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PRACTICAL APPROACH TO IDENTIFYING AND HANDLING SUSPECT HIGH BIOSAFETY RISK AGENTS IN THE ROUTINE CLINICAL MICROBIOLOGY LABORATORY

INTRODUCTION

The UHN/SHS clinical microbiology laboratory is a Containment Level 2 facility licensed to safely possess and handle most Risk Group 2 organisms. However, as the laboratory processes unknown specimens, an inherent risk exists to isolate Risk Group 3 organisms. Hence, the microbiology laboratory plays an essential role in identifying and limiting the spread of potentially dangerous infectious agents. The following procedures are in place to safely identify and handle organisms of high biosafety risk.
WHEN TO SUSPECT HIGH BIOSAFETY RISK AGENTS:

A. Presumptive diagnosis provided

SUSPECT CJD or other relevant agents listed below

B. Gram smear

Bacteria:
- Small Gram-negative bacilli or coco-bacilli from all sites
  SUSPECT Brucella, Francisella, Yersinia pestis or Burkholderia pseudomallei
- Gram-negative diplococci from sterile sites
  SUSPECT Neisseria meningitidis
  Note: *N. meningitidis* is a Risk Group 2 organism, but given the potential for serious infection, culture should only be opened in a BSC.

Mould:
- Large thick-walled broad-based budding yeast from all sites
  SUSPECT Blastomyces dermatitidis

C. Culture

Bacteria:
- Slow-growing Gram-negative bacilli/coco-bacilli from all sites
  SUSPECT Brucella, Francisella, Yersinia pestis, or Burkholderia pseudomallei
- Rapid-growing non-hemolytic large spore-forming Gram-positive bacilli from all sites
  SUSPECT Bacillus anthracis

Mould:
- White mould growing on cycloheximide-containing agar after $\geq 3$ days of incubation
  SUSPECT Histoplasma, Blastomyces, Coccidioides, or Paracoccidioides
- Black, olivaceous green or brown mould from brain tissue
  SUSPECT Cladophialophora bantiana or Rhinocladiella mackenziei

**IF YOU ENCOUNTER ANY OF THE ABOVE, NOTIFY SENIORS IMMEDIATELY AND FOLLOW THE BIOSAFETY MANUAL FOR FURTHER INSTRUCTIONS.**

See “Profile of Relevant Risk Group 3 Organisms” in the section below for complete characteristic profile of common RG 3 pathogens.

For a complete list of Risk Group 3 and 4 organisms, see PHAC’s ePATHogen – Risk Group Database available online.
WHAT TO DO IF A RISK GROUP 3/4 ORGANISM IS SUSPECTED
Small Gram-negative bacilli or coco-bacilli from all sites (Brucella, Francisella, Yersinia, or B. pseudomallei)

Should a suspect Risk Group 3 concern be provided from the clinical team or recognized from the Gram stain or culture, follow steps below:

1. **Notify Senior/Charge** of potential Risk Group 3 organism.

2. **Process samples offline** within a BSC (DO NOT load into WASP / WASPLAB)
   - Incubate BC for 21 days; for cultures add *Staph* streak to BA plate
   - Immediately seal all plates with parafilm circumferentially from all relevant specimens for the patient with the isolate in question. This will require expunging any already processed plates within WASPLAB.
   - Label plates and plate rack with RG3 Alert labels.

3. Continue **incubating all plates offline** until growth is observed.
   - Any further handling of sealed plates must be done in a Class 2 Biological Safety Cabinet (BSC) with an N95 respirator and gloves.

4. **If suspicious growth is observed:**
   a. Work-up all organisms as per the Small Gram negative bacilli/cocco-bacilli Workup Flowchart. NOTE: Perform oxidase and catalase only. DO NOT SET UP MALDI OR MANIPULATE ANY FURTHER.
   b. Notify seniors, who shall:
      i. Ensure notification Biological Safety Officer, Microbiologist, and Infection Control occurs.
      ii. Notify the Local Public Health Unit when a preliminary ID is available.
      iii. Send LIS email to all staff with patient demographics warnings.
      iv. Add RG3ESO flag.
      v. Post patient demographics in specimen processing and bacteriology and incubators containing culture plates using the RG3 Alert signs.
   c. Send isolate to PHOL:
      i. Notify PHOL of incoming RG3 organism.
      ii. Package according to Transportation of Dangerous Goods regulations (Only certified staff are permitted to do the packaging)

5. **Store plates in the Seniors RG3 Basket** pending PHOL confirmation.

