Recognize, Rule-in, Rule-out, Refer

Orange County Sentinel Clinical Laboratory
Laboratory Response Network Training

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Objectives

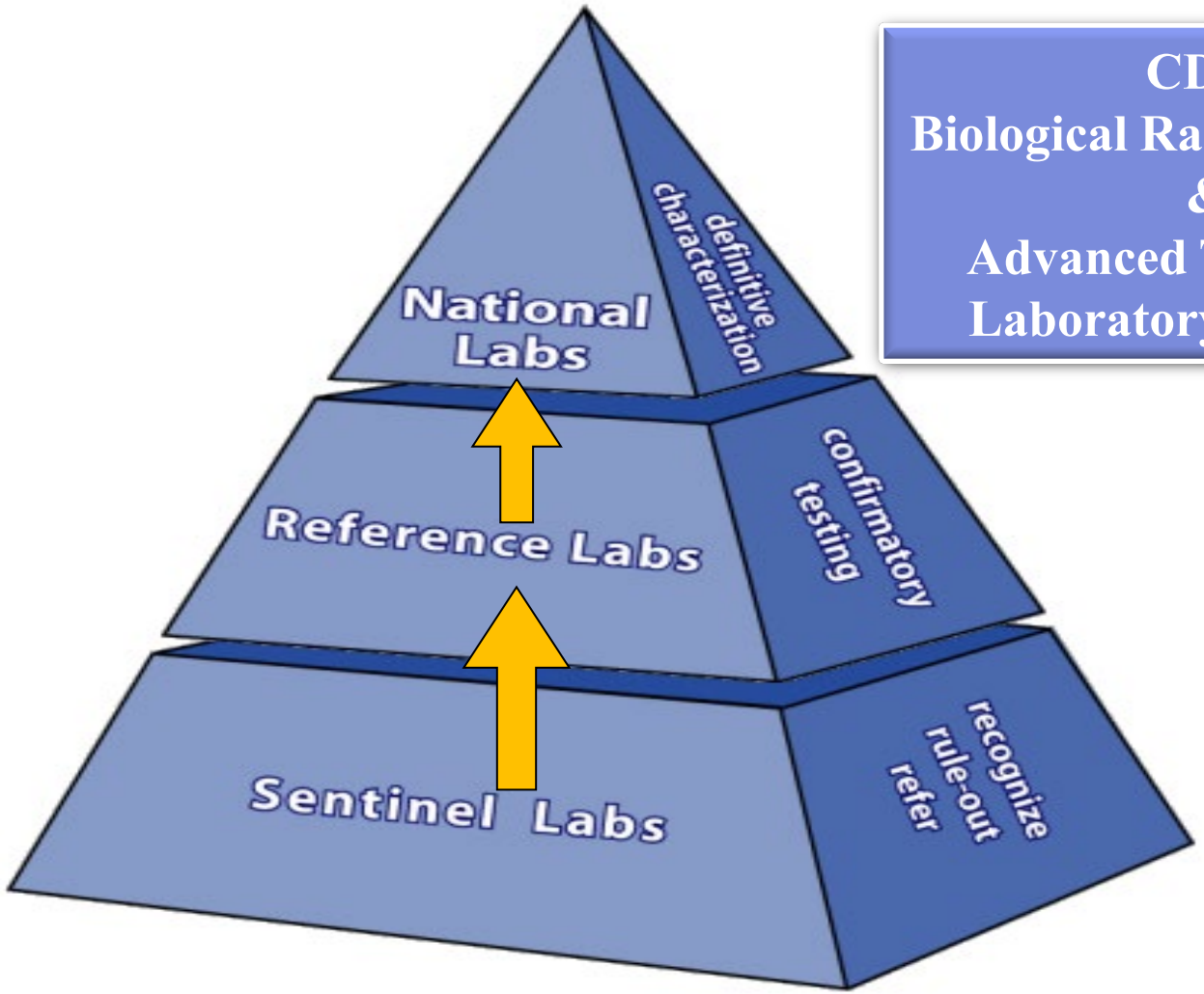
- Describe Sentinel Laboratory role in Laboratory Response Network (LRN)
- Locate and utilize LRN Sentinel Clinical Laboratory level Protocols
- Recognize potential select agents
- Distinguish key biochemicals to rule in, rule out, or refer the select agents

LRN Definition and Purpose

- Established to respond to biological, chemical, radiological threats, and public health emergencies
- Strengthen the response to public health threats
- Interconnected system, a novel approach to public health preparedness

LRN Structure

Types, Testing, Specimen Flow



CDC
Biological Rapid Response
&
Advanced Technology
Laboratory (BRRAT)

Agent Specific Laboratory

Sentinel Laboratory

- **Foundation for quickly recognizing and reporting potential threat agents**
  - Suspected biological threat agent should be directed to the nearest LRN Reference Laboratory **immediately**
- **Not required to register with the Select Agent Program**
  - Follow the FSAP policies for retaining an isolate when a select agent cannot be ruled out
  - Initiate documentation showing notification and transfer of SA to your LRN B lab, and destruction or transfer cultures once ID is complete
- **Direct Nonclinical specimens to LRN Reference Laboratory**
- Link for Responsibilities

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Role and Responsibly: Sentinel Laboratory

1. Follow Reportable Disease Guidelines
2. Follow federal packing and shipping regulation
3. Refer biothreat agent specimens/isolates
4. Comply with BMBL
5. Follow ASM Sentinel Level Guidelines and demonstrates competency (CAP LPX Exercise)

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Demonstrate Annual Competency

LABORATORY PREPAREDNESS EXERCISE - LPX

Analytes/procedures in **bold** type are regulated for proficiency testing by the Centers for Medicare & Medicaid Services (CMS).

Analyte	Challenges per Shipment	Number of Shipments
Live organisms	3	Two shipments per year

The Laboratory Preparedness Exercise (LPX) was developed as a collaborative effort between the College of American Pathologists, the Centers for Disease Control and Prevention (CDC), and the Association of Public Health Laboratories (APHL). Laboratories will be sent live organisms that either exhibit characteristics of bioterrorism agents or demonstrate epidemiologic importance and will be expected to respond following Laboratory Response Network Sentinel Laboratory Guidelines if a bioterrorism agent is suspected. All agents provided are excluded from the CDC's select agent list. These may include strains of *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, and *Brucella abortus* that have been modified and are safe for testing in a laboratory that contains a certified Class II Biological Safety Cabinet and is capable of handling Category A and B agents.

Program Information

- Three swab specimens with diluents
- Not available to international customers due to United States export law restrictions

Shipping Schedule

- Shipment A: April 6
- Shipment B: September 8

BIOHAZARD CDC Centers for Disease Control and Prevention APHL

Education through Practice

COLLEGE of AMERICAN PATHOLOGISTS

LABORATORY PREPAREDNESS EXERCISE-LPX

Role and Responsibly: Sentinel Laboratory

6. Follows OSHA regulations
7. Complies with Federal Select Agent Program regulations
8. Biosafety and biosecurity risk assessment policy
9. Utilizes Class II or higher certified BSC
10. Never test environmental, animal, food, or water for biological threat without approval

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Role and Responsibly: Reference Laboratory

- Formally registered with the LRN, often called LRN-B
- Responsible for investigation and/or referral of specimens
- Include 120 domestic state and local laboratories
- Perform rapid molecular tests to detect biological agents
- May process environmental samples
- Provide training and guidance

Role and Responsibly: National Laboratory

- CDC, USAMRIID
- Have highest biosafety level to work with highly dangerous/infectious agents (smallpox, Ebola)
- They have methods to further characterize isolates of biological agents

Sentinel Laboratory Protocols

- ASM Sentinel Level Guidelines
- Integrate protocols into laboratory SOP
- Review annually along with other laboratory documents

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Recognize-Rule In – Rule Out – Refer

- *Bacillus anthracis*
- *Bacillus cereus* biovar *anthracis*
- *Brucella* species

Break

- *Francisella tularensis*
- *Yersinia pestis*
- *Burkholderia mallei*
- *Burkholderia pseudomallei*

Bacillus anthracis

ANTHRAX

Anthrax and Bioterrorism

- Potential use as a bioterrorism agent
- Used by many countries, primarily for military purposes in the conduct of biowarfare.
- Inhalation of *B. anthracis* spores can occur following an intentional aerosol release, as was evident in the 2001 anthrax event.
- Aerosolization of anthrax spores is the most likely method to be used in a bioterrorism event.

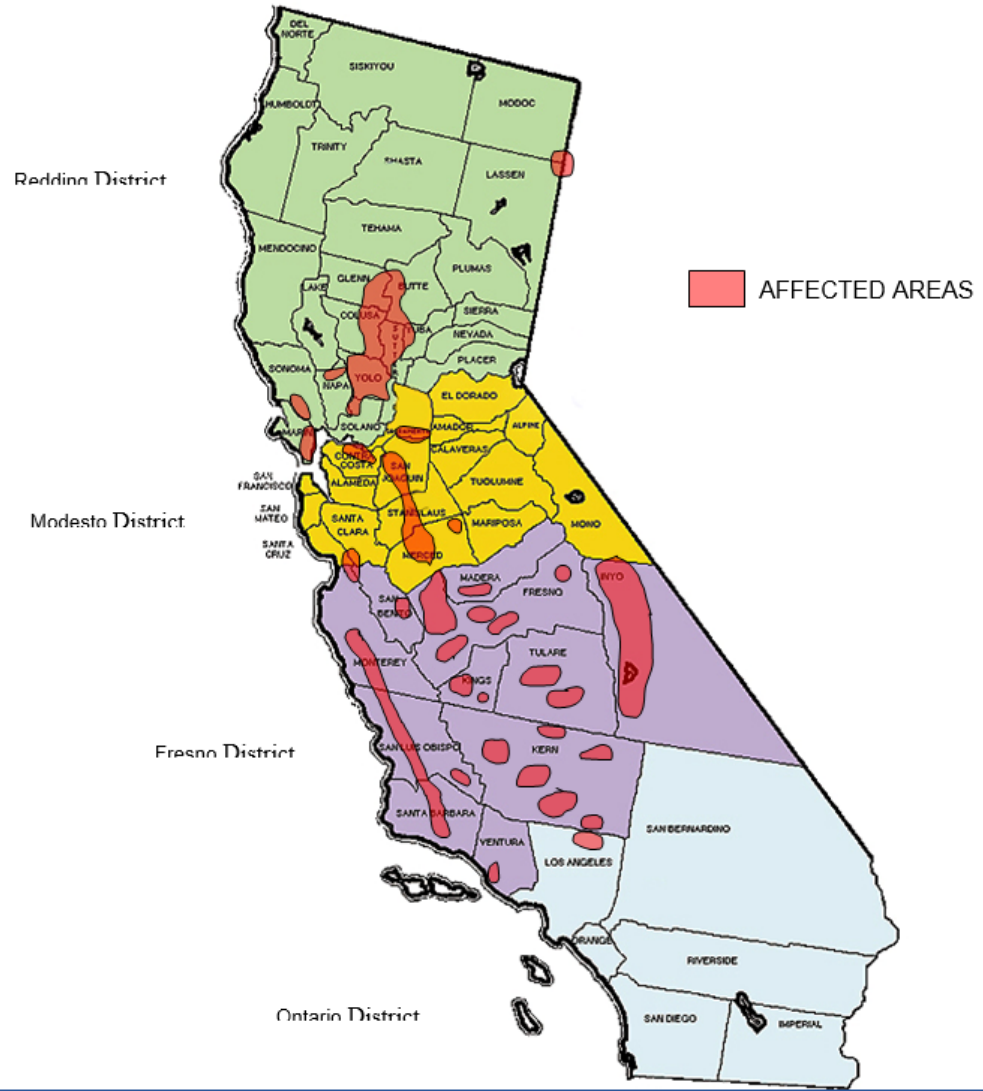
Bacillus anthracis

- **Naturally occurring in the environment.**
- **Anthrax is a zoonotic disease that occurs most frequently in herbivorous animals**
- **Human disease is less common and results from contact with infected animals or with commercial products derived from them, such as wool and hides.**
- **Four naturally occurring cases of human anthrax have been reported in the US since 2006: one gastrointestinal, one cutaneous, and two inhalational**

California Department of Food and Agriculture™

Animal Health and Food Safety Services
Animal Health Branch

Known Areas in which Anthrax Outbreaks in Livestock Have Occurred in the Last Century



Bacillus cereus biovar anthracis (Bcbva)

- **Identified in the early 2000's in Cameroon (strains CA) and Cote d'Ivoire (strains CI)**
- **These strains were recovered in gorillas and chimpanzees with anthrax-like disease**
- **This organism has since been recovered from elephants, goats and blow flies in other countries of Africa**

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Anthrax infections may occur in one of four forms:

1. Cutaneous
2. Ingestion/Gastrointestinal
3. Inhalation anthrax
4. Injection

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Sentinel Laboratory Procedures for *B. anthracis*

In a Sentinel Laboratory, you could encounter this organism in:

1. **Direct specimen smears**
2. **Blood culture or CSF**
3. **Vesicle fluid, swab or biopsy of eschar**
4. **Stool**
5. **Postmortem tissue**

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Sentinel Laboratory Procedures for *B. anthracis*

1. Culture characteristics
2. Gram stain
3. Hemolysis
4. Catalase
5. Motility

Sentinel Laboratory Procedures for *B. anthracis*

Culture characteristics

1. This organism will grow on most routine culture media BAP, CHOC, but NOT MAC
2. Will grow in routine blood culture systems
3. Growth on plated media may be evident as early as eight hours

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Sentinel Laboratory Procedures for *B. anthracis*

Colony on BAP @ 35°C, 18-24 hours

1. Flat or slightly raised, white to grey
2. Irregular edges with; “Medusa head”, “comet tail”, or “comma-shaped projections”
3. Surface has “ground glass” appearance
4. Tenacious or sticky consistency
5. B. anthracis is non-hemolytic (gamma hemolytic)
6. B .cereus biovar anthracis may exhibit weak hemolysis upon extended incubation

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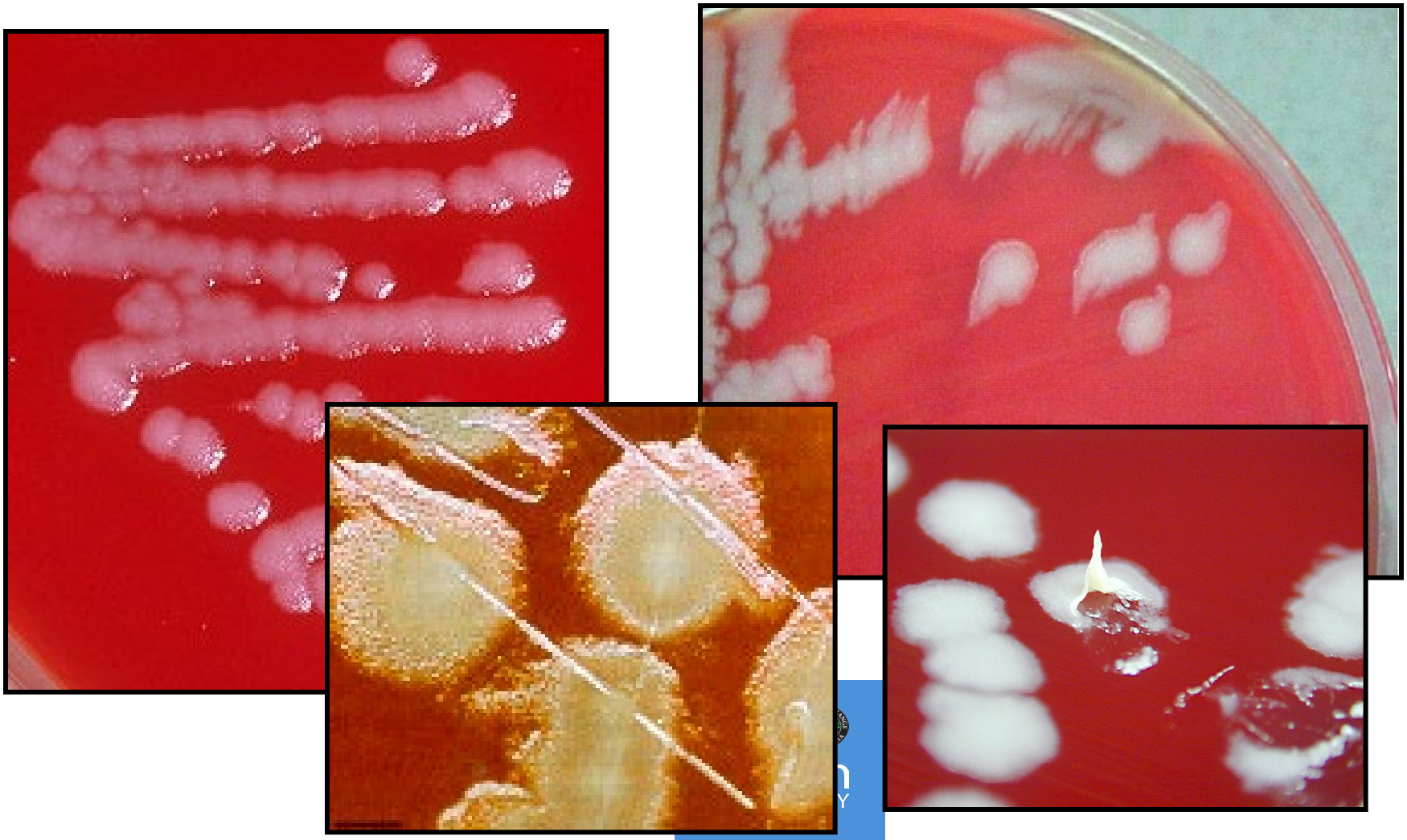
B. anthracis colonies on blood agar



B. cereus biovar *anthracis* on blood agar

Photo courtesy of APHL

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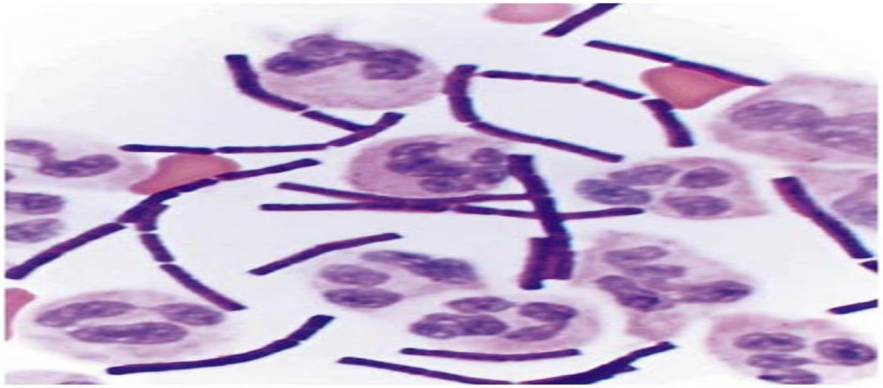
Sentinel Laboratory Procedures for *B. anthracis*

