# Identifying Select Agents

Shannon Sharp
Bioterrorism Training Coordinator
MDHHS Bureau of Laboratories

# Objectives

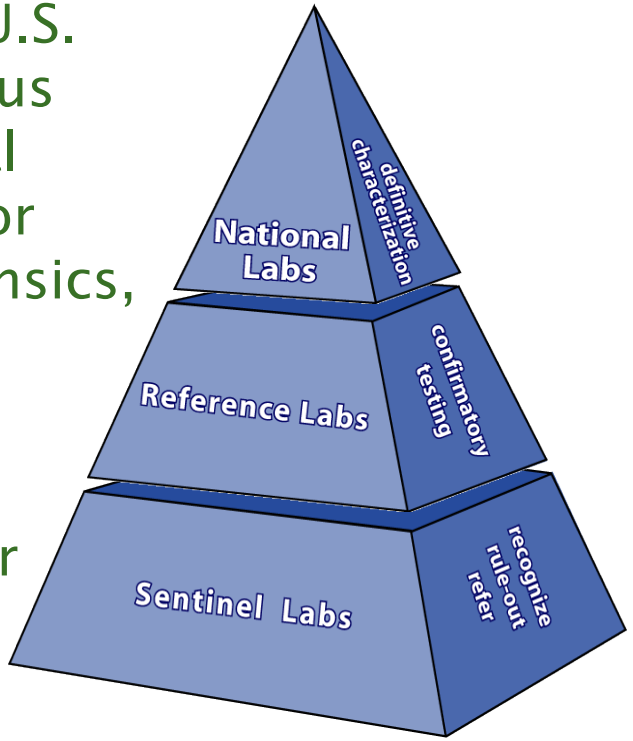
1. Define the responsibilities of a sentinel laboratory.
2. Describe the types of samples to collect to rule out select agents.
3. Determine the tests to perform to rule out suspected select agents.
4. List the steps to take once a select agent cannot be ruled out.

# 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, radiological threats and other high priority public health emergencies, including emerging infectious diseases

# LRN-B cont.

- **National Laboratories**, including the CDC, U.S. Army Medical Research Institute of 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**, are responsible for investigation and confirmatory testing.

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

American Society for Microbiology (asm.org)

# Sentinel Laboratory Definition

The laboratory is certified to perform high complexity testing under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) by the Centers for Medicare & Medicaid Services (CMS) for the applicable Microbiology specialty or the laboratory is a Department of Defense (DoD) Laboratory certified under the DoD Clinical Laboratory Improvement Program or the laboratory is a veterinary medical diagnostic laboratory that is fully accredited by the American Association of Veterinary Laboratory Diagnosticians (AAVLD).

**Laboratory in-house testing includes Gram stains and at least one of the following: lower respiratory tract, wound or blood cultures.***

* American Society for Microbiology (asm.org)

# Responsibilities of Sentinel Lab

- Familiar with reportable disease guidelines in its jurisdiction; has policies and procedures to refer specimens or isolates suspected to contain biothreat agents to the local/state public health laboratory.
- Ensures personnel meet applicable federal regulations for packaging and shipping of infectious substances.
- 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.

American Society for Microbiology (asm.org)

# Responsibilities of Sentinel Lab cont.

- 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.

- Has a Class II or higher Certified Biological Safety Cabinet (BSC).

- Complies with Biological Safety Level II (BSL-2) practices as outlined in the current edition of the Biosafety in Microbiological and Biomedical Laboratories guidelines (BMBL).

American Society for Microbiology (asm.org)

# Responsibilities of Sentinel Lab cont.

- Complies with applicable Occupational Safety and Health Administration (OSHA) regulations for a respiratory protection program.
- Complies with the rules and regulations of the Federal Select Agent Program.


American Society for Microbiology (asm.org)

# American Society for Microbiology (ASM)
# https://www.asm.org/

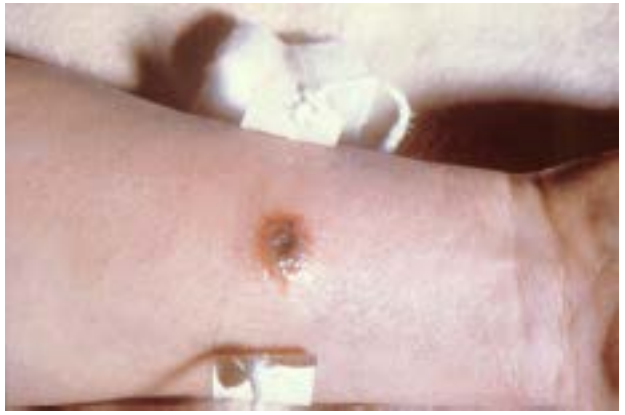
| Select Agent Guidelines Available on the ASM Website | |
|---|---|
| Anthrax (*Bacillus anthracis*) | Novel Influenza Viruses |
| *Brucella* | Plague (*Yersinia pestis*) |
| Botulinum Toxin | Smallpox |
| *Burkholderia* | Staphylococcal Enterotoxin B |
| *Coxiella burnetii* | Tularemia (*Francisella tularensis*) |

**Additional guidelines:** BT Readiness Plan & Packaging and Shipping

# Select Agents: Information & Identification

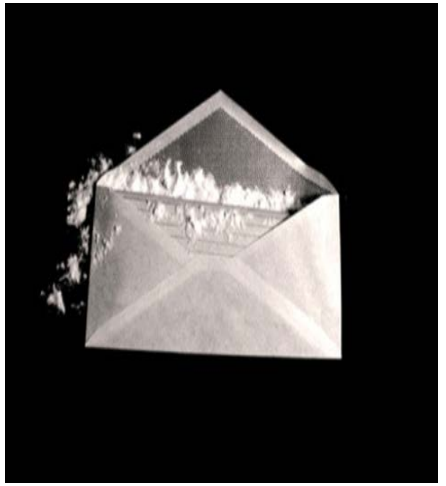
# *Bacillus anthracis* "Anthrax"

- Zoonotic organism (primarily herbivores)
- Soil reservoir
  - Also found in water and plants
- No person to person transmission
- Safety Consideration:
  - As soon as *B. anthracis* is suspected in the laboratory, perform ALL further work within a Class II Biological Safety Cabinet (BSC) using BSL-3 practices.



# *Bacillus anthracis*

- Infection Types–Based on Entry Into Body:
  - Inhalation (most deadly form of anthrax)
    - Inhale spores which can occur following intentional aerosol release
      - I.e. 2001 anthrax biocrime
    - If untreated, 85 to 90% patients will not survive
  - Cutaneous
    - Most common form of naturally occurring cases
    - Spores enter skin through cuts and abrasions
  - Gastrointestinal
    - Consume contaminated undercooked meat
  - Injection
    - Identified in heroin-injecting drug users in northern Europe.



# Sample Collection for *Bacillus anthracis*

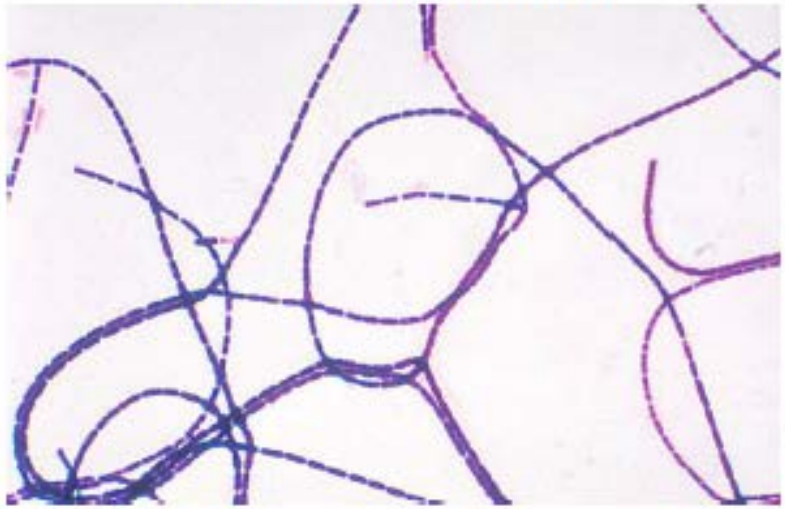
| A. Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing* | | |
|---|---|---|
| Cutaneous | Vesicular (early) stage | Unroof vesicle and aspirate fluid or collect with two sterile swabs (dacron) |
| | Eschar (late) stage | Insert swab (dacron) beneath the edge of the eschar, rotate swab or obtain an aspirate. Transport specimens at room temperature. |
| Gastrointestinal | | Stool (> 5 grams…pecan size), collect and transport in a leak proof sealed container |
| | | Collect blood (late stage of infection) directly into an appropriate blood culture bottle (aerobic and anaerobic) |
| | | Transport specimens and bottles at room temperature |
| Inhalational | | Sputum |
| | | Blood: collect directly into an appropriate blood culture bottle (aerobic and anaerobic) |
| | | Cerebral Spinal Fluid only if signs of meningitis occur |
| | | Transport specimens and bottles at room temperature |
| Postmortem Tissue | | Tissue pieces should be collected and kept moist |
| | | Transport in sterile container at room temperature within 1 hour of collection |

APHL Clinical Laboratory Preparedness and Response Guide pg. 77

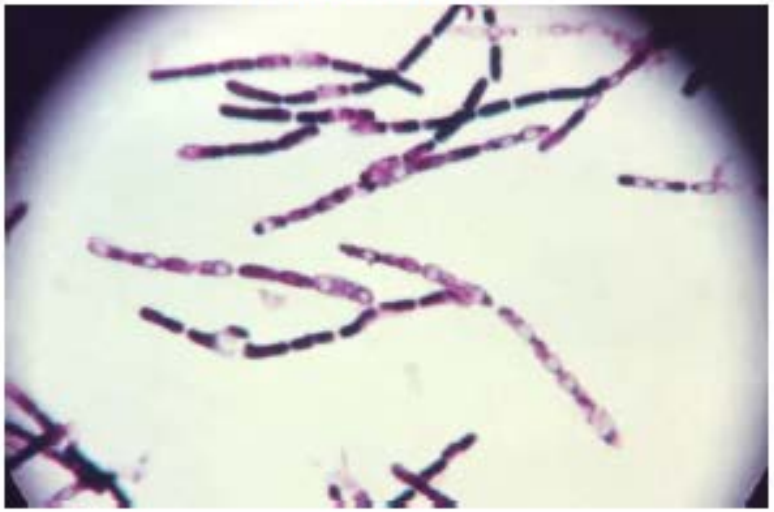
# *Bacillus anthracis*

- Gram Stain
  - Large Gram positive rods
  - Spores may be found in cultures grown in 5% $CO_2$ but are not usually seen in clinical samples

Gram stain of *Bacillus anthracis*



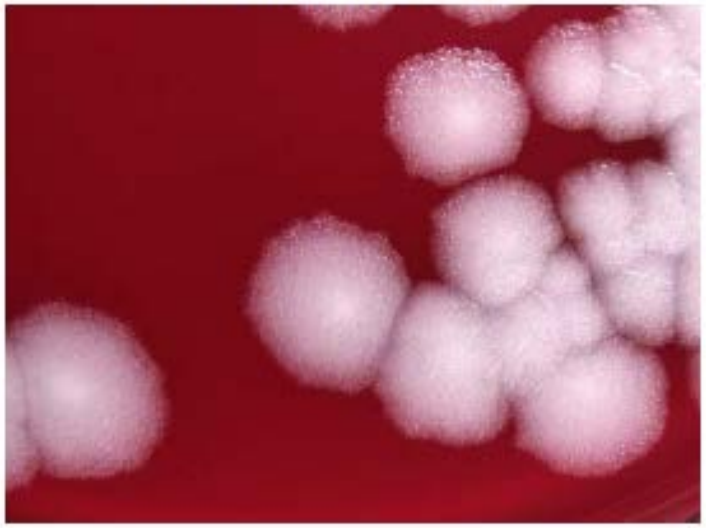
Gram positive, endospore-forming *Bacillus anthracis*



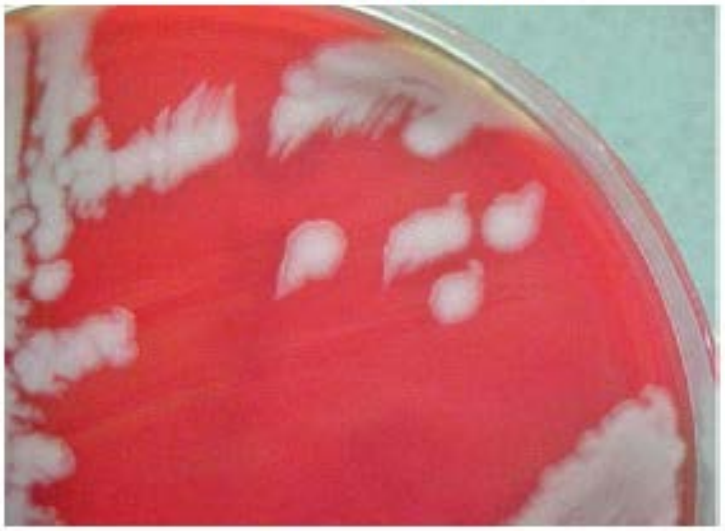
# *Bacillus anthracis*

- Colony Morphology
  - Grows well on Blood (BAP) & Chocolate (CHOC) agar
    - Non-hemolytic on BAP
  - Does not grow on MacConkey (MAC)

24 HOURS ON BAP @ 35° C



Colonies on BAP showing
"Medusa Head" morphology



# *Bacillus anthracis*

- Additional Tests
  - Catalase: Positive
  - Motility: Non-motile

- Common Misidentifications
  - May not be identified in common automated ID systems, including MALDI TOF, and possible misidentifications may include: *Bacillus megaterium* and other *Bacillus* species.