6. Ensure all plates with **confirmed RG3 organisms are autoclaved and disposed.**
Gram-negative diplococci from sterile sites (*N. meningitidis*)

*Note: N. meningitidis is not Risk Group 3 organism but given the potential for serious infection, culture should only be opened in a BSC.*

Should a suspect *Neisseria meningitidis* concern be provided from the clinical team or recognized from the Gram stain or culture, follow steps below:

1. **Notify Senior/Charge** of potential Risk Group 3 organism.

2. **Process samples offline** within a BSC (DO NOT load into WASP / WASPLAB).
   - Immediately seal all plates with parafilm circumferentially from all relevant specimens for the patient with the isolate in question. This will require expunging any already processed plates within WASPLAB.
   - Label plates and plate rack with RG3 Alert labels.

3. **Incubate all plates offline** observing for growth at 24hrs.
   - *Any further handling of sealed plates must be done in a Class 2 Biological Safety Cabinet (BSC) with gloves.*

4. **If suspicious growth is observed:**
   - a. **Process culture in BSC with gloves for identification and susceptibilities.**
   - b. **Notify Seniors, who shall:**
     - i. Ensure notification to Biological Safety Officer, Microbiologist, Infection Control occurs.
     - ii. Notify the Local Public Health Unit when a preliminary ID is available.
     - iii. Send LIS email to all staff with patient demographics warnings.
     - iv. Add RG3 ESO flag.
     - v. Post patient demographics in specimen processing and bacteriology and incubators containing culture plates using the RG3 Alert signs.
   - c. **Send isolate to PHOL:**
     - i. Notify PHOL of incoming RG3 organism.
     - ii. Package according to Transportation of Dangerous goods regulations (Only certified staff are permitted to do the packaging)

5. **Store plates in the Seniors RG3 Basket** pending PHOL confirmation.

6. Plates do NOT need to be autoclaved.
Non-hemolytic large aerobic spore-forming Gram-positive bacilli from all sites (*B. anthracis*)

Should a suspect anthrax (*B. anthracis*) concern be provided from the clinical team or recognized from the Gram stain or culture, follow steps below:

1. **Notify Senior/Charge** in area of potential Risk Group 3 organism.
2. **Process further relevant samples offline** within a BSC (DO NOT load into WASP / WASPLAB).  
   **Immediately seal all plates with parafilm** circumferentially from all relevant specimens for the patient with the isolate in question. This will require expunging any already processed plates within WASPLAB.  
   **Label** plates and plate rack with **RG3 Alert labels**.
3. **Any further handling of sealed plates must be done in a Class 2 Biological Safety Cabinet (BSC) with gloves.**
4. **If suspicious growth is observed:**
   a. **Perform catalase and motility testing on all suspicious colonies.**
      i. If catalase negative, continue processing culture routinely.  
      ii. If catalase positive and motile, continue processing culture routinely.  
      iii. If catalase positive and non-motile, STOP testing (possible anthrax).
   b. **Notify Seniors, who shall:**
      i. Ensure notification to Biological Safety Officer, Microbiologist, Infection Control occurs.  
      ii. Notify the Local Public Health Unit when a preliminary ID is available.  
      iii. Send LIS email to all staff with patient demographics warnings.  
      iv. Add RG3ESO flag.  
      v. Post patient demographics in specimen processing and bacteriology and incubators containing culture plates using the **RG3 Alert signs**.
   c. **Send isolate to PHOL:**
      i. Notify PHOL of incoming RG3 organism.  
      ii. Package according to Transportation of Dangerous goods regulations  
(Only certified staff are permitted to do the packaging)
5. **Store plates in the Mycology/Seniors RG3 Basket** pending PHOL confirmation.
6. Ensure all plates with **confirmed RG3 organism are autoclaved and disposed.**
Suspect Risk Group 3 Moulds

A) Suspect white mould growing on cycloheximide-containing agar after $\geq 3$ days of incubation from all sites (*Histoplasma*, *Blastomyces*, *Coccidioides*, *Paracoccidioides*)

B) Black, olivaceous green/black mould from brain tissue (*Cladophialophora bantiana*, *Rhinocladiella mackenziei*)

Should a suspect Risk Group 3 mould concern be provided from the clinical team or recognized from the Gram stain or culture, follow steps below:

1. **Notify Senior/Charge** of potential Risk Group 3 organism.

