Recognize. Rule-Out. Refer.

Biothreat Agent Bench Cards for the Sentinel Clinical Laboratory

For questions, contact your designated LRN Reference Level Laboratory

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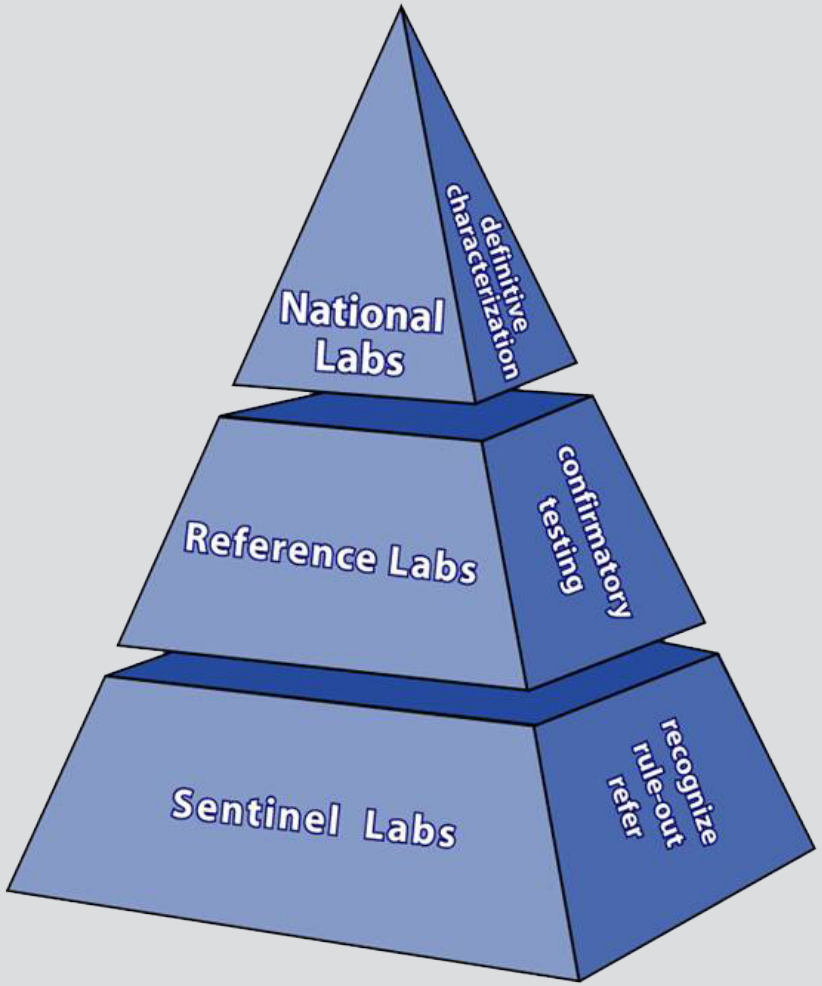
Laboratory Response Network for Biological Threats (LRN-B)

The LRN-B was founded in 1999 by the Centers for Disease Control and Prevention (CDC), Federal Bureau of Investigation (FBI) and the Association of Public Health Laboratories (APHL) to coordinate laboratory response to biological, chemical and emerging threats.

- **National Laboratories**, including the CDC, U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID), and the Naval Medical Research Center (NMRC), are responsible for specialized strain characterization, bioforensics, select agent activity and handling of highly infectious biological agents.

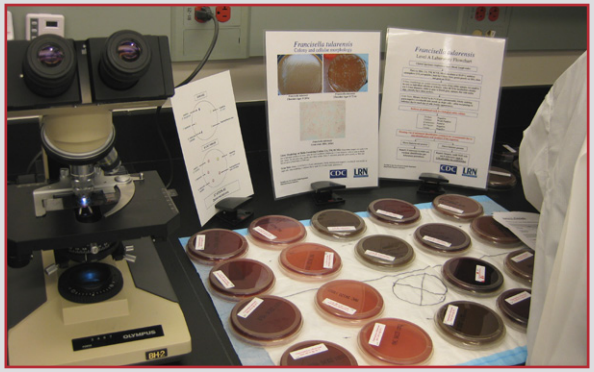
- **Reference Laboratories**, including over 150 state and local public health, military, international, veterinary, agriculture, food and water testing laboratories, are responsible for investigation and confirmatory testing.

- **Sentinel Clinical Laboratories**, comprised of hospital-based and commercial laboratories, are responsible for the early detection and the rule-out or referral of potential biothreat agents.



Responsibilities of the Sentinel Clinical Laboratory

1. Familiar with reportable disease guidelines; has policies and procedures to refer specimens or isolates suspected to contain biothreat agents to the local/state public health laboratory
2. Ensures personnel meet applicable federal regulations for packing and shipping of infectious substances
3. Has policies and procedures for referral of suspect biothreat agent specimens and/or isolates reflecting the American Society for Microbiology (ASM) Sentinel Level Clinical Laboratory Protocols for Suspected Biological Threat Agents and Emerging Infectious Diseases
4. Maintains capability to perform testing outlined in the ASM Sentinel Level Clinical Laboratory Protocols for Suspected Biological Threat Agents and Emerging Infectious Diseases and demonstrates annual competency by participation in proficiency testing or exercises
5. Has a Class II or higher Certified Biological Safety Cabinet
6. Complies with Biological Safety Level II (BSL-2) practices
7. Complies with applicable Occupational Safety and Health Administration (OSHA) regulations for a respiratory protection program
8. Complies with the rules and regulations of the Select Agent Program



Bacillus anthracis

Gram Stain

- Large Gram-positive rods (1-1.5 µm x 3-5 µm)
- Short chains in clinical specimens (2-4 cells); Long chains in culture
- Spores are oval, central to subterminal (not commonly seen in direct smears of clinical specimens)
- Capsules may be present in clinical specimens