Gram stain morphology

From clinical smears : Large Gram-positive rods in chains, 2 - 4 cells

Spores are NOT usually present



Bush, et al. 2001. N Engl J Med 345(22):1607-1610

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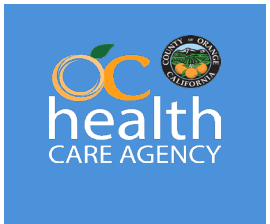
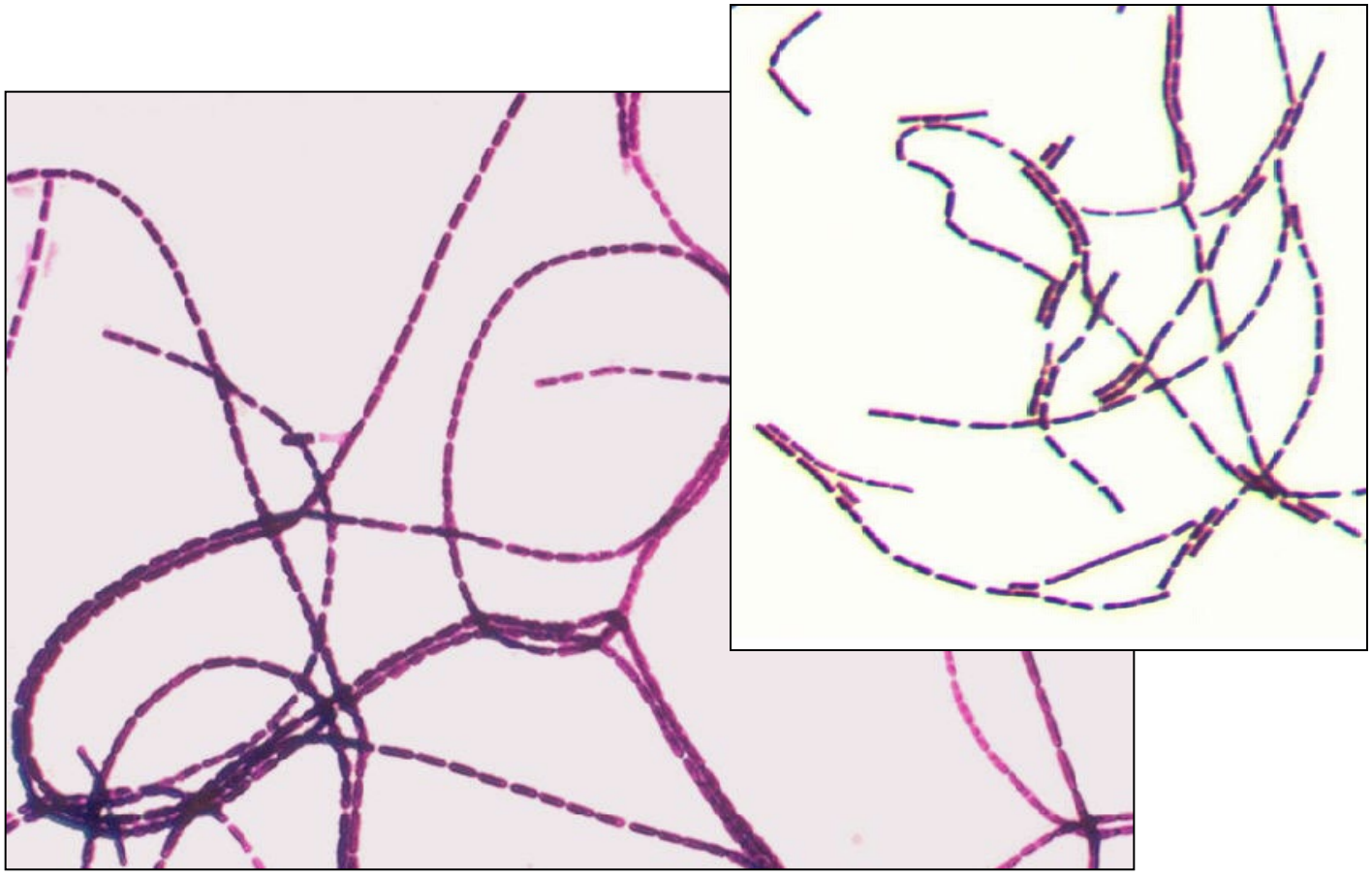
Sentinel Laboratory Procedures for *B. anthracis*

Gram stain morphology – from *culture*

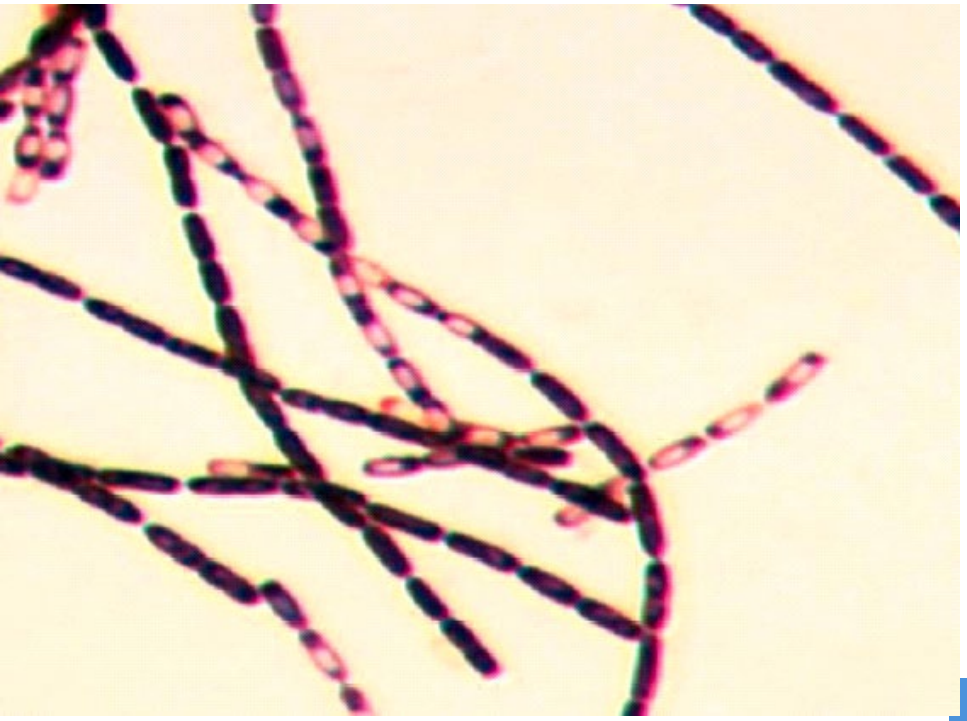
1. Large Gram-positive rod
2. Non-encapsulated, often in long chains
3. Cells are more readily decolorized with age
4. Central to sub-terminal oval spores, with no swelling of the cell
5. Presence of spores increases with age of culture



Sentinel Laboratory Procedures for *B. anthracis*



Sentinel Laboratory Procedures for *B. anthracis*



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Sentinel Laboratory Procedures for *B. anthracis*

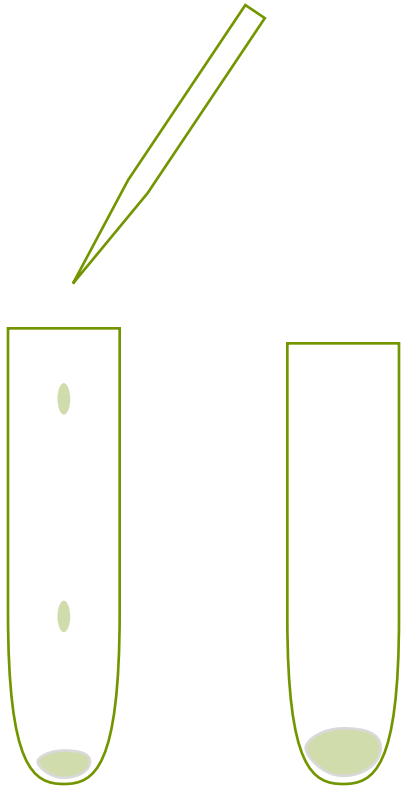
Screening Test—Catalase

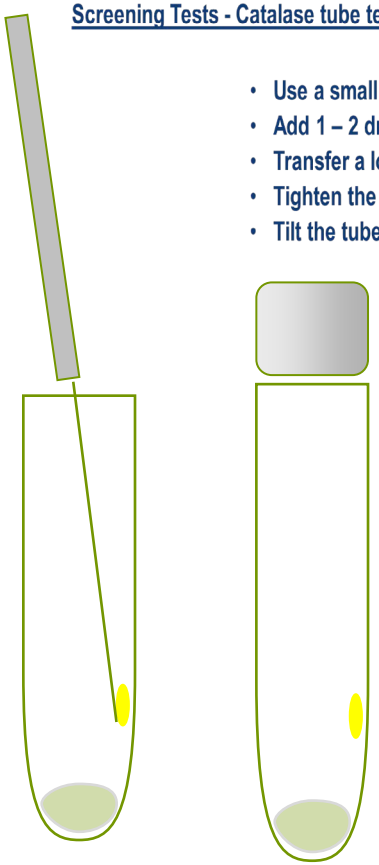
Catalase - Positive

The catalase test is very dangerous—do not perform as a slide test on the open bench

Recommend performing the tube test in a BSC

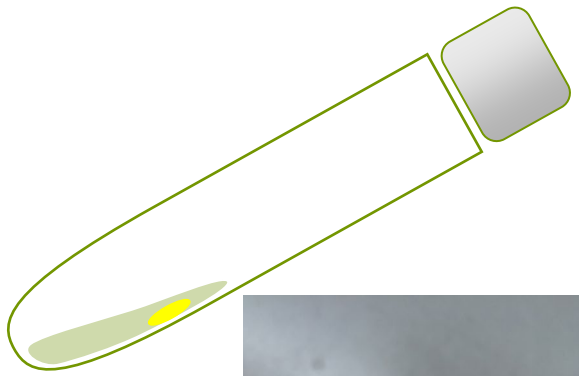
Sentinel Laboratory Procedures for *B. anthracis*

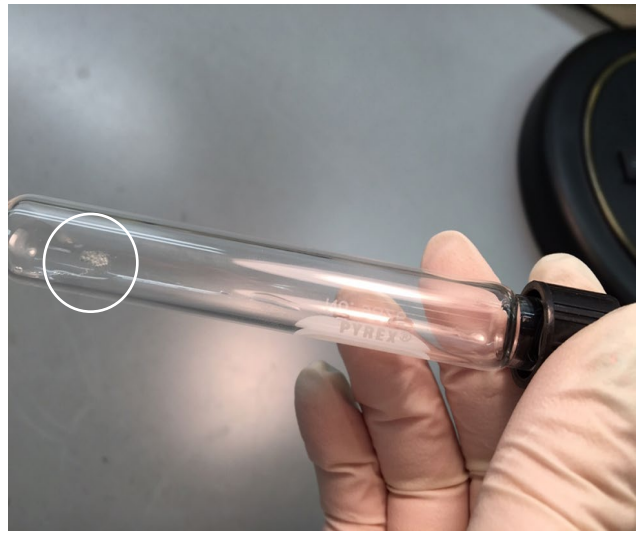




Screening Tests - Catalase tube test

- Use a small test tube with a screw cap
- Add 1 – 2 drops of 3% hydrogen peroxide to the tube
- Transfer a loopful of the culture from the plate to the wall of the tube just above the level of peroxide
- Tighten the screw cap
- Tilt the tube until the peroxide washes over the culture on the wall and observe for evidence of bubbling





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Sentinel Laboratory Procedures for *B. anthracis*

Motility

- Motility in semi-solid medium– B. anthracis is non-motile; Bcbva strains are usually motile

Characteristic	*B. anthracis*	*B. cereus*	*Bcbva CI*[1]	*Bcbva CA*[2]
Hemolysis[4]	-	+	-	-
Motility[5]	-	+	+/-	+/-

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Bacillus anthracis Identification Flowchart

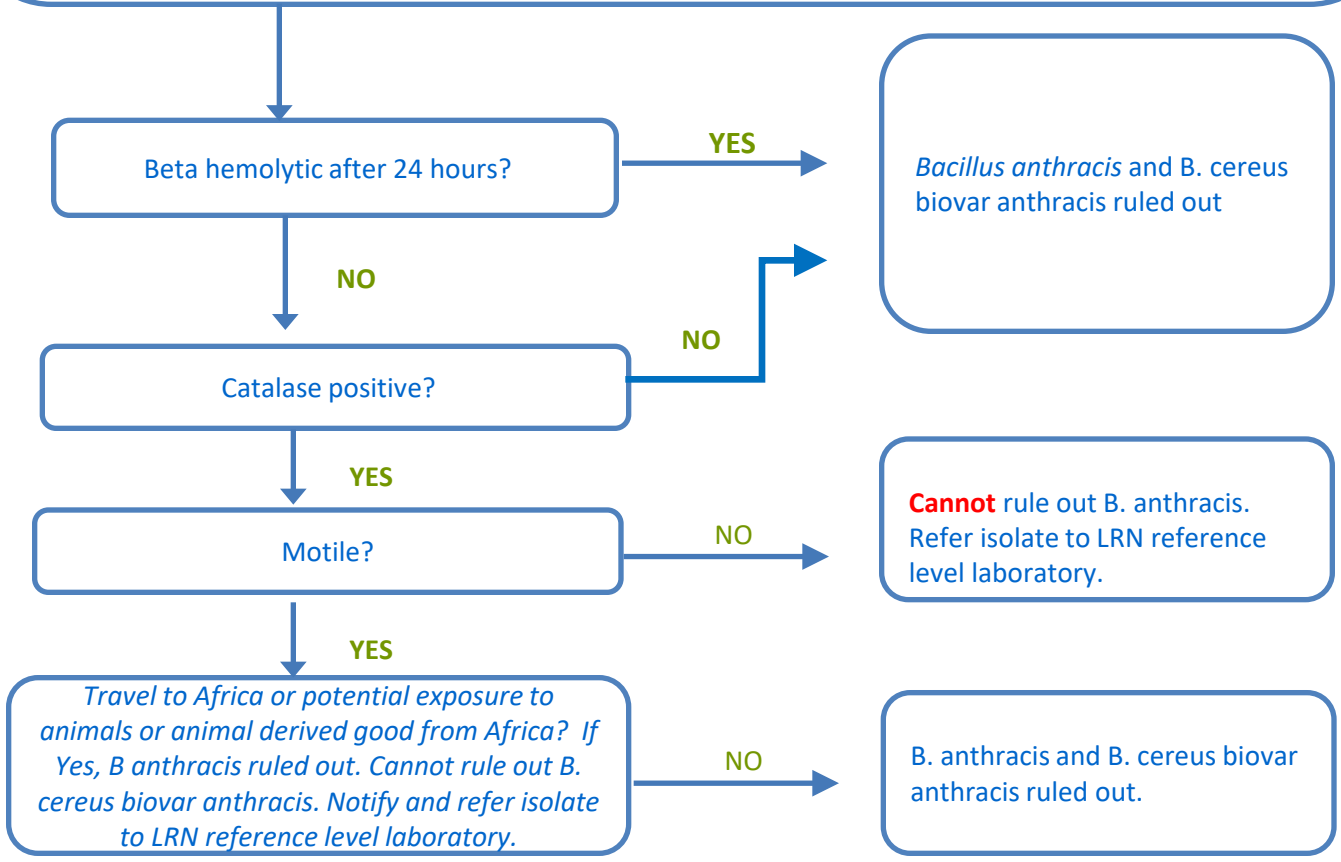
Major characteristics of *Bacillus anthracis and B. cereus biovar anthracis*:

Gram stain morphology: Large, Gram-positive rods. Spores may be found in cultures, but not usually in clinical samples

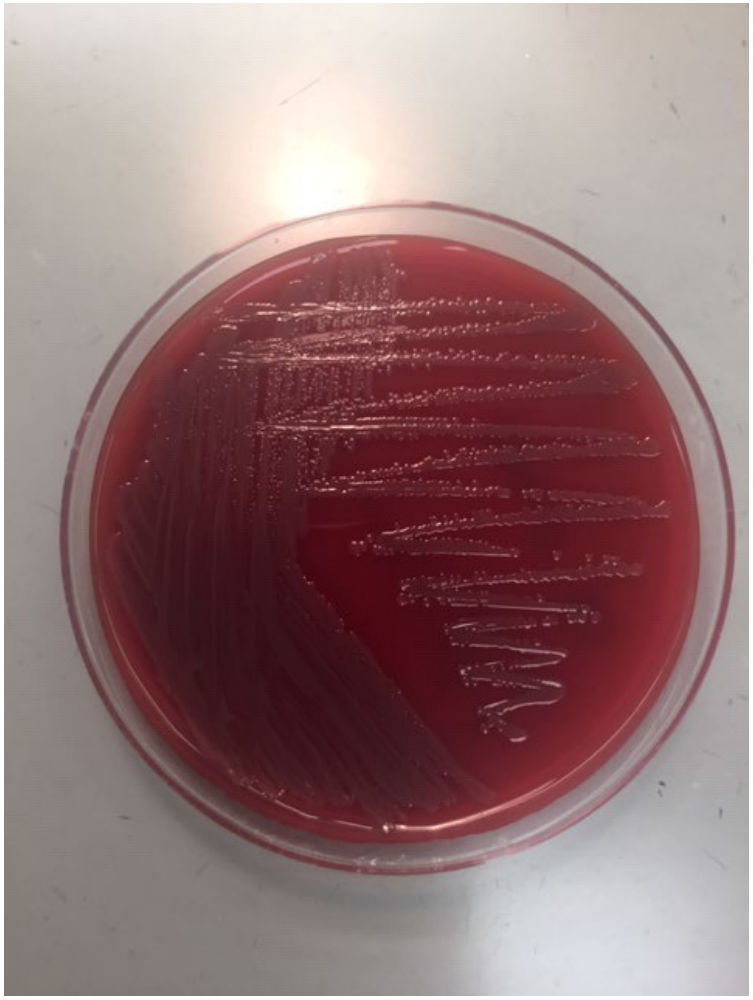
Colony morphology: Ground glass appearance, non-pigmented, gamma hemolytic (no hemolysis) on BAP (some strains of B. cereus biovar anthracis may be weakly hemolytic after 48h of incubation)

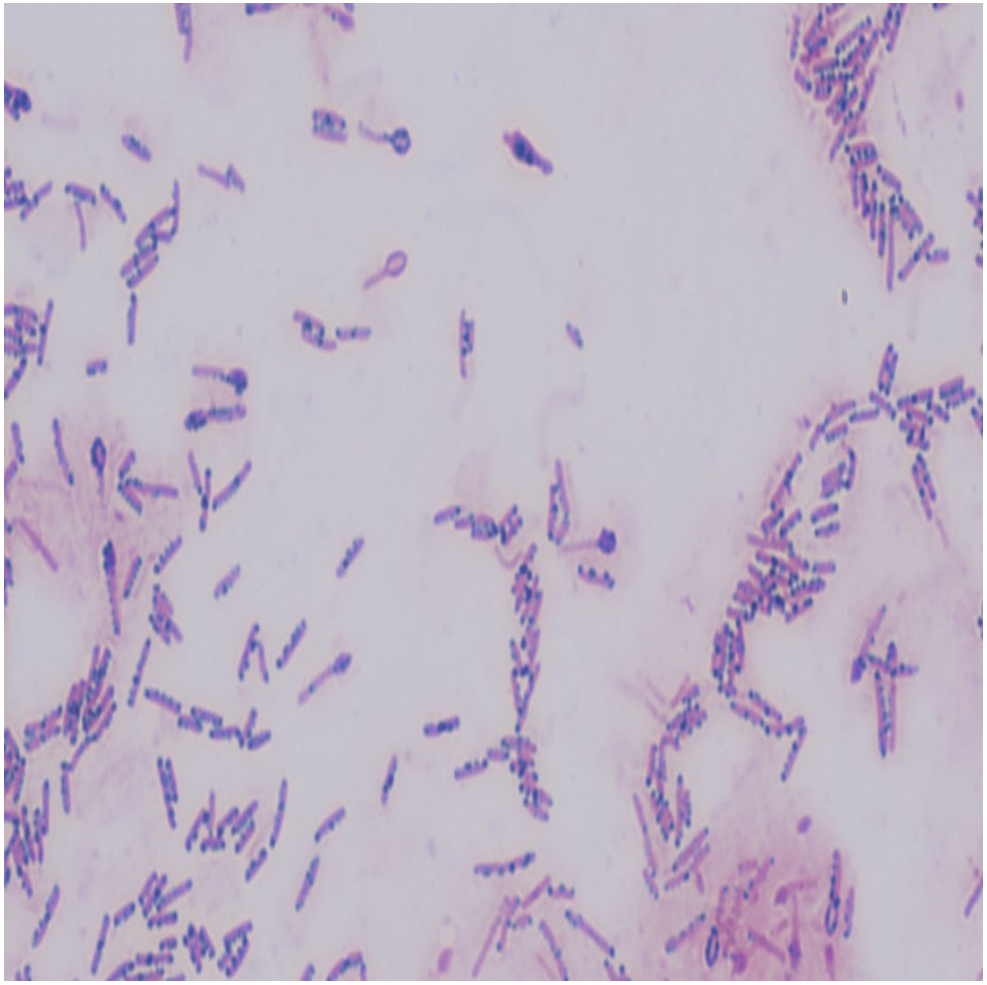
No growth on MAC (or EMB)

Perform all work in a biosafety cabinet using BSL-3 precautions.