2. **Process samples offline within a BSC** (DO NOT load into WASP / WASPLAB)
   - **Immediately seal plates with parafilm** circumferentially from all relevant specimens for the patient with the isolate in question. This will require expunging all plates within WASPLAB.
   - **Label** plates and plate rack with RG3 Alert labels.

3. Continue **incubating all plates offline** until growth is observed.
   - **Any further handling of sealed plates must be done in a Class 2 Biological Safety Cabinet (BSC) with gloves.**

4. **If suspicious growth is observed:**
   - **a.** DO NOT PERFORM ANY SMEARS OR MANIPULATE ANY FURTHER.
   - **b.** Notify Seniors, who shall:
     - i. Ensure notification to Biological Safety Officer, Microbiologist, Infection Control occurs.
     - ii. Notify the Local Public Health Unit when a preliminary ID is available.
     - iii. Send LIS email to all staff with patient demographics warnings.
     - iv. Add RG3 ESO flag.
     - v. Post patient demographics in specimen processing and bacteriology and incubators containing culture plates using the RG3 Alert signs.
   - **c.** Send isolate to PHOL:
     - i. Notify PHOL of incoming RG3 organism.
     - ii. Package according to Transportation of Dangerous goods regulations (Only certified staff are permitted to do the packaging)

5. **Store plates in the Mycology** section pending PHOL confirmation area.

6. Ensure all plates with **confirmed RG3 organism are autoclaved and disposed.**

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Creutzfeldt-Jakob Disease (CJD)

Should a suspect CJD concern be provided from the clinical team, follow steps below:

1. DO NOT PROCESS CSF for microbiology tests other than CJD.

2. Notify Senior technologist in area of CJD request. Apply CJD ESO flag.

3. Senior technologist shall
   - Notify Microbiologist & Infection Control of request
   - Provide requestor with SPECIMEN COLLECTION INSTRUCTIONS

4. Process specimen according to CJD CSF SPECIMEN PROCESSING INSTRUCTIONS
   aliquoting 2mL of CSF in a sample vial.
   Freeze specimen immediately at -20°C to -80°C in the designated virology freezer.

5. Store remaining CSF in the Seniors RG3 Basket until result confirmation is received.

6. Send frozen aliquot to NML according to Transportation of Dangerous Goods regulations.

7. If CJD is confirmed, autoclave and dispose of specimen. Ensure CJD ESO flag has been applied.
   Provide autoclave report stapled to LIS printout of relevant order to BSO
CJD Specimen Collection Instructions

While routine practices can be used to perform the lumbar puncture, we use additional precautions in the microbiology laboratory given the potential for aerosolization and contamination with how we process the CSF.

C. Collection and Transporting CSF from Patients with suspect CJD:
Routine collection containers can be used to collect CSF. They must be labelled and placed in the usual biosafety transport bags.
The only differences are:
1) To be extra safe in case of leakage or spillage while in transit, we ask that the biosafety transport bags be placed into a hard screw-top transport container (available for UHN through TWH Specimen Management at 13-5011), one container per lab (i.e. the tubes for microbiology should be placed in a separate container for microbiology, and the tubes for core lab/cytology if relevant be placed in a separate container). The clinical team collecting the specimen for UHN can call specimen management at 13-5011 and arrange for two screw top containers to be sent to the relevant ward.
2) We ask that specimen collection be done during day hours so that our experienced microbiology technologists can process them and arrange for them to be sent to NML before our FedEx shipment pick up (which occurs between 4:30-5pm). So it would be ideal to have the LP scheduled before 2pm to give enough time for specimen management to send them to us and for us to process them and package them for send-out.

B. Ordering Non-Microbiology Lab Tests:
For UHN, Non-microbiology lab tests can be ordered and processed per routine practices.

C. Ordering Microbiology Tests:

CJD Ordering:
CJD is orderable in EPR and Cerner. During downtimes, please send a downtown paper requisition along with the CSF and specify r/o CJD on the requisition. We will order the test in the lab and you will receive the results in EPR/Cerner.

CSF Volume Required for CJD Testing:
The National Lab requests that 2mL of CSF (with no visible blood) be sent for CJD testing.