Colony Morphology

- Growth on BAP and CHOC; No growth on MAC (or EMB)
- Colonies are 2-5 mm on BAP at 18-24h; Growth can be observed as early as 4-8h
- Colonies are round with irregular edges, flat or slightly convex with ground glass appearance; May have comma-like projections
- Colonies are tenacious and adhere to the agar surface
- Non-hemolytic on BAP

Biochemical/Test Reactions

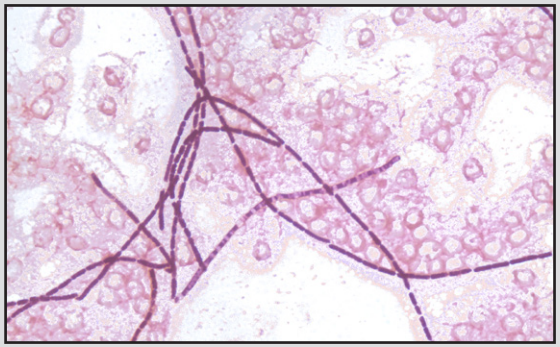
- Catalase positive
- Motility negative (via wet mount or tube media)

Additional Information

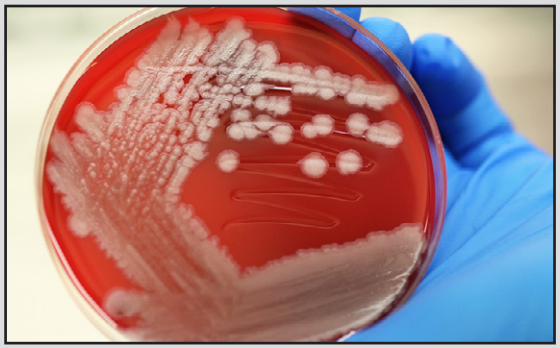
- Biosafety precautions: BSL-2 agent; recommend performing all manipulations in a Class II BSC
- **Commonly misidentified as *Bacillus megaterium* and other *Bacillus* species by automated ID systems**
- Transmission: inhalation, ingestion, direct contact (spored enter the skin through cuts or abrasions)

Acceptable Specimen Types

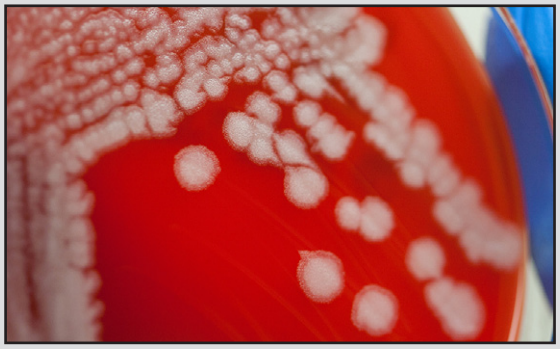
- Swab of vesicular fluid or edge of eschar from cutaneous lesion
- Stool (> 5 g)
- Sputum (≥ 1 mL)
- Whole blood in an appropriate blood culture bottle
- CSF (only if signs of meningitis occur)
- Postmortem tissue



Gram stain of blood culture



24h growth on BAP



Irregular edged colonies

B. anthracis Algorithm

WARNING: See Additional Information Regarding Misidentification with Automated Systems

Large Gram-positive rods (1-1.5 µm x 3-5 µm); Spores may be found in cultures grown in 5% CO_2 (but not usually in clinical samples)

↓

Ground glass appearance;
No hemolysis or pigment on BAP; No growth on MAC (or EMB)

↓

Perform all additional work in a certified Class II BSC
Catalase positive
Motility negative (via wet mount or tube media)

↙ No

B. anthracis ruled out. Continue with routine identification.

↘ **Yes, STOP**

B. anthracis not ruled out. Call the LRN Reference Level Laboratory and send suspect agent.

24h growth on BAP

24h growth on CHOC

Brucella species

Gram Stain

- Faintly staining, tiny Gram-negative coccobacilli (0.4 x 0.8 µm)
- May be mistaken for cocci

Colony Morphology

- Growth on BAP and CHOC (CO_2 may be required by some strains); No growth on MAC
- Pinpoint colonies at 24h; Discrete, white colonies (0.5-1.0 mm) evident at 48h
- Non-hemolytic

Biochemical/Test Reactions

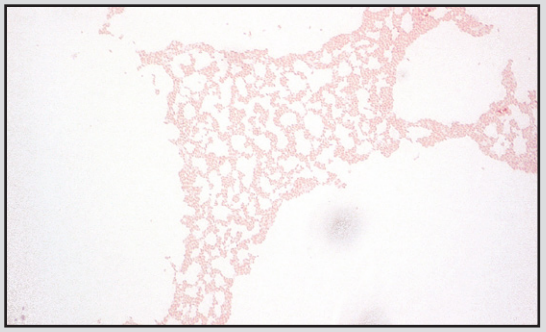
- Oxidase positive
- Catalase positive
- Urease positive
- Satellite negative

Additional Information

- Biosafety precautions: BSL-3 agent; perform all manipulations in a Class II BSC
- **Commonly misidentified as *Moraxella* spp., *Micrococcus* spp. *Corynebacterium* spp., "slow growing" *Staphylococcus* spp., *Oligella ureolytica*, *Bordetella bronchiseptica*, *Haemophilus* spp., or *Pasteurella* spp. by automated ID systems**
- Transmission: inhalation, consumption of unpasteurized dairy products, direct contact with skin
- **Common laboratory-acquired infection due to the generation of aerosols during manipulation**

Acceptable Specimen Types

- Bone marrow or whole blood in an appropriate blood culture bottle
- Joint or abdominal fluid in an appropriate blood culture bottle
- Spleen or liver abscesses
- Serum (≥1 mL, without anticoagulant)



Gram stain

48h growth on BAP

48h growth on CHOC

Brucella species Algorithm

WARNING: See Additional Information Regarding Misidentification with Automated Systems

Tiny, Gram-negative coccobacilli (0.4 x 0.8 µm)