Rule In or Rule Out?





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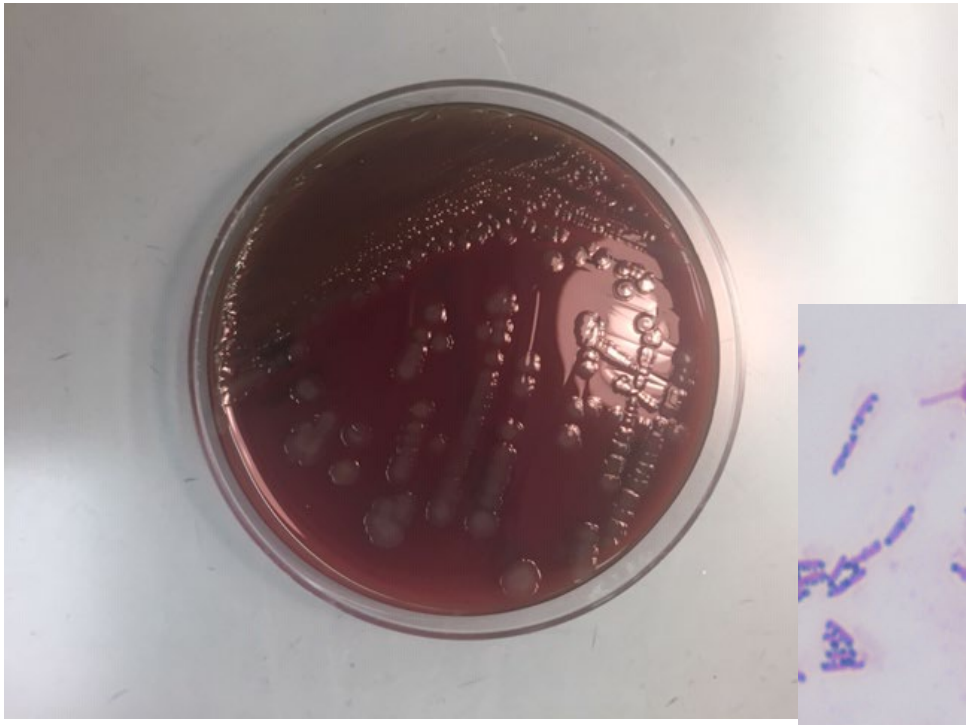

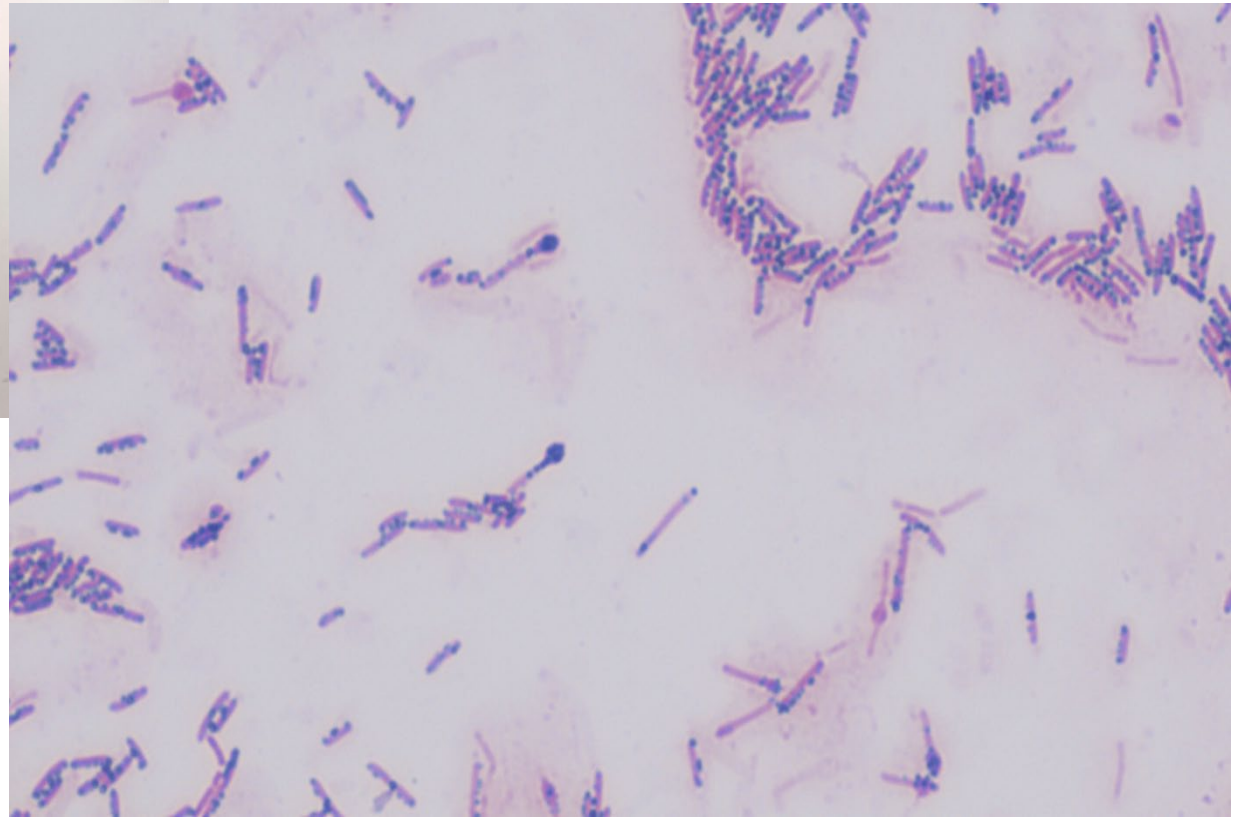

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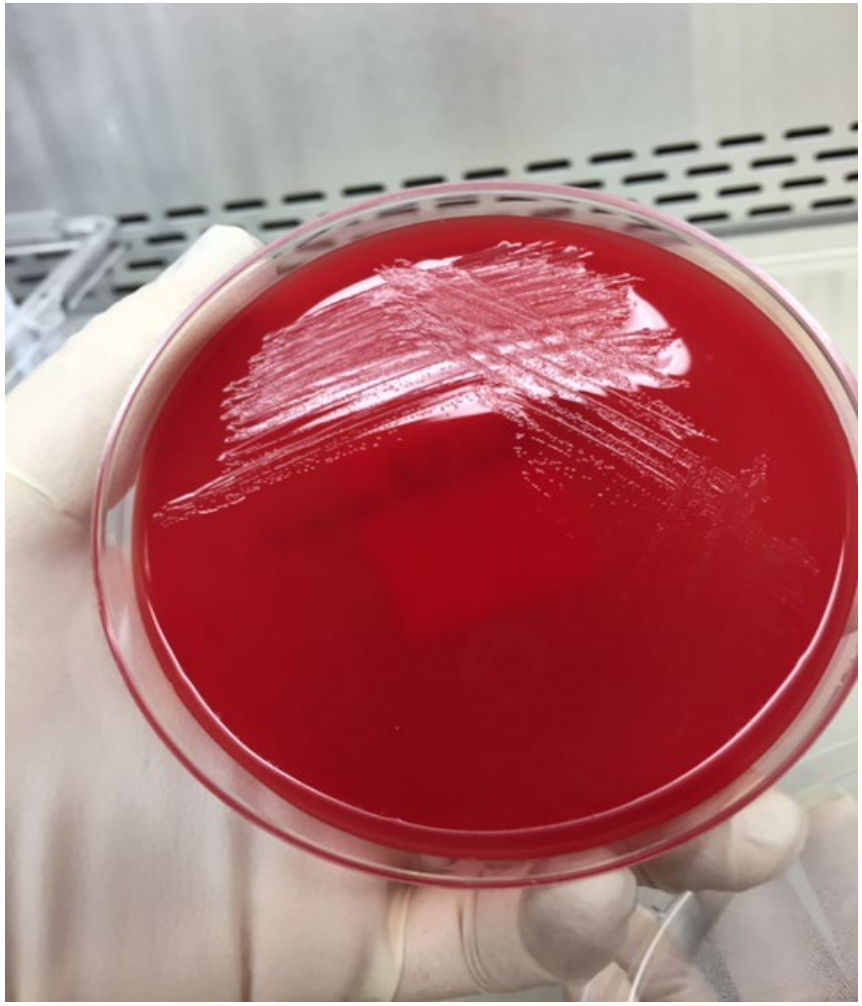

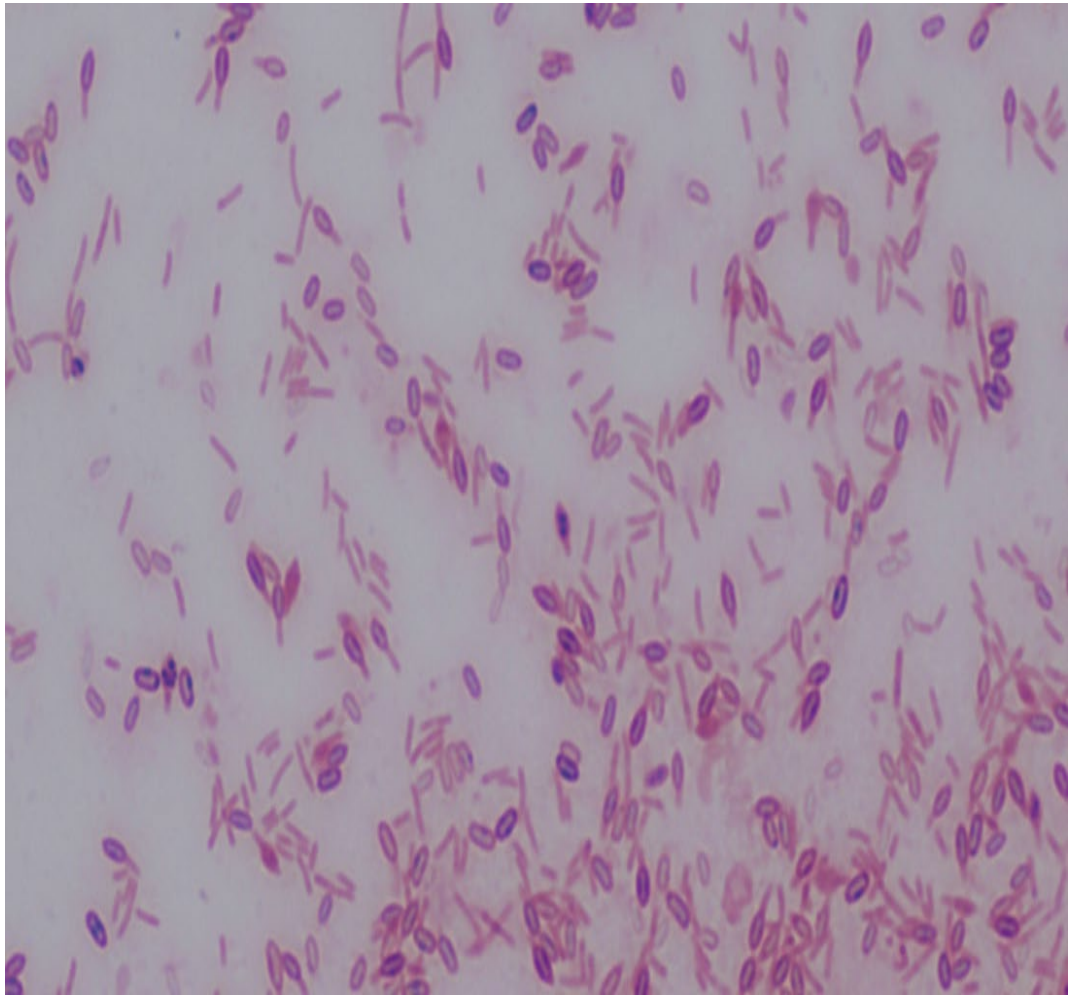

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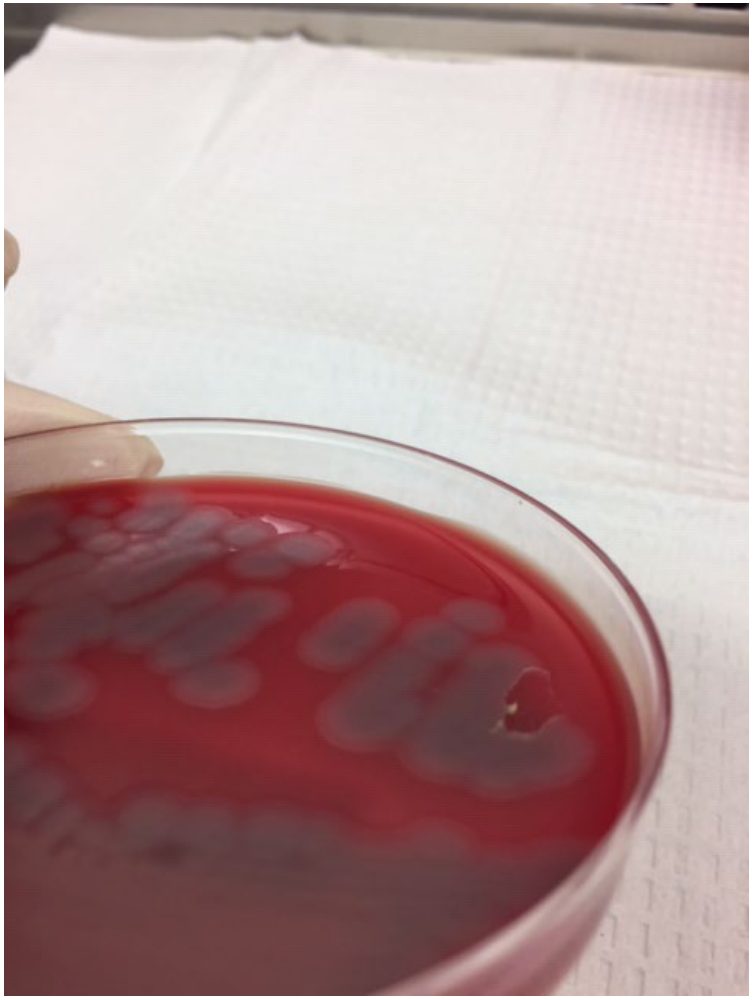

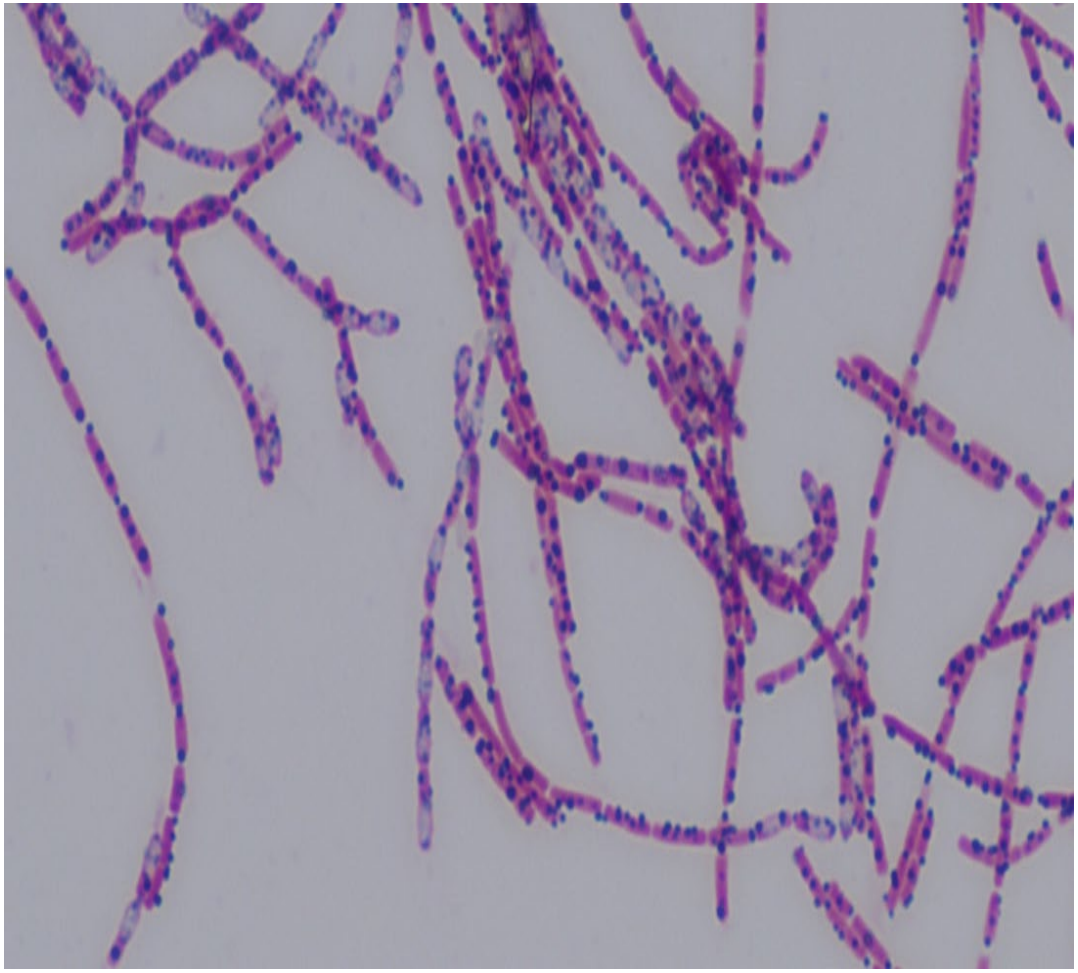

Sentinel Laboratory Procedures for *B. anthracis*

IF YOU SEE:

- **Rapidly growing, large, flat, "ground-glass" colonies on BAP**
- **Large Gram-positive rods, spores do not swell the cell**
- **Non-hemolytic**
- **Catalase positive**

And cannot rule out *B. anthracis* using the protocol flow chart…….

