STANDARD OPERATING PROCEDURE (SOP) for

ISOLATION OF *Burkholderia pseudomallei*

FROM CLINICAL SAMPLES

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1. Aim

Burkholderia pseudomallei is a non-fermenting Gram-negative bacillus that is oxidase positive. The culture of *B. pseudomallei* from any specimen remains the gold standard for diagnosis of melioidosis. Samples of blood, urine and a throat swab should be obtained for culture from all patients, together with respiratory secretions, pus and wound swab where relevant. All specimens from patients with suspected melioidosis should be handled with the appropriate safety precautions (see Risk Assessment). The organism grows well on most routine laboratory media but specimens from non-sterile sites can benefit from the use of selective media (such as Ashdown's agar and selective broth), which permit the growth of *B. pseudomallei* and suppress other organisms. The methods for isolation, identification and susceptibility testing of *B. pseudomallei* are detailed in the following sections.

2. Method

2.1 Blood culture

- Aseptically collect 8-10 mL blood and inject into BACTEC™ Plus Aerobic/F Culture Vials catalogue number 442192 (Becton Dickinson, Cockeysville, MD, USA). The principle use of these media is with the BD BACTEC fluorescent series instruments.
- Incubate the bottles at 35°C in the BACTEC 9120 instrument for 7 days, or until detected as positive by the machine.
- Positive bottles are evaluated with Gram stain and subcultured on blood agar.
- Incubate the plates at 37°C in air for 2 days and inspect the plates daily.

Note: The inoculated BACTEC vial should be transported to the laboratory as quickly as possible.

2.2. Respiratory secretions, pus, fluid and wound swab culture

- Plate the neat sample (≥10μl) or wound swab onto one half each of blood agar, MacConkey agar and Ashdown's agar then inoculate the sample or swab into selective broth. Streak all inoculated plates to obtain single colonies.
- Incubate the agar plates at 37°C in air for 2 days (for blood agar and MacConkey agar) or 4 days (for Ashdown's agar). Inspect the plates daily.
- Incubate the selective broth at 37°C in air for 2 days, and then sub culture 10μl from the surface of the broth onto half an Ashdown's agar plate. Then, incubate the Ashdown's agar plates at 37°C in air for 4 days and inspect the plates daily.

2.3. Throat swab

- Plate the swab directly onto half an Ashdown's agar plate, and then place the swab into selective broth. Streak the inoculated plate to obtain single colonies.
- Incubate the plates at 37°C in air for 4 days, and inspect the plates daily.
- Incubate the selective broth at 37°C in air for 2 days, and then subculture 10μl from the surface of the broth onto half an Ashdown's agar plate. Then, incubate the Ashdown's agar plates at 37°C in air for 4 days and inspect the plates daily.

2.4. Urine culture

- Spread 1μl of fresh, unprocessed urine using a sterile calibrated loop onto one half each of a MacConkey agar plate and an Ashdown's agar plate
- Centrifuge the remaining urine sample at 3,000 rpm for 5 min
- Collect the urine pellet (in the bottom) using a sterile transfer pipette, and use one drop of the urine pellet for culture on half an Ashdown's agar plate.
- Incubate the plates at 37°C in air for 2 days (MacConkey agar) or 4 days (Ashdown's agar)
- Inspect the plates daily, and record the quantitative count of *B. pseudomallei*.

Record urine culture results with quantitative count (CFU/ml) based on the presence and number of *B. pseudomallei* colonies observed as follows:

Culture positive only from the centrifuged pellet ($<10^3$ CFU/ml)
Culture positive from neat sample:
1-9 colonies (10^3-10^4CFU/ml)
10-99 colonies (10^4-10^5 CFU/ml)
≥ 100 colonies (≥ 10^5 CFU/ml)

Note: B. pseudomallei is not a member of the human commensal flora, and the isolation of even a single colony in urine (or any other sample) is both significant and sufficient to confirm the diagnosis of melioidosis.

3. Identification of *Burkholderia pseudomallei*

3.1 Basic colony morphology

- Perform Gram stain: *B. pseudomallei* is a Gram-negative bacillus.

  Note: *B. pseudomallei* frequently does not show bipolar-staining on Gram stain, but it is often pleomorphic and usually stains slightly unevenly.

- Evaluate colony morphology: *B. pseudomallei* colonies are usually cream coloured with a

metallic sheen and may become dry and wrinkled after >24 hours' incubation on blood agar, although considerable variation is seen. On MacConkey agar, *B. pseudomallei* colonies will initially be colourless and opaque with a metallic sheen but become pink after 48 hours (thought to be due to the uptake of dye from the medium), frequently with a central umbo. *B. pseudomallei* on Ashdown's agar grows as very small (pin point) colonies by 18 hours, which are usually purple, flat, dry and wrinkled after 48 hours of incubation.

3.2 Latex agglutination test

Initial screening of suspect colonies from any agar medium is undertaken by latex agglutination using latex particles coated with monoclonal antibodies specific for the 200-kDa exopolysaccharide of *B. pseudomallei*.

