raised concerns for intentional use of biological agents, and the LRN was created in late 1999 to aid in controlling misuse of biological agents. However, its role and responsibilities in preparing for, and responding to, bioterrorism increased dramatically during and after the 2001 Amerithrax incident in which *B. anthracis* spores were distributed in letters via the U.S. postal system. The lessons learned from this national event demonstrated the serious need for training of sentinel level clinical laboratories and preparing them to play a key role in the LRN.

In the United States, four naturally occurring cases of human anthrax have been reported since 2006, and include one gastrointestinal, one cutaneous, and two inhalational cases [9-13]. These are examples of the critical role that sentinel laboratories, using the LRN designated tests and algorithms, play in ruling out *B. anthracis* and *Bcbva* from suspicious *Bacillus* spp. isolates.

C. Diseases and Clinical Presentation

Anthrax is a zoonotic disease that occurs most frequently in herbivorous animals (e.g., cattle, sheep, and goats), which acquire endospores from contaminated soil. Human disease is less common and results from contact with infected animals or with commercial products derived from them, such as wool and hides. Infection can occur in one of four forms:

1. **Cutaneous** anthrax is responsible for >95% of naturally occurring cases and is initiated when the bacterium or spores enter the skin through cuts or abrasions, such as when handling contaminated hides, wool, leather, or hair products (especially goat hair) from infected animals [11, 14, 15]. Skin infection begins as a raised itchy bump or papule that resembles an insect bite. Within 1 to 2 days, the bump develops into a fluid-filled vesicle, which ruptures to form a painless ulcer (eschar), usually 1 to 3 cm in diameter with a characteristic black necrotic area in the center. Pronounced edema is often associated with lesions due to the release of edema toxin, a major virulence factor produced by the organism. Lymph nodes 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 antimicrobial therapy, with lesions resolving several weeks later [21]. There are a few case reports of transmission by insect bites, presumably after the insect fed on an infected carcass [16].

2. **Ingestion** anthrax may occur 1 to 7 days following consumption of contaminated undercooked meat from infected animals and is characterized by 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 bloody diarrhea. If not treated, the mortality rate ranges from 25 to 60%. Pharyngeal lesions may also occur from ingestion of contaminated food [10].

3. **Inhalational** anthrax results from the inhalation of *B. anthracis* spores and can occur following an intentional aerosol release as was evident in the 2001 anthrax biocrime. Depending on the quantity of aerosolized spores, the incubation period ranges from 1 to 6 days. Aerosolization of *B. anthracis* spores is regarded as the

most likely method to be used in a bioterrorism or biocrime event [17-21]. Though the minimum infectious inhaled dose has not been specifically determined, the U. S. Department of Defense (DoD) estimates that the 50% lethal dose for humans is between 8,000 and 10,000 spores [19]. Following inhalation, the spores enter pulmonary macrophages and are carried to the mediastinal lymph nodes. Germination and vegetative growth result in the production of an antiphagocytic capsule and a toxin comprised of three proteins--edema factor, lethal factor, and protective antigen--which play major roles in the virulence and resultant infection and clinical manifestations [22]. Disease onset is gradual and nonspecific. Fever, malaise, and fatigue may be present initially (prodromal stage) and be accompanied by a nonproductive cough and chest discomfort. These initial symptoms are often followed by a short period of severe respiratory distress with labored breathing (dyspnea), profuse sweating (diaphoresis), high-pitched whistling respiration (stridor), and bluish skin color (cyanosis). Fatal sepsis with generalized hemorrhage, massive hemorrhagic mediastinitis, and necrosis ensue. Shock and death usually occur within 24 – 36 h following the onset of respiratory distress. If therapy is not initiated within 48 h following the onset of symptoms, mortality reaches 95 – 100% [21-23]. Physical examination is usually nonspecific. The chest X-ray often reveals a widened mediastinum (not always present) with pleural effusions, while infiltrates are commonly absent. However, this is not diagnostic as other diseases, such as tuberculous mediastinitis and Hodgkin lymphoma, may also cause a widened mediastinum. A single case of inhalational anthrax should alert all healthcare workers to the possibility of a bioterrorism or biothreat event [17]. Person-to-person transmission of inhalational anthrax has not been confirmed [18, 24].

