Bench Guide
for
*B. anthracis*,
*Brucella spp.*, 
*Burkholderia mallei* & *pseudomallei*, 
*Francisella tularensis*, 
& *Yersinia pestis*

Wisconsin State Laboratory of Hygiene
24/7 Emergency Messaging Center: 
608-263-3280
WARNING! PERFORM ALL ADDITIONAL WORK IN A BSC ONCE A BIOTERRORISM AGENT IS SUSPECTED.

WARNING! MANIPULATE CULTURES NO MORE THAN NECESSARY ONCE A BIOTERRORISM AGENT IS SUSPECTED.

WARNING! USE OF AUTOMATED ID SYSTEMS IS NOT RECOMMENDED IF A BIOTERRORISM AGENT IS SUSPECTED.

Warnings
## BIOTERORRISMS AGENT WORKUP TABLE

<table>
<thead>
<tr>
<th>Gram Stain Morphology</th>
<th>Growth</th>
<th>Sheep Blood Agar</th>
<th>Chocolate</th>
<th>MAC/EMB</th>
<th>Rule out</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram Positive Rod</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large gram positive rods; subterminal spores may be present with no significant swelling of the cell. Capsule may be seen as a clear area around the bacillus.</td>
<td>Good growth at 24 hrs. 2-8 mm slightly convex colonies with irregular borders, “Medusa head” shape, ground glass color. No or very little hemolysis.</td>
<td>See SBA</td>
<td>No Growth</td>
<td><em>B. anthracis</em></td>
<td></td>
</tr>
<tr>
<td><strong>Gram Negative Coccobacilli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small gram negative coccobacillus</td>
<td>Dust like growth at 24 hrs. Smooth, convex, nonpigmented, nonhemolytic, punctate colonies at 48 hrs.</td>
<td>See SBA</td>
<td>Some strains may grow</td>
<td><em>Brucella</em></td>
<td></td>
</tr>
<tr>
<td>Gram negative coccobacillus or small rod</td>
<td>Poor growth at 24 hrs; smooth, nonpigmented, gray, translucent colonies at 48 hrs.</td>
<td>See SBA.</td>
<td>May or may not grow on MAC.</td>
<td><em>B. mallei</em></td>
<td></td>
</tr>
<tr>
<td>Tiny, pleomorphic, faintly staining gram negative coccobacillus.</td>
<td>Grows poorly on SBA - requires cysteine supplementation.</td>
<td>1-2 mm, white to gray to blue-gray, opaque, flat, entire, smooth and shiny colonies at ≥48 hrs.</td>
<td>No growth</td>
<td><em>F. tularensis</em></td>
<td></td>
</tr>
</tbody>
</table>

Choc = Chocolate Agar; MAC/EMB = MacConkey Agar / EMB Agar

WORK UP ALL SLOW GROWING, GRAM NEGATIVE ORGANISMS IN A CLASS 2 BSC.

**Summary 1**
## BIOTERRORISM AGENT WORKUP TABLE

<table>
<thead>
<tr>
<th>Gram Stain Morphology</th>
<th>Growth</th>
<th>Rule out</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram Negative Bacilli</strong></td>
<td>Sheep Blood Agar</td>
<td>Chocolate</td>
</tr>
<tr>
<td>Gram negative rod, small, straight or slightly curved, may demonstrate bipolar morphology at 24 h and peripheral staining, like endospores, when cultures are older.</td>
<td>Poor growth at 24 h; good growth of smooth, white, nonpigmented colonies at 48 h, which become dry, wrinkled colonies over time; often demonstrates strong musty, earthy odor.</td>
<td>See SBA</td>
</tr>
<tr>
<td>Gram negative rod, “enteric-like”. Single, pairs, short chains. “safety pin” stain may occasionally be seen.</td>
<td>No or very little detectable growth at 24 hours. Gray-white to slightly yellow and opaque, nonhemolytic colonies (1-2) mm in diameter appear after 48 hrs. Colonies have a “fried egg” appearance at 4x magnification.</td>
<td>See SBA</td>
</tr>
</tbody>
</table>

