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PRACTICAL APPROACH TO BIOSAFETY AND BIOTERRORISM IN THE ROUTINE
CLINICAL MICROBIOLOGY LABORATORY

INTRODUCTION

The UHN/MSH microbiology laboratory is a level 2 clinical laboratory licensed to possess and safety handle Risk group 2 organisms. As a clinical laboratory processing unknown specimens, an inherent risk exists to isolate Risk group 3 organisms. Hence, the microbiology laboratory plays an essential role in the initial identification and control of spread of potentially infectious agents. The following procedures are in place to safety identify and handle potential Risk Group 3/4 organism.

WHEN TO SUSPECT A RISK GROUP 3/4 ORGANISM:

A. Presumptive diagnosis provided
   - Suspect CJD (or other Risk Group 3/4 agent)

B. Gram smear
   - Small/tiny Gram-negative bacilli/cocco-bacilli from all sites
     \( (Brucella \text{ spp. Francisella tularencis}) \)
   - Gram-negative diplococci from sterile sites Note: \( N. \text{ meningitidis} \) is not Risk Group 3 but given the potential for serious infection, culture should only be opened in a BSC.

C. Culture
   Bacteria:
     - Slow-growing Gram-negative bacilli/cocco-bacilli from all sites
     - Non-hemolytic \( Bacillus \) spp from all sites
   Mould:
     - White mould growing on cycloheximide-containing agar after three days of incubation \( (Histoplasma, Blastomycyces, Coccidioides) \)
     - Black, olivaceous green or brown mould from brain tissue \( (Cladophialophora bantiana, Ramichloridium mackenziei) \).

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

See “Profile of Risk Group 3 Organism Cultures” in the bioterror manual for complete characteristic profile of common level 3 pathogens.

For a complete list of RG3/4 organisms See PHAC’s EPathogen Risk Group Database
WHAT TO DO IF A RISK GROUP 3/4 ORGANISM IS SUSPECTED

Creutzfeldt-Jakob Disease - CJD

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

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

3. DO NOT PROCESS CSF for other microbiology tests.

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

5. Store remaining CSF here 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 complete the LP, we use additional precautions in the microbiology laboratory given the potential for aerosolization and contamination with how we process the CSF.

A. Collection and Transporting CSF from Patients with Query 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 arranged 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 in microbiology our experienced 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 also be deferred until negative CJD testing results
are available. When processed, results will have a statement that the delay in set up may reduce the sensitivity of the tests. In there is a high pre-test probability that there is another infectious cause, 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). 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, 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
- For 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 other labs should be communicated to the 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 CJD diagnosis has not still been kept. 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:

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<tr>
<th>Test Code</th>
<th>Test Comment</th>
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</thead>
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<tr>
<td>CJDD</td>
<td>“This test has been deferred until CJD is ruled out. Please contact the microbiologist-on-call with any questions.”</td>
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For CJD confirmed positive cases, finalize other pending tests with test comment:

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<tr>
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<td>“This test was CANCELLED for biosafety reasons given positive CJD test results. Please contact the microbiologist-on-call with any questions.”</td>
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For CJD confirmed negative cases, process other pending tests with test comment:

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<th>Test Comment</th>
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<tbody>
<tr>
<td>CJDR</td>
<td>“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.”</td>
</tr>
</tbody>
</table>
Small/tiny Gram-negative bacilli/cocco-bacilli from all sites - suspect *Brucella*, *Francisella*, *Yersinia*

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

1. **Notify Senior/Charge** in area of potential risk group 3 organism.

2. **Process samples offline** within a BSO (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 a N95 mask and gloves.*

4. Work-up all organisms as per [Small or Tiny Gram negative bacilli/cocco-bacilli 3 Workup Flowchart](#).
   NOTE: for slow-growing tiny Gram-negative bacilli/cocco-bacilli, **perform oxidase and catalase only. DO NOT SET UP MALDI OR MANIPULATE ANY FURTHER.**

5. **Once suspicious growth appears, seniors shall:**
   a. Ensure notification to Biological Safety Officer, Microbiologist, Infection Control occurs.
   b. Notify the Local Public Health Unit when a preliminary ID is available.
   c. Send LIS email to all staff with patient demographics warnings.
   d. Add RG3ESO flag.
   e. Post patient demographics in specimen processing and bacteriology and incubators containing culture plates using the **RG3 Alert signs**.