**Note:** *Bacillus cereus* Group includes *B. anthracis*, but automated ID systems may not alert microbiologist beyond this group identification.

# *Bacillus anthracis* IDENTIFICATION FLOWCHART

**SAFETY:** As soon as *B. anthracis* is suspected, perform ALL further work in a Class II BSC using BSL-3 practices.

Major characteristics of *Bacillus anthracis*:

**Gram stain morphology:** Large, Gram positive rods. Spores may be found in cultures grown in 5% $CO_2$ but not usually in clinical samples

**Colony morphology:** Ground glass appearance, non-pigmented, gamma hemolytic (no hemolysis) on BAP,
No growth on MAC (or EMB)

**Yes**

Gamma hemolytic (no hemolysis)? **No** → *Bacillus anthracis* is ruled out. Continue with routine identification.

**Yes**

Catalase positive? **No** → *Bacillus anthracis* is ruled out. Continue with routine identification.

**Yes**

Nonmotile? **No** → *Bacillus anthracis* is ruled out. Continue with routine identification.

**Yes**

*Bacillus anthracis* is not ruled out.
Contact your LRN Reference Level Laboratory to refer isolate.

**Report:** Possible *Bacillus anthracis* submitted to LRN Reference Laboratory

# *Bacillus cereus* biovar *anthracis*

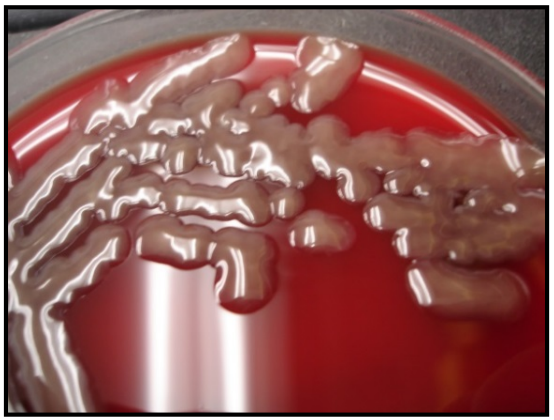
- Centers for Disease Control and Prevention (CDC) added organism to the Tier 1 select agent list on Oct. 14, 2016
- First described in gorillas and chimps in Cameroon & Côte d'Ivoire
  - Has been isolated from elephants & goats in Africa
  - No human infections to date
- Genetically similar to *B. anthracis* and produces all of the primary *B. anthracis* virulence factors

# *Bacillus cereus* biovar *anthracis*

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

- 1:CI = Côte d'Ivoire strains,
  - from chimpanzees
- 2:CA = Cameroon strains
  - from gorillas/chimpanzees
- 3:Hemolysis:
  - +=beta hemolytic on SBA; -=non-hemolytic
- 4: Motility:+ = motile; - = non-motile
- *B. cereus* biovar *anthracis* strains are usually motile with exception of those recovered from goat strains from Democratic Republic of the Congo which were non-motile (3)
- 5: Gamma phage susceptibility:
  - += susceptible; - = resistant
- 6. S=susceptible; R=resistant

24 h growth on BAP, 5% $CO_2$



Cameroon (CA) strain

Côte d'Ivoire (CI) strain

Chart courtesy of American Society for Microbiology (asm.org)

# *Bacillus cereus* biovar *anthracis*

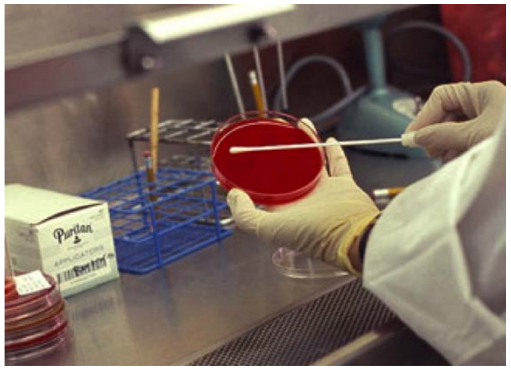
- This organism is very rare and travel history plays a key role if the physician suspects this organism.
- Recommendations:
  - Follow local public health guidelines to assess whether the public health lab or clinical lab should contact the patient's physician to determine likely clinical significance
  - Does the patient have an anthrax-like clinical syndrome

- If *Bacillus cereus* biovar *anthracis* is suspected, contact your LRN laboratory for further guidance.

American Society for Microbiology (asm.org)

# *Brucella spp.*

- Found worldwide
  - Especially found in countries that lack effective public health & domestic animal health programs
    - I.e. Mediterranean Basin, Mexico, Eastern Europe
- Modes of transmission:
  - Ingestion–Most common method
    - Eating unpasteurized dairy products
  - Inhalation
    - Laboratorians–Most commonly reported laboratory-associated bacterial infection
  - Cutaneous & Mucous Membrane
    - Contact with infected animals



# *Brucella spp.*

Red arrows indicate select agent species

| Host Animals for *Brucella* Species | |
|---|---|
| Species | Main Animal Host(s) |
| *B. abortus* | Cattle http://www.cdc.gov/brucellosis/veterinarians/cattle.html |
| *B. canis* | Dogs http://www.cdc.gov/brucellosis/veterinarians/dogs.html |
| *B. ceti* | Cetaceans (dolphins, porpoises, whales) |
| *B. melitensis* | Goats, sheep, camels |
| *B. microti* | Common vole |
| *B. neotomae* | Wood rats |
| *B. ovis* | Sheep, goats |
| *B. pinnipediae* | Pinnipeds (seals, sea lions, walruses) |
| *B. suis* | Pigs |

# *Brucella spp.*

- Safety considerations
  - As soon as Brucella is suspected in the laboratory, perform ALL further work within containment such as a Class II Biological Safety Cabinet (BSC) and follow BSL-3 practices.

# *Brucella spp.*

- A number of factors contribute to the risk of an accidental *Brucella* laboratory exposure, including:
  - Lack of experience working with the organism
  - Unknown or unidentified samples that arrive for analysis
  - Work performed on a *Brucella* isolate on an open bench, not under containment such as within a Class II BSC, or in BSL-3 conditions.

# *Brucella spp.*

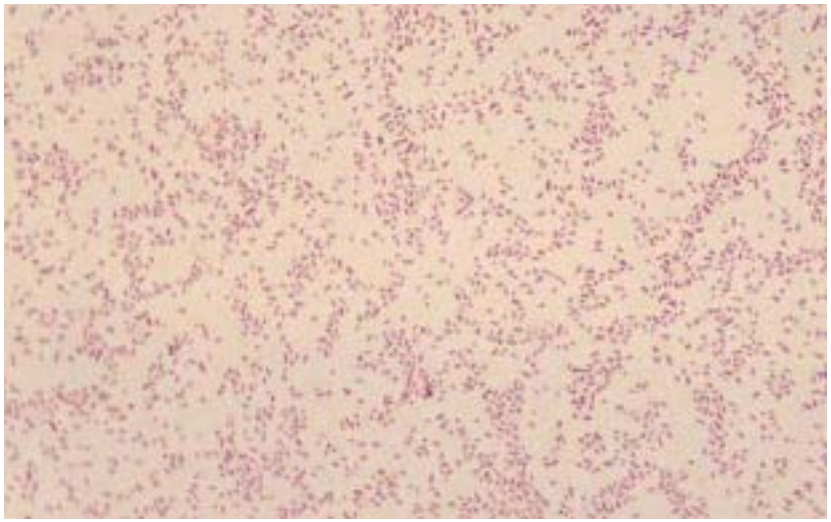
- Minimize Exposure Risks:
- Manipulate isolates of small gram-negative or gram-variable coccobacilli /rods within a Biological Safety Cabinet (BSC).
- Use primary barriers such as sealed safety centrifuge cups, PPE, and Class II or higher BSC for procedures with a high likelihood of producing aerosols.
- Restrict access to the laboratory when work is being performed
- Minimize the creation of splashes or aerosols.
- Prohibit sniffing of opened culture plates

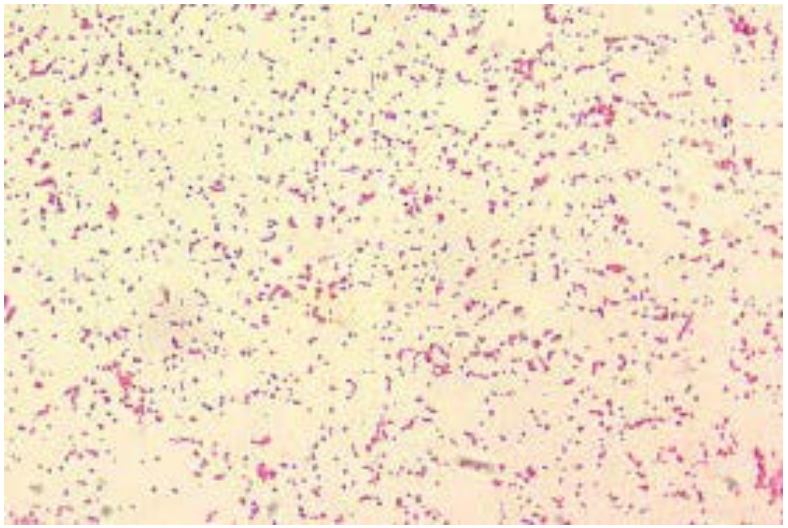
# *Brucella spp.*

| Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing | |
|---|---|
| Bone marrow or whole blood | Considered the best specimen for culture but can be limited in chronic cases. Collect directly into an appropriate blood culture bottle. Aseptically inoculate liquid blood culture bottles with maximum amount of blood or body fluid per manufacturers' instructions. Incubate at 35°C. Transport bottles at room temperature as soon as possible to obtain the diagnosis. Alternatively, follow the manufacturer's instructions for the lysis-centrifugation method and inoculate pellet to BAP, CHOC and MAC. Incubate plates at 35°C in a humidified incubator with 5 to 10% $CO_2$. |
| Joint or abdominal fluid | Collect directly into an appropriate blood culture bottle. Transport bottles at room temperature as soon as possible to obtain the diagnosis. |
| Spleen, liver abscesses | Tissue pieces (at least the size of a pea) should be collected and kept moist. Transport in sterile container at room temperature within 1 hour of collection. May add 1-2 drop of sterile saline to keep moist. |
| Serum | Collect at least 1 mL without anticoagulant for serologic diagnosis.[1] Store at 4°C until testing is performed. Acute specimen is collected as soon as possible after onset of disease. Convalescent-phase should be collected >14 (14 – 21) days after the acute specimen. |
| Tissues | Inoculate BAP, CHOC and MAC and incubate at 35°C in a humidified incubator with 5 to 10% $CO_2$ for 7 days (MAC need only be incubated for 3 days at 35°C in ambient air or 5 to 10% $CO_2$.) Humidity may be maintained by placing a pan of water in the bottom of the incubator or by wrapping the plates with gas permeable tape. |

# *Brucella spp.*

- Gram Stain
  - Small gram negative coccobacilli or tiny GNR
  - Sometimes will take up the crystal violet and appear as gram positive

