Chapter 9: Testing Workflows for the Identification of *B. pseudomallei*

Chapter Overview

Samples from patients or animals that meet the case definition for suspected melioidosis should undergo testing for *B. pseudomallei*. This testing protocol should follow a workflow or algorithm. Laboratories should decide what type of workflow works best for their setup, however, keep in mind culture detection is generally accepted as the gold standard for laboratory diagnosis of *B. pseudomallei*. Some suggested workflows and rule-out flowcharts that laboratories may utilize when encountering a suspected *B. pseudomallei* isolate from clinical specimens are shown below.

Testing Workflow 1 (APHL 2016):

Microscopy Characteristics

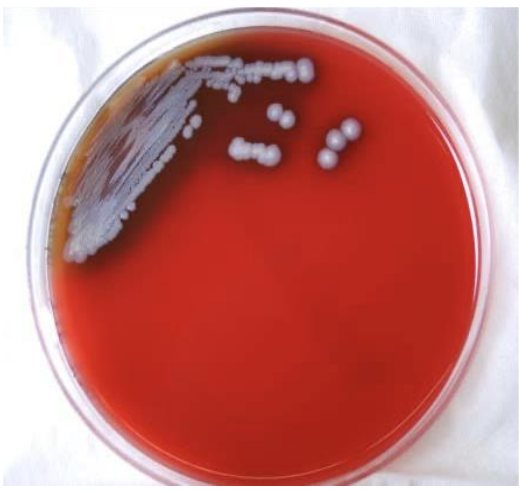
1. Gram Stain
    a) Small gram-negative rods (2-5 x 0.4-0.8 µm)
    b) Organized in long, parallel bundles (smooth form) or irregularly (rough form)
    c) Bipolar staining (not seen in mucoid strains)
2. Colony Morphology and Growth
    a) On Sheep Blood agar (BAP) and Chocolate agar (CHOC):
        - Pinpoint colonies at 24 hours becoming smooth, creamy white colonies. May exhibit metallic sheen >48 hours
        - Colonies may show mucoid or dry and wrinkled morphology after 48-72 hours
    b) Pink on MacConkey agar (MAC) or Eosin Methylene Blue agar (EMB) at 24-48 h due to oxidation of lactose

c) Growth at 42°C

d) Characteristic musty or earthy smell (do not sniff plate)

24 HOURS ON BAP

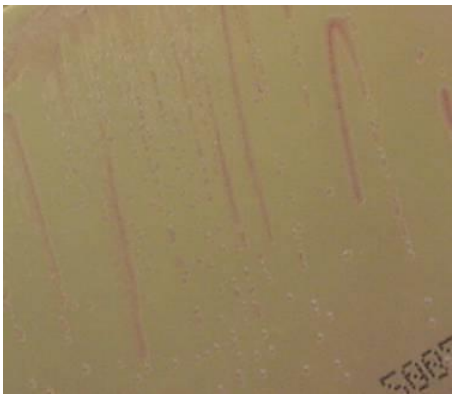
24 HOURS ON CHOC



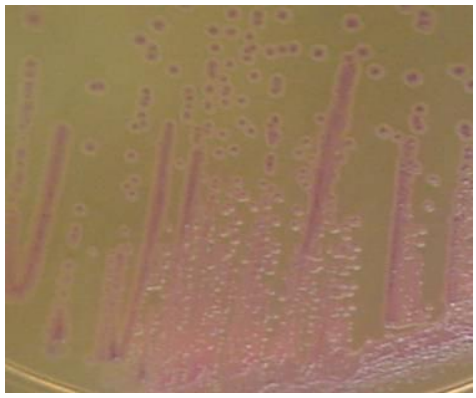
72 HOURS ON BAP

72 HOURS ON CHOC

24 HOURS ON MAC



48 HOURS ON MAC



3. Biochemical Testing

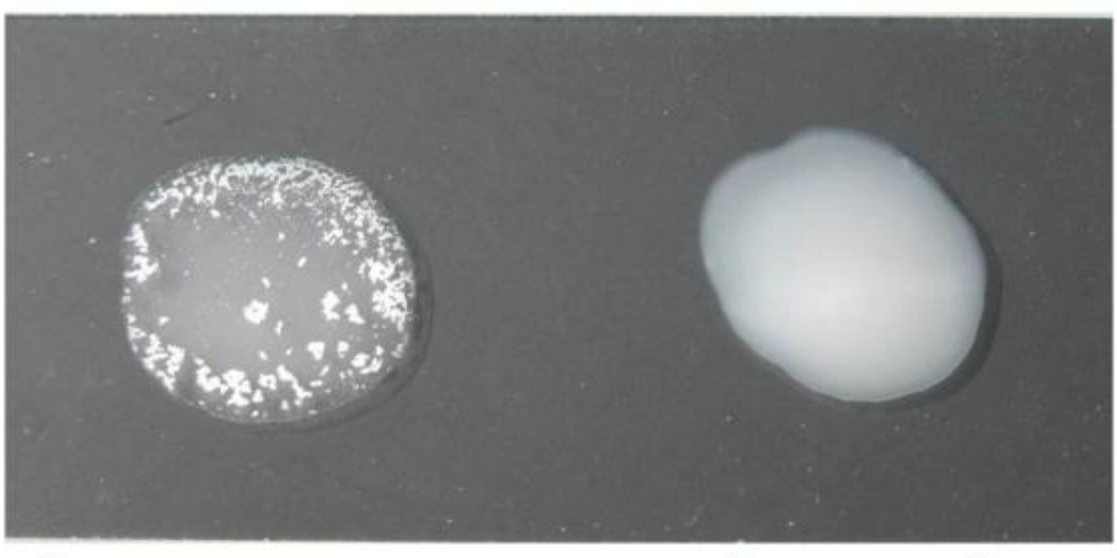
    a. Oxidase- *Burkholderia pseudomallei* is oxidase positive (+).

4. 3-Disk Diffusion Antimicrobial Susceptibly Testing

    1. Ensure inoculum is prepared in a biological safety cabinet (BSC) where possible. Disk diffusion method may be used for presumptive identification in resource-limited laboratories, but if possible, testing should also include broth-dilution MICs or Etest since disk-diffusion method can overestimate resistance.

2. Use interpretative criteria for MICs published by Clinical and Laboratory Standards Institute (CLSI) for *Pseudomonas aeruginosa* and *Enterobacteriaceae* (CLSI 2015) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for MIC and disk diffusion zone diameter criteria of *B. pseudomallei* (EUCAST 2020), available at:
    a. https://clsi.org/standards/products/microbiology/documents/m45/
    b. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0, 2021. http://www.eucast.org.
3. *B. pseudomallei* is susceptible to amoxicillin-clavulanic acid but resistant to colistin, polymyxin B and gentamicin. This AST profile can be used for presumptive identification of any gram-negative oxidase-positive bacilli.

4. <u>Monoclonal Antibody-Based Latex Agglutination</u>
    1. Positive latex agglutination test (left) (Melioidosis.Info 2012)



If you see the following characteristics:

• Gram-negative coccobacilli that may demonstrate bipolar staining

• Growth of creamy white colonies on BAP/CHOC and possible metallic sheen at 48 h

- Pink growth on MAC/EMB at 48 h
- Growth at 42°C
- May have distinctive musty odor
- Oxidase (+)
- Resistant to colistin and polymyxin B and susceptible to amoxicillin/clavulanic acid
- Positive latex agglutination test
- And cannot rule out *Burkholderia pseudomallei* using the protocol flow chart **then you cannot rule out *B. pseudomallei.***

Testing Workflow 2 (APHL 2016; ASM 2016)**-**

***Burkholderia pseudomallei* should be suspected and cannot be ruled out if the isolate fulfils the following characteristics:**

1. Gram Stain:
    a. *B. pseudomallei* is a Gram-negative rod that may exhibit bipolar staining.

2. Colony Morphology and Growth:
    a. On blood agar, *B. pseudomallei* appears as grey-white creamy colonies, which may gradually change after >48 h to dry, wrinkled colonies.
    b. Colonies are non-hemolytic.
    c. *B. pseudomallei* does not show any pigment on Mueller Hinton agar.
    a. On MacConkey agar, colonies are initially lactose nonfermenting, and colorless, and may develop a metallic sheen with a pinkish appearance after ≥48 h (mimicking lactose fermenters).

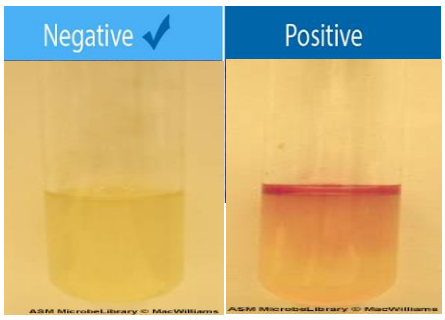
d. On Ashdown agar, colonies are typically pinpoint in size at 24 h and become purple, flat, and wrinkled at 48 h.

e. Often produces a characteristic musty or earthy odor (do not sniff plates!).

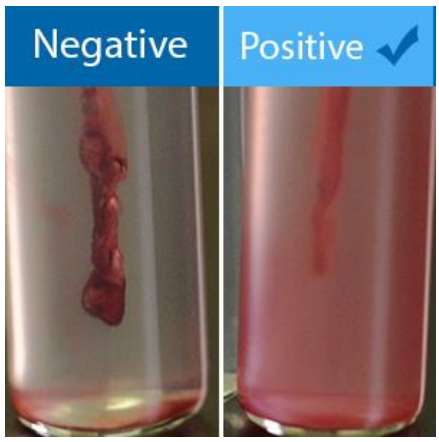
f. Growth at 42°C.

3. Biochemical Tests:

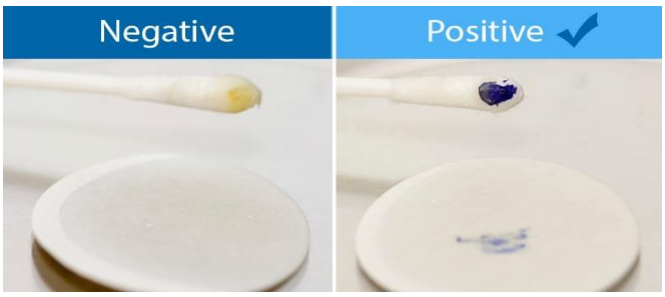
a. Indole- *Burkholderia pseudomallei* is indole negative (-).



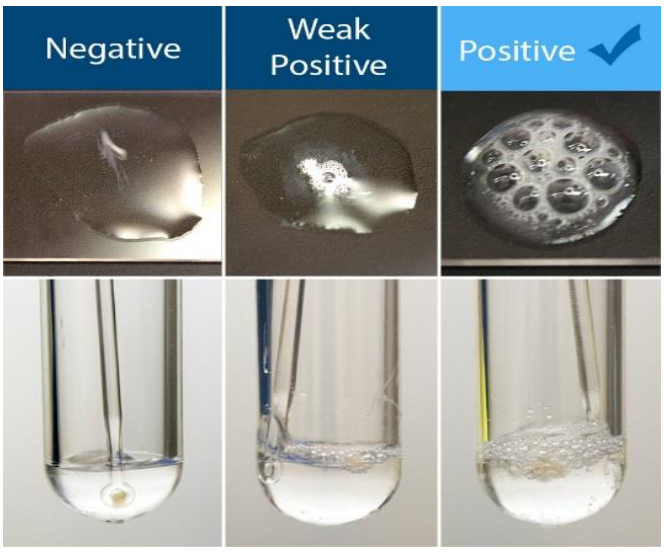
b. Motility- Use Motility Test Medium with 2,3,5-triphenyltetrazolium chloride (TTC). *Burkholderia pseudomallei* is motile (+).



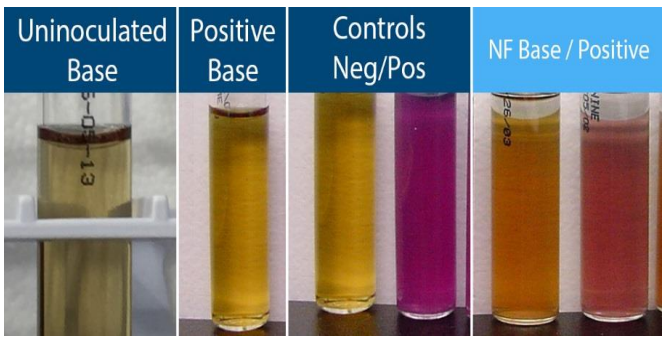
c. Oxidase- *Burkholderia pseudomallei* is oxidase positive (+).



d. Catalase- *Burkholderia pseudomallei* is catalase positive (+). **NOTE- It is recommended that this test be performed in a BSC or in a covered dish or tube to ensure the containment of aerosols that are produced when the test organism generates a positive result.**



e. Arginine dihydrolase (decarboxylase)-*Burkholderia pseudomallei* is arginine positive. (see NF Base/Positive)



4. Commercial Gram-negative identification panels (such as API 20NE and Vitek 1 or 2)
   a. Identifications of *Chromobacterium violaceum* for organisms that are not violet in color or are nonhemolytic could be *B. pseudomallei*.
   b. Identifications of *B. cepacia* for organisms that are susceptible to amoxicillin-clavulanate could be *B. pseudomallei.*
   c. Identifications of *B. cepacia* recovered from the blood or tissue of a non-cystic fibrosis patient could be *B. pseudomallei.*
   d. Commercial systems that give “no identification” for an organism that screens as potentially *B. pseudomallei* could be *B. pseudomallei.*

5. Polymerase Chain Reaction (PCR)

   a. PCR-positive using a validated *B. pseudomallei*-specific assay (e.g., TTS1).

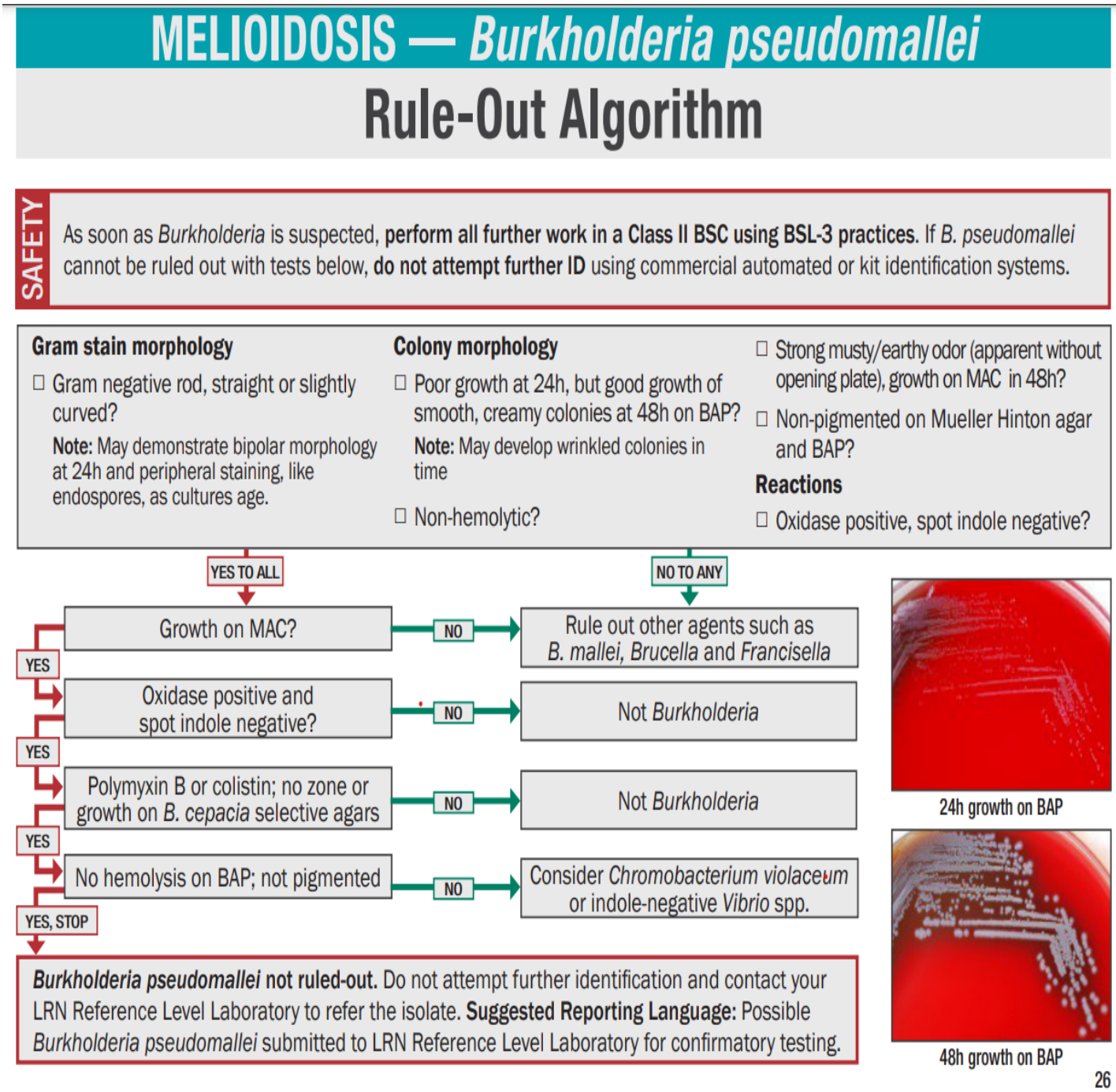


Figure 30- *B. pseudomallei* rule-out testing algorithm (APHL 2017).