Method:

- Pipette 10 µl of control and test latex onto a glass slide

  Note: Controls do not have to be tested with every sample but should be run in tandem on each testing day.

- Using a toothpick, touch the suspected colony and emulsify the colony in the test latex.
- Rock the samples to mix to allow the reaction to occur.

Interpretation:

- Observe for agglutination. Agglutination (positive) may be rapid or may take up to 20 secs. Observe for at least 2 mins before declaring the status of the sample as positive or negative.

Note: Any Gram-negative bacillus that is oxidase positive and not Pseudomonas aeruginosa isolated from any clinical specimens (including blood, sputum, urine, pus and fluid collected from normally sterile sites) should ideally be screened by ***latex agglutination****, particularly if it is found to be resistant to aminoglycosides and colistin or polymyxin but susceptible to co-amoxiclav.*

3.3 Colistin (CT) and gentamicin (CN) antibiotic screening test

*Method***:**

- Colonies that are positive by latex agglutination or morphologically typical on Ashdown's agar should be evaluated with CT and CN antibiotic screening test
- Streak colonies onto half of a Columbia agar plate.
- Apply 10 µg Colistin (CT10) disc on the 1st streak area and 10 µg Gentamicin (CN10) disc on the 2nd steak area.
- Incubate the plates at 37°C in air for 24 hours

Interpretation:

- *B. pseudomallei* is normally resistant to colistin and gentamicin (no zone) and so any latex positive isolate with this susceptibility pattern can be reported as presumptive *B. pseudomallei*. If any clear zone is detected, do drug susceptibility testing on Mueller Hinton agar (standard method) for confirmation. Further identification will be carried out for any presumptive isolates in the Bangkok lab using either API 20NE or MLST.

3.4 Assessment of antibiotic susceptibility of *B. pseudomallei*

To determine the level of relative antibiotic susceptibility or resistance of individual *B. pseudomallei* isolates.

Method:

- Select 5-7 colonies of pure *B. pseudomallei* from culture-positive 48-hour Ashdown's agar or 24-hour Columbia agar plates with a sterile cotton swab.
- Emulsify colonies in 3 ml of normal saline (0.85% w/v NaCl). The suspension is adjusted to achieve a turbidity equivalent to a 1.0 McFarland standard (Densimat). This results in a suspension containing approximately 1 to $2x10^8$ CFU/mL.
- Within 15 minutes after adjusting the turbidity of the suspension, dip a new sterile cotton swab into the adjusted suspension. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level. This removes excess fluid from the swab.
- Inoculate the swab onto a Mueller-Hinton agar plate using a rotary spreader, starting at the centre and moving the swab slowly to the edge of the agar surface so that the whole plate is equally inoculated.
- Allow the plate to dry for 5-10 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the disks.
- Dispense the suggested set of antimicrobial disks (detailed below) onto the surface of the inoculated agar plate by using the disk dispenser. Each disk must be pressed down to ensure complete contact with the agar surface.
- Invert the plates and place in an incubator set at 37°C in air within 15 minutes after the disks are applied.
- After 18 to 24 hrs of incubation examine each plate. If the plate was satisfactorily inoculated the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. Measure the zones to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate. Any discernible growth within the zone of inhibition is indicative of resistance or contamination and needs to be identified by latex agglutination test and, if positive, repeat susceptibility test.

Note: no interpretive Clinical and Laboratory Standards Institute (CLSI) guidelines exist for disk diffusion testing of B. pseudomallei. Zone diameters have been adapted from data for related organisms, such as non-Enterobacteriaceae or Pseudomonas species. Caution needs to be exercised in interpreting zone diameters for trimethoprim/sulfamethoxazole as

overgrowth and indistinct endpoints can lead to the incorrect reporting of resistance. Isolates assigned as 'susceptible' or 'intermediate' to co-trimoxazole by disc diffusion may be viewed as 'susceptible'; those assigned as 'resistant' require further evaluation by MIC methodology or Etest. E-test, though not specifically recommended by the CLSI, uses the same breakpoints as broth microdilution minimal inhibitory concentration (MIC)

Interpretation:

Table 1: Zone diameter (mm) Limits for Individual Tests on Mueller-Hinton Medium for *Burkholderia pseudomallei*.

Antimicrobial Agent	**Disk (μg) Content**	**Resistant**	**Intermediate**	**Susceptible**
Amoxycillin/clavulanic acid	30	≤13	14-17	≥18
Ceftazidime	30	≤14	15-17	≥18
Doxycycline	30	≤12	13-15	≥16
Imipenem	10	≤13	14-17	≥18
Meropenem	10	≤12	13-15	≥16
Trimethoprim/sulfamethoxazole*	1.25/23.75	≤10	11-15	≥16

* Drug susceptibility for co-trimoxazole may be tested by Etest for every isolate if budget allows. Isolates classified as resistant by disc diffusion need to be re-tested by E-Test.

