



Bench Guide for Hazardous Pathogens

Nebraska Public Health Laboratory 24/7 Emergency Pager
402-888-5588

Warnings



- All specimens received in a biosafety level (BSL) 2 or higher facility are to be processed in a biological safety cabinet (Class II Type A BSC, at a minimum) to adhere to safe BSL2 practices. If a BSC is unavailable in the laboratory, employ an effective splash shield and continue to follow universal precautions. Additional precautions may be necessary if warranted by site-specific risk assessments.
- “Sniffing” of plates is dangerous and should NOT be done. A strong distinctive odor will be apparent without sniffing.
- Wet prep for motility and slide catalase are discouraged, as potential exposure to dangerous pathogens is great. Tube motility and tube catalase are strongly recommended.
- Select Agents are infectious substances that have been determined to have the potential to pose a severe threat to humans. The Category A classification contains both select agents and non-select agents. Shiga-toxin positive E.coli is an example of a Category A non-select agent organism. Additional precautions such as respiratory protection should be added when suspecting a select agent.
- References: Biosafety in Microbiology and Biomedical Laboratories; http://www.cdc.gov/biosafety/publications/bmbl5/BMbl5_introduction.pdf. Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories, MMWR/January 6, 2012/Vol.61.

Warnings

Prevent Laboratory Acquired Infections (LAI)

Safety requirements when working with culture plates at an open bench:

- Wear appropriate PPE
- Wash hands frequently
- Keep hands away from nose, mouth and eyes
- Cover cuts and hangnails with adhesive bandages
- Do NOT use personal items in lab (cell phones, lip balm, etc.)
- Immediately seal any plate with mold or fungus
- Never sniff plates!
- Use aerosol-tight rotors for all centrifugations – open rotor with in BSC

- **Watch for trigger points!**
- Work up all Gram negative diplococci and coccobacilli seen in original Gram stain of sterile sites in biosafety cabinet (despite likelihood of *Haemophilus influenzae*)
- Work up all slow growing organisms in biosafety cabinet, especially if no growth or poor growth on MAC. DO NOT USE these organisms on AUTOMATED systems because of lack of accuracy and danger of aerosols
- Cultures growing suspicious organisms should be manipulated only in the biosafety cabinet – use class II biosafety cabinet with BSL-3 precautions including respiratory protection

**Laboratory Acquired
Infections**

Trigger Points

A trigger point is a recognized combination of diagnostic findings used to determine when to heighten precautions for handling a specimen or culture.

Trigger points are indicators of possible high-risk pathogens that may require manipulation in a biosafety cabinet (BSC):

- Patient history of travel, hunting, farming, immigration
- Growth from sterile sites - Blood, CSF, Body Fluid
- Gram stain of clinical specimen:
 - ➔ Sterile site with Gram negative diplococci or coccobacilli
 - ➔ Large Gram positive rods
 - ➔ Many WBC, no organisms seen
- Poor growth after 48-72 hours incubation
- Growth only on chocolate or better growth on chocolate compared to SBA
- Growth of Gram negative rod (GNR) or coccobacilli (GNCB) on SBA/Choc with no or poor growth on MacConkey
- Any culture with mold
- Rapid growth of flat, nonhemolytic, irregular colonies with comma projections and ground-glass appearance; Gram stain shows large Gram positive rods, may decolorize.
- GNR with Bipolar staining (safety pin shape) in Gram stain
- GNR with “Fried Egg” or “Hammered Copper” appearance in older cultures

HAZARDOUS PATHOGENS WORKUP TABLE

Select Agents

Gram Stain Morphology	Growth at 35° C			Rule out
	Sheep Blood Agar	Chocolate	MAC	
Gram Positive Rods				
<ul style="list-style-type: none"> Large Gram positive rods (1-1.5 µm x 3-5µm), may be in chains Capsule may be seen as a clear area around the rod in direct clinical specimen Gram stain of culture may show subterminal or centralized spores with no significant swelling of the cell May be easily decolorized 	<ul style="list-style-type: none"> Good growth at 15-24 hr, growth may be observed as early as 8 hr 2-8 mm flat or slightly convex colonies with irregular edges Ground glass appearance May have comma shape projections (Medusa head) No or very little hemolysis Tenacious – stands up like beaten egg white 	See SBA	No Growth	<i>Bacillus anthracis</i>
Gram Negative Rods				
<ul style="list-style-type: none"> Gram negative slender rod (0.8 x 2-5 µm) small, straight or slightly curved May demonstrate bipolar (safety pin) morphology. 	<ul style="list-style-type: none"> Poor growth at 24 hr Smooth, white, nonpigmented colonies at 48 hr May become dry, wrinkled colonies Growth at 42°C 	See SBA	<ul style="list-style-type: none"> Growth at 24-48 hr May appear wrinkled at 24-72 hr Varied Morphology 	<i>Burkholderia pseudomallei</i>
<ul style="list-style-type: none"> Gram negative plump rod (0.5-0.8 x 1-3 µm) Single, short chains in broth Bipolar stain (safety pin) may occasionally be seen 	<ul style="list-style-type: none"> Pinpoint growth at 24 hr Gray-white to opaque, nonhemolytic colonies at 48 hr May have a “fried egg” appearance after 48-72 hr Growth at room temperature (25°C) 	See SBA	Small non-lactose fermenting colonies at 48 hr	<i>Yersinia pestis</i>

