Chapter 5: Clinical Specimen Collection

Chapter Overview

The following chapter focuses on the collection of clinical specimens from humans and animals suspected of having melioidosis. The appropriate specimens required to make an accurate diagnosis, collection procedures as well as specimen handling, storage and viability are discussed in detail. Methods of specimen testing for the presence of *B. pseudomallei* are described in subsequent manual sections.

Specimen Collection in Humans

B. pseudomallei is not part of the normal colonizing microbiota in humans and its isolation from any site is considered diagnostic (Wuthiekanun et al. 2001). Culture is considered the current gold-standard for diagnosis and culture-confirmation should always be sought in patients where disease is suspected (see more about culture diagnostic protocols for *B. pseudomallei* in Chapter 10). The likelihood of diagnosing melioidosis is maximized when appropriate clinical samples from a variety of sites and specimen types are sent to the microbiology laboratory for microscopy and culture (Hoffmaster et al. 2015).

Where available, all patients with suspected melioidosis should have the following samples taken for culture (Currie 2014):

1. Blood cultures
2. Sputum
3. Urine
4. Abscess fluid or pus
5. Swab of an ulcer or skin lesion
6. Throat swab

7. Rectal swab

Table 8- Specimen types, collection and instructions appropriate for human diagnostic testing for melioidosis (ASM 2016).

Sample Type	Collection and Transport
Blood	• Collect the volume and appropriate number of sets as dictated by the local protocols. • Collect specimens prior to starting antimicrobial therapy. • Transport bottles for culture as soon as possible.
Sputum or bronchoscopically obtained specimens	• Collect the specimen into sterile cup or closeable container or collect during bronchoscopy procedure. • Transport at room temperature within 2 hours of collection. • If the specimen will be transported 2-24 hours after collection store and transport at 2-8°C.
Tissue specimens (biopsies, abscess aspirates, pus) and swabs	• Tissue pieces (at least the size of a pea) should be collected and kept moist. • Transport in sterile container at room temperature within 1 hour of collection • Alternatively, swab samples can be submitted in a hospital transport tube with medium to stabilize specimen (e.g. Amies transport medium). Refrigerate swabs in transport media at 4°C and test within 24 hours after collection; otherwise, freeze at -70°C.
Urine	• Collect at least 1 ml into leak-proof, sterile container. • Transport at room temperature up to 2 hours after collection. • If the specimen will be transported more than 2 hours after collection refrigerate until culture inoculation.

Blood culture should be performed for all patients with suspected melioidosis where possible. The *B. pseudomallei* bacterial count in venous blood can reach high levels in septic patients, making conventional blood culture an effective means of establishing a bacteriological diagnosis (Wuthiekanun et al. 1990). Samples from non-sterile sites are less helpful since *B. pseudomallei* is often outcompeted by commensal species and the bacterial count may be much lower. However, this problem can often be overcome through the use of

selective culture media such as Ashdown's media (see Chapter 10 for further detail) (Wuthiekanun et al. 1990).

Urine and throat swab specimens should be cultured using selective media, even in those patients where pharyngitis or urinary symptoms are not present (Cheng et al. 2006; Wuthiekanun et al. 2001). The sensitivity of urine culture may be improved by centrifuging the specimen and culturing the resulting pellet (Limmathurotsakul et al. 2005). In patients with localized lesions, abscesses or pneumonia, sputum specimens, surface lesions swabs, and sterile aspirates (pus, pleural fluid, peritoneal fluid, CSF) should be collected when available and cultured using selective media. Rectal swabs should also be cultured using selective media (Currie 2014). It is recommended that cultures are repeated for any culture-negative patient whose symptoms are strongly suggestive of melioidosis.

While specimens can be *B. pseudomallei*-culture positive even in patients pre-treated with antimicrobial therapy, specimens should still be obtained before starting antimicrobials where possible. Prior treatment may interfere with bacterial growth (Hoffmaster et al. 2015). Absence of any clinical disease has been described in several culture-positive patients with cystic fibrosis and bronchiectasis; however, an attempt at eradication is still recommended for these patients (Holland et al. 2002; Howe et al. 1997).

A chest radiograph (X-ray) should also be performed on all patients with suspected melioidosis. Computed tomography (CT) scans may also be performed in adult patients with confirmed or suspected melioidosis to detect the presence of abscesses (e.g. in the prostate, liver, spleen, kidneys, urinary tract), or detect occult foci of infection (Hoffmaster et al. 2015). Abdominal ultrasound may be used as an alternative for pregnant women and children. In patients with central nervous system (CNS) involvement, magnetic resonance

imaging (MRI) is preferred over CT imaging, as it may indicate areas of hyperintense infection including micro-abscesses, leptomeningeal enhancement, or trigeminal nerve involvement (Smith et al. 2018).

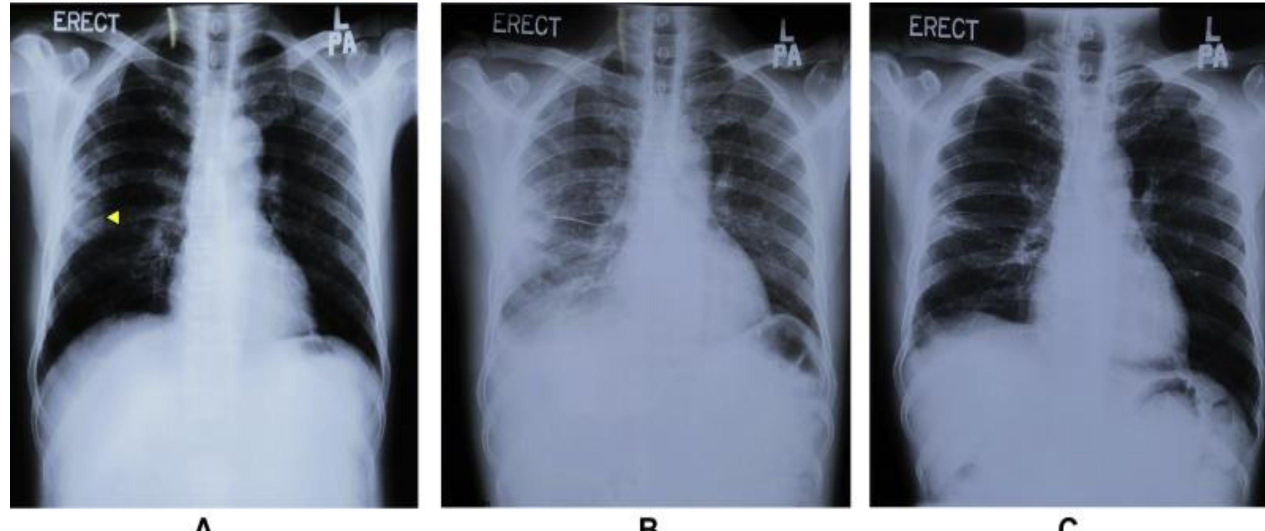


Figure 10- A) Right pulmonary opacity with cavity at right midzone (arrow) on first admission. B) Worsening of right pulmonary lesion compared to first admission with right-sided pleural effusion. C) Resolving right pulmonary lesion and resolved pleural effusion after 1 month of treatment (Chang et al. 2020).

Diagnosis of melioidosis (i.e. active disease) is not made based on a positive serology result since it does not provide a definitive diagnosis, however, melioidosis serology should still be ordered if melioidosis is suspected. The indirect hemagglutination antibody (IHA) test is most commonly used for the sero-diagnosis of melioidosis. While it cannot distinguish between patients with past exposure to the bacterium, latent infection or active disease, higher titers (>1:640) may suggest an active infection (Muttarak et al. 2009). Additionally, acute- and convalescent-phase serum specimens taken at least two weeks apart may be useful for diagnosis, with a fourfold or greater rise in *B. pseudomallei* antibody titer likely indicating infection.

Blood Culture Specimen Collection Procedures (CDC 2019)

1. Disinfect bottle tops with 70% isopropyl alcohol (alcohol pad), clean puncture site with alcohol and allow to dry.
2. For adults, collect at least 10-20 cc and 1-3 cc for suspected paediatric cases for each blood culture set (or as instructed by specific hospital laboratory protocol).
3. Two or three blood cultures from different sites per septic episode is usually sufficient.

Sputum Specimen Collection Procedures (CDC 2019)

1. The patient should rinse their mouth with water before collecting the specimen to remove excess oral flora.
2. Position the patient upright and have them cough deeply. A sodium chloride nebulizer can help to loosen secretions if needed.
3. Collect the expectorated specimen into a sterile, closable transport container, or collect during bronchoscopy procedure as dictated by the local protocols.
4. The specimen can be transported at room temperature if done within 2 hours. If the specimen is will transported more than 2 hours after collection it should be stored and transported at 2-8°C.

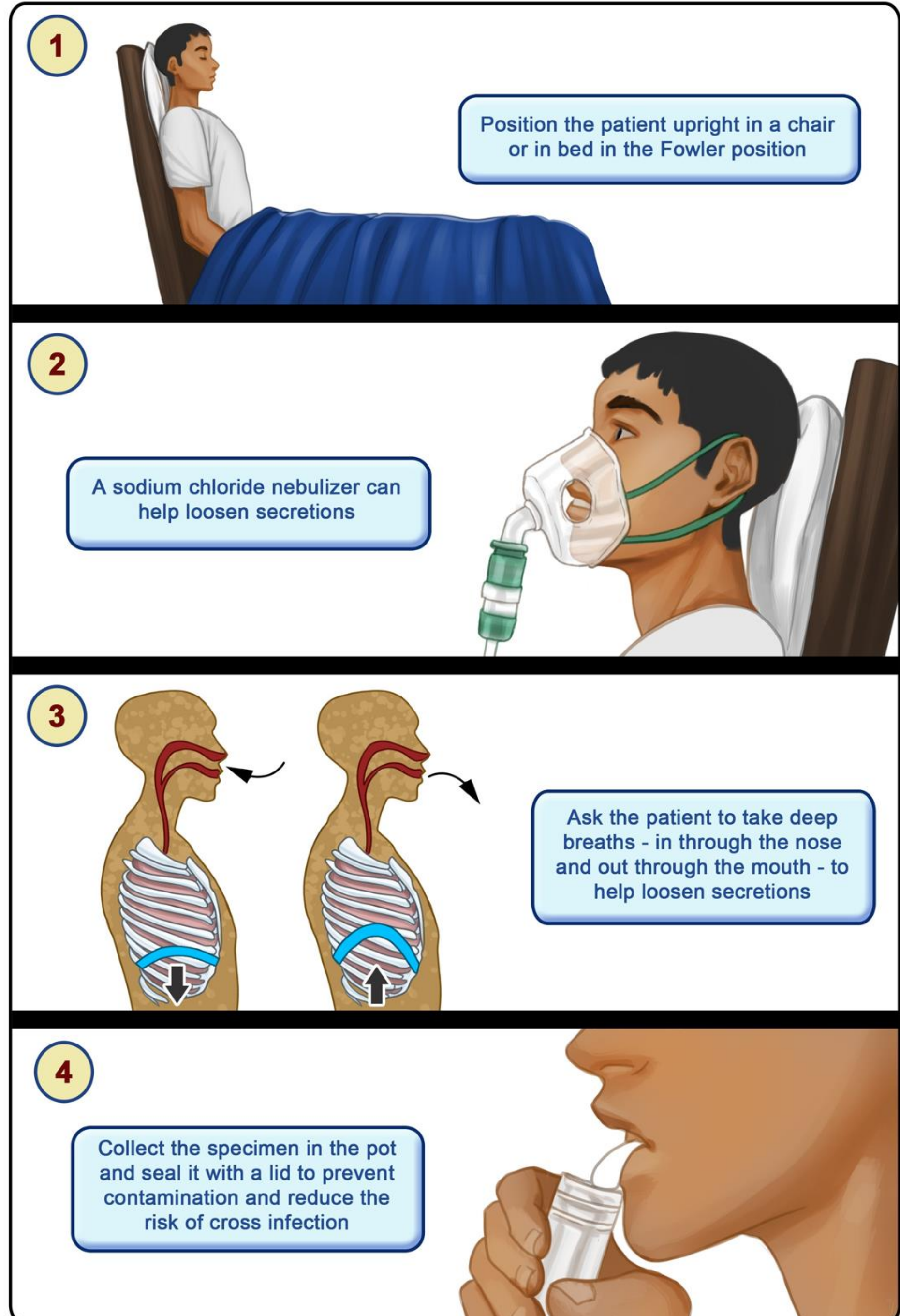


Figure 11- Procedure for obtaining a sputum specimen.

Cutaneous Lesion and Abscess Specimen Collection Procedures

1. Swab the base of the lesion using a sterile moist swab (pre-moistened with sterile saline or appropriate transport medium) and place in a sterile transport vial/tube

containing transport medium as instructed by the local protocols. Refrigerate at 4°C and test within 24 hours after collection; otherwise, freeze samples at -70°C.

2. Aspirate any pus or fluid if any and either transport in syringe or in a sterile transport vial/container.
3. For tissue specimens, try to obtain a deep culture or biopsy (at least the size of a pea) for culture whenever possible. Do not culture chronic superficial lesions. Store at room temperature and transport within 1 hour of collection.

Throat Swab Collection Procedure

1. Pass the swab across tonsils and back of oropharynx ensuring not to touch other oral surfaces (e.g., tongue, teeth, gums). Use of a tongue depressor may aid in collection.
2. Place into a sterile tube or vial with transport medium. The medium should be deep enough so that it covers the tip of the swab.
3. Break off top portion of the swab to close to container.
4. Refrigerate swabs in transport media at 4°C and test within 24 hours after collection; otherwise, freeze samples at -70°C.

