

**SENTINEL LEVEL CLINICAL LABORATORY GUIDELINES**  
**FOR**  
**SUSPECTED AGENTS OF BIOTERRORISM**  
**AND**  
**EMERGING INFECTIOUS DISEASES**

**Glanders: *Burkholderia mallei***

**and**

**Melioidosis: *Burkholderia pseudomallei***

American Society for Microbiology (ASM)

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**For latest revision, see web site below:**

<https://www.asm.org/Articles/Policy/Laboratory-Response-Network-LRN-Sentinel-Level-C>

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## PREANALYTICAL CONSIDERATIONS

### I. PRINCIPLE

#### A. Introduction

*Burkholderia mallei* is the etiologic agent of glanders, a febrile illness typically seen in equines: i.e., horses, mules, and donkeys (2, 4). It is a nonmotile, aerobic Gram-negative coccobacillus, which may or may not be oxidase positive or grow on MacConkey agar.

*Burkholderia pseudomalleus* was thought to cause a glanders-like illness in humans (30), and was called “*pseudomallei*” by Stanton and Fletcher (8). The disease in humans due to *B. pseudomallei* is now referred to in the medical literature as “melioidosis,” from the Greek word “melis,” which was the term for distemper in donkeys (20). *B. pseudomallei* is an oxidase-positive, aerobic Gram-negative bacillus that is straight or slightly curved. The organism will grow on most standard laboratory media, such as BAP, CHOC and MAC, and produces a characteristic musty odor (13). The recent sequencing of the genomes suggests that *B. mallei* is a recently evolved clone of *B. pseudomallei*. *B. mallei* has a smaller genome which makes it much less environmentally adaptable (15) Both species were classified as a member of the genus *Pseudomonas*, but in 1992, they were reclassified into the genus *Burkholderia* (35).

As of October 2012, *Burkholderia mallei* and *B. pseudomallei* are considered Tier 1 select agents because they present the greatest risk of deliberate misuse with most significant potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence. ([www.gpo.gov/fdsys/pkg/FR-2012-10-05/html/2012-24389.htm](http://www.gpo.gov/fdsys/pkg/FR-2012-10-05/html/2012-24389.htm)) *Burkholderia mallei* and *B. pseudomallei* are also classified as “Overlap select agents” because they not only have the potential to pose a threat to public health and safety, but they also pose a threat to animal health and animal products. ([www.selectagents.gov/select%20agents%20and%20toxins%20list.html](http://www.selectagents.gov/select%20agents%20and%20toxins%20list.html))

This procedure describes the steps to rule out, recognize, and presumptively identify these organisms in clinical specimens in Sentinel Clinical Laboratories. Such laboratories are defined as those certified to perform high complexity testing under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) by the Centers for Medicare and Medicaid Services for the applicable Microbiology specialty. Laboratory in-house testing includes Gram stains, and at least one of the following: lower respiratory tract, wound or blood cultures.

Sentinel Clinical Laboratories are not required to register with the Select Agent Program to conduct diagnostic testing for Select Agents, both Tier 1 and non-Tier 1. Testing for Select Agents may be performed by laboratories as long as the laboratory follows the policies listed in the reporting section of this document when a Select Agent cannot be ruled out. Consult with your designated LRN Reference Laboratory or refer to the CDC Division of Select Agents and Toxins website at <http://www.selectagents.gov> for questions.

## **B. Geographic distribution**

Glanders is a rare disease acquired by humans from exposure to infected horses. Because of a program of compulsory slaughter of infected or seropositive horses or other animals, *B. mallei* has been eradicated in the United States and western Europe. The last naturally acquired human case of glanders in the United States was seen in 1945 but a case of laboratory-acquired glanders occurred in 2000 (2). Equines are the primary reservoir of the rare cases of glanders still seen in Eastern Europe, the Middle East, Asia, and Africa (2).

Melioidosis is a disease endemic in the tropical regions of the world, with the majority of cases in the medical literature being reported from rice-growing regions of Southeast Asia and the tropical, northern regions of Australia (7, 16). The organism has been detected in very high concentrations in water found in rice paddies in both Vietnam and Thailand (13). There are data to suggest that this organism is also endemic in both the Philippines and the Indian subcontinent (8). There is little known about the prevalence of this organism in tropical regions of Africa and the Americas although cases have been recently documented in Brazil, countries either bordering the Caribbean and potentially the United States (3, 19, 31).

Infections with this organism in the United States and Western Europe are almost certainly imported from regions of endemicity (3, 13). Since the Vietnam War, melioidosis has been referred to as the “Vietnam time bomb” because the disease, much like tuberculosis, can reactivate in returning soldiers after remaining latent for decades (6, 8, 21). The recognition of multiple cases of melioidosis in North America or Western Europe in patients without an appropriate travel history requires a thorough investigation of the possibility of a bioterrorism attack.

## **C. Diseases and Clinical Presentation**

*Burkholderia mallei* infections can present either as cutaneous or systemic (2). The incubation period is typically 1 to 14 days. Patients with cutaneous infection will have nodules with accompanying localized lymphadenitis. The systemic illness usually manifests itself either as broncho- or lobar pneumonia. Bacteremia may also occur, resulting in lesions being seen in the liver and spleen. Infection in humans with *B. mallei* is often fatal without antimicrobial treatment (12).

Melioidosis can manifest as an asymptomatic, acute, subacute, or chronic process (6, 7). The typical presentation of acute infection is pneumonia, with high fever, dyspnea, and pleuritic chest pain. The most severe manifestation of acute melioidosis is septicemic pneumonia. Genitourinary infections are well described. Subacute infections can mimic those of *Mycobacterium tuberculosis*. Chronic infection is similar to millitary tuberculosis in that the infection is disseminated, and granulomatous lesions can be seen in a variety of tissues. Since melioidosis has a wide range of signs and symptoms, it can be mistaken for other respiratory diseases.

Two organisms which are very similar to *B. pseudomallei* phenotypically have recently been described in the literature (9, 10). *Burkholderia thailandensis* is an environmental organism found in rice paddy water and soil in Thailand. It has been shown to be of comparatively low virulence in animal models and has been infrequently reported to be a cause of human disease (9, 10). The second organism is *Burkholderia oklahomensis*. It has been reported from two cases in the US and it has been found to be essentially avirulent in animal models (9). Phenotypic testing is unable to differentiate *B. pseudomallei* from *B. oklahomensis*. Arabinose assimilation differentiates *B. thailandensis* (positive) from both *B. pseudomallei* and *B. mallei* (negative) but is not practical because few laboratories will have this capability. *B. thailandensis* and *B. oklahomensis* will not be differentiated from *B. pseudomallei* by the algorithm supplied in this protocol. However given the rarity of isolation of these two organisms in clinic settings, it is unlikely that they will be encountered in critically ill individuals.