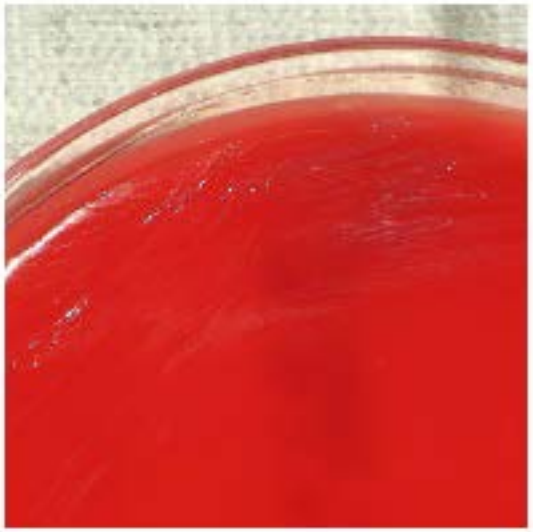




# *Brucella spp.*

- Colony Morphology
  - **Very slow growing** (may look like haze at 24 h)
  - Can take 2-4 days to grow in blood cultures
  - Grows on BAP or CHOC; No growth on MAC

24 Hours on BAP

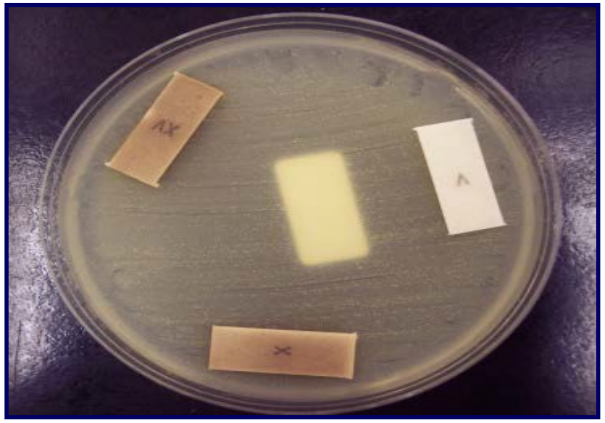


48 Hours on BAP

APHL Clinical Laboratory Preparedness and Response Guide

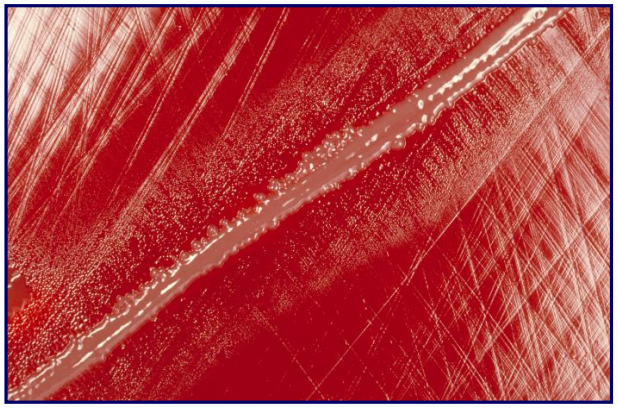
# *Brucella spp.*

- Biochemical Tests
  - Catalase positive
  - Oxidase positive
  - Urea positive
  - Satellite test negative



*Brucella* growing on the entire plate. Negative for X and V factors.

- Sometimes misidentification include:
  - *Haemophilus spp.*
  - *Oligella ureolytica*
  - *Psychrobacter phenylpyruvicus*
  - *Psychrobacter immobilis*
  - *Bordetella bronchiseptica*



*Haemophilus* demonstrating growth only around the *Staphylococcus aureus* streak.

# Major characteristics of *Brucella* species:

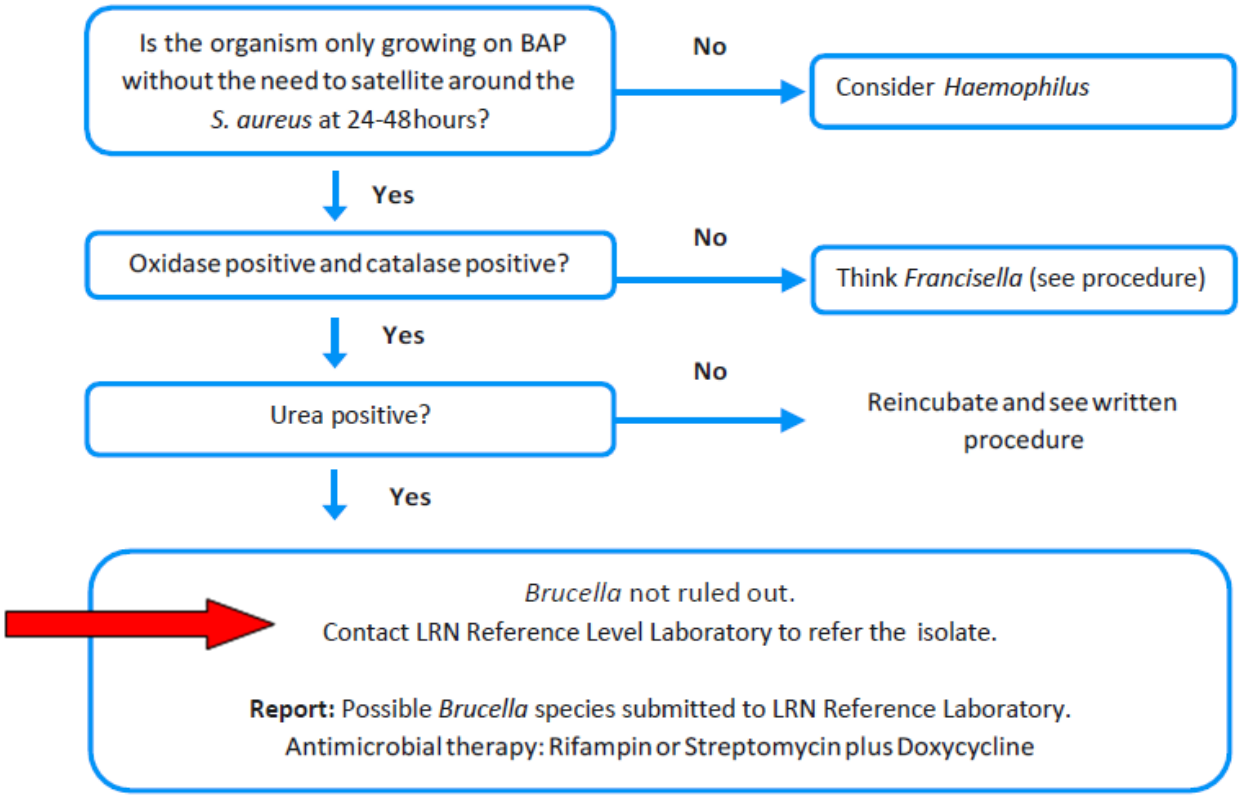
**Gram stain morphology:** Small (0.4 x 0.8µm), Gram negative coccobacillus

THINK BRUCELLA

**Colony morphology:** Subculture positive aerobic blood culture to BAP, CHOC. Incubate in 5-10% $CO_2$ at 35°C, Spot BAP with S. *aureus* ATCC 25923 for satellite test. Note poorly growing colonies after 24 hour incubation on BAP and CHOC.

Incubate plates for at least two additional days if no growth in 24 hours.

Organism does NOT grow on MAC/EMB. (Pinpoint colonies infrequently after extended Incubation; 7 days)



# *Burkholderia mallei* (Glanders)

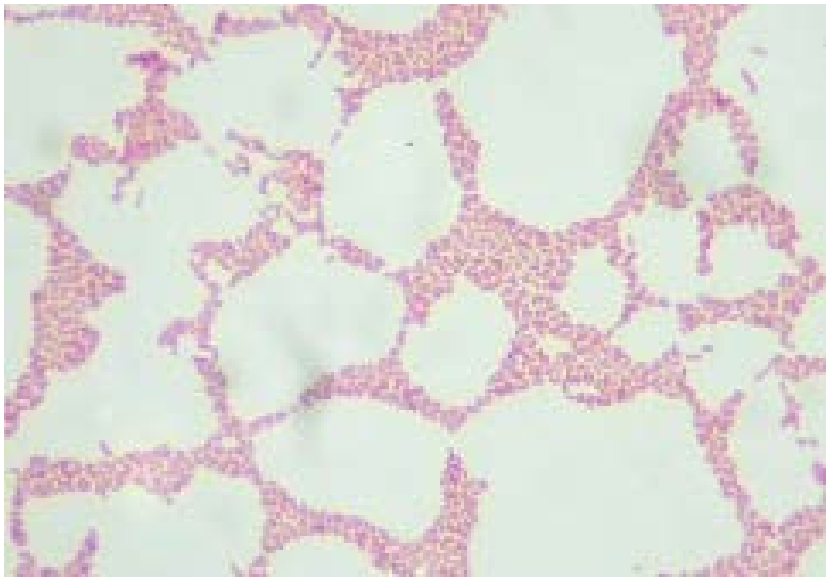
- Equines are primary reservoir but can effect other animals
- Used during WWI as biowarfare agent
- 4 types of infection:
  - Localized
  - Pulmonary
  - Bloodstream
  - Chronic

# *Burkholderia mallei*

| Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing | |
|---|---|
| Bone marrow or whole blood | • Considered the best specimen for culture<br>• Collect directly into an appropriate blood culture bottle<br>• Transport bottles at room temperature as soon as possible to obtain the diagnosis |
| Sputum or bronchoscopically obtained specimens | • Collect expectorated specimen into sterile transport cup or collect during bronchoscopy procedure.<br>• Transport at room temperature up to 2h<br>• If it is known that material will be transported from 2-24 h after collection, then store and transport at 2-8°C. |
| Tissue specimens (biopsies, abscess aspirates) and wound swabs | • Tissue pieces (at least the size of a pea) should be collected and kept moist<br>• Transport in sterile container at room temperature within 1 hour of collection<br>• Alternatively a swab from a tissue sample can be submitted in hospital transport tube with medium to stabilize specimen (e.g. Amies charcoal). |
| Urine | • Collect at least 1 mL into leak-proof container<br>• Transport at room temperature up to 2 h<br>• Refrigerate 2 up to 24 h until culture inoculation |

# *Burkholderia mallei*

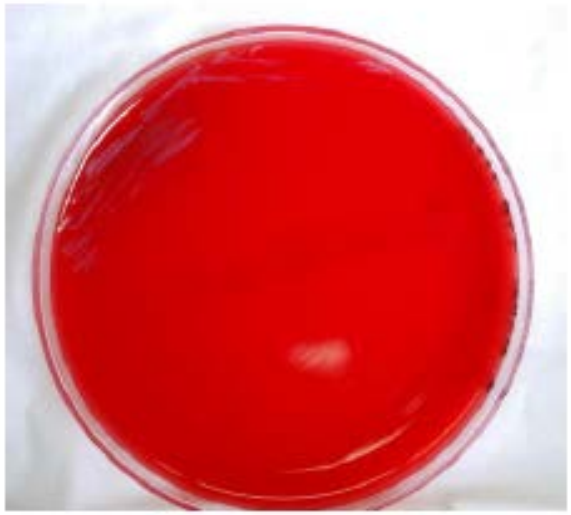
- Gram Stain
  - Small straight or slightly curved Gram negative coccobacilli with rounded ends
  - Cells arranged in pairs, parallel bundles, or the Chinese letter form



# *Burkholderia mallei*

- Colony Morphology
  - On BAP, pinpoint to small grey colonies at 24h that may become smooth, grey, and translucent at 48h with no distinctive odor
  - Non-hemolytic on BAP
  - No growth or pinpoint colorless colonies (which may uptake light pink dye) on MAC after 48h