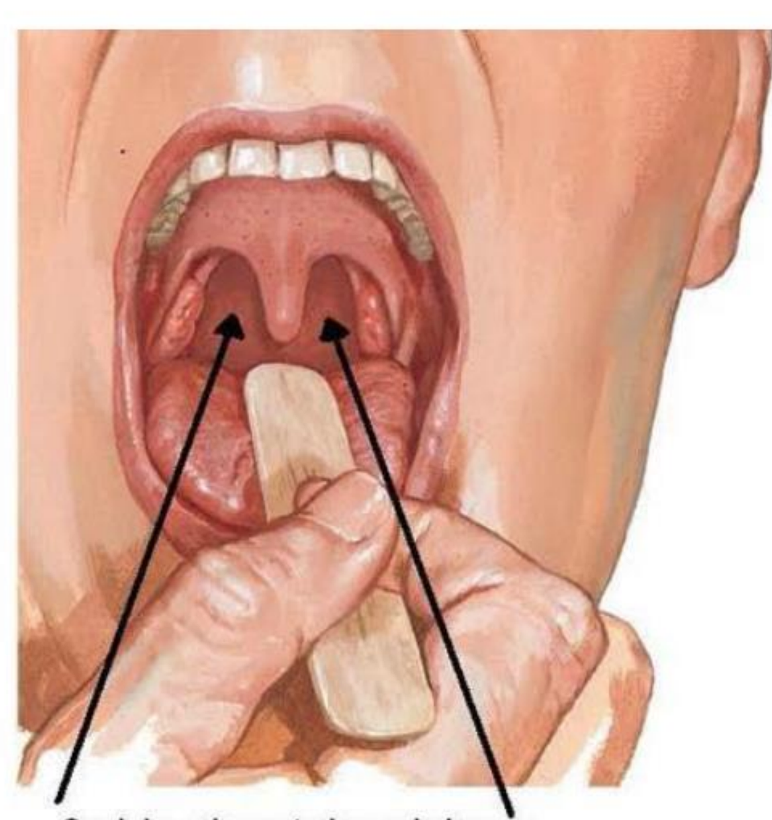


Figure 12- Appropriate throat swab specimen collection technique (APHL).

Rectal Swab Collection Procedure

1. Moisten swab in sterile saline solution or an appropriate transport medium (e.g., Amies transport medium).
2. Insert swab 1.5-2 inches into rectum and gently rotate (or follow the local collection protocol).
3. Place swab into a sterile tube or vial deep enough that medium covers the swab tip.
4. Break off the top portion of the swab to close the container.
5. Refrigerate swabs in transport media at 4°C and test within 24 hours after collection; otherwise, freeze at -70°C.

Urine Specimen Collection Procedure

1. Follow the local guidelines for urine specimen collection. Collect at least 1 mL urine in a leak-proof sterile container. Urine should ideally be a midstream clean-catch specimen to reduce any possible cellular or microbial contamination.
2. Transport at room temperature up to 2 hours. If urine will not be transported until 2-24 h after collection refrigerate until culture inoculation.

Labelling specimens

All specimens potentially harboring *B. pseudomallei* should be documented and appropriately labelled upon collection and arrival at the laboratory. To improve traceability, samples should be assigned a unique specimen identification number. This number should be clearly labelled on both the testing forms and the specimen container. Label all specimens clearly, preferably with a permanent marking pen (alcohol-resistant). Include the:

1. Patient's name and date of birth
2. Patient identification number
3. Date and time of specimen collection
4. Specimen type

Appropriate Handling of Specimens

Clinicians should inform laboratories when melioidosis is suspected so staff can perform the appropriate tests. Samples should be thoroughly inspected before being accepted for testing and should be rejected if any of the following are found (ASM 2016):

1. Submitter was not authorized to submit sample.
2. Incomplete or missing labels or paperwork.
3. Insufficient specimen volume.
4. Broken or damaged tubes/containers or improperly packaged samples.
5. Nonviable cultures.

If specimens are held awaiting transport, any fresh material, sera and swabs in transport media should be refrigerated, not frozen. However, specimens will deteriorate under refrigeration and should be transported as soon as possible.

Specimen Collection in Animals

Diagnosis of melioidosis in animals is usually obtained through post-mortem examination unless the presenting clinical sign is an active lesion or abscess of the skin. *B. pseudomallei* may be isolated from the sputum, blood, wound exudates, tissue, and feces. In some species, serum may also be collected for serologic tests. Upon detection of the disease, environmental sampling of the surrounding soil and water where the infected animal is kept is recommended to determine a potential infection source (Sim et al. 2018; Sprague & Neubauer 2004).

Recommended Specimen Types (Queensland Gov 2019)

1. Swabs or unpreserved samples of lesions or discharges.

2. Formalin-fixed lesions and full range of tissues.
3. Blood and serum for serology- complement fixation test (CFT) and indirect hemagglutination antibody (IHA) test.

Collecting Animal Specimens

The time after death that specimens are collected is critical, since blood samples from dead animals are unsuitable for hematology and clinical biochemistry and decomposing tissues are typically not satisfactory for histopathology. Collect samples from necropsies as soon as possible after death and immediately after opening the carcass. Collect samples aseptically and submit them promptly in individual sterile leak-proof screw-top plastic containers. Do not use non-sterile containers or fragile containers such as zip-lock bags when submitting material for bacteriological examination. The rate of decomposition varies with seasonal and weather conditions and is faster with some animals than with others. In aquatic animals such as fish and marine mammals, post-mortem decomposition is rapid. These specimens should be collected from humanely euthanized animals that are preserved in fixative, held in ice or kept refrigerated. Whole sick animals should be sent live in aerated and cooled transportation (Queensland Gov 2019). Proper personal protective equipment (PPE) should always be worn before collecting any clinical specimens. Refer to the PPE section for more information on appropriate PPE for clinical specimen collection.

Before collecting a specimen, it is important to consider the following:

1. The use of suitable containers/tubes for each test.
2. The correct storage of each specimen before sending.
3. Specimen transport requirements.
4. Providing the correct and most relevant clinical information.

Blood and Serum Collection Procedure

1. Fill tubes containing anticoagulants first so that any clot formation is minimized.
2. Collect blood from the jugular vein (cattle, sheep, pigs and horses), tail vein (cattle), ear vein (pigs) or wing vein or heart (poultry).
3. Use plain tubes or serum separator tubes (SST) for the collection of serum (used for CFT or IHA- see more below). These should be at least 5 mL in volume. Fill the tubes to two-thirds capacity to provide a minimum of 2 mL of serum. Allow the blood in the plain tubes to clot at room temperature and separate the serum by pouring off or centrifugation before transport to the laboratory. This is particularly important if the samples will not be received at the laboratory within 18 hours of collection. Hemolysis and leakage of red cell components affects most analyses and can occur if serum is not separated promptly.
4. Transport all samples to the laboratory at 4°C within 24 hours of collection. Attach a completed specimen advice sheet to each submission. Pack the specimen advice sheet externally to the samples and clearly indicate on it that melioidosis is suspected (Queensland Gov 2019).

Melioidosis Serology in Animals (CFT and IHA test)

The *B. pseudomallei* CFT indicates active infection, while the IHA test indicates past and current infection. Animals in melioidosis-endemic areas tend to have higher background IHA titers, so the interpretation and diagnosis of serological tests should be read with caution. A more accurate diagnosis of melioidosis is likely if both the CFT and the IHA test are run simultaneously and the results are compared (shown in Table 9). Titers are variable and do not necessarily correlate with the clinical signs observed. If melioidosis is suspected, samples suitable for culture should also be submitted.

Table 9- Interpretation of CFT and IHA titers for the diagnosis of melioidosis (Queensland Gov 2019).

Test/titer		Interpretation of Result
CFT	IHA	
Negative	Negative	Not infected (does not exclude infection in the 6 days prior to sampling)
Negative	40 to 160	Possibly natural agglutinins or cross-reaction with other infectious organisms though may indicate past infection or exposure
Negative	320 or greater	Possibly melioidosis; symptoms may or may not be apparent. Re-bleed in 14–21 days to check for a rising titre. May indicate a chronic infection
8 or 16	40 to 160	Possibly melioidosis; symptoms may or may not be apparent. Re-bleed in 14–21 days to check for a rising titre
32 or greater	320 or greater	Infected Likely to have clinical signs of acute melioidosis

Figure 13- In a positive CFT test, the complement is bound to an antigen-antibody complex and will remain unlysed. These cells will settle to the bottom of the well and form a button. Likewise, in a negative test, there is no antigen-antibody complex so the complement will interact with the sensitized RBCs causing them to lyse (Texas DSHS 2010).

Unfixed Abscesses and Lesion Swabs Collection Procedures

1. Sample fresh, active lesions. For smaller lesions, submit the entire lesion or organ. For larger or widespread lesions, submit a portion of the affected tissue containing the lesion and surrounding tissue.

2. Alternatively, take an aspirate of the lesion and transfer it to a small sterile container or use swabs and store in a bacterial transport medium.
3. If using swabs, sear the surface of the lesion or tissue with a hot spatula (heated using a small portable gas burner) and cut through the seared surface using a sterile scalpel blade, then swab the cut surface of the tissue.
4. Swabs of lesions or tissue samples should be homogenized with saline before being cultured.
5. To aid in diagnosis, take and fix samples for histopathology at the same time (Baszler & Graham 2017).

Formalin-Fixed Lesions and Tissue Collection

Tissues for microscopic examination collected either via biopsy or during necropsy can be crucial to obtaining a diagnosis, however, autolyzed tissues are generally useless for histopathologic examination. Timely examination and organ sampling are essential. Tissue specimens should never be frozen before fixation. Important things to consider when collecting these specimens include (Bildfell 2016):

1. Histological specimens should not be >1 cm thick (preferably 5–7 mm) and should be placed into ≥10 times their volume of phosphate-buffered 10% formalin to ensure adequate fixation immediately after collection.
2. Tissues should be representative and centered directly on the existing visible lesions.
3. Wedge biopsies and tissue specimens should include some of the normal surrounding tissue where possible. This margin can provide key information.

4. Larger abscesses should be sliced so that formalin can penetrate the full specimen. Alternatively, collect multiple representative samples that include both normal and abnormal tissue.

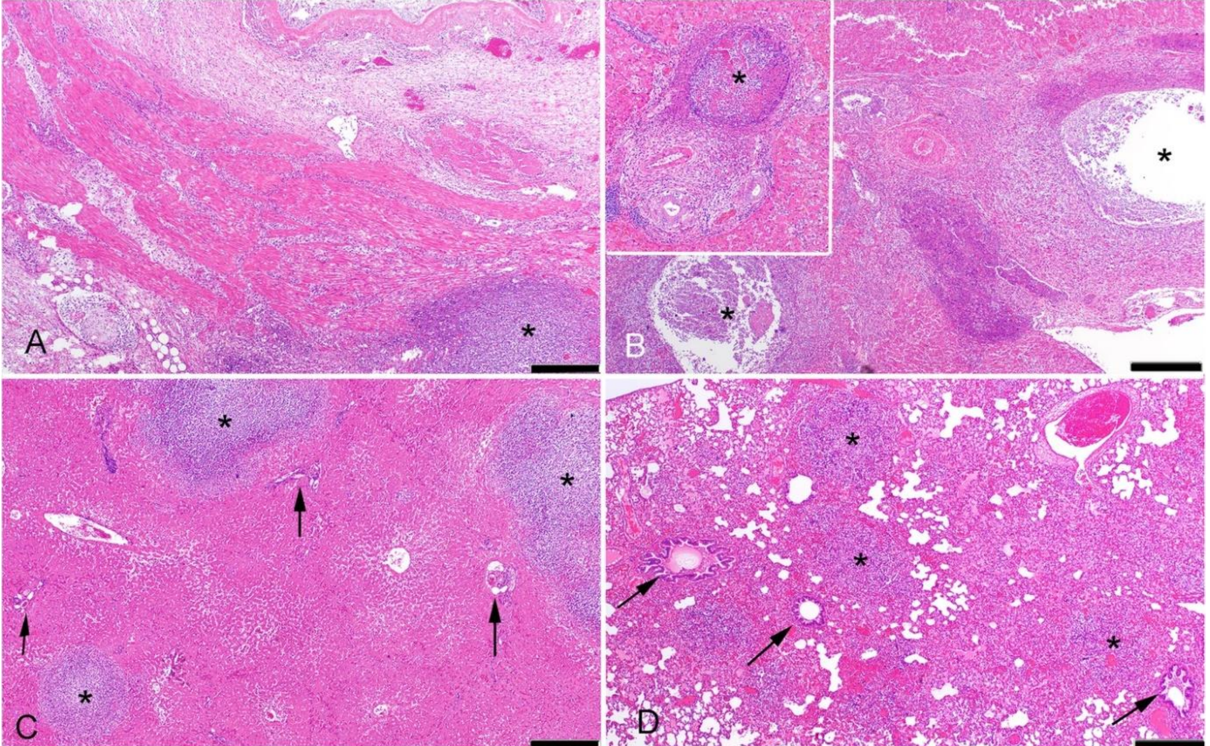


Figure 14- Formalin-fixed lesions taken for histopathology from meerkats with melioidosis showing (a.) The gastric wall with marked congestion, vascular thrombosis and foci of necrotic neutrophils (asterisk). (b.) Entrance of portal vein (asterisks indicate two sections of the lumen) at the hepatic hilus. The architecture of the vein is obliterated by a dense infiltrate of necrotic neutrophils expanding into the surrounding tissue. (c.) Liver showing embolic showering of bacteria from the systemic circulation (d.) Embolic showering of the lung and multiple random foci of neutrophil infiltration (asterisks) with surrounding haemorrhage (Rachlin et al. 2019).