NOTE- It is important that a reference laboratory always confirms identification of *B. pseudomallei* due to biosafety concerns and the dangers of misidentification.

Chapter Summary

All specimens from patients and animals that meet the case definition for suspected melioidosis infection should be tested for *B. pseudomallei.* The preceding chapter provides some suggested workflows and rule-out flowcharts that laboratories may utilize when encountering a suspected *B. pseudomallei* isolate from clinical specimens.

Chapter 10: *B. pseudomallei* Bacterial Culture Detection

Chapter Overview

Bacterial culture is the current "gold standard", although imperfect, method recommended for the detection and isolation of *B. pseudomallei* from clinical specimens. *B. pseudomallei* is never found as part of the normal human microbiota and isolation from any clinical sample is therefore considered diagnostic for melioidosis (Hoffmaster et al. 2015). The following chapter discusses the current recommended consensus methodologies used for bacterial culture detection of *B. pseudomallei* from clinical and environmental specimens.

Laboratory Media used for *B. pseudomallei* Culture

Standard laboratory media (e.g. 5% sheep blood and chocolate agar) can support the growth of *B. pseudomallei*. However, the use of selective media is critical, particularly for environmental samples and clinical specimens contaminated with normal microbiota, such as sputum, throat or wound swabs or tissue (Hoffmaster et al. 2015) and where the numbers of organisms can be very low (e.g. urine).

Ashdown's medium is one such selective medium frequently used in areas where melioidosis is endemic and is cost-effective (Currie et al. 2000), but it is not commercially available in many countries. It is composed of a tryptone soya agar base supplemented with glycerol, crystal violet, neutral red, and gentamicin. Glycerol is incorporated to produce the characteristic wrinkled colonies of *B. pseudomallei,* which develop a pink/purple color due to the absorption of the crystal violet and neutral red dyes. The addition of crystal violet also inhibits the growth of Gram-positive bacteria. The use of gentamicin inhibits other aerobic and facultative Gram-negative bacteria (Ashdown 1979). It is worth noting that as antimicrobial resistance increases, many other Gram-negative bacteria will be able to grow on Ashdown's, and it may need to be modified in the future

to suit local needs. The use of an enrichment broth, such as Ashdown's broth or Threonine Basal Salt Solution (TBSS-C50) containing 50 mg/L colistin prior to plating on Ashdown's agar may further increase the bacterial yield (Cheng et al. 2006). Ashdown's agar can be ordered directly from ThermoFisher Scientific in Australia, however it is not available in all countries. In this instance, it can be prepared in-house. See below for reagents and procedures to prepare Ashdown's and TBSS-C50 enrichment broth.

The best and most effective way to sterilize media before use is to heat it in an autoclave, however in resource or space-limited settings a pressure cooker can also be used for small batches of media. To sterilize lab media using a pressure cooker:

1. Add 1-2 inches (2-5 cm) of water to the pressure cooker.
2. Set containers on top of something inside of the cooker so they are slightly raised off of the bottom. Glass can crack if it is placed directly on the bottom.
3. Following the manufacturer's instructions, close the pressure cooker so it is adequately sealed and heat the cooker on a burner. A stove, electric fifth burner, or kitchen hot plate may be more effective than laboratory hot plates.
4. Leave to cook for at least 15 minutes. Media containing more than 15% or more salt may need to cook for 5-10 minutes longer in order for the agar to fully dissolve.
5. Remove the pressure cooker from the burner and allow the pressure to decrease before opening.

Media Preparation

Ashdown's Selective Agar Plates (grams/liter)

Ingredients	Amount
Tryptone Soya Broth	10 g
Glycerol	40 mL
1 % aqueous neutral red	5 mL

0.1 % crystal violet	5 mL
Agar (e.g Agar #3)	15 g
Gentamicin	8 mg
Distilled water	950 mL

Procedure

1. Mix all ingredients **except gentamicin** in a sterile autoclavable bottle. Fill with distilled water to 1L mark and mix thoroughly.
2. Autoclave at 121°C for 15 minutes.
3. Cool to 50-55°C, add gentamicin to a final concentration of 5 mg/L for clinical specimens and 8 mg/L if using for environmental specimens.
4. Dispense the agar into Petri dishes and allow to set.

Note: For optimal coloration, the crystal violet 0.1% solution should be incubated at 37°C for two weeks prior to use.

Ashdown's Selective Broth (grams/liter)

Ingredients	Amount
Tryptone Soya Broth	10 g
0.1 % crystal violet	5 mL
Colistin	50 mg
Distilled water	Up to 1000 mL

Procedure

1. Add all reagents **except colistin** to sterile autoclavable container. Fill with distilled water to 1L mark and mix thoroughly.
2. Autoclave at 121°C for 15 min and allow to cool to 50°C. Store bottles in clean refrigerator until ready to use.
3. Add colistin aseptically before use and mix thoroughly.

See the YouTube link for a step-by-step video demonstrating how to prepare selective Ashdown's Media https://youtu.be/5sI-gbiZokw, also available from melioidosis.info here: https://www.melioidosis.info/info.aspx?pageID=104&contentID=1040209.

Threonine Basal Salt Solution (TBSS-C50)

1. **Solution A (to be added to base solution)**

Ingredients	Common Name	Amount
H_3PO_4 85%	Phosphoric acid	2.306 ml
$FeSO_4.7H_2O$	Iron (II) sulfate heptahydrate/Ferrous sulphate	0.556 g
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate	0.297 g
$CuSO_4.5H_2O$	Copper (cupric) sulfate pentahydrate	0.0218 g
$MnSO_4.H_2O$	Manganese sulfate, monohydrate	0.125 g
$Co(NO_3)2.6H_2O$	Cobalt (II) nitrate hexahydrate	0.030 g
$Na_2MoO_4.2H_2O$	Sodium molybdite dihydrate	0.030 g
H_3BO_3	Boric acid	0.062 g
Distilled Water		10000 mL

Procedure

1. Mix all the above ingredients in a 1 liter bottle on a hot plate with a magnetic stirrer until all are dissolved.
2. Sterilize by autoclaving at 121°C for 20 minutes.

Ingredients	Common name	Amount
KH_2PO_4	Monopotassium phosphate/Potassium dihydrogen phosphate	0.451 g
K_2HPO_4	Dipotassium phosphate	1.730 g
$MgSO_4.7H_2O$	Magnesium sulfate	0.123 g
$CaCl_2$.$2H_2O$	Calcium chloride	0.0147 g

NaCl	Sodium chloride	10 g
Nitrilotriacetic acid	Nitrilotriacetic acid (NTA)	0.200 g
Solution A		20 mL
Distilled Water		1000 mL

2. Solution B- Base medium

Procedure

1. Mix all the above ingredients in a 1 liter autoclavable bottle.
2. Adjust pH to 7.2 with 1 normal (N) potassium hydroxide (KOH).
3. Sterilize by autoclaving at 121°C for 20 minutes.

3. Solution C- L-Threonine Solution

Ingredients	**Amount**
L-Threonine	5.956 g
Distilled Water	100 mL

Procedure

1. Mix the above ingredients in a 100 ml bottle.
2. Sterilize by filtration through 0.20 µm filter (e.g. 0.20 µm Millipore filter).

4. TBSS-C50 Solution Procedure

1. Add 100 ml of L-Threonine solution (solution C) to 900 mL of the base medium (final concentration of L-Threonine of 0.05M).
2. Add colistin to a final concentration of 50 mg/L.

Media Quality Control (Wuthiekanun & Dance 2012)

1. Sterility test- A sample of each batch of medium should be incubated for two days in air at 35-37°C. Use two agar plates or two tubes of broth per 1 liter of medium. If there no growth occurs after 2 days, the batch of medium may be used.
2. Growth test- Test the ability of the medium to support the growth of a positive reference strain. If possible, use of *B.thailandensis* or Select Agent-exempt strains of *B. pseudomallei* are preferrable since they can be used at BSL-2 and present a safer option.
    a. Touch 5-7 colonies of a pure bacterial culture with a cotton swab.
    b. Place the colonies in 3 mL of saline (0.85% NaCl) and adjust to match a 1.0 McFarland turbidity standard to a concentration of $3x10^8$ CFU/mL.
    c. Make serial 1 in 10 dilutions of the 1.0 MacFarland suspension to get 6 dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions).
    d. Drop 10 µL of the 10^{-4}, 10^{-5}, 10^{-6} dilutions onto an Ashdown's agar plate or into 10 mL Ashdown's broth or TBSS-C50 broth in duplicate.
    e. Incubate broth for two days in air at 37°C then subculture 10 µL of the surface layer onto an Ashdown's agar plate.
    f. Incubate agar plates in air at 37°C for four days.
    g. Plates should show good growth for all dilutions.

Colony Morphology

Laboratory identification of *B. pseudomallei* can be difficult, particularly in countries where the bacteria is rarely observed. The large wrinkled colonies are often assumed to be environmental contaminants and are often discarded. The most discernible feature of *B. pseudomallei* is its

metallic sheen and subsequent progression to dry and wrinkled colonies after 48 hours, however different colony morphologies are common and may include smooth and shiny, mucoid or dry, or colonies that appear as different shades of purple (Chantratita et al. 2007). *B. pseudomallei* often produces a distinctive musty or earthy odor. This is normally very noticeable when opening an agar plate containing the bacterium and may sometimes be observed when opening an incubator that contains a positive plate. **Note- sniffing of agar plates containing *B. pseudomallei* is a biohazard and should not be done. This odor is evident without sniffing.**

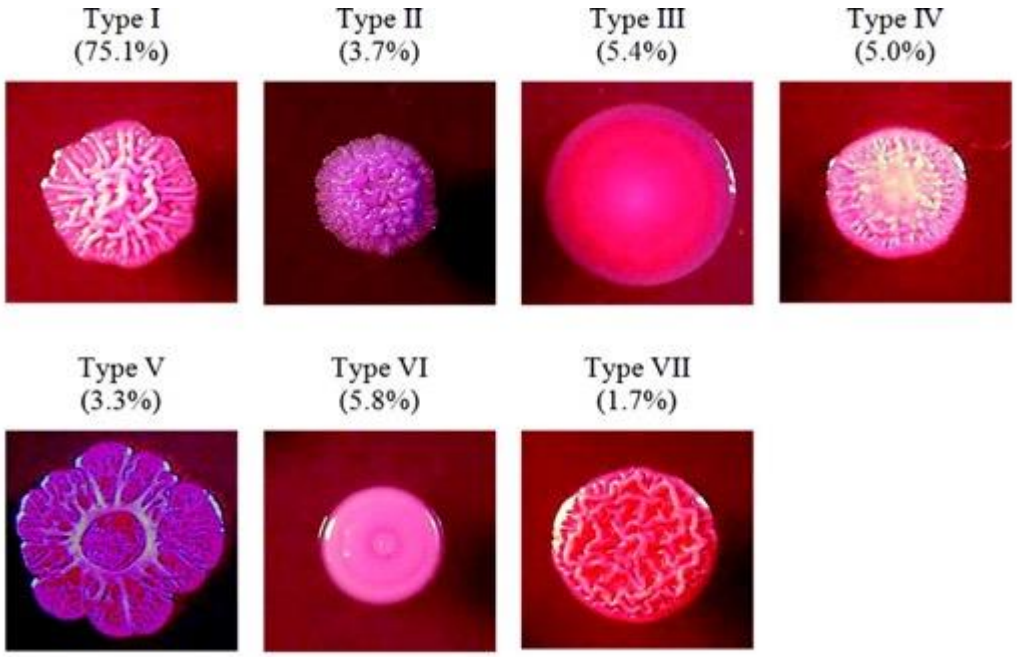


Figure 31- Morphological variants of *B. pseudomallei* colonies grown on Ashdown's agar for 4 days at 37°C in air (Chantratita et al. 2007).

Any colony with a morphology suggestive of *B. pseudomallei* should be further tested using additional preliminary microbiological tests (e.g Gram stain, positive oxidase test) followed by confirmatory tests such as three-disk antibiotic susceptibility testing (see Chapter 8), *B. pseudomallei* latex agglutination test (see the next chapter covering *B. pseudomallei* serology testing for the protocol) (Wuthiekanun et al. 2002), PCR assay (Novak et al. 2006), or commercial identification kits such as API20NE or Vitek system. For rapid identification, latex agglutination or PCR assay could be used as an initial confirmatory test (see the previous chapter on *B. pseudomallei* diagnostic methods for more details and examples of testing algorithms) (Limmathurotsakul et al. 2013).

Morphology on Different Culture Media

- Blood agar

B. pseudomallei normally grows as small cream-colored colonies with a metallic sheen. These can develop a dry or wrinkled appearance after 48 hours of incubation.

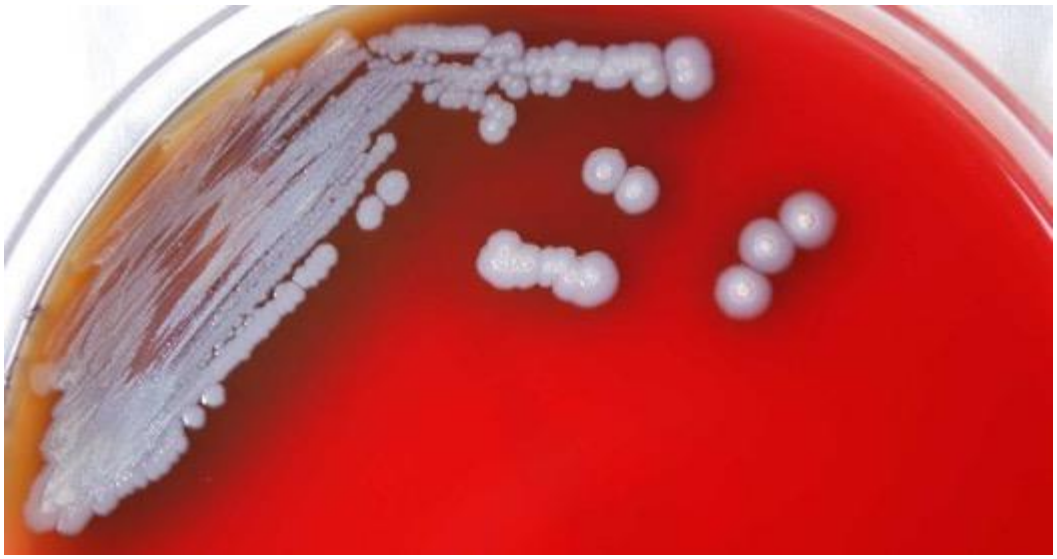


Figure 32- *Burkholderia pseudomallei* bacteria grown on a medium of sheep's blood agar (FDA 2019: CDC/Parker and Marsh 2019).