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

HAZARDOUS PATHOGENS WORKUP TABLE

Select Agents

Gram Stain Morphology	Growth			Rule out
	Sheep Blood Agar	Chocolate	MAC	
Gram Negative Coccobacilli or Small Gram Negative Rods				
<ul style="list-style-type: none"> • Tiny, pleomorphic, poorly stained Gram negative coccobacillus (0.2x0.2-0.7 μm) • Mostly single cells 	<ul style="list-style-type: none"> • May initially grow on SBA if cultured from nutrient rich specimen (blood culture) • Usually no growth upon subculture - requires cysteine supplementation 	<ul style="list-style-type: none"> • Slow growth at ≤ 48 hr • Gray-white, opaque, shiny or wet colonies 	No growth	<i>Francisella tularensis</i>
<ul style="list-style-type: none"> • Small Gram negative coccobacillus (0.5x0.6-1.5 μm), faintly staining • May be slow to decolorize, can appear as Gram positive cocci 	<ul style="list-style-type: none"> • Slow/no growth at 24 hr • Smooth, convex, nonpigmented, nonhemolytic at 48 hr 	See SBA	• No growth	<i>Brucella</i>
<ul style="list-style-type: none"> • Straight or slightly curved small Gram negative rods or coccobacilli (0.5 x 1.5-3 μm) • May have rounded ends or wavy sides • May be in parallel bundles 	<ul style="list-style-type: none"> • Slow/no growth at 24 hr • Smooth/gray, translucent colonies at 48 hr • No growth at 42°C in 48 hr 	See SBA	• Poor growth or no growth on MAC	<i>Burkholderia mallei</i>

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

HAZARDOUS PATHOGENS WORKUP TABLE

Non-Select Agents

Gram Stain Morphology	Growth			Rule out
	Sheep Blood Agar	Chocolate	MAC	
<i>Gram Negative Diplococci</i>				
<ul style="list-style-type: none"> Gram negative cocci in singles or pairs (1-2 μm) Possibly intracellular in PMN's 	<ul style="list-style-type: none"> Smooth, entire edges about 1mm diameter at 18 hr Gray, convex, glistening, occasionally mucoid Blood agar beneath colony may display gray/green color 	(See SBA)	No growth	<i>N. meningitidis</i>
<i>Mycobacteria</i>				
<ul style="list-style-type: none"> Faint staining "ghost-like" beaded Gram positive bacilli Difficult to stain due to high lipid content Acid fast staining required (carbol fuchsin and fluorochrome stains) Slender, slightly curved or straight, rod-shaped organisms (0.2-0.6 x 1-10 μm) 	<ul style="list-style-type: none"> Rapid growing <i>Mycobacteria</i> sp. appear as tiny, dry or "chalky" colonies within 3-5 days; branching filaments may be present on periphery of colonies Other clinically significantly <i>Mycobacteria</i> (TB complex) require 2-6 wk incubation on complex media (such as Lowenstein-Jensen) or automated broth system 	(See SBA)	<ul style="list-style-type: none"> No growth except for <i>M. fortuitum</i> or <i>M. Chelonae</i> 	<ul style="list-style-type: none"> <i>Rapid growing Mycobacteria</i> <i>M. tuberculosis- bovis complex</i>

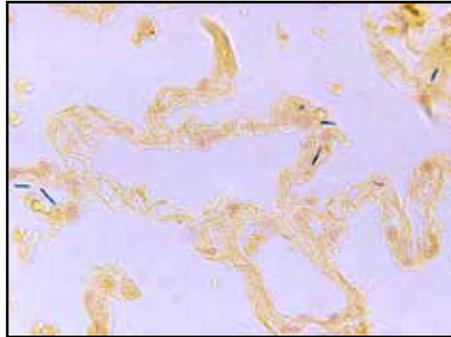
WORK UP ALL SLOW GROWING ORGANISMS IN A CLASS II BSC

GRAM POSITIVE BACILLI

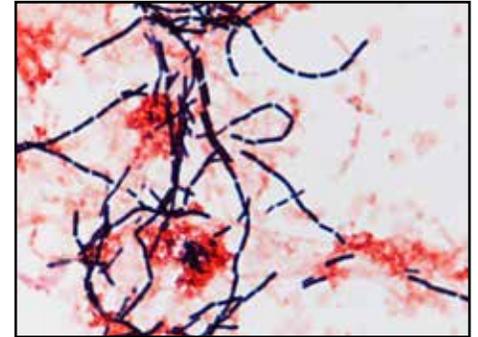
- Encountered in blood, skin lesion, sputum, CSF, rarely stool
- Large; single or in chains; seen in direct smear; may be easily decolorized
- Usually spores not seen in patient specimen; may be seen after extended growth in vitro
- Capsule may be seen from a direct patient specimen but not seen from culture



Bacillus anthracis, Gram stain.
Direct smear from blood, 1000x
(NPHL)



Bacillus anthracis, Gram stain.
Direct smear from lung, 1000x
(CDC)