Because travelers to endemic regions of Southeast Asia and Northern Australia may obtain *B. pseudomallei* from the environment, finding of this organism may not represent an intentional release of this organism. Regardless, organisms suspected of being either *B. mallei* or *B. pseudomallei* should be referred to the appropriate LRN Reference Laboratory, for confirmation.

## II. SAFETY CONSIDERATIONS

*Burkholderia mallei* and *B. pseudomallei* are infectious pathogens that can infect through the aerosol route (27). Infections following laboratory exposure have been documented with both organisms from exposure to cultures of the organisms in the research setting (2, 27). In reported cases, breaks in standard laboratory safety protocols were reported, emphasizing the importance of adhering to standard BSL 2 safety practices with these organisms. In one case, precautions were not taken because the organism was misidentified as *Burkholderia cepacia* (28). The danger is especially serious for persons with a diagnosis of diabetes mellitus (2, 27, 29). High risk of exposure in the clinical laboratory can be the result of exposure to culture aerosols outside a BSC; this would include splashes, centrifugation and vortexing (27).

*Burkholderia mallei* and *B. pseudomallei* require the use of biological safety level BSL-3 or BSL-2 with BSL-3 precautions (29) for all manipulations of cultures. Initial culture of specimens can be performed with BSL-2 practices. All patient specimens and culture isolates should be handled while wearing gloves and PPE in a BSC. Subcultures should be performed in a Class II BSC. Plates should be taped shut when incubating. All further testing should be performed only in the BSC while wearing gloves to protect from infections through the skin (27). Any procedure that can generate an aerosol, such as preparing standard inoculums for identification systems, must be performed in a BSL-2 BSC with BSL-3 precautions. Centrifugation and vortexing should be avoided. It is recommended that if *B. pseudomallei* or *B. mallei* is suspected that they be transferred to a BSL-3 facility as soon as possible (27).

Do not process nonclinical (environmental or animal specimens) in hospital laboratories. Restrict processing to human clinical specimens only. Veterinary laboratories are equipped to handle animal specimens. Nonclinical specimens should be directed to the designated LRN

Reference Laboratory.

Decontamination of laboratory surfaces is easily accomplished using a fresh solution of 10% bleach. Plates and specimens should be destroyed as directed by the LRN reference laboratory when the identification is confirmed.

### III. MATERIALS

#### A. Media

1. One of the following blood culture systems should be used for bone marrow aspirates and blood specimens.
  - a. Standard liquid blood culturing system with manual or instrument detection
  - b. Lysis-centrifugation system of ISOLATOR (Wampole Laboratories, Cranbury, NJ)
2. Media.
  - a. BAP
  - b. CHOC
  - c. MAC or EMB
  - e. Selective agars for *Burkholderia pseudomallei* including Ashdown and *Burkholderia cepacia* selective agars (13, 26). Standard enrichment broth (thioglycollate or brain heart infusion broth, may substitute others if they support the growth of environmental gram negative rods.)

#### a. Reagents

- i. Appropriate disinfectant such as phenol or 10% bleach
- ii. Gram stain reagents

**NOTE: Separate procedures for the biochemical tests listed below are located in the last section of the *General Introduction, Recommendation and Biochemical Procedures Document*.**

- iii. Catalase (3% hydrogen peroxide)
- iv. Oxidase (0.5 % tetramethyl-p-phenylenediamine)
- v. Spot indole reagent (see procedure for options)
- vi. Motility semisolid medium with 2,3,5-triphenyltetrazolium chloride (TTC) indicator
- vii. Disks:
  1. Colistin 10 µg or polymyxin B 300 U.
  2. Amoxicillin-clavulanic acid 20/10 µg
  3. Penicillin 10 U
- viii. Mueller Hinton agar
- ix. Optionally, for *Burkholderia pseudomallei* only, commercial gram-negative rod identification system;
  1. API NFT, also called the 20 NE or Vitek 2 with GN card

(BioMerieux, Durham, N.C.) have been studied extensively.

2. *Burkholderia pseudomallei* and *B. mallei* are not in the database of the Phoenix system (Becton Dickinson Microbiology Systems, Cockeysville, MD).

b. Equipment and supplies

- i. Biosafety cabinet
- ii. PPE (gloves, solid front gown)
- iii. 35-37°C incubator (5-10% CO<sub>2</sub> )
- iv. Light microscope with 100x objective and 10x eyepiece
- v. Microscope slides and cover slips
- vi. Pipettes, inoculating loops and swabs
- vii. Blood culture instrument (optional)

#### IV. QUALITY CONTROL

Perform quality control of media and reagents according to package inserts, most recent CLSI document M22, and CLIA standards, using positive and negative controls. Examine culture plates for contamination, poor hemolysis, cracks, and drying. Confirm the ability of CHOC to support growth of fastidious organisms. Quality control of biochemical tests using a positive and negative reacting organism should generally be performed on each lot of reagent. For frequency of quality control, refer to manufacturer guidelines and state and federal regulations. Refer to the biochemical test section for procedures and quality control organisms for each test.

Quality control of *Burkholderia cepacia* selective agars or Ashdown medium requires testing with an ATCC strain of *Burkholderia cepacia* as a positive growth control and *E. coli* as a negative growth control. Quality control with *B. pseudomallei* strains is not necessary and presents a safety hazard.

It is desirable for Sentinel Laboratories to subscribe to a proficiency program designed to test the competency of Sentinel Laboratories in detection of agents of bioterrorism. Should the laboratory identify a select agent, the laboratory is required to fill out and submit Form 4B within 90 days of receipt of the sample (<http://www.selectagents.gov>).