- MacConkey agar

B. pseudomallei colonies on MacConkey agar are initially colorless and have a metallic sheen but will become pink after 48 hours incubation. This is believed to be caused by the uptake of dye from the medium. Colonies are typically lactose and nonfermenting.

Figure 33- Growth of *Burkholderia pseudomallei* on MacConkey agar after 48 hours showing pink, rugose colonies with a metallic sheen (Dhodapkar et al. 2008).

- Ashdown's agar

  B. pseudomallei grows as very small (pinpoint) colonies by 18 hours, which typically become purple, flat, dry and wrinkled after 48 hours of incubation. However, there may be considerable variation both within and between strains and it is not unusual for cultures to appear mixed, with more than one colony type. (Chantratita et al. 2007; Hoffmaster et al. 2015).

Figure 34- Growth of *Burkholderia pseudomallei* on Ashdown's agar showing purple, dry, wrinkled colonies with a metallic sheen.

Clinical Specimen Culturing

It is important that the appropriate clinical samples are collected and sent to laboratories familiar with culturing the bacterium. Blood, throat, and urine cultures should be performed on all patients with suspected melioidosis, regardless of symptoms. Throat swabs and a centrifuged deposit of urine should be cultured on selective media for optimal sensitivity (Dance et al. 2019). Specimens from localized disease, such as sputum, wound swabs, and aspirates from abscesses, should also be collected (Hoffmaster et al. 2015). Blood specimens should be inoculated into blood culture bottles as soon as possible. Since culture detection for *B. pseudomallei* has low sensitivity (approximately 60% overall, although this will vary from patient to patient depending on the methods used and the sampling of accessible foci of infection), repeating cultures, particularly of blood, sputum, urine and pus, should be considered in patients with strong indications of disease. It is not uncommon to find successive samples positive even when initial results are negative (Limmathurotsakul et al. 2010).

Clinical Culturing Protocols

Specimens

- Urine
- Sputum or bronchoscopy specimens
- Abscess material and wound swabs
- Serum or fluid
- Blood

Equipment

- Incubator set at 37°C

- Class II Biological Safety Cabinet (BSC)
- Adjustable pipettes
- Sterile pipette tips
- Sterile universal tubes
- Sterile loops
- Vortex mixer
- Racks
- Personal Protective Equipment according to local safety guidelines

Media

- Blood agar (BAP), MacConkey agar (MAC) or Cystine–Lactose–Electrolyte-Deficient (CLED) agar, Chocolate agar (CHOC)
- Selective agar (such as Ashdown's agar (ASH), *Burkholderia cepacia* selective agar)
- Selective broth media (such as Ashdown's medium or Threonine basal salt solution (TBSS-C50) – see above).
    - If Ashdown's or TBSS-C50 are unavailable thioglycollate or brain heart infusion broth may be substituted (ASM 2016).

***B. pseudomallei* Urine Culture Protocol**

1. Urine cultures should be performed according to the CLSI or local relevant standard semi-quantitative urine streaking technique. Lab staff should be familiar with the appearance of *B. pseudomallei* on the local standard media used.
2. In addition, since *B. pseudomallei* bacteriuria may occur at 'non-significant' levels in patients with melioidosis and urine may be the only positive sample in some patients, selective agar

(e.g. ASH or *Burkholderia cepacia* selective agar) should be used, if available, where a high clinical suspicion of melioidosis exists and cases have been reported previously.

3. Label agar plate with the date, patient and sample details.
4. Shake the urine container and remove the lid.
5. Spread 10 µL of fresh, unprocessed urine using a sterile calibrated loop onto one half of each agar plate.
6. Centrifuge the remaining urine sample at 4000 rpm for 10 minutes.
7. Tip out most of the supernatant, leaving approximately 1 mL, and resuspend the pellet in the remaining supernatant.
8. Inside a biosafety cabinet, use a sterile pipette and place 1 drop (approx. 10 mL) of the resuspended pellet onto the agar plate.
9. Streak out the plates as in Figure 36 below and incubate all plates at 37^{o}C.
10. Check all plates daily for growth, up to 7 days.

***B. pseudomallei* Sputum Culture Protocol**

1. Sputum cultures should be performed according to the CLSI or local relevant standard semi-quantitative streaking technique. Lab staff should be familiar with the appearance of *B. pseudomallei* on the local standard media used.
2. Selective agar (e.g. ASH or *Burkholderia cepacia* selective agar) should be added, where available, if a high clinical suspicion of melioidosis exists and cases have been reported previously. To improve the isolation of *B. pseudomallei* from respiratory specimens that are likely to contain additional microbiota, a colistin disk or polymyxin B disk may be placed in the initial inoculation area on the BAP to help select for *Burkholderia spp*., since they are intrinsically resistant (Hemarajata et al. 2016).

3. Where possible, selective enrichment broth containing colistin (Ashdown's or TBSS-C50) should also be inoculated with the sputum specimen to check for bacterial growth.
4. Label agar plates and enrichment broth containers with the date and appropriate patient and sample details.
5. Culture undiluted sputum according to local protocols and incubate plates and broth at 37°C degrees in air.
6. Check the plates and broths daily for growth.
7. Subculture broths onto agar plates after 2 days and incubate. Check the plates and broths daily for growth.

***B. pseudomallei* Abscess or Pus Culture Protocol**

1. Cultures should be performed according to the CLSI or local relevant standard semi-quantitative streaking technique. Lab staff should be familiar with the appearance of *B. pseudomallei* on the local standard media used.
2. Where available, selective agar (e.g. ASH or *Burkholderia cepacia* selective agar) should be used for ruptured abscess or wound specimens, especially where a high clinical suspicion of melioidosis exists and cases have been reported previously. Aspirates of an unruptured abscesses, which normally grow organisms in pure culture, do not require selective media.
3. Where possible, selective enrichment broth containing colistin (Ashdown's or TBSS-C50) can also be inoculated with ruptured abscess or pus specimens to check for bacterial growth. Incubate the selective broth at 37°C in air for 2 days, and then sub-culture 10 µL from the surface of the broth according to local protocols. Incubate the Ashdown's agar plates at 37°C in air for 2 days and inspect the plates daily.
4. Check all plates daily for growth for 7 days.

***B. pseudomallei* Throat and Rectal Swab Culture Protocol**

1. Selective agar (e.g. ASH or *Burkholderia cepacia* selective agar) should be used for these specimen types if available, especially where a high clinical suspicion of melioidosis exists and cases have been reported previously (Wuthiekanun et al. 1990).
2. Plate the swab directly onto 1/3rd of an agar plate. Streak out the inoculated plate to obtain single colonies. Incubate plates in air at 37°C and check plates daily for growth, up to 7 days.
3. Where possible, selective enrichment broth containing colistin (Ashdown's or TBSS-C50) can also be inoculated with the wound or pus specimen to check for bacterial growth. Incubate the selective broth at 37°C in air for 2 days, and then subculture 10 µL from the surface of the broth onto 1/3rd of an agar plate and streak out. Incubate the plates at 37°C in air for 2 days and inspect the plates daily.
    a. If swabs are received in standard transport media, place them into labelled selective enrichment broth before incubating.

Serum and Fluid (**cerebrospinal, synovial, pericardial, pleural fluid) *B. pseudomallei* Culture Protocol**

1. Cultures should be performed according to the CLSI or local relevant standard semi-quantitative urine streaking technique. Lab staff should be familiar with the appearance of *B. pseudomallei* on the local standard media used. Selective agar (e.g. ASH or *Burkholderia cepacia* selective agar) may be used, especially where a high clinical suspicion of melioidosis exists, but is not essential as these specimens are normally sterile (with the exception of effusions that have been repeatedly aspirated).
2. Incubate plates at 37^{o}C degrees.
3. Check the plates daily for growth, up to 7 days.

***B. pseudomallei* Blood Culture Protocol**

1. Aseptically inoculate blood culture bottles with the maximum amount of blood and incubate according to the specific manufacturer instructions and local protocols (typically 8-10 mL for adults and 1-2 mL for neonates). See commonly used automated blood culture systems and bottles routinely used for *B. pseudomallei* blood culture detection in the Table below (Table 13).
    a. Incubate automated systems for 5 days.
    b. Incubate non-automated (manual) broth blood cultures for 7 days, with direct observations for turbidity daily. Subculturing should be done (at a minimum) on day 1, day 2, and day 7 following incubation. Figure 35 below provides examples of growth in blood culture bottles.
        i. For blind subculturing, inoculate to BAP and incubate plates for 3 days before reporting as negative.
2. For positive blood culture bottles, inoculate to routine media using local methods. Incubate plates at 37°C and check daily for growth. Ensure that all oxidase-positive Gram-negative rods are identified, at least to exclude the possibility of *B. pseudomallei* (e.g. using latex agglutination or 3-disc susceptibility test as described in Chapter 8).

Table 13- Automated blood culture systems and bottles routinely used to detect *B. pseudomallei* in blood specimens (Gonzalez et al. 2020).

Blood Culture System	Method for Monitoring Growth	Bottle Types	Additional Information	Max Volume
BacT/ALERT (bioMérieux)	Colorimetric change caused by drop in pH from increased CO_2 levels	BacT/ALERT FA PLUS	Aerobic media with adsorbent polymeric resin beads	10 mL

		BacT/ALERT PF PLUS	Pediatric, standard aerobic	4 mL
BACTEC FX (Becton, Dickson and Company)	Change in fluorescence caused by a drop in pH from increased CO_2 levels	BACTEC Plus Aerobic	Aerobic media with adsorbent polymeric resin beads	10 mL
		BACTEC Peds Plus	Pediatric, aerobic media w/adsorbent polymeric resin beads	5 mL
VersaTREK (Thermo Scientific)	Measures pressure changes caused by gas consumption or production	REDOX 1	Aerobic	10 mL
		REDOX 1 EZ Draw	Aerobic, direct blood inoculation	5 mL

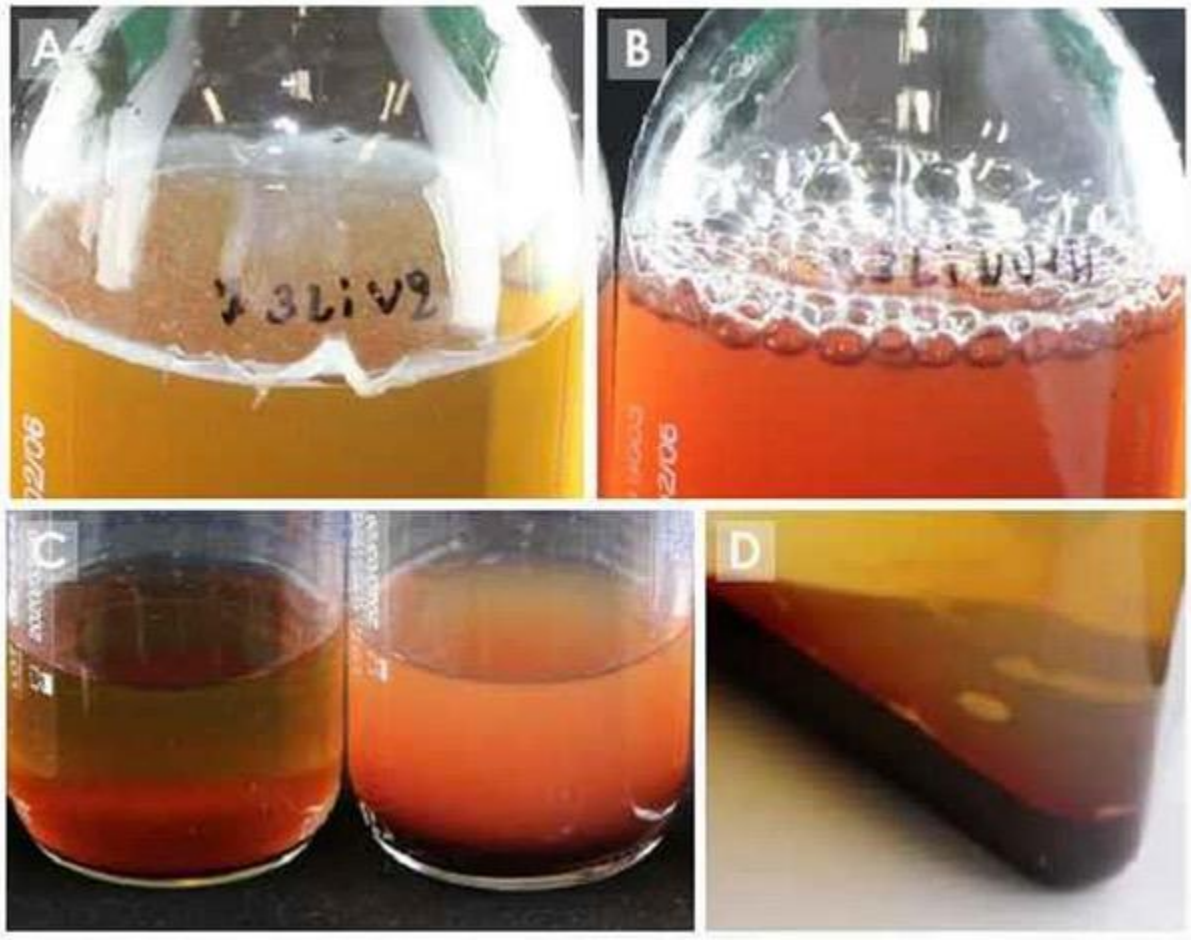


Figure 35- Signs of growth in blood culture bottles. (A) pellicle formation on surface; (B) gas production; (C) turbidity (left bottle: no growth; right bottle: turbidity); (D) puff balls. A and C are more typical observed with *B. pseudomallei* grown in blood culture (Ombelet et al. 2019).

B. pseudomallei **Environmental Sample Culturing**

Detection of *B. pseudomallei* in the environment can be used to establish the risk for acquiring melioidosis and increase understanding about a potential infection source. The methods currently recommended for the detection of *B. pseudomallei* from environmental samples include bacterial culture with selective enrichment media, followed by confirmation using a *B. pseudomallei*-specific PCR (Kaestli et al. 2007; Novak et. al 2006). PCR detection and confirmation of *B. pseudomallei* is discussed further in Chapters 12-15.

It is important to note that although a consensus culture method has been described and has been successful in isolating *B. pseudomallei* from soil in many endemic regions, it appears not to have equivalent sensitivity everywhere (Limmathurotsakul et al. 2013). The reasons for this variation are not completely understood but may involve differences in the amount of *B. pseudomallei* present in the soil, the volume of sample processed, variations in the number of competing microorganisms present in a sample, and differences in the soil type (Dance et al. 2018). Other methods have been described (for example a semi-quantitative method known as the 'conventional method' (Dance et al. 2018) and in some cases have a higher sensitivity than the consensus method, but no method is perfect in detecting *B. pseudomallei* in environmental samples. Additionally, the conventional method is time-consuming and necessitates highly trained laboratory staff to detect colonies of *B. pseudomallei* amid a range of competing environmental microorganisms. Furthermore, molecular methods, usually with prior enrichment culture, have been shown to have a higher sensitivity than

culture alone (Dance et. Al, 2018). **Consequently, it is important that researchers conducting environmental surveys are aware that a failure to isolate *B. pseudomallei* from the environment using the consensus culture method does not mean that it is not present.** Rather, a modified approach for those looking for *B. pseudomallei* could be to use enrichment culture followed by PCR as an initial screening method, then implement different culture methods on PCR-positive samples until one is shown to successfully isolate the bacterium (Dance et al. 2018).