Other Microbiology Tests:
Given the possible biosafety concerns with processing CJD CSF samples for microbiology tests, any other tests for microbiology ordered will be deferred until negative CJD testing results are
available. When processed, results will have a statement expressing that the delay in set up may reduce the sensitivity of the tests. In there is a high pre-test probability of another infectious etiology, you may want to consider a repeat LP. If CJD results are positive, the other tests will not be processed; if there are clinical suggestions that there may be more than one diagnosis and results of these other tests will change management plans, please contact the microbiologist-on-call to discuss.

**D. Notification of Toronto Public Health and the Canadian CJD Surveillance System**

The clinical team should contact local Public Health to notify them of the suspect CJD case. Additionally, The Canadian CJD Surveillance System should be also contacted via the following phone # 1-888-489-2999 ([https://www.canada.ca/en/public-health/services/surveillance/blood-safety-contribution-program/creutzfeldt-jakob-disease.html](https://www.canada.ca/en/public-health/services/surveillance/blood-safety-contribution-program/creutzfeldt-jakob-disease.html)). This should be done for any suspect CJD cases, not just confirmed CJD cases.
CJD CSF Specimen Processing Instructions

Specimen processing of CSF must be performed with increased safety precautions within a biological safety cabinet due to the risk group and infectious nature of CJD following the instructions below.

- **The specimens should only be opened in a BSC**
- Have only one tech involved in processing this specimen (or limit the number to as few as possible), preferably having only experienced technicians involved in any specimen processing
- The tech involved should wear single-use gloves, gown, and mask
- Single-use lab instruments/equipment should be used when possible
  - Used single-use lab equipment, residual specimens/specimen containers, and other laboratory waste should be sealed in a leak proof, puncture-resistant container, labelled “Biohazardous” and disposed of by incineration
  - Used non-disposable lab instruments should be cleaned and decontaminated as per the PHAC recommendations
- Any further test requested other than CJD on a CSF shall be deferred pending CJD results. (See Reporting Instructions)
- Any CSF being sent out to labs other than the MSH microbiology lab should be communicated to those labs prior to sending. The CSF should be sent in a sealed, leak proof, puncture-resistant container that is clearly labelled as “Caution - high risk for CJD”
  
  *Note: Other MSH labs do not accept any suspect CJD samples. If CJD negative, consultation with Microbiologist should occur prior to sending sample to ensure that a clinical diagnosis CJD has been completely rule out. UHN laboratories will accept all suspect CJD samples.*
- For hard surfaces (e.g. BSC): remove visible soil; flood with 2N NaOH or undiluted sodium hypochlorite; let stand for 1 hour; then mop up and rinse with water
- If any residual contaminated specimens/waste sealed as above are to be transferred to us in microbiology to be incinerated along with our waste, these must be walked to our lab – please do NOT use the tube system.

Remaining specimens are kept in the **Seniors RG3 Basket** until test results are obtained.

Once NML has identified the patient to be positive for CJD, dispose all specimens from the patient by incineration.
CJD Reporting Instructions

Report pending test(s) with test comment:

  )CJDD  “This test has been deferred until CJD is ruled out. Please contact the microbiologist-on-call with any questions.”

For CJD confirmed positive cases, finalize other pending tests with test comment:

  )CJDX  “This test was CANCELLED for biosafety reasons given positive CJD test results. Please contact the microbiologist-on-call with any questions.”

For CJD confirmed negative cases, process other pending tests with test comment:

  )CJDR  “Testing was delayed until CJD was ruled out which may reduce the sensitivity of this test. Please take this into consideration when interpreting this result. Please contact the microbiologist-on-call with any questions.”
Profile of Relevant Risk Group 3 Organisms

*Bacillus anthracis*

**Direct Gram stain from clinical samples:**

- large (1.0 to 1.5 μm by 3 to 5 μm) encapsulated Gram-positive bacilli in short chains.
- Gram stain can demonstrate clear zones (capsule) around bacilli.
- Spores usually not present in clinical specimens unless exposed to atmospheric O₂.

**Gram stain from Sheep Blood Agar (SBA) or other routine nutrient medium**

Photo courtesy of U.S. Army Medical Research Institute of Infectious Diseases, 2009.
- Large Gram-positive bacilli in long chains, usually non-encapsulated.