4. **Injection** anthrax usually presents as a severe soft tissue infection, including necrotizing fasciitis and cellulitis or abscess, particularly if associated with edema, which is often marked. Compartment syndrome has also been noted which is manifested as increased pressure in a muscle compartment leading to muscle and nerve damage and impaired blood flow. Injection anthrax may include signs of severe sepsis, with or without evidence of soft tissue infection. No eschar is visible. Between December 2009 and December 2010, drug users in Scotland, England and Germany acquired injection anthrax through using heroin contaminated with *B. anthracis* spores. There were 47 confirmed cases (119 including probable cases) with 13 deaths in Scotland; 5 cases with 4 deaths in England; and 1 fatal case in Germany [25]. This resulted in a mortality rate of 34%. Prior to the 2009-2010 outbreak, one case of injection anthrax was reported in a heroin skin-popper in Norway in 2000. In 2012, a second outbreak of injection anthrax occurred in intravenous (IV) drug users in northern Europe resulting in 13 confirmed cases and 5 deaths [26].

All four forms of anthrax listed above can lead to anthrax meningitis. Meningitis may also be the primary sign of anthrax in patients lacking an obvious source of exposure.

D. CDC Case Definition (https://ndc.services.cdc.gov/case-definitions/anthrax-2018/)

A case that meets the clinical criteria and has confirmatory laboratory test results.

Confirmatory laboratory criteria for *Bacillus anthracis* or *Bacillus cereus* expressing anthrax toxins (testing performed at LRN reference laboratory or CDC) require meeting one of the following:

- Culture and identification from clinical specimens by the LRN;
- Demonstration of *B. anthracis* antigens in tissues by immunohistochemical staining using both *B. anthracis* cell wall and capsule monoclonal antibodies;
- Evidence of a four-fold rise in antibodies to protective antigen (PA) between acute and convalescent sera or a four-fold change in antibodies to protective antigen in paired convalescent sera using CDC quantitative anti-PA immunoglobulin G (IgG) ELISA testing in an unvaccinated person;
- Detection of *B. anthracis* or anthrax toxin genes by the LRN-validated polymerase chain reaction and/or sequencing in clinical specimens collected from a normally sterile site (such as blood or cerebrospinal fluid [CSF]) or lesion of other affected tissue (skin, pulmonary, reticuloendothelial, or gastrointestinal);
- Detection of lethal factor (LF) in clinical serum specimens by LF mass spectrometry.

II. Safety Considerations

A. Sentinel laboratories (human or animal) must restrict processing to specimens within their level of expertise. Do not process non-clinical (environmental) specimens in hospital or commercial reference laboratories; restrict processing to clinical specimens only. All other specimens should be directed to the designated LRN reference laboratory.

B. Primary patient specimens can be handled using BSL-2 practices, and working in a certified Class II biological safety cabinet (BSC) is recommended when performing activities having a high potential for aerosol production (e.g. catalase test, opening vortexed tubes). If *B. anthracis* or *Bcbva* is suspected, BSL-3 precautions must be taken, such as wearing gloves, a solid front gown, and respirator (N95, powered air purifying respiratory [PAPR], etc.).

C. Subcultures of suspected *B. anthracis* or *Bcbva* must be performed in a certified BSC. Plates should be secured (e.g., taped/shrink sealed) and clearly marked.

D. Decontamination of laboratory surfaces is easily accomplished using a fresh (made daily) solution of 10% (approximately 5,000 ppm free available chlorine) household bleach or commercially available stabilized bleach disinfectant. Low concentrations of free chlorine (<5,000 ppm) are sufficient to kill vegetative cells within seconds while five minutes wet contact time is sufficient to kill ≥ 99% of *Bacillus atrophaeus* spores; however, contamination with organic matter (e.g. blood) may inactivate bleach [28]. Since *B. atrophaeus* spores have been found to be slightly more resistant to bleach than the spores of *B. anthracis*, the above contact time and available chlorine concentration is effective to inactivate *B. anthracis* spores. If an autoclave is not available on-site, pipettes, needles, plastic loops, and microscopic slides should be soaked in 10% bleach or 10 – 30% formalin for 24 h and then discarded in sharps/medical waste. Phenolics are not sporicidal at the usual working dilutions [27].

E. A biological risk assessment should be performed by each laboratory to evaluate the hazards and risks of performing testing on suspected anthrax specimens and to implement mitigations, if necessary, which reduce the likelihood of exposure and illness. Risk assessments should be reviewed regularly and when new methods or equipment are initiated, or when an exposure or near-miss is reported.