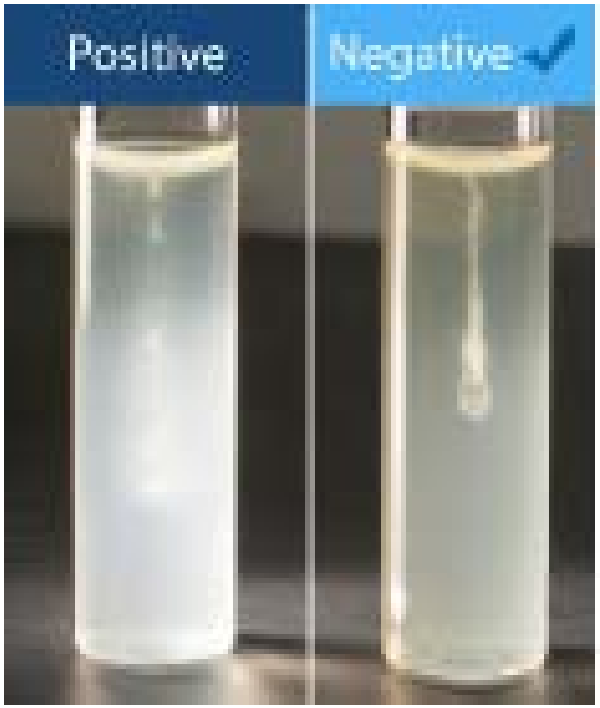
24 HOURS ON BAP



48 HOURS ON BAP

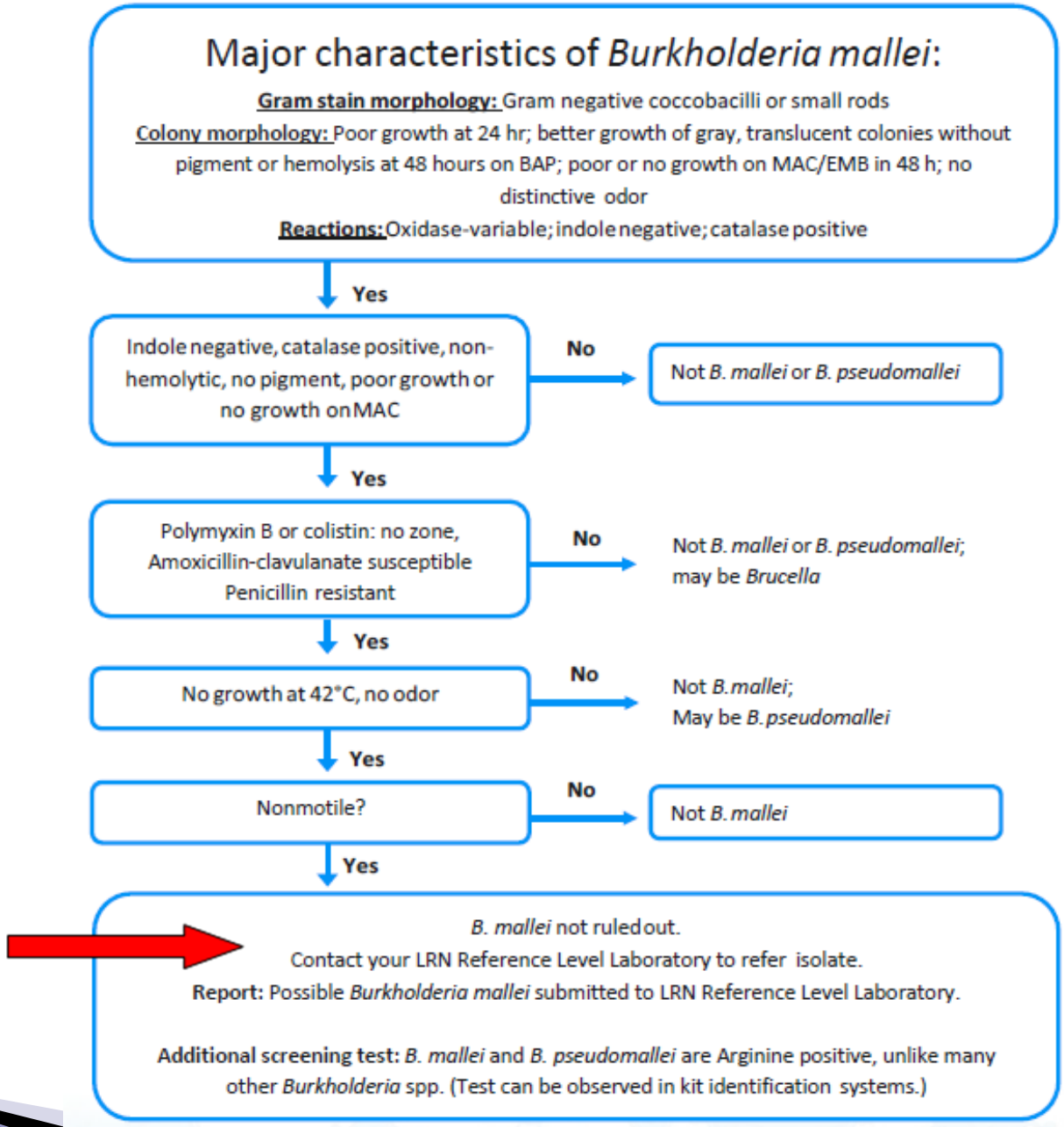
# *Burkholderia mallei*

- Biochemical Tests
  - Catalase positive
  - Oxidase variable; most are negative
  - Spot indole negative
  - Non-motile *(Recommend tube test not wet mount due to potential aerosol production)*
  - No growth at 42˚C
  - Polymyxin B and colistin no zone, penicillin resistant, amoxicillin-clavulanate susceptible



# *Burkholderia mallei*

| Possible Misidentifications for *Burkholderia mallei* Include: | |
|---|---|
| Organism | Differential Test |
| *Burkholderia cepacia* | Resistant to amoxicillin-clavulanic acid, lactose fermenter (LF) on MacConkey and EMB, motile, arginine negative |
| *Chromobacterium violaceum* | Hemolysis, violet pigment on BAP, motile |
| *Pseudomonasstutzeri* | Growth on MacConkey, arginine negative |
| *S. maltophilia* | Growth on MacConkey, arginine negative |
| *Bacillus* spp. may appear Gram negative | Sensitive to penicillin |
| *Pandoraea* spp. | Growth on MacConkey |
| *Ralstonia* spp. | Growth on MacConkey |


Major characteristics of Burkholderia mallei:
Gram stain morphology: Gram negative coccobacilli or small rods
Colony morphology: Poor growth at 24 hr; better growth of gray, translucent colonies without pigment or hemolysis at 48 hours on BAP; poor or no growth on MAC/EMB in 48 h; no distinctive odor
Reactions: Oxidase-variable; indole negative; catalase positive
Yes
Indole negative, catalase positive, non-hemolytic, no pigment, poor growth or no growth on MAC
No
Not B. mallei or B. pseudomallei
Yes
Polymyxin B or colistin: no zone, Amoxicillin-clavulanate susceptible Penicillin resistant
No
Not B. mallei or B. pseudomallei; may be Brucella
Yes
No growth at 42°C, no odor
No
Not B. mallei; May be B. pseudomallei
Yes
Nonmotile?
No
Not B. mallei
Yes
B. mallei not ruled out.
Contact your LRN Reference Level Laboratory to refer isolate.
Report: Possible Burkholderia mallei submitted to LRN Reference Level Laboratory.
Additional screening test: B. mallei and B. pseudomallei are Arginine positive, unlike many other Burkholderia spp. (Test can be observed in kit identification systems.)

# *Burkholderia pseudomallei (Melioidosis)*

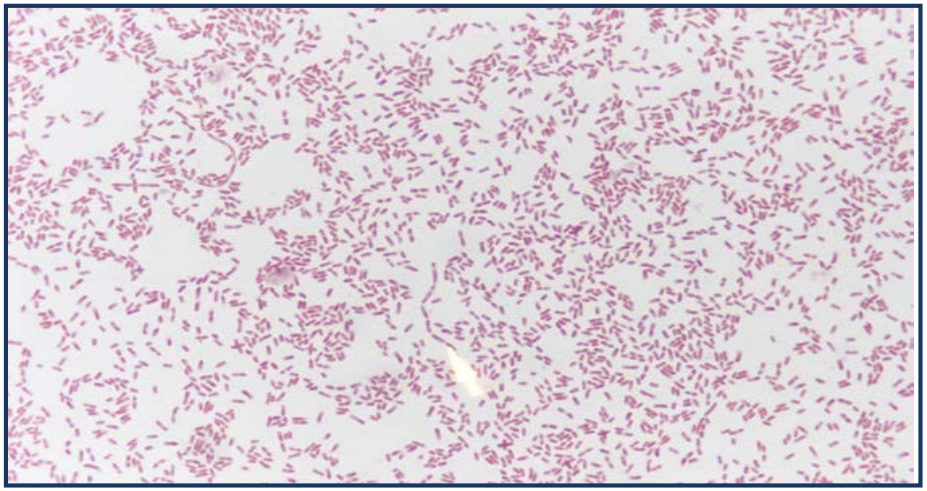
- Found in tropical areas
  - Southeast Asia & Australia
- Known as "Vietnam time bomb"
  - High levels in water of rice paddies–Vietnam & Thailand
  - Known to reactivate after many years of latency
- Types of infection:
  - Localized
  - Pulmonary
  - Bloodstream
  - Disseminated

# *Burkholderia pseudomallei*

| Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing | |
|---|---|
| Bone marrow or whole blood | • Considered the best specimen for culture<br>• Collect directly into an appropriate blood culture bottle<br>• Transport bottles at room temperature as soon as possible to obtain the diagnosis |
| Sputum or bronchoscopically obtained specimens | • Collect expectorated specimen into sterile transport cup or collect during bronchoscopy procedure.<br>• Transport at room temperature up to 2h<br>• If it is known that material will be transported from 2-24 h after collection, then store and transport at 2-8°C. |
| Tissue specimens (biopsies, abscess aspirates) and wound swabs | • Tissue pieces (at least the size of a pea) should be collected and kept moist<br>• Transport in sterile container at room temperature within 1 hour of collection<br>• Alternatively a swab from a tissue sample can be submitted in hospital transport tube with medium to stabilize specimen (e.g. Amies charcoal). |
| Urine | • Collect at least 1 mL into leak-proof container<br>• Transport at room temperature up to 2 h<br>• Refrigerate 2 up to 24 h until culture inoculation |

# *Burkholderia pseudomallei*

- Gram Stain
  - Straight, or slightly curved Gram negative rods
  - Colonies may demonstrate bipolar morphology in direct specimens and peripheral staining in older cultures, which can mimic endospores



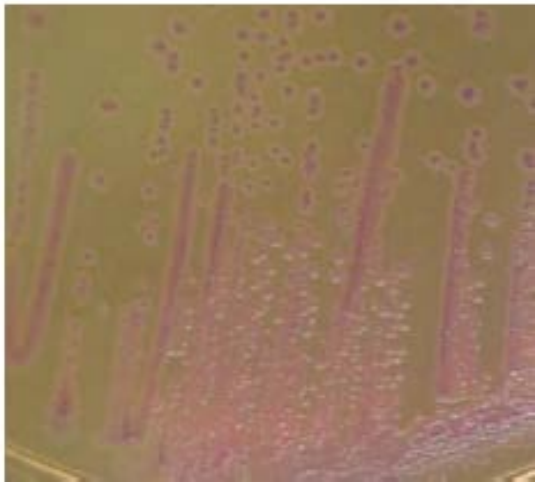
# *Burkholderia pseudomallei*

- Colony Morphology
  - On BAP, small, smooth, creamy colonies in the first 1-2 days, that may gradually change in time to dry, wrinkled colonies (similar to *Pseudomonas stutzeri*)
  - Poor growth at 24h, good growth at 48h
  - Colonies are non-hemolytic and not pigmented on BAP. Organism grows on MAC
  - Distinctive musty earthy odor
    - (the odor is apparent without sniffing or opening plate)