Choc = Chocolate Agar; MAC/EMB = MacConkey Agar / EMB Agar

**WORK UP ALL SLOW GROWING, GRAM NEGATIVE ORGANISMS IN A CLASS 2 BSC.**

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**Summary 2**
GRAM POSITIVE (+) ROD
(large; single or in chains; central to subterminal spores may be present in cultures with no significant swelling of the cell)

B. anthracis, Gram stain. Direct smear from cutaneous lesion (CDC)

B. anthracis from culture. Single cells & short chains, (http://www.state.sd.us/doh/Lab BT/bacillusGram.htm)

B. anthracis, Gram stain. with spores, 1000X (ASM)

REFER TO B. ANTHRACIS

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G+ Rod
GRAM NEGATIVE (-) ROD

(Medium-sized; plump; mostly single cells; pairs or short chains from liquid media; stains well; bipolar “safety pin” stain may occasionally be seen)

Note: Bipolar staining reported with other enteric bacteria, e.g., Pasteurella spp.

REFER TO Y. PESTIS
GRAM NEGATIVE (-) ROD
(Small, straight or slightly curved, may demonstrate bipolar morphology at 24 hrs and peripheral staining, like endospores, when cultures are older.)

Note: Bipolar staining reported with other enteric bacteria, e.g., Pasteurella spp.

REFER TO B. PSEUDOMALLEI

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GRAM NEGATIVE (-) COCCOBACILLUS
(tiny, stains poorly)

REFER TO F. TULARENSIS and BRUCELLA SPP.

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G- Coccobacilli
**B. ANTHRACIS-1**

**GRAM POSITIVE (+) ROD**

Growth on Sheep Blood Agar
Rapid, non-pigmented, flat, slightly convex, irregular edge, comma projections, ground-glass surface, tenacious (beaten egg white)

**Hemolysis**

**B. anthracis**
Ruled Out

**No Hemolysis**

REFER TO Pg 7-**B. ANTHRACIS-2**

**B. anthracis -1**
B. anthracis

Ruled Out

GRAM POSITIVE (+) ROD
+ Growth on Sheep Blood Agar

HEMOLYSIS

POSITIVE
NEGATIVE

CATALASE

NEGATIVE
POSITIVE

MOTILITY

POSITIVE
NEGATIVE

WARNING!

B. anthracis produces
VARIABLE
OXIDASE REACTIONS:
87% POSITIVE
13% NEGATIVE

CANNOT RULE OUT
B. ANTHRACIS
CONTACT WSLH
REFER TO LRN
REFERENCE LAB

B. anthracis - 2
**Y. PESTIS**

- **GRAM NEGATIVE (-) ROD**
  - SLOW-GROWING, PINPOINT COLONIES, Gray-White to Opaque on Sheep Blood Agar after 24 hours

**NO**
- Proceed with Routine ID Procedures

**YES**
- OXIDASE: NEGATIVE
- CATALASE: POSITIVE
- UREASE: NEGATIVE
- INDOLE: NEGATIVE

- **WARNING!**
  - Y. pestis NOT present
- **CANNOT RULE OUT Y. PESTIS**
  - CONTACT WSLH
  - REFER TO LRN REFERENCE LAB

- **Sheep Blood Agar - 48 hr**

**Y. pestis**

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**BRUCELLA spp.**

- **GRAM NEGATIVE TINY COCCOBACILLI**
  - POOR GROWTH on Sheep Blood Agar after 48 hr
  - Non-pigmented, moist, convex, non-hemolytic

  **YES**

  **X & V Factors NOT required.**
  - OXIDASE: POSITIVE
  - CATALASE: POSITIVE
  - UREASE: POSITIVE

  **NO**

  - **Brucella Species Ruled Out**

  **YES**

  - CANNOT RULE OUT BRUCELLA SPECIES
  - CONTACT WSLH
  - REFER TO LRN
  - REFERENCE LAB

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**F. TULARENSIS**

**GRAM NEGATIVE COCCOBACILLI**
TINY, STAINS POORLY,
POOR GROWTH on Sheep Blood Agar after 48 hours
Blue-white to gray, flat, smooth, shiny, pleomorphic