CONTACT YOUR LRN REFERENCE LABORATORY

BRUCELLOSIS

Removal of *Brucella* species from Select Agent toxin list

- Took into effect on January 16th ,2025
- This includes Brucella abortus, melitensis and suis
- Still reportable per Title 17
- Send sample to LRN lab for confirmation
- Risks and safety concerns have NOT changed

Brucella spp. Biosafety Alert

- **Brucellosis has been the most commonly reported *laboratory-associated bacterial infection*, aerosols are highly infectious. Infective dose = 10 -100 organisms**

- **Laboratory workers can also acquire the disease from direct exposure to cultures of the organism**

- **Cases have occurred in clinical laboratory settings by "sniffing" cultures, direct skin contact with cultures, and aerosol generating procedures.**

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CLINICAL SIGNIFICANCE

- ***B. melitensis***: (goats, sheep, camels) most severe and more acute
- ***B. abortus***: (cattle) more chronic
- ***B. suis***: (pigs) severe, associated with osteomyelitis
- ***B. canis***: (dogs) very rare in humans

BRUCELLOSIS: TRANSMISSION

Ingestion-Unpasteurized dairy products

- The most common mode of transmission

Direct skin contact

- Occupational hazard for farmers, butchers, veterinarians, hunters, and laboratory personnel

Aerosols

- Highly infectious; easily aerosolized

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Sentinel Laboratory Procedures for *Brucella* spp.

In a Sentinel Laboratory, you could encounter this organism in:

- **Bone Marrow or whole blood**
- **Joint or abdominal fluid**
- **Spleen, liver abscesses**
- **Serum**
- **Rarely sputum**

Sentinel Laboratory Procedures for *Brucella* spp.

- Culture characteristics
- Gram stain morphology
- Oxidase
- Catalase
- Urease
- Satellite phenomenon

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Sentinel Laboratory Procedures for *Brucella* spp.

Culture characteristics

- Grows slowly on BAP and CHOC, (NOT ON MAC)
- Grows in routine blood culture systems, but may require extended incubation
- Some strains benefit from an incubation atmosphere enriched with CO_2

Sentinel Laboratory Procedures for *Brucella* spp.

Colony on BAP at 35°C in 5% CO_2

Small (0.5-1.0mm), convex, glistening

Non-hemolytic and non-pigmented

Visible growth may take 48 - 72 hours

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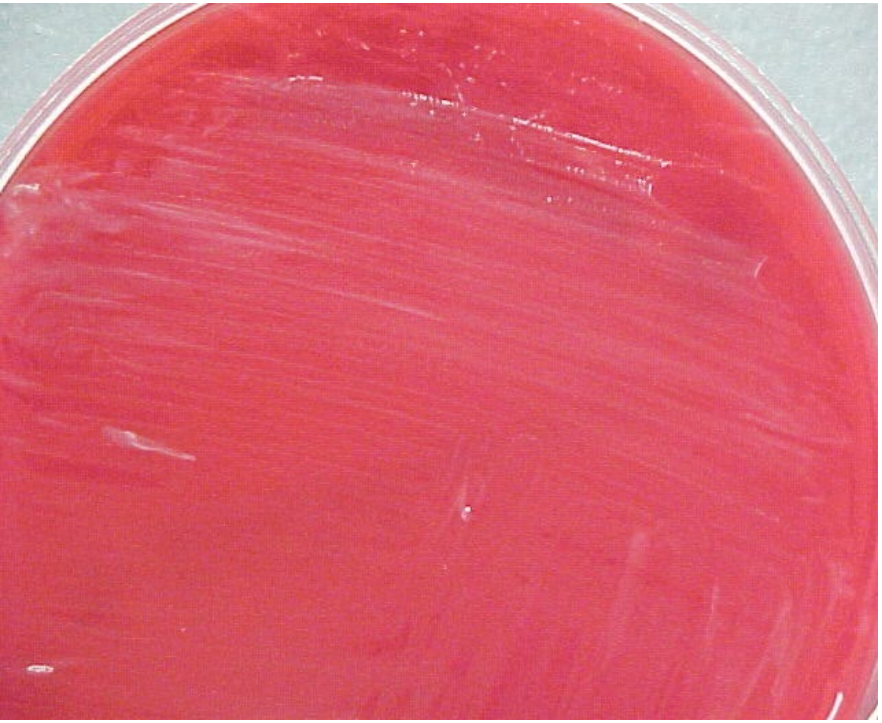
Sentinel Laboratory Procedures for *Brucella* spp.

Growth on blood agar @35C

After 24 – 48 hrs



After 72 hrs

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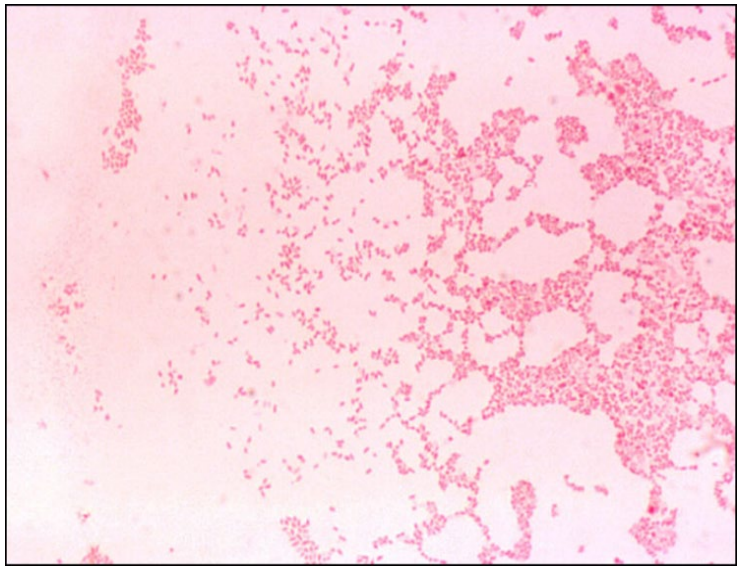
Sentinel Laboratory Procedures for *Brucella* spp.

Gram stain morphology

Very small (0.4 by .08µm), faintly staining, Gram-negative coccobacilli

Larger than *F. tularensis*



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Sentinel Laboratory Procedures for *Brucella* spp.

Screening Tests Results

Oxidase - positive

Catalase - positive

Urease - positive

Satellite phenomenon – growth on BAP without need to satellite around *S. aureus*

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Sentinel Laboratory Procedures for *Brucella* spp.

Urease hydrolysis – positive

Different strains of Brucella and their reaction to urea

- ***B. suis, B. canis* < 5 minutes**
- ***B. abortus* and *melitensis* >5 min to 24 hours**

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Sentinel Laboratory Procedures for *Brucella* spp.

- *Achromobacter* grp B
- *Acidovorax* spp.
- *Agrobacterium* spp.
- *Methylobacterium* spp.
- ★ *Psychrobacter phenylpyruvicus*
- ★ *Psychrobacter immobilis*
- ★ *Oligella urealytica*
- ★ *Bordetella bronchiseptica*
- *Haemophilus spp.*
- *Paracoccus yeei*

	Brucella spp.	*Psychrobacter immobilis*	*Paracoccus yeei*	*Psychrobacter phenylpyruvicus*	*Methylobacterium* spp.	*Oligella ureolyticaa*	*Bordetella bronchiseptica, B. hinzii, Cupriavidus pauculus*	*Haemophilus* spp.
Gram stain morphology	tiny ccb, stains faintly	ccb, rods	cocci in packets	ccb, rods, retains crystal violet	Vacuolated rods	tiny ccb	rods	Tiny ccb
Catalase	+	+	+	+	+	+	+	v
Oxidase	+	+	+	+	+	+	+	v
Urea	+	v	+	+	v	+	+	v
Motility	-	-	-	-	-	+,delayed	+	-
BAP distinctions	-	Prefers 20°C, Odor of roses	Mucoid	-	Pink, mucoid	-	-	No growth
MAC-48 h	-	-	-	-	-	-	+	-

Sentinel Laboratory Procedures for *Brucella* spp.

In 2020 *Ochrobactrum* spp. were reclassified to the Brucella genus

Classical *Brucella* species

1. Brucella abortus (Schmidt 1901) Meyer and Shaw 1920 (Approved Lists 1980)
2. Brucella canis Carmichael and Bruner 1968 (Approved Lists 1980)
3. Brucella ceti Foster et al. 2007
4. Brucella inopinata Scholz et al. 2010
5. Brucella melitensis (Hughes 1893) Meyer and Shaw 1920 (Approved Lists 1980)
6. Brucella microti Scholz et al. 2008
7. Brucella neotomae Stoenner and Lackman 1957 (Approved Lists 1980)
8. Brucella pinnipedialis Foster et al. 2007
9. Brucella ovis Buddle 1956 (Approved Lists 1980)
10. Brucella papionis Whatmore et al. 2014
11. Brucella suis Huddleson 1929 (Approved Lists 1980)
12. Brucella vulpis Scholz et al. 2016

There are several novel *Brucella* strains that have been described from frogs, bats, Australian rodents and a sting ray that have not been designated as species.

New *Brucella* species, previously Ochrobactrum

13. Brucella anthropi (Holmes et al. 1988) Hördt et al. 2020
14. Brucella ciceri (Imran et al. 2010) Hördt et al. 2020
15. Brucella cytisi (Zurdo-Piñeiro et al. 2007) Hördt et al. 2020
16. Brucella daejeonensis (Woo et al. 2011) Hördt et al. 2020
17. Brucella endophytica (Li et al. 2016) Hördt et al. 2020
18. Brucella gallinifaecis (Kämpfer et al. 2003) Hördt et al. 2020
19. Brucella grignonensis (Lebuhn et al. 2000) Hördt et al. 2020
20. Brucella haematophila (Kämpfer et al. 2007) Hördt et al. 2020
21. Brucella intermedia (Velasco et al. 1998) Hördt et al. 2020
22. Brucella lupini (Trujillo et al. 2006) Hördt et al. 2020
23. Brucella oryzae (Tripathi et al. 2006) Hördt et al. 2020
24. Brucella pecoris (Kämpfer et al. 2011) Hördt et al. 2020
25. Brucella pituitosa (Huber et al. 2010) Hördt et al. 2020
26. Brucella pseudintermedia (Teyssier et al. 2007) Hördt et al. 2020
27. Brucella pseudogrignonensis (Kämpfer et al. 2007) Hördt et al. 2020
28. Brucella rhizosphaerae (Kämpfer et al. 2008) Hördt et al. 2020
29. Brucella thiophenivorans (Kämpfer et al. 2008) Hördt et al. 2020
30. Brucella tritici (Lebuhn et al. 2000) Hördt et al. 2020

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Sentinel Laboratory Procedures for *Brucella* spp.

Current Recommendations

- **If isolate is identified as a NBBS (*Brucella(Ochromobactrum) antropi* or *Brucella (Ochromobactrum) intermedium* or as *Brucella spp***
- **Evaluate using the ASM rule-out testing**
- **If unable to differentiate using microbiological methods, refer to LRN Laboratory for rule-out testing**

Brucella and Ochrobactrum Taxonomic Updates for Laboratories | ASM.org

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SAFETY: As soon as *Brucella* is suspected, perform ALL further work in a Class II Biosafety Cabinet using BSL-3 practices

Brucella Identification Flowchart

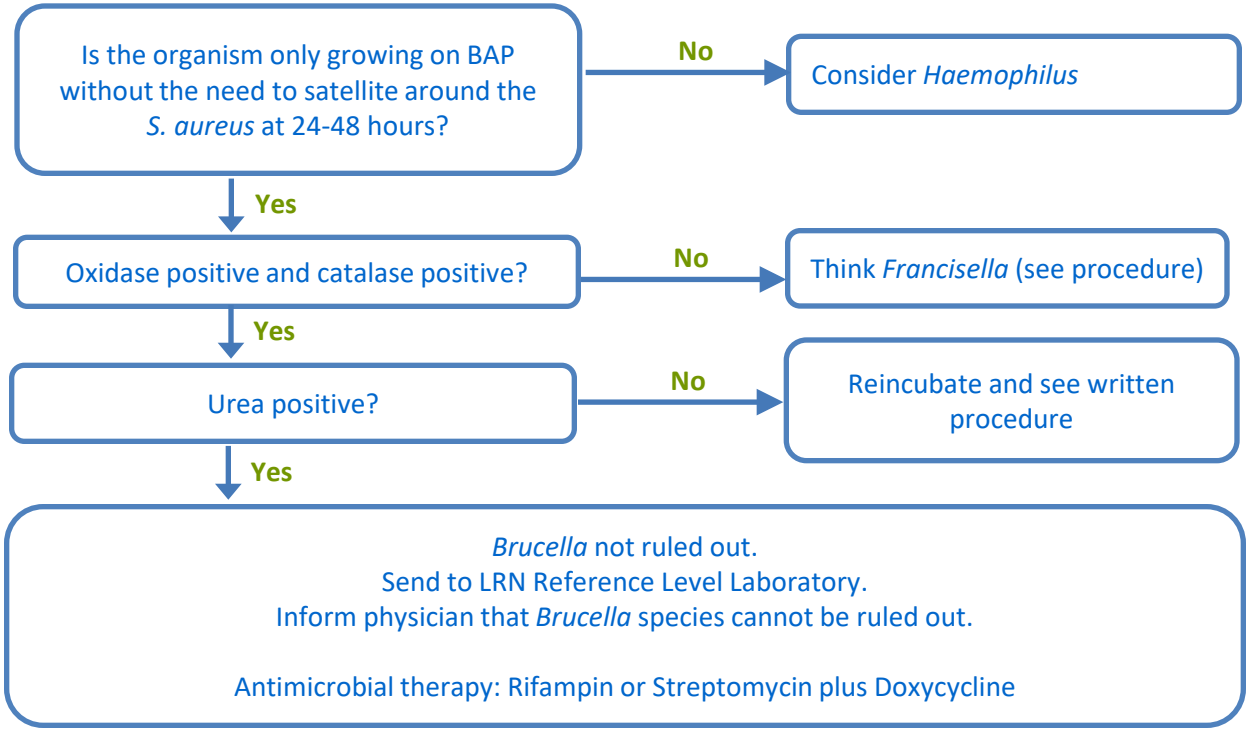
Major characteristics of *Brucella* species:

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

THINK BRUCELLA

Growth: 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.



Sentinel Laboratory Procedures for *Brucella* spp.

IF YOU SEE:

- **Very small, faintly staining, Gram-negative coccobacilli from blood, bone marrow, or lymphoid tissue**
- **Slow growth on BAP, CHOC needing 2-3 days for colonies to appear**
- **Oxidase (+), urease (+), catalase (+)**
- **Satellite phenomenon- growth on BAP without need to satellite around *S. aureus***

And you cannot rule out *Brucella* spp. using the protocol flow chart…….