General Safety Considerations

- All procedures should follow local safety rules and regulatory authorities.
- All inspection of culture plates and manipulations of microbiological organisms should be carried out in a biological safety cabinet (Class I or II).
- Change gloves regularly.
- Clean pipettes before and after use.
- Clean the hood before and after use.
- Clean centrifuges before and after use with ethanol.
- **Do not process environmental specimens in biosafety cabinets used for clinical specimen processing.**

Equipment

- Incubator set at 40°C (Incubator set at 37°C is optional)
- Shaking incubator (if available)
- Class II Biological Safety Cabinet (BSC)
- Adjustable pipettes
- Sterile pipette tips
- Sterile universal tubes

- Sterile loops
- Vortex mixer
- Racks
- Personal Protective Equipment according to local safety guidelines
- 70% ethanol

Media

- Selective agar (such as Ashdown's agar (ASH), *Burkholderia cepacia* selective agar)
- Selective enrichment broth (such as Ashdown's broth or Threonine basal salt solution (TBSS-C50) – see above).

***B. pseudomallei* Soil Sample Culture**

The methodology used to detect *B. pseudomallei* in soil samples has two primary stages involving bacterial detachment from soil particles and subsequent detection using culture and/or PCR. The process of bacterial detachment includes the addition of a solution, typically selective enrichment media (TBSS-C50 or Ashdown's broth) to the soil, followed by homogenization (Limmathurotsakul et al. 2013). Here, soil is mixed with an equal volume of *B. pseudomallei* enrichment medium and incubated at 40°C in air for 48 hours (although it can grow up to 42°C). This incubation temperature is based on evidence that *B. pseudomallei* is able to grow well at 40°C (Chen et al. 2003), while being inhibitory to some other soil biota. However, incubation at 37°C is acceptable in the event that resources are not available to incubate at 40°C. In well-resourced laboratories, a shaking incubator may also be used to achieve improved mixing and homogenization but is not required. Additionally, if available, bio-reaction tubes with a 0.22 µm hydrophobic vented membrane allow for improved air exchange and enhanced safety within the shaking incubator. The enrichment medium is then streaked to Ashdown's agar to achieve single colonies, which is examined every 24 hours for 7 days.

Additional culture methods that involve mixing a quantity of soil with distilled water and shaking for 1-2 days prior to inoculation into selective enrichment broth have also been studied and are well-validated. However, these methods can increase the time to detection, involve additional consumables, and the rate of bacterial detection has not been shown to be consistently greater overall (Dance et al. 2018; Limmathurotsakul et al. 2013; Rachlin et al. 2020). Both methods are described below.

B. pseudomallei **Soil Culture Protocol** (Figure 37, Table 14)

1. Weigh 10 gram of soil using laboratory scales and place in a sterile 25 ml universal tube.
2. Add 10 mL of TBSS-C50 or Ashdown's enrichment broth to the universal tube.
3. Ensure that the lid is replaced and is tight and vortex the universal tube for 30 seconds.
4. Incubate the universal tube at 40°C in air or place in shaking incubator at 220rpm at 40°C for 48 hours.
5. If the solution has been in a shaking incubator, let stand for 1 to 2 hours to settle before removing and subculturing.
6. Subculture 10 μL of the upper layer of the medium onto an Ashdown's agar plate in a BSC, streaking to achieve single colonies. Place the enrichment broth back in the incubator.
7. Incubate the Ashdown's agar plate at 40°C in air.
8. Examine the Ashdown's agar plate daily for 7 days in a BSC for colonies suspected to be *B. pseudomallei,* subculturing suspected colonies to an additional Ashdown's plate as per Figure 36 below to achieve single colonies.
9. After the broth has been incubating for 7 days, repeat steps 6 to 8 by subculturing 10 μL of the upper layer of the enrichment medium onto an Ashdown's agar plate. Incubate the plate

and examine daily for 7 days in a BSC for colonies suspected to be *B. pseudomallei.* See Figure 37 for a soil culturing flow diagram.

10. Any bacterial colony suspected to be *B. pseudomallei* should be further identified and confirmed using the methods listed below.
11. If colony is identified as *B. pseudomallei* store a large sweep of growth in a sterile closable tube at -80°C in tryptone soy broth containing 20% glycerol. If a -80°C freezer is not available, store at -20°C and immediately refer to the local reference laboratory.

OR

1. Weigh out 20 grams of soil using laboratory scales and place in a sterile 50 mL universal tube.
2. Add 20 mL distilled sterile water, tighten tube lid and vortex the tube for 30 seconds to mix thoroughly.
3. Incubate the universal tube at 40°C in air or place in shaking incubator at 220rpm at 40°C for 48 hours.
4. If the solution has been in a shaking incubator, let stand for 1 to 2 hours to settle before removing from the shaker.
5. Remove top 10 mL of liquid and place into 30ml of selective TBSS-C50 or Ashdown's enrichment broth.
6. Incubate the universal tube at 40°C in air for 48 hours. After 48 hours incubation subculture 10 mL of the upper layer of the medium onto an Ashdown's agar plate in a BSC, streaking to achieve single colonies and place the broth back into the incubator.
7. Examine the Ashdown's agar plate daily for 7 days in a BSC for colonies suspected to be *B. pseudomallei*.
8. After the broth has been incubating for 7 days, repeat steps 6 and 7 by subculturing 10 μL of the upper layer of the enrichment medium onto an Ashdown's agar plate. Incubate the plate and examine daily for 7 days in a BSC for colonies suspected to be *B. pseudomallei.*

9. Any bacterial colony suspected to be *B. pseudomallei* should be further identified and confirmed using the methods described below.
10. If colony is identified as *B. pseudomallei* store a large sweep of growth in a sterile closable tube at -80°C in tryptone soya broth containing 20% glycerol. If a -80°C freezer is not available, store at -20°C and immediately refer to the local reference laboratory.

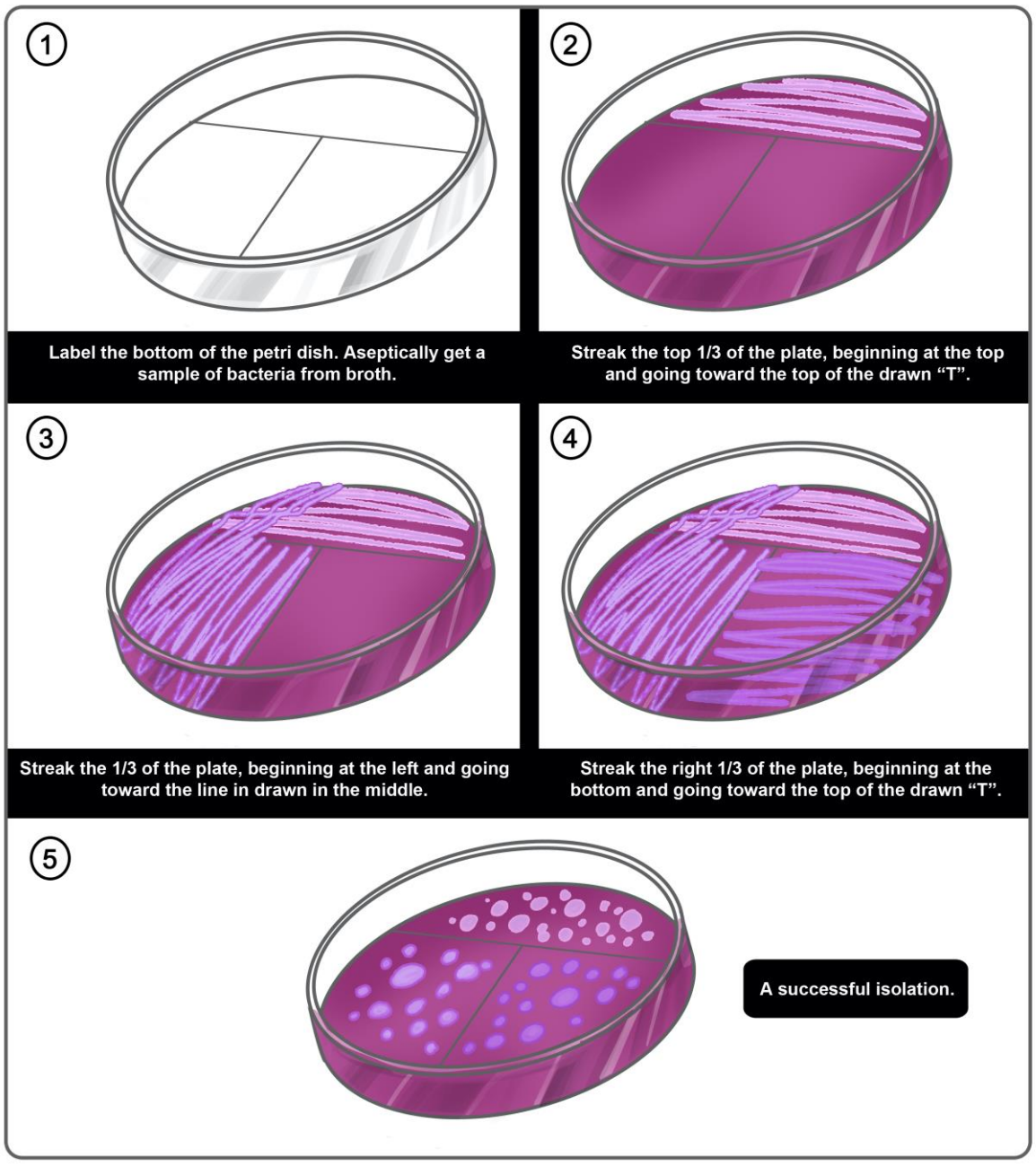


Figure 36- Subculture streaking technique for suspected *B. pseudomallei* colonies to Ashdown's agar to achieve single colonies.

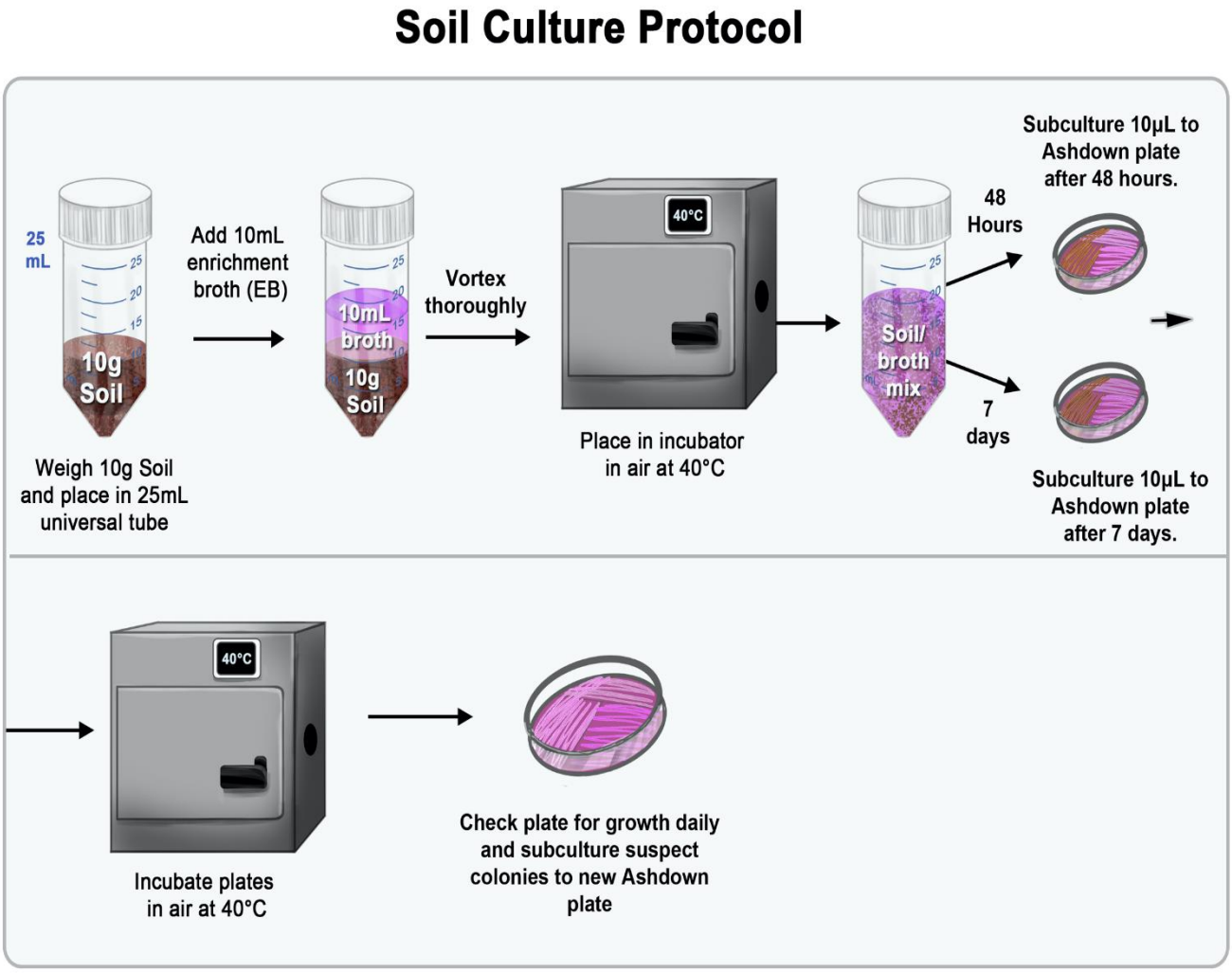


Figure 37- Diagram demonstrating the steps involved in culturing *B. pseudomallei* from soil specimens.

Table 14- Recommended methods for the isolation of *B. pseudomallei* from soil (Limmathurotsakul et al. 2013).

Method	**Consensus guideline**
***B. pseudomallei* enrichment broth**	• Threonine-basal salt plus colistin 50 mg/L (TBSS-C50 broth) or • Ashdown's broth containing 50mg/L colistin and crystal violet as an alternative
Quantity of soil and detachment solution	• 10 grams of soil added to 10 mL of TBSS-C50 or Ashdown broth and vortexed for 30 seconds

Method	Consensus guideline
	OR • 20 mL distilled water added to 20 grams of soil, vortexed for 30 seconds. After 48 hours incubation remove top 10 mL of water and place in 30mL of TBSS-C50 or Ashdown's enrichment broth.
Incubation	• 40°C is recommended, 37–42°C can be used as an alternative option. • A shaking incubator may also be used to achieve improved mixing and homogenization but is not required. Set the incubator to run at 220rpm at 40°C for 48 hours.
Protocol for sub-culture	• Subculture 10 µL of supernatant onto an Ashdown's agar plate, and streak to achieve single colonies. • Incubate plate for 48 hours and examine daily for 7 days.
Confirmation of *B. pseudomallei* from cultures	• Basic microbiological tests (which include typical colony morphology, Gram stain, positive oxidase test, inability to assimilate arabinose, resistance to gentamicin and colistin with susceptibility to co-amoxiclav). • Confirmatory tests (API20NE, Vitek system, specific latex agglutination test, lateral flow immunoassay (AMD) or *B. pseudomallei*-specific PCR assay).

B. pseudomallei Culture Detection from Water Samples

Recent environmental surveys have indicated that water is a significant reservoir for *B. pseudomallei* (Ribolzi et al. 2016; Vongphayloth et al. 2012; Zimmermann et al. 2018). Since stormwater is known to capture and leach what is in the soil, including particulates, contaminants and bacteria, water may provide an accurate indication of *B. pseudomallei* distribution and presence in a region. *B. pseudomallei* has also been isolated in groundwater and domestic bore/well water in Northern Australia (Baker et al. 2011; Draper et al. 2010) and these isolates have since been linked to clinical isolates using molecular typing (Baker et al. 2011).