**X & V Factors Not Required**
OXIDASE: NEGATIVE
CATALASE: WEAK POSITIVE
B-lactamase: POSITIVE
UREASE: NEGATIVE

**YES**

**NO**

**F. tularensis**
Ruled Out

**YES**

CANNOT RULE OUT
F. TULARENSIS
CONTACT WSLH
REFER TO LRN
REFERENCE LAB

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**F. tularensis**
BURKHOLDERIA MALLEI

Gram negative coccobacilli or small rods, arranged in pairs, parallel bundles or Chinese-letter forms. POOR GROWTH at 24 hr. on SBA. Gray, translucent colonies at 48 hr. on SBA.

No pigment, Indole negative, Catalase positive
Oxidase variable, Motility negative
Polymyxin B or Colistin: No Zone
No Growth at 42°C at 48 hr.

Burkholderia mallei
Ruled Out

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B. mallei
**BURKHOLDERIA PSEUDOMALLEI**

- **Sheep Blood Agar – 24 hr.**
  - Gram negative rods, small, straight or slightly curved, may demonstrate bipolar morphology at 24 h., peripheral staining (like endospores) in older. Poor growth at 24 h; good growth of white colonies at 48 h on SBA, may develop nonpigmented, wrinkled colonies.

- **Sheep Blood Agar – 72 hr.**
  - No pigment, Indole negative, Catalase positive
  - Oxidase positive, Motility positive, Polymyxin B or Colistin: No Zone; Growth on B. cepacia selective agars, Growth at 42°C at 48 hr.

**YES**

- **Burkholderia pseudomallei Ruled Out**

**NO**

- CANNOT RULE OUT BURKHOLDERIA PSEUDOMALLEI
  - CONTACT WSLH & REFER TO LRN REFERENCE LAB

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**B. pseudomallei**
### SPOT TESTS OF SELECTED ORGANISMS (adapted from CDC)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gram Stain Morphology</th>
<th>Growth</th>
<th>Hemolysis</th>
<th>Motility</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Indole</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus anthracis</strong></td>
<td>Gram positive broad rod</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td><strong>Brucella spp.</strong></td>
<td>Gram negative coccobacillus</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td><strong>Burkholderia mallei</strong></td>
<td>Gram negative coccobacillus</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>V</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td><strong>Burkholderia pseudomallei</strong></td>
<td>Gram negative bacillus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>V</td>
</tr>
<tr>
<td><strong>Francisella tularensis</strong></td>
<td>Gram negative coccobacillus</td>
<td>—V</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td><strong>Yersinia pestis</strong></td>
<td>Gram negative rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
</tbody>
</table>

+ = ≥90% positive; — = ≤10% positive; +V = most species/strains positive; —V = most species/strains negative; V = 11-89% positive