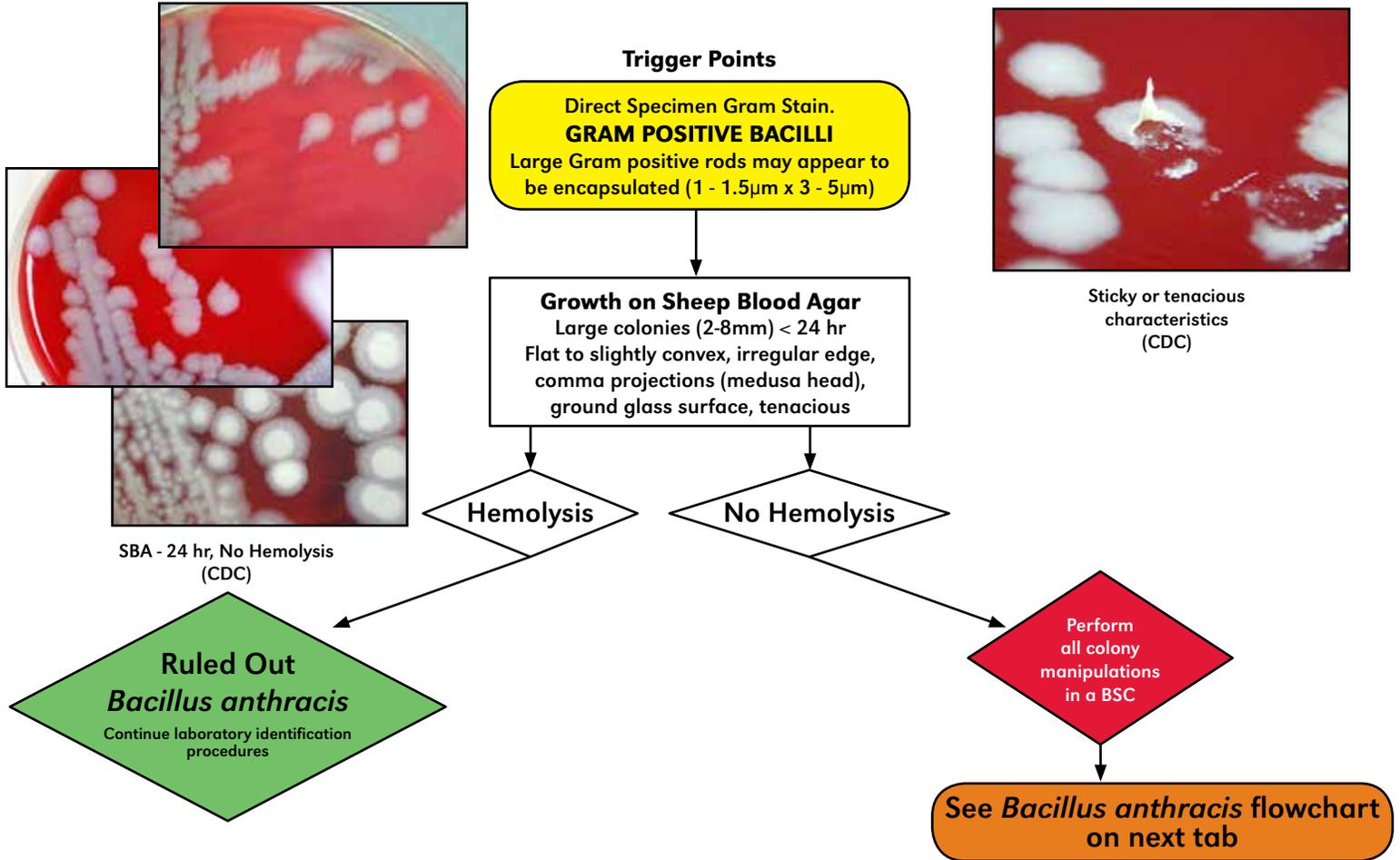


Bacillus anthracis, Gram stain.
Direct smear from blood, 1000x
(NPHL)

REFER TO *Bacillus anthracis* Tab 1

Nebraska Public Health Laboratory 24/7 Emergency Pager • 402-888-5588

Bacillus anthracis Tab 1



Bacillus anthracis Tab 2

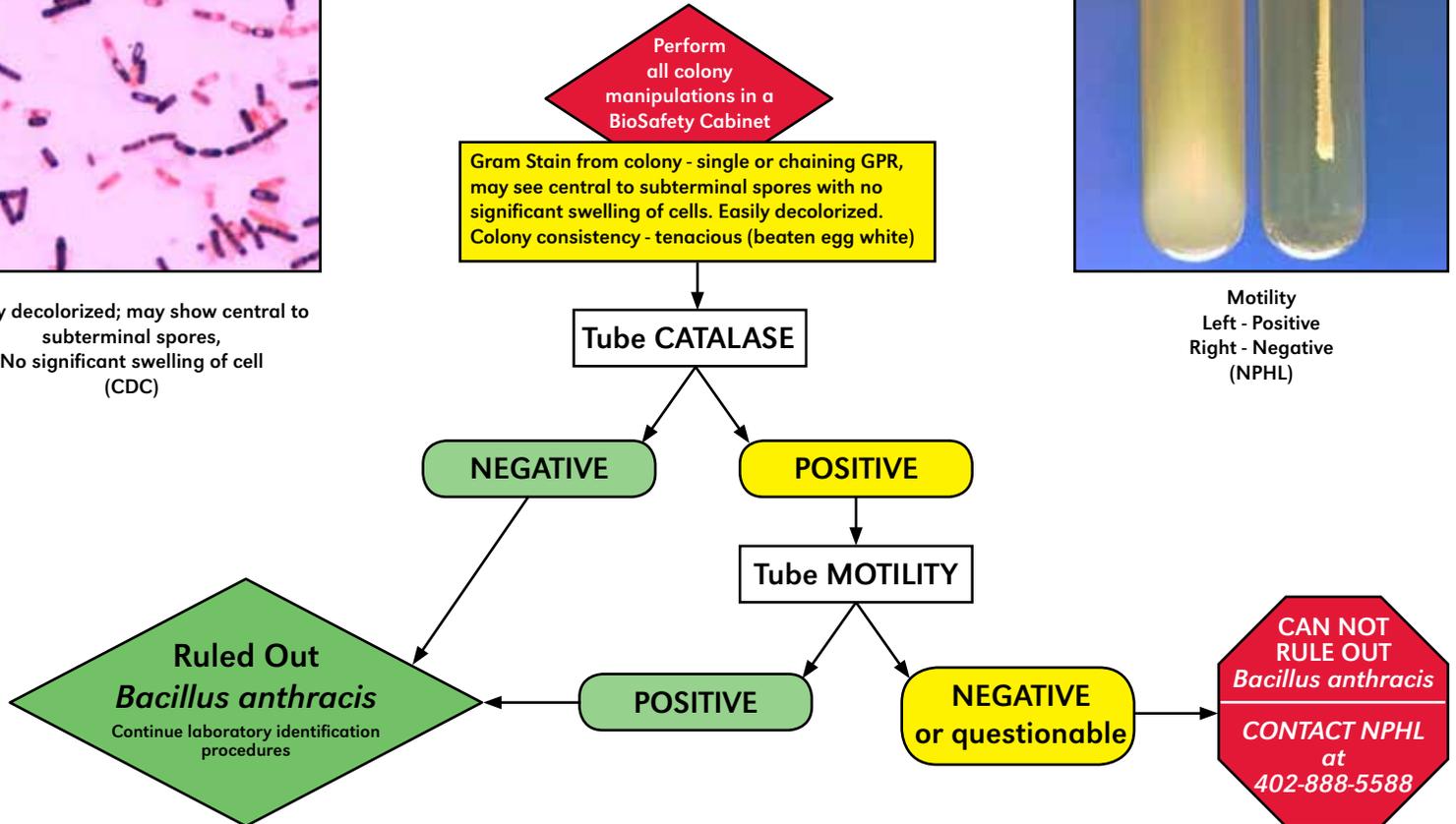
Wet prep Motility, India Ink and Slide Catalase NOT RECOMMENDED