6. **Once suspicious growth appears, send isolate to PHOL**
   a. Notify PHOL of incoming RG3 organism.
   b. Package according to Transportation of Dangerous goods regulations (Only certified staff are permitted to do the packaging)

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

8. Ensure all plates with **confirmed RG3 organism are autoclaved and disposed**.
Gram-negative diplococci from sterile sites (suspect *N.meningitidis* - Risk Group 2)

*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 *N.meningitidis* concern be provided from the clinical team, recognized from the Gram or culture, follow steps below:

1. **Notify Senior/Charge** in area of potential risk group 3 organism.
2. **Process samples offline** within a BSO (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.**

Growth on BA and/or CHOC: Process culture in BSC with gloves for ID and AST.

4. **Once suspicious growth appears, seniors shall:**
   a. Ensure notification to Biological Safety Officer, Microbiologist, Infection Control occurs.
   b. Notify the Local Public Health Unit when a preliminary ID is available.
   c. Send LIS email to all staff with patient demographics warnings.
   d. Add RG3 ESO flag.
   e. Post patient demographics in specimen processing and bacteriology and incubators containing culture plates using the **RG3 Alert signs**.

5. **Once suspicious growth appears, send isolate to PHOL**
   a. Notify PHOL of incoming RG3 organism.
   b. Package according to Transportation of Dangerous goods regulations (Only certified staff are permitted to do the packaging)

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

7. Plates do NOT need to be autoclaved.

---

*NOTE: This document is Uncontrolled When Printed. Any documents appearing in paper form that are not stamped in red "MASTER COPY" are not controlled and should be checked against the document (titled as above) on the server prior to use.*
Non-hemolytic Bacillus spp from all sites - suspect *B.anthracis*

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

1. **Notify Senior/Charge** in area of potential risk group 3 organism.

2. **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.  
   - **Process further relevant samples offline** within a BSO (DO NOT load into WASP / WASPLAB).

3. **Any further handling of sealed plates must be done in a Class 2 Biological Safety Cabinet (BSC) with gloves.**  
   - On all non-hemolytic *Bacillus* colonies, perform catalase and motility testing.  
     - Catalase negative - continue processing culture routinely  
     - Catalase positive, motile - continue processing culture routinely  
     - Catalase positive, non motile - suspicious for *B.anthracis*, STOP testing.

4. **Once suspicious growth appears, seniors shall:**  
   - k. Ensure notification to Biological Safety Officer, Microbiologist, Infection Control occurs.  
   - l. Notify the Local Public Health Unit when a preliminary ID is available.  
   - m. Send LIS email to all staff with patient demographics warnings.  
   - n. Add RG3ESO flag.  
   - o. Post patient demographics in specimen processing and bacteriology and incubators containing culture plates using the **RG3 Alert signs**.

5. **Once suspicious growth appears, send isolate to PHOL**  
   - b. Package according to Transportation of Dangerous goods regulations  
      (Only certified staff are permitted to do the packaging)

6. **Store plates in the Mycology/Seniors RG3 Basket** pending PHOL confirmation.

7. 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 three days of incubation from all sites (Histoplasma, Blastomyces, Coccidioides)
B. Black, olivaceous green/black mould from brain tissue from all sites (Cladophialophora bantiana, Ramichloridium mackenziei)

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

1. **Notify Senior/Charge** in area of potential risk group 3 organism.

2. **Process samples offline** (DO NOT load into WASP/WASPLAB) within a BSO
   *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.*
   DO NOT PERFORM ANY SMEARS OR MANIPULATE ANY FURTHER.

4. **Once suspicious growth appears, notified senior shall:**
   a. Ensure notification to Biological Safety Officer, Microbiologist, Infection Control occurs.
   b. Notify the Local Public Health Unit when a preliminary ID is available.
   c. Send LIS email to all staff with patient demographics warnings.
   d. Add RG3 ESO flag.
   e. Post patient demographics in specimen processing and bacteriology and incubators containing culture plates using the **RG3 Alert signs**.

5. **Once suspicious growth appears, send isolate to PHOL**
   a. Notify PHOL of incoming RG3 organism.
   b. Package according to Transportation of Dangerous goods regulations (Only certified staff are permitted to do the packaging)

6. **Store plates in the Mycology** pending PHOL confirmation area.

7. Ensure all plates with **confirmed RG3 organism are autoclaved and disposed.**
Profile of Risk Group 3 Organism Cultures

*B. anthracis*

**Gram Stain:**

Photo courtesy of Dr. James Rudrick, Michigan Department of Community Health
https://www.asm.org/images/PSAB/LRN/Anthrax%20LRN%20091217.pdf

**Direct smear 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 rods.
- Spores usually not present in clinical specimens unless exposed to atmospheric O₂.