SBA = Sheep Blood Agar; Choc = Chocolate Agar; MAC/EMB = MacConkey / Eosin-Methylene Blue Agar
## BIOSAFETY & MOST COMMONLY ENCOUNTERED AGENTS OF BIOTERRORISM
(adapted from CDC and ASM)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Biosafety Level</th>
<th>Specimen Exposure/Risk</th>
<th>Recommended Precautions for Sentinel Laboratories</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus anthracis</strong></td>
<td>2</td>
<td>Blood, skin lesion exudates, CSF, pleural fluid, sputum; rarely urine &amp; feces.</td>
<td>BSL2: Activities involving clinical material collection &amp; diagnostic quantities of infectious cultures. BSL3: Activities with high potential for aerosol or droplet production.</td>
</tr>
<tr>
<td><strong>Brucella spp.</strong></td>
<td>2</td>
<td>Blood, bone marrow, CSF, tissue, semen, occasionally urine.</td>
<td>BSL2: Activities limited to collection, transport &amp; plating of clinical material. BSL3: All activities involving manipulations of cultures.</td>
</tr>
<tr>
<td><strong>Burkholderia mallei &amp; pseudomallei</strong></td>
<td>2</td>
<td>Blood, sputum, CSF, tissue, abscesses, and urine</td>
<td>BSL2: Activities limited to collection, transport &amp; plating of clinical material. BSL3: All activities involving manipulations of cultures.</td>
</tr>
<tr>
<td><strong>Clostridium botulinum</strong></td>
<td>2</td>
<td>Toxin may be present in food specimens, clinical material (serum, gastric &amp; feces), &amp; environmental samples (soil, surface water). TOXIN IS EXTREMELY POISONOUS!</td>
<td>BSL2: Activities with materials known or potentially containing toxin must be handled in a Class II BSC with lab coat, disposable gloves, &amp; face shield (as needed). BSL3: Activities with high potential for aerosol or droplet production.</td>
</tr>
<tr>
<td><strong>Francisella tularensis</strong></td>
<td>2</td>
<td>Skin lesion exudates, respiratory secretions, CSF, blood, urine, tissues from infected animals &amp; fluids from infected arthropods.</td>
<td>BSL2: Activities limited to collection, transport &amp; plating of clinical material. BSL3: All activities involving manipulations of cultures.</td>
</tr>
<tr>
<td><strong>Yersinia pestis</strong></td>
<td>2</td>
<td>Bubo fluid, blood, sputum, CSF, feces, urine.</td>
<td>BSL2: Activities involving clinical material collection &amp; diagnostic quantities of infectious cultures BSL3: Activities with high potential for aerosol or droplet production.</td>
</tr>
</tbody>
</table>

Safety 1
# Biosafety & Less Commonly Encountered Agents of Bioterrorism

(adapted from CDC and ASM)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Biosafety Level</th>
<th>Specimen Exposure/Risk</th>
<th>Recommended Precautions for Sentinel Laboratories</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alphaviruses</strong></td>
<td>2 3</td>
<td>Blood, CSF. Tissue culture and animal inoculation studies should be performed at BSL-3 and are NOT Sentinel (Level A) laboratory procedures.</td>
<td>BSL-2: Activities involving clinical material collection and transport. Biosafety levels variable by agent; may require BSL3 or more.</td>
</tr>
<tr>
<td><strong>Coxiella burnetii</strong></td>
<td>2 3</td>
<td>Blood, tissue, body fluids, feces. Manipulation of tissues from infected animals and tissue culture should be performed at BSL-3 and are NOT Sentinel laboratory procedures.</td>
<td>BSL2: Activities limited to collection and transport of clinical material, including serological examinations. BSL3: Activities involving inoculation, incubation, harvesting of eggs or cell cultures, animal necropsy, manipulation of infected tissues.</td>
</tr>
<tr>
<td><strong>Smallpox</strong></td>
<td>4 4</td>
<td>Lesion fluid or crusts, respiratory secretions, or tissue</td>
<td>BSL-2: Packing and shipping. Do NOT put in cell culture. Contact public health and WSLH</td>
</tr>
<tr>
<td><strong>Staphylococcal enterotoxin B</strong></td>
<td>2 2</td>
<td>Toxin may be present in food specimens, clinical material (serum, gastric, urine, respiratory secretions, and feces), and isolates of S. aureus.</td>
<td>BSL-2: Activities involving clinical material collection and diagnostic quantities of infectious cultures. BSL3: Activities with high potential for aerosol or droplet production.</td>
</tr>
<tr>
<td><strong>Viral Hemorrhagic Fever (VHF)</strong></td>
<td>4 4</td>
<td>Blood, urine, respiratory, and throat secretions, semen, and tissue</td>
<td>BSL-2: Packing and shipping. Do NOT put in cell culture. Contact public health and WSLH</td>
</tr>
</tbody>
</table>

**Safety 2**