Easily decolorized; may show central to subterminal spores,
No significant swelling of cell
(CDC)



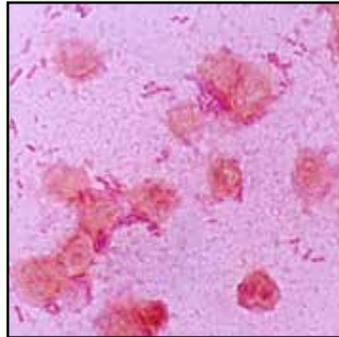
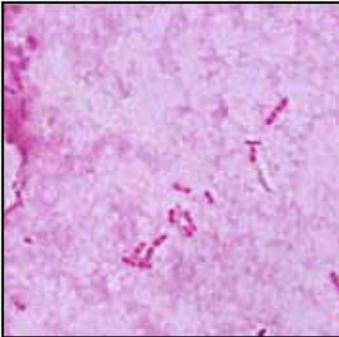
Motility
Left - Positive
Right - Negative
(NPHL)



GRAM NEGATIVE ROD

Burkholderia pseudomallei

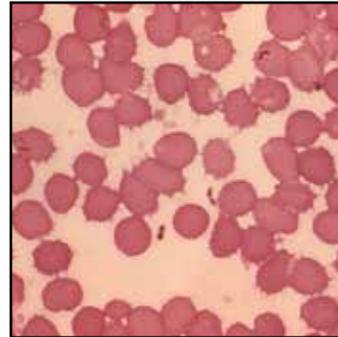
- Cause of melioidosis, presents as pneumonia and systemic with wide-spread abscesses in lungs, liver, spleen and kidney
- Encountered in bone marrow or blood, tissue, urine or respiratory specimens
- Small, straight or slightly curved Gram negative rods
- May demonstrate bipolar staining, resembling safety pins, however this is not relied upon for a presumptive clinical diagnosis



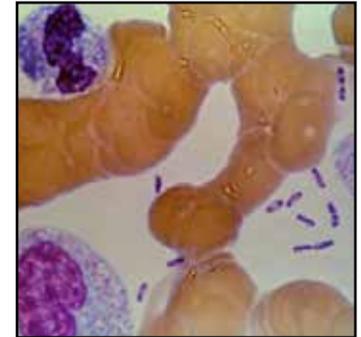
B. pseudomallei
Gram stain, 1000x
(ASM)

Yersinia pestis

- Cause of plague, presents as bubonic, septicemic or pneumonic; sudden onset of fever, weakness, painful swollen lymph nodes; extremities turn black. Transmitted by flea bite
- Encountered in blood, lymph node aspirate, respiratory tract
- Medium-sized; plump; mostly single cells, short chains in broth
- Stains well; bipolar “safety pin” stain may occasionally be seen with Wright or Giemsa stain, however, this is not reliable; hard to see on Gram stain



Yersinia pestis,
Gram stain, 1000x
(CDC)



Yersinia pestis, Wright stain,
Bipolar staining, 1000x
(CDC)

Note: Bipolar staining reported with other enteric bacteria, e.g., *Pasteurella spp*, Enteric GNR, other *Yersinia spp*.

REFER TO *Burkholderia pseudomallei* Tab

REFER TO *Yersinia pestis* Tab

Nebraska Public Health Laboratory 24/7 Emergency Pager • 402-888-5588

Gram Negative Rod

Burkholderia pseudomallei



Sheep Blood Agar - 24 hr
(CDC)

Direct Specimen Gram Stain.
GRAM NEGATIVE RODS
GNR, slender, straight or slightly curved in direct specimen gram stain. May appear bipolar.

Poor growth at 24 hr; good growth of white colonies at 48 hr on SBA, nonpigmented, non-hemolytic may be wrinkled ≥ 72 hr. MAC in 48 hr, color may vary.