24 HOURS ON BAP

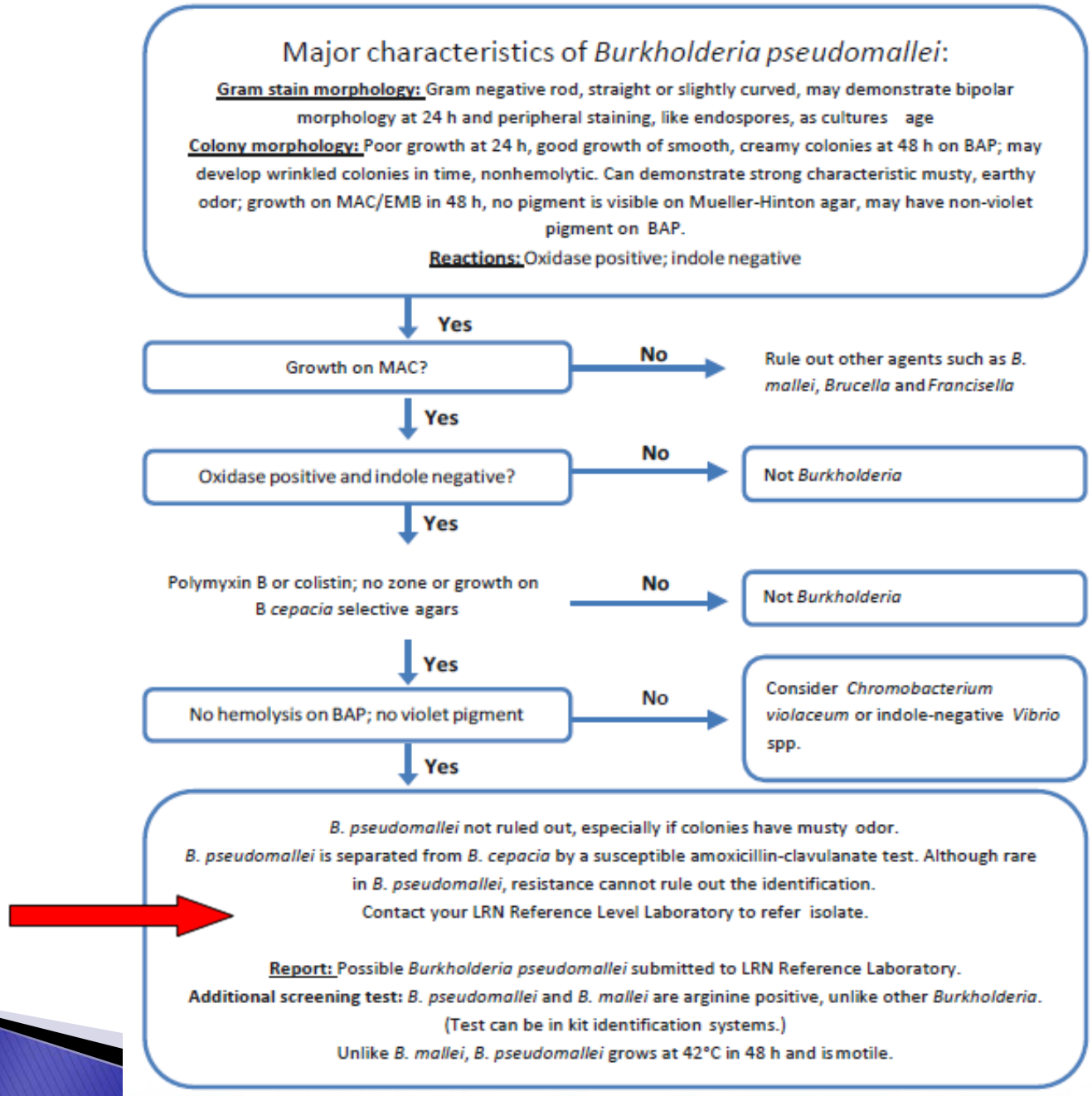
48 HOURS ON BAP

48 HOURS ON MAC



# *Burkholderia pseudomallei*

- Biochemical Tests
  - Oxidase positive
  - Spot indole negative
  - Motile
  - Growth at 42˚C
  - Polymyxin B and colistin no zone, penicillin resistant, amoxicillin-clavulanate susceptible
- Possible Misidentification:
  - *Burkholderia cepacia, Chromobacterium violaceum, Pseudomonas aeruginosa, Pseudomonas stutzeri, S. maltophilia* and other nonfermenting Gram negative bacilli.


Major characteristics of *Burkholderia pseudomallei*:
**Gram stain morphology:** Gram negative rod, straight or slightly curved, may demonstrate bipolar morphology at 24 h and peripheral staining, like endospores, as cultures age
**Colony morphology:** Poor growth at 24 h, good growth of smooth, creamy colonies at 48 h on BAP; may develop wrinkled colonies in time, nonhemolytic. Can demonstrate strong characteristic musty, earthy odor; growth on MAC/EMB in 48 h, no pigment is visible on Mueller-Hinton agar, may have non-violet pigment on BAP.
**Reactions:** Oxidase positive; indole negative
Yes
Growth on MAC?
No
Rule out other agents such as *B. mallei*, *Brucella* and *Francisella*
Yes
Oxidase positive and indole negative?
No
Not *Burkholderia*
Yes
Polymyxin B or colistin; no zone or growth on B *cepacia* selective agars
No
Not *Burkholderia*
Yes
No hemolysis on BAP; no violet pigment
No
Consider *Chromobacterium violaceum* or indole-negative *Vibrio* spp.
Yes
*B. pseudomallei* not ruled out, especially if colonies have musty odor.
*B. pseudomallei* is separated from *B. cepacia* by a susceptible amoxicillin-clavulanate test. Although rare in *B. pseudomallei*, resistance cannot rule out the identification.
Contact your LRN Reference Level Laboratory to refer isolate.
**Report:** Possible *Burkholderia pseudomallei* submitted to LRN Reference Laboratory.
**Additional screening test:** *B. pseudomallei* and *B. mallei* are arginine positive, unlike other *Burkholderia*. (Test can be in kit identification systems.)
Unlike *B. mallei*, *B. pseudomallei* grows at 42°C in 48 h and is motile.

# *Francisella tularensis* (Tularemia)

- Highly virulent
  - Infectious dose 10-50
- Zoonotic-widespread throughout Northern Hemisphere
  - Hosts include: mammals, birds and amphibians
  - Primary host-small rodents
  - Has been reported in every state except Hawaii

# *Francisella tularensis*

## Forms of Tularemia

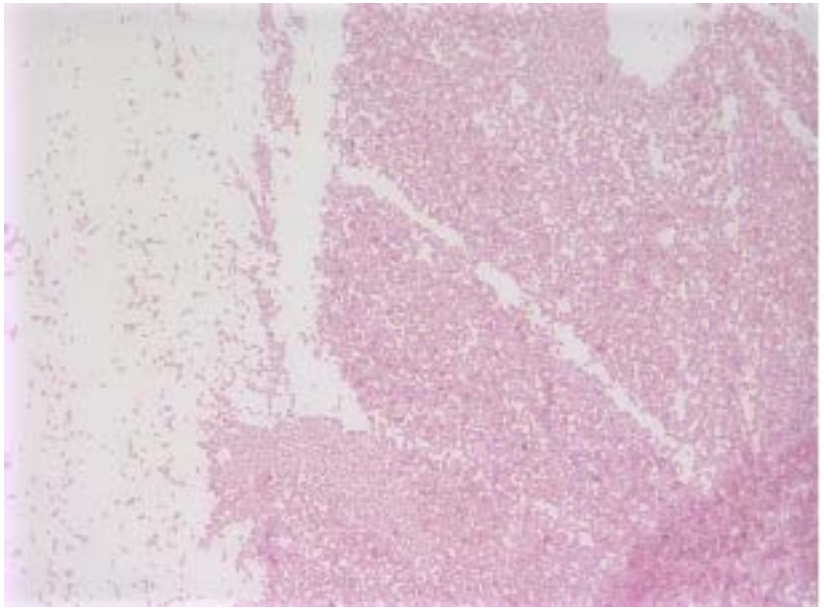
- **Ulceroglandular** This is the most common form of tularemia and usually occurs following a tick or deer fly bite or after handling of an infected animal.
- **Glandular** Similar to ulceroglandular tularemia but without an ulcer.
- **Oculoglandular** This form occurs when the bacteria enter through the eye.
- **Oropharyngeal** This form results from eating or drinking contaminated food or water.
- **Pneumonic** This is the most serious form of tularemia. Symptoms include cough, chest pain, and difficulty breathing.

# *Francisella tularensis*

| Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing | |
|---|---|
| **Tissue**<br>Biopsy, scraping of an ulcer, or conjunctival swab | • For small tissue samples, add several drops of sterile normal saline into a sterile container to keep the tissue moist<br>• Transport at room temperature for immediate processing<br>• If processing of specimen is delayed beyond 2 hours, keep specimen chilled (2-8°C)<br>• Amies transport media is an appropriate transport medium |
| **Aspirate**<br>Lymph node or lesion | • Submit in a sterile container<br>• Transport at room temperature for immediate processing.<br>• If processing of specimen is delayed beyond 2 hours, keep specimen chilled (2-8°C) |
| **Bone Marrow** | • Submit in a sterile container<br>• Transport at room temperature for immediate processing<br>• If processing of specimen is delayed beyond 2 hours, keep specimen chilled (2-8°C) |
| **Blood** | • Transport inoculated bottles directly to laboratory at room temperature<br>• Hold at room temperature until placed into automated, continuous monitoring blood culture incubators<br>• Do not refrigerate |
| **Respiratory Secretions** | • Submit in a sterile container<br>• Transport at room temperature for immediate processing<br>• If processing of specimen is delayed beyond 2 hours, keep specimen chilled (2-8°C) |
| **Serum** | • Collect at least 1 mL without anticoagulant for serologic diagnosis<br>• Store at 4°C until testing is performed.<br>• Acute specimen is collected as soon as possible after onset of disease<br>• Convalescent-phase should be collected >14 days after the acute specimen. |

# *Francisella tularensis*

- Gram Stain:
  - Tiny, Gram negative coccobacilli
  - Poorly counterstaining with safranin (basic fuchsin counterstain may increase resolution)
  - Pleomorphic
  - Mostly single cells

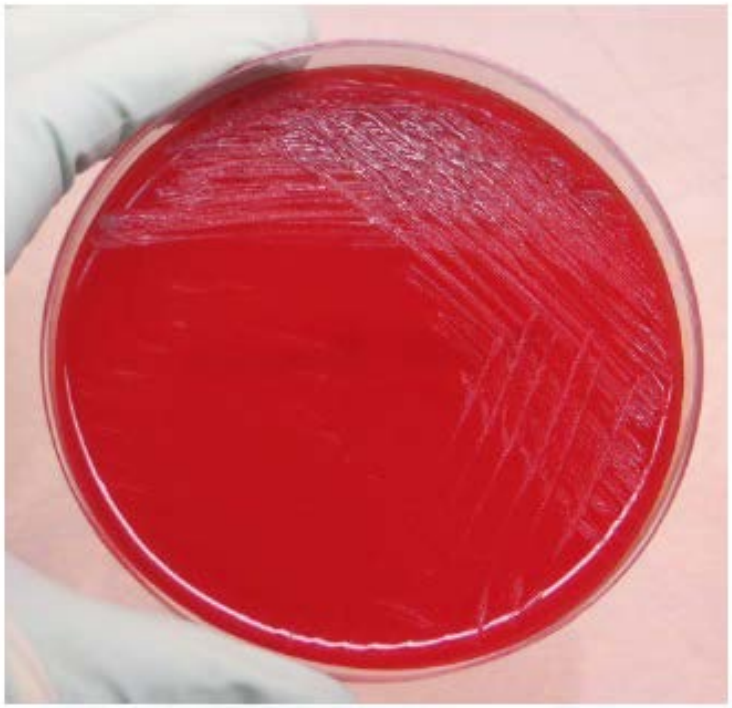


# *Francisella tularensis*

- Colony Morphology
  - Aerobic, fastidious
  - No growth on MAC or EMB
  - Scant or no growth on BAP; may grow on primary culture, not well on subculture
  - Slow growing on CHOC, TM or BCYE: 1-2 mm after 48h
  - Colonies are opaque, grey-white, butyrous with smooth and shiny surface

48 hours on BAP

- After 48 h: <1 mm, gray-white, opaque, no hemolysis.