The methodology used to detect *B. pseudomallei* in water samples has two main stages involving bacterial concentration, followed by detection using bacterial culture techniques and/or *B. pseudomallei*-specific PCR (Limmathurotsakul et al. 2013). PCR detection and confirmation of *B. pseudomallei* is discussed further in Chapters 12-15. Bacterial concentration is typically achieved through sample filtration using fine membrane filters (e.g. cellulose Millipore filters). Hand pumps or electric vacuum pumps can both be used to filter water samples, however electric vacuum pumps are recommended when testing large numbers of samples. Funnels and filtering systems should be cleaned and disinfected thoroughly with distilled water followed by 70% ethanol and dried after every sample has been filtered to ensure cross-contamination of samples does not occur.

Water Sample Filtration and Water Culture Protocol

Equipment (Additional to List Above)

- Hand or electric vacuum pump and filtration system
- 0.22 mm pore size cellulose filters (diameter will be based on laboratory-specific filtration system- typically 47 mm diameter)
- Urine specimen jars or sealable sterile containers
- Forceps/tweezer (for handling filters)

Figure 38- Example of stainless-steel filtration funnels used to filter water samples.

Procedure (Figure 39)

1. Ensure funnels, forceps/tweezers and filtration apparatuses have been thoroughly disinfected with distilled water followed by 70% ethanol before use.
2. Remove the filter from its package, ensuring only the tweezers touch the filter, and place on funnel base.
3. Ensure the funnel is securely in place before pouring the water sample into the funnel.
4. Turn on the pump, ensuring filter valves are open, or use hand pump to begin filtering samples. NOTE- the exact protocol will vary based on the filter pump system being used. Be sure to follow the specific manufacturer instructions.

5. Once all of the water has been filtered through, place the filters in 30 mL enrichment broth (TBSS-C50 or Ashdown's broth with colistin).
6. Clean funnel thoroughly with distilled water followed by 70% ethanol and wipe clean before filtering the next sample.
7. Incubate the enrichment broths containing the filters at 40°C.
8. After 48 hours incubation subculture 10 μL of the surface of the broth onto an Ashdown's agar plate. Streak out to achieve single colonies.
9. Check agar plates after 2 days growth and then daily for growth for suspected colonies, subbing suspected colonies to an additional Ashdown's plate as per Figure 36 above.
11. Repeat steps 8 and 9 after the enrichment broth has been incubating for 7 days by subculturing an additional 10 mL of the surface of the broth onto a new Ashdown's agar plate. Incubate plate and check after 2 days growth and then daily for growth or suspected colonies.
12. Any bacterial colony suspected to be *B. pseudomallei* should be further identified and confirmed using the methods listed below.
13. If colony is identified as *B. pseudomallei* store a large sweep of growth in a sterile closable tube at -80°C in tryptone soya broth containing 20% glycerol. If a -80°C freezer is not available, store at -20°C and immediately refer to the local reference laboratory.

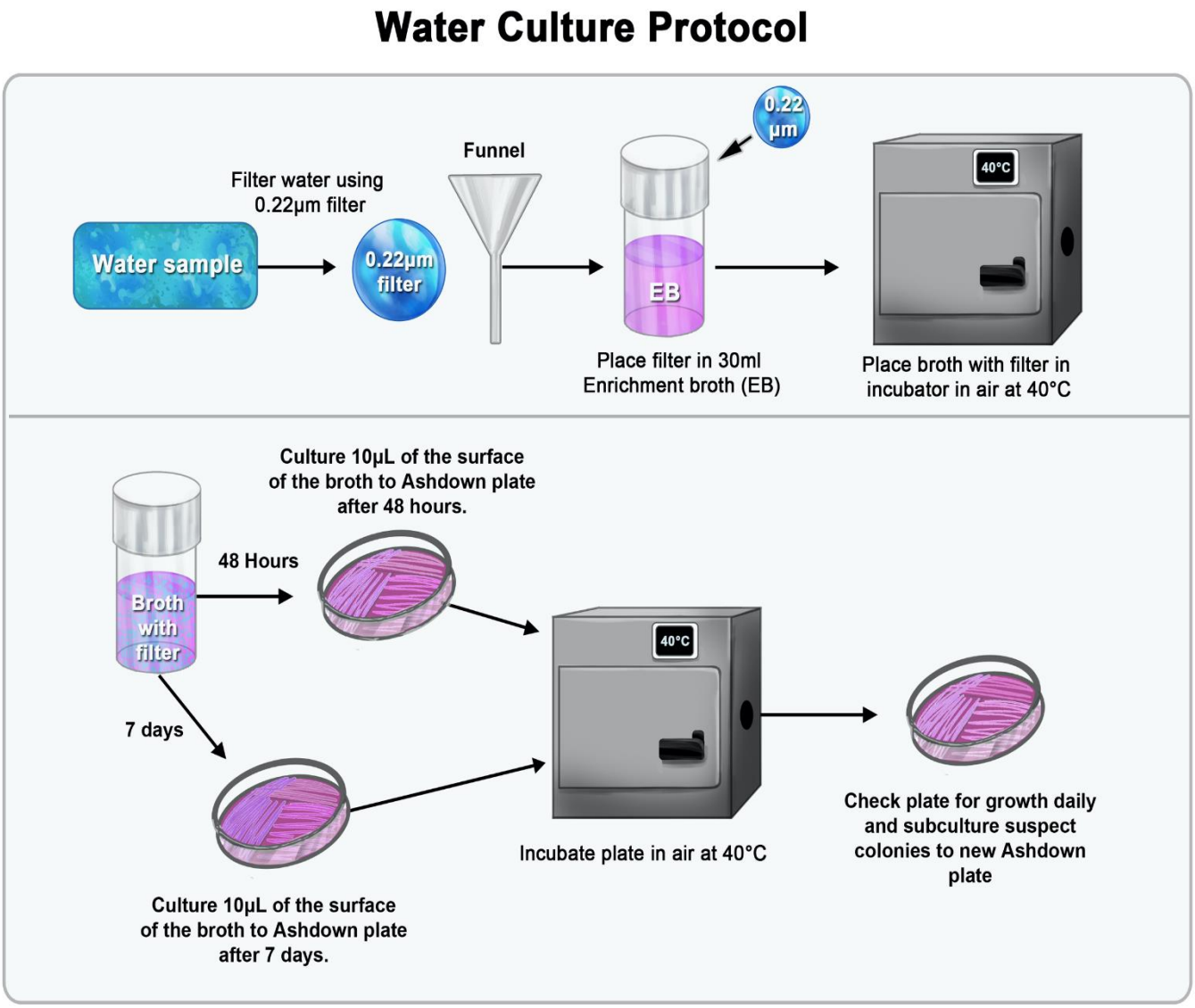


Figure 39- Diagram demonstrating the steps involved in culturing *B. pseudomallei* from water specimens.

B. pseudomallei Culture Detection from Air Samples

B. pseudomallei can aerosolize during severe weather events such as tropical monsoons or hurricanes. There is now increasing evidence for the occurrence of melioidosis acquired by inhalation after heavy rainfall and strong winds. Microbial air samplers can be used in the field to collect a predetermined volume of air and capture the microorganisms onto a filter or an agar-based

growth medium (Whyte & Albisu 2007). While isolation of *B. pseudomallei* from air has rarely been successful, it has been reported on several occasions and may still be worth trying where resources permit (Chen et al. 2015; Currie et al. 2015). Isolation may be improved if samples are collected during storms or severe weather events, or when there is increased risk of bacterial aerosolization, such as high-pressure hosing. In these instances, a mask should be worn to prevent aerosol exposure.

Air Sample Culturing Protocol

1. If selective agar plates have been used to collect air specimens, place plates directly in a 40°C incubator and check plates after 2 days for growth, then daily.
2. Otherwise, if using portable air sample filter, place the filter in a clean labelled urine specimen jar or sealable container with sterile forceps/tweezers.
3. Add 30 mL of selective enrichment broth (TBSS-C50 or Ashdown's broth) to the container with the filter. Ensure the filter has completely dissolved in the broth (if using a gelatine filter) or is completely submerged in the broth.
4. Incubate the enrichment broth containing the filter at 40°C.
5. After 48 hours incubation subculture 10 mL of the surface of the broth onto an Ashdown's agar plate. Streak out to achieve single colonies.
6. Check agar plates after 2 days for growth and then daily for growth for suspected colonies.
7. Repeat steps 5 and 6 after the enrichment broth has been incubating for 7 days by subculturing an additional 10 mL of the surface of the broth onto a clean Ashdown's agar plate. Incubate plates and check after 2 days growth and then daily for growth for suspected colonies.
8. Any bacterial colony suspected to be *B. pseudomallei* should be further identified and confirmed using the methods listed below.

9. If colony is identified as *B. pseudomallei* store a large sweep of growth in a sterile closable tube at -80°C in tryptone soya broth containing 20% glycerol. If a -80°C freezer is not available, store at -20°C and immediately refer to the local reference laboratory.

Identification of *Burkholderia pseudomallei*-Suspected Colonies

Any colony with a morphology typical of *B. pseudomallei* should be further tested. Initial screening of suspect colonies from Ashdown's agar can be done via latex agglutination using latex particles coated with monoclonal antibodies that are specific to the 200-kDa exopolysaccharide of *B. pseudomallei* (Wuthiekanun et al. 2002*).* See the next chapter covering *B. pseudomallei* serology diagnostic testing for the latex agglutination protocol. Latex-positive colonies can then be screened for antimicrobial susceptibility using the 3-disk susceptibility method described in Chapter 8. Any oxidase-positive colony that has an appearance typical of *B. pseudomallei* on Ashdown's agar and is resistant to colistin and susceptible to co-amoxiclav can be presumptively identified as *B. pseudomallei* if it is positive for latex agglutination. Additional methods are also available for the identification of isolates presumptively identified as *B. pseudomallei* including biochemical test kits such as the API 20NE or molecular identification tests such as PCR.

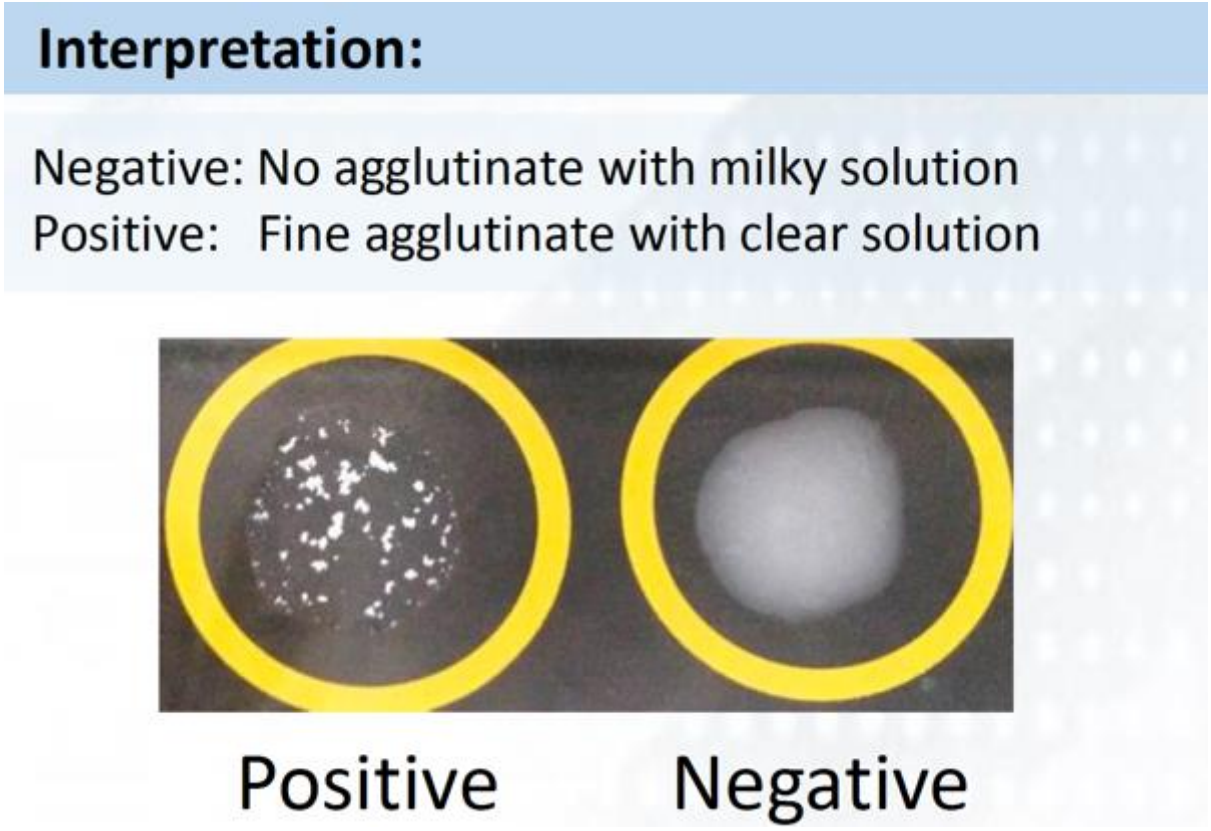


Figure 40- Interpretation of latex agglutination assay for suspected *B. pseudomallei* colonies (Melioidosis.Info 2016).

Chapter Summary

Bacterial culture is the current "gold standard" method recommended for the detection and isolation of *B. pseudomallei* from clinical and environmental specimens. The above chapter describes the current conventional consensus methods for bacterial culture identification of *B. pseudomallei.*

It is recommended that isolates presumptively identified as *Burkholderia species* for the first time in a country or region should be referred to a national or international reference laboratory or, in the U.S.A, a LRN Reference Laboratory for definitive identification.

Chapter 11: *B. pseudomallei* Antibody Serology and Direct Antigen Detection Methods

Chapter Overview

The following chapter describes common serological and direct antigen detection methods used for the diagnosis of melioidosis. Direct detection latex agglutination and the rapid lateral flow immunoassay are used in some endemic areas and have the potential for use as both rapid and sensitive *B. pseudomallei* antigen detection methods globally. New serological assays relying on purified antigens have also shown some evidence of improved sensitivity and specificity compared to the indirect haemagglutination assay (IHA), however these are still currently lower than for culture diagnosis. Moreover, interpretation of a positive antibody test is also difficult in endemic areas with high background seroprevalence rates and because the time to seroconversion upon exposure to *B. pseudomallei* can vary. As a result, bacterial culture should still be sought for any patient with clinical presentations strongly suggestive of melioidosis.

Antibody Serology Detection

Even amongst culture-positive patients, isolation and confirmation of *B. pseudomallei* takes time and expertise, resulting in delayed treatment. Serological tests are often used as a preliminary test in endemic areas to speed up the time to diagnosis. Since a positive result suggests exposure to *B. pseudomallei*, serological assays can be useful in determining if certain immunologically naïve populations, such as laboratory workers, military personnel, and other returning travellers from non-endemic countries, have been exposed to the bacterium (Wiersinga et al. 2012). They are particularly useful when paired acute and convalescent serum samples taken at least two weeks apart are available. There are many antibody detection methods in use, which are generally unstandardized or only used in reference laboratories in endemic countries (Lau et al. 2015). Interpretation of a positive antibody test is also difficult in endemic areas with high background

seroprevalence rates and because the time to seroconversion upon exposure to *B. pseudomallei* is variable (Cheng et al. 2006). Thus, bacterial culture should always be sought for any patient with clinical presentations strongly suggestive of melioidosis.