## V. SPECIMEN COLLECTION

A. Collection and Transport of Clinical Specimens for Laboratory Rule-Out Testing	
Bone marrow or whole blood	<ul style="list-style-type: none"> <li>• Considered the best specimen for culture.</li> <li>• Collect directly into an appropriate blood culture bottle</li> <li>• Transport bottles at room temperature as soon as possible to obtain the diagnosis</li> </ul>
Sputum or bronchoscopically obtained specimens	<ul style="list-style-type: none"> <li>• Collect expectorated specimen into sterile transport cup or collect during bronchoscopy procedure.</li> <li>• Transport at room temperature up to 2 h</li> <li>• If it is known that material will be transported from 2-24 h after collection, then store and transport at 2-8°C.</li> </ul>
Tissue specimens (biopsies, abscess aspirates) and wound swabs	<ul style="list-style-type: none"> <li>• Tissue pieces (at least the size of a pea) should be collected and kept moist</li> <li>• Transport in sterile container at room temperature within 1 hour of collection</li> <li>• Alternatively, a swab from a tissue sample can be submitted in hospital transport tube with medium to stabilize specimen (e.g. Amies charcoal).</li> </ul>
Urine	<ul style="list-style-type: none"> <li>• Collect at least 1 ml into leak-proof container</li> <li>• Transport at room temperature up to 2 h</li> <li>• Refrigerate 2 up to 24 h until culture inoculation</li> </ul>

### B. Rejection of specimens

1. Use established laboratory criteria for rejection of specimens for culture
2. Do not process saliva per laboratory protocol.
3. Do not process urine from contaminated collections per laboratory protocol
4. Environmental or non-clinical samples are not processed by Sentinel laboratories; contact your designated LRN Reference Laboratory state public health laboratory directly.

## ANALYTICAL CONSIDERATIONS

### VI. SPECIMEN PROCESSING

#### A. Blood and bone marrow

1. Aseptically inoculate liquid blood culture bottles with the maximum amount of blood or body fluid per manufacturers' instructions. Incubate at 35°C.
2. Alternatively, follow the manufacturer's instructions for the lysis-centrifugation method and inoculate pellet to BAP, CHOC and MAC. Incubate plates at 35°C.

- B. For respiratory specimens and tissues, inoculate BAP, CHOC and MAC and incubate at 35°C. Incubation in CO<sub>2</sub> is acceptable.
1. Enrichment broth should be inoculated for tissue specimens and wound swabs.
  2. Selective agar should be used if available and a high clinical suspicion exists (i.e. other cases have been reported) for the isolation of *B. pseudomallei* but not *B. mallei*. *Burkholderia cepacia* selective agar has been shown to be equivalent to Ashdown agar for this use (26).
  3. For improved isolation of specimens likely to contain indigenous microflora such as wounds and respiratory secretions, a colistin disk or polymyxin B disk may be placed in the initial inoculation area of the BAP if isolation of *Burkholderia* spp. is specifically requested.
  4. A smear for Gram stain should be made
- C. Urine cultures should be performed according to the institutions standard quantitative urine streaking technique on BAP and MAC.

## VII. INCUBATION AND EXAMINATION OF CULTURES

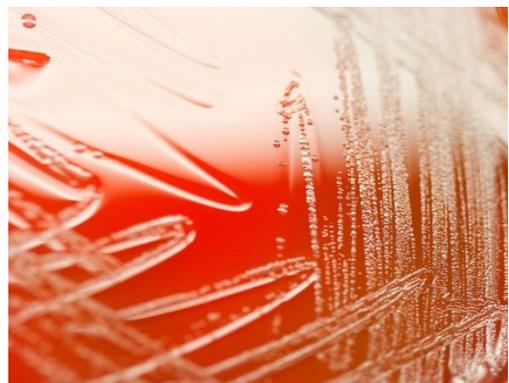
- A. *B. mallei* will not grow as rapidly as *B. pseudomallei* and may require extended incubation to 14 days for any specimen that is identified as possibly containing this organism. Otherwise, follow the standard laboratory recommendations below.
- B. Blood culture bottles
1. Incubate non-automated broth blood cultures for 7 days, with direct observations for turbidity daily and terminal blind sub-culturing.
  2. Incubate automated systems for 5 days.
  3. Plates from the lysis-centrifugation system should be incubated for 5 days.
  4. Daily observation for growth in the bottles is either automated or visual, depending on the system.
  5. For blind subculturing, inoculate to BAP and incubate plates at 35°C for 3 days before reporting as negative.
  6. For positive broth cultures, inoculate to BAP, CHOC and MAC. Place a colistin or polymyxin B disk on the BAP to differentiate *Burkholderia* from other organisms. Incubate plates at 35°C until growth appears.
  7. The recovery of *B. pseudomallei* from blood culture within the first 24 h of incubation indicates fulminant sepsis, which has a very high (90%) mortality rate.
- C. Plate culture incubation times:
1. Recommended incubation of BAP and CHOC is 5 days at 35°C.
  2. MAC need only be incubated for 3 days at 35°C in ambient air.
- D. All plates either from direct inoculation of specimens or from subculture of broths should be examined daily for growth of colonies.

## VIII. CULTURE IDENTIFICATION

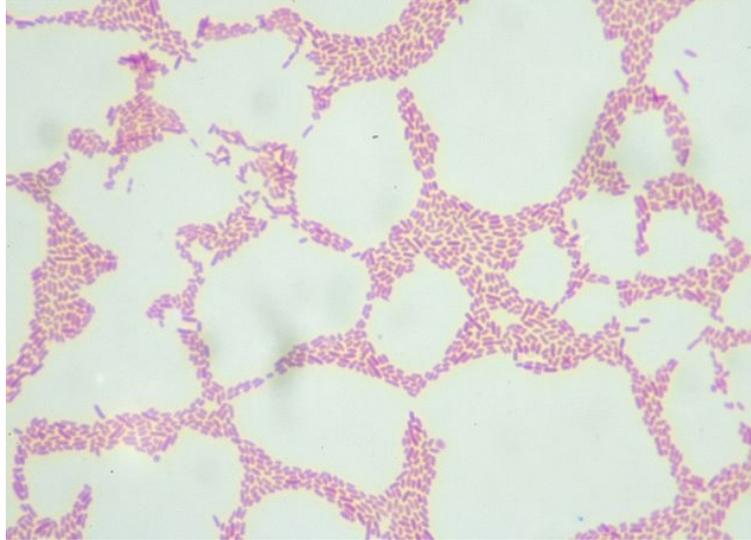
If your laboratory uses Mass Spectrometry (MALDI-TOF or bioMerieux) for bacterial identification and if the manufacturer provides your facility with an alternate tube extraction method, it is recommended that the resulting extract be filtered using a 0.2  $\mu$  (or less) filter. This additional step is recommended to reduce the risk of laboratory contamination with viable bacteria and spores.

Using automated systems, including Mass Spectrometry (MALDI-TOF, bioMerieux) technology may result in exposure to dangerous pathogens, and could result in erroneous identification, Ex: *Bacillus anthracis* misidentified as *B. cereus*; *Yersinia pestis* misidentified as *Y. pseudotuberculosis*.