III. Materials

1. Media

a. Standard liquid blood culturing system

b. 5% Sheep Blood Agar Plates (BAP)

c. Chocolate Agar Plates (CHOC)

d. MacConkey (MAC) or Eosin Methylene Blue (EMB) Plates

e. Semi-solid motility medium without triphenyltetrazolium chloride (TTC); while many laboratories no longer use this medium in clinical practice, it is highly encouraged they continue to stock motility media for purposes of ruling out B. anthracis. **NOTE: It is preferred that motility is assessed in a semi-solid medium instead of a wet mount.**

f. Trypticase soy broth (TSB), or equivalent, for cerebrospinal fluid (CSF) only.

2. Reagents

a. Appropriate disinfectant [28]

i. 10% bleach

ii. Bleach Rite® (Current Technologies)

iii. SporGon® (Decon Labs, Inc)

b. Gram stain reagents

c. Catalase (3% hydrogen peroxide). **NOTE: It is preferred that a tube catalase be performed instead of a slide catalase.**

3. Equipment and Supplies

a. Certified Biological Safety Cabinet (BSC) (normally a Class II)
b. Personal Protective Equipment (PPE); eye protection, gloves, solid-front gown
c. 35 - 37°C incubator (ambient air, 5 – 10% CO2)
d. Light microscope with 100x objective and 10x eyepiece
e. Microscope slides
f. Pipettes, pipette tips, inoculating loops and needles
g. Blood culture instrument (optional)
h. Centrifuge with sealed rotor (for CSF cytospin procedure only)

IV. Quality Control

Perform quality control of media and reagents according to the manufacturer's package inserts, and applicable accreditation regulations (e.g. CLIA, College of American Pathologists [CAP]). Do not use *B. anthracis* or *Bcbva* as a control organism due to their infectious nature and requirements of the Federal Select Agent Program. Quality control of biochemical tests using a positive and negative reacting organism should generally be performed on each new lot or shipment of reagent or according to the laboratory's Individual Quality Control Plan (IQCP), if applicable. For frequency of quality control, refer to manufacturer's guidelines, state and federal regulations, and accrediting body requirements.

It is desirable for sentinel laboratories to subscribe to or enroll in a proficiency program or exercise designed to assess competency for the rule-out and/or referral of agents of bioterrorism. Sentinel laboratories should inquire with their designated LRN reference laboratory about participation in a proficiency program or exercise if they are not currently subscribed. Proficiency programs will send an exempt, avirulent, or attenuated strain to mimic an actual Category A or B agent, and will not send a true select agent.

V. Specimen Collection

A. Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing*	
Cutaneous Vesicular (early) stage	• Unroof vesicle and aspirate fluid or collect with two sterile, dry polyester (e.g. Dacron®) or nylon swabs
Eschar (late) stage	• Insert sterile, premoistened polyester (e.g. Dacron®) or nylon swab beneath the edge of the eschar, rotate swab or obtain an aspirate • Collect cerebrospinal fluid only if signs and/or symptoms of meningitis occur • Transport in accordance with institutional procedures and processes
Gastrointestinal	• Stool (≥5 grams [pecan size]), collect and transport in a leakproof sealed container. Fresh stool transport time ≤ 1hr; stool in Cary-Blair at 2-8°C <24 hr. • Blood: Collect 2 sets directly into appropriate blood culture bottles. Preferably, collect the maximum amount of blood acceptable per the blood culture bottle manufacturer. • Collect cerebrospinal fluid only if signs and/or symptoms of meningitis occur • Transport specimens and bottles at room temperature
Inhalational	• Sputum, if present • Blood: Collect 2 sets directly into appropriate blood culture bottles. Preferably, collect the maximum amount of blood acceptable per the blood culture bottle manufacturer. • Collect cerebrospinal fluid only if signs and/or symptoms of meningitis occur • Transport specimens and bottles at room temperature
Injection	• Blood: Collect 2 sets directly into appropriate blood culture bottles. Preferably, collect the maximum amount of blood acceptable per the blood culture bottle manufacturer. • Obtain tissue biopsy from localized lesion tissue following debridement • Transport specimens and bottles at room temperature
Postmortem Tissue	• Collect tissue in a sterile container and keep moist by adding a few drops of sterile saline. • Transport to the laboratory at room temperature within 1 hour of collection

References [14, 17, 28, 29]

* Additional specimens that may be requested by the designated LRN reference laboratory include: plasma, pleural fluid, serum

A. Rejection of specimens

1. Use established laboratory criteria for rejection of specimens.

2. Environmental specimens are not to be processed by sentinel laboratories; contact the designated LRN reference laboratory directly.