CO-TRIMOXAZOLE ETEST:

MIC ≤2/38 mg/L = susceptible
MIC ≥4/76 mg/L = resistant

4. Quality Control

The goals of a quality control programme are to monitor the following:

- the precision and accuracy of the susceptibility test procedure;
- the performance of reagents used in the test; and
- the performance of persons who carry out the tests and read the results.

Every time a new lot of agar or a new lot of antimicrobial disks is introduced, it must be tested with the appropriate quality control strains, otherwise the control organisms should be tested weekly and the zones of inhibition recorded in mm.

Table 2: Quality-Control Limits for Monitoring Antimicrobial Disk Susceptibility test

Reference strain	**ATCC ref.**	**AMC 20/10 µg**	**CAZ 30 µg**	**SXT 1.25/23.75 µg**	**MEM 10 µg**	**IMP 10 µg**	**DO 30 µg**
E. coli fully-sensitive	ATCC 25922	18-24	25-32	23-29	28-34	26-32	18-24
E. coli β-lactamase positive, AMC-sensitive	ATCC 35218	17-22	-	-	-	-	-
Staphylococcus aureus	ATCC 25923	28-36	16-20	24-32	29-37	-	23-29
Pseudomonas aeruginosa	ATCC 27853	-	22-29	-	27-33	20-28	-

Co-trimoxazole Etest results for *E. coli* 25922 should be between 0.064 - 0.25 µg/mL.

5. Storage of *Burkholderia pseudomallei* isolates

Storage criteria

a. All primary isolates from every site or sample of similar origin (*i.e.* T/S and sputum)
b. Subsequent isolates only if sensitivity changes
c. Other isolates if significant variation in colonial morphology. *e.g.* mucoid.
Note: Isolates should be grown on agar (24 hour) without antibiotics before collection for storage.

Method:

- Inoculate *Burkholderia pseudomallei* from Mueller-Hinton agar to one "TSB plus 20% Glycerol" vial.
- Use heavy inoculum and store at –80°C.

***Records*:**

a. Record details of all stored isolates in the "stored isolates" file.
b. Each patient should be given a strain number and a running alphabet letter in the order that they were stored (*e.g.* HBPUB10001A, HBPUP10001B, HBPUB10002A, etc.) according to the storage criteria above.
c. Samples from relapsed patients must use the original Ubon strain number. Also, record details of antibiotic treatment and the presence of atypical colonial morphology.

6. COSHH risk assessment - University of Oxford COSHH Assessment Form	
Department Microbiology **Location of work** Ubon	**Persons involved** Microbiology staff
Description of procedure Culture and in vitro isolation of *B. pseudomallei*	**Substances used** All clinical samples from suspected patients
Quantities used Various	**Frequency of use** Daily
Hazards identified Possible infection risk via puncture wound, mucous membranes and aerosol. The infectious dose is not known but is thought to be low via the aerosol route	**Could a less hazardous substance be used instead?** No.

What measures have you taken to control risk?

i. Transport specimens from ward to laboratory using proof-leaking plastic zip bag
ii. Perform all manipulations in Class II Biological Safety Cabinet (BSCII)
iii. Centrifuge samples in bio-contained buckets and opened in BSCII
iv. Transport agar plates, slides, APIs etc. either in plastic sealed container or carry rack before removal from the BSC to incubator or microscope.

PPE:

i. As designated for blood collection
ii. Designated gown for BSCII work
iii. N95 Mask (in case of spill)
iv. Gloves
v. Hair net (as requested)
vi. Covered shoe

Special practices and procedures:

i. Minimize the use of glassware and sharps as far as possible
ii. Have a durable, puncture proof sharps container near the disposal area for glass, needles and other sharps
iii. Change gloves if they are contaminated, and wash hands between glove changes and prior to leaving the laboratory.
iv. Do not touch common items in the laboratory while wearing gloves (i.e. phone, computer keyboard)

Checks on control measures

Annual certification of BSCII
Observation and supervision by senior staff

Is health surveillance required? No.	**Training requirements:** i. BSL3 and BSCII. ii. The procedure for donning and doffing PPE iii. Centrifugation iv. Waste disposal v. Accident reporting procedures vi. Spill clean up procedures vii. Signs and symptoms of infection viii. Sample handling and transportation
Emergency procedures: i. Eyes – Eyewash procedure - Report ii. Aerosol – Report iii. Needle stick- Apply mild pressure to wound to induce bleeding or expression of material. Wash exposed skin area thoroughly with soap and water being careful not to further abrade skin. Contact CSO/DSO and follow MORU-CS-002. iv. Report incident to CSO/DSO/Area supervisor v. Contact Clinical Safety Adviser whenever staff are thought to have been exposed for a risk assessment regarding the need for antibiotic prophylaxis **Spills** i. In BSCII – clean using 1% Virkon ii. Outside BSCII – Do not clean up – evacuate the lab and contact Area supervisor immediately.	**Waste disposal procedures**: 1% Virkon overnight then autoclave 121°C/15 minutes
Name and position of assessor: *Dr.Stuart Blacksell, Biological Safety Officer*  **Signature:** **Name of head of department or nominee:** *Dr. Direk Limmathurotsakul*  **Signature:**	