- A. The procedures described below are to be used to rule out the presence of *B. mallei* and *B. pseudomallei* in clinical specimens. These procedures will not differentiate *B. pseudomallei* from *B. thailandensis* or *B. oklahomensis* (9, 10). The latter 2 species are not considered pathogenic. Because the 3 species share >99% homology at the nucleotide level, differentiation among them at the molecular level is challenging (1). Phenotypic testing is unable to differentiate *B. pseudomallei* from *B. oklahomensis*. Arabinose assimilation differentiates *B. thailandensis* (positive) from both *B. pseudomallei* and *B. mallei* (negative) (10). If the arabinose assimilation is positive with the API NE, the 2 species can possibly be differentiated (34). Other systems have not been reported to differentiate *B. thailandensis* from other species.
- B. Colony and Gram stain morphology
  1. *Burkholderia mallei*
    - a) On BAP, *B. mallei* shows smooth, gray, translucent colonies in 2 days, without pigment or distinctive odor. Colonies may or may not be present on MAC, but if present, they do not look like typical gram negative rods, but rather are pinpoint colonies in 48 h. The photographs below are taken from a 48 h culture on BAP.



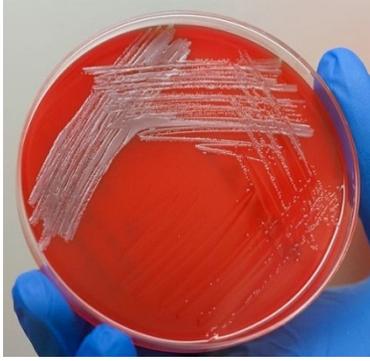
- b) *B. mallei* is a small, straight or slightly curved gram-negative coccobacilli measuring 1.5 to 3  $\mu\text{m}$  in length and 0.5 to 1  $\mu\text{m}$  in diameter with rounded ends. Cells are arranged in pairs, parallel bundles, or the Chinese-letter form. See photograph below.



**WARNING: If a Gram negative rod is isolated in a Sentinel Laboratory and it grows on BAP but does not grow on MacConkey agar, it should not be processed for identification by a multi-test kit or automated system, because of the danger of aerosol production due to preparing high concentrations of organisms. In addition, the identification by these systems can produce false results due to biochemically related organisms in the database. See below for preliminary identification of *Burkholderia mallei*.**

2. *Burkholderia pseudomallei*

- a) On BAP, *B. pseudomallei* will form small, smooth creamy colonies in the first 1 (photograph on left) to 2 days (photographs in center and on right), which may gradually change after a few days to dry, wrinkled colonies similar to *Pseudomonas stutzeri*. Colonies are greyish-white, non-hemolytic and are without violet pigment. Colonies are present on both BAP and MAC. *B. pseudomallei* often produces a distinctive musty or earthy odor that is very pronounced on opening a Petri dish growing the microorganism or even opening an incubator door when a positive plate is present. "Sniffing" of plates containing *B. pseudomallei* is dangerous and should not be done. The odor will be apparent without sniffing.



24 h

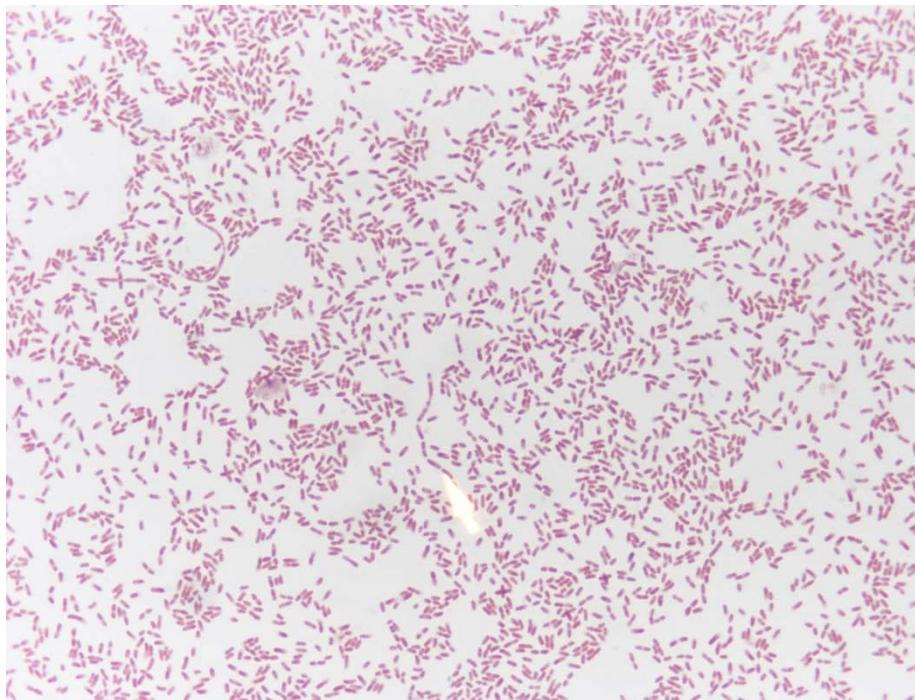


48 h



48 h

- b) *B. pseudomallei* is a straight, or slightly curved Gram-negative rod; may demonstrate bipolar morphology in direct specimens. It measures 2 to 5  $\mu\text{m}$  in length and 0.4 to 0.8  $\mu\text{m}$  in diameter.



All images are courtesy of the APHL.

- C. Perform the following biochemical tests in a BSC. See section on biochemical tests for methodology and interpretation of results.
1. Catalase test
    - a) If the isolate is *not* growing well on MacConkey agar in 48 hr perform catalase test.