Fecal Specimen Collection

Fecal specimens may be useful in the diagnosis of melioidosis in animals, since *B. pseudomallei* has been detected in feces from several species previously, including wallabies and chickens (Hoger et al. 2016; Sprague & Neubauer 2004). In animals, the identification of *B. pseudomallei* in fecal specimens does not always indicate active disease. As bacterial shedding is possible even in the absence of clinical disease, take care when interpreting results.

Protocol for Fecal Collection

1. Fecal specimens (at least 100g) should be collected with sterile disposable gloves or a trowel/spade.
2. Trowels and spades should be cleaned with 70% ethanol after each collection.
3. Try to collect fresh specimens that have been kept out of direct sunlight.
4. Samples can be stored in biohazard bags kept at room temperature and should be processed within 7 days.

Labelling and Appropriate Handling of Specimens

Label all specimens clearly, preferably with a permanent alcohol and water-resistant marking pen. Include the following information:

1. Owner's name
2. Animal number or ID
3. Tissue from which the sample was taken

If specimens are held awaiting transport, any fresh material and sera and swabs in transport media should be refrigerated, not frozen. However, specimens will deteriorate under refrigeration and should be dispatched as soon as possible. Package specimens to prevent leakage in transit and include sufficient absorbent material to contain any leakage that might occur.

Factors leading to unsuitable samples or unclear results may include:

1. Hemolysis of blood specimens or serum/plasma separation.

2. Decomposition of dead animals- Marked changes occur in blood composition after death. Sample only live animals or animals immediately before euthanasia when possible.
3. Treated animals- Take samples needed to confirm the diagnosis before giving treatment. If this is not possible, record the treatment given on the specimen advice sheet.
4. Inappropriate sample storage.
5. Bacterial contamination- Collect samples using aseptic techniques and keep them cool in transit.

Removal and Decontamination of Infected Animals

Ensure appropriate PPE is used when handling affected animals and when working in the area where animals may have become infected.

Commercial incineration is the recommended method for removal of infected carcasses. To lessen the risk of infection during transport the carcass should be wrapped in heavy, leakproof plastic or bins. Disinfect all equipment and vehicles used during removal. Ten percent bleach, Virkon and 70% ethanol are all suitable disinfectants. Ensure that the potential for environmental contamination is also minimized by decontaminating the disposal site. Consider any environmental restrictions when using chemicals or disinfectants and be sure to contact the local environmental authority as necessary (Baszler & Graham 2017; Queensland Gov 2019; Government of Western Australia 2018).

Chapter Summary

There are a variety of specimen types that can be collected for the diagnosis of melioidosis in humans and animals. The procedures for collecting and processing these clinical specimens

differs based on the type of sample being examined and the site of infection. All specimens should be appropriately documented, labelled and stored in appropriate leak-proof, sterile containers and sent to the laboratory for processing as soon as possible for the most accurate diagnosis.

Chapter 6: Clinical Specimen Packaging and Transport

Chapter Overview

The following chapter focuses on the packaging and shipping of diagnostic specimens for melioidosis testing. Regulations placed on the transportation of biological goods are necessary to ensure the integrity of specimens as well as the safety of staff and transport workers. This is primarily achieved through careful storage and packaging that can withstand rough handling and leakage, as well as appropriate labelling and documentation of any hazardous contents. Packaging should be durable enough to prevent any content leakage and be able to withstand rough handling, shocks, pressure changes, humidity, and vibration. Each package must be correctly marked, labelled and contain appropriate documentation to ensure the timely and accurate processing of specimens (BMBL 6th ed. 2020).

Storage of Specimens for Transport

The conditions of transport can greatly affect the viability and reactivity of the specimens, resulting in lower testing sensitivity or, in some instances, false-negative results. As specimens may need to be transported long distances before being tested, it is important to store samples properly to maintain viability.

Specimens for Culture

Samples for culture should be kept from overheating to ensure viability. Blood specimens for culture should be incubated the same day as collection (although this should be done immediately where possible) but should be kept at 4C° if shipped overnight. Other samples for culture should be transported either with wet ice, ice packs or, if the distance is long, dry ice. Ideally, the package should have enough cold packaging to arrive at its destination and

still be cool to the touch. The recipient of the package should make note of the condition of the specimens after transport.

Other Specimen Types

Swab specimens stored in transport medium and slides may be transported and stored at room temperature. Some specimens may require frozen transport. If this is necessary, the package should contain dry ice or liquid nitrogen to maintain this temperature long term (described in further detail below)(WHO 2015). Serum is particularly sensitive to heat and the reactivity to antibody detection decreases at higher temperatures and should always be transported on ice.

Specimen Type	Short Term Storage (days)	Long Term Storage (weeks to months)
Whole Blood	4C°	-80C°
Serum	4C°	≤ -20C°
Tissues	4C°	≤ -20C°
Urine	4C°	≤ -20C°
Swabs	4C° in transport medium	-20C°

Table 10- Storage conditions for common melioidosis specimen types. Storage conditions typically change depending on the length of time the specimen will need to be kept viable.

Refrigerants

If ice or dry ice are used during transport, they should be placed outside of the secondary packaging. If wet ice is used it should be placed inside of a leakproof container and the outer package should also be leakproof. This is done to prevent damage once the refrigerant has melted or has dissipated. An overpack (a special type of insulated packaging) can be used to hold dry ice. Dry ice should never be placed directly inside the primary or secondary packaging because of the risk of explosion. If dry ice is used, the outer package should permit the release of carbon dioxide gas. Failure to use a breathable outer box could result

in the build-up of too much pressure which can damage the package, the specimen and potentially harm personnel. If dry ice is used, this should be included on the shipping label (see Figure 17).

If liquid nitrogen is used as a coolant, arrangements with the transport carrier should be made in advance. Primary packages must be able to withstand very low temperatures and appropriate packaging requirement of the carrier must be checked and followed (IATA 2019; WHO 2015).

Specimen Packaging and Transport Overview

Regardless of the type of specimen being transported, there should always be three layers of packaging. The first layer, called the primary packaging, is what the specimen itself is contained in. Primary packaging typically consists of a plastic tube such as a blood vial or a 2ml watertight container with a closure such as a screwcap or snap-on lid. Samples should be taped or parafilmed for additional protection and appropriately labelled. Primary containers should each contain less than 500ml liquid or 500g solids. The second layer, termed secondary packaging, is a second receptacle that the primary packaging is placed in. This is usually a larger tube or plastic bag. Several wrapped primary receptacles may be placed in a single secondary packaging, however, volume and/or weight limits for shipping infectious substances should be checked with the relevant transport authority prior to shipment. Secondary packaging should also be watertight and properly sealed. It must be large enough to hold any absorbent material placed inside the package. Absorbent material is necessary when shipping liquids to soak up all contents of primary receptacles in the event of a leak or spillage. Acceptable absorbent material typically includes cotton, paper towels or wadding. The third and final layer of packaging is the outer packaging. This should be rigid and sturdy. Ideally, it should be a material that is easy to decontaminate such as smooth

plastic, or something easy to dispose of. The outer packaging should also be tightly sealed and an itemized list of contents must be enclosed in a waterproof plastic bag inside. Additional forms and any information that might identify or describe the specimen and the individual(s) shipping and receiving should be taped to the outside of the package. Both dry ice and wet ice must be placed in an overpack or leakproof receptacle inside the outer packaging and should not contain more than 4 litres of liquid or 4kg solid (BMBL 6th ed. 2020; IATA 2019; WHO 2004; WHO 2015).

Figure 15- Specimens should have three layers of packaging for transport to the laboratory. Primary packaging can include tubes or blood vials. It should be placed into a secondary packaging with absorbent material. The outer packaging should be sturdy and easy to decontaminate or dispose of and labeled with a biohazard symbol (CDC 2019).

Appropriate Packaging for Shipping Diagnostic Specimens

Regardless of the specimen type, there must be primary packaging, secondary packaging and outer packaging (Figure 15) (BMBL 6th ed. 2020; DOT 2011; WHO 2015).

- Primary packaging should always consist of the following:
    a. The primary receptacle must be waterproof.
    b. All containers should also be sealed with adhesive tape, parafilm or something similar.

c. If multiple containers are being shipped they should be wrapped individually to prevent breakage.
d. Primary containers should be less than 500mL (16.9 ounces) for liquid specimens; or be less than 500g (1.1 pounds) for a solid specimen. Transport media is considered part of the diagnostic specimen for shipping purposes.
e. If being shipped by air, the primary or secondary container must be able to withstand an internal pressure difference of no less than 95 kPa (14 psi) between -40° C to 55° C (-40° F to 130° F).

- Secondary packaging should consist of the following:
    a. Secondary packaging must be waterproof. An example of appropriate secondary packaging is a leakproof biohazard bag.
    b. Enough absorbent material should be placed inside the secondary container to absorb the entire contents of all primary containers in case of leakage or any spillage. Acceptable absorbent material includes cotton balls or wads, paper towels or cellulose wadding.
    c. Do not over pack the secondary receptacle. This can lead to breakage of the primary receptacles.
- Outer packaging should consist of the following:
    a. Outer packaging should be sturdy and be appropriately sized for the contents inside. Examples of appropriate outer packaging include corrugated fiberboard, wood, metal, or rigid plastic.
    b. Packing should be able to withstand a 1.2meter (4 foot) drop test.
    c. One dimension/side of the package must be at least 4 inches in width.
    d. The outer packaging should not contain more than 4 liters of fluid or 4 kg of solids.
    e. Both dry and wet ice should be placed outside the secondary packaging.

   a. Dry ice: packaging should permit the release of carbon dioxide gas and not allow pressure to build-up, as this can break the packaging.
   b. Wet ice: the packaging must be leak-proof.
   c. An overpack or leakproof receptacle should be used to hold cold packs or dry ice.

f. An itemized description of contents should be included and should be placed in a sealed plastic bag to protect from moisture or leakage.

g. The name, address, and telephone number of the shipper and receiver must be on the package and the air waybill. You must also include the sample types being shipped and the hazard classification code on the air waybill (see more about hazard classifications and appropriate labelling of packages below).

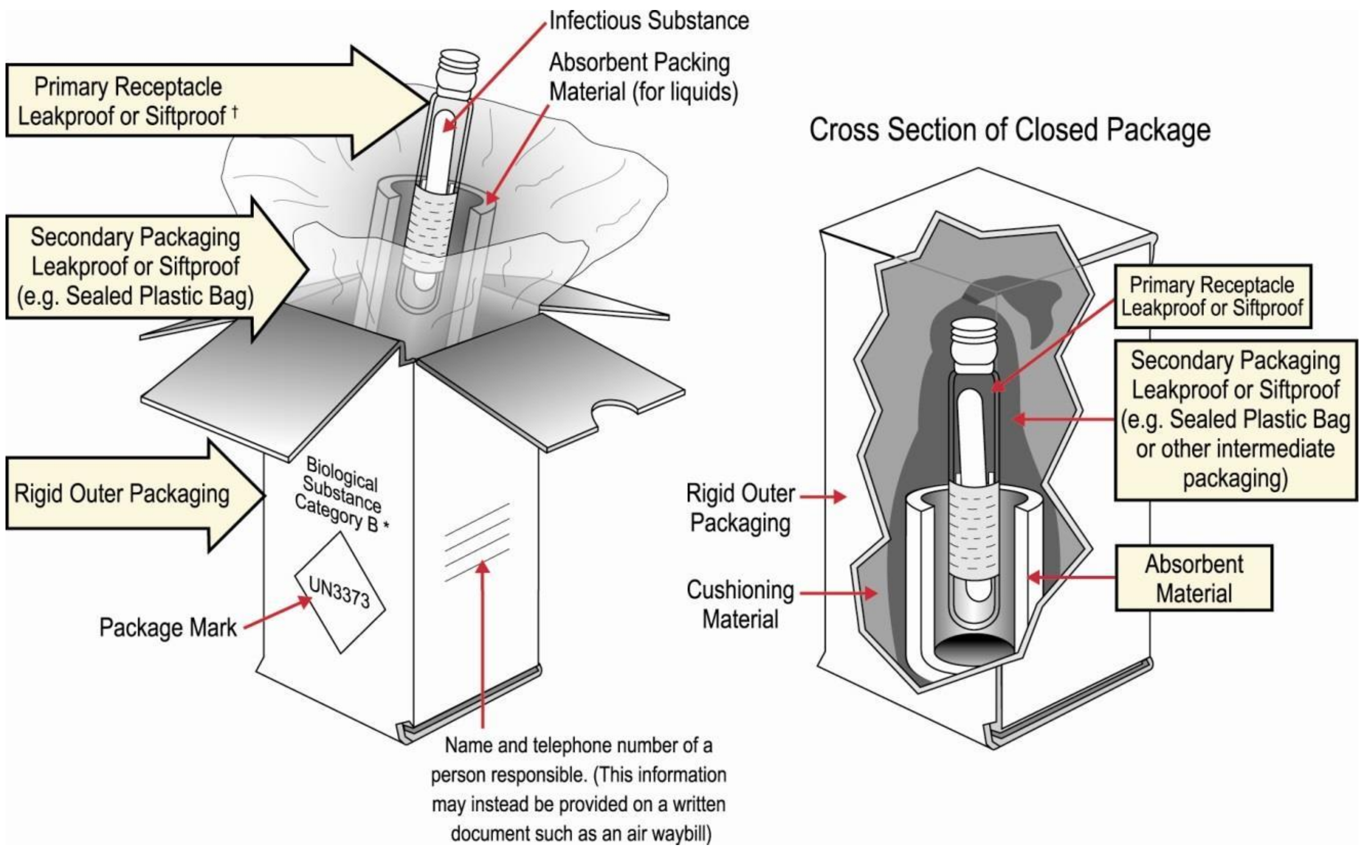


Figure 16- Category B packaging schematic demonstrating triple packaging of diagnostic specimens (CDC 2014).