Analytical Considerations

VI. Specimen Processing

A. Cutaneous, tissues and stool - Plate cutaneous, tissue, and stool specimens onto BAP and MAC (or EMB). With the exception of stool specimens, prepare smears for Gram stain.

B. Respiratory - Plate respiratory specimens onto BAP, CHOC, and MAC (or EMB) and prepare a smear for Gram stain.

C. Blood - At time of collection, aseptically inoculate liquid blood culture bottles with the appropriate blood volume per manufacturer's instructions.

D. Cerebrospinal fluid (CSF)

1. Prepare a cytospin smear for Gram stain, AND
2. Centrifuge CSF specimens at 1,500 x g for 15 min using a clinical centrifuge equipped with a sealed rotor. Open the sealed rotor only in a BSC. Plate the sediment onto BAP and CHOC; aspirate the remaining sediment and inoculate TSB or equivalent.

VII. Incubation and Examination of Cultures

A. Incubate (broth if used) all plated specimens, except MAC (or EMB), at 35 to 37°C in 5-10% CO_2; incubate MAC (or EMB) in ambient air at 35 to 37°C. Incubate blood culture bottles for continuous monitoring systems as directed by the manufacturer and using standard incubation time.

B. Examine all cultures within 18-24 h of incubation. Growth of *B. anthracis* or *B. cereus* biovar *anthracis* may be observed as early as 8 h following incubation. This can be helpful when looking for *B. anthracis* or *B. cereus* biovar *anthracis* from mixed cultures (sputum, stool).

VIII. Culture-based Rule Out

Sentinel laboratories should NOT intentionally use automated systems for identification of possible select agents but should use the established sentinel laboratory procedures instead. Using automated systems, including MALDI-TOF MS technology, may result in exposure to dangerous pathogens, and could result in erroneous identification, Ex: *Yersinia pestis* misidentified as *Y. pseudotuberculosis*, human brucellosis-causing species of *Brucella* misidentified as *Brucella anthropi*, or *Bacillus anthracis* misidentified as *B. cereus*. It should be noted that an identification of "*Bacillus cereus* group" is not a misidentification of *Bacillus anthracis*, as *B. anthracis* is a member of the *Bacillus cereus* group. However, from a biosafety and biosecurity perspective, in addition to patient management, it is critical that the microbiologist is knowledgeable of these differences and takes the appropriate action when interpreting such a result.

The rule out or referral of *B. anthracis* or *B. cereus* biovar anthracis is accomplished by following the algorithm below and the associated tests.

Prepare smears for Gram-staining suspicious colonies from agar plates and blood cultures in a certified BSC. *B. anthracis* and *Bcbva* are large (1 to 1.5 by 3 to 5 µm) gram-positive rods. Vegetative cells seen on Gram-stained smears of clinical specimens often occur in short chains of two to four cells that are encapsulated. Gram stains from colonies grown on BAP appear as long chains of non-encapsulated gram-positive bacilli. Note that older cultures may decolorize readily and appear gram-negative. If present, endospores are oval and located centrally or subterminally and do not cause swelling of the vegetative cell (Fig 1). Spores are rarely seen in direct smears of clinical specimens; their presence may indicate a delay in transportation of the specimen to the laboratory.

Fig. 1. Gram stain of a blood culture containing *B. anthracis*.