- b) Both *B. pseudomallei* and *B. mallei* are catalase-positive.
- 2. Oxidase test
  - a) *B. mallei* is usually oxidase-negative but may be oxidase positive
  - b) *B. pseudomallei* is oxidase positive
- 3. Spot indole test – both species are indole negative.
- 4. Disk tests performed by Kirby Bauer method on Mueller Hinton agar: Colistin or polymyxin B, penicillin, and amoxicillin/clavulanic acid. Prepare inoculum using BSL-3 or BSL-2 with BSL-3 precautions. See separate procedure at end of protocols for interpretation of zone sizes.
  - a) Some species of *Bacillus*, which do not grow on MAC, can appear as Gram negative rods; however, it is either hemolytic or is penicillin susceptible, which differentiates it from *Burkholderia mallei* that do not grow on MAC and are resistant to penicillin. *B. pseudomallei* are also resistant to penicillin but easily grow on MAC.
  - b) *B. mallei* and *B. pseudomallei* are resistant to polymyxin B and colistin having no zone of inhibition around the disks.
    - (1) *Chromobacterium violaceum* is resistant to polymyxin B and colistin, but has a large zone of hemolysis on BAP and often has a violet pigment.
    - (2) *Ralstonia* are resistant to colistin or polymyxin B.
    - (3) As an alternative to polymyxin B or colistin testing, growth on *B. cepacia* selective agars or modified Thayer Martin may substitute for the colistin or polymyxin B disk test, because these media contain polymyxin B or colistin. However, the lack of growth on these media should be confirmed by the disk test.
    - (4) *Vibrio* are resistant to polymyxin B but are rarely confused with *Burkholderia* because they are glucose-fermenting rods.
  - c) Amoxicillin-clavulanic acid (20/10 mg) is helpful to differentiate from *B. cepacia* (14, 33) that are identified by commercial systems. Greater than 99% of *B. pseudomallei* are susceptible with  $\geq 18$  mm zones. *B. cepacia* are resistant.
  - d) *Burkholderia mallei* and *B. pseudomallei* do not show any pigment on Mueller Hinton agar.
- 5. Growth at 42°C should be tested by inoculation of BAP at 35°C and 42°C.
  - a) *B. mallei* does not grow at 42°C
  - b) *B. pseudomallei*, most *B cepacia* and *Ralstonia* grow at 42°C
- 6. Motility
  - a) The motility test should be performed if the isolate has the colony morphology and Gram stain reaction of *B. mallei* and

is resistant to colistin or polymyxin B, and penicillin but susceptible to amoxicillin-clavulanic acid.

- b) Because of the danger of laboratory-acquired infection, the wet mount motility should **not** be performed.
- c) The tube test is recommended using motility semisolid medium, preferably with indicator dye (see separate procedure).
- d) A diffusible red-colored growth spreading away from the stab line indicates motility.
- e) *B. mallei* is nonmotile, and *B. pseudomallei* is motile.

**WARNING: If *B. mallei* cannot be ruled out with the tests indicated above, further identification should not be attempted with commercial automated or kit identification systems, because of the danger of aerosol production due to preparing high concentrations of organisms. In addition, the identification by these systems produces false results due to biochemically related organisms in the database.**

#### 7. Commercial identification systems:

If the organism is growing on MAC, a commercial gram negative rod identification system is generally used in Sentinel Laboratories. These systems can sometimes be helpful in the identification, if safety precautions are used and their limitations are taken into consideration.

- a) If the system reports an organism that is colistin or polymyxin B resistant as *Burkholderia cepacia*, *Chromobacterium violaceum* or *B. pseudomallei*, it could be *B. pseudomallei*.
- b) If the colonies of such an organism are beta-hemolytic, the organism is not correctly identified as *B. pseudomallei*. It is likely either a *Vibrio* or *Chromobacterium violaceum*.
- c) If the Phoenix system is used, *B. pseudomallei* will not be separated from *B. cepacia*. All identifications of *B. cepacia* must be investigated to rule out *B. pseudomallei* using other biochemical tests, such as those listed here.
- d) Any isolate that screens as potentially *B. mallei*, should be referred to the LRN Laboratory for identification regardless of the identification by a commercial system.

#### Procedure note:

There is only one study that describes the reliability of commercial systems for the identification *B. mallei*. Both API 20 NE and RapID NF Plus (Remel, Lenexa, KS) either incorrectly identified or failed to identify 23 *B. mallei* isolates. (11).

While only the API 20NFT and the Vitek system have been studied extensively, commercial systems currently in use do not reliably identify *B. pseudomallei* (11, 17, 18, 23, 24). Initial studies suggested that the API 20E and 20NE systems and Vitek 2 were reasonably reliable systems for the identification of *B. pseudomallei* (17, 23). More recent studies suggest that the API 20NE is not as reliable for identification of *B. pseudomallei* when a greater variety of isolates were tested (11, 18); however, as with all manual methods, reliability is dependent on user expertise. *B. pseudomallei* is not always reliably separated from *B. cepacia* in the Vitek II system (36). In a recent study, when colorimetric rather than fluorometric based identification system was used in the VITEK 2, the identification of *B. pseudomallei* was still not particularly accurate (24, 32).

*B. pseudomallei* is not in the Phoenix database and has been reported to call the isolates *B. cepacia* (22, 32). For a review of the performance of commercial systems, see reference 25. In general, commercial systems may misclassify *B. pseudomallei* isolates as *B. cepacia* complex and *Chromobacterium violaceum* (17, 18, 23). The converse is also true.

#### **D. Presumptive identification of *Burkholderia* species**

##### **1. *Burkholderia mallei*:**

- a. Gram stain: small gram-negative coccobacilli measuring 1.5 to 3  $\mu\text{m}$  in length and 0.5 to 1  $\mu\text{m}$  in diameter with rounded ends.
- b. Colony morphology:
  - i. On BAP, *B. mallei* shows smooth, gray, translucent colonies in 2 days, without hemolysis or distinctive odor.
  - ii. Organism grows weakly or not at all in 48 h on MAC.
  - iii. Organism has no pigment, even on Mueller Hinton agar.
- c. Biochemical test results:
  - i. Catalase-positive
  - ii. Oxidase reactions are variable; most are negative.
  - iii. Spot indole-negative
  - iv. No zone with polymyxin B or colistin disk
  - v. Susceptible to amoxicillin-clavulanic acid but not penicillin
  - vi. Non-motile
  - vii. Does not grow at 42°C
- d. If the above reactions do not rule out *B. mallei*, do not use a commercial system or any results from a commercial system to further identify *B. mallei*. Submit to LRN Reference Laboratory for confirmatory identification.