Shipping Hazard Classifications

Under shipment guidelines, pathogens are classified into categories based on the likelihood of causing severe or potentially fatal infection in humans if accidental exposure occurs. While the category determines what package labelling is required, always check with the relevant shipping or transport authority to determine if anything additional is required. If the package will be sent internationally, also check to ensure the appropriate shipping permits are in place.

Anything listed as category A is considered the most hazardous to individuals and the community. This usually relates to shipment of pure cultures and isolates. Diagnostic specimens are generally considered Category B, as the microorganisms are not in a form that can easily cause illness (DOT 2011; IATA 2019; WHO 2015). Human and animal diagnostic specimens for melioidosis are normally considered Category B Substances for the purposes of packaging and shipment.

Domestic and International Shipping of *B. pseudomallei* Isolates and Suspected Specimens

Domestic shipping and transport of *B. pseudomallei* or suspected specimens should follow local or national guidelines and regulations. Whether submitting specimens to diagnostic laboratories within the same site/facility, or if shipping specimens between sites, ensure that any local and laboratory-specific transfer protocols are followed and the receiving laboratory has been notified beforehand. All specimens, regardless of whether they are being transported within or between sites, should be well-labelled (see below) and packaged to ensure viability of the specimen and safety of the those handling the package. Ensure the package also contains appropriate documentation to guarantee the timely and accurate processing of specimens.

For global shipping guidelines, the International Air Transport Association (IATA) Dangerous Goods Regulations (DGR) is the worldwide gold standard for international shipping. Following the IATA DGR will ensure that the package and specimen being shipped will meet international transport regulations. See the latest edition of the DGR at https://www.iata.org/en/publications/dgr/.

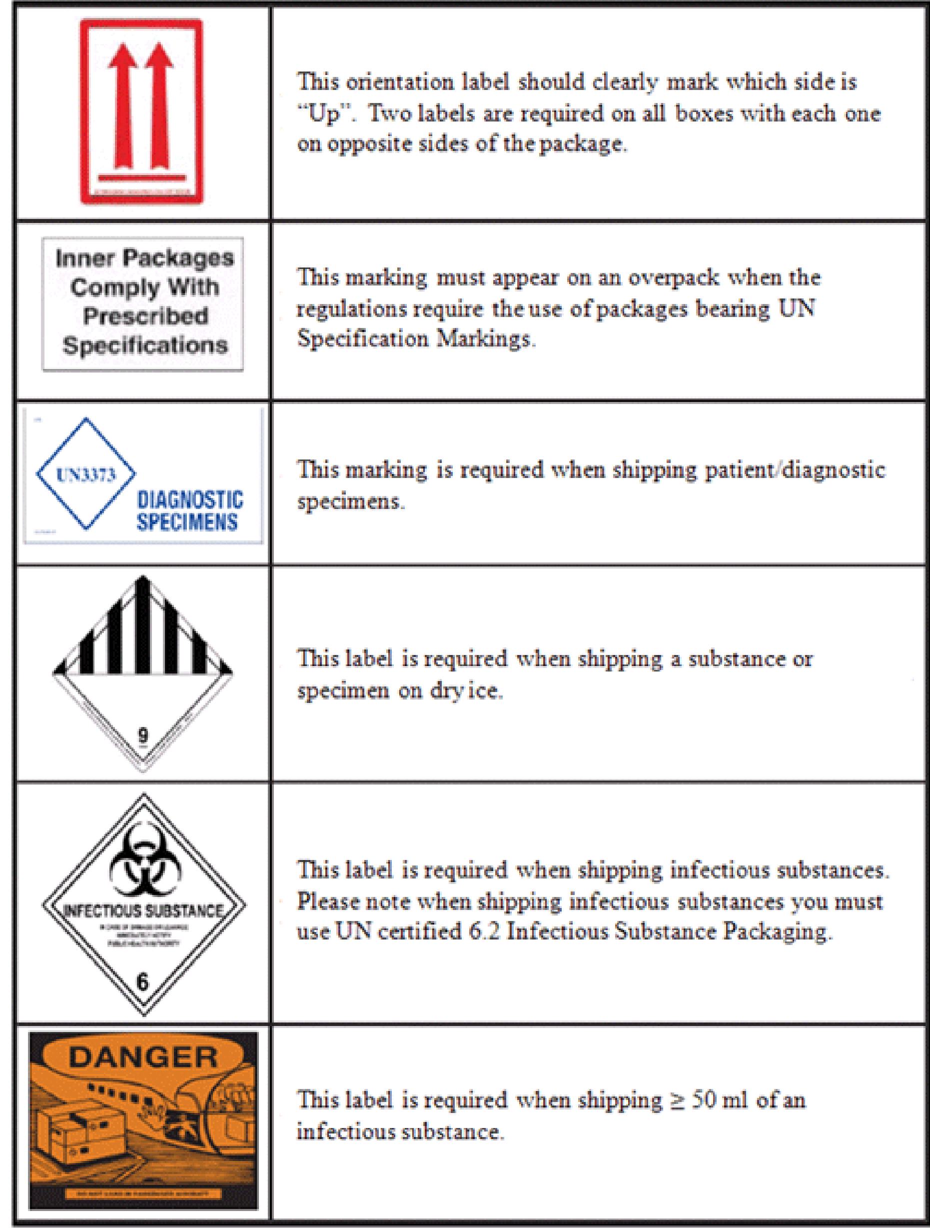
Appropriate Labelling and Packaging Documentation

It is important to track the movement of diagnostic specimens from the point of collection, specimen processing, storage, and disposal. The time and date of collection should be recorded on the Case Report Form; the time the samples are received in the laboratory and the time of testing should be recorded by the laboratory. All samples that are to be stored prior to shipment to an off-site laboratory or another site must be stored under appropriate conditions and a log of the stored samples should be maintained (e.g. a freezer or refrigeration log). These logs are considered essential study documents and they should be kept with the rest of the study documents following study completion. When samples are shipped, a copy of the courier form should be retained (WHO 2015; CDC 2016).

Package Labelling

For most diagnostic specimens (e.g. Category B- see Figure 16) outer packaging should be labelled with:

1. The name, address and telephone number of BOTH the sender and receiver of package
2. UN number (e.g. UN 3373)
3. Appropriate shipping name (e.g. “Biological Substance Category B”)
4. Total volume/weight of the specimens being shipped and total weight of package
5. Packages that contain more than 50 mL or more of liquid should have two orientation labels affixed to the outside of the outer packaging. Each label should be placed on opposite sides of the package and arrows should point upwards.
6. Diagnostic specimen shipments DO NOT require an Infectious Substance label or a Declaration for Dangerous Goods.



Label	Description
	This orientation label should clearly mark which side is "Up". Two labels are required on all boxes with each one on opposite sides of the package.
Inner Packages Comply With Prescribed Specifications	This marking must appear on an overpack when the regulations require the use of packages bearing UN Specification Markings.
UN3373 DIAGNOSTIC SPECIMENS	This marking is required when shipping patient/diagnostic specimens.
9	This label is required when shipping a substance or specimen on dry ice.
INFECTIOUS SUBSTANCE 6	This label is required when shipping infectious substances. Please note when shipping infectious substances you must use UN certified 6.2 Infectious Substance Packaging.
DANGER	This label is required when shipping ≥ 50 ml of an infectious substance.

Figure 17- Description of specific labels and markings required for safe and proper shipping of various package types (CDC 2016).

Checklist for Packaging and Shipping of Diagnostic Specimens

Check if Complete	Item/Activity
	All primary packaging containing specimen containers should be waterproof and be closable (e.g., screw-on caps).
	All caps should be wrapped with Parafilm or tape.
	Primary packing should be labelled with the patient's name or other individual identifiers and the date the sample was collected.
	For liquid specimens, the primary packaging should be leakproof and hold no more than 500ml liquid and 500g solid.
	The primary or secondary packaging must be able to withstand an internal pressure difference of no less than 95kPa (14 psi) between -40°C to 55°C (-40°F to 130°F) if being shipped by air.
	Primary containers should be individually wrapped or separated and placed inside leakproof secondary packaging.
	Absorbent material should be placed between the primary and secondary container. Enough material should be used to absorb the entire contents inside the primary receptacles.
	The secondary container should not be over packed.
	An itemized list of the contents should be included with the shipment. This should include a telephone number and email address where any issues with the shipment can be reported.
	A sturdy outer package should be used.
	For liquid, the outer packaging should contain less than 4 liters of liquid and 4 kg solids.
	The outer packaging should include an appropriate label.
	Ice packs and insulated outer packaging should be used for specimen integrity.

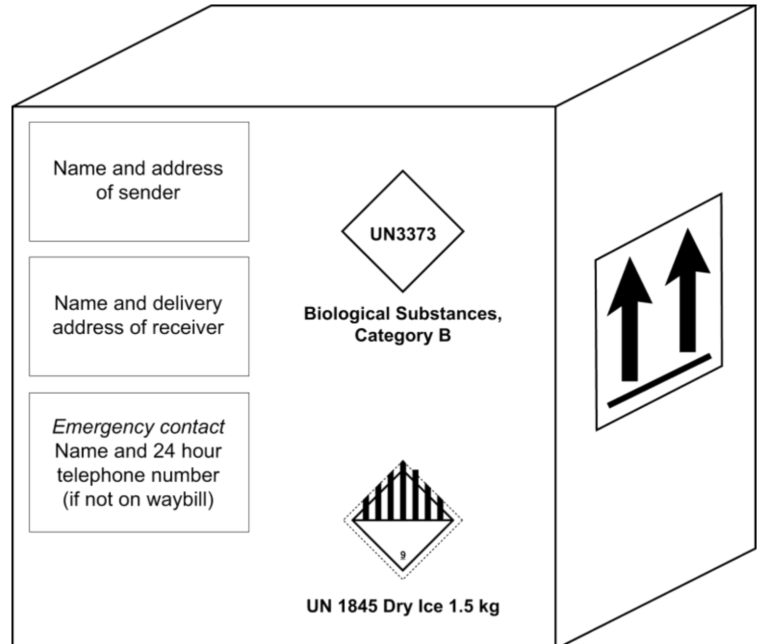


Figure 18- An example of appropriately labelled package for transporting Category B Biological Substances by air with dry ice.

Chapter Summary

It is important to ensure that packaging and shipping conditions preserve the integrity of specimens and minimize the potential for damage during transport. Regardless of the type of specimen being shipped or the Hazard Classification, there should always be three layers of packaging. This should be comprised of a waterproof, sealed primary receptacle containing the specimen, a second watertight, leak-proof packaging to enclose and protect the primary receptacle(s), and durable outer packaging that protects the contents from outside influences incurred during transport. Each package must be correctly marked, labelled and contain appropriate documentation to ensure the timely and accurate processing of specimens.

Chapter 7: Environmental Sampling for the Detection and Surveillance of *B. pseudomallei*

Chapter Overview

Environmental sampling for *B. pseudomallei* can be used to detect geographic areas where humans and animals are at risk of acquiring melioidosis and for creating a global risk map of the infection. While *B. pseudomallei* is primarily found in tropical soils, the bacteria can also be leached out into the water table during periods of heavy rainfall and aerosolize during severe weather events. Cases of melioidosis are frequently attributed to contaminated water sources, and increasingly, inhalation of contaminated aerosols. The following chapter reviews the current protocols for soil, water, air, and swab sampling for *B. pseudomallei* in the environment. Downloadable protocols and mechanisms for data collection and sharing can also be found through the International Melioidosis Network (IMN) webpage at www.melioidosis.info.

B. pseudomallei Soil Sampling

Environmental sampling can be used to determine the presence and geographic distribution of *B. pseudomallei.* It can also help to identify geographic regions where humans and animals are at risk of exposure and examine the associated risk of infection before cases are recognized (Kaestli et al. 2009; Limmathurotsakul et al. 2010; Rachlin et al. 2020).

The most appropriate strategy is dependent on the objectives of the study and whether any information about the presence of *B. pseudomallei* in the geographical area to be sampled is already known. For preliminary studies in regions where sampling has not been done previously, investigators should examine any available data about previously confirmed or suspected melioidosis cases in the area. Selection of sites to sample should focus on patient

residences or places of work. In the absence of such information, GIS (geographic information system) software may be used to randomly select several preliminary sampling locations using a less targeted approach. For large environmental surveys in areas where *B. pseudomallei* is already known to exist, randomly selecting sampling sites using GIS software can also be performed (Limmathurotsakul et al. 2013). Likewise, depending on the study goal, locations that may have a higher probability of isolating *B. pseudomallei,* such as rice paddy fields, riparian zones (land alongside creeks, streams, rivers etc.) or recent construction areas can also be targeted.

Soil sampling should be the subject of a safety risk assessment prior to starting any sampling activity. Safety assessments should act a starting point for project planning.
Before commencing it is recommended that sampling staff:

- Identify potential risks and any biological hazards that may be located at the proposed sampling areas.
- Locate the presence of all utilities in the sampling areas before any digging begins.
- Know the sampling address and make sure others are aware of this address.
- Use the buddy system.
- Take a phone (or preferred method of portable communication) and first aid kit to the sampling area in case of emergency.