OC health CARE AGENCY

CONTACT YOUR LRN REFERENCE LABORATORY

Will Return in *15 Minutes*

Francisella tularensis

TULAREMIA

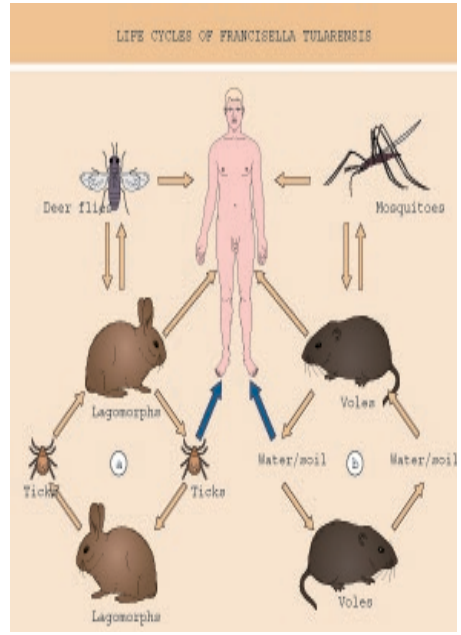
Biosafety Alert

- Dangerous, highly virulent organism
- Select Agent
- Infectious dose very low 10-50 organisms
- Laboratory-acquired infections
- Do not manipulate on an open bench

OC health CARE AGENCY

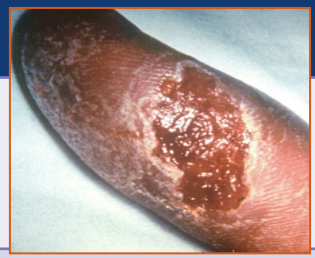
Disease Transmission

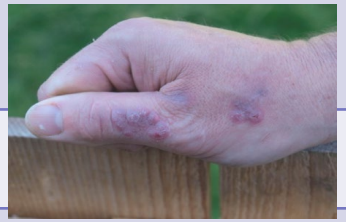
- *Zoonotic pathogen*
  - Naturally occurring infection rodents, rabbits, and their ectoparasites
- Humans are accidentally infected
  - Dead-end Host
  - No evidence for person to person
- Incubation period 3-5 days
- Exposure
  - Certain occupational groups
  - Bite of an infected arthropod
  - Contact with infected animal or carcasses
  - Ingestion of contaminated food or water
  - Inhalation of infectious aerosols

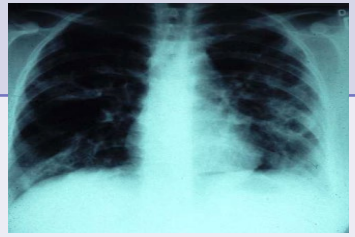


Clinical Presentation

Ulceroglandular	·Most common form (45 to 80% of cases) ·Ulcer at site of exposure with inflamed lymph nodes
Glandular	·Regional lymphadenopathy ·Ulcer is undetectable
Oculoglandular	·Conjunctivitis with regional lymphadenopathy
Oropharyngeal	·Ingestion of contaminated food or water ·Acute septicemia and cervical lymphadenitis ·Pharyngitis
Intestinal	·Ingestion of contaminated food or water ·Vomiting, abdominal pain, and diarrhea
Typhoidal	·Febrile illness ·Route of infection is unknown
Pneumonic	·Inhalation of infectious aerosols ·Most severe and lethal form ·May present as unresponsive community acquired pneumonia







Clinical specimens

- Ulcer scraping
- Tissue biopsy or aspiration
- Lymph node biopsy
- Gastric washes
- Sputum
- Blood

OC health CARE AGENCY

Sentinel Laboratory Procedures

- Culture characteristics
- Gram stain
- Oxidase
- Catalase
- β-lactamase
- Satellite phenomenon

F. tularensis: Culture Characteristics

- Slow growing, nutritionally fastidious
- Grows on SBA initially, poorly or not at all on subcultures
- Cysteine-enriched media
- Will NOT grow on MAC or EMB plates

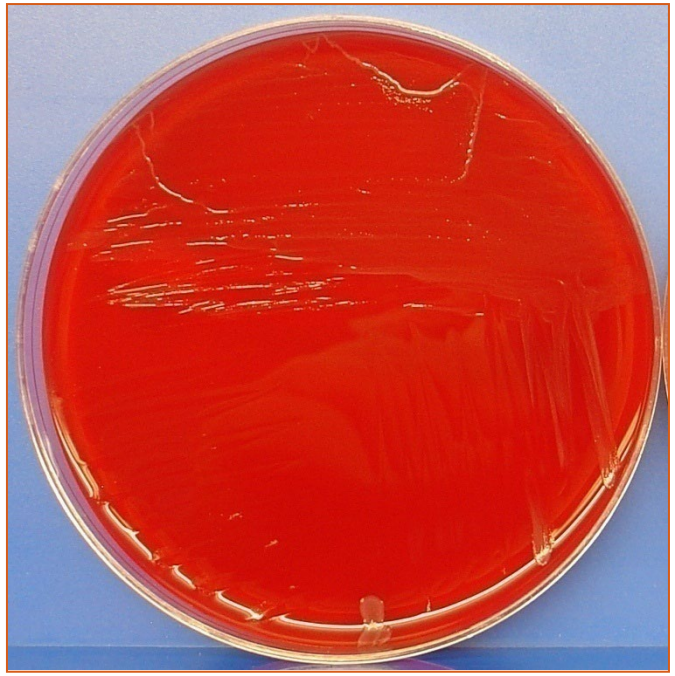
OC health CARE AGENCY

F. tularensis: Culture Characteristics

Colonies on SBA @ 35° C in 5% CO2

Note: Growth on SBA becomes progressively weaker with subsequent subcultures

24 hours

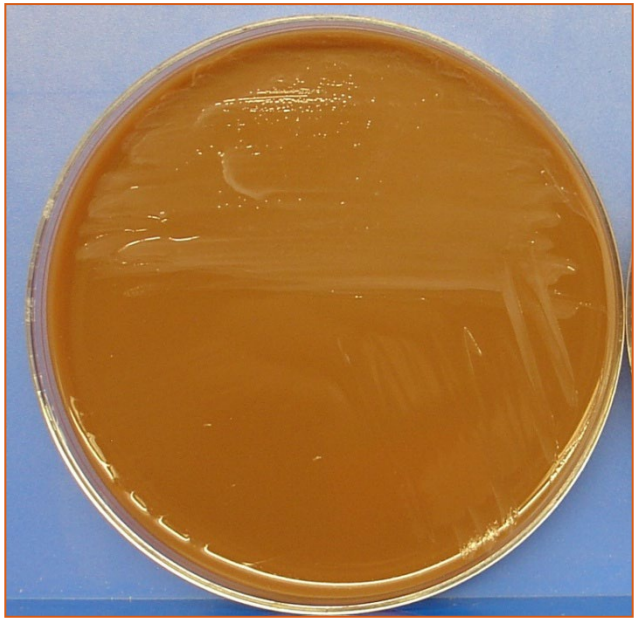


48 hours

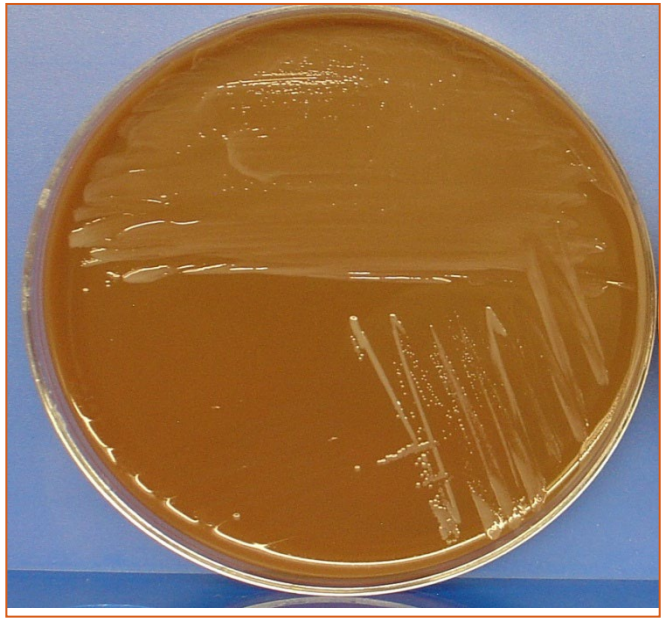
OC health CARE AGENCY

F. tularensis: Culture Characteristics

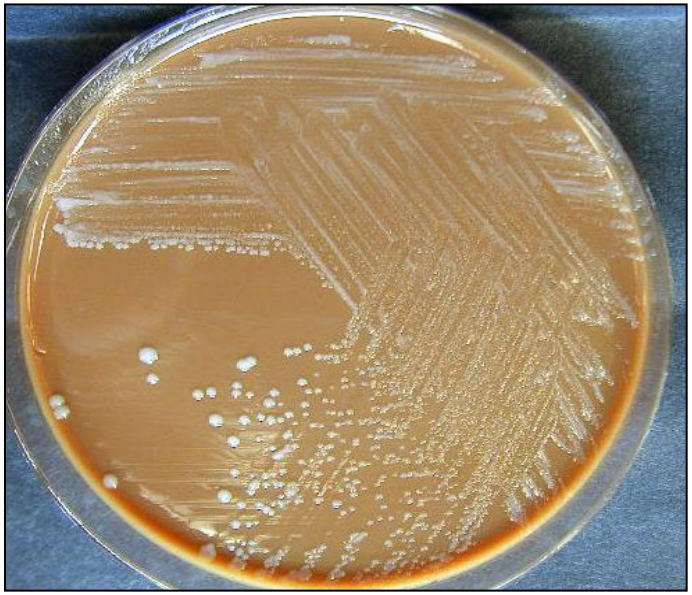
Colonies on CHOC @ 35° C in 5% CO2



After 24 hrs



After 48 hrs

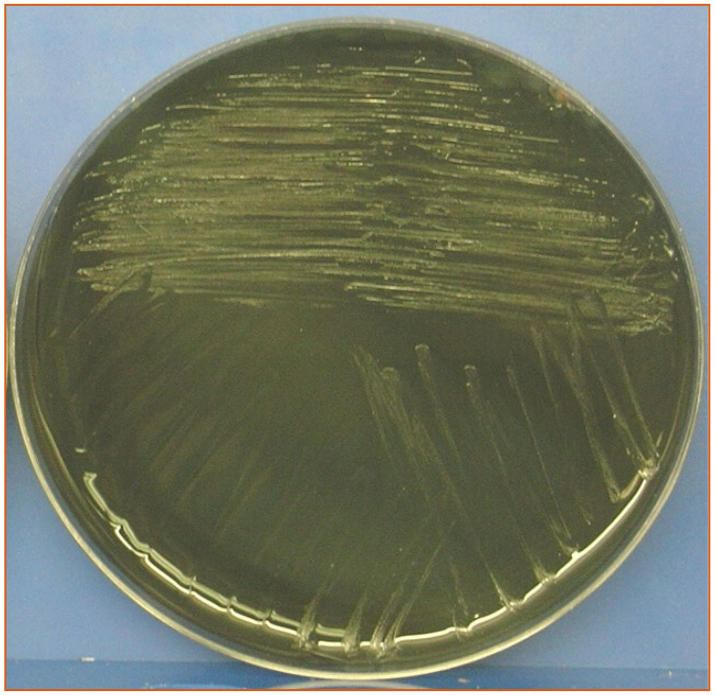


After72 hrs

OC health CARE AGENCY

F. tularensis: Culture Characteristics

Colonies on Cysteine-enriched media @ 35° C in 5% CO2



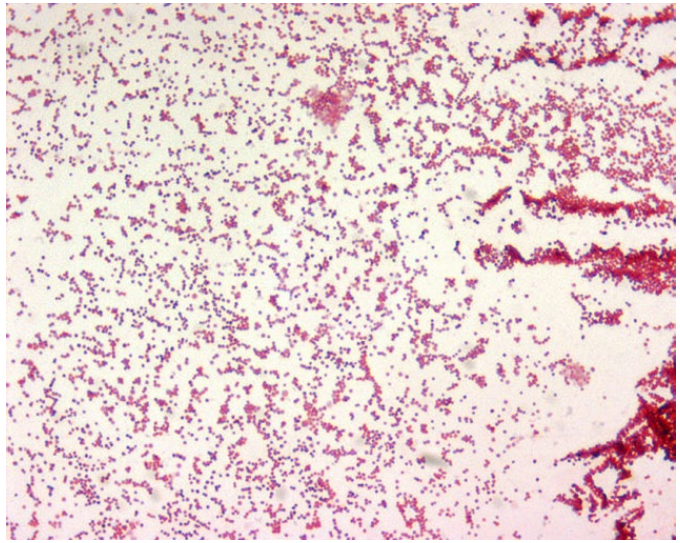
After 24 hrs

After 48 hrs

OC health CARE AGENCY

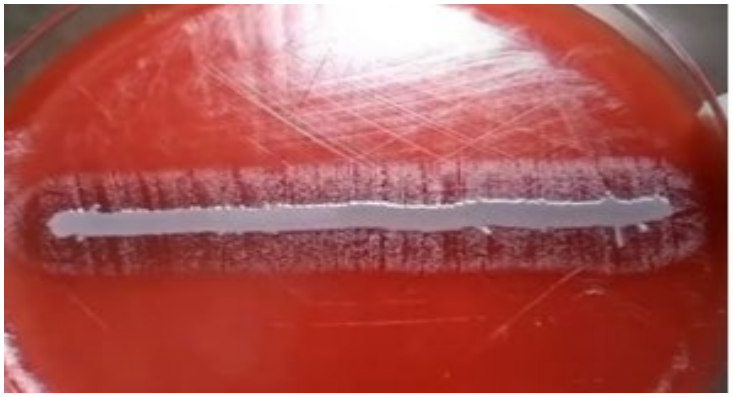
F. tularensis: Gram Stain Morphology

- Very small Gram-negative coccobacillus
- Very tiny, smaller than Brucella
  - "grains of sand"
- Faint staining
- Usually seen as single

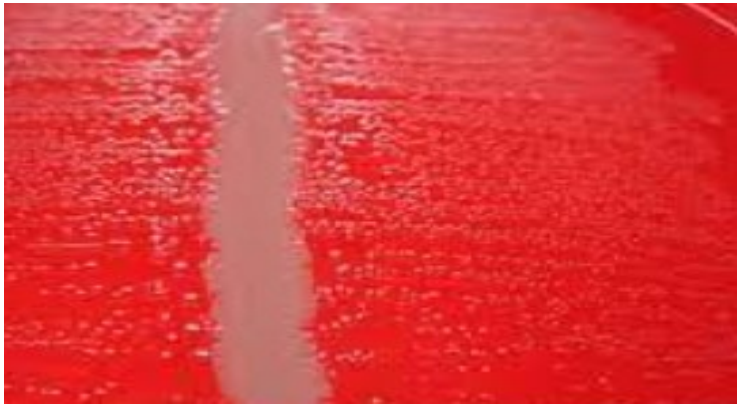


OC health CARE AGENCY

Satellite Phenomenon



Positive



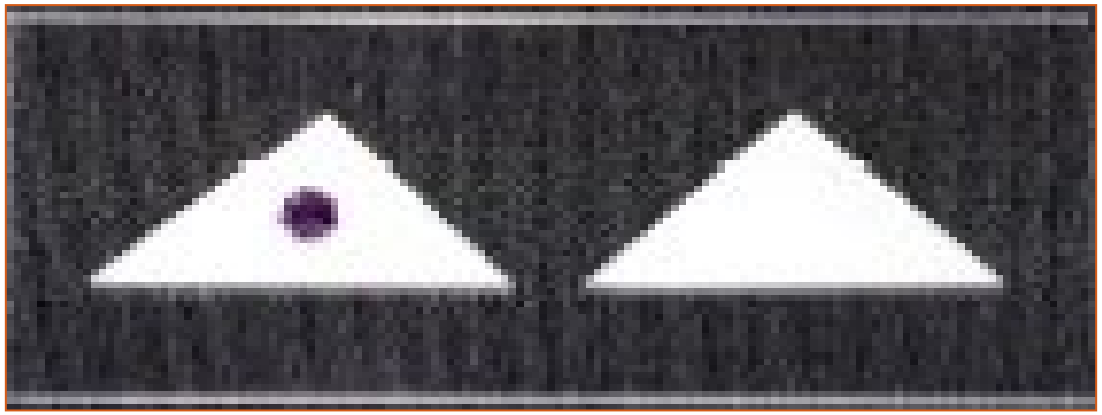
Negative

Look very carefully at the plate

Note: *F. tularensis* does not need hemin or NAD (X or V), however, it may not grow on BAP or it may grow as a very fine film since the medium does not contain cysteine.

OC health CARE AGENCY

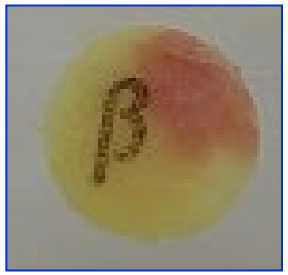
Oxidase Test



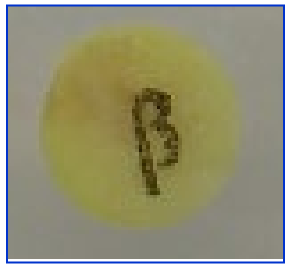
Positive Negative

health CARE AGENCY

β-lactamase Test



Positive



Negative

F. tularensis : Tests Summary

Oxidase – negative

Catalase – negative (or weakly positive)

β-lactamase – positive

Satellite phenomenon - negative

OC health CARE AGENCY

Misidentifications for *F. tularensis.*

MOST LIKELY	LEAST LIKELY
Acinetobacter spp. (oxidase negative)	*Dysgonomonas* spp. (DF-3)
Aggregatibacter spp.	*Brucella* spp. (gram stain, catalase positive)
H. influenzae (satellite or XV positive)	*Psychrobacter phenylpyruvicus*
Bordetella, CDC Grp. IV (urea pos)	*Oligella ureolytica*
Pasteurella spp. (non-sticky, MAC +)	*Haemophilus spp*

Presumptive ID Chart[a]

	***Brucella* spp.**	***Francisella tularensis***	*Psychrobacter phenylpyruvicus*	*Oligella ureolytica*a	*Haemophilus* spp. [b]
Gram stain morphology	tiny ccb, stains faintly	Tiny ccb, stains faint	ccb, rods, retains crystal violet	tiny ccb	Tiny ccb
Catalase	+	- , or weakly +	+	+	v
Oxidase	+	-	+	+	v
Motility	-	-	-	+, delayed	-
BAP distinctions	-	+ (scant growth)	-	-	No growth [b]
MAC-48 h	-	-	-	-	-

a Reactions extracted from ASM Sentinel Protocols for *Brucella* spp. and *F. tularensis*; NA, not applicable; v, variable; ccb, coccobacilli. *O. ureolytica* is primarily a uropathogen.

b Only grows on CHOC (requires X & V); or on BAP associated with Staphylococcus colony (satellite test).

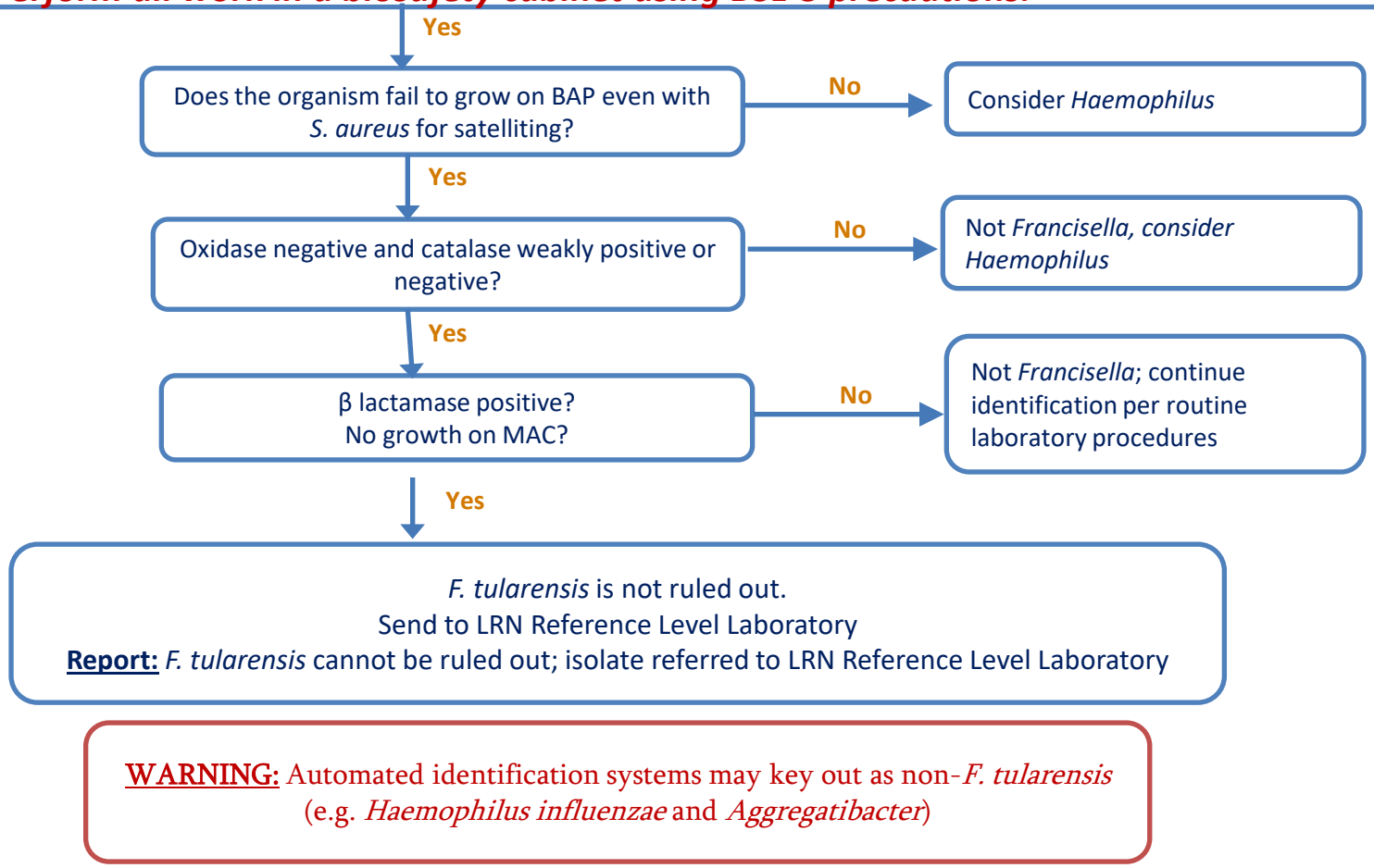
Francisella tularensis Identification Flowchart

Major characteristics of *Francisella tularensis*

Gram stain morphology: Aerobic, 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, scant to no growth on BAP after > 48 h. Produces 1-2 mm gray to grayish-white colonies on CHOC after > 48 h

Perform all work in a biosafety cabinet using BSL-3 precautions.