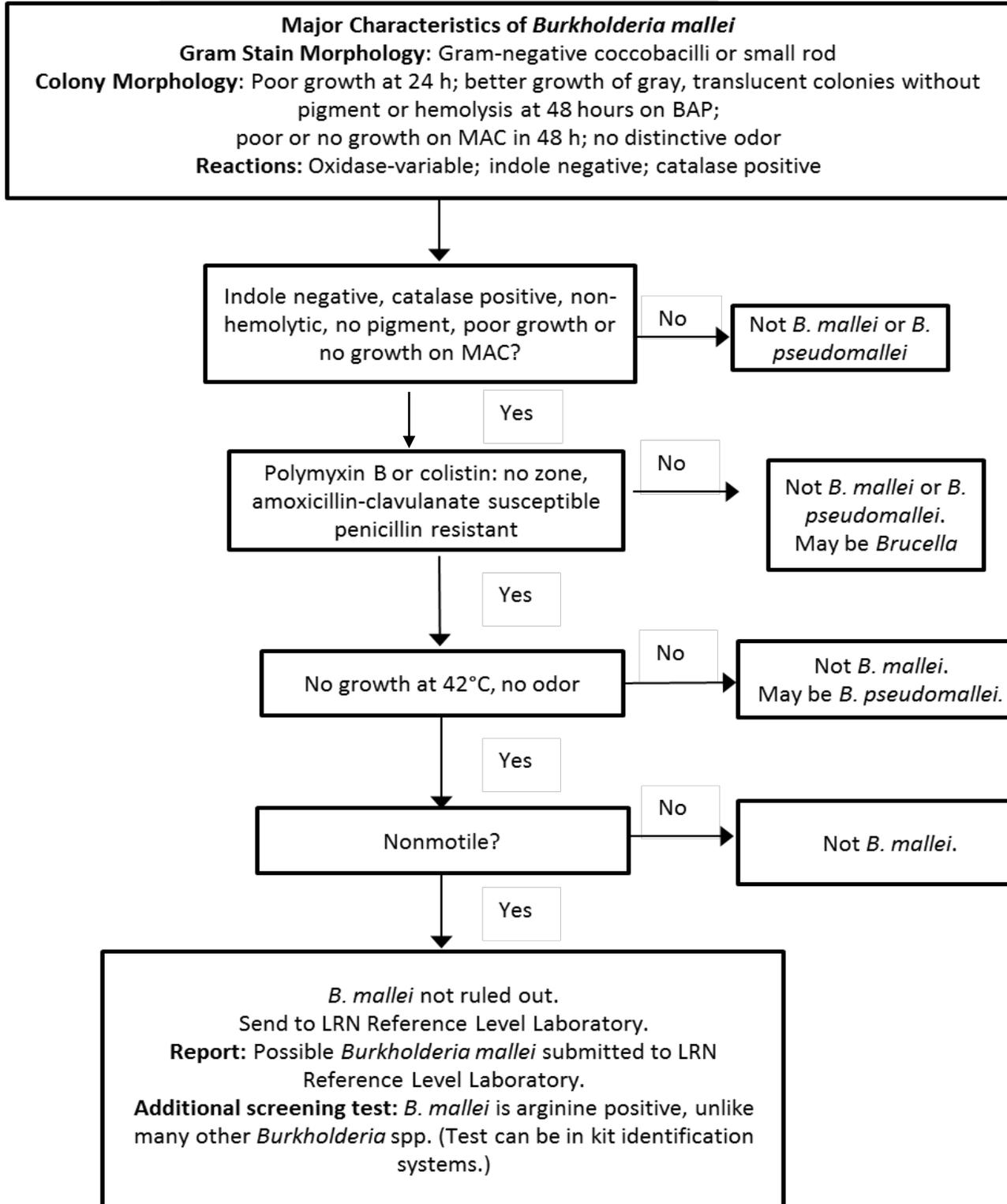
##### **2. *Burkholderia pseudomallei***

- a. Gram stain: *B. pseudomallei* is a typical gram negative rod that can demonstrate bipolar staining in direct specimens and peripheral staining in older cultures, which can mimic endospores.
- b. Colony morphology:
  - i. On BAP, *B. pseudomallei* appears as creamy colonies, which may gradually change after a few days to dry, wrinkled colonies. Colonies are non-hemolytic, and do not produce violet pigment on Mueller Hinton agar.
  - ii. Organism grows on MAC.
  - iii. *B. pseudomallei* often produces a distinctive musty or earthy odor, which is diagnostic.
- c. Biochemical reactions
  - i. Oxidase-positive
  - ii. Spot indole-negative
  - iii. No zone with polymyxin B or colistin disk
  - iv. Susceptible to amoxicillin-clavulanic acid but not penicillin
  - v. Motile
  - vi. Grows at 42°C
- d. Commercial gram-negative identification panels can be used for preliminary identification of *Burkholderia pseudomallei*, such as API 20NFT, also called 20NE, the API 20E or Vitek II, but the results should be evaluated compared to the known limitations of these systems.
  - i. Identifications of *Chromobacterium violaceum* for organisms that are not violet in color or are nonhemolytic could be *B. pseudomallei*.
  - ii. Identifications of *B. cepacia* for organisms that are susceptible to amoxicillin-clavulanate could be *B. pseudomallei*.
  - iii. Identifications of *B. cepacia* recovered from the blood or tissue of a non-cystic fibrosis patient, could be *B. pseudomallei*
  - iv. Commercial systems that give “no identification” for an organism that screens as potentially *B. pseudomallei*, could be *B. pseudomallei*.
- e. If the above reactions do not rule out *B. pseudomallei*, submit to LRN reference laboratory for confirmatory identification.

**NOTE:** Confirmatory identification is made by an LRN Reference Level Laboratory; refer to <http://www.bt.cdc.gov/lrn/biological.asp>