↓

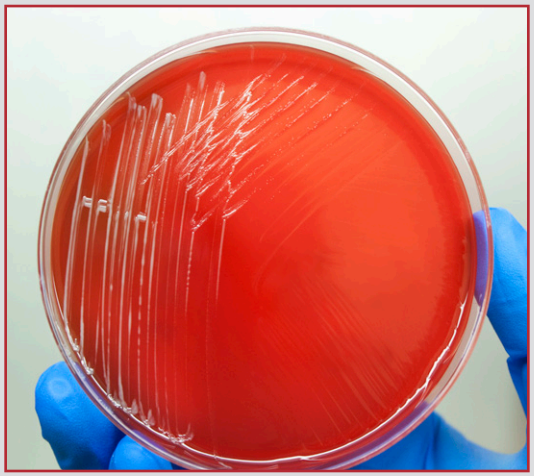
Poor growth at 24h on BAP and CHOC; Discrete colonies at 48h; Non-hemolytic; Non-pigmented; No growth on MAC

↓

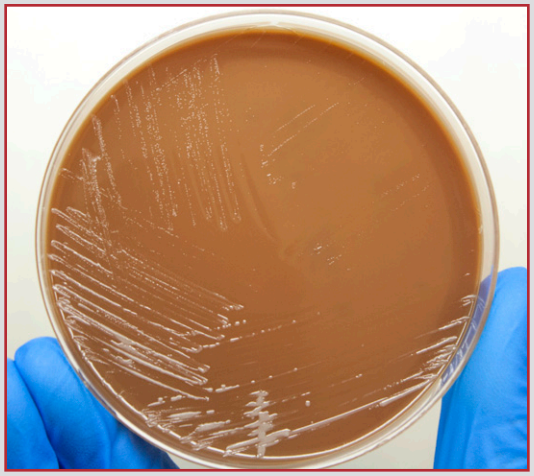
Perform all additional work in a certified Class II BSC

Oxidase positive
Catalase positive
Urease positive
Satellite negative

No	Yes, STOP
↓	↓
Brucella ruled out. Continue with routine identification.	*Brucella* not ruled out. Call the LRN Reference Level Laboratory and send suspect agent.



48h growth on BAP



48h growth on CHOC

Burkholderia mallei

Gram Stain

- Faintly staining, Gram-negative bacilli or slightly curved coccobacilli (1.5-3 µm x 0.5-1 µm)
- Cells are arranged in pairs, parallel bundles or the Chinese-letter form

Colony Morphology

- Pinpoint to small grey colonies on BAP at 24h; May become smooth, grey, and translucent on BAP at 48h
- No growth or colorless to light pink colonies on MAC at 48h
- Non-hemolytic

Biochemical/Test Reactions

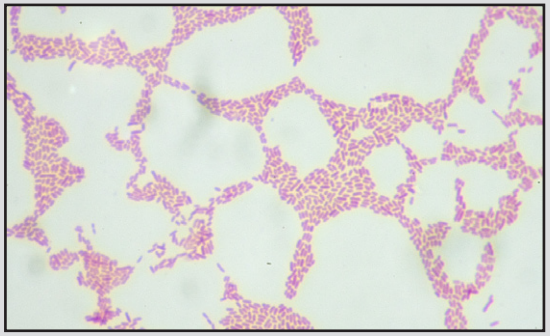
- Oxidase variable
- Catalase positive
- Indole negative
- Motility negative (via tube media)
- Polymyxin B and colistin resistant; penicillin resistant; amoxicillin-clavulanate susceptible
- No growth at 42 °C

Additional Information

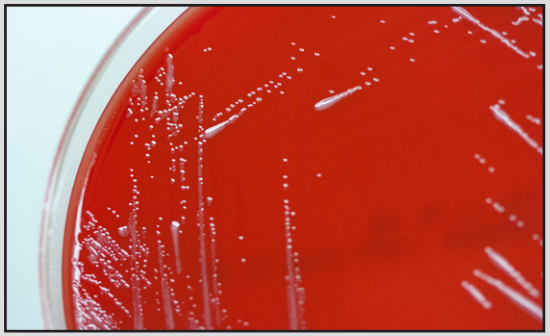
- Biosafety precautions: BSL-3 agent; perform all manipulations in a Class II BSC
- **Commonly misidentified as other non-fermenting Gram negative bacilli by automated ID systems**
- Transmission: inhalation, wound contamination, direct contact with infected animals

Acceptable Specimen Types

- Bone marrow or whole blood in an appropriate blood culture bottle
- Sputum or bronchoscopically obtained specimens
- Tissue specimens (abscess aspirates, biopsies) and wound swabs
- Urine (≥ 1 mL)



Gram stain



24h growth on BAP

48h growth on BAP

B. mallei Algorithm

WARNING: See Additional Information Regarding Misidentification with Automated Systems

Gram-negative coccobacilli or small rods (1.5-3 µm x 0.5-1 µm)

↓

Poor growth at 24h; Better growth of grey, translucent, non-pigmented colonies at 48h on BAP; Non-hemolytic; Poor or no growth on MAC at 48h

↓

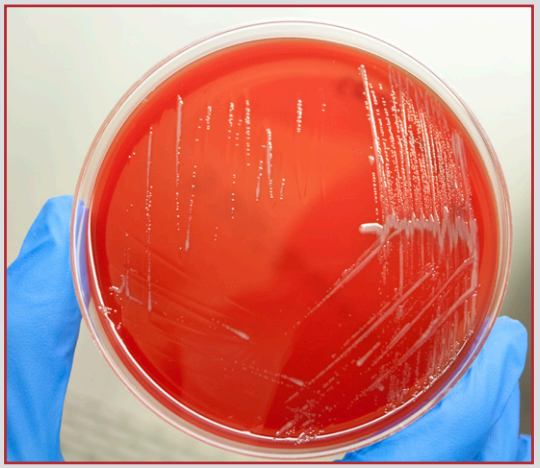
Perform all additional work in a certified Class II BSC
Oxidase variable
Catalase positive
Indole negative
Motility negative (via tube media)
Polymyxin B and colistin resistant; penicillin resistant; amoxicillin-clavulanate susceptible
No growth at 42°C