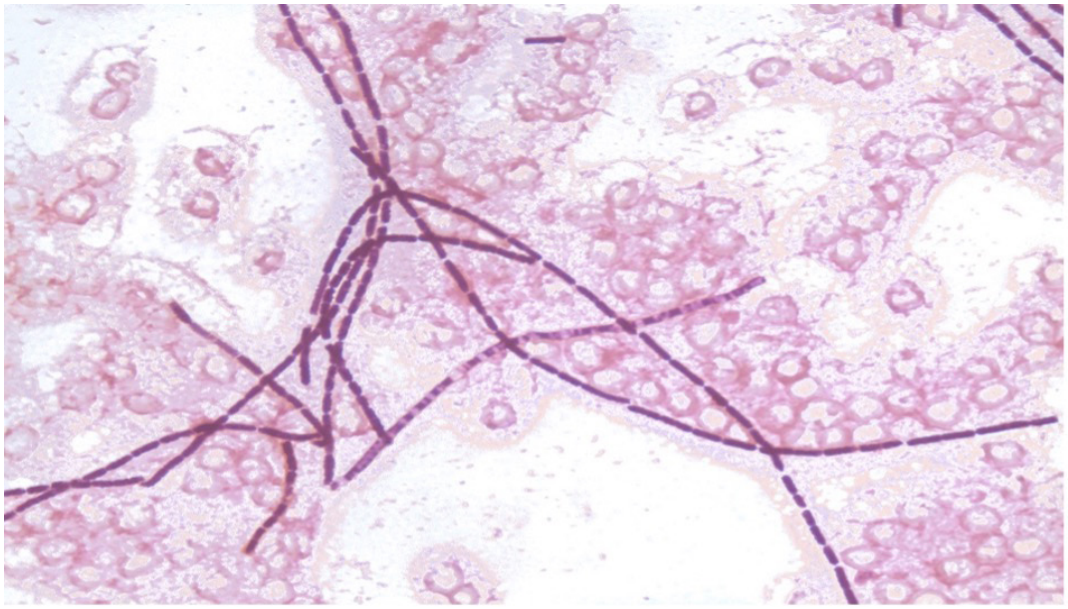


Photo courtesy of Dr. James Rudrik, Bureau of Laboratories, Michigan Department of Health and Human Services

A. Both *B. anthracis* and *Bcbva* grow well on BAP and CHOC, but do not grow on MAC (or EMB). After 18–24 h of incubation, colonies of *B. anthracis* are grey to white, 2-5 mm in diameter, round with irregular edges, flat or slightly convex with a ground glass appearance. There are often projections from the edge of the colony – from a single projection forming "comma-shaped" colonies to multiple projections producing the "Medusa head" appearance. The colonies are non-hemolytic on BAP and have a tenacious consistency that will stand up like beaten egg whites when teased with a loop. Some *B. cereus* biovar *anthracis* strains may exhibit weak hemolysis upon extended incubation (48 h), particularly when incubated in CO_2. Capsulated *Bcbva* organisms may appear mucoid on BAP when incubated in CO_2 (Fig. 3), but not when incubated in ambient atmosphere.

Fig. 2. Colonies of *B. anthracis* on blood agar.

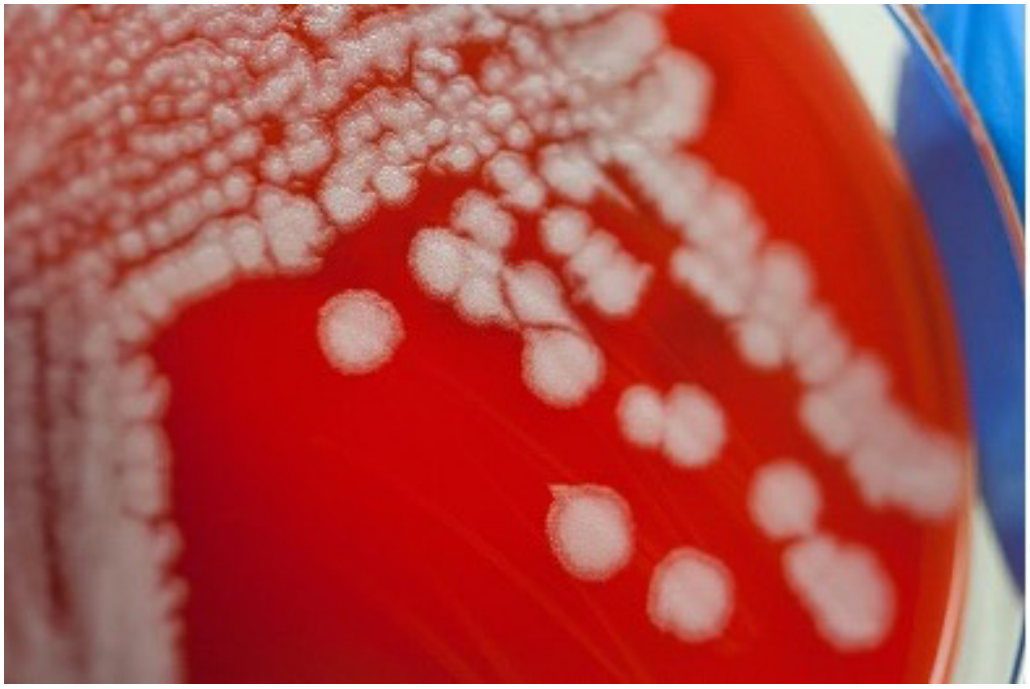


Photo courtesy of APHL

Fig. 3. Colonies of *B. cereus* biovar *anthracis* CA on blood agar, 5% CO_2.