Sheep Blood Agar - 72 hr
(CDC)

Trigger Points

Growth on MAC?

YES

NO

Perform all colony manipulations in a BioSafety Cabinet

Consider *B. mallei*, *Brucella* and *Francisella*

Gram Stain on colony - GNR may demonstrate bipolar staining, long parallel bundles or irregular shapes.

Ruled Out
Burkholderia pseudomallei
Continue laboratory identification procedures



MacConkey Plate - 48 hr
(CDC)

Oxidase: positive
Tube Catalase: positive
Indole: negative
Tube Motility: positive

WARNING!

Commercial Systems should NOT be attempted. May key out as *B.cepacia*, *B.thailandensis*, *Ps.aeruginosa*, *Ps.fluorescens*, *S.maltophilia*, *Ps.alcaligenes*, *C.violaceum*

Optional Testing
Arginine: positive
42°C Growth: positive

NO

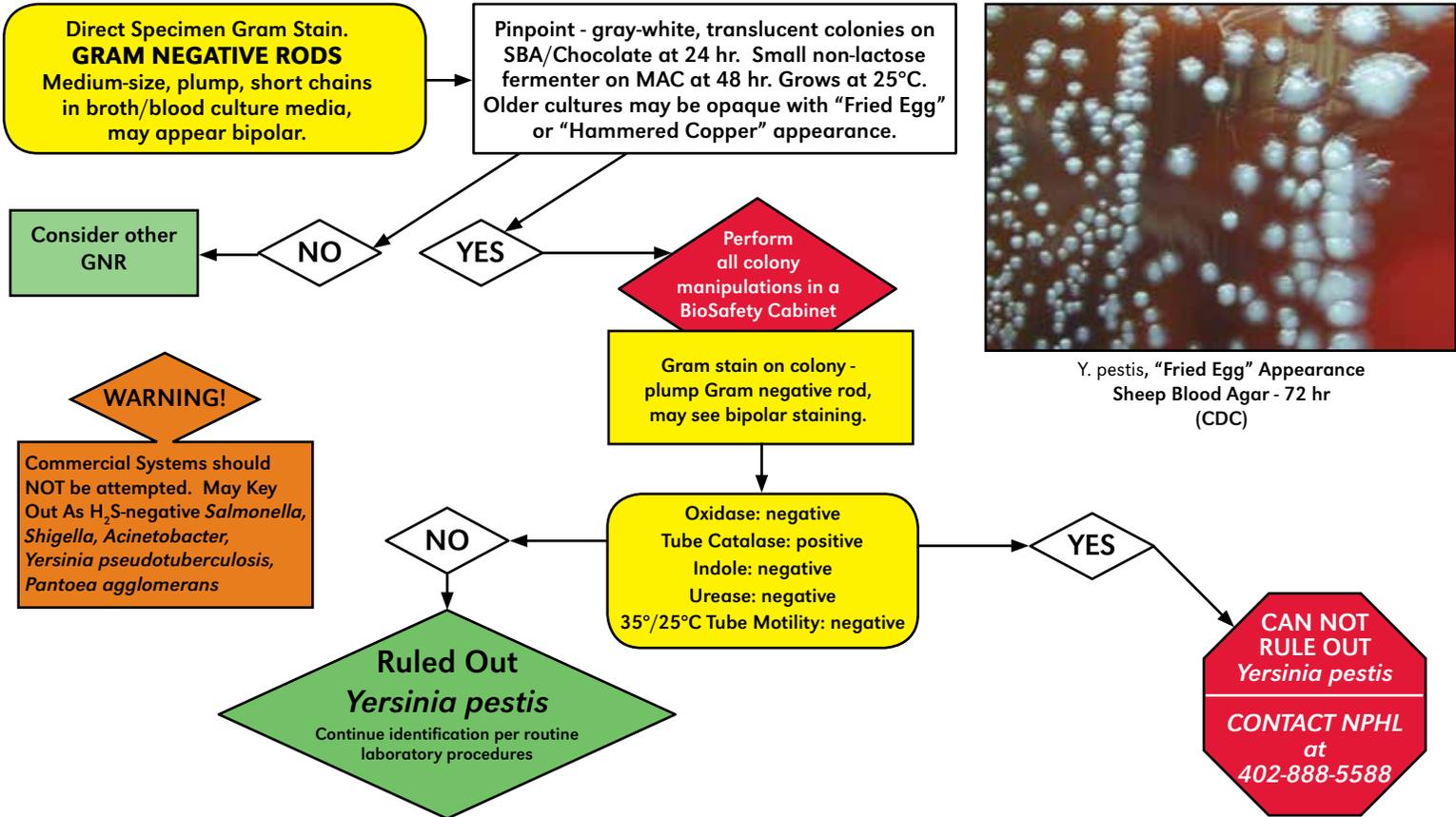
CAN NOT RULE OUT
Burkholderia pseudomallei
CONTACT NPHL
at
402-888-5588

YES

Burkholderia pseudomallei

Yersinia pestis

Trigger Points

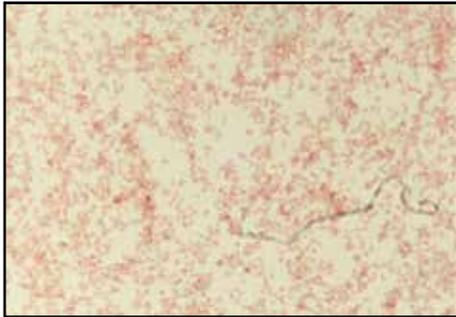


Yersinia pestis

GRAM NEGATIVE COCCOBACILLI

Francisella tularensis

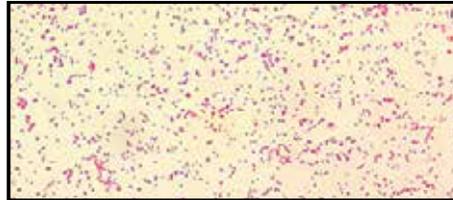
- Cause of tularemia, multiple presentations, sudden onset, persists for weeks if not treated
- Encountered in blood, CSF, lymph node, respiratory, abscess/wound, tissue
- Very tiny GN CB, weakly staining, difficult to see individual cells
- Interpretation very difficult due to minute size, often reported as NOS



Francisella tularensis
Gram stain 1000x
(NPHL)

Brucella spp.