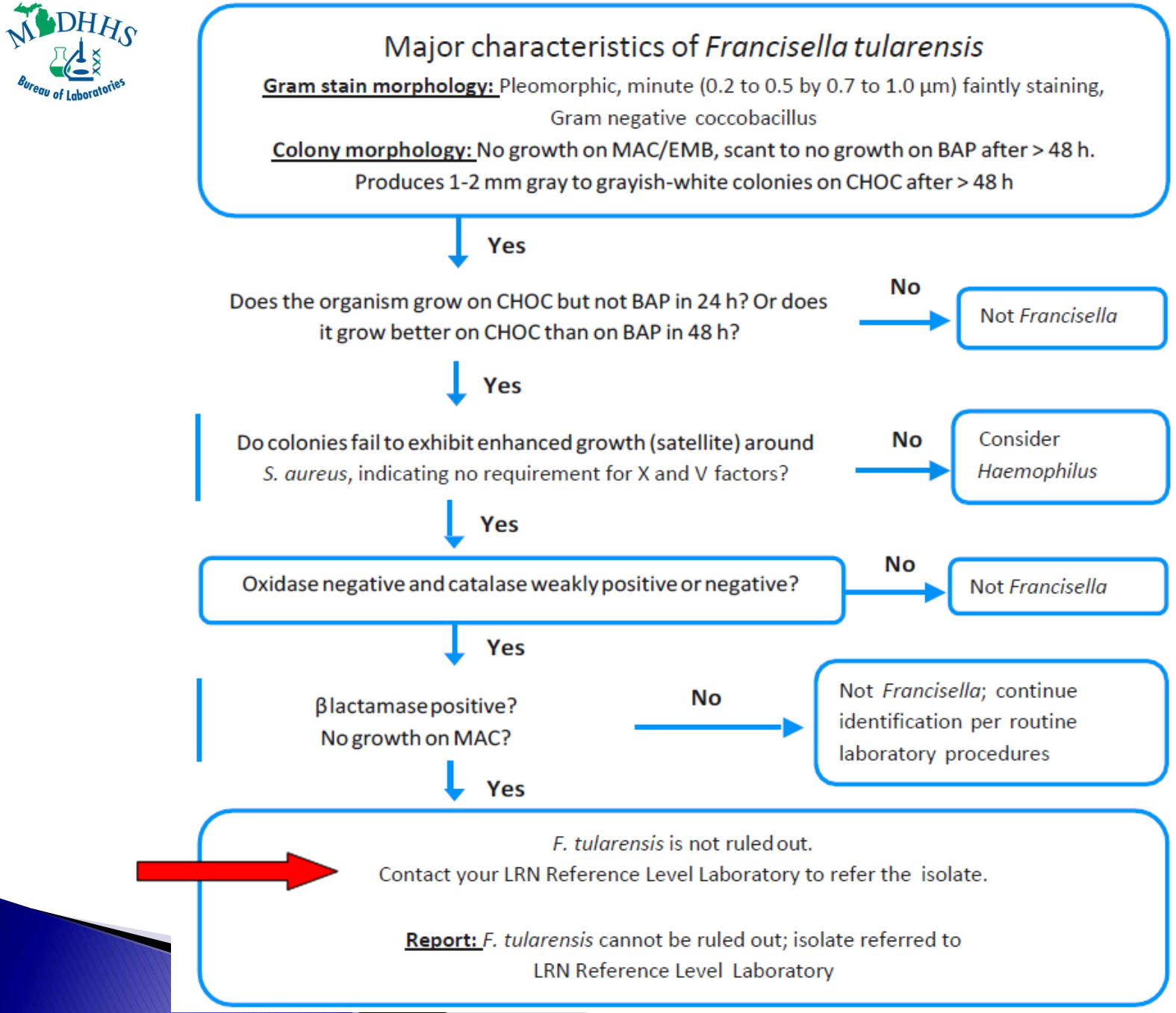


# *Francisella tularensis*

- Biochemical Tests
  - Oxidase negative
  - Catalase negative or weakly positive
  - Satellite negative
  - Beta-lactamase positive

*F. tularensis* is beta-lactamase positive (+).

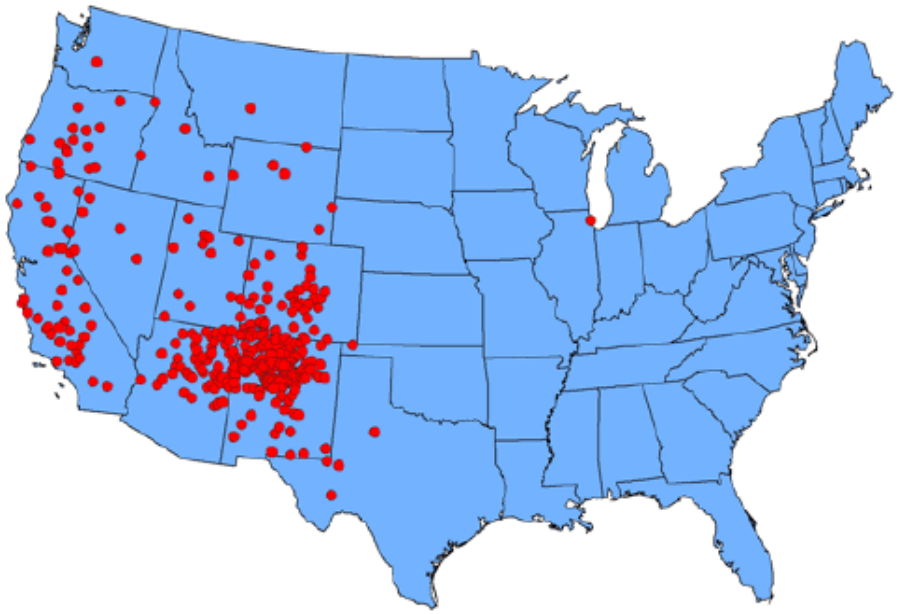
- Common Misidentifications: *Acinetobacter*, *Aggregatibacter actinomycetemcomitans*, *Haemophilus influenzae*, *Oligella* spp. and *Psychrobacter* spp


Major characteristics of *Francisella tularensis*
**Gram stain morphology:** Pleomorphic, minute (0.2 to 0.5 by 0.7 to 1.0 µm) faintly staining, Gram negative coccobacillus
**Colony morphology:** No growth on MAC/EMB, scant to no growth on BAP after > 48 h. Produces 1-2 mm gray to grayish-white colonies on CHOC after > 48 h
Yes
Does the organism grow on CHOC but not BAP in 24 h? Or does it grow better on CHOC than on BAP in 48 h?
No
Not *Francisella*
Yes
Do colonies fail to exhibit enhanced growth (satellite) around *S. aureus*, indicating no requirement for X and V factors?
No
Consider *Haemophilus*
Yes
Oxidase negative and catalase weakly positive or negative?
No
Not *Francisella*
Yes
βlactamase positive? No growth on MAC?
No
Not *Francisella*; continue identification per routine laboratory procedures
Yes
*F. tularensis* is not ruled out.
Contact your LRN Reference Level Laboratory to refer the isolate.
**Report:** *F. tularensis* cannot be ruled out; isolate referred to LRN Reference Level Laboratory

# *Yersinia pestis* (Plague)

- Plague is a zoonotic disease
- There are 1-17 human cases per year in the U.S. (50% are from New Mexico)

Reported cases of human plague--United States, 1970-2012



1 dot placed in county of exposure for each plague case

# *Yersinia pestis*

- Host range:
  - A number of species of rodent fleas act as natural vectors.
  - Rodent reservoir (squirrels, prairie dogs, chipmunks, deer mice, voles, rats, occasionally hares & rabbits).
  - Most mammals, including humans and carnivores (such as bobcats, mountain lions, dogs & cats) are accidental hosts; not birds or reptiles.
  - Cats & Dogs

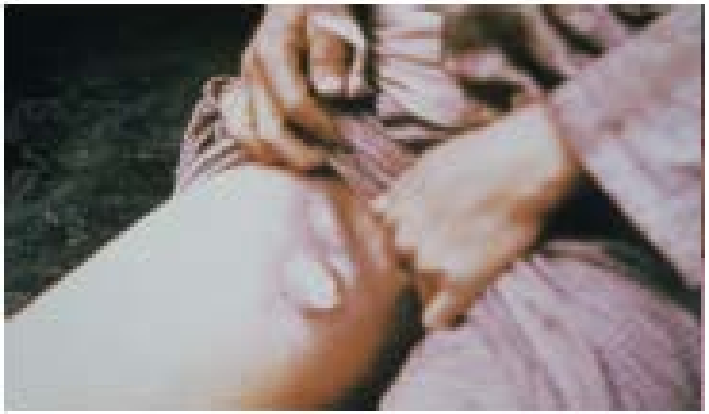
APHL Clinical Laboratory Preparedness and Response Guide

# *Yersinia pestis*

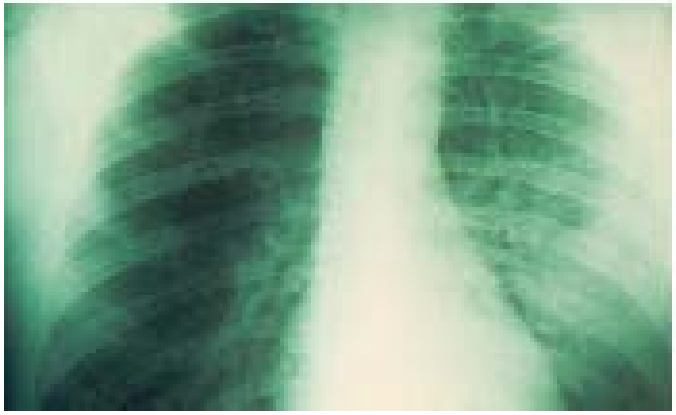
- **Bubonic**
  - Transmitted by the bite of an infected flea
  - Untreated mortality ~60%,
  - Not transmitted person-to-person
- **Septicemic**
  - Results from contact with, or being bitten by, an infected animal
  - Petechiae, necrosis and gangrene of the extremities ("the black death")
  - Untreated mortality ~100%
  - Not transmitted person-to-person
- **Pneumonic**
  - Inhalation- deadliest form-intentional release as an aerosol
  - May be transmitted person-to-person, or animal-to-person, extremely contagious

# *Yersinia pestis*

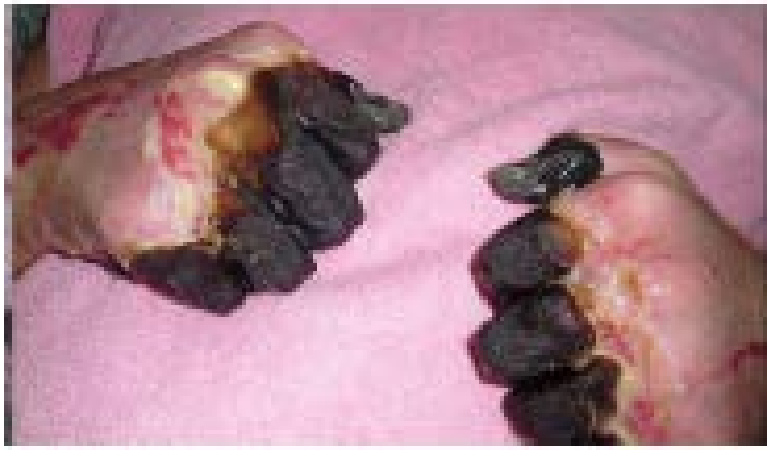
Bubonic Plague



Pneumonic Plague



Septicemic Plague



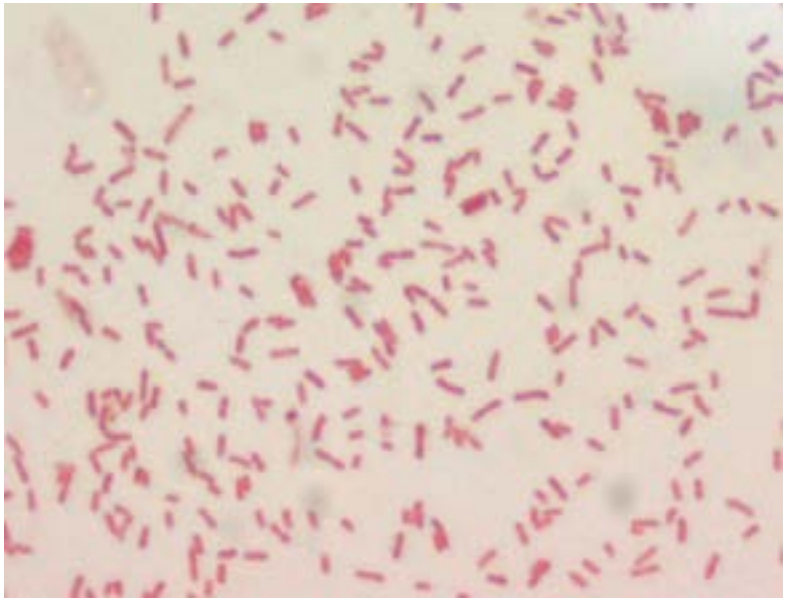
# *Yersinia pestis*

| Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing | |
|---|---|
| Lower respiratory tract | • Transport specimens in sterile, screw-capped containers at room temperature.<br>• If it is known that material will be transported from 2-24 h after collection, then store and transport at 2-8°C |
| Blood | • Transport samples directly to the laboratory at ambient temperature and place onto the blood culture instrument<br>• Do not refrigerate<br>• Follow established laboratory protocols for processing blood cultures |
| Aspirate, tissue or biopsy specimen | • Submit tissue or aspirate in a sterile container.<br>• For small samples, add 1–2 drops of sterile normal saline to keep the tissue moist.<br>• Transport sample at room temperature for immediate processing.<br>• Keep the specimen chilled if processing will be delayed (> 2 h). |
| Swabs | • A swab of tissue is not recommended.<br>• However, if a swab specimen is collected, the swab should be reinserted into an appropriate transport package and sent to the laboratory at room temperature for immediate processing. Keep the specimen chilled if processing will be delayed (>2 h). |