Photo courtesy of APHL

B. Perform all testing in a certified BSC. Refer to the Biochemical Test Procedures listed in the General Introduction, Recommendations and Biochemical Procedures document.

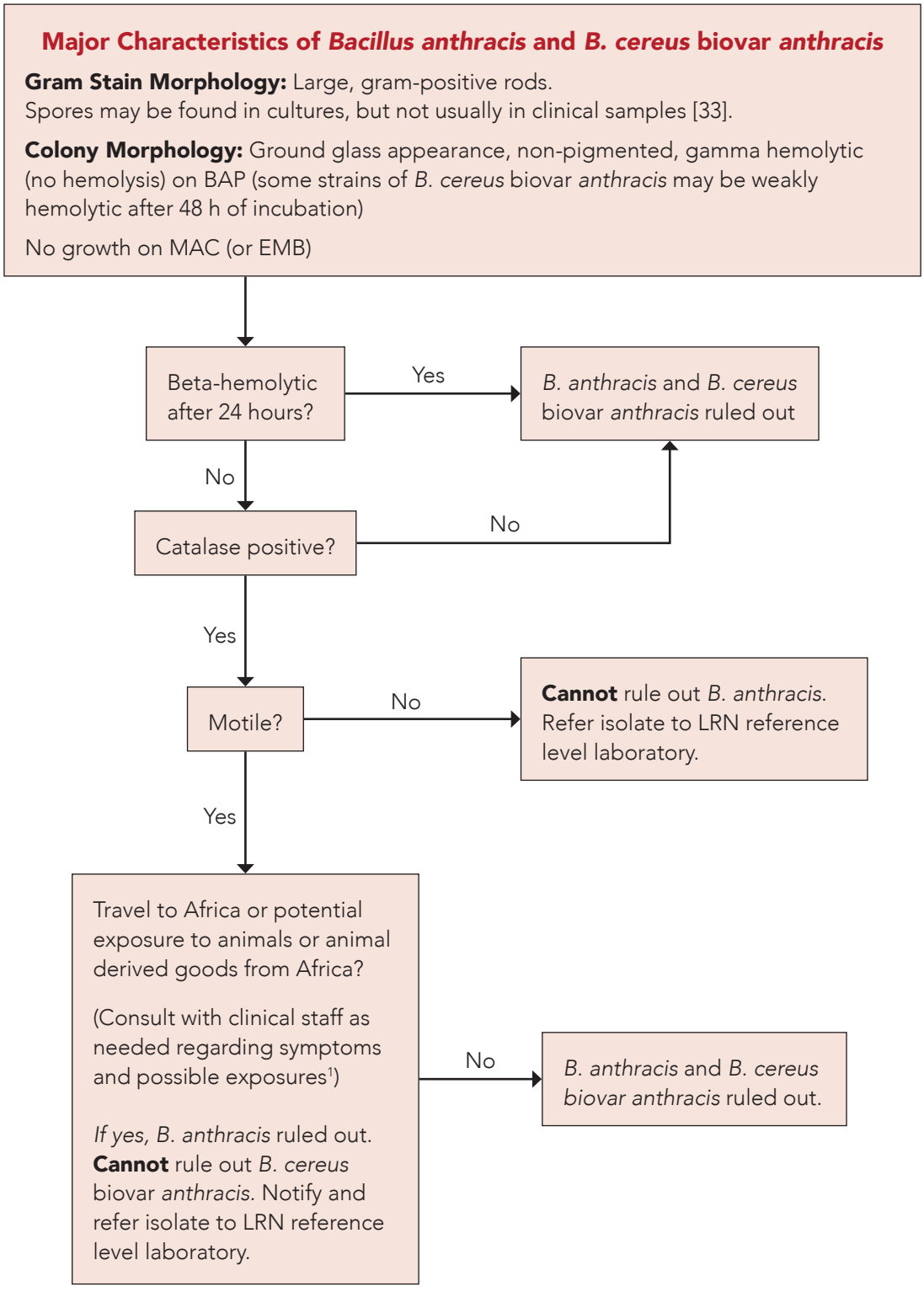
1. Catalase – positive
2. Motility in semi-solid medium– *B. anthracis* is non-motile; *Bcbva* strains are usually motile (Table 1).

NOTE: Performing a catalase test may be hazardous due to the potential for the creation of an aerosol. The catalase test should therefore be performed in a BSC. Tube catalase is recommended. It is also recommended to perform motility in semi-solid medium instead of a wet mount since results are less subjective in semi-solid medium and the potential for creating aerosols is greatly reduced.