↓ No

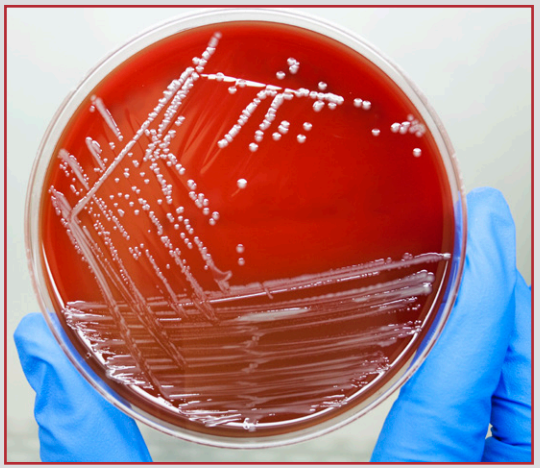
B. mallei ruled out. Continue with routine identification.

↓ **Yes, STOP**

B. mallei not ruled out. Call the LRN Reference Level Laboratory and send suspect agent.



24h growth on BAP



48h growth on BAP

Burkholderia pseudomallei

Gram Stain

- Straight, or slightly curved Gram-negative rods (2-5 µm x 0.4-0.8 µm)
- May demonstrate bipolar staining in direct specimens and peripheral staining in older cultures

Colony Morphology

- Smooth, creamy white colonies at 24h; may become dry or mucoid on BAP at 48h
- Growth on MAC
- Non-hemolytic
- Often produces distinctive musty or earthy odor (DO NOT SNIFF PLATE)

Biochemical/Test Reactions

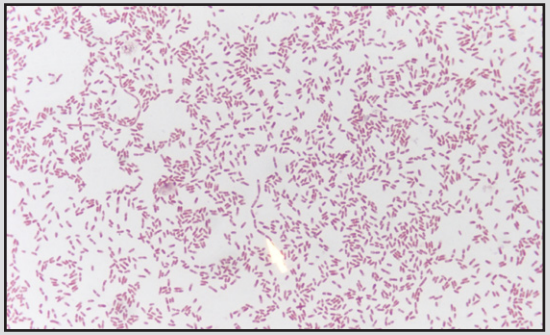
- Oxidase positive
- Catalase positive
- Indole negative
- Motility positive (via tube media)
- Polymyxin B and colistin resistant; penicillin resistant; amoxicillin-clavulanate susceptible
- Growth at 42°C

Additional Information

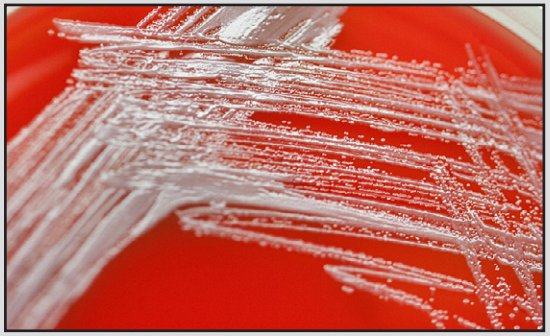
- Biosafety precautions: BSL-3 agent; perform all manipulations in a Class II BSC
- **Commonly misidentified as *Chromobacterium violacium* (hemolytic and may have violet pigment on BAP) and other non-fermenting Gram negative bacilli by automated ID systems**
- Transmission: inhalation, wound contamination

Acceptable Specimen Types

- Bone marrow or whole blood in an appropriate blood culture bottle
- Sputum or bronchoscopically obtained specimens
- Tissue specimens (abscess aspirates, biopsies and wound swabs)
- Urine (≥ 1 mL)



Gram stain



24h growth on BAP

48h growth on BAP

B. pseudomallei Algorithm

WARNING: See Additional Information Regarding Misidentification with Automated Systems

Gram-negative rods, may have bipolar staining (2-5 µm x 0.4-0.8 µm)

↓

Poor growth at 24h, good growth at 48h on BAP, may become wrinkled; Non-hemolytic; Growth on MAC at 24h; Musty or earthy odor

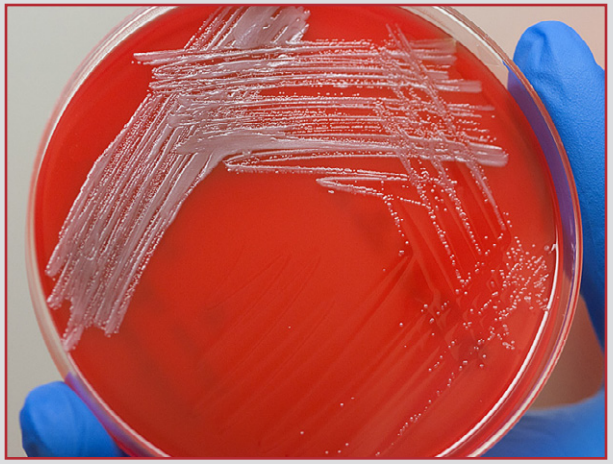
↓

Perform all additional work in a certified Class II BSC

Oxidase positive
Catalase positive
Indole negative
Motility positive (via tube media)
Polymyxin B and colistin resistant; penicillin resistant; amoxicillin-clavulanate susceptible
Growth at 42°C

No → *B. pseudomallei* ruled out. Continue with routine identification.

Yes, STOP → *B. pseudomallei* not ruled out. Call the LRN Reference Level Laboratory and send suspect agent.