# *Yersinia pestis*

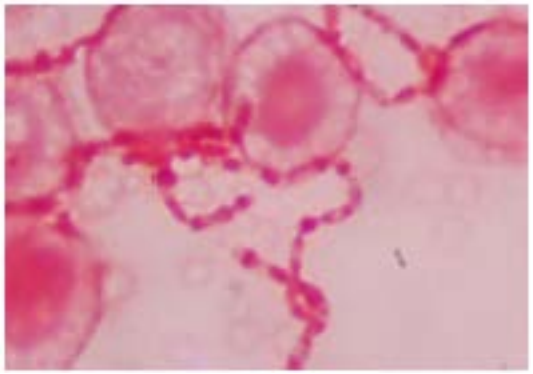
- Gram Stain

- Plump Gram negative rods seen mostly as single cells or pairs, and may demonstrate short chains in liquid media
- May exhibit bipolar, "safety-pin" appearance that is not seen on Gram stain, may be exhibited by Giemsa stain or Wrights stain



Gram stain of *Yersinia pestis* from a patient blood culture bottle

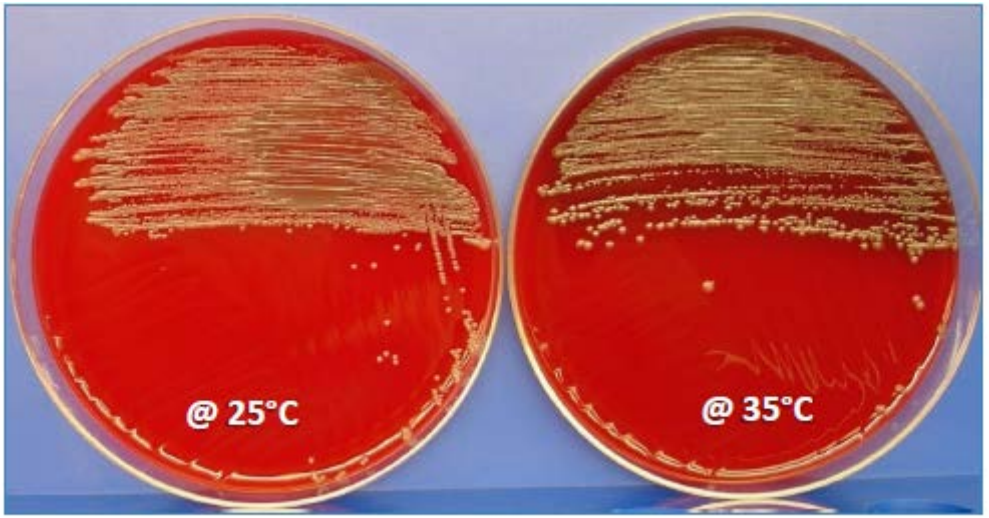
Note chaining and bipolar-staining Gram negative rods



# *Yersinia pestis*

- Colony Morphology

- Facultative anaerobe
- Slow growing at 35˚C, better growth at 25-28˚C
- Grey-white, translucent pinpoint colonies at 24h
- At 48 h, colonies on BAP gray-white to slightly yellow and opaque
- "Fried egg" on BAP in older cultures (96h)
- At 48h, lactose non-fermenter on MAC or EMB

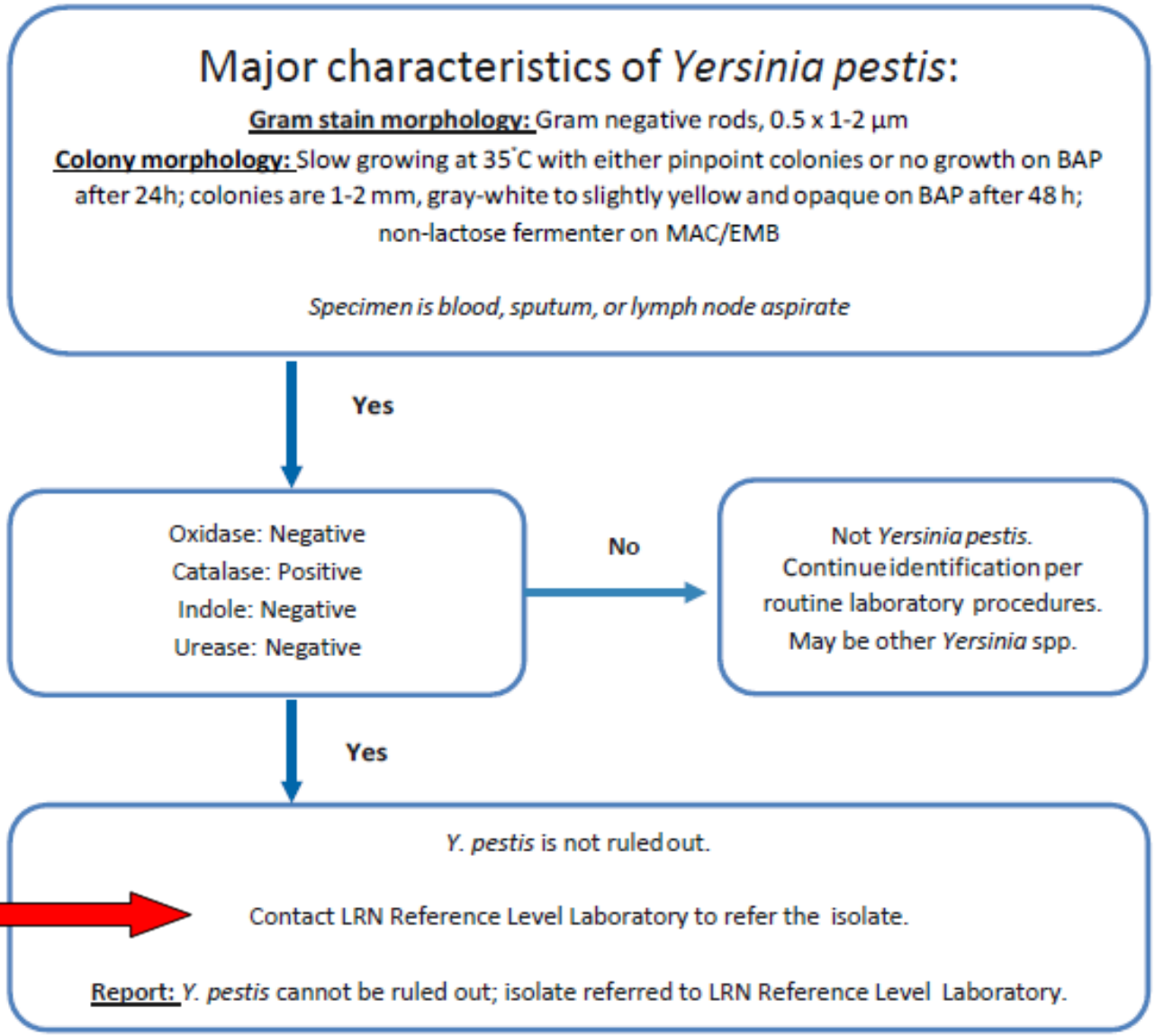



48 HOURS ON BAP at 25°C and 35°C

# *Yersinia pestis*

- Biochemical Tests
  - Catalase positive
  - Oxidase, urease (at 35˚C) and indole: negative

- Common Misidentifications:
  - *Shigella* spp., $H_2S$(–) *Salmonella* spp., *Acinetobacter* or *Pseudomonas* spp., or *Yersinia pseudotuberculosis*.
  - May not be identified in common automated ID systems, including MALDI TOF


Major characteristics of Yersinia pestis:
Gram stain morphology: Gram negative rods, 0.5 x 1-2 µm
Colony morphology: Slow growing at 35˚C with either pinpoint colonies or no growth on BAP after 24h; colonies are 1-2 mm, gray-white to slightly yellow and opaque on BAP after 48 h; non-lactose fermenter on MAC/EMB
Specimen is blood, sputum, or lymph node aspirate
Yes
Oxidase: Negative
Catalase: Positive
Indole: Negative
Urease: Negative
No
Not Yersinia pestis. Continue identification per routine laboratory procedures. May be other Yersinia spp.
Yes
Y. pestis is not ruled out.
Contact LRN Reference Level Laboratory to refer the isolate.
Report: Y. pestis cannot be ruled out; isolate referred to LRN Reference Level Laboratory.

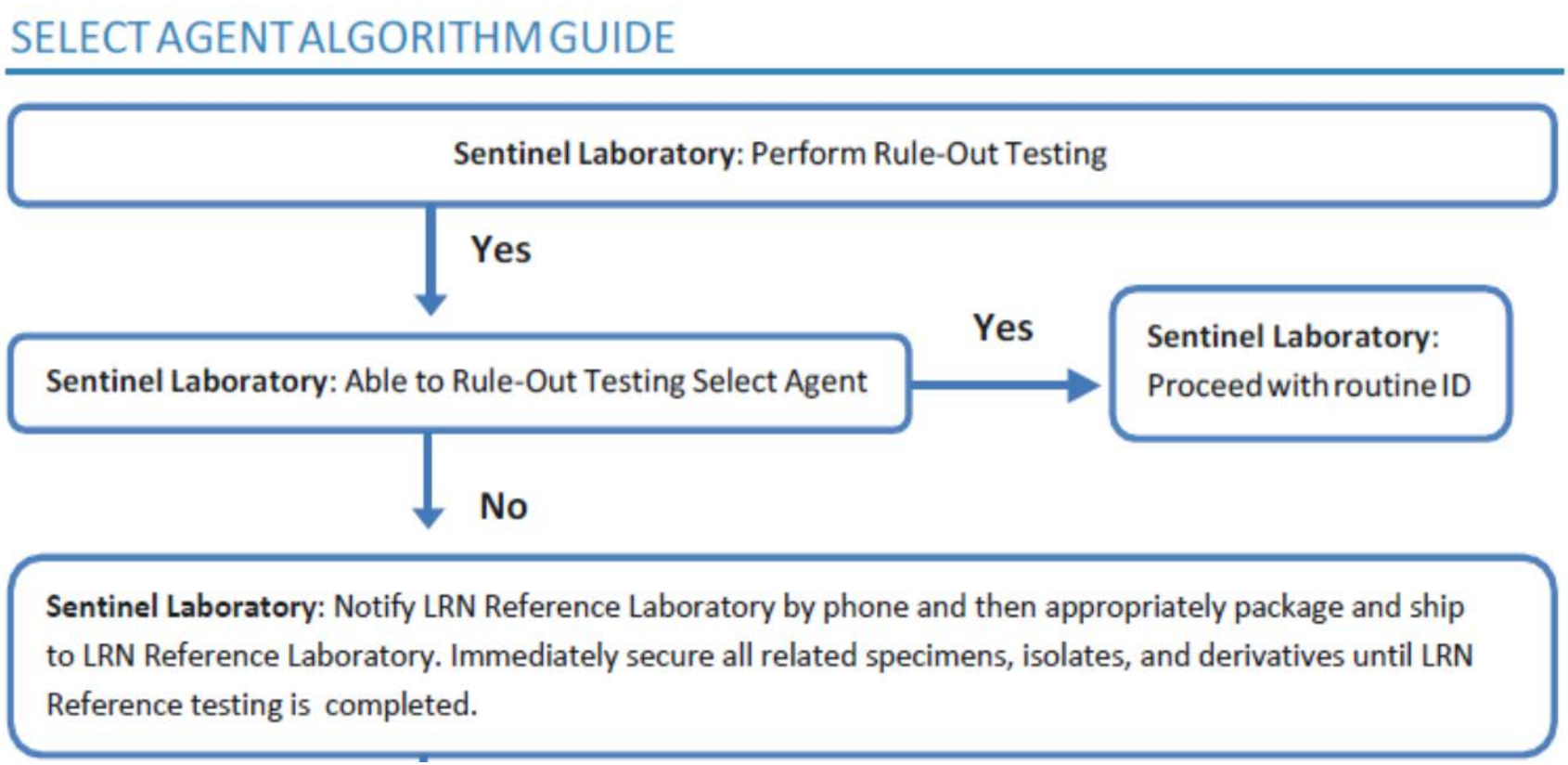
# Next Steps for Select Agent Identification