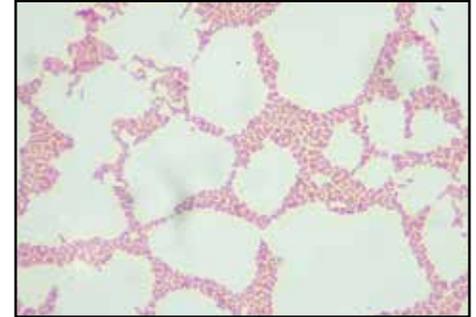
- Cause of brucellosis, presentation is non-specific and systemic, with fever, sweats, fatigue, muscle weakness, weight loss, can become chronic
- Encountered in blood, lymph node, bone marrow, liver or spleen, joint fluid, abscess
- Small GN CB, faint but discrete cells will be evident in direct smear
- May retain crystal violet stain, can be mistaken for Gram positive cocci
- 10 days sufficient incubation time in automated blood culture system; 21 days for manual



Brucella spp.
Gram stain 1000x
(CDC)

Burkholderia mallei

- Cause of glanders, presents as cutaneous with lymphadenitis or systemic, manifesting as pneumonia or lesions in spleen and liver, often fatal if not treated
- Encountered in bone marrow or blood, respiratory, tissue, abscess/wound specimens or urine
- Faintly staining Gram negative, straight or slightly curved rod with rounded ends or coccobacilli
- May be arranged in pairs end-to-end, in parallel bundles



Burkholderia mallei
Gram stain 1000x
(CDC)

REFER TO *Francisella tularensis*, *Brucella spp.* or *Burkholderia mallei* Tab

Nebraska Public Health Laboratory 24/7 Emergency Pager • 402-888-5588

Francisella tularensis

Note: Tularemia is a common laboratory acquired infection;
all work on suspect cultures should be performed under BSL2 conditions