Common Serological Assays for Melioidosis

While the indirect haemagglutination assay (IHA) is the most widely used test, there is no "gold-standard" serological assay used to detect a melioidosis infection. Rather, the choice of serology test still typically depends on available resources and local technical knowledge. Immunoglobulin G (IgG) and immunoglobulin M (IgM) enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays (IFAs) have also been developed for the serodiagnosis of melioidosis (Suttisunhakul et al. 2016; Wuthiekanun et al. 2004). Several studies have suggested that immunoglobulin G (IgG) assays using whole-cell antigens are more specific for acute infection (Chantratita et al. 2007). More recently, a hemolysin co-regulated protein 1 (Hcp1) ELISA has also been shown to be a highly effective for the detection of antibodies directed against *B. pseudomallei* and may be used to replace the IHA for the detection of melioidosis in resource-limited areas in the future (Phokrai et al. 2018; Pumpuang et al. 2017).

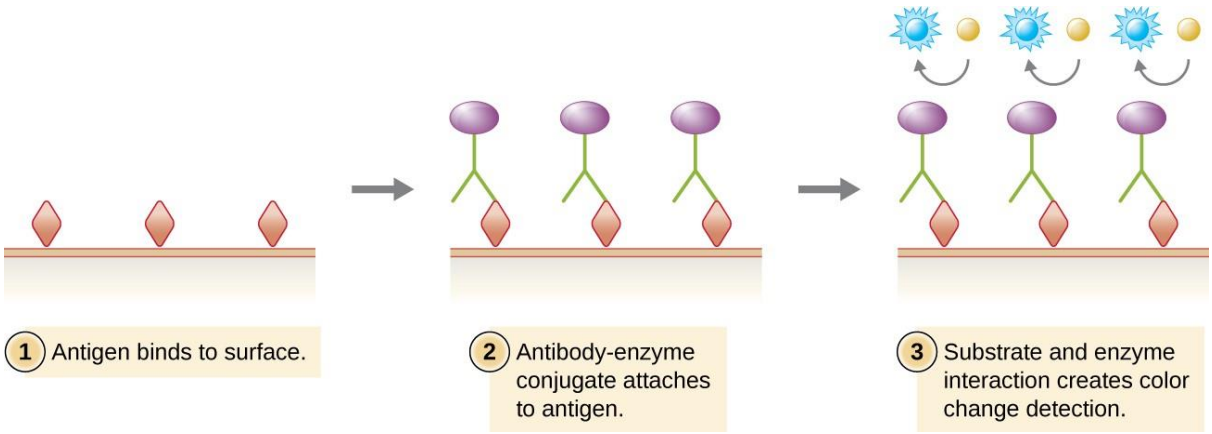


Figure 41- Direct ELISA, such as the Hcp1 ELISA, use an enzyme-antibody conjugate to deliver a detectable substrate to the site of an antigen. The substrate may be colorless and converted into a

colored product or may be a fluorescent molecule that will give off a detectable signal after activation of the enzyme. ("Cavitri"/ Wikimedia Commons).

Indirect Haemagglutination Assay

The indirect haemagglutination assay (IHA) is the main serologic assay for melioidosis used worldwide. It is a simple and inexpensive serological test used to detect antibodies raised by humans to *B. pseudomallei* (Hoffmaster et al. 2015). Here, sheep red blood cells are sensitized with antigen originating from local clinical *B. pseudomallei* reference strains (Ileri 1965; Lau et al. 2015). The sensitized blood cells are then added to serial dilutions of heat-inactivated patient serum. The IHA titer is identified as being the highest dilution of serum that results in agglutination of the blood cells (Cheng et al. 2006).

Figure 42- If enough antibody is present to agglutinate, the antibody-antigen complex will form a mat at the bottom of the well. If not enough antibody is present, the cells will fall to the bottom of the well and form a pellet or button (McGill University 2005).

While the IHA is routinely used in melioidosis-endemic areas it has several disadvantages, including a short shelf-life and unstandardized antigen preparations. The sensitivity and specificity are also low due to high background seropositivity in some endemic areas, likely because of repeated exposure to *B. pseudomallei* (Chaowagul et al. 1989; Cheng & Currie 2005). As a result, many patients presenting with febrile illness are misdiagnosed with melioidosis in highly endemic regions (e.g. northeast Thailand) due to a positive IHA test. Cut-off values are normally assigned based on background seropositivity in the population to account for this. In Australia, for example, a positive titer is normally defined as being 1:40 or higher, while in

Thailand probable cases are defined as clinically compatible illness with an IHA titer ≥1:160 (Hantrakun et al. 2019). In contrast, some patients with melioidosis never mount a good antibody response, possibly due to a compromised immune system. Thus, melioidosis diagnosis should never rely on a positive IHA result alone.

IHA Protocol

Materials

- *B. pseudomallei* antigen from local clinical *B. pseudomallei* reference strains (stored at -20°C)
- Phosphate Buffered Saline (PBS) pH 6.5
- Sheep Red Blood Cells stored in Alsever's solution (see below for solution components)
- 96-well U-bottom microtiter plates
- Sterile plastic tubes
- Pipettes
- Sterile pipette tips

Controls

Positive and negative control serum specimens should be used when carrying out the IHA test, all of which should be prepared in the laboratory from selected clinical specimens. Controls should be tested with each new run to validate performance of the test.

- The negative control should be comprised of pooled sera from 3 patients with no detectable IHA titer.
- The positive control should be comprised of pooled sera from 3 patients with known positive IHA titer of >1:160 (aim for control positive value of around 1:1280).

Determination of optimal *B. pseudomallei* antigen concentrations

Sheep red blood cells must first be sensitized with antigen originating from local clinical *B. pseudomallei* reference strains prior to patient testing. The optimal bacterial antigen concentration must be calculated for every batch of bacterial antigens used. This is typically established by testing the agglutination known positive serum with red cells sensitized with a serial dilution of the bacterial antigen. Antigens from two separate local reference strains of *B. pseudomallei* should be tested separately. Using two strains rather than one is done to account for any antigenic variation between strains. The optimal antigen concentration is the one that gives the correct (previously known) IHA titer for the pooled serum. This concentration is determined by a block titration. The optimal concentration of antigen is typically in the range of 1:80 to 1:120, but this can vary between bacterial antigens. Antigen preparation and dilution can often be done at a local reference laboratory, where there is access to local clinical *B. pseudomallei* reference strains.

See the link below for an example of a method used for antigen preparation and antigen titration for *B. pseudomallei* IHA in Thailand: https://www.melioidosis.info/download/MICRO_SOP_IHA_ENG_v1%203_8Dec11_SDB.pdf.

Procedure (Melioidosis.Info 2011)

1. Collection and preparation of sheep red cells
    a. Collect sheep blood aseptically into an equal volume of sterile Alsever's solution (see below for components). Label with the date and store at 4°C for less than one month.
    b. Prior to use, cells require washing and suspension in Phosphate Buffered Saline (PBS) to give an initial working dilution of 10%. Prepare these fresh each time cells are to be sensitized. To prepare a 10% suspension of sheep blood cells:
        i. Centrifuge 20 mL sheep red blood cell/Alsever's solution at 3000rpm for 10 minutes.

ii. Remove the supernatant (this should be clear – not lysed).

iii. Wash the pellet with 20 mL PBS. Mix/invert and centrifuge as above 3 times. Discard the supernatant.

iv. After the third wash use blood cell pellet to make a 10% solution in PBS (e.g., 2 mL blood cells in 18 mL PBS).

2. Preparation of serum specimens- This is done to remove complement and non-specific sheep cell agglutinins.

    a. Add 50 µL of each specimen and controls to an appropriately labelled plastic tube (e.g. Eppendorf tube).
    b. Inactivate 50 µL serum samples by placing them in a 56°C water bath for 30 minutes.
    c. Remove the specimens from the water bath and allow them to return to room temperature.
    d. Add 350 µL PBS and 100 µL 10% non-sensitized sheep cells to each tube.
    e. Incubate at room temperature for 1 hour, mixing every 15 minutes.
    f. Spin in a centrifuge for 3 minutes at 13,000 rpm and retain the supernatant (supernatant will be used for the actual dilution).
    g. Be sure to include the prepared positive and negative controls.
    h. Store at 4°C for up to 24 hours or freeze at -80°C (good for up to 5 years).

3. Preparation of 10% sensitized sheep red cells

    a. Ensure the *B. pseudomallei* antigens have already been diluted to the optimum concentration before starting.
    b. Label two 10 mL plastic centrifuge tubes “Sensitized Cells”.
    c. Add 9.2 mL PBS to the tubes.

d. Add 50 µL of the diluted *B. pseudomallei* antigens to the tubes and mix by inverting the tubes.
e. Add 750 µL of the prepared 10% sheep blood cell suspension to the tubes (this results in a final antigen dilution of 1 in 250).
f. Incubate the tubes at 37°C for 1 hour, mixing every 15 minutes.
g. After 1 hour centrifuge the cells at 4,000rpm for 5 minutes and wash 3 times with 10 mL of PBS, removing the supernatant after each wash.
h. After the third wash dilute the pellet with 10 mL of PBS and resuspend the pellet to achieve a suspension.
i. The sensitized cells are now ready for use.
j. Store at 4°C until use (24-48 hours).

4. Preparation of 10% unsensitized sheep red cells
   a. Label one 10 mL plastic centrifuge tube "Unsensitized Cells".
   b. Add 9.25 mL of PBS to the tube.
   c. Add 750 µL of the prepared 10% sheep blood cell suspension to the tube and vortex tube to mix.
   d. The unsensitized cells are now ready for use.
   e. Store at 4°C until use (24-48 hours).

5. Testing unknown patient serum samples- The bacterial antigens that have been prepared from two local strains should now be pooled. The volume of each antigen must be adjusted so that the optimal antigen concentration of each is the same after dilution. This is especially important if the antigens do not have identical concentrations.
   a. Add 50 µL of PBS into all wells of columns 2 to 11 of a 96-well U-shaped microtiter plate.

b. Add 50 µL of the prepared supernatant from each serum specimen being tested into all wells of columns 1, 2 and 12. The final 2 rows should contain the negative and positive control serum, respectively. If multiple plates are used these controls are only required on one plate.

c. Make a two-fold dilution series from column 2 through to column 11 (using 50 µL volumes) and discard the remainder. The final volume should be 50 µL in all wells. The serum dilution achieved should be as shown in Table 15 below. Try not to produce any bubbles inside of the wells.

d. Add 25 µL of 1% sensitized cells to all wells of columns 1 – 11.

e. Add 25 µL of non-sensitized cells to all wells in column 12. This will act as another negative control.

f. Tap plate to mix, cover with foil and incubate 2 hours at room temperature. Store plates at 4°C if leaving overnight.

g. Read agglutination patterns at either time point and record titers. These should be the same at 2 hours and overnight.

6. Interpretation of results

a. Negative wells (no red cell agglutination) have an intact button at the bottom of the well.

b. Positive wells (agglutination) will show red cells settled as a fine carpet or as a loose button with ragged edges (Figures 42-43). Plates can also be read with a mirror using light transmitted from below if required. The correct titer is defined as the first clearly positive well. If results are indeterminate they should be recorded as equivocal and not used.

c. Controls: This is a control for non-specific red cell agglutination and should show no agglutination. Ensure rows containing the negative control show no agglutination and

positive control show agglutination. The positive control should also be identical or be no more than one dilution different from the known IHA result (1:2560).

Table 15- Example of an IHA plate setup. Note that there should be two negative controls and one positive control.

Serum dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	Unsensitized cells (Negative Control) 1:10
Column	1	2	3	4	5	6	7	8	9	10	11	12
Patient A												
Patient B												
Patient C												
Patient D												
Patient E												
Patient F												
Negative control												
Positive control												

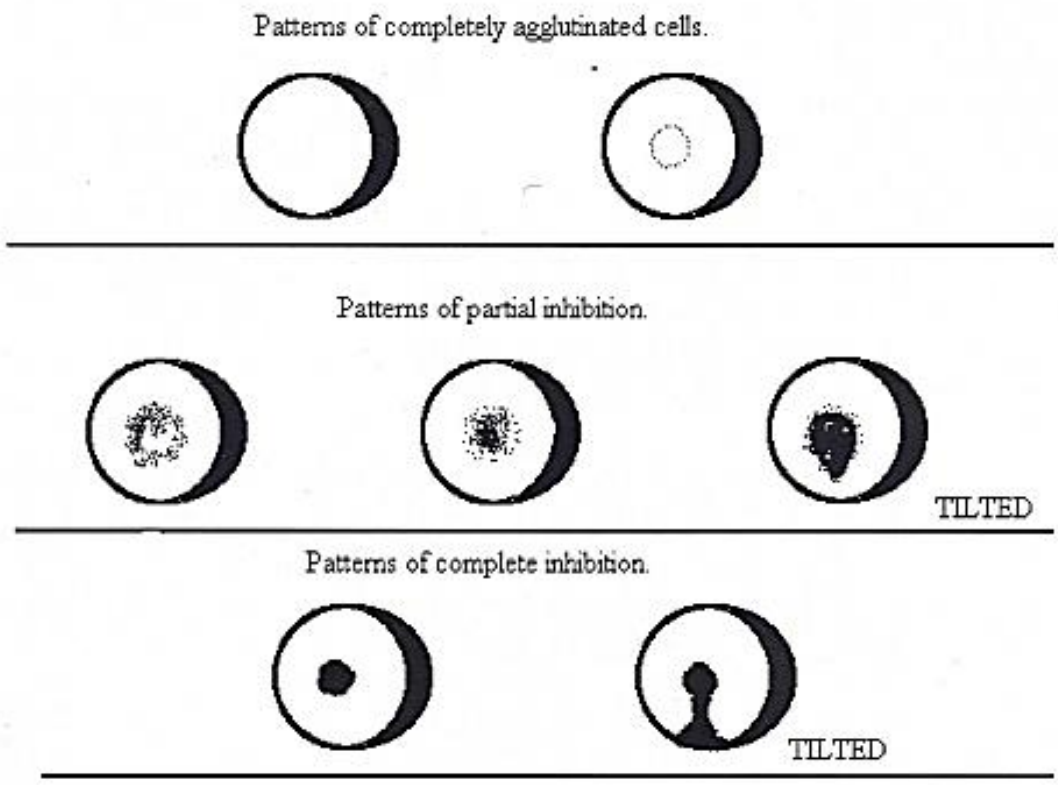


Figure 43- Examples of settled cell patterns showing full and partial agglutination, as well as a negative IHA result (complete inhibition) (APHA).

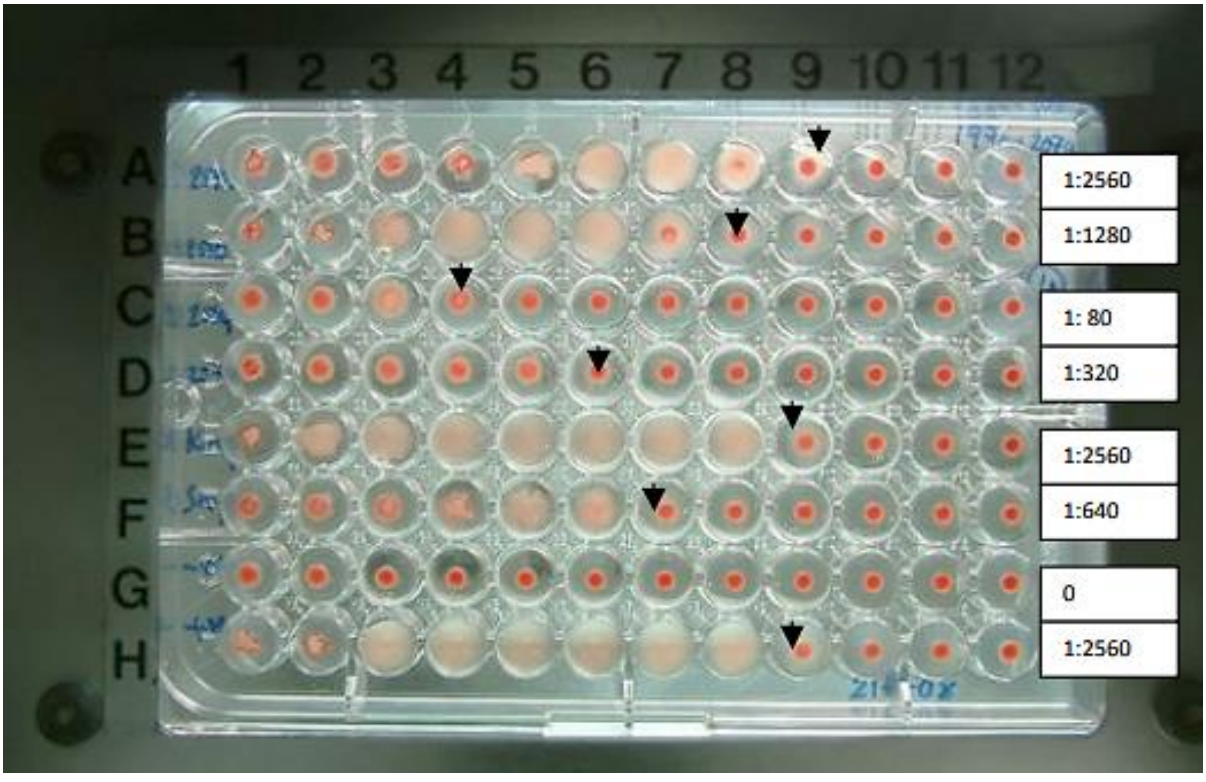


Figure 44- Example of an IHA plate setup and interpretation of agglutination results from different patient samples. Row G and column 12 both serve as negative controls, while Row H functions as the positive control (Melioidosis.Info 2011).