C. See flowchart below for summary of major characteristics for rule out of *B. anthracis* and *Bcbva*.

NOTE: Confirmatory identification is made by an LRN reference laboratory; consult with the designated LRN reference laboratory.

Bacillus Anthracis and *B. Cereus* Biovar *Anthracis* Rule Out Flowchart



[1] Clinical laboratories should consult with the clinical team if the patient has anthrax-like symptoms prior to submitting to an LRN reference laboratory.

Post Analytical Considerations

IX. Reporting, Notification, and Transfer

A. Sentinel clinical laboratories are not required to register with the Federal Select Agent Program to conduct presumptive diagnostic testing for select biological agents and toxins. Testing to rule out select agents may be performed by sentinel clinical laboratories as long as the laboratory transfers the presumptive select agent sample(s) to another laboratory (e.g., LRN reference laboratory) registered with the Federal Select Agent Program for confirmation.

B. *B. anthracis* or *Bcbva* is suspected and cannot be ruled out if the isolate fulfills the following characteristics:

- Direct Gram staining reveals large gram-positive rods
- Grows on BAP as non-pigmented, non-hemolytic, white or grey-white colonies having a characteristic ground glass appearance with edges that are slightly undulate ("Medusa heads"; comma-shaped)
- Does not grow on MAC (or EMB)
- Non-hemolytic on BAP (some strains of *B. cereus* biovar *anthracis* may be weakly hemolytic after 48 h of incubation)
- Catalase – positive
- Motility – *B. anthracis* strains are non-motile; most *Bcbva* strains are motile

C. Notification and submission of cultures if *B. anthracis* or *Bcbva* cannot be ruled out based on the above characteristics.

1. If *B. anthracis* cannot be ruled out, contact the LRN reference laboratory and refer for confirmatory testing.
2. If *Bcbva* cannot be ruled out, the sentinel laboratory should contact the clinical team to discuss the result and the patient's symptoms and travel history to determine if the specimen should be referred to an LRN reference laboratory for confirmatory testing.
3. Do not attempt full identification and susceptibility testing in the sentinel level clinical laboratory.
4. Preserve original specimens in anticipation of a criminal investigation and transfer to the designated LRN reference laboratory in accordance with state and local requirements. In particular, the appropriate material, including blood culture bottles, tubes, plates, and clinical specimens (e.g., aspirates, biopsies, sputum specimens) should be documented and either submitted to the LRN reference laboratory or securely retained until the reference laboratory confirms the identification.
5. Consult with the LRN reference laboratory bioterrorism coordinator regarding additional clinical specimens that may be submitted for testing.
6. Do not ship specimens or cultures to the LRN reference laboratory without prior notification and arrangements.
7. Notify other public health authorities (e.g., state public health department Epidemiologist/Health Officer) as required by local and state communicable disease reporting requirements. The state public health laboratory/department will notify law enforcement officials (state and federal), such as the local Federal Bureau of Investigation (FBI), as appropriate.
8. In accordance with your institution's policies, notify relevant services (e.g., infectious diseases, infection prevention) so that the appropriate treatment and infection control measures can be taken.
9. Initiate documentation showing the specimen identification, notification and transfer to the designated LRN reference laboratory, and documentation of all plates and tube cultures which will need to be destroyed or transferred if identification of Select Agent is confirmed.

D. Sentinel level clinical laboratories should consult with their designated LRN reference laboratory prior to or concurrent with testing if *B. anthracis* or *Bcbva* is requested by the clinician, or a bioterrorist event is suspected. Obtain guidance from the state public health laboratory or LRN reference laboratory as appropriate (e.g., requests from local law enforcement or other local government officials). The FBI and state public health laboratory/state public health department will coordinate the transfer of isolates/specimens to a higher level LRN laboratory (e.g., CDC) as appropriate.

E. If *B. anthracis* or *Bcbva* is ruled out, proceed with efforts to identify using established criteria.

F. If other cases are suspected, collect appropriate specimens and submit them to the designated LRN reference laboratory for additional testing.

G. If a select agent is identified, sentinel laboratories should investigate and evaluate staff for potential exposures. Sentinel laboratories should coordinate with their designated LRN reference laboratory, occupational health and/or local public health agency for exposure-related testing and post-exposure prophylaxis, as appropriate. The sentinel laboratory must complete an APHIS/CDC Form 3 and immediately report any laboratory exposure. For further guidance and access to the necessary forms, consult with the designated LRN reference laboratory or refer to the Federal Select Agent Program website: https://www.selectagents.gov.