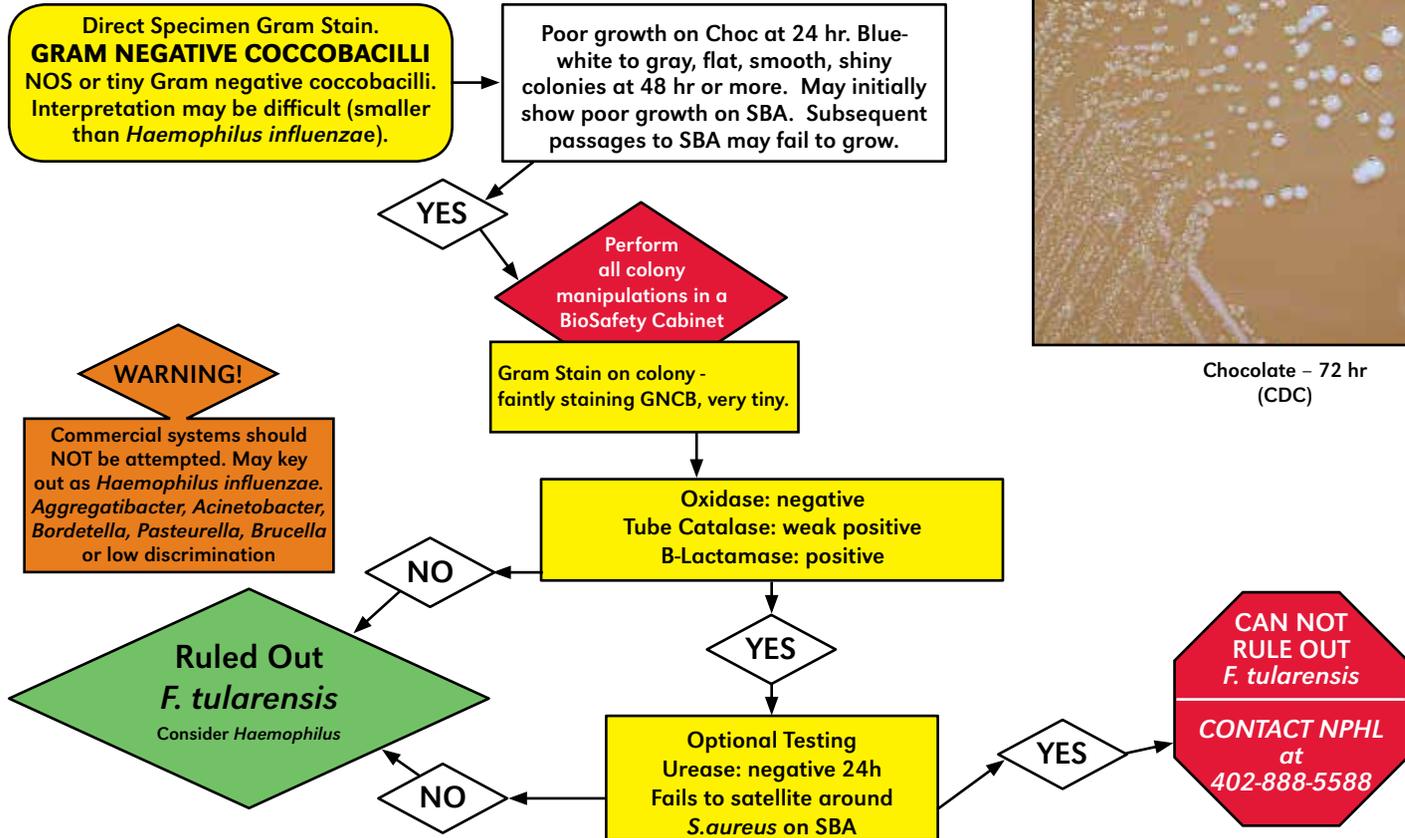
Trigger Points

Direct Specimen Gram Stain.
GRAM NEGATIVE COCCOBACILLI
NOS or tiny Gram negative coccobacilli.
Interpretation may be difficult (smaller than *Haemophilus influenzae*).

Poor growth on Choc at 24 hr. Blue-white to gray, flat, smooth, shiny colonies at 48 hr or more. May initially show poor growth on SBA. Subsequent passages to SBA may fail to grow.



Chocolate – 72 hr
(CDC)



Francisella tularensis

Brucella spp.

Note: Brucellosis is a common laboratory acquired infection;
all work on suspect cultures should be performed under BSL2 conditions.



Sheep Blood Agar – 48 hr.
(CDC)



Sheep Blood Agar – 72 hr
(CDC)

Trigger Points

Direct Specimen Gram Stain.
GRAM NEGATIVE COCCOBACILLI
Small, faint, GNGB, discrete cells evident.
May retain crystal violet stain.

Growth on SBA, Choc
may take 48-72 hr.
Non-pigmented,
non-hemolytic, moist,
convex.

YES

Perform
all colony
manipulations in a
BioSafety Cabinet

Gram Stain on colony
GNGB

Oxidase: positive
Tube Catalase: positive
Urease: positive (rapid to 24 hr)

NO

YES



Positive Urease – 1 hr (Top)
(NPHL)

**Ruled Out
Brucella spp.**
Consider *Francisella*

WARNING!

Commercial systems should NOT
be attempted. May key out as
Ochrobactrum anthropi,
Psychrobacter, *Oligella ureolytica*,
Bordetella bronchiseptica

**CAN NOT
RULE OUT
Brucella spp.**
**CONTACT NPHL
at
402-888-5588**

Brucella spp.

Burkholderia mallei

Trigger Points

Direct Specimen Gram Stain.

GRAM NEGATIVE COCCOBACILLI OR SMALL ROD

Faintly staining, slightly curved, arranged in singles, end-to-end pairs, parallel bundles.

Smooth, gray, translucent colonies at 48 hr.
Poor or no growth MAC?

YES

Perform all colony manipulations in a BioSafety Cabinet

Gram Stain on colony
GNCB or small GNR

WARNING!

Commercial systems should NOT be attempted. May key out as *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*

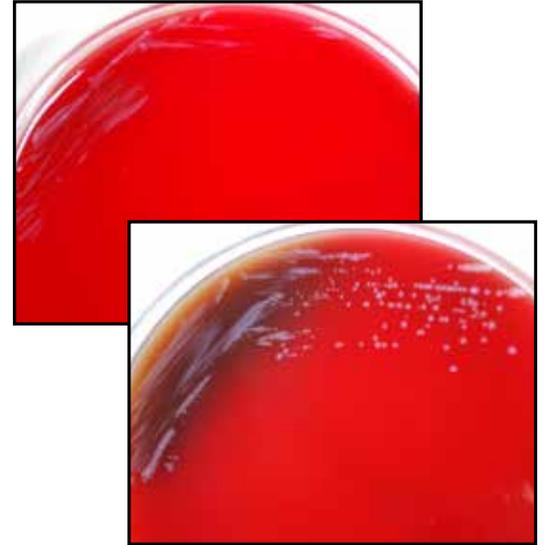
NO

***B.mallei*
Ruled Out
Consider Brucella**

Continue laboratory identification procedures

Oxidase: negative or variable
Tube Catalase: positive
Indole: negative
Urease: negative 24 hr
Tube Motility: negative

Optional Testing
Arginine: positive
42°C Growth: negative



Sheep Blood Agar – 24-48 hr (CDC)

YES

**CAN NOT
RULE OUT
*Burkholderia mallei***

**CONTACT NPHL
at
402-888-5588**

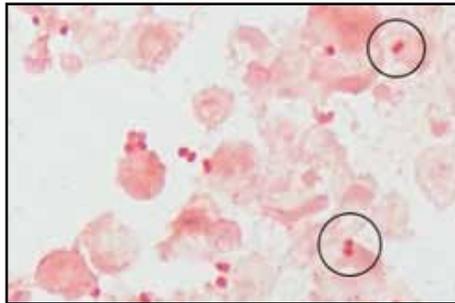
Burkholderia mallei

GRAM NEGATIVE DIPLOCOCCI

Neisseria meningitidis

- Cause of Invasive meningococcal disease (IMD) presents as meningitis or acute sepsis, with petechial lesions which coalesce. Mortality 30% with meningococcal septic shock. Complications include arthritis, pericarditis, pneumonia.
- Encountered in CSF, blood, joint aspirates, biopsy. Organism can be carried in pharyngeal area
- Gram negative diplococci seen in direct Gram stain can be intracellular in PMN's. May resist decolorization.
- Positive direct smears for gram negative diplococci is sufficient for presumptive diagnosis of meningococcal meningitis.