Number of Soil Samples

Taking an inadequate number of soil samples or using random sampling methods with too small a sample size can run the risk of a false-negative result and may be associated with a lower power of detection (Klironomos et al. 1999). This is partially related to the heterogeneous distribution *B. pseudomallei* has in the environment, which can give rise to bacterial hot spots (Limmathurotsakul et al. 2010; Rachlin et al. 2020). This issue can be

avoided by increasing the number of samples taken at a given site. Based on statistical considerations, a minimum of 100 sampling points is suggested in an area of around 50×50 square meters in order to determine the presence of *B. pseudomallei* (Limmathurotsakul et al. 2013). If the area of interest is large (e.g., across one or multiple provinces or country-wide) or where sampling resources are more limited, collecting fewer samples at more sites can also be done. Here, the number of samples taken per site and the number of sites investigated could be determined based on a statistical power sample size calculation (Cressie 2015).

If a region is already known or is suspected to be positive for *B. pseudomallei*, an adaptive approach may be used. Here, a preliminary study can first be performed in a specified area where a quantity of random points are sampled. If any of these samples are positive for *B. pseudomallei*, this suggests that the organism is present and is enough to label this area as a potential risk to humans and animals. If all samples are negative, a further round of sampling can be done where 100 samples are taken from the same site using a fixed interval grid approach (see Figure 19 below).

Figure 19- Example of a fixed-interval systematic grid for sampling of *B. pseudomallei* in soil (Image courtesy of Menzies School of Health Research, Australia).

Distance Between Samples

The presence of bacterial hot spots in the environment gives rise to an effect termed "spatial autocorrelation". This affects the distance needed between each sampling point and means that adjacent sampling points are more likely to yield the same result (e.g. a sample next to a negative sample is likely to be negative) (Limmathurotsakul et al. 2013). Studies from Thailand have suggested that the distance between samples should be between 2.5 and 5 meters apart to adjust for *B. pseudomallei* spatial autocorrelation. While 2.5 to 5 meters is the suggested distance between samples, variability in *B. pseudomallei* count in the soil both within and across different countries and regions is well described (Kaestli et al. 2007; Limmathurotsakul et al. 2010). Consequently, the proposed sampling distance may not hold

true in areas where the *B. pseudomallei* presence in the soil is markedly different. In this instance, the ideal sampling distance for the specific region can also be estimated based on the results of a pilot study containing 100 sampling points at one or more sites (Cressie 2015).

Soil Sample Depth and Storage

A depth of 30cm for soil samples is recommended. This is based on evidence that the proportion of samples that are positive for *B. pseudomallei* is higher at 30 cm than at shallower depths, but is comparable to samples taken at greater depth (Palasatien et al. 2008; Limmathurotsakul et al. 2013). However, the optimum depth may vary based on the geographic area (Manivanh et al. 2017).

The amount of soil collected per sample has also varied in published studies, but there is currently no indication that collecting a greater weight of soil is associated with improved sensitivity. However, given the heterogeneity of *B. pseudomallei* in soil, there is some chance that small sample quantities may have an increased risk of yielding false negative results. A weight of 10 grams per sample is suggested based on feasibility and ease, however, more may be collected and weighed out for sample processing back in the laboratory (Limmathurotsakul et al. 2012). Soil samples should be kept at room temperature (24 to 32°C) and away from direct sunlight during transport. Samples should be processed as soon as possible.

Table 11- Recommended sampling strategies for the isolation of *B. pseudomallei* from soil (Limmathurotsakul et al. 2013).

Sampling strategy	Consensus guideline
Sample size calculation	• Sample size calculation should correspond with study objectives and may vary based on whether any previous information about *B. pseudomallei* presence in the region is known. • In areas where presence is unknown, a minimum of 100 sampling points is suggested in an area of around 50×50 square meters.
Site selection	• For pilot studies in areas where sampling has not been done, sites should be chosen using the information available (e.g. areas or fields around patient households). If this information is not available, use a GIS program to randomly select sites. • In areas where *B. pseudomallei* is already known to exist a GIS program can be used to randomly select sites across the region of interest.
Sampling points per site	• Use a fixed interval sampling grid. • To determine the presence of *B. pseudomallei* in one field the sampling area should be approximately 50×50 square meters and have 100 sample points per site. • The number of sampling points per site and number of sites can be calculated based on a geostatistical sample size calculation for studies covering large geographic areas.
Distance between sampling points	• Take samples at a distance of 2.5 to 5 meters apart.
Soil sampling depth	• 30 cm depth
Weight of each soil sample	• Minimum 10 grams of soil put into a sterile universal tube or self-sealing plastic bag.

Soil Collection Procedures

Equipment

- Shovel
- Augur
- Disposable latex gloves
- Rubber boots
- 70% ethanol spray
- Paper towels
- Sterile containers or bags (e.g. 25 mL plastic universal containers, self-sealing plastic bags)
- String and stakes

- Weighing scales (if soil will be weighed on site)
- Waterproof labelling markers and pens/pencils
- Insulated container for sample storage (e.g. polystyrene box)
- Tape measure (at least 5 meters but preferably longer)
- Study forms
- Camera
- GPS (global positioning system) or satellite navigation device

Personal Safety in the Field

Wear protective gloves and closed shoes and change gloves regularly, particularly if dirty. This will also help to avoid cross-contamination of samples and equipment. Clean hands and arms regularly with 70% ethanol solution or antibacterial soap.

Soil Sampling Collection Methods

1. Select the location of the sampling site as described above. Ensure you have recorded the GIS location of each sampling site.
    a.) **NOTE- It is important that you obtain the appropriate permissions and clearances from relevant councils/governing bodies/landowners PRIOR to sampling commencement!**
2. Fill in preliminary information about the site/study (e.g., date, study name, location, etc.) in the study form (see example soil sampling study form below). Take great care in filling in the form. The same person should fill in forms to avoid observer bias.
3. Record the GPS position of each study site.

4. Take pictures of the study site that shows the surrounding landscape, size of the site, proximity to people/livestock/water sources. You should also take photos of the sampling grid as well as the individual holes/samples as you work.
5. Divide the sampling site into a 10 x 10 grid of squares that are 5 meters x 5 meters (total 100 squares). Measure these using a tape measure and mark squares with stakes and strings.
6. Mark the center of each square using string. This is where the sample will be collected.
7. Dig a hole using a clean, pointed metal gardening shovel. Collect the soil sample from a depth of 30 cm. An augur can also be used if available.
8. Check that the shovel/auger has gone down to 30 cm, use a ruler if necessary. If the hole does not go down to 30 cm, note the actual depth on the study form (e.g. 15 or 20cm, etc.).
9. Discard the top 10cm of soil and transfer remaining into a sterile container or self-sealing plastic bag to be weighed out upon return to the laboratory (alternatively 10 grams of soil may be weighed using measuring scales in the field directly into sterile 50 ml universal tubes if preferred). Do not use your hands to manipulate the soil, as this may cross-contaminate samples.
10. Cap and label the container with study name, sample ID and date and place it in an insulated container in the shade.
11. To prevent cross-contamination, clean all instruments with water and remove all visible dirt, spray with 70% ethanol and allow to air dry, then wipe off residual wetness with a clean paper towel prior to collecting the next sample. It is important to clean and disinfect all equipment after EACH sample is collected.
12. Transport samples to the laboratory at ambient temperature without exposure to direct sunlight. Samples can be stored at room temperature in a dark area. They

should not be left in direct sun or in the heat/cold for multiple days. Process samples as soon as possible.

Figure 20- Sampling collection techniques used for the isolation of *B. pseudomallei* from soil (Images courtesy of Menzies School of Health Research, Australia).

Study form – Burkholderia pseudomallei soil sampling

Date (Day, Month, Year) [|] [|] [|] m m y y

Study No's ______ to ______

Area

General location parameters

GIS (codes)		
Address		
Diagram of place + collection sites		
General **comments** to place		
Pics taken?	**Pic of area:**	**Pic of hole+soil/veg + study number**
Any **waterlogging** < ~25m? If yes, where?	1(yes), 0 (no), 2 (in wetseason), 3 (don't know)	where?
Distance to stream / run off 1(<10m), 2 (10-50m), 3 (50-100m), 4 (>100m), 0 (don't know)		comments

Sun accessibility 0(shade most of day), 1(shade half a day), 2(in full sun)		comments?
Vegetation around site 1 (grass), 2 (low bush), 3 (crops), 4 (single trees), 5 (open forest), 6 (forest), 7 (other)		comments
In Slope? / steepness? 1(flat) 2 3 4		0 (flat), 1(slightly rising), 2 (steep)

Signs of environmental change?

any obvious **irrigation?**	1(yes), 0 (no), 3 (don't know)	**where?**
Used regularly by **humans?**	1(yes), 0 (no), 3 (don't know)	**specify**
Used regularly by **animals?**	1(yes), 0 (no), 3 (don't know)	**what animals?**

Soil sample parameters

Depths of samples (cm) A: shallow B: deeper		
Soil Water Status 0 (dry), 1 (moderately moist), 2 (moist), 3 (wet), 4 (soaked)		

Figure 21- Example of a study form used when collecting soil samples for the presence of *B. pseudomallei*. The form is based on a study form from Menzies School of Health Research and may be altered based on specific study questions and requirements.

Water Sampling for the Detection of *Burkholderia pseudomallei*

Choice of Water Sampling Sites

If the aim of water sampling is to investigate the potential source of infection in a melioidosis patient, a thorough history of patient activities within the last 2-3 weeks before the onset of disease is important. Recording any activities that may have resulted in potential exposure to *B. pseudomallei* (e.g., rice farming, construction work, swimming in stream/creek areas with open wounds, aspiration events, drinking unchlorinated well water) will provide critical information about potential water sampling sites. Possible collection sites include: unchlorinated tank/well water (directly from the tank or from a tap), pooled still water, creek/stream water, or surface runoff e.g. drain water (Draper et al. 2010; Limmathurotsakul et al. 2013).

In areas where melioidosis cases are rare, it is especially important to collect as many samples as possible from different water sources to increase the chances of *B. pseudomallei* detection. Ideally, a minimum of 3-5 water samples per site should be collected, though if the area is larger or there are multiple water sources at a site (e.g. taps, drains, bore heads, untreated well/tank water, etc.) more samples (>10) should be collected.

Equipment and Consumables

- For personal safety: gloves, closed shoes, antibacterial soap or 70 % ethanol to clean hands and arms
- 1 liter sterile screw top bottles or containers
- Extendable sampling pole (if collecting water from stream/drains)
- Waterproof markers to label bottles

- Sturdy box to store bottles
- Camera
- GPS
- Study form

Water Sampling Procedure

1. Label each bottle or container clearly with a waterproof pen. Include the date, study and sample number.
2. Record study information (e.g. date, study name, location, etc.) in the study form. Take pictures of the surrounding area and at each water sample site. Record GPS location data of the site.
3. Collect water in a sterile 1L bottle or container (if 1L of water is not available collect at least 300mL). Avoid collecting water directly from the surface where possible, as *B. pseudomallei* is UV-sensitive.
4. Store samples in a dark box or room at air temperature and process as soon as possible, and at least within one week of collection.
5. All equipment should be cleaned with water and 70% ethanol between uses to prevent cross-contamination.

Study forms should contain additional information such as:

- Date
- Location of site
- Type of water samples collected (well or tank water, creek, drain etc.)
- Comments about the area e.g., if there are any animals or livestock present, or if the water is unchlorinated
- Water color (*B. pseudomallei* presence has been shown to correlate with increased levels of iron and turbid/particle rich water)

Figure 22- Water sampling for *B. pseudomallei* in (a) tank water (b) bore heads (c) drain water. (d) An example of an appropriately labelled water collection bottle (Images courtesy of Menzies School of Health Research, Australia).

Air Sampling

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. Attempts to isolate *B. pseudomallei* from air samples in melioidosis-endemic regions have been largely unsuccessful in the past. However, the use of portable air filters that concentrate air flow has been more effective for the detection of *B. pseudomallei* (Chen et al. 2015; Currie et al. 2015). Microbial air samplers are used to collect a large predetermined volume of air and capture the microorganisms onto a filter or an agar-based growth

medium (Whyte et al. 2007). Where air filtration devices are not available, sterile *B. pseudomallei*-selective agar plates can also be held at shoulder level to oncoming winds. Ideally, air samples for *B. pseudomallei* should be collected during storms or severe weather events, or when there is increased risk of bacterial aerosolization, such as high-pressure hosing.

Figure 23- Air sampling for *B. pseudomallei* using portable electric filtration device (Sartorius MD8 Air Sampler) and disposable gelatine membrane filters (Sartorius 80mm) (Images courtesy of Menzies School of Health Research, Australia).

Equipment and Consumables

- For personal safety: gloves, closed shoes, face mask (to prevent possible inhalation of *B. pseudomallei* during storms), antimicrobial soap to clean hands
- 70% ethanol
- Sterile dry membrane or gelatine filters (<0.8μm pore size)- diameter of filter will depend upon the individual air sampler
- Tweezers/forceps for filter removal
- Sterile screw top containers or self-sealing plastic bags for filter storage
- Waterproof markers to label bags/containers
- Sturdy box to store bags/containers
- Camera
- GPS

- Study forms

Air Sampling Protocol

NOTE- The exact protocol will vary based on the specific air sampler model and filter type

1. Place sterile dry or gelatine membrane filter into air filter system using clean gloves or sterile forceps/tweezers.
2. Place air filter onto sturdy, flat surface at least one meter above the ground.
3. Turn the machine on and run for a minimum of 20 minutes (minimum 1000L fixed sample volume).
4. Record study information (e.g., date, study name, location, etc.) in the study form. Take pictures of the surrounding area and at each water sample site. Record GPS location data of the site.
5. After 20 minutes, remove the filter with sterile forceps/tweezers and place it in labelled sterile screw top container or self-sealing plastic bag.
6. Disinfect tweezers/forceps between uses.
7. Keep samples in a portable box or container until returning to the lab, do not refrigerate or freeze.
8. Process filters immediately upon returning to the laboratory.