WARNING: Automated identification systems may key out as non-*F. tularensis* (e.g. *Haemophilus influenzae* and *Aggregatibacter*)

Francisella tularensis:

Recognize-Rule In – Rule Out

- Tiny Gram-negative coccobacilli from specimens
- Slow growth on chocolate agar
- Poor growth or not at all on blood agar at 72 hours
- No growth on MAC
- Oxidase (-), catalase (- or w+), β-lactamase (+), satellite (-)
- Cannot rule out *F. tularensis* using identification flow chart

Francisella tularensis : Refer

CONTACT YOUR LRN
REFERENCE LABORATORY

Yersinia pestis

PLAGUE

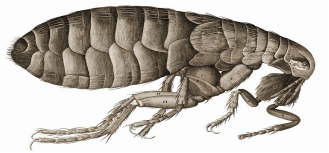
Yersinia pestis: Biosafety Alert

- Dangerous, highly virulent organism
- **Select Agent**
- Laboratory-acquired infections
- Do not manipulate on an open bench

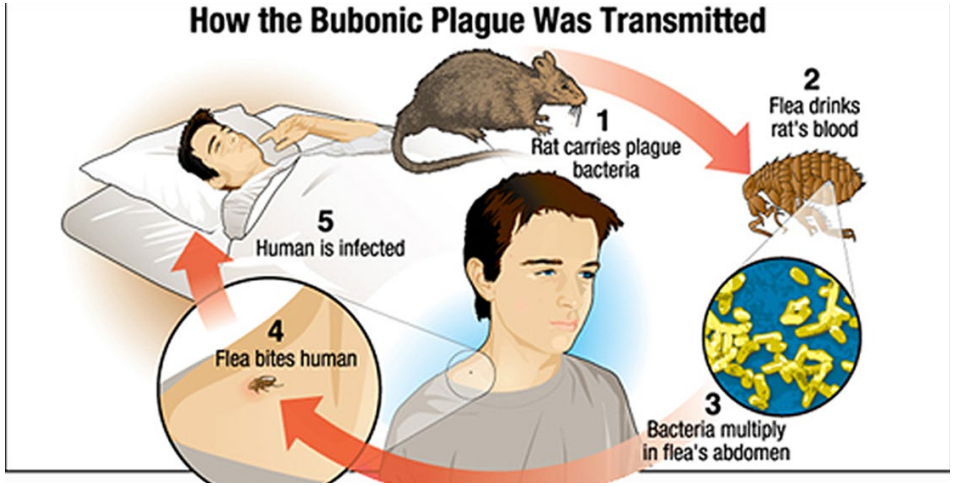
OC health CARE AGENCY

Disease Transmission

- Zoonotic disease
- Transmitted from animals and their infected fleas
- Associated with flea contact
- Human is accidental host

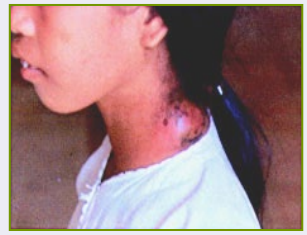
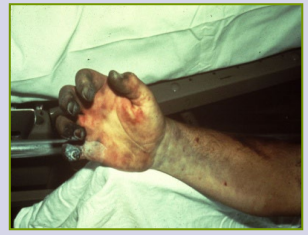
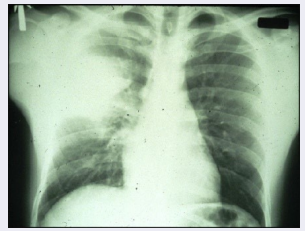


- **Bioterrorism**



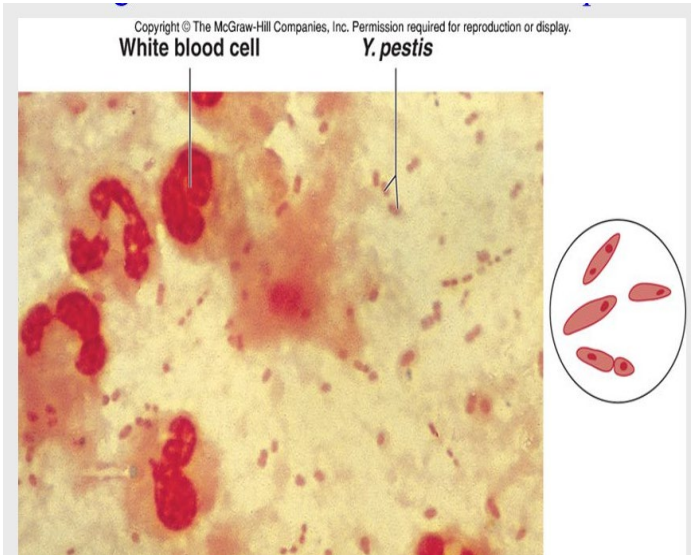
OC health CARE AGENCY

Clinical Presentations of Plague

Bubonic	• Results from bite infective flea or direct skin contact • Lymph nodes become inflamed (Bubo) • Most common clinical presentation 
Septicemic	• Results from bite infective flea or direct skin contact • Similar to bubonic without swollen lymph nodes • Blood-borne organisms 
Pneumonic	• Results from aerosol transmission • Contagious, communicable • Deadliest, least common 

Clinical Specimens

- Lower respiratory tract
- Blood
- Aspirate, tissue or biopsy specimen
- Swabs of tissue (not recommended)



OC health CARE AGENCY

Sentinel Laboratory Procedures

- Culture characteristics
- Gram stain morphology
- Oxidase
- Catalase
- Urease
- Indole
- Motility at 35°C and 25°C (room temperature)

Y. pestis : Culture characteristics

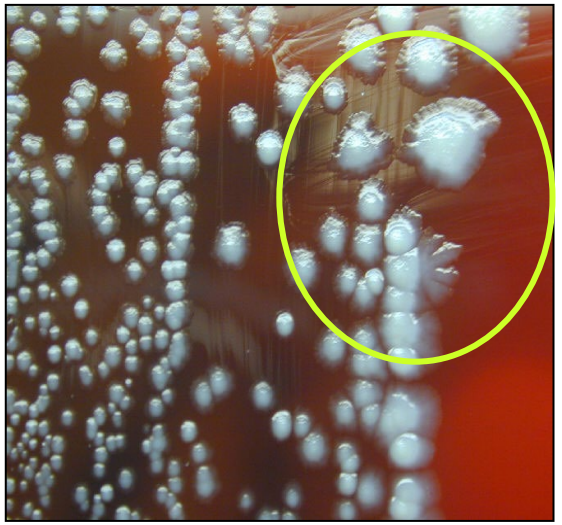
- Grows on SBA, CHOC, and MAC
- Resembles other *Enterobacterales*
  - *EXCEPT* grows more slowly
- Slow growing at 35-37°C and at 25- 28°C
  - Grows faster at RT
- Non-lactose fermenter on MAC/EMB
- Grows on Cefsulodin Irgasan Novobiocin (CIN) agar
- Grows in blood culture systems

OC health CARE AGENCY

Y. pestis : Culture characteristics

After 48 hrs

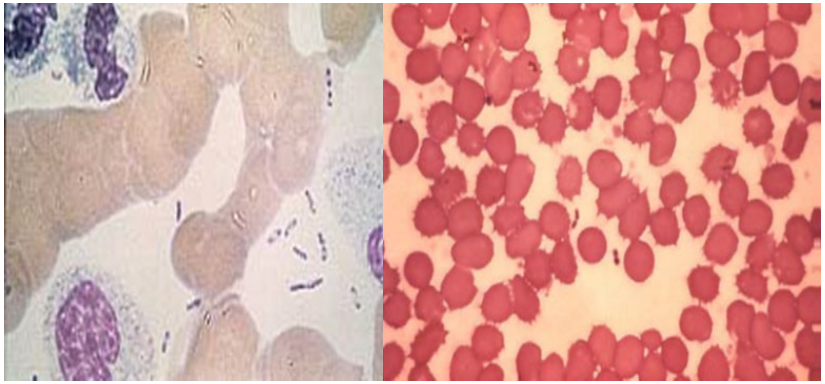
After 72 hrs
Fried egg appearance

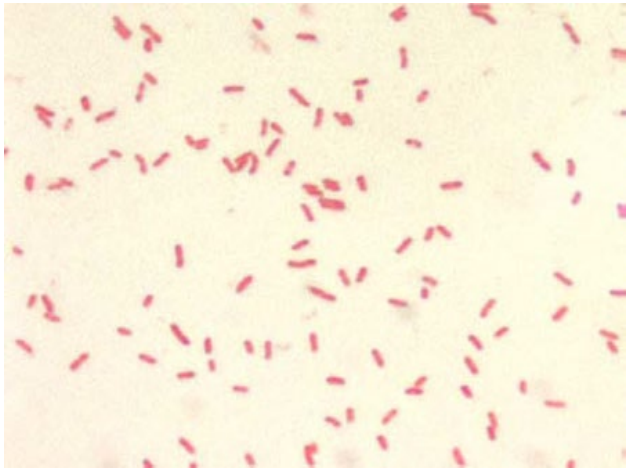


Hammered copper appearance

Y. pestis : Gram stain morphology

- Gram-negative rod
- May not show bipolar characteristics
- May be detected in peripheral blood smears
  - Bipolar staining :Wayson, Wright, Giemsa, or Methylene blue stains





Y. pestis : Screening Tests

Oxidase
Catalase
Urease
Spot indole
Motility 35°C and 25°C

Y. pestis : Tests Summary

Oxidase - negative
Catalase - positive
Urease - negative
Spot indole – negative
Motility* – negative

* *Y. pestis* is the only *Yersinia* that is non-motile at RT

OC health CARE AGENCY

Differentiation of *Yersinia species*

Yersinia species	Oxidase	Catalase	Urea	Indole
Y. pseudotuberculosis	negative	positive	positive	negative
Y. enterocolitica	negative	positive	positive	variable
Y. frederiksenii	negative	positive	positive	positive
Y. kristensenii	negative	positive	positive	variable
*Y. ruckeri**	negative	positive	negative	negative
Y. pestis	**negative**	**positive**	**negative**	**negative**

* Infections in fish

Yersinia pestis Identification Flowchart

Major characteristics of *Yersinia pestis*:

Gram stain morphology: Gram-negative rods, 0.5 x 1-2 mm

Colony morphology: Slow growing, pinpoint colonies 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; growing both at 25-28°C and at 35-37°C.

Specimen is blood, sputum, or lymph node aspirate

Perform all work in a biosafety cabinet using BSL-2 precautions.

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. Send to LRN Reference Level Laboratory.
Report: *Y. pestis* cannot be ruled out; isolate referred to LRN Reference Level Laboratory.

WARNING: Some of the automated identification systems do not identify *Y. pestis* adequately. *Y. pestis* has been falsely identified as *Y. pseudotuberculosis, Shigella*, H_2S negative *Salmonella*, *Acinetobacter,* and *Pseudomonas* species.

Yersinia pestis

Recognize-Rule In – Rule Out

- Gram-negative rods from specimens
- Colonies resemble enteric, but grow much more slowly
- Non-lactose fermenter on MAC
- Catalase (+), oxidase (-), urease (-), indole (-), Motility(-)
- Cannot rule out *Y. pestis* using identification flow chart

OC health CARE AGENCY

Yersinia pestis: Refer

CONTACT YOUR LRN REFERENCE LABORATORY

Burkholderia

B. mallei GLANDERS

B. pseudomallei MELIOIDOSIS

Burkholderia: Biosafety Alert

- Dangerous, highly virulent organism
- Select Agent
- Laboratory-acquired infections
- Do not manipulate on an open bench

****NOT Sniff Plates****

OC health CARE AGENCY

Why *Burkholderia*?

- Both have been "weaponized" in the past
- Highly infectious as aerosols
- Mortality rate for untreated glanders (*B. mallei*) is high
- *B. pseudomallei* has a low infectious dose

- Rare diseases in the USA
  - Limited experience in diagnosis and treatment
  - Specialized testing capability

Working Safely with *Burkholderia*

- Remember—Laboratory Acquired Infections reported
  - Laboratory-Acquired Human Glanders Maryland, May 2000
    - MMWR Vol. 49 (24):532 06/23/2000
  - Laboratory Exposure to Burkholderia pseudomallei Los Angeles, California, 2003
    - MMWR Vol. 53 (42):988 10/29/2004
- For Initial processing of diagnostic specimens use BSC (Class II) and follow BSL-2 practices
- All manipulations of cultures including test procedures require the use of BSL-3 or BSL-2 with BSL-3 precautions

OC health CARE AGENCY

B. pseudomallei in USA

- Multistate outbreak of melioidosis, 2021
  - Georgia, Kansas, Minnesota, and Texas
- Involved four cases, two of the cases were fatal.
- Linked to an imported aromatherapy spray from India.

- Burkholderia pseudomallei Laboratory Exposure, Arizona, 2023
  - Emerging Infectious Diseases journal, Vol 29, (5): 1061 5/2023

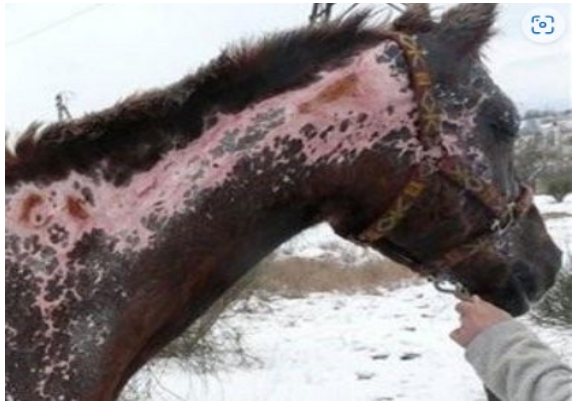
B. mallei: Disease Transmission

- Glanders disease-RARE
- Zoonotic disease-Infects equines and other mammals
- Direct contact through abraded skin or aerosol inhalation
- No naturally occurring cases since the 1940's
- Rarely person to person
- Risk factors-Occupational
- Ricks factor in the US - Laboratorians

OC health CARE AGENCY

Glanders

- Animals: Causes nodules and ulcerations in the respiratory tract and lungs. A skin form, known as ‘farcy’, also occurs.



- Humans: nasal, localized with nodules and abscesses, pulmonary, and septicemia with disseminated or chronic infection, respectively.

Glanders was first described as a disease of horses in 450 BC by Hippocrates

Glanders - WOAH Middle-East

B. pseudomallei : Disease Transmission

- Melioidosis: Bacterial infection in people and animals.
- Found in water and soil in tropical regions
- Contracted through aerosol transmission, direct contact or ingestion of contaminated water
- High risk for rice farmers
- Cases may increase after hurricanes, heavy rain
- No evidence of person to person spread
- Incubation 2-5 days, months, years (can reoccur years later)
- Risk factors where endemic: diabetes, alcoholism, renal impairment, penetrating wounds
- High Risk group in the US:
  - Laboratorians

OC health CARE AGENCY

Melioidosis

- Both humans and animals can get melioidosis, but people can't get it from animals. Melioidosis is also sometimes called Whitmore's disease.