X. Summary/Special Considerations

A. Antimicrobial Susceptibility

1. Antimicrobial susceptibility testing (AST) of *B. anthracis* or *B. cereus* biovar *anthracis* is neither needed nor appropriate for sentinel level laboratories to perform.
2. Consult with the local LRN reference laboratory regarding antimicrobial susceptibility testing. The LRN reference laboratory will either conduct the AST themselves or refer to CDC if needed.
3. Treatment of anthrax depends on factors such as presence of meningitis, production of toxin, potential for antimicrobial resistance, and the potential presence of spores (32).
4. Consult with public health regarding post-exposure prophylaxis for personnel (adults and children), including laboratory personnel suspected of exposure to *B. anthracis* or *B. cereus* biovar *anthracis* spores. Prophylaxis may include ciprofloxacin, levofloxacin (adults) or doxycycline. Once started, antibiotic therapy should be continued for 60 days post-exposure [32].

B. Select Agent Reporting and Compliance

1. Though sentinel level laboratories are not required to register with the Federal Select Agent Program (FSAP) under the Select Agent Rule (7 CFR Part 331, 9 CFR Part 121, and 42 CFR Part 73), reporting of all identified select biological agents and toxins is required.
2. The laboratory that confirms the identification of a select agent must complete Sections A and B of the APHIS/CDC Form 4A within one week (7 calendar days) of the confirmed identification along with the laboratory that originated the specimen completing Sections C and D. For *B. anthracis* or *B. cereus* biovar *anthracis*, immediate notification to the Federal Select Agent Program is required. The LRN reference laboratory will notify FSAP within 24 hours of confirmation. For further guidance and access to the necessary forms, consult with the designated LRN reference laboratory or refer to the Federal Select Agent Program website at https://www.selectagents.gov.
3. If the organism is transferred following a confirmed identification, the sentinel laboratory must complete the APHIS/CDC Form 2 before the transfer. Transfers of a confirmed select agent may only be sent with pre-approval to an LRN laboratory registered with the Federal Select Agent Program. For further guidance and access to the necessary forms, consult with the designated LRN reference laboratory or

refer to the CDC Division of Regulatory Science and Compliance website: https://www.selectagents.gov.

4. Any laboratory that has a potential theft, loss or release (exposure) of a select agent must immediately notify FSAP and complete an APHIS/CDC Form 3.
5. The designated LRN reference laboratory will advise and assist the laboratory with completion of required forms (e.g. APHIS/CDC Forms 2, 3, and 4). Always refer to https://www.selectagents.gov for the latest guidance and versions of these forms.

C. Destruction

1. Once the identification of a select agent has been confirmed, the Federal Select Agent Regulations require that the residual specimen(s) and culture(s) of the specimen be destroyed or transferred to a laboratory registered with the FSAP (an approved select agent entity) within 7 calendar days of a confirmed identification. The designated LRN reference laboratory can advise the laboratory on destruction or transfer requirements of remaining material.
2. All plates, tubes, and clinical material that contain the select agent organism must be either destroyed on-site by a recognized sterilization or inactivation process, or submitted to the designated LRN reference laboratory for disposal with appropriate documentation. Transfer of confirmed select agent material is only permitted to a FSAP-registered laboratory.
3. Recognized sterilization and inactivation processes for *B. anthracis* and *Bcbva* include steam sterilization (autoclave), or submersion of all cultures and residual specimens in 10% bleach. If the bleach method is used, organisms in blood culture bottles can be inactivated by injecting a sufficient volume of undiluted bleach to obtain the 10% dilution. The minimum contact time to inactivate large numbers of *B. anthracis* or *Bcbva* spores is 60 minutes (34). However, due to the presence of high concentrations of organic matter in the culture media and residual specimens that can neutralize the bactericidal and sporicidal effect of the hypochlorite in the bleach, it is recommended that the material remain in contact with the 10% bleach for 24 hours.

D. Packing and Shipping

1. Refer to the ASM Packing and Shipping Infectious Substances guidelines (https://www.asm.org/ASM/media/Policy-and-Advocacy/LRN/Sentinel%20Files/PackAndShip.pdf).
2. All materials sent to the designated LRN reference laboratory must be shipped in compliance with International Air Transport Association (IATA) requirements, Department of Transportation (DOT) regulations, and LRN reference laboratory specimen submission requirements.

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