B. pseudomallei Environmental Swab Sampling

Swab samples can be used when sampling environmental surfaces for the presence of *B. pseudomallei*. They are particularly useful when sampling small, non-porous surfaces or objects prone to *B. pseudomallei* contamination or biofilm formation, including some indoor household areas (e.g., laundry taps, sink faucets, showerheads), the insides of water pipes or wells/bores, and have also been used to isolate *B. pseudomallei* from animal enclosures, incubators and tanks previously (Dawson et al. 2020; Kaestli et al. 2019; McRobb et al. 2013; Rachlin et al. 2019).

Swab Sampling Protocol

Equipment

- For personal safety: gloves, closed shoes, antibacterial soap or 70% ethanol, for cleaning hands/arms
- Sterile non-cotton swabs (e.g. rayon, polyester or macrofoam)
- Sterile moistening agent such as sterile water, saline solution, or phosphate-buffered saline (PBS)
- Sterile closable conical vial to store swab specimens
- Sterile sealable plastic bag to store vials
- Waterproof markers to label vials and bags
- Camera
- GPS
- Study form

Protocol (CDC 2012)

1. Wearing a clean pair of gloves, remove the swab from the packaging. Hold the swab near the top of the handle ensuring the absorbent head is not touched.
2. If the sterile swab is not pre-moistened, moisten the sterile swab by dipping it in the 10 mL container of sterile water, saline or PBS solution. Remove excess liquid by gently pushing the swab head on to the inside of the container.
3. Swab the surface to be sampled using the moistened sterile swab. The recommended wipe area is <100 cm^2 making enough vertical S-strokes to cover the entire sample area. Avoid letting the swab dry completely.

4. Rotate the swab once more and swab the same area using diagonal 'S'-strokes.

5. Place the swab in a sterile screw-capped container and break off the tip. Ensure the end of the swab, which has been handled by the individual collecting the sample, does not touch the inside of the tube. Tighten the cap to secure and label the tube with the appropriate sample identifiers and date and time of collection.
6. Place the container in a sealable plastic bag and label the bag accordingly.
7. Record study information in the study form. Take pictures of the sampled area and of the swab specimen for study records. Record GPS location data of the site.

Chapter Summary

Environmental sampling can be used to determine the presence and distribution of *B. pseudomallei* and identify any risk of infection before cases are recognized. Cases of melioidosis are frequently attributed to contaminated water sources, and increasingly, inhalation of aerosols. The preceding chapter describes the current protocols for soil, water, air, and swab sampling for *B. pseudomallei* detection in the environment. Visit www.melioidosis.info to access downloadable protocols and additional mechanisms for data collection and sharing.

Chapter 8: Specimen Processing and Diagnostic Testing Methods Overview

Chapter Overview

Clinical specimens collected from patients or animals that meet the case definition for suspected melioidosis infection should undergo testing for the presence of *B. pseudomallei.* The following chapter presents an overview of the current recommended methods for *B. pseudomallei* specimen processing and diagnosis of melioidosis. With some modifications, these methods can also be used to process environmental samples. In many melioidosis-endemic areas, a simple three-disk diffusion antimicrobial susceptibility test and latex agglutination antigen detection are the most useful and widely used initial tests for screening oxidase-positive, Gram-negative rods that are not *Pseudomonas aeruginosa.* Protocols for the most widely used methods are described in detail later in the manual. Examples of common workflows used for the detection and identification of *B. pseudomallei* are shown in Chapter 9.

Specimen Processing Safety

Early recognition of a potential *B. pseudomallei* isolate and appropriate specimen processing are key to minimizing laboratory exposure and allow for more prompt, effective treatment. Testing protocols should follow a workflow or algorithm and laboratories should decide what type of workflow works best based on their setup and the local laboratory regulations. Do not process nonclinical (environmental or animal) specimens in hospital laboratories unless no other option is available. Veterinary laboratories should handle all animal specimens, while nonclinical specimens should be sent to the designated reference laboratory with experience in environmental specimen processing (ASM 2016).

All patient specimens suspected of containing *B. pseudomallei* and culture isolates should be handled in a Biosafety Cabinet (BSC) wearing gloves and appropriate Personal Protective Equipment (PPE). When available, BSL-3 containment equipment and facilities are recommended for all manipulations of suspect cultures, animal necropsies, research involving the deliberate propagation of *B. pseudomallei*, and for experimental animal studies. BSL-3 practices are also recommended when preparing cultures and contaminated materials for automated identification systems, or when performing any procedure that can generate an aerosol. If BSL-3 facilities are not available, such work should only be undertaken following a thorough and detailed risk assessment. BSL-2 practices, containment equipment, and facilities are recommended for primary inoculation of cultures from potentially infectious clinical materials (BMBL 6th ed. 2020; Peacock et al. 2008). See Chapters 3 and 4 for further guidance on *B. pseudomallei* biosafety.

Quality Control

Perform quality control of media and reagents according to manufacture instructions, recent Clinical & Laboratory Standards Institute (CLSI) document M22 and Clinical Laboratory Improvement Amendments (CLIA), or European Committee on Antimicrobial Susceptibility Testing (EUCAST; Europe/UK) standards using positive and negative controls. Ideally, labs undertaking work on *B. pseudomallei* should be accredited under an appropriate national or international scheme (e.g. ISO 15189).

Examine culture plates for contamination, any cracks and dried out areas. Quality control checks should utilize a positive and negative control organism and should be done for each batch of reagents when possible. For frequency and specific protocols regarding quality control testing, refer to manufacturer guidelines and local and/or national regulations (ASM 2016).

NOTE- Quality control testing with *B. pseudomallei* strains is not recommended since this presents an unnecessary safety hazard. Substituting with avirulent strains or using near neighbors, like *B. thailandensis* should be encouraged.

Diagnostic Testing Options

1. **Gram Staining**

The most common stain for presumptive identification of bacteria under the microscope is the Gram stain, which is used to distinguish between gram-positive and gram-negative bacteria. Gram-positive bacteria stain purple because of to the presence of a thick layer of peptidoglycan in their cell wall, while gram-negative bacteria stain red/pink because of a thinner peptidoglycan wall that does not retain the crystal violet stain.

Gram staining involves three steps: staining with a water-soluble dye (crystal violet), decolorization, and counterstaining. Cells are initially stained with crystal violet dye. Gram's iodine solution is then added and a complex is formed between the crystal violet and iodine. A decolorizer (e.g., ethyl alcohol or acetone) is then added to the sample. Lastly, a counterstain such as safranin is added to the sample (Versalovic et al. 2011). The light microscope can then be used to distinguish between different bacterial shapes. While there are a variety of shapes, the most commonly seen are coccus, bacillus, and spiral. Bacillus, such as *B. pseudomallei*, are rod-shaped bacteria that usually occur in single rods but may also form chains.

Upon Gram staining, *B. pseudomallei* appears as a gram-negative (red/pink in color) rod-shaped bacillus often with a characteristic "safety pin" appearance (bipolar staining). However, it is important to note that the microscopic morphology of *B. pseudomallei* is not always suggestive and there may be absence of any bacterial structure, even in an appropriately stained smear of a good

quality sample. This is particularly true in patients receiving antimicrobial therapy, where the shape may be highly atypical, filamentous, or may appear similar to that of yeasts. Additional forms of diagnostic tests are always necessary to confirm the presence of *B. pseudomallei* (Inglis et al. 2005).

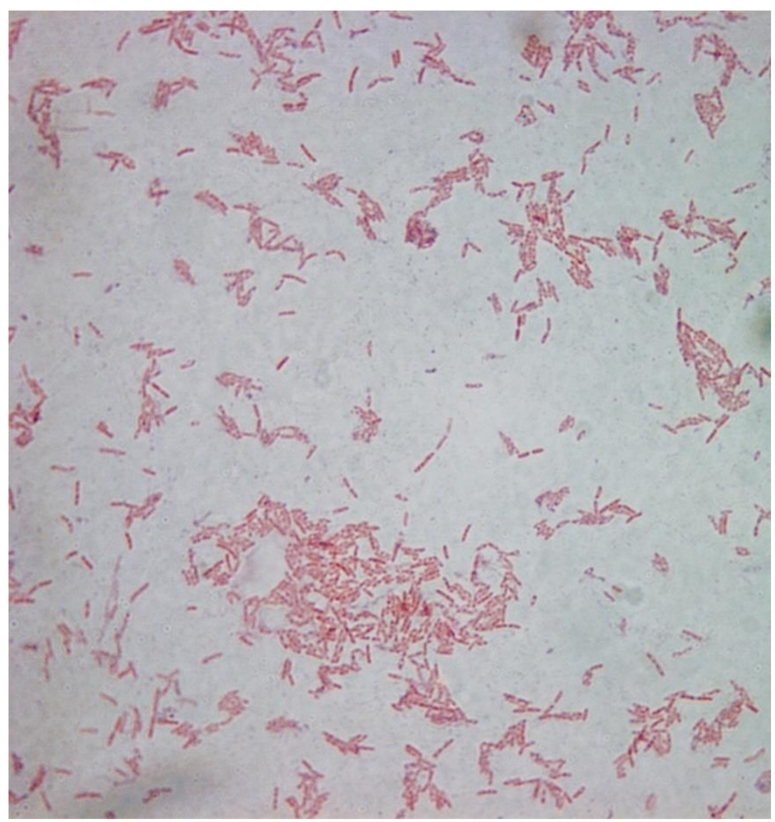


Figure 24- Typical Gram stain of *B. pseudomallei* under the microscope. *B. pseudomallei* is a gram-negative rod, often with a characteristic bipolar staining.

Gram Stain Protocol

Labs may use their own local Gram staining protocols where available. An example protocol is given below.

Reagents

- Fixative (methanol)
- Crystal violet solution
- Iodine solution (e.g. Lugol's iodine)
- Decolorizer
- Safranin solution

Equipment

- Glass microscope slides (and Coverslips if available)
- Lens paper
- Immersion oil
- Autoclavable waste container
- Compound light microscope with 40x and 100x objectives
- Specimen
- Tissue
- Water and alcohol resistant marker pen
- Gloves
- Lab coat/gown
- Eye protection

Procedure

1. Clinical specimens should initially be handled in a BSC (where available) with appropriate PPE. Prepare smears of specimens or bacterial cultures according to local protocols.
2. Inside the BSC, drop a thin film of fixative (ethanol) on the smear. Open flames are not recommended inside the BSC. Allow to dry for a few minutes. Make sure the fixative is completely dry (Calfee & Wendling 2015). Following fixation, the slide may be removed from the BSC.

1. Over an autoclavable waste container, hold the end of the slide with a gloved hand and flood the slide with crystal violet solution. Allow to stain for 1 minute.
2. Wash off the crystal violet solution with iodine solution. Then flood the slide with more iodine solution and let stand for 1 minute.
3. Gently wash the slide with an indirect stream of water for 2-3 seconds.
4. Flood the slide with decolorizer until no more purple color runs off the slide.
5. Gently wash the slide with an indirect stream of water for 2-3 seconds.
6. Flood the slide with safranin solution and let stand for 1 minute.
7. Gently wash the slide with an indirect stream of water until no color runs off the slide. Drain the

   access liquid by blotting slide on a tissue.
8. Gently overlay a coverslip. If not using coverslips, proceed directly to the microscope.
9. Place slide securely on the stage and turn on microscope. Examine the slide under 40x and move the focus knob until the bacteria are in view.
10. If needed, add a small drop of immersion oil to the slide and examine under the 100x objective. Be sure to only use the fine focus when looking under 100X lens.
    a. If you use the 100x objective, after examining the slide be sure to clean the objective lens with lens paper to remove excess oil.
11. Note the shape (coccoid, bacillus or spiral) and color of the bacteria in the sample on the lab worksheet for that specimen.
12. When complete, remove the slide from the stage and place in a sharps container for proper disposal according to local protocol (preferably by incineration).