- Fever and myalgias
- Localized infection: ulcer, nodule, or skin abscess
- Lung infection: cough, chest pain, high fever, headache, loss of appetite

Melioidosis: Causes, Symptoms, Transmission & Treatment

Clinical Specimens

- Bone marrow or whole blood
- Sputum or bronchoscopically obtained specimens
- Tissue Specimens
  - Biopsies, abscess aspirates, and wound swab
- Urine

OC health CARE AGENCY

Sentinel Laboratory Procedures

- Culture characteristics
- Gram stain
- Hemolysis
- Oxidase
- Catalase
- Indole (Spot)
- Polymyxin B or Colistin disk
- Amoxicillin-clavulanate acid and Penicillin discs
- Motility
- Growth at 42°C

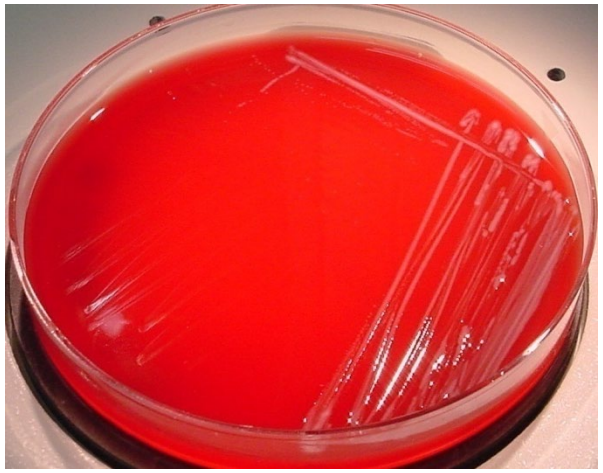
OC health CARE AGENCY

B. mallei: Culture Characteristics

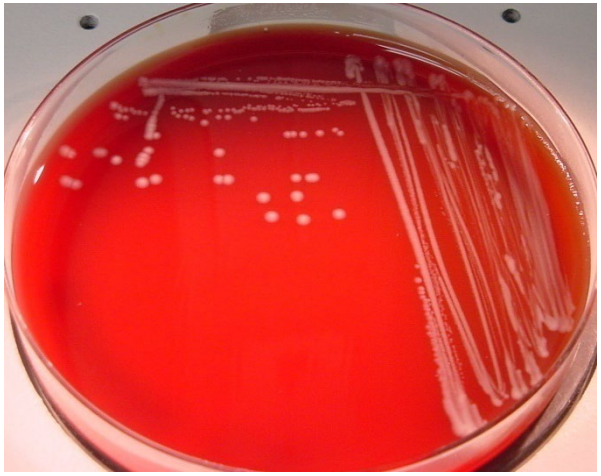
- Grows slow on SBA and CHOC @ 35°C
  - Smooth, gray, translucent colonies
  - non-hemolytic
  - Non-pigmented
- Poor or No growth on MAC @ 35°C
- No growth @ 42°C on SBA by 48 hours

B. mallei: Culture Characteristics

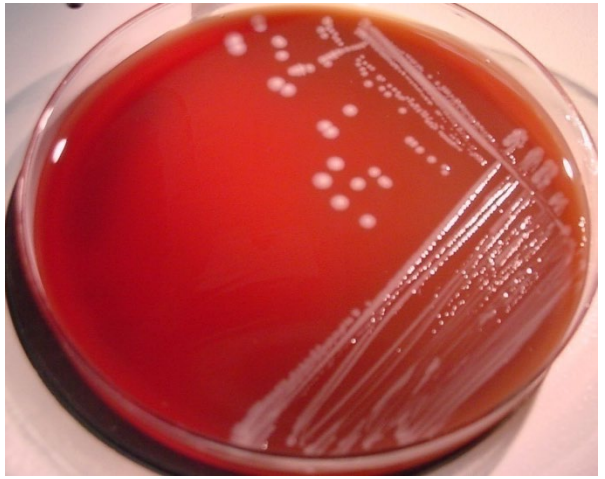
Colonies on SBA @ 35˚ C in 5% CO2



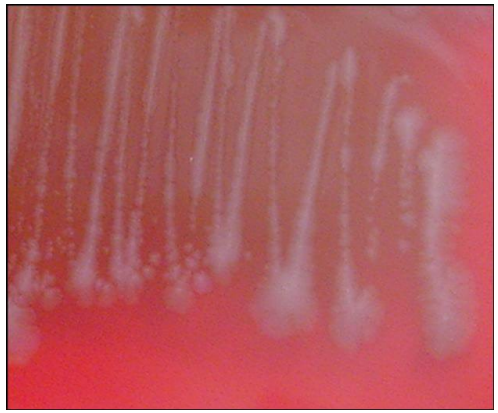
24 hrs



48 hrs



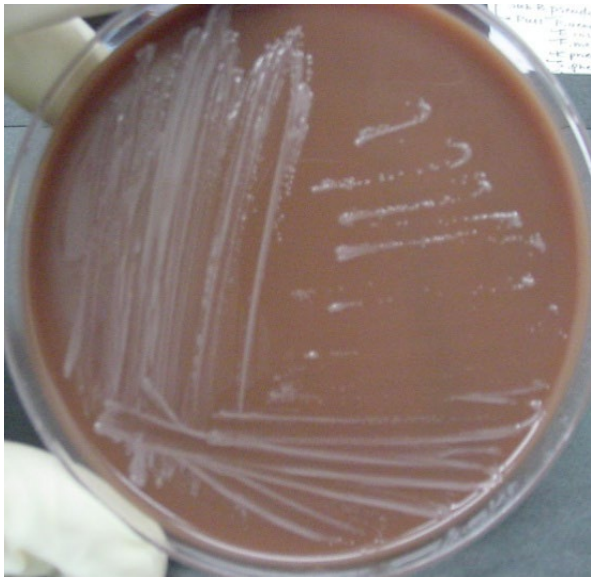
72 hrs



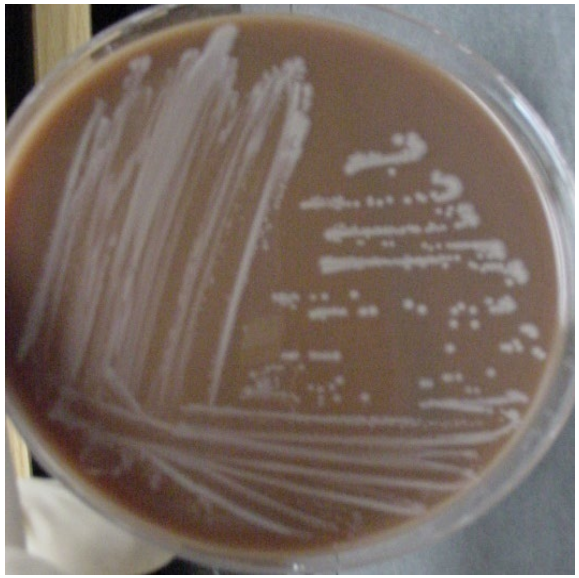
After 72 hrs

B. mallei: Culture Characteristics

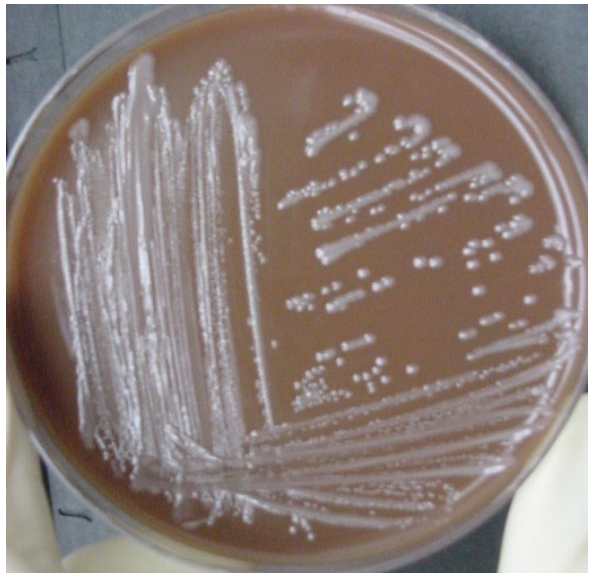
Colonies on CHOC @ 35° C in 5% CO2



24 hrs



48 hrs



72 hrs

OC health CARE AGENCY

B. mallei: Culture Characteristics

Colonies on MacConkey agar @ 35° C in O2

24 hrs

48 hrs

72 hrs

B. pseudomallei: Culture Characteristics

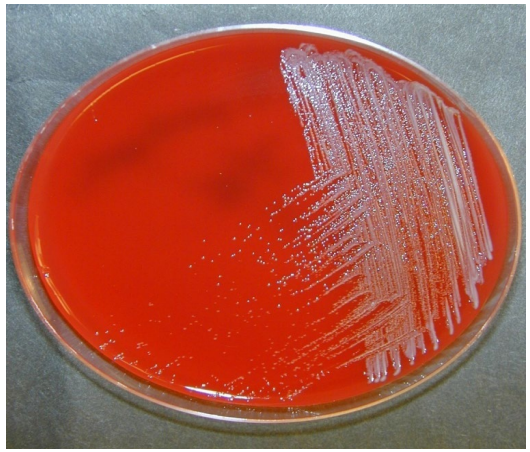
- Grows on SBA , CHOC, and MAC, @ 35°C
- Mature colonies at 48 to 72 hours
- Non-hemolytic
- Colonies become rough and wrinkled with time
- Growth at 42°C

OC health CARE AGENCY

B. pseudomallei: Culture Characteristics

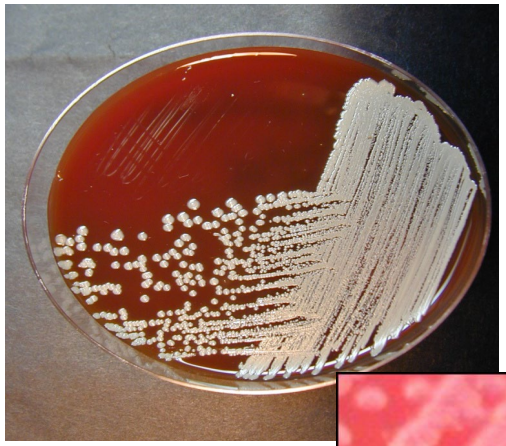
Colonies on SBA @ 35˚ C in 5% CO2

24 hrs



48 hrs

72 hrs



After 72 hrs

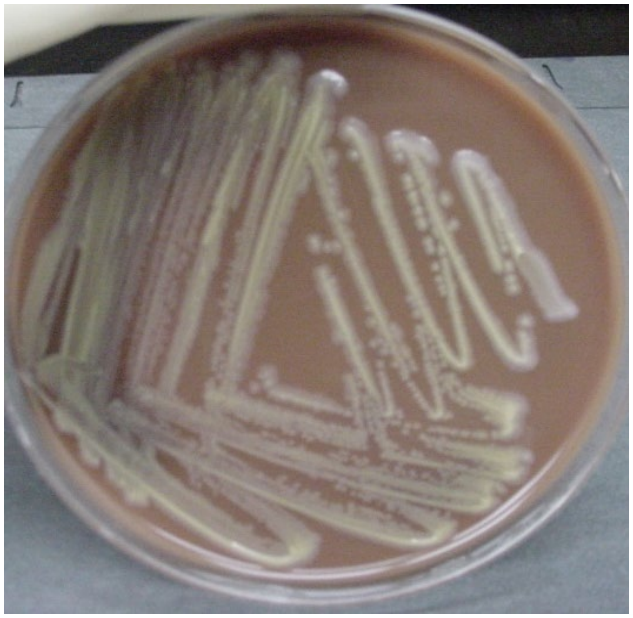
OC health CARE AGENCY

B. pseudomallei: Culture Characteristics

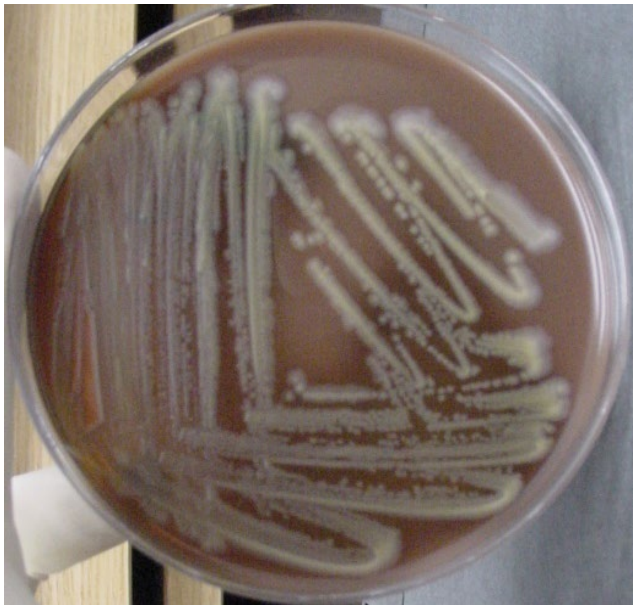
Colonies on CHOC @ 35° C in 5% CO2

24 hrs

48 hrs



72 hrs



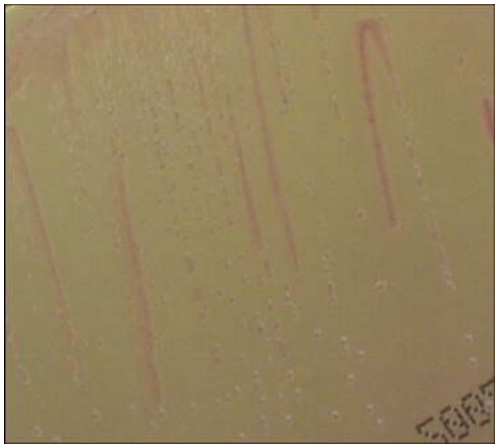
Burkholderia pseudomallei
mature growth at 24 hrs

OC health CARE AGENCY

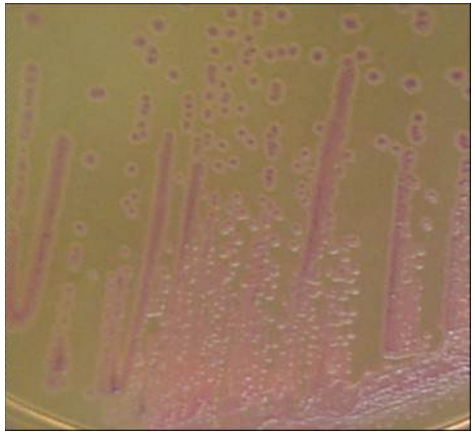
B. pseudomallei: Culture Characteristics

Colonies on MacConkey agar @ 35° C in O2

24 hrs



48 hrs



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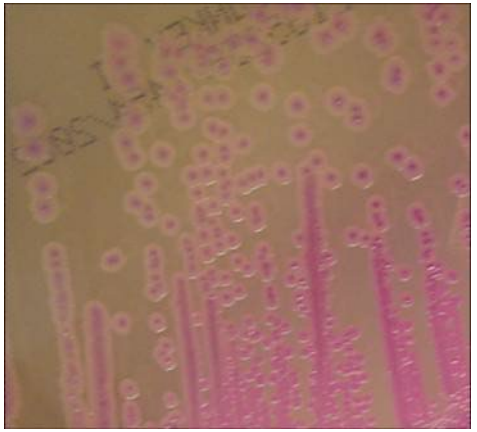


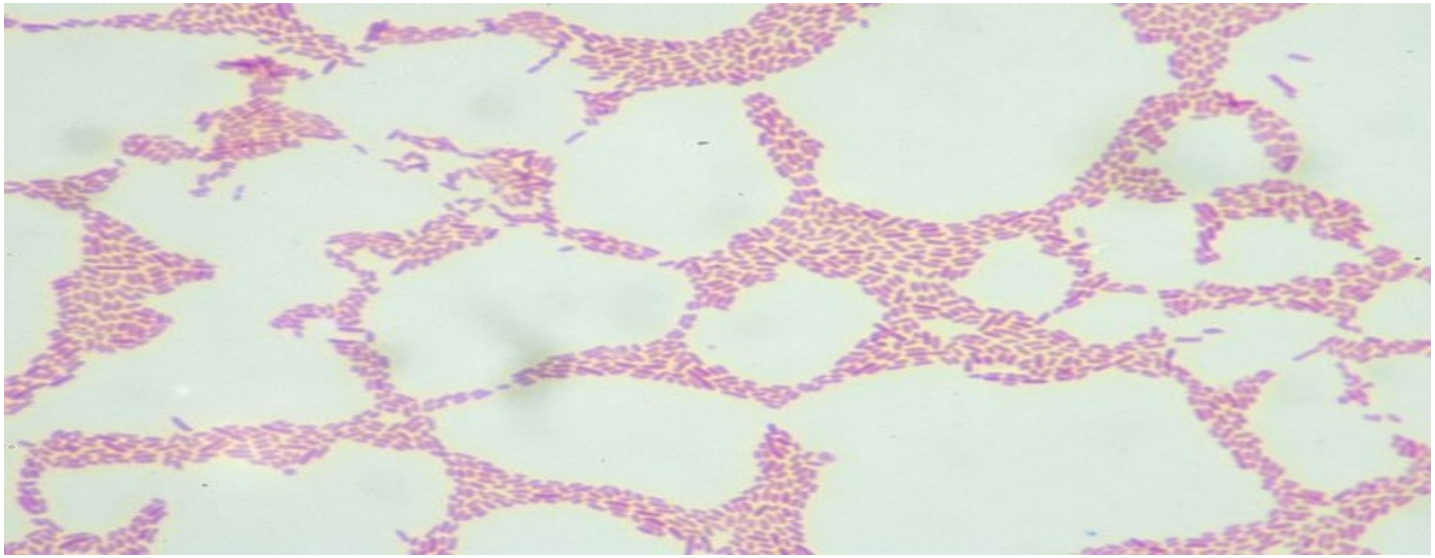
Colorless Colonies with Slight Pink Centers

OC health CARE AGENCY

B. mallei: Gram stain morphology

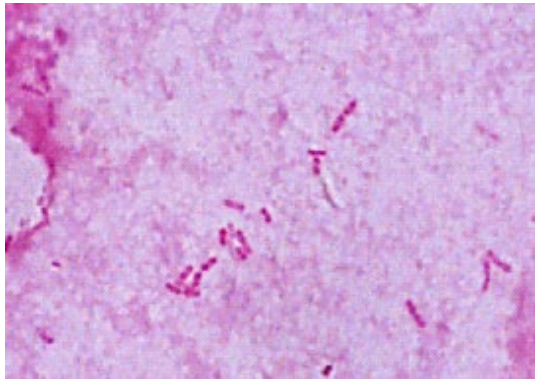
- Gram-negative coccobacilli- rods
- Small, straight or slightly curved with rounded end

- Cells are arranged in pairs or parallel bundles



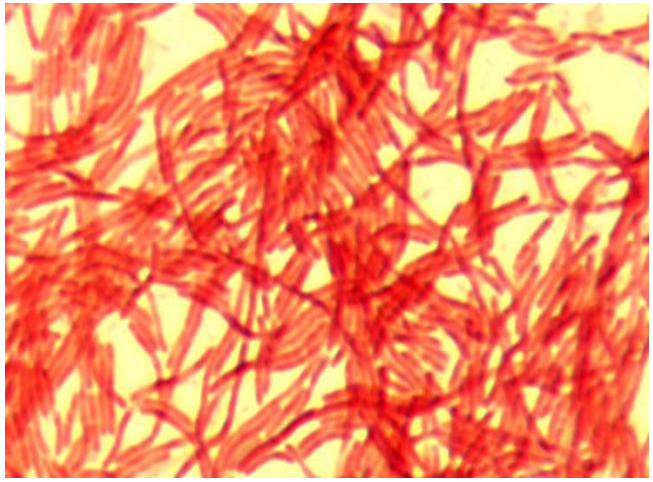
B. pseudomallei: Gram stain

- Gram-negative rods
  - Straight or slightly curved
  - Smooth form and Rough form
- Demonstrate Bipolar morphology