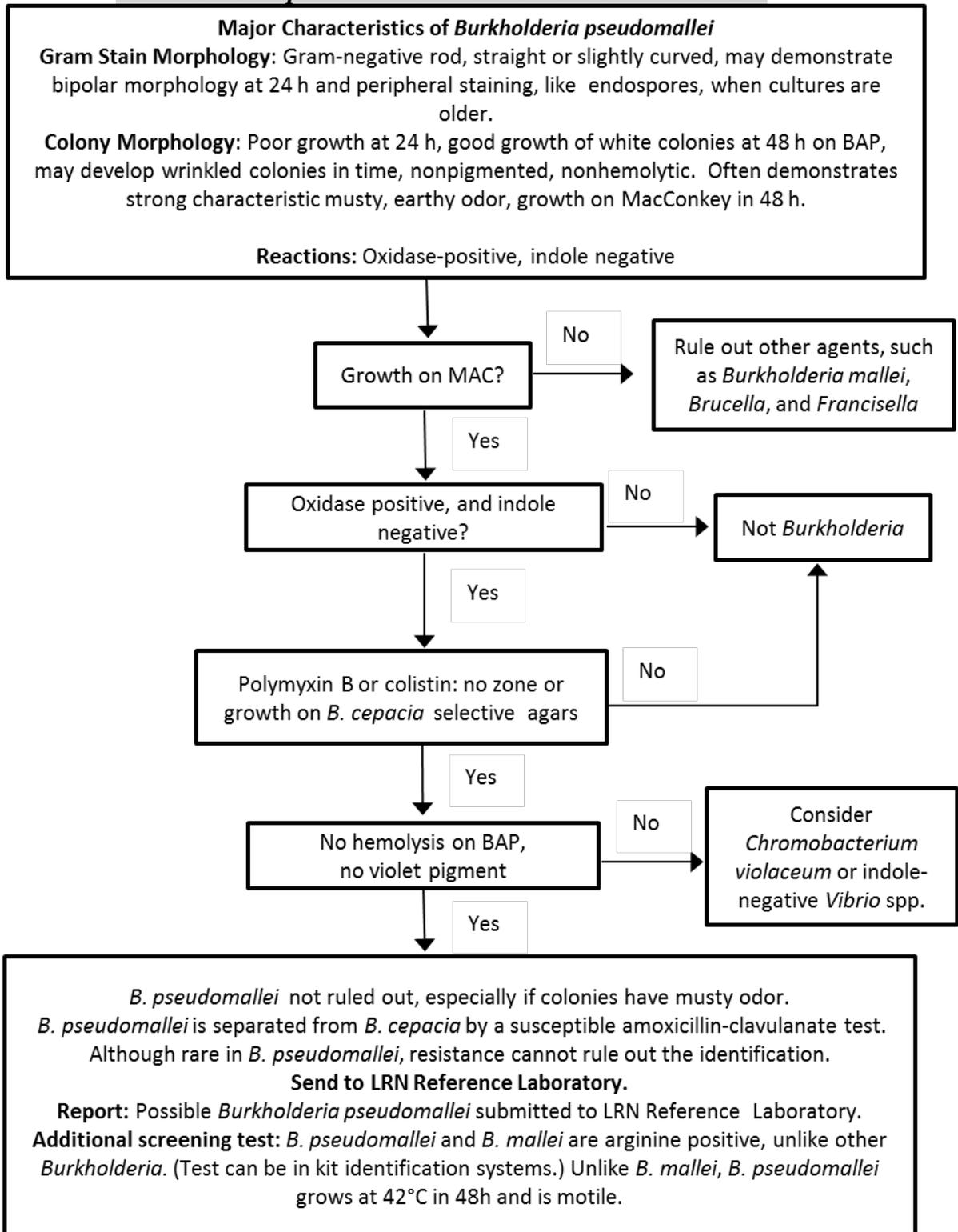
See flowcharts below for summary of major characteristics for presumptive diagnosis of *Burkholderia mallei* and *B. pseudomallei*

## *Burkholderia mallei* Identification Flowchart



**Note:** Biochemical test procedures and quality control instructions can be found at the end of the *General Recommendations and Biochemical Testing Procedures* document.

## ***Burkholderia pseudomallei* Identification Flowchart**



**Note:** Biochemical test procedures and quality control instructions can be found at the end of the *General Recommendations and Biochemical Testing Procedures* document.

## POST ANALYTICAL CONSIDERATIONS

### IX. REPORTING, NOTIFICATION, AND TRANSFER

A. *Burkholderia mallei* is suspected and cannot be ruled out if the isolate fulfills the following characteristics:

- Gram negative coccobacillus
- Growing on BAP as gray, translucent colonies that are non-hemolytic, non-pigmented, and odorless.
- Not growing or growing poorly on MAC in 48 h
- Oxidase-variable, catalase-positive, and indole-negative.
- Resistant to polymyxin B or colistin, resistant to penicillin and susceptible to amoxicillin-clavulanate
- No growth at 42°C
- Non-motile

B. *Burkholderia pseudomallei* is suspected and cannot be ruled out if the isolate fulfills the following characteristics:

- Gram negative rod that may demonstrate bipolar staining
- Growing on BAP as greyish-white colonies that are non-hemolytic and may be wrinkled.
- Growing on MAC in 48 h
- Oxidase-positive, catalase-positive, and indole-negative.
- Resistant to polymyxin B or colistin or growing on Selective agar
- Colonies are non-hemolytic without violet pigmented on Mueller Hinton agar
- May have musty odor. **Not all *B. pseudomallei* have the characteristic odor, which cannot be used to rule out the organism.**
- Additional screening to increase specificity of identification, if available:
  - a. 99% are amoxicillin-clavulanate susceptible
  - b. Motility positive
  - c. Grows at 42°C
  - d. Glucose oxidizer
  - e. Arginine-positive, lysine and ornithine-negative

The above biochemical reactions can even be evaluated from the API 20E, if the laboratory has no system specific for glucose non-fermenting organisms. If any identification system is not incubated for 48 h or a heavy inoculum used, the arginine can be negative, leading to misidentifications.

- If an identification system yields an identification of *Burkholderia pseudomallei*, *B. cepacia* or *Chromobacterium violaceum*, or “no identification” and the above listed tests are consistent with *B. pseudomallei*, *B. pseudomallei* cannot be ruled out.

Because of the danger of misidentification, Sentinel Laboratories should confirm the identification of *B. cepacia*, especially if it is recovered from the blood or tissue of a non-cystic fibrosis patient. *B. cepacia* are never susceptible amoxicillin clavulanate, but 99% of *B. pseudomallei* are susceptible.

- C. Notifications and submission of cultures if *B. mallei* or *B. pseudomallei* cannot be ruled out by above characteristics.
1. Generate a report to the physician that *Burkholderia pseudomallei* or *B. mallei* cannot be ruled out.
  2. Do not attempt full identification and susceptibility testing in the Sentinel Clinical Laboratory.
  3. Immediately notify your designated LRN Reference Laboratory, which will provide the referring laboratory with guidance and recommendations for retaining the specimen or isolate and submission for confirmative identification.
  4. Preserve original specimens pursuant to a potential criminal investigation and transfer to your designated LRN Reference Laboratory in accordance with state and local requirements. In particular, the appropriate material, including blood culture bottles, tubes and plates, and actual clinical specimens (aspirates, biopsies, sputum specimens) should be documented, and either submitted to the LRN Reference Laboratory or saved until the Reference Laboratory confirms the identification.
  5. Do not ship specimens or cultures to LRN Reference Laboratories without prior arrangements.
  6. Notify other public health authorities (e.g. state public health department epidemiologist/health officer) as required by local and state communicable disease reporting requirements. The state public health laboratory/state public health department will notify law enforcement officials (state and federal), such as local FBI agents, as appropriate.
  7. Within the hospital setting, immediately notify the infection preventionists and/or infectious disease service so that the patient can be treated appropriately, infectious precautions can be taken, and a further investigation of the patient’s history can be made.
  8. Consult with the LRN Reference Level Lab about additional clinical specimens that may be submitted for testing
  9. Initiate documentation, showing the specimen identification control, notification and transfer to the designated LRN Reference Laboratory, and documentation of all plates and tube cultures, which will need to be destroyed or transferred once identification has been completed.