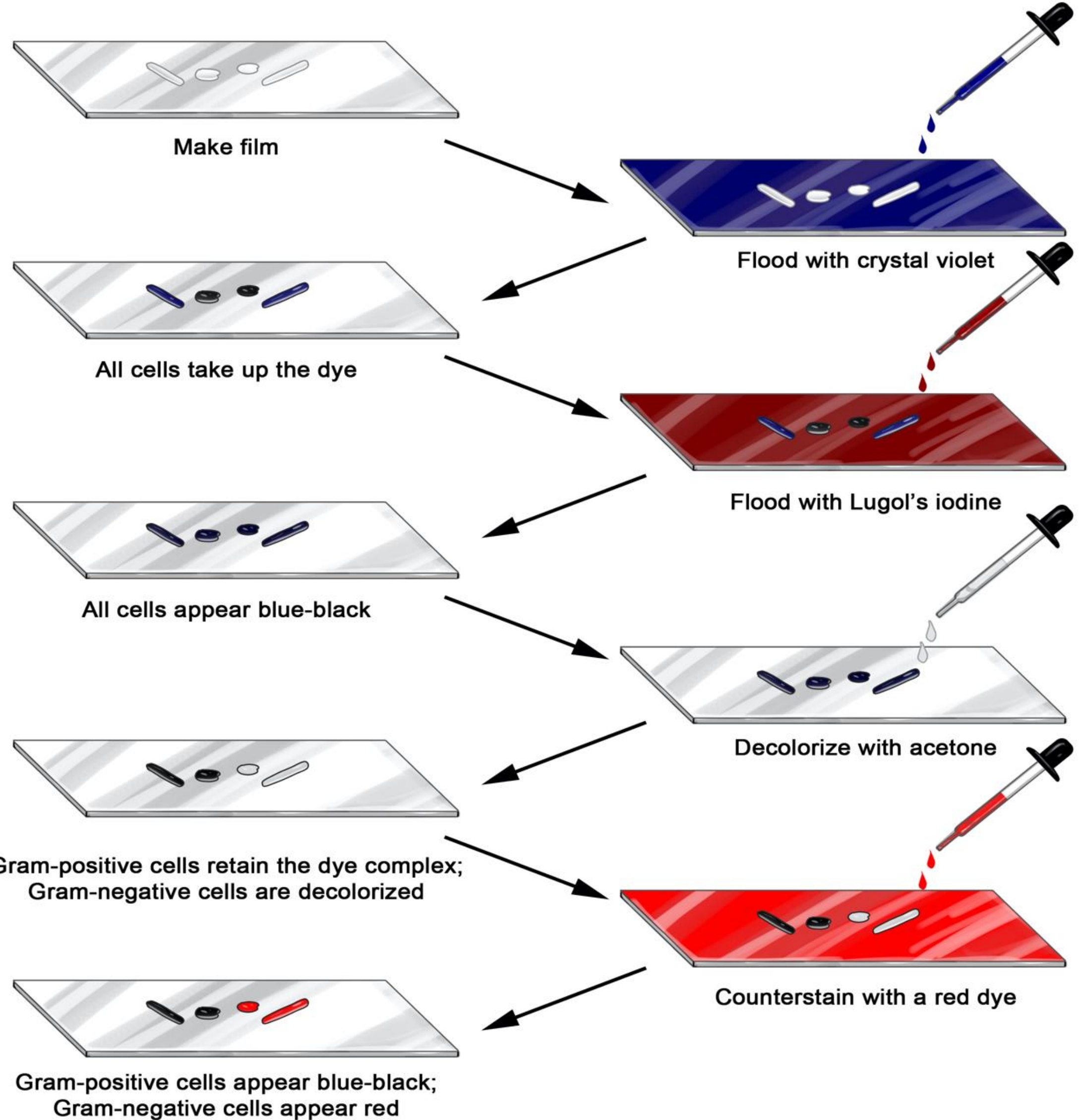


Figure 25- Example of a protocol for performing a Gram Stain in the microbiology lab.

Result	Reaction
Gram-positive	Cells appear blue to purple

Gram-negative	Cells appear pink to red

2. Bacterial Culture Identification

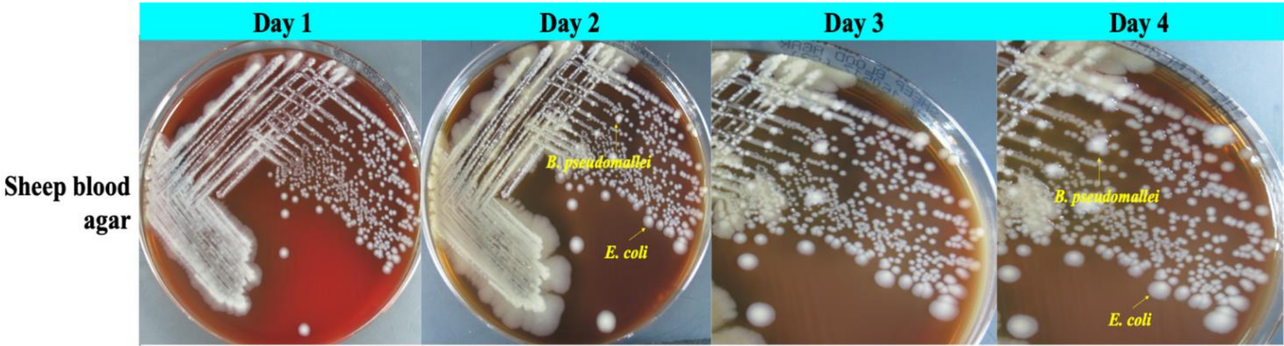
Bacterial culture is the current "gold standard" method used for the diagnosis of melioidosis. Since *B. pseudomallei* is never found as part of the normal human microbiota, its isolation from any clinical sample is considered diagnostic for the infection (Hoffmaster et al. 2015). It is crucial that the correct clinical specimens are collected and sent to laboratories familiar with the disease for culture identification. Blood, throat, and urine cultures should be done for all patients with suspected melioidosis and specimens from localized infection (e.g., sputum, wound swabs, and aspirates from abscesses) should also be collected when available (Hoffmaster et al. 2015). Blood should be inoculated into blood culture bottles as soon as possible to ensure specimen viability. The organism grows well on most routine laboratory media. However, sensitivity depends on correct sampling of the infected site, specimen transport and prompt processing, and method of culture (Limmathurotsakul et al. 2010). Since culture detection for *B. pseudomallei* has low sensitivity (60% overall), repeat cultures (especially of blood, sputum, urine and pus samples) may need be performed for patients with strong indications of disease (Limmathurotsakul et al. 2010).

Standard laboratory media (e.g. 5% sheep/horse blood and chocolate agar) support the growth of *B. pseudomallei*. However, the use of selective media is critical, particularly for tissues or specimens contaminated with normal human microbiota, such as sputum, throat or wound swabs, or following centrifugation where low numbers of organisms may be present, such as urine (Hoffmaster et al. 2015). Ashdown agar is frequently used in endemic regions (Currie et al. 2000; Dance et al. 2019), but it is not commercially available in most countries currently. The use of an enrichment broth similar to Ashdown medium without agar but including colistin for 48 hours at 37°C prior to plating on Ashdown agar may further increase bacterial yield but also increases time to laboratory diagnosis (Cheng et al. 2006). Alternative selective media may also be used for the

isolation of *B. pseudomallei*, including *B. cepacia* selective agar and *B. pseudomallei* selective agar (Peacock et al. 2005). In the absence of specialized media, a colistin or polymyxin B disk can also be positioned in the first quadrants of blood agar plates to help select for *B. pseudomallei*, as the bacterium is intrinsically resistant to these antimicrobials (Hemarajata et al. 2016) (See Chapter 10 for further detail).

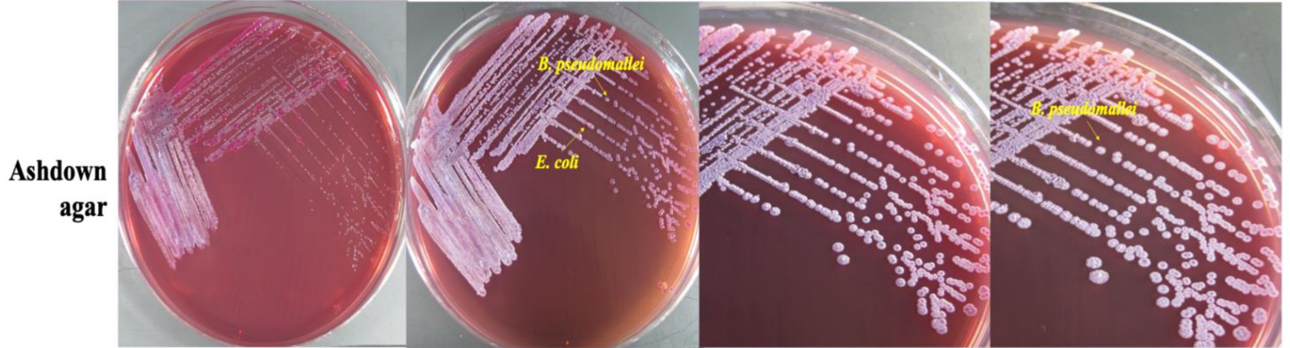
B. pseudomallei colonies normally appear as small, pinpoint and cream-colored with a metallic sheen on blood agar. Colonies may develop a dry or wrinkled appearance following 48 hours incubation. On MacConkey agar, colonies are lactose nonfermenting and are originally colorless. However, they often develop a pinkish appearance after 48 hours and may also develop a metallic sheen. On Ashdown agar, colonies typically develop into mauve to purple, flat, wrinkled colonies at 48 hours (Hoffmaster et al. 2015) (Figure 26). Laboratory identification of *B. pseudomallei* can be difficult and the large, wrinkled colonies are often mistaken as environmental contaminants, particularly in countries where the bacterium is rarely observed. Colony morphology is also highly variable and one strain may exhibit multiple colony types. This can include colonies with a smooth appearance, so inexperienced laboratory staff may misidentify the organism or discard the growth assuming it is environmental contamination (Chantratita et al. 2007; Greer et al. 2019).

Detailed protocols for the culture detection of *B. pseudomallei* from clinical and environmental samples are discussed in further detail in Chapter 10.



B. pseudomallei forms creamy colonies which are non-haemolytic and resemble a coliform. Slight metallic sheen. Becoming dry and wrinkled after 2 days of incubation. *E. coli* has similar morphology and tends to overgrow *B. pseudo.*

B. pseudomallei resembles a non-lactose fermenting coliform (colourless). Becoming dry and wrinkled after 2 days of incubation. *E. coli* is a lactose fermenter (pink to red colour) with colonies that become colourless on longer incubation.



First visible colonies of *B. pseudomallei* are pinpoint with clear to pale pink color. They become darker pink to purple, flat, slightly dry and wrinkled with a definite metallic sheen after 2 days of incubation. *E. coli* fails to grow because it is inhibited by gentamicin in the agar.

Figure 26- Colony morphology of *Burkholderia pseudomallei* on different culture media (image modified from Melioidosis.Info 2016).

3. <u>Conventional Biochemical Tests</u>

The difference in protein, fat and carbohydrate metabolism, enzyme production and compound utilization are several features that can be used for bacterial identification. Biochemical tests are

also simple, inexpensive and can be easily applied to most laboratories. There are a variety of biochemical tests that can aid in the diagnosis of *B. pseudomallei*. Some of these tests and characteristic biochemical properties of *B. pseudomallei* are shown in the table below:

Basic Characteristics	**Properties (*Burkholderia pseudomallei*)**
Arginine Dihydrolase	Positive (+ve)
Catalase	Positive (+ve)
Citrate	Positive (+ve)
Gelatin Hydrolysis	Positive (+ve)
Gram Staining	Gram-negative
H_2S	Negative (-ve)
Indole	Negative (-ve)
Lipase	Positive (+ve)
*Maltose	Positive (-ve)
Motility	Positive (+ve)
Nitrate Reduction	Positive (+ve)
Oxidase	Positive (+ve)
Pigment	Negative (-ve) (Some strains can produce pigmented colonies after prolonged incubation)
Shape	Rod-shaped; “safety pin” appearance (bipolar staining)
Spore	Negative (-ve)
*Sucrose	Negative (-ve)
TSIA (Triple Sugar Iron Agar)	K/NC/H_2S -ve
Growth at 42°C	Positive (+ve)

* Using Remel™ OF King Medium

Any oxidase-positive, indole-negative, gram-negative rod cannot be ruled out as *B. pseudomallei*. The organism is also catalase-positive, motile and displays no violet pigment on Mueller-Hinton agar (Inglis et al. 2005). *B. pseudomallei* motility may be confirmed using triphenyl tetrazolium chloride (TTC) indicator, which is convenient and can be safer than testing using the hanging-drop method. The colonies often produce a distictive musty or earthy odor, however plates should not

be sniffed due to the risk of exposure (CDC 2004). Another non-pathogenic bacterium, *B. thailandensis*, which is rarely encountered in clinical practice but may be detected in environmental samples, often resembles *B. pseudomallei* based on appearance and biochemical profiles. However, *B. Thailandensis* is able to utilize L-arabinose as its sole carbon source, distinguishing it from *B. pseudomallei* (Chaiyaroj et al. 1999). *B. mallei*, the causative agent of glanders, can be distinguished from *B. pseudomallei* by its nonmotility and inability to grow at 42°C (ASM 2016).

4. **Commercial Biochemical Identification Tools**

Commercially available systems provide fast and convenient identification to the species level as well as detect novel or unusual strains. Commonly used and well-studied systems for the identification of *B. pseudomallei* include the API 20NE (Figure 27) and bioMérieux Vitek 1 or Vitek 2 systems. These systems differ in whether they are manual, such as the API20NE, or automated, such as the Vitek systems (Glass & Popovic 2005; O'Hara 2005).

Commercially available identification systems perform generally well and in reference laboratories, if the bacterial isolate of question can grow on MacConkey Agar, a commercial gram-negative rod commercial identification system is normally used for non-fermenters. However, fresh cultures should always be used for accurate testing (Lowe et al. 2002). Commercial bacterial identification kits often still fail to distinguish between *B. pseudomallei* and closely related species such as *B. thailandensis* and members of the *B. cepacia* complex (BCC) even if there is a positive culture. Misidentification may also be caused by incorrect interpretation of tests, which can be difficult to read (Inglis et al. 2005) or can be the result of limited panels used to create profiles. If a commercial system reports an organism that is colistin or polymyxin B resistant as *Burkholderia cepacia, Chromobacterium violaceum* or *B. pseudomallei*, it may still be *B. pseudomallei* and this identification should be investigated. All identifications of *B. cepacia* or non-pigmented *C.*

violaceum must be investigated to rule out *B. pseudomallei* using other testing methods (ASM 2016).