24h growth on BAP

48h growth on BAP

Francisella tularensis

Gram Stain

- Tiny, poorly counterstaining, Gram-negative coccobacilli (0.2-0.5 µm x 0.7-1.0 µm)

Colony Morphology

- Little to no growth on BAP; No growth on MAC
- Growth on CHOC: grey-white, smooth, flat, opaque colonies with shiny surface
- Slow growing – colonies are 1-2 mm in diameter on CHOC at 48h

Biochemical/Test Reactions

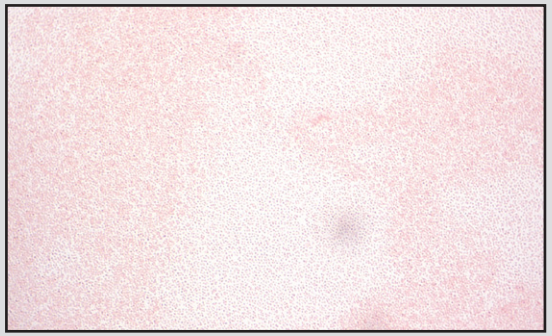
- Oxidase negative
- Catalase negative or weakly positive
- Satellite negative
- β-lactamase positive

Additional Information

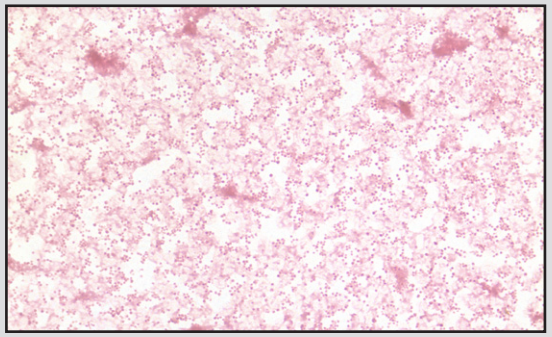
- Biosafety precautions: BSL-3 agent; perform all manipulations in a Class II BSC
- **Commonly misidentified as *Aggregatibacter actinomycetemcomitans*, *Haemophilus influenzae*, *Oligella* spp., or *Psychrobacter* spp. by automated ID systems**
- Transmission: inhalation, insect bite, contact with tissues or bodily fluids of infected animals
- **Common laboratory-acquired infection due to the generation of aerosols during manipulation**

Acceptable Specimen Types

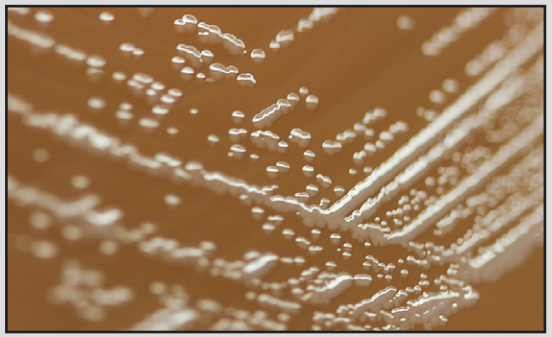
- Tissue biopsy or scraping of ulcer; tissue swab (not preferred)
- Lymph node or lesion aspirate
- Bone marrow or whole blood in an appropriate blood culture bottle
- Respiratory secretions
- Serum (≥1 mL, without anticoagulant)



Gram stain



Gram stain of a blood culture



48h growth on CHOC

F. tularensis Algorithm

WARNING: See Additional Information Regarding Misidentification with Automated Systems

Tiny, pleomorphic, faintly staining, Gram-negative coccobacilli (0.2-0.5 µm x 0.7-1.0 µm)

↓

Little to no growth on BAP >48h; Produces 1-2 mm grey to greyish-white colonies on CHOC >48h; No growth on MAC

↓

Perform all additional work in a certified Class II BSC

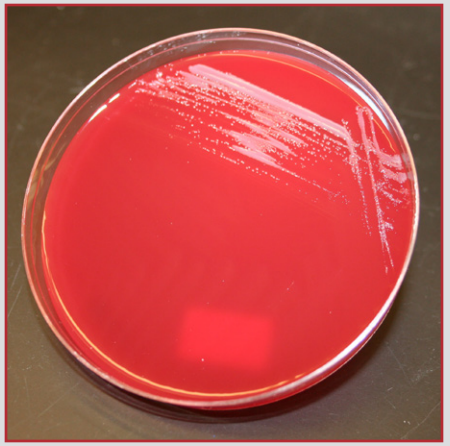
Oxidase negative
Catalase negative or weakly positive
Satellite negative
β-lactamase positive

↓ No

F. tularensis ruled out. Continue with routine identification.

↓ **Yes, STOP**

F. tularensis not ruled out. Call the LRN Reference Level Laboratory and send suspect agent.



48h growth on BAP

48h growth on CHOC

Yersinia pestis

Gram Stain

- Gram-negative rods (1-2 µm x 0.5 µm)
- Arranged singly, in pairs, or short chains
- May exhibit bipolar ("safety-pin") appearance that may be seen with Giemsa or Wright's stains

Colony Morphology

- Grey-white, translucent, pinpoint colonies on BAP at 24h
- Grey-white to slightly yellow, opaque, 1-2 mm colonies on BAP at 48h
- Little to no hemolysis on BAP
- Non-lactose fermenter on MAC/EMB

Biochemical/Test Reactions

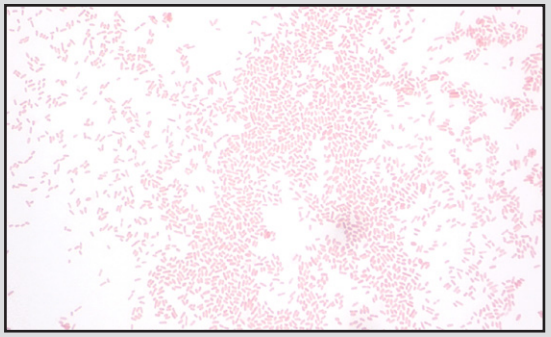
- Oxidase negative
- Catalase positive
- Indole negative
- Urease negative