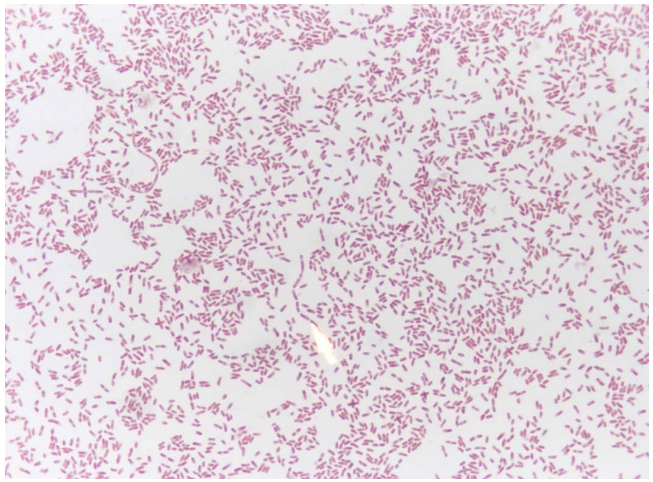


Bipolar morphology

Smooth form



Rough form

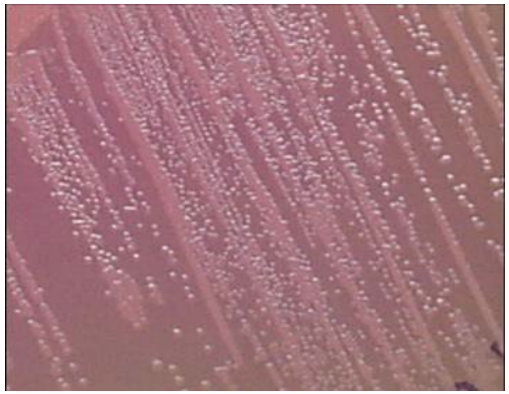


Disk Diffusion Assay Screening Tests

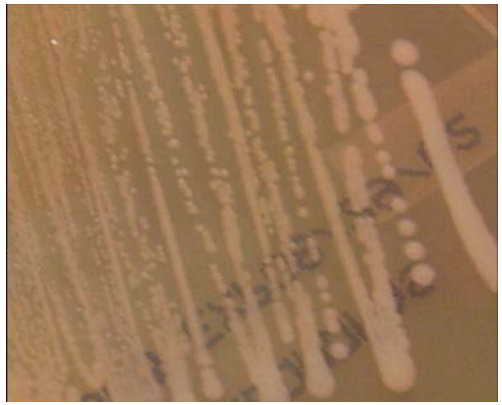
- **KB method disk diffusion assay using Mueller-Hinton agar**
  - **Colistin 10ug**
  - **Polymyxin B 300U,**
  - **Amoxicillin-clavulanate acid 20/10 ug**
  - **penicillin 10 U**
- **Read the test as zone (S), no zone (R)**
  - Optional : colistin test on PC agar, BC agar, or Modified Thayer-Martin agar.
    - Read for growth/no growth because those media contain colistin
    - Negative result →Confirm with disc assay

Polymyxin B using PC agar

Burkholderia pseudomallei resistance to polymyxin B using PC agar or BCSA @ 35° C in O2



24 hrs



48 hrs

72 hrs

OC health CARE AGENCY

Motility: An optional test

Burkholderia pseudomallei

Motile

Positive Negative

Burkholderia mallei

Non-motile

wet mount

health CARE AGENCY

Arginine dihydrolase: An optional Test

B. mallei / Burkholderia pseudomallei

Arginine Positive

Base Positive Negative

OC health CARE AGENCY

B. mallei: Tests Summary

- Oxidase - variable (mostly negative)
- Catalase - positive
- Spot indole - negative
- Colistin- resistant or Polymyxin B - resistant
- Amoxicillin-clavulanate acid - susceptible
- Penicillin- resistant
- Motility - negative
- 42°C - no growth

OC health CARE AGENCY

B. pseudomallei: Tests Summary

- Oxidase - positive
- Catalase - positive
- Spot indole - negative
- Colistin- resistant or Polymyxin B - resistant
- Amoxicillin-clavulanate acid – susceptible
- Penicillin- resistant
  - resistance cannot rule out the identification
- Motility - positive
- 42°C - growth
- Arginine - positive
- Glucose oxidizer

OC health CARE AGENCY

Burkholderia: Comparison Chart

	B. pseudomallei	B. mallei
Gram stain	Small, straight or slightly curved Gram-negative rod	Small, straight or slightly curved Gram-negative coccobacillus
SBA Colony morphology	Smooth, creamy -white after 24hrs incubation; may become dry and wrinkled after 48 hrs.	Smooth, gray, translucent only after 48 hours incubation
Oxidase	Positive	Variable
Indole	Negative	Negative
Growth at 42ºC in 48 hrs	Growth	No Growth
Motility	Positive	Negative
Musty/earthy odor	Yes	No
Catalase	Positive	Positive

Organisms Resemble B. mallei and B. pseudomallei

Burkholderia cepacia
Burkholderia gladioli
Burkholderia thailandensis
Pseudomonas mendocina
Pseudomonas stutzeri
Ralstonia picketii
Stenotrophomonas maltophilia
***Acinetobacter* spp.**

Identification Flowchart

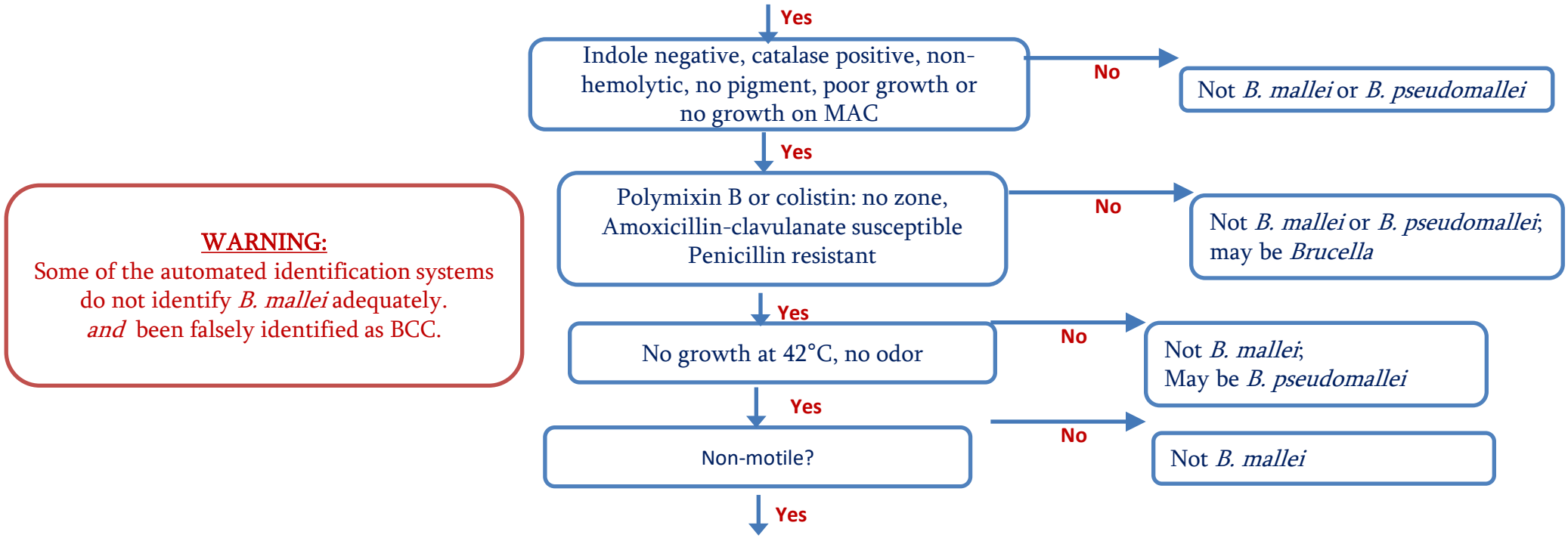
Major characteristics of *Burkholderia mallei*

Gram stain morphology: Gram-negative coccobacilli or small rods

Colony morphology: Poor growth at 24 hr; better growth of tray, translucent colonies without pigment or hemolysis at 48 hours on BAP; poor or no growth on MAC in 48 h; no distinctive odor

Reactions: Oxidase-variable; indole negative; catalase positive

Perform all work in a biosafety cabinet using BSL-3 precautions.



WARNING:
Some of the automated identification systems do not identify *B. mallei* adequately. *and* been falsely identified as BCC.

B. mallei not ruled out.
Send to Reference Level Laboratory
Report: Possible *Burkholderia mallei* submitted to LRN Reference Level Laboratory.
Additional screening test: *B. mallei* is Arginine positive, unlike many other *Burkholderia* spp. (Test can be kit identification systems)

Identification Flowchart

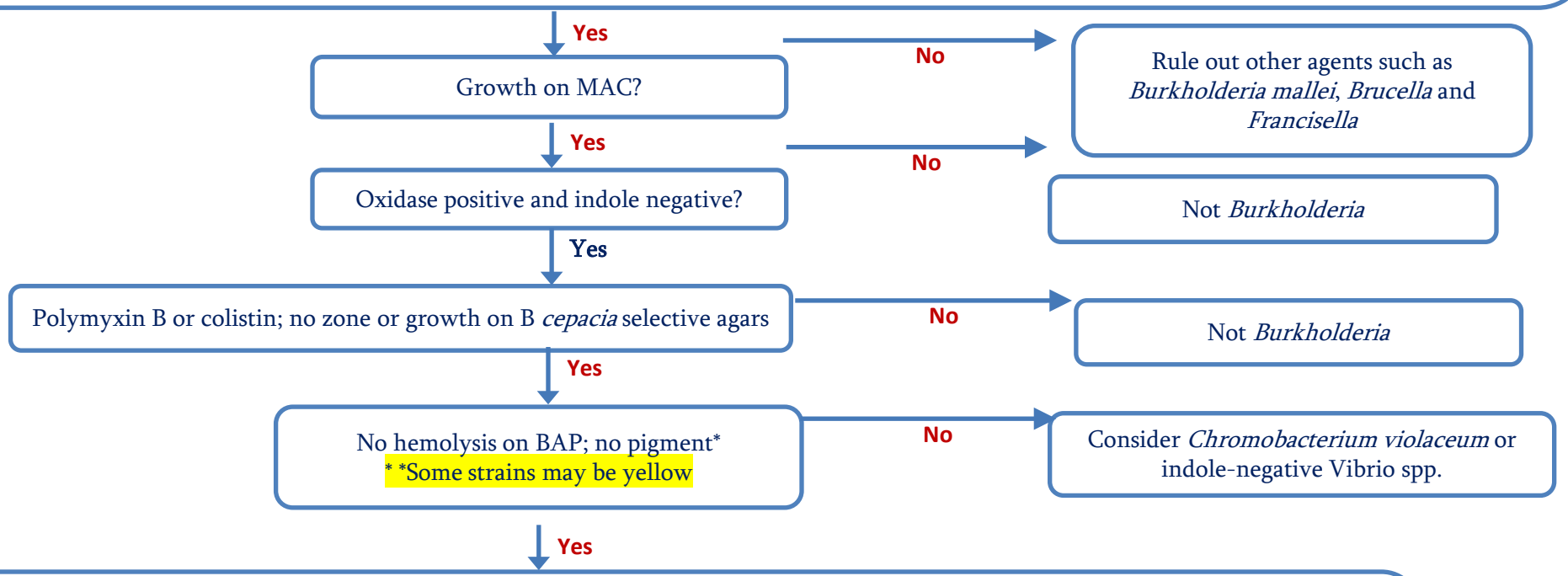
Major characteristics of *Burkholderia pseudomallei*

Gram stain morphology: Gram-negative rod, straight or slightly curved, may demonstrate bi-polar morphology at 24 h and peripheral staining, like endospores, when cultures are older

Colony morphology: Poor growth at 24 hr; good growth of white colonies at 48 hours on BAP; may develop wrinkled colonies in time, no violet pigment, nonhemolytic. Often demonstrates strong characteristic musty, earthy odor; growth on MAC in 48 h.

Reactions: Oxidase positive; indole negative

Perform all work in a biosafety cabinet using BSL-3 precautions.



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.
Send to LRN Reference Level Laboratory

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.

Burkholderia mallei

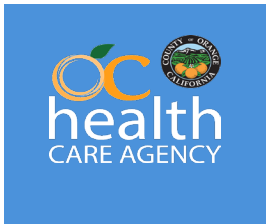
Recognize-Rule In – Rule Out

- Gram-negative coccobacilli
- Very slow growth on SBA, CHOC and little if any growth MAC
- Nonpigmented, non-hemolytic, and no growth at 42°C
- Oxidase variable
- Catalase positive
- Motility negative
- Resistant to polymyxin B or colistin
- Amoxicillin-clavulanate acid susceptible, Penicillin resistant
- Cannot rule out *Burkholderia mallei* using the protocol flow chart

OC health CARE AGENCY

Burkholderia mallei: Refer

CONTACT YOUR LRN REFERENCE LABORATORY



Burkholderia pseudomallei

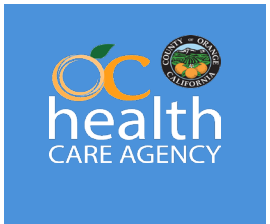
Recognize-Rule In – Rule Out

- Gram-negative rods
- may demonstrate bipolar staining
- Slow to moderate growth on SBA and MAC
- Oxidase positive
- Catalase positive
- Indole negative
- Resistant to polymyxin B or colistin or growing on Selective agar
- Cannot rule out *Burkholderia pseudomallei* using the protocol flow chart

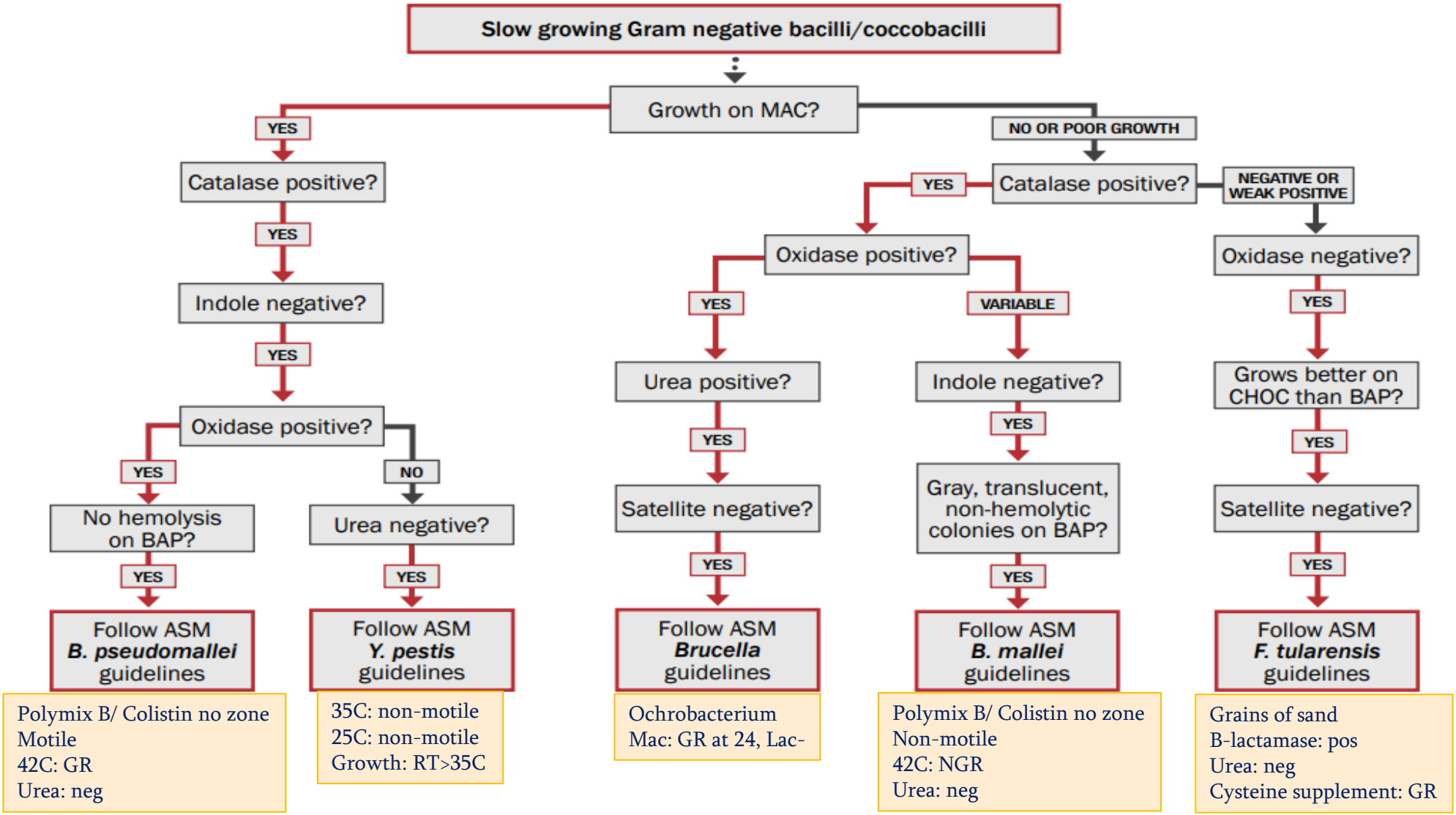
OC health CARE AGENCY

Burkholderia pseudomallei: Refer

CONTACT YOUR LRN REFERENCE LABORATORY



Gram Negative Bacilli/Coccobacilli Rule-Out Algorithm



Polymix B/ Colistin no zone
Motile
42C: GR
Urea: neg

35C: non-motile
25C: non-motile
Growth: RT>35C

Ochrobacterium
Mac: GR at 24, Lac-

Polymix B/ Colistin no zone
Non-motile
42C: NGR
Urea: neg

Grains of sand
B-lactamase: pos
Urea: neg
Cysteine supplement: GR

Biothreat Agents Identification Bench Cards for Sentinel Laboratories

Commercial Identification Systems

Commercial systems are not suitable for:

- Slow growing
- Nutritionally fastidious
- Limited number of isolates in system database
- Highly infectious in an aerosol

OC health CARE AGENCY

SA in Commercial Systems ID Database

Considering the danger presented by aerosols generated during test preparations,
THEY SHOULD NOT BE USED

	Biolog	MicroScan	MIDI	Vitek		API
				I	II	
B. anthracis	Yes	No	Yes	No	Yes	Yes
Brucella spp.	Yes	Yes	Yes	No	Yes	No
F. tularensis	Yes	No	Yes	No	Yes	No
Y. pestis	Yes	Yes	Yes	Yes	Yes	Yes
B. pseudomallei	Yes	Yes	Yes	Yes	Yes	Yes

Yes: included in the system

No: not included in the system

Select Agent Misidentification

Select Agent Misidentification by Commercial Systems

Y. Pestis	*F. tularensis*	*Brucella spp.*	*Burkholderia pseudomallei*
Y. pseudotuberculosis *Shigella boydii* *Pantoea agglomerans* *Acinetobacter lwoffii*	*P. multocida*	*Moraxella spp.* *Micrococcus spp.* *Haemophilus spp.* *Ochrobactrum anthropi* *Acinetobacter spp.* *Chryseobacterium indologenes*	*B. cepacia* *Chromobacterium violaceum* *Pseudomonas spp.* *Stenotrophomonas maltophilia* *Other non-fermenters*

Valuable Insight

Following Guidance From Your LRN Reference Laboratory

Do

- Incorporate protocols into your SOPs
- Advise clinical staff on appropriate specimen selection, collection, storage
- Establish a chain of custody, if necessary
- Assist in packaging and preparing to transport specimens
- Know whom to call
- How and where to refer suspect isolates

Don't

- Do not attempt to recover from or detect these agents in clinical specimens
- Do not accept or process environmental samples

Role of the Sentinel Laboratory

Be Vigilant

- Have a response plan in place and practice it

- Be able to rule out threat agents

or

- Contact your LRN Reference Laboratory

OC health CARE AGENCY

Thank you

Special thanks to:

- our partners at CDPH- High Risk Pathogens Section
- The CDC in Atlanta, GA
- The CDC Fort Collins, CO
- All of you who attended this webinar today.

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