  - Oval, central to subterminal spores: 1 x 1.5 µ with no significant swelling of cell.
Culture:

On Sheep Blood Agar (SBA):
- **Rapid growth.** Heavily inoculated areas may show growth on a blood agar plate within 6-8 h and individual colonies may be detected within 12-15 h. This trait can be used to isolate *B. anthracis* from mixed cultures containing slower-growing organisms.
- **Colonies are nonhemolytic** (hemolysis on SBA excludes *B. anthracis*), flat or slightly convex, with ground-glass appearance and tenacious consistency.
- **Colonies often** exhibit comma-shaped protrusions from colony edge (“Medusa head” colonies).

If isolate is non-hemolytic, perform motility test using motility test media. Presumptive identification of *B. anthracis* is based on identification of large Gram-positive bacilli that are **nonhemolytic** on SBA and **non-motile**. If presumptive diagnosis of *B. anthracis*, proceed as **WHAT TO DO IF A RISK GROUP 3 ORGANISM IS SUSPECTED**. Otherwise, report as "Bacillus species isolated" (from sterile sites) or as part of "Commensal flora" (from non-sterile sites such as wounds).

If a presumptive *B. anthracis* colony is identified and suspected as a bioterrorism threat agent, **preserve original specimens** pursuant to a potential criminal investigation.

N.B. *B. anthracis* does not typically grow on McConkey (MAC) agar containing crystal violet, but the MAC plate used at our microbiology lab does not contain crystal violet, hence this characteristic is not particularly useful here. (This is why we do not include MAC as a media for primary isolation to avoid confusion).

Photo courtesy of APHL.
https://www.asm.org/images/PSAB/LRN/Anthrax%20LRN%20091217.pdf
**Francisella tularensis**

**Gram stain:**

![Gram stain image](https://www.asm.org/images/PSAB/LRN/Tularemia316.pdf)

Tiny (0.2 to 0.5 μm by 0.7 to 1.0 μm), poorly staining pleomorphic Gram-negative bacilli / coccobacilli.

**Culture:**

![Culture images](https://www.asm.org/images/PSAB/LRN/Tularemia316.pdf)

SBA

CHOC

Photo courtesy of MAJ Todd Kijek, USAMRIID

- On Sheep Blood Agar (SBA): Non-hemolytic, gray-white colonies, 1-2 mm after 48h.
- On MacConkey agar (MAC): No growth.

When tiny gram negative bacilli/coccobacilli are identified, follow Small/tiny Gram-negative bacilli/cocco-bacilli from all sites.

Report as "Gram negative bacillus / coccobacillus isolated. Further identification to follow".
Brucella spp.

Gram Stain:
Tiny (0.5 to 0.7 μm by 0.6 to 1.5 μm), faintly staining, Gram-negative coccobacilli

Culture:
- On Sheep Blood Agar (SBA): Small (0.5 to 1.0 mm) glistening, non-hemolytic, non-pigmented colonies after 2 to 3 days incubation.
- On MacConkey agar (MAC): Some strains may grow slowly.

When tiny gram negative bacilli/coccobacilli are identified, follow Small/tiny Gram-negative bacilli/cocco-bacilli from all sites. Report as "Gram negative bacillus / coccobacillus isolated. Further identification to follow".
**Yersinia pestis**

**Gram Stain:**
Gram-negative bacilli (1.0 by 0.5 µm) that may exhibit bipolar staining

![Gram Stain Image](https://phil.cdc.gov/details_linked.aspx?pid=1915)

**Culture:**
- On Sheep Blood Agar (SBA):
gray/white/slightly yellow opaque colonies after 48 h incubation, with little or no hemolysis. Colonies develop fried egg appearance beyond 48 h incubation.
- On MacConkey agar (MAC): small, lactose negative colonies after 24 h incubation.

![Culture Image](https://www.asm.org/images/PSAB/LRN/Ypestis316.pdf)

When slow growing gram negative bacilli as per growth characteristics are described, follow Small/tiny Gram-negative bacilli/cocco-bacilli from all sites. Report as "Gram negative bacillus / coccobacillus isolated. Further identification to follow".
**Burkholderia pseudomallei**

- Small (1-3 µm) Gram-negative bacilli with bipolar staining ("safety pin" appearance) with irregular arrangement (occasionally as long thin bundles).