Additional Information

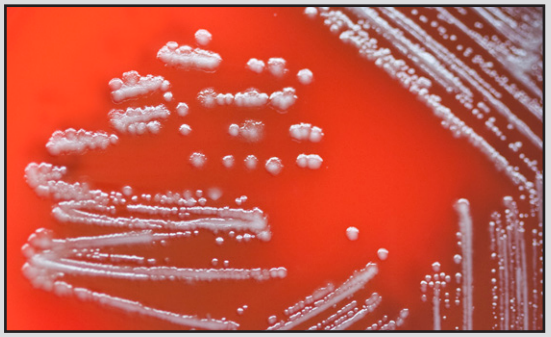
- Biosafety precautions: BSL-2 agent; recommend performing all manipulations in a Class II BSC
- **Commonly misidentified as *Y. pseudotuberculosis*, *Shigella* spp., $H_2S(-)$ *Salmonella* spp., *Acinetobacter* spp., or *Pseudomonas* spp. by automated ID systems**
- Transmission: inhalation, flea bite and contact with contaminated tissue
- Bipolar staining may be seen in the Gram stain of older cultures
- "Fried egg" appearance on BAP in older cultures
- Motility negative at 25-28°C and at 35°C (via wet mount or tube media)

Acceptable Specimen Types

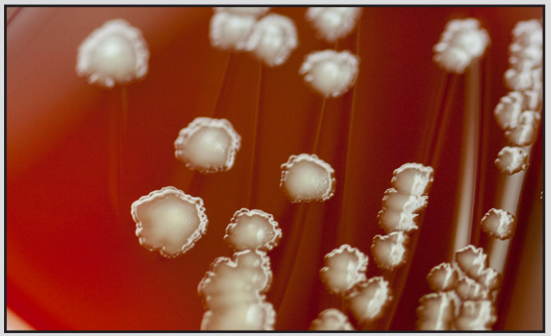
- Lower respiratory tract specimens
- Whole blood in an appropriate blood culture bottle
- Aspirate, tissue, or biopsy specimen
- Tissue swab (not preferred)



Gram stain



48h growth on BAP



Fried egg appearance at 96h

Y. pestis Algorithm

WARNING: See Additional Information Regarding Misidentification with Automated Systems

Gram-negative rods (1-2 µm x 0.5 µm)

↓

Slow-growing, pinpoint, grey-white to opaque colonies on BAP after 24h; Non-lactose fermenter on MAC/EMB; Growth at 25-28 °C and 35-37 °C