# Select Agent ID

- Next steps once you cannot rule out a select agent:

1. Notify the LRN (MDHHS Bureau of Laboratories)
2. Properly package and ship isolate to MDHHS for further testing– (i.e. Category A)
3. Secure all related specimens and isolates until confirmatory testing is complete by the LRN

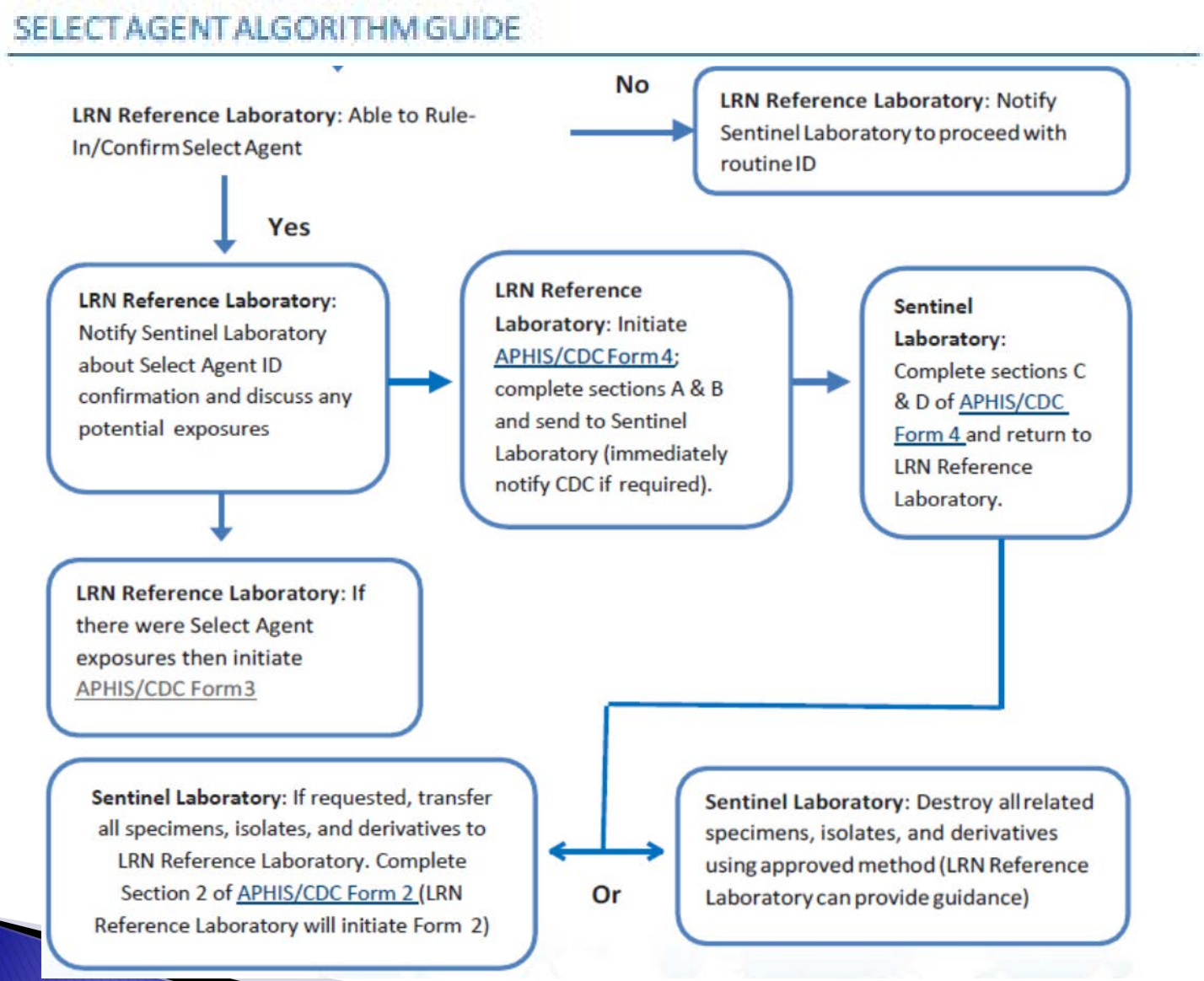
# Select Agent ID



# Select Agent ID

- Next steps once MDHHS confirms select agent
  1. MDHHS will contact sentinel lab with results
  2. MDHHS Lab/EPI will discuss potential lab exposures
     1. Exposed lab staff may require monitoring & prophylaxis
     2. If exposures occurred-CDC form 3 will need to be completed
  3. MDHHS will notify the CDC-CDC form 4 is completed
     1. MDHHS fills out section A & B of CDC form 4
     2. Sentinel Lab fills out section C & D of CDC form 4
  4. Sentinel lab destroys all isolates, specimens and derivatives within 7 days of MDHHS select agent confirmation

# Select Agent ID



# Automated Systems Study

# Preparation Safety Study

- Determined viability of BT agents using direct, extended direct, and tube extraction
  - *Bacillus anthracis* Sterne, *Brucella abortus* Strain 19, *Francisella tularensis* LVS, *Yersinia pestis* A1122, *Burkholderia thailandensis* ATCC 70038, *Clostridium* spp (*botulinum* types A, B, E, and *perfringens)*
- Some isolates of *B. anthracis*, *C. botulinum*, *F. tularensis*, *Y. pestis*, and *B. abortus* survived the direct and extended direct prep methods
- None survived the tube extraction
- *B. anthracis, B. thuringiensis, B. cereus, B. subtilis* survived tube extraction in other studies
- Use tube extraction AND 0.2 µm filter for "hazardous organisms"

# Automated Systems Study

- Determine ability of Bruker and Vitek software libraries to accurately identify BT agents
  - Test IVD, ROU, and Security Libraries independently
- Panel of 6 strains and 6 near neighbors for each agent
  - *B. anthracis*, *Y. pestis*, *F. tularensis* (5), *C. botulinum* (4), *B. mallei*, *B. pseudomallei*, *Brucella* spp.
  - Select isolates from AOAC Stakeholder Panel on Agent Detection Assays (SPADA) – inclusivity and exclusivity panels
  - Each isolate tested in triplicate
  - Acceptable ID:
    - Bruker sample score $\geq$2.0; Vitek ID score $\geq$ 60%
- Challenges
  - No SPADA panels for *C. botulinum* or *Brucella* spp.
  - Isolate availability and shipping
  - No tube extraction procedure for Vitek MS

# Study Participants

- Michigan Department of Health and Human Services
- New York Department of Health, Wadsworth Center, Biodefense Laboratory
- North Carolina State Laboratory of Public Health
- New York City Department of Health and Mental Hygiene
- Florida Department of Health, Bureau of Public Health Laboratories – Jacksonville Branch
- Minnesota Department of Health
- State Hygienic Laboratory at the University of Iowa
- Texas Department of State Health Services, Laboratory Services Section, Austin
- Center for Microbial Genetics and Genomics, Northern Arizona University

# Study Limitations

- Samples were prepared by four labs, shipped and stored frozen
- Some participants tested extracts beyond the 45 day time frame established in the study protocol
- Some participants prepared new targets for testing each software library

| Organism (# of strains tested) | Reported Identification | IVD Library | | RUO Library | | Security Library | |
|---|---|---|---|---|---|---|---|
| | | Mean Score (# replicates) | Species level ID % (>2.0) | Mean Score (# replicates) | Species level ID % (>2.0) | Mean Score (# replicates) | Species level ID % (>2.0) |
| *B. anthracis* (6) | No reliable ID | 1.13 (90) | | 1.38 (45) | | 1.1 (40) | |
| | *B. cereus* | | | 1.88 (62) | 8.3 | | |
| | *B. anthracis* | | | | | 2.08 (68) | 50 |
| | *B. pseudomycoides* | | | 1.56 (1) | | | |
| *B. thuringiensis* (1) | No reliable ID | 1.1 (12) | | 1.33 (9) | | 1.12 (9) | |
| | *B. cereus* | | | 2.01 (9) | 22.2 | | |
| | *B. anthracis* | | | | | 2.06 (9) | 38.9 |
| *B. circulans* (1) | No reliable ID | 1.06 (12) | | 1.33 (5) | | 1.01 (18) | |
| | *B. circulans* | | | 1.87 (13) | 16.7 | | |
| *B. cereus* (1) | No reliable ID | 1.15 (12) | | 1.35 (9) | | 1.23 (9) | |
| | *B. cereus* | | | 2.08 (9) | 44.4 | | |
| | *B. anthracis* | | | | | 2.14 (9) | 50 |
| *B. mycoides* (1) | No reliable ID | 1.15 (15) | | 1.61 (7) | | | |
| | *B. mycoides* | | | 1.77 (7) | | | |
| | *B. anthracis* | | | | | 1.77 (18) | |
| | *B. weihenstephanensis* | | | 1.19 (4) | | | |
| *B. megaterium* (1) | No reliable ID | 1.12 (12) | | 1.35 (3) | | 1.06 (18) | |
| | *B. megaterium* | | | 1.98 (15) | 55.6 | | |
| *B. subtilis* (1) | No reliable ID | 1.04 (15) | | 1.47 (13) | | 0.98 (18) | |
| | *B. subtilis* | | | 1.68 (5) | | | |

| Organism (# of isolates tested) | Reported Identification | IVD Library | | RUO Library | |
|---|---|---|---|---|---|
| | | Mean Score (# replicates) | Species level ID % (≥60%) | Mean Score (# replicates) | Species level ID % (≥60%) |
| *Y. pestis* (6) | No Identification | 0 (2) | | 0 (23) | |
| | *Y. pseudotuberculosis/Y. frederiksenii* | 51.7/48.2 (7) | | | |
| | *Y. ruckerii/Y. pseudotuberculosis/Y. frederiksenii* | 33.3/33.4/33.2 (1) | | | |
| | *Y. pseudotuberculosis* | 99.9 (17) | 58.6 | 87.8 (18) | 33.3 |
| | *Y. pestis* | 99.9 (1) | 3.4 | | |
| | *Yersinia* spp. | | | 88.8 (13) | |
| *Y. ruckeri* (1) | No Identification | 0 (5) | | | |
| | *Y. ruckerii* | 99.7 (4) | 44.4 | | |
| | *Yersinia* spp. | | | 85.7 (9) | |
| *Y. pseudotuberculosis* (3) | No ID | 0 (1) | | | |
| | *Y. pseudotuberculosis* | 99.6 (20) | 95.2 | 91.9 (20) | 95.2 |
| | *Y. enterocolitica* | | | 94.1 (1) | 4.8 |
| *Y. enterocolitica* (2) | No Identification | 0 (1) | | | |
| | *Y. enterocolitica* | 99.9 (8) | 66.7 | 97.5 (11) | 91.7 |
| | *Y. pseudotuberculosis* | | | 86 (1) | |
| | *Y. pseudotuberculosis/Y. enterocolitica* | 49.1/50.9 (3) | | | |

# Identification Errors

| Organism | Concern | Organism | Concern |
|---|---|---|---|
| *B. anthracis* | *B. cereus*<br>*B. cereus* group<br>Split identifications | *B. mallei* | *B. pseudomallei* |
| *B. thuringiensis* | *B. anthracis* | *B. pseudomallei* | *B. mallei* |
| *B. cereus* | *B. anthracis* | *B. thailandensis* | *B. pseudomallei*<br>*B. mallei* |
| *Y. pestis* | *Y. pseudotuberculosis*<br>*Yersinia* spp.<br>Split identifications | *C. botulinum* | *C. sporogenes* |
| *Y. pseudotuberculosis* | *Y. pestis* | *B. melitensis* | *Brucella* spp. |
| *Y. enterocolitica* | *Y. pestis* | *B. abortus, suis, canis, ovis, ceti pinnipedialsis* | *B. melitensis*<br>*Brucella* spp. |
| *F. philomiragia* | *F. tularensis* | | |

# Study Summary

- Software libraries for BT agents need improvement
  - **Currently not reliable for BT agents**
  - Addition of BT agents to IVD software
  - Availability of alternative libraries i.e. MicrobeNet
- Laboratories must be aware of software limitations
  - Partial or incorrect identifications
  - Additional training by manufacturer or BT coordinator
- Continue use of the rule out and refer protocols

MDHHS Bureau of Laboratories