Photo courtesy of Erasmus MC University Medical Center Rotterdam Dept. Medical Micro. and Inf. Dis.
| Section: Bacteriology Procedures | Subject Title: Suspect Risk Group 3_4 Biosafety Manual |

Culture:

Photo courtesy of US CDC. Left, colonies on SBA at 48 h. Right, colonies on CHOC at 72 h.
- Sheep Blood Agar (SBA): Variable, smooth, creamy, white colonies growing within 24 h of incubation; may become wrinkled (“cornflower” appearance), metallic, and dry with purple hue over time.

- MacConkey agar (MAC): Variably lactose-fermenting or colorless colonies at 24-48 h of incubation.
**Blastomyces dermatitidis**

**Gram stain** (from clinical samples, or after incubation at 37°C):

Large (8-15 µm), globose, thick-walled (“double wall”, refractile) yeast cell, often with single broad-based (4-5 µm) budding daughter cell.

Photo courtesy of Yin Ping Tse, Mount Sinai Hospital Dept. of Microbiology

**Culture:**
- BHIM or BAP at 37°C: White or beige, wrinkled, pasty, and moist colonies seen at 3 days to 4 weeks of incubation.

Photos courtesy of Yin Ping Tse, Mount Sinai Hospital Department of Microbiology

- IMA at 25°C: Floccose, white mold (turning tan to yellow with age) with tan to brown reverse seen at 3 days to 4 weeks of incubation.

**Lactophenol cotton blue stain** (after incubation at 25°C):

Septate hyaline hyphae with microconidia (2 – 10 µm) at right angles. No macroconidia.

Photo courtesy of Yuri, Fun With Microbiology Blog (http://thunderhouse4-yuri.blogspot.com)
**Histoplasma capsulatum**

**Gram stain** (from clinical samples, or after incubation at 37°C):

Small (2-4 µm) elongate, narrow budding yeast cells, variably stained, occasionally encapsulated, and often intracellular (e.g. within macrophages).

- **Culture:**

  - BHIM or BAP at 37°C: White, creamy, smooth, moist, and round colonies seen at 3 days to 4 weeks of incubation.

  - IMA at 25°C: Suede, white mold ((turning tan to buff-brown with age) with yellow to tan reverse seen at 3 days to 4 weeks of incubation.

  Photo courtesy of Sarah Kidd et al, National Mycology Reference Centre, University of Adelaide 2016

**Lactophenol cotton blue stain** (after incubation at 25°C)
Septate hyaline hyphae with large, tuberculate, thick-walled, round, and unicellular macroconidia having finger-like projections on the surface. Also has round, unicellular microconidia with smooth or rough wall.

Photo courtesy of Joy King and Lisa Stempak, University of Mississippi Medical Centre
Coccidioides immitis

**Gram stain** (from clinical samples, or after incubation at 37°C):

Photo courtesy of Subhash Mohan, Mount Sinai Hospital Department of Microbiology. Left and middle immature spherules; right, ruptured mature sphere with released endospores.

Large, thick-walled spherules (10-80 µm) containing multiple endospores (2-5 µm); immature spherules with no endospores may mimic *Blastomyces*. No true budding.

**Culture:**

BHIM or BAP at 37°C, or IMA at 25°C: Variable morphology from greyish, moist, glabrous, and membranous colonies to abundant, floccose, and white mold (turning tan to red with age) with pale brown to orange reverse seen at 3 days to 4 weeks of incubation.
**Lactophenol cotton blue stain** (after incubation at 25°C)

Thin, septate hyaline hyphae, with one-celled, cylindrical to barrel-shaped, thick and smooth-walled arthroconidia (2-8 x 3-5 µm) alternating with thin-walled empty disjunctor cells. True arthroconidia eventually fragment and disperse.

Photo courtesy of Audrey Schuetz et al, Diagn Cytopathol 2012
Paracoccidioides brasiliensis

**Gram strain** (from clinical samples, or after incubation at 37°C):

Large (4-60 µm), thick-walled (1 µm), double-countered, globose cells with multilateral narrow budding of daughter cells (“steering wheel” appearance) which may produce smaller secondary buds.