↓

Perform all additional work in a certified Class II BSC

Oxidase negative
Catalase positive
Indole negative
Urease negative

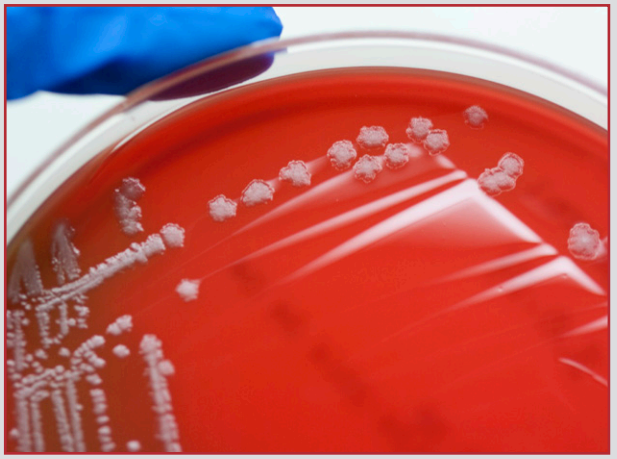
↓ No

Y. Pestis ruled out. Continue with routine identification.

↓ **Yes, STOP**

Y. pestis not ruled out. Call the LRN Reference Level Laboratory and send suspect agent.

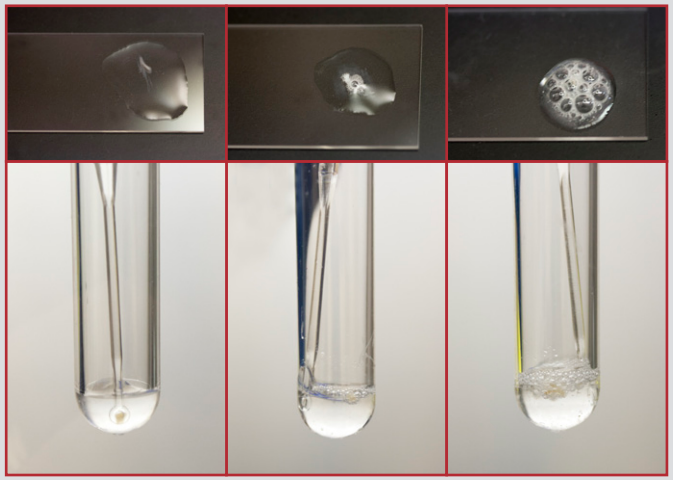
48h growth on BAP



48h growth on BAP

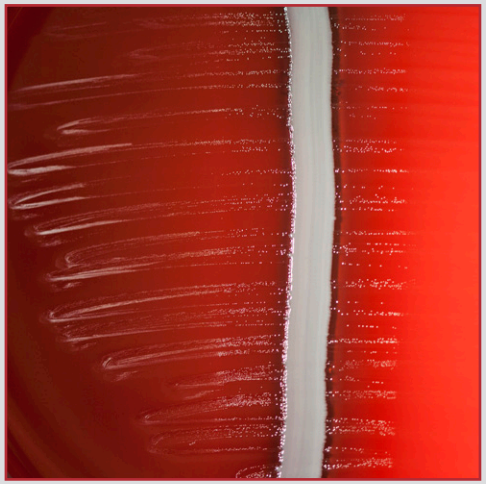
Biochemical Reactions

Catalase



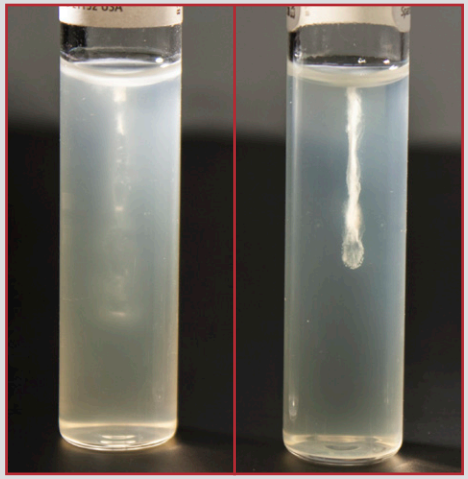
Negative | Weak Positive | Positive

Satellite Growth



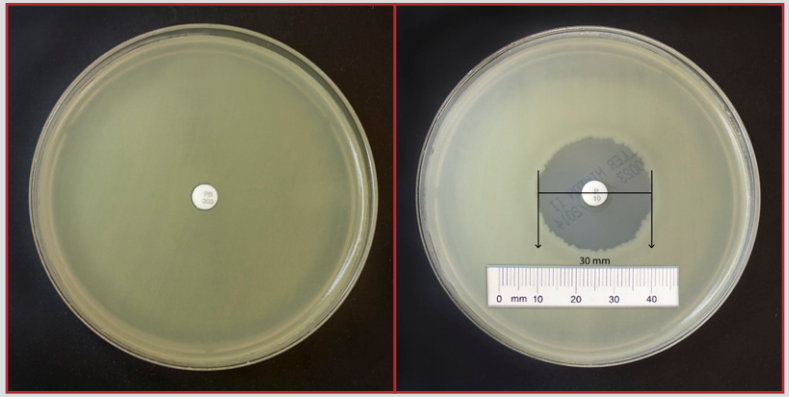
Note: Positive growth is observed around *Staphylococcus aureus* streak

Motility



Positive | Negative

Antibiotic Susceptibility



No Zone of Inhibition:
Resistant

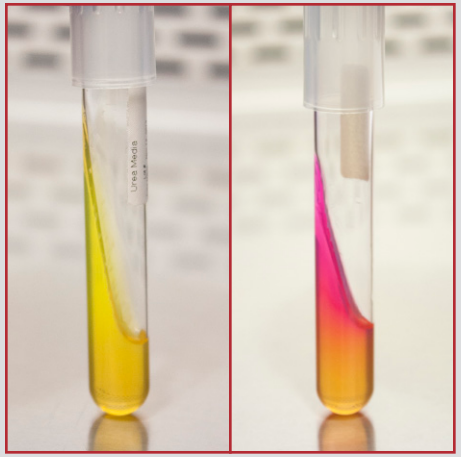
Zone of Inhibition:
Check for Susceptibility

Biochemical Reactions

Oxidase

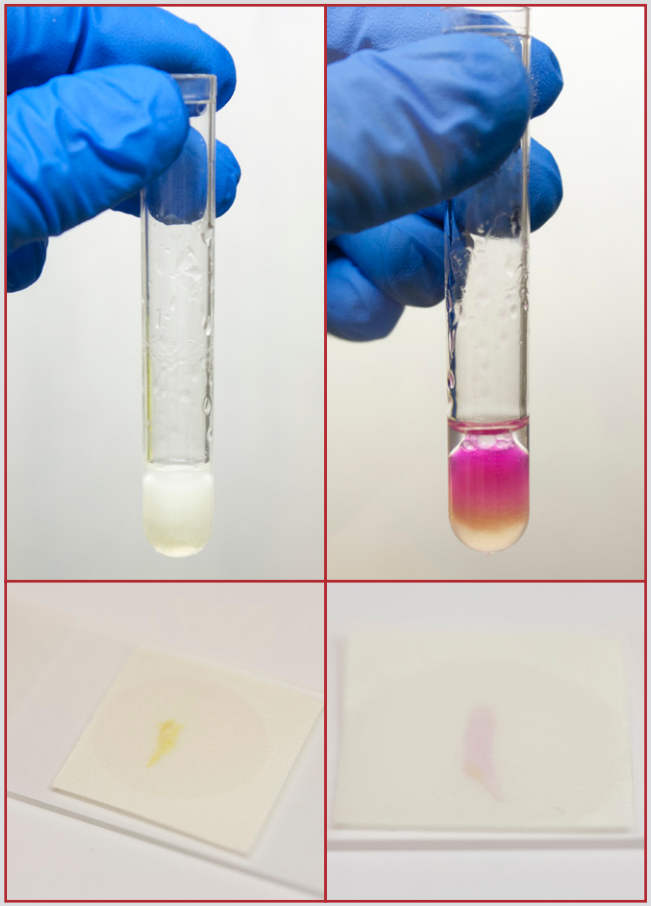
Negative Positive

Urease



Negative Positive

Indole



Negative Positive

Checklist for Sentinel Clinical Laboratories

Preparedness in the Sentinel Clinical Laboratory

Plans

- ❑ Institutional Emergency Response Plan
- ❑ Specific Bioterrorism Response Plan

Training

- ❑ Packaging and Shipping
- ❑ Rule-Out of Select Agents/BT Agents
- ❑ Select Agent Regulations
- ❑ Communications and Messaging

Proficiency Testing

- ❑ Proficiency test/exercise (e.g., CAP LPX)
- ❑ Maintain supplies for rule-out testing

Updates

- ❑ Review ASM's website for updated Sentinel Level Clinical Laboratory Protocols
- ❑ Trainings from the Association of Public Health Laboratories (APHL)

What to do if you have a:

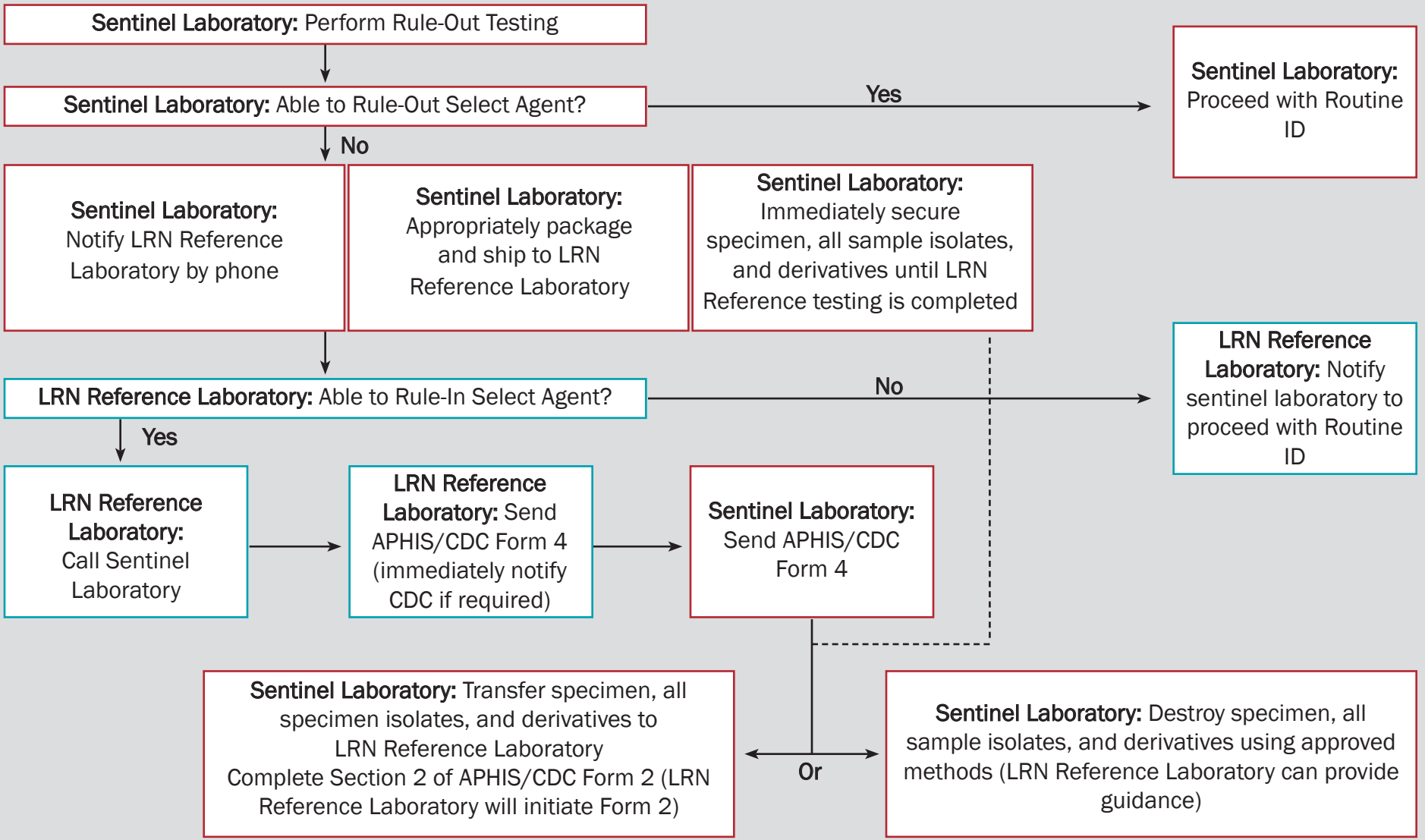
Suspect BT Agent

- ❑ Follow rule-out procedures
- ❑ Initiate/maintain communication with departmental/hospital leadership and infection control
- ❑ Contact BT personnel at designated LRN Reference Level Laboratory
- ❑ Ship isolate to designated LRN Reference Level Laboratory
- ❑ Document courier transfer (e.g., institutional or commercial courier tracking number)
- ❑ Secure all potential select agent(s) and residual samples
- ❑ Document personnel with access to potential select agent(s) (biosecurity)
- ❑ Document personnel who have worked with suspect select agent and those present in laboratory if exposure occurred (biosafety)

Confirmed BT Agent

- ❑ Follow directions from designated LRN Reference Level Laboratory for the destruction or transfer of all isolates/specimens
- ❑ Document identification of select agent(s)
  - APHIS/CDC Form 4
- ❑ Document disposition of select agent(s)
  - APHIS/CDC Form 2 for the transfer of select agent(s)
  - APHIS/CDC Form 4 for the destruction of select agent(s)
- ❑ Document any laboratory exposures
  - Work with designated LRN Reference Level Laboratory or health department for post-exposure prophylaxis
  - APHIS/CDC Form 3

Select Agent Response Algorithm



References

- APHL's Public Health Preparedness & Response Program: http://www.aphl.org/aphlprograms/preparedness-and-response/Pages/default.aspx
- APHL's Training Department: http://www.aphl.org/training/Pages/default.aspx
- American Society for Microbiology's (ASM) Sentinel Level Clinical Laboratory Protocols for Suspected Biological Threat Agents and Emerging Infectious Diseases: http://www.asm.org/index.php/guidelines/sentinel-guidelines
- CDC Morbidity and Mortality Weekly Report: Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories http://www.cdc.gov/mmwr/preview/mmwrhtml/su6101a1.htm
- Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition: http://www.cdc.gov/biosafety/publications/bmbl5/
- Definition of Sentinel Clinical Laboratories: http://www.aphl.org/aphlprograms/preparedness-and-response/partnerships-and-outreacDocuments/PHPR_2013Nov_Sentinel-Laboratory-Definition.pdf
- APHL State Public Health Laboratories Emergency Contact Directory: http://www.aphl.org/AboutAPHL/publications/Documents/PHPR_2012April_State-Public-Health-Laboratories-Emergency-Contact-Directory.pdf
- National Select Agent Registry: http://www.selectagents.gov/
- Wadsworth Center at the New York State Department of Health Basic Select Agent Flow Chart & Evaluation (B-SAFE) Bench Cards: http://www.health.ny.gov/guidance/oph/wadsworth/

APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES®

For more information about these benchcards, please contact the Public Health Preparedness and Response Team at APHL:
emergency.preparedness@aphl.org