**Smears from sheep blood agar or other routine nutrient medium**
- 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:

![Culture Image](https://www.asm.org/images/PSAB/LRN/Anthrax%20LRN%20091217.pdf)

*B. anthracis* grows rapidly; 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.

On Sheep Blood Agar (SBA) - Nonhemolytic, flat or slightly convex colonies with ground-glass appearance; tenacious consistency (Hemolysis on SBA excludes *B. anthracis*). Often have comma-shaped protrusions from colony edge (“Medusa head” colonies).

If isolate is non-hemolytic, perform motility test using motility test media (*B. anthracis* is non-motile).

*B. anthracis* will not grow on McConkey (MAC) agar with crystal violet. Since the MAC plate we use is without crystal violet, this characteristic is not useful; this is why we do not include MAC as a media for primary isolation to avoid confusion.

Presumptive identification:

Presumptive identification of *B. anthracis* is based on identification of large gram positive bacilli that are [nonhemolytic](https://www.asm.org/images/PSAB/LRN/Anthrax%20LRN%20091217.pdf) on SBA and [non-motile](https://www.asm.org/images/PSAB/LRN/Anthrax%20LRN%20091217.pdf). If presumptive diagnosis of *B. anthracis*, proceed as [WHAT TO DO IF A RISK GROUP 3 ORGANISM IS SUSPECTED](https://www.asm.org/images/PSAB/LRN/Anthrax%20LRN%20091217.pdf). 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 bioterrorist threat agent: Preserve original specimens pursuant to a potential criminal investigation.
**F. 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)

Photo courtesy of MAJ Todd Kijek, USAMRIID

SBA - Non-hemolytic, gray-white colonies, 1-2 mm after 48 hrs  MAC - No growth

*Francisella* are also catalase positive, oxidase negative and urease negative which grow on BA & BCYE but not MAC.

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:**

![Gram Stain Image](https://www.asm.org/images/PSAB/LRN/Brucella316.pdf)

Tiny (0.5 to 0.7 μm by 0.6 to 1.5 μm), faintly staining, gram negative coccobacilli

**Culture:**

![Culture Image](https://www.asm.org/images/PSAB/LRN/Brucella316.pdf)

SBA - Small (0.5 to 1.0 mm) glistening, non-hemolytic, non-pigmented colonies after 2 to 3 days incubation

MAC - Some strains may grow slowly

*Brucella* spp. are also are oxidase positive and urea hydrolysis positive.
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".
**Y. pestis**

**Gram Stain:**

![Gram negative bacilli (1.0 by 0.5 μm) that may exhibit bipolar staining](https://phil.cdc.gov/details_linked.aspx?pid=1915)

**Culture:**

![SBA - gray-white to slightly yellow opaque colonies after 48 hrs incubation; Beyond 48 to 72 hrs incubation, colonies develop fried egg appearance. Little or no hemolysis. MAC - small, lactose negative colonies after 24 hrs incubation.](https://www.asm.org/images/PSAB/LRN/Ypestis316.pdf)
When slow growing gram negative bacilli as per growth characteristics described, follow Small/tiny Gram-negative bacilli/cocco-bacilli from all sites.

Report as "Gram negative bacillus / coccobacillus isolated. Further identification to follow".
APPENDIX I: Flowchart Work-Up To Rule Out Small or tiny gram negative bacilli / cocco-bacilli Potential Risk Group 3 Organisms

RG3 ALERT

Small or tiny gram negative bacilli / cocco-bacilli

Satelliting? YES

Growth at 24 hrs?

NO / Poor Growth

Perform Oxidase & Catalase

?Haemophilus Process routinely

YES

OX+ CAT+

R/O Brucella

R/O B. pseudomallei

Send to PHOL

OX- CAT-/weak+

R/O Francisella

OX- CAT+

R/O Y. pestis

OX v CAT+

R/O B. mallei

OX + CAT-

R/O Unusual ID
APPENDIX II: Flowchart OF *Bacillus* sp. Work-Up To Rule Out *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

  - **POS**
  - **MOTILITY** (tube motility test medium with TTC)
    - **NEG**
    - **Suspect B. anthracis**
    - **POS**
      - **Follow routine practices**

  - **NEG**
    - **NOT B. anthracis**
      - **NOT**
      - **Follow routine practices**
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

<table>
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<td>March 1 2002</td>
<td>Dr. T. Mazzulli</td>
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<td>May 12 2003</td>
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<td>April 4, 2018</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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