Photo courtesy of Priscila Marques de Macedo et al, Rev Soc Bras Med Trop 2018

**Culture:**
- BHIM or SBA at 37°C: White, heaped, wrinkled or folded colonies seen at 10 days to 4 weeks of incubation.

Photo courtesy of Jessica Gomes-Rezende thesis 2017

- IMA at 25°C: White cream filamentous, leathery, flat to wrinkled, wolly or cottony or glabrous mold (turning to tan or brown with age) with yellowish to brown reverse seen at 10 days to 4 weeks of incubation. Similar appearance to Blastomyces.
**Lactophenol cotton blue** (after incubation at 25°C):

Photo courtesy of R. C. Cruz et al, J Clin Microbiol 2012

Hyaline septate hyphae, often sterile, or with rare oval, unicellular, truncate conidia with broad base and round apex. Arthroconidia and intercalary chlamydomphores may also be observed. *Cladophialophora bantiana* (previously *Cladosporium bantiana*)

**Culture:**

- IMA at 30°C: Powdery, woolly, or velvety, olivaceous green to black with black reverse mould often growing after 2 weeks of incubation. Supports growth at 42°C.
Lactophenol cotton blue stain (after incubation at 30°C):

Septate brown hyphae with long, smooth, lemon-shaped unicellular conidia (6-11 x 2.5-5 μm) in sparsely-branched chains emerging from undifferentiated conidiophores, and occasional chlamydoconidia. The youngest conidia are found at the apex of the chain (acropetal conidium formation). No attachment scars, comparatively to other Cladophialophora species. No shield cells, comparatively to Cladosporium species.
**Rhinocladiella mackenziei** (previously *Ramichloridium mackenziei*):

**Culture:**

- IMA at 30°C: Velvety, olivaceous to brown with olivaceous black reverse with occasionally elevated center mould often growing after 2 weeks of incubation.

**Lactophenol cotton blue stain** (after incubation at 30°C)

- Dark-pigmented, smooth septate hyphae with short, thick, brown conidiophores at right angles leading to cylindrical denticles producing oval sympodial conidia (8-10 x 4-5 µm, “Mickey Mouse” appearance) with prominent basal scar.
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<td>Bacteriology Procedures</td>
<td>Suspect Risk Group 3_4 Biosafety Manual</td>
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</table>
APPENDIX I: Flowchart work-up for suspect small or tiny gram negative bacilli / coccobacilli potential Risk Group 3 organisms

rg3 alert

small or tiny gram negative bacilli / coccobacilli

growth at 24 hrs?

NO / Poor Growth

perform oxidase & catalase

ox+ cat+

R/O Brucella

R/O Francisella

R/O B. pseudomallei

ox- cat -/weak+

ox- cat+

R/O Y. pestis

R/O B. mallei

ox v cat+

R/O Unusual ID

send to phol
APPENDIX II: Flowchart of Bacillus sp. work-up for suspect B. anthracis

GRAM AND SPORE STAIN OF CULTURE
Large Gram positive bacilli with spores (central, paracentral, may be delayed)

Haemolysis on sheep BA?

WORK IN BSC

Catalase

MOTILITY (tube motility test medium with TTC)

Suspect B. anthracis

NOT B. anthracis

Follow routine practices

POS

NEG

NEG

NO

YES
REFERENCES

2. CDC Guidelines for State Health Departments (Revised October 14, 2001)
3. CDC Basic protocol for the presumptive identification of Bacillus anthracis
### Record of Edited Revisions

**Manual Section Name:** Bioterrorism Procedure Manual

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<tr>
<th>Page Number / Item</th>
<th>Date of Revision</th>
<th>Signature of Approval</th>
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<tr>
<td>Entire document: update title to RG 3/4 organisms clarify procedures for safe handling Appendix I, II - change &quot;rule out&quot; to &quot;suspect&quot;</td>
<td>01Nov2019</td>
<td>Dr. T. Mazzulli</td>
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<td>Addition of Biosafety procedures: How to Identify and what to do when potential RG3 organism are suspected. Addition of Flowchart of suspect RG3 organisms. Addition of gram and culture images. Removal of instruction to perform any testing including oxidase, catalase, urease on suspect RG3 organisms.</td>
<td>January 28, 2019</td>
<td>Dr. T. Mazzulli</td>
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<td>April 4, 2018</td>
<td>Dr. T. Mazzulli</td>
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<td>April 7, 2017</td>
<td>Dr. T. Mazzulli</td>
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<tr>
<td><strong>Pathology &amp; Laboratory Medicine - Emergency Preparedness Plan D0004273.doc</strong> Link Added</td>
<td>September 17, 2014</td>
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