Alsever's solution

Alsever's solution is used for the collection and storage of sheep red cells. Ensure sheep blood cells are collected under aseptic conditions and mixed into an equal volume of sterile Alsever's solution prior to using in the IHA. This product can also be ordered commercially depending on local availability (e.g., Sigma-Aldrich #A3551).

Alsever's solution Ingredients:

- Glucose- 2.05 g
- NaCl- 0.42 g
- Sodium citrate dehydrate- 0.8 g
- Anhydrous citric acid- 0.055 g
- Distilled water- Up to 100 mL

Mix ingredients and sterilize at 121°C for 15 minutes. Check that the pH is 6.1 before use.

Direct Antigen Detection Methods

Direct antigen tests are immunoassays used to detect the presence of a particular *B. pseudomallei* antigen. They are both rapid, relatively inexpensive and can be indicative of an active *B. pseudomallei* infection. Direct IFA and sandwich ELISAs, which make use of monoclonal antibodies raised against *B. pseudomallei* crude whole cell antigens, are utilized in some highly endemic areas. While the sensitivity is lower than for culture, they are useful for rapid screening of clinical specimens from severely ill patients, particularly those patients with a high bacterial load

(Anuntagool et al. 1996; Lau et al. 2015; Walsh et al. 1994). A monoclonal antibody-based latex agglutination assay is also available to aid in identifying *B. pseudomallei* from cultures or blood culture broth (Duval et al. 2014) and a rapid diagnostic lateral flow immunoassay (LFI), called the Active Melioidosis Detect, has become more widely available (Houghton et al. 2014). These tools are now routinely used as point-of-care diagnostic screening methods in several endemic areas.

1. Lateral Flow Immunoassay

Lateral flow immunoassays (LFIs) are ideal for rapid diagnostic testing and are ideal for resource-limited settings in that they are inexpensive, stable at room temperature and can accept multiple specimen types. LFIs function by running a liquid sample along the surface of a pad with reactive molecules that show a visual positive or negative result. The pads are designed using a series of capillary beds, such as porous nitrocellulose paper (Koczula & Gallotta 2016). LFIs are typically made of two parts: 1) A chromatographic system that is used to separate components of a mixture based on of differences in their movement through the membrane and 2) an immunochemical reaction that occurs between the antibody-antigen complex.

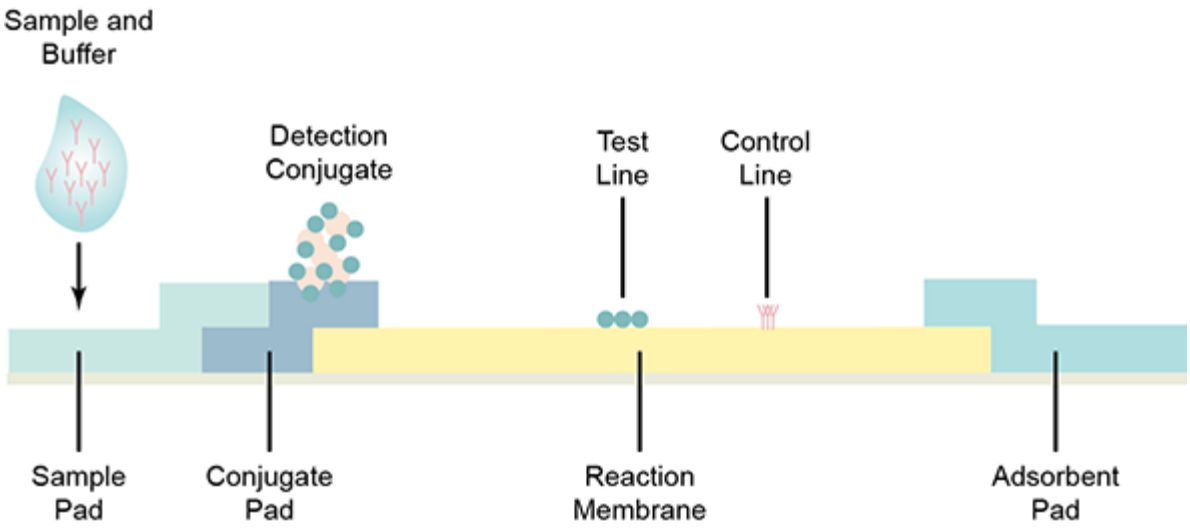


Figure 45- The basic structure of lateral flow assay. The sample pad is where sample is dropped; The conjugate pad which transfers the detector reagent and test sample onto the membrane; The reaction membrane contains the test line and control line demonstrating the antigen-antibody interaction; The absorbent pad reserves waste and increases the total volume of sample that enters the test strip (Koczula & Gallotta 2016).

Recently a rapid diagnostic LFI, called the Active Melioidosis Detect ™ (AMD), has become more widely available in melioidosis endemic areas (Houghton et al. 2014). The membrane is pre-coated with a specific monoclonal antibody to the *B. pseudomallei* capsular polysaccharide (CPS). During testing, the specimen reacts with a gold conjugate that has been pre-coated in the test device. The mixture then travels through the membrane and complexes with CPS from the sample and the monoclonal antibody on the membrane to produce a line in the test region. This line indicates a positive result, while its absence indicates a negative result. Regardless of whether a test line appears, a line at the control line region should always appear. The presence of this control is used to verify that there enough sample volume has been used and the reagents are working as excepted.

While the rates of sensitivity and specificity have been shown to be comparable to that of PCR detection methods when used on whole blood culture specimens (upwards of 95-99% sensitivity and 100% specificity), they have varied widely for other clinical specimen types (Peeters et al. 2018; Rizzi et al. 2019; Robertson et al. 2015; Woods et al. 2019). Thus, while the LFI may be used to both aid in and reduce the time to diagnosis, a negative test result should not completely rule out the possibility of melioidosis.

Active Melioidosis Detect ™ LFI Protocol

Materials and equipment

- Test kit containing LFI strips and buffer (chase) vials

- Sterile test tubes
- Pipette
- Sterile pipette tips
- Centrifuge
- Biosafety cabinet (class II) and appropriate PPE

Blood Specimens

Human whole blood and serum can be tested with this test strip. Use finger pricked blood, or collect blood in presence of anti-coagulants citrate, EDTA, or heparin. Testing should be performed as soon as possible after sample collection but blood specimens may be stored overnight at 2-8°C.

1. Remove the test strip from the foil pouch or vial.
2. Add 35 µL of whole blood, serum or plasma to the test strip and onto the colloidal gold conjugate coated area beneath the sample arrow tape.
3. Add 150 µL (approximately 3-4 drops) of Chase buffer to the sample pad.
4. Results should be read after 15 minutes.

Sputum Specimens

<u>If sample is chunky or thick:</u>

1. Add 150 µL (3-4 drops) of lysis buffer to 20 µL of sputum and mixed well by vortex mixer.
2. Add 20 µL of sputum/lysis buffer to test strip followed by 150 µL (3-4 drops) of Chase buffer to the sample pad.
3. Results should be read after 15 minutes.

<u>If sputum is thin:</u>

1. Add 100 µL (2-3 drops) of lysis buffer to 50 µL of sputum and mixed well by vortex mixer.
2. Add 20 µL of sputum/lysis buffer to test strip followed by 150 µL (3-4 drops) of Chase buffer to the sample pad.

3. Results should be read after 15 minutes.

Pus Specimens

1. Add 100 µL (2-3 drops) of lysis buffer to 20 µL of pus and mixed well by vortex mixer.
2. Add 20 µL of pus/lysis buffer to test strip followed by 150 µL (3-4 drops) of Chase buffer to the sample pad.
3. Results should be read after 15 minutes.

Urine Specimens

1. Combine 50 µL of unfiltered urine and 2-3 drops of chase buffer, pipette up and down to mix.
2. Add to LFI sample pad and let test run for 15 min; read test and control line.
3. If urine is highly concentrated:
    a. Spin down maximum of 10 mL of urine at 3000- 4000 rpm for 10 minutes.
    b. Pour off supernatant and resuspend pellet in 50 µL (1-2 drops) lysis buffer.
    c. Add 20 µL of urine/lysis buffer to test strip followed by 150 µL (3-4 drops) of Chase buffer.
    d. Results should be read after 15 minutes.

Culture specimens

<u>From blood culture bottles:</u>

1. Spin down maximum of 10 mL of culture at 3000-4000 rpm for 10 minutes.
2. Pour off supernatant and resuspend pellet in 50 µL (1-2 drops) lysis buffer.
3. Add 20 µL of culture/lysis buffer to test strip followed by 150 µL (3-4 drops) of Chase buffer to the sample pad.
4. Results should be read after 15 minutes.

From bacterial isolates:

1. Pick a single colony and resuspend in 2 drops of lysis buffer and mix in a sterile plastic tube or vial.
2. Transfer bacterial suspension to test strip and immediately add 150 µL (3-4 drops) of Chase buffer to the sample pad.
3. Results should be read after 15 minutes.

Results Interpretation

Positive result

The test is positive when a control line (C) and test line (T) appear in the test area. A positive result indicates that capsular polysaccharide (CPS) antigen for *B. pseudomallei* is present in the sample. A faint line is considered a positive result. The red color in the test region will vary depending on the concentration and affinity of antigens present.

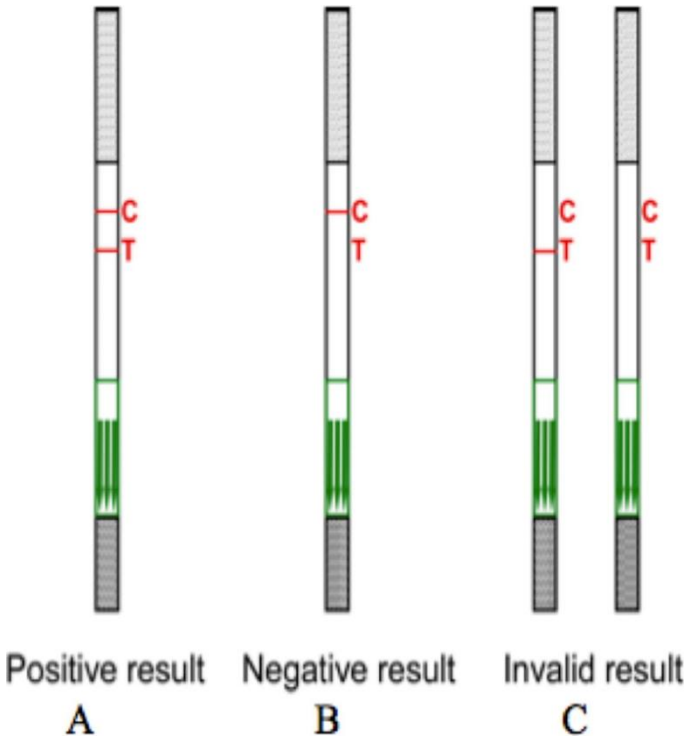


Figure 46- Interpretation according to the manufacturer instructions (InBios). C, control line; T, test line.

Negative result

The test is negative when only the control line appears. A negative result indicates that the CPS antigen of *B. pseudomallei* has not been detected.

An Invalid Result

The test is invalid if no control line appears, regardless of the presence of a test line. The test should be repeated with a fresh sample, if possible.

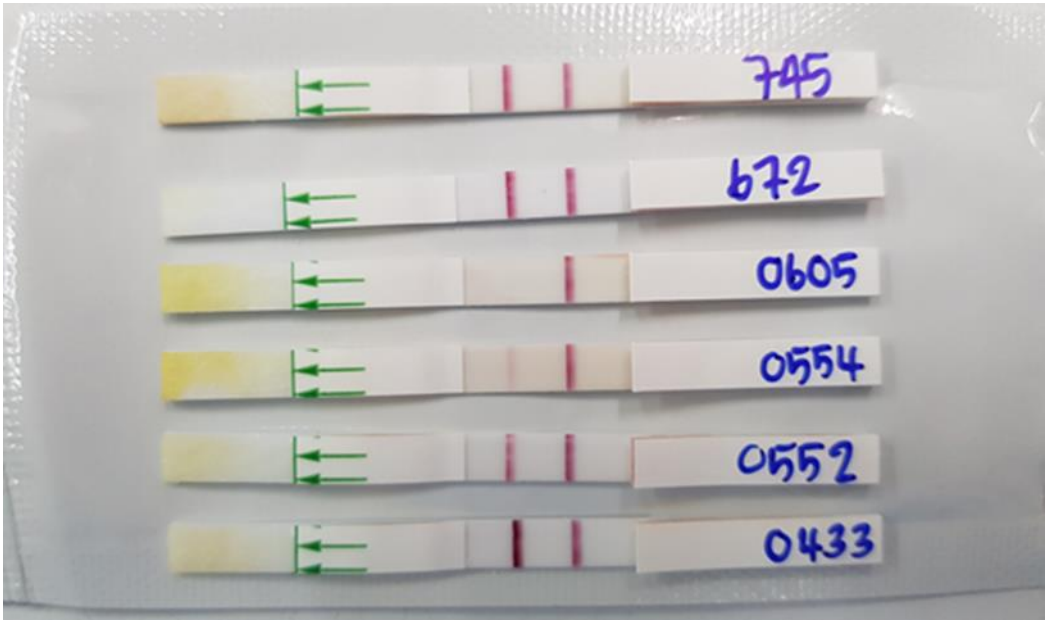


Figure 47- Positive, weak positive and negative LFI results. Test strips 745, 672, 0552, and 0433 are positive, test 0554 is a weak positive result and test 0605 shows a negative test result (Wongsuvan et al. 2018). Note: An updated cassette version of the AMD LFI is also now available through InBios. See https://inbios.com/active-melioidosis-detect-rapid-test-kit-intl/ for more information about the Active Melioidosis Detect rapid test and ordering information.

2. Latex Agglutination

Latex agglutination is a rapid diagnostic test for the identification of *B. pseudomallei* isolates grown on agar, liquid culture or from blood culture fluid. The most widely used latex agglutination assay is based on a monoclonal antibody specific to the *B. pseudomallei* 200-kDa exopolysaccharide (Anuntagool et al. 2000; Samosornsuk et al. 1999). Latex agglutination is particularly useful in poorly-resourced clinical diagnostic settings in that it is rapid (<5 minutes), inexpensive, simple to learn, and is both sensitive and specific (Hoffmaster et al. 2015).