- D. Sentinel Laboratories should consult with the designated LRN Reference Laboratory prior to or concurrent with testing, if *Burkholderia mallei* or *B. pseudomallei* is requested by the physician or a bioterrorist event is suspected. Obtain guidance from the state public health laboratory as appropriate (e.g., requests from local law enforcement or other local government officials). FBI and state public health laboratory/state public health department will coordinate the transfer of isolates/specimens to a higher-level LRN laboratory as appropriate.
- A. If *Burkholderia mallei* and *B. pseudomallei* species is ruled out, proceed with efforts to identify using established procedures.
- B. If other cases are suspected or there is a laboratory exposure, collect samples to submit to the designated LRN Reference Laboratory for serological testing.

## X. SUMMARY/SPECIAL CONSIDERATIONS

### A. Antimicrobial susceptibility

1. Antimicrobial susceptibility testing of *Burkholderia mallei* and *B. pseudomallei* is only performed by the broth method in a reference laboratory equipped to test agents of bioterrorism in a BSL level 3 facility (5, 12). It is not appropriate for Sentinel Laboratories to perform testing for either *B. mallei* or *B. pseudomallei*.

- a. Therapy

There are no case controlled trials for treatment of either *B. mallei* or *B. pseudomallei*. Therapeutic choices are based on case series and expert clinical experience (27, 31). Additionally there is a paucity of clinical experience for treating *B. mallei* infections with “modern” antimicrobials such as third generation cephalosporins, carbapenems, beta-lactamase inhibitors/beta-lactams, and fluoroquinolones so antimicrobial recommendations for treatment of *B. mallei* are the same as those for *B. pseudomallei*.

Current recommendations for treatment of *B. pseudomallei* are to initially administer ceftazidime, meropenem, or imipenem for 10 to 14 days, followed by prolonged oral eradication therapy with trimethoprim/sulfamethoxazole for 3 to 6 months. In trimethoprim/sulfamethoxazole allergic individuals, doxycycline and amoxicillin-clavulanate are alternatives. Drug dosages are available in reference 27 and 31.

2. Post-exposure prophylaxis (PEP) for laboratory workers is recommended following laboratory exposure for both *B. mallei* and *B. pseudomallei*. There are no efficacy data on any antimicrobial agents for PEP. Based on animal data for *B. pseudomallei*, the Centers for Disease Control and Prevention recommends the

use of oral agents given over a three week period for both *B. pseudomallei* and *B. mallei*. Trimethoprim/sulfamethoxazole is the drug of choice; for patients who cannot tolerate this agent, amoxicillin-clavulanic acid or doxycycline may be used as alternatives. Dosing recommendations can be found in reference 27. Because of high failure rates in the treatment of clinical cases of melioidosis, ciprofloxacin is not recommended as PEP.

## **B. When to refer to the LRN Reference Laboratory**

1. Naturally occurring cases of *B. mallei* are extremely rare in humans and should be referred to LRN Reference laboratories in all cases.
2. Naturally occurring cases of *B. pseudomallei* may be observed in any of the following situations. Confirmation of the identification of these isolates may be requested from a LRN Reference laboratory, but they are unlikely to represent a bioterrorism event. Patients who cannot be classified into any of the following patient populations may represent a bioterrorism event.
  - i. Patients with acute infection who have a recent history of travel to the region of endemicity. This includes Southeast Asia (in particular Thailand, Vietnam, Myanmar, or Taiwan), the Philippines, the Indian subcontinent, or the northern coast of Australia. Recent US cases have also been imported from countries either bordering the Caribbean. Cases have been reported in Brazil so *B. pseudomallei* may also be considered, for biothreat reasons, as endemic in tropical regions of Central and South America.
  - ii. Recent immigrants or visitors from the region of endemicity.
  - iii. Patients with recent onset of diabetes, renal failure, or immunosuppressed states who have travelled in the region of endemicity mentioned above even if that travel occurred decades before.
  - iv. Individuals (such as zoo employees) who work with animals that have recently been imported from regions of endemicity.
  - v. Individuals who work in laboratories where they may be exposed to this organism.

## **C. Select Agent reporting and compliance**

1. Reporting of all identified Select Agents is still required, even though Sentinel laboratories are not required to register under the Select Agent Rule.

2. The laboratory must complete Form 2 within one week (7 days) following notification of the confirmed identification. For further guidance and access to the necessary forms, consult with your designated LRN Reference Laboratory or refer to the CDC Division of Select Agents and Toxins website at [www.selectagents.gov](http://www.selectagents.gov).
3. Reporting all identified Select Agents is required by completing Form 4 A within 7 days of confirmed identification. If the isolate is from a Proficiency test sample, Form 4 B is to be completed within 90 days of receipt of the sample.
4. Your designated LRN Reference Laboratory will advise you with completion of required forms (e.g., Forms 2, 3, and 4). Always refer to [www.selectagents.gov](http://www.selectagents.gov) for the latest guidance and versions of these forms.

#### **D. Destruction**

1. Once the identification of the isolate is confirmed, the Sentinel Laboratory Select Agent regulations require that the residual specimen and cultures of the isolate be destroyed or transferred to an approved Select Agent entity within 7 days of confirmed identification. Your designated LRN Reference Laboratory must advise you on destruction or transfer of isolates.
2. Generally all plates and clinical material that contain the organism should be autoclaved, incinerated on-site or submitted to the designated LRN Reference Laboratory for disposal.
3. Alternatively, contaminated items should be soaked in 10% bleach or 10% formalin for 24 h.

#### **E. Packing and shipping**

1. Refer to the ASM Packing and Shipping Sentinel Guidelines.
2. All materials sent to your designated LRN Reference Laboratory must be shipped in compliance with IATA and DOT regulations

#### **X. Limitations**

1. Do not attempt to identify gram-negative rods that do not grow on MAC using a commercial identification system because of their lack of accuracy and danger of aerosols.
2. Understand that *B. pseudomallei* infections may be naturally occurring and may not represent a biocrime. The infected patient's potential exposure to naturally occurring *B. pseudomallei* either due to travel or workplace exposures should be determined

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