Note: meningococemia can be a common laboratory acquired infection (LAI); all work on suspect culture should be performed under BSL2 conditions with BSL3 practices.

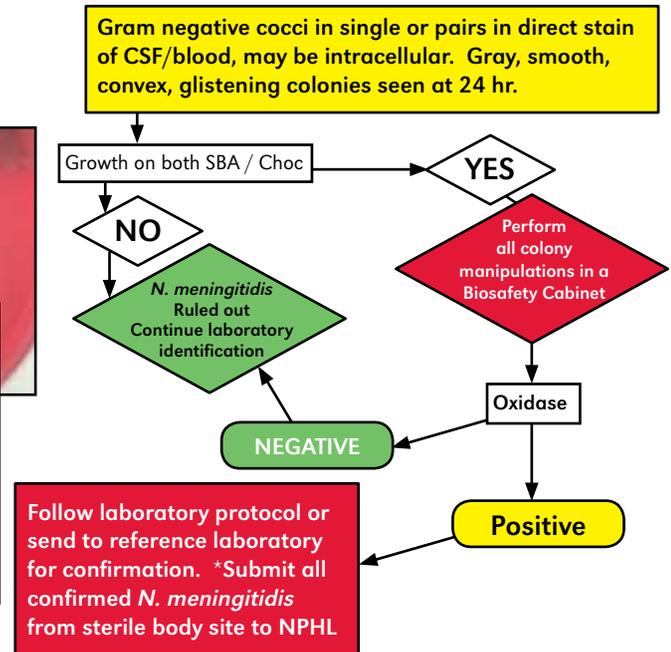


Neisseria meningitidis
Gram stain, 1000x
(NPHL/FAMC)



Neisseria meningitidis
(NPHL/FAMC)

Trigger Points



Gram Negative Diplococci

SPOT TESTS OF HAZARDOUS ORGANISMS (adapted from CDC)

Organism	Gram Stain Morphology	Growth			Motility	Oxidase	Catalase	Indole	Urease	Beta - Lactamase
		SBA	Choc	MAC						
<i>Bacillus anthracis</i>	GPR	+	+	—	—	NA	+	NA	NA	
<i>Yersinia pestis</i>	GNR	+	+	+	—	—	+	—	—	
<i>Burkholderia pseudomallei</i>	GNR	+	+	+	+	+	+	—	V	
<i>Burkholderia mallei</i>	GNCB	+	+	V	—	V	+	—	V	
<i>Francisella tularensis</i>	GNCB	—V	+	—	NA	—	Weak +	—	—	+
<i>Brucella spp.</i>	GNCB	+	+	V	NA	+	+	—	+	
<i>Neisseria meningitidis</i>	GNDC	+	+	—	NA	+	NA	NA	NA	

+ positive; — negative; +V most species/strains positive; —V most species/strains negative; V variable SBA Sheep Blood Agar; Choc = Chocolate Agar; MAC = MacConkey

Key Tests

BIOSAFETY & MOST COMMONLY ENCOUNTERED HAZARDOUS PATHOGENS (adapted from CDC and ASM)

Organism	Biosafety Level		Specimen Exposure/Risk	Recommended Precautions for Sentinel Laboratories	
	Specimen Handling	Culture Handling			
<i>Bacillus anthracis</i>	2	3	Blood, skin lesion exudates, CSF, pleural fluid, sputum; rarely urine & feces.	BSL2: Activities involving clinical material collection & diagnostic quantities of infectious cultures.	BSL3: Activities with high potential for aerosol or droplet production.
<i>Brucella spp.</i>	2	3	Blood, bone marrow, CSF, tissue, semen, occasionally urine.	BSL2: Activities limited to collection, transport & plating of clinical material.	BSL3: <u>All</u> activities involving manipulations of cultures.
<i>Burkholderia mallei & pseudomallei</i>	2	3	Blood, sputum, CSF, tissue, abscesses, and urine	BSL2: Activities limited to collection, transport & plating of clinical material.	BSL3: <u>All</u> activities involving manipulations of cultures.
<i>Francisella tularensis</i>	2	3	Skin lesion exudates, respiratory secretions, CSF, blood, urine, tissues from infected animals & fluids from infected arthropods.	BSL2: Activities limited to collection, transport & plating of clinical material.	BSL3: <u>All</u> activities involving manipulations of cultures.
<i>Yersinia pestis</i>	2	3	Bubo fluid, blood, sputum, CSF, feces, urine.	BSL2: Activities involving clinical material collection & diagnostic quantities of infectious cultures.	BSL3: Activities with high potential for aerosol or droplet production.

RESOURCES

Packaging and shipping requirements must be met for all samples and are the responsibility of the shipper. Shipping materials can be obtained by calling NPHL at (402) 559-3590. Shipping instructions can be found www.nphl.org

BT preparedness information can be found on the CDC website www.bt.cdc.gov
ASM - <http://www.asm.org/index.php/issues/sentinel-laboratory-guidelines>

Biosafety in Microbiological and Biomedical Laboratories (BMBL); DHHS/CDC – 5th Edition, Revised 2009

Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories - MMWR, supplement/Vol 61, January 6, 2012

Manual of Clinical Microbiology, 10th Edition, Versalovic, J; ASM Press

Cyber Infrastructure Group at Virginia Bioinformatics Institute Train the Trainer: Laboratory Preparedness for the Sentry Laboratory, NLTN, 2012

Special thanks to the Wisconsin State Laboratory of Hygiene for letting us use their idea for this Bench Guide.
Updated by NPHL, 2013