Latex agglutination testing for *B. pseudomallei* is typically done as an adjunct to culture diagnosis on suspect colonies growing on Ashdown agar. Latex positive colonies can then be screened for antimicrobial susceptibility. Any colony that has an appearance typical of *B. pseudomallei* on Ashdown agar and is resistant to colistin and susceptible to co-amoxiclav can be presumptively identified as *B. pseudomallei* if it is positive for latex agglutination.

Anyone interested in obtaining the *B. pseudomallei*-specific latex agglutination reagent can contact melioidosis.info@tropmedres.ac or Menzies School of Health Research, Australia at mark.mayo@menzies.edu.au.

Latex Agglutination Protocol

1. Use a sterile toothpick to pick a suspected colony and mix the colony with 5-10 µL of the latex reagent on a glass slide or latex agglutination card.
2. Gently rock the slide/card continually for 2 minutes, ensuring none of the suspension spills.
3. Observe for positive agglutination (fine, but distinct clumping).

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

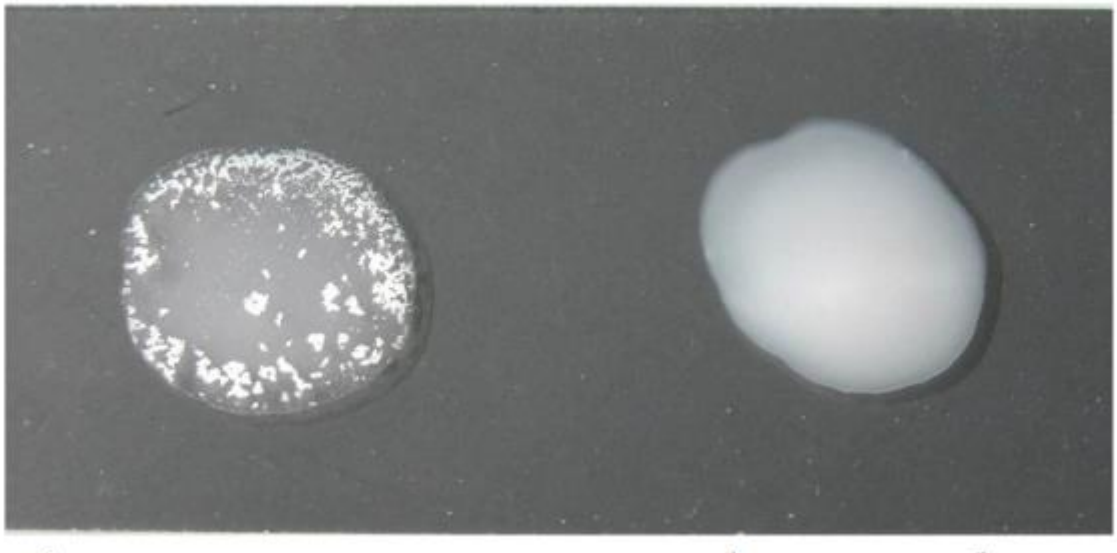


Figure 48- Positive latex agglutination test (left) shown after 10 µL of latex reagent is mixed with 10 µL of positive control reagent. A negative latex agglutination test is shown on the right after mixing 10 µL latex reagent with 10 µL negative control reagent (Melioidosis.Info 2012).

3. Immunofluorescence Assay (IFA)

The direct IFA is a rapid, inexpensive and simple method for the direct detection of *B. pseudomallei* antigens. It uses a monoclonal antibody specific for an exopolysaccharide of *B. pseudomallei* and is particularly useful for specimens with high bacterial density (e.g. pus, sputum, and urine) and blood culture fluid (Chantratita et al. 2013; Tandhavanant et al. 2013). *B. pseudomallei* IFA is not commercially available and is not normally used for routine diagnosis of melioidosis, however it can often be found in specialized laboratories in melioidosis-endemic regions for providing rapid diagnosis, such as those in northeast Thailand. Here, probable cases of melioidosis are defined as clinically compatible illness with an IFA result >1:400, or a 4-fold increase in titer in confirmed cases (Hantrakun et al. 2019). While IFA is simple and only needs minimal time for a diagnosis, it requires a UV microscope and experienced technicians. Additionally, while specificity of the IFA is high, the diagnostic sensitivity of IFA (45%–66%) is lower than that of culture and many other diagnostic tests for *B. pseudomallei* (Hoffmaster et al. 2015; Wuthiekanun et al. 2005). Thus, the diagnosis of melioidosis should never rely on IFA results alone.

For more information about *B. pseudomallei*-specific IFA reagents and how to obtain them contact the melioidosis group at Mahidol Oxford Tropical Medicine Research Unit (MORU), Bangkok, Thailand at melioidosis.info@tropmedres.ac.

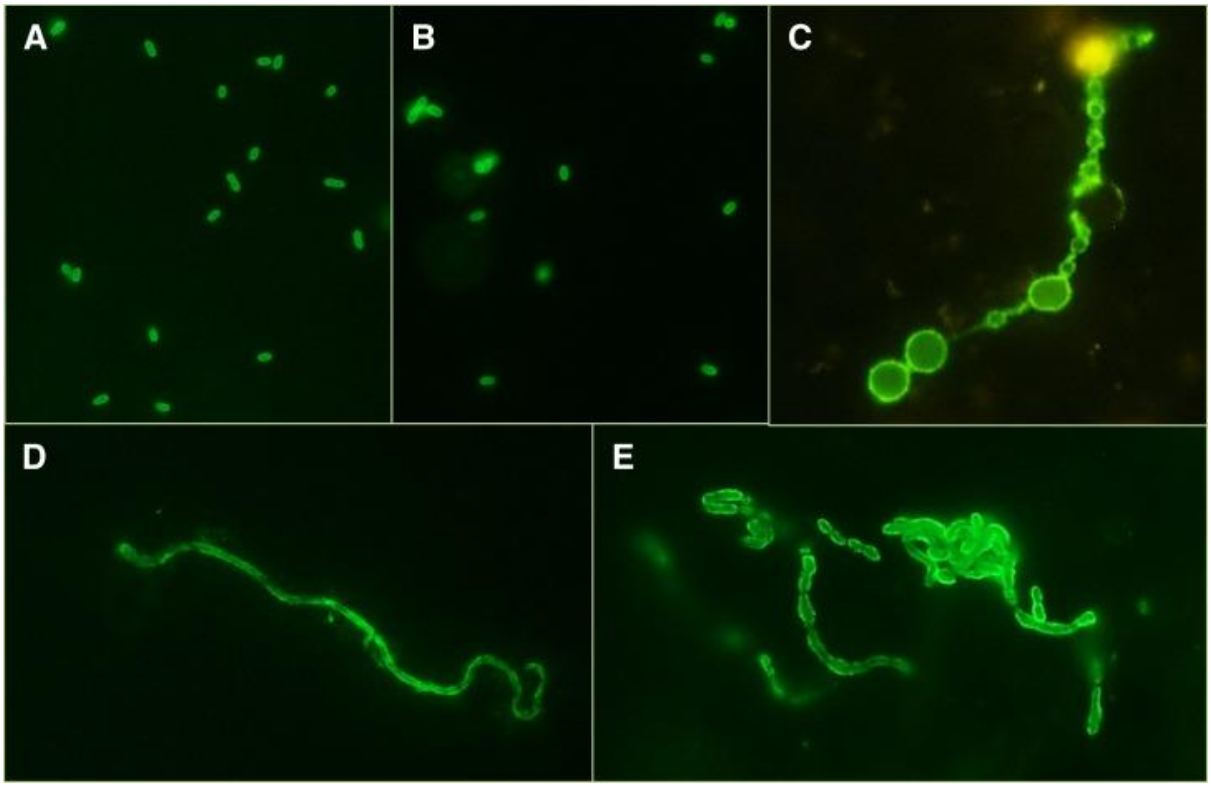


Figure 49- Fluorescent microscopy of *Burkholderia pseudomallei* stained with monoclonal antibody-IFA reagent. Bacterial cultures were grown on Columbia agar (A) LB broth (B), or from clinical samples (urine [C], pus [D], or sputum [E]) from patients with melioidosis (Tandhavanant et al. 2013).

Enzyme-Linked Immunosorbent Assays (ELISAs)

ELISA is used to detect the presence of either an antibody or antigen in a sample. While IgG and IgM ELISAs are commonly used for the serological diagnosis *of B. pseudomallei*, several antigen detection ELISAs, such as sandwich "antigen capture" ELISAs, have be used to detect *B. pseudomallei* antigens directly from clinical specimens (Anuntagool et al. 1996). Here, a monoclonal antibody directed against the antigen of interest is linked to a solid support (e.g., 96 well plate or a bead). The specimen is then added and if the bacterial antigens of interest are present, they are captured by

the bound antibody. The bound antigen can be detected using a second antibody that is linked to an enzyme. Lastly, a chromogenic molecule is converted by the enzyme to a produce a detectable signal (Figures 50-51) (Lequin 2005; Shah & Maghsoudlou 2016).

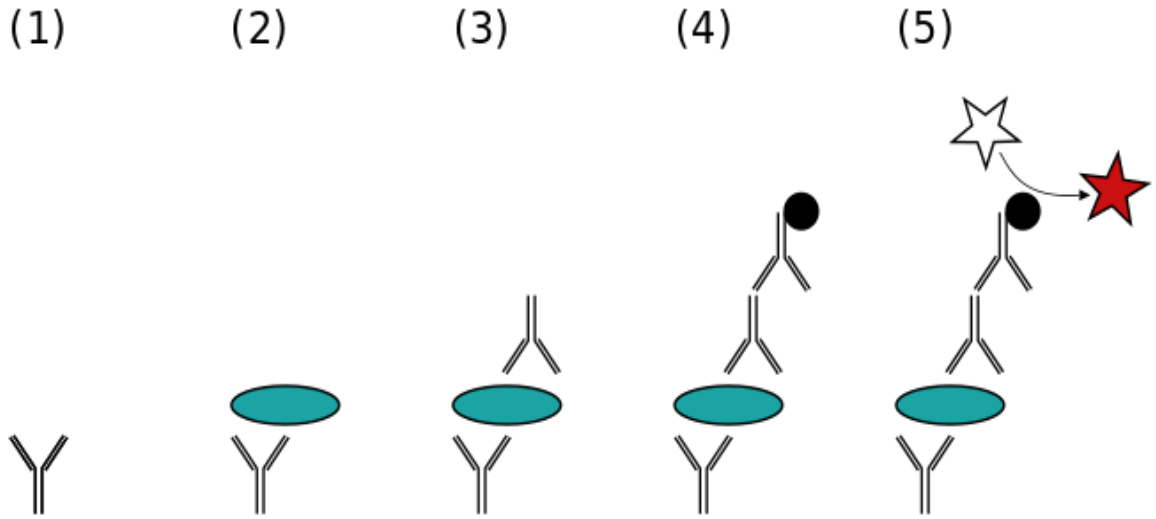


Figure 50- Example of a sandwich ELISA (1) the plate is coated with a capture antibody; (2) the sample is added and will bind to the capture antibody if the antigen is present; (3) a detection antibody is then added and will bind to the antigen; (4) a secondary antibody that is linked to an enzyme is added and binds to the detector antibody; (5) a chromogenic substrate is added and is then converted by the enzyme to give a detectable signal (Jeffrey M. Vinocur 2008 CC BY-SA 3.0 via Wikimedia Commons).

Several of these ELISAs have now been developed for the rapid direct detection of melioidosis and have targeted the *B. pseudomallei* recombinant truncated flagellin, type III secretion system (TTSS-3) Bip proteins, and cell envelope proteins (Anuntagool et al. 1996; Druar et al. 2008; Ma et al. 2014). However, most of these remain “in-house” assays and are not currently used for routine diagnosis of melioidosis.

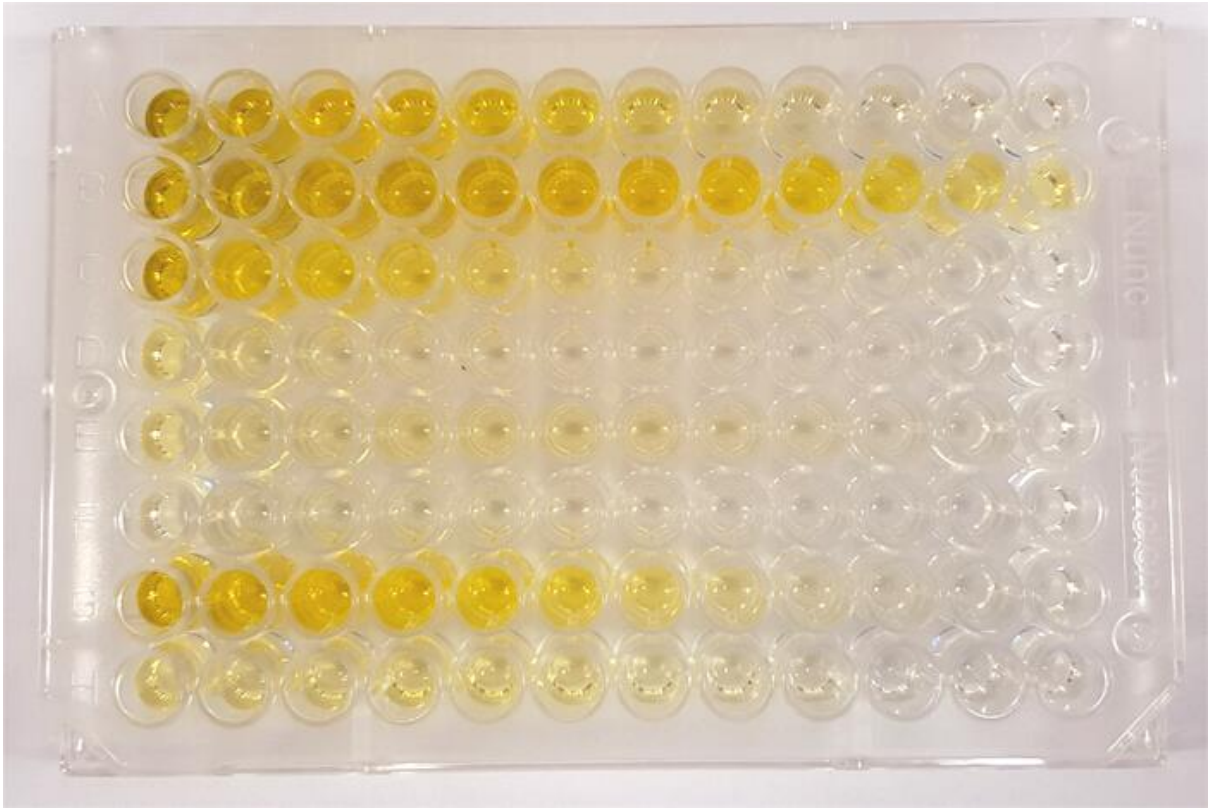


Figure 51- The yellow color indicates the target antigen is present. The higher the degree of color, the greater the concentration of the target antigen.

Chapter Summary

B. pseudomallei antibody serology and antigen immunoassays may be useful for rapid screening of clinical specimens and for determining if exposure to *B. pseudomallei* has occurred. The preceding chapter describes common serodiagnostic and direct antigen detection methods for melioidosis. Direct detection latex agglutination and the rapid lateral flow immunoassay have become more widely available in endemic areas (Houghton et al. 2014) and have the potential for use as rapid, point-of-care *B. pseudomallei* antigen detection methods globally. New serological assays relying on purified antigens have shown some evidence of improved sensitivity and specificity compared to the IHA, however these are still currently lower than for culture diagnosis. Moreover, interpretation of a positive antibody test is also difficult in endemic areas with high background seroprevalence rates and because the time to seroconversion upon exposure to *B. pseudomallei* can vary. As a result,

bacterial culture should still be sought for any patient with clinical presentations strongly suggestive of melioidosis.