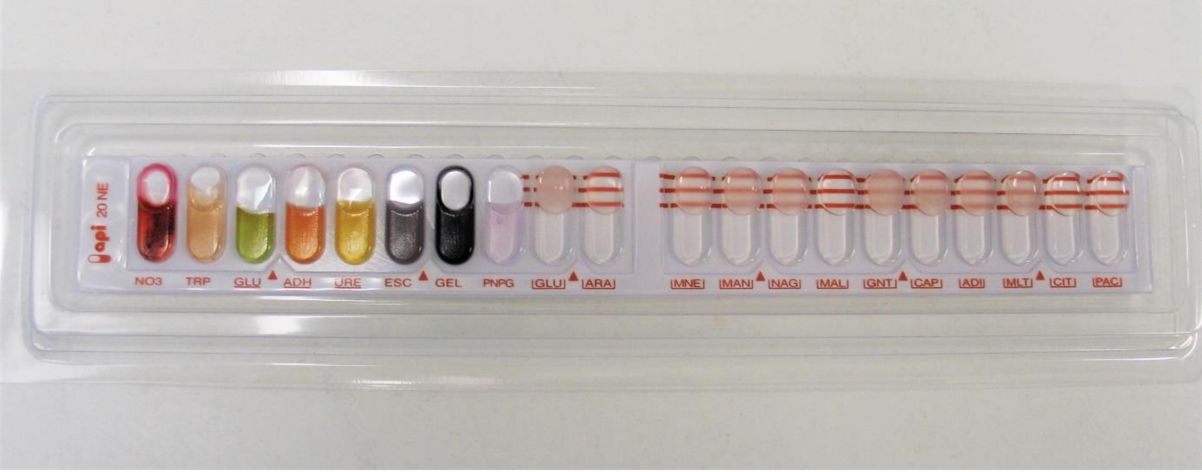


Figure 27- API 20NE commercial detection system showing characteristic profile of *B. pseudomallei.*

Examples of common workflows implementing biochemical testing used for the identification of *B. pseudomallei* are shown in Chapter 9.

5. Polymerase Chain Reaction (PCR)

PCR is a laboratory technique used to amplify a single copy of DNA or RNA across several orders of magnitude. This type of analysis can be useful with clinical or environmental isolates and specimens, where the pathogen is unknown or only suspected, or to confirm purity or identity of a sample or isolate. Using PCR, unique gene sequences found in the DNA of *B. pseudomallei* can be copied or "amplified" to determine the presence of the bacterium in a sample with a high sensitivity and accuracy. It is important to remember that PCR is used to detect the presence of bacterial DNA but does not represent active growth of the organism (ThermoFisher 2012).

Methods for PCR detection of *B. pseudomallei* are covered in further detail in Chapters 12-15.

6. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) is routinely used in melioidosis-endemic areas to identify *B. pseudomallei* and to distinguish it from closely related species including *B. cepacia*. The antimicrobial susceptibility pattern of *B. pseudomallei* is very characteristic. In resource-limited areas three disc-diffusion AST is routinely used to screen for resistance to gentamicin and colistin (or polymyxin B) and susceptibility to amoxicillin-clavulanic acid (co-amoxiclav) in gram-negative, rod-shaped bacteria that produce cytochrome oxidase (Dance et al. 2017; Wiersinga et al. 2012). This unusual pattern is enough to presumptively identify an oxidase-positive Gram-negative bacillus as probable *B. pseudomallei* (Trinh et al. 2018).

Where available, AST can also utilize minimum inhibitory concentration (MIC)-based broth microdilution method, however this can be arduous and costly in endemic countries with high case numbers (Karatuna et al. 2020; Jenney et al. 2001; Wuthiekanun et al. 2005). Graduated antibiotic strips (Etests) may also be used, but trimethoprim-sulfamethoxazole and tetracycline endpoints can be difficult to interpret. It should also be noted that the majority of strains in Sarawak, Malaysia are susceptible to aminoglycosides and macrolides, meaning typical recommendations isolation and AST do not apply there (Podin et al. 2014). It is not known whether other such foci exist, although this is possible.

Disk-Diffusion Susceptibility Testing Protocol (Karatuna et al. 2020)

1. Perform the antimicrobial susceptibility testing in a biological safety cabinet (BSC) where possible.
2. Prepare inoculum of *B. pseudomallei* isolated colonies selected from an 18-24 hour non-selective agar plate to get a concentration of approximately 1.5×10^8 CFU/mL (equivalent to a 0.5 McFarland standard).
3. Swab the bacterial inoculum onto the entire surface of Mueller-Hinton agar in three different directions (horizontal, vertical, and diagonal), to form a uniform lawn of growth.

4. Dispense preferred set of antimicrobial disks.
    a. EUCAST guidelines specify amoxicillin-clavulanic acid, ceftazidime, imipenem, meropenem, doxycycline, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole for AST.
    b. For presumptive identification, a simple three-disk testing method can be applied using gentamicin, colistin and amoxicillin-clavulanic acid (Trinh et al. 2018). See the Table below for the recommended disk concentrations.
5. Incubate plates at 35°C (+/- 2) for 18-24 hours.
6. The resulting zones of inhibition need to be uniformly circular with a confluent lawn of growth.
7. 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) (Table 12), 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.

NOTE- The new EUCAST criteria is the only internationally validated criteria for disc diffusion testing that is specific to *B. pseudomallei*.

8. Caution should be used when interpreting zone diameter for trimethoprim-sulfamethoxazole (SXT) since disk diffusion can overcall resistance. MICs or Etests are a more precise alternative and should be used for any isolate that appears resistant or intermediate by disk diffusion.
9. *B. pseudomallei* is normally susceptible to co-amoxiclav/amoxicillin-clavulanic acid and resistant to colistin or polymyxin B and gentamicin. This AST profile can be used for presumptive identification of any gram-negative oxidase-positive bacilli.

Table 12- MIC and zone diameter clinical breakpoints set by EUCAST.

For susceptibility testing purposes, the concentration of clavulanic acid is fixed at 2 mg/L.

*In EUCAST methodology, tetracycline disc diffusion is used to infer doxycycline susceptibility (Karatuna et al. 2020).

Antimicrobial agent	**EUCAST MIC and zone diameter clinical breakpoints for *B. pseudomallei***				
	Disc content (µg)	**MIC breakpoints (mg/L)**		**Zone diameter breakpoints (mm)**	
		S ≤	**R >**	**S ≥**	**R <**
Amoxicillin-clavulanic acid#	20-10	0.001	8	50	22
Ceftazidime	10	0.001	8	50	18
Imipenem	10	2	2	29	29
Meropenem	10	2	2	24	24
*Doxycycline/Tetracycline	30	0.001	2	*50	*23
Chloramphenicol	30	0.001	8	50	22
Trimethoprim-sulfamethoxazole	1.25-23.75	0.001	4	50	17

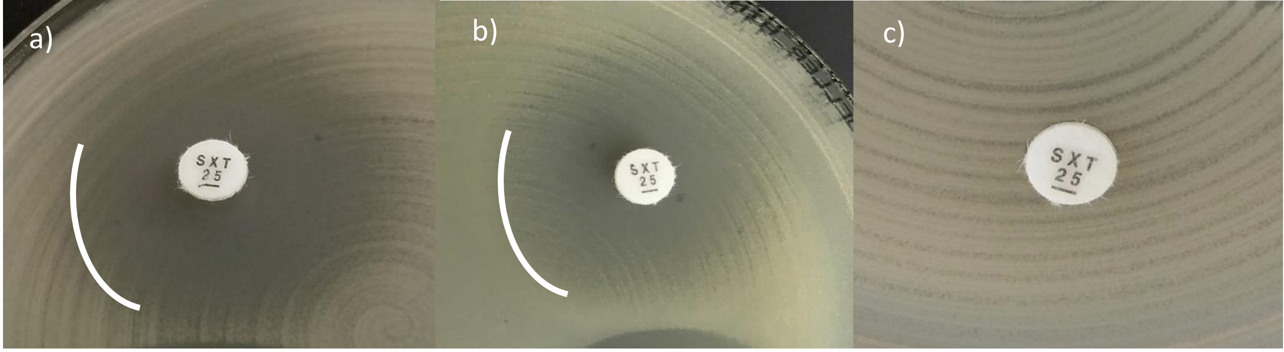


Figure 28- Examples of inhibition zones for *Burkholderia pseudomallei* with trimethoprim-sulfamethoxazole. a-b) An outer zone can be seen. Read the outer zone edge and interpret according to the breakpoints. c) Growth up to the disk and no sign of inhibition zone. Report resistant (EUCAST 2020).

It is important to note that recent reclassifications by EUCAST have meant that many of the agents used to treat melioidosis are now classified as “I” (Susceptible, increased exposure) rather than “S” (Susceptible, standard dosing regimen). Since *B. pseudomallei* is less susceptible to many antimicrobial agents than many other bacterial species, this has resulted in a shift of wild-type isolates from the ‘S’ category to the ‘I’ category for several antibiotics, including ceftazidime and trimethoprim-sulfamethoxazole (Dance et al. 2021).

Despite this, laboratories should still adopt new EUCAST criteria, as they are the only internationally validated criteria for testing *B. pseudomallei* by disc diffusion. Clinicians should not change prescribing practices when treating melioidosis on the basis of a laboratory report that says ‘I’ based on disc diffusion using the new EUCAST criteria. Ceftazidime should still be used as the treatment of choice during the initial intensive phase, even when reported as ‘I’ (which will be the majority of cases), as long as appropriately high doses are used (Dance DAB 2014; Sullivan et al. 2020). Trimethoprim–sulfamethoxazole should still be used for the eradication phase if it is reported as ‘I’ (in the majority of patients), unless there are prior contraindications. Isolates should not be reported as resistant on the basis of disc diffusion alone. MICs or Etests are a more accurate alternative and should be undertaken for any isolate that appears resistant by disk diffusion (Wuthiekanun et al. 2005).

7. ***B. pseudomallei* Antibody and Antigen Detection**

Antibody Detection

Even amongst culture-positive patients, isolation and confirmation of *B. pseudomallei* takes time and a high level of skill, which can result in delayed treatment. Serological tests are often used as a preliminary test in endemic areas to expedite melioidosis diagnosis. The sensitivity is significantly lower than culture diagnosis and should only ever be used as an adjunct to culture-based diagnosis, since interpretation of a positive antibody test is difficult in endemic areas with high background

seroprevalence rates (Charoenwong et al. 1992; Lau et al. 2015; Vandana et al. 2016). Some patients with melioidosis also fail to develop antibodies *to B. pseudomallei,* especially if immunosuppressed (Cheng et al. 2006). Since a positive result suggests exposure to *B. pseudomallei*, tests may also be useful in determining if laboratory workers, military personnel, and other returning travelers have been exposed to the bacterium (Hoffmaster et al. 2015; Wiersinga et al. 2012). However, a negative result should not rule out the disease and bacterial culture should always be attempted in any patient with clinical presentations strongly suggestive of melioidosis. There are several antibody serology tests currently in use for the detection of *B. pseudomallei*, which are often only available in reference laboratories. The indirect haemagglutination assay (IHA) is the most widely used serological assay for melioidosis worldwide, although it lacks standardization and has poor sensitivity (Hoffmaster et al. 2015). Other tests include IgM and IgG immunofluorescence assays (IFA) and enzyme-linked immunosorbent assays using a range of different antigens (ELISA), which may help to avoid the observer bias of IHAs (Suttisunhakul et al. 2016).

B. pseudomallei **Rapid Antigen Detection**

Antigen tests are immunoassays that can be used to detect the presence of a specific bacterial antigen and can indicate an active infection. They are comparatively inexpensive and several can also be used at the point-of-care. Direct IFA and sandwich “antigen capture” ELISAs, which typically utilize monoclonal antibodies raised against *B. pseudomallei* whole cell extract, are employed in some highly endemic areas (Lau et al. 2015). A monoclonal antibody-based latex agglutination assay is also available which can help to identify *B. pseudomallei* in cultures or blood culture broth (Duval et al. 2014) and a rapid diagnostic lateral flow immunoassay (LFI), called the Active Melioidosis Detect, has been widely evaluated in endemic areas (Houghton et al. 2014; Shaw et al. 2018).

***B. pseudomallei* antibody serology and rapid antigen detection assays and protocols are described in further detail in Chapter 11.**

8. Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS is increasingly being implemented as a diagnostic tool for rapid bacterial identification. It relies on the comparison of mass spectroscopy profiles against a database of isolates of a known species. There are two main types of databases: 1) a closed database where isolates have been previously verified and uploaded by the manufacturer and 2) an open database where isolates can be added by the user.

MALDI-TOF MS is a molecular technique used to separate proteins or peptides in a sample, which are portrayed as peaks on a spectrum (Figure 29). The tool has been shown to be effective in the identification of various non-fermenting, gram-negative bacilli, including several *Burkholderia spp.*. Several studies have now evaluated different MALDI-TOF MS systems for the detection of *B. pseudomallei*, including the Bruker MALDI Biotyper, Vitek MS (bioMérieux), and Andromas systems (Inglis et al. 2012; Lau et al. 2012; Suttisunhakul et al. 2017). The widespread implementation of MALDI-TOF MS for *B. pseudomallei* laboratory diagnosis is currently hindered by the scarcity of isolate profiles in existing closed databases (Karger et al. 2012). Efforts to add *B. pseudomallei* isolates to local open databases are under way in some melioidosis-endemic areas. One such open database is the U.S. Centers for Disease Control and Prevention “MicrobeNet”, which allows laboratories to search isolates by protein profiles generated by MALDI-TOF MS instruments. The free online database also provides additional information on genetic sequence information, biochemical and morphological characterization, and antibiotic resistance profiles for many rare and unusual pathogens. Visit https://www.cdc.gov/microbenet/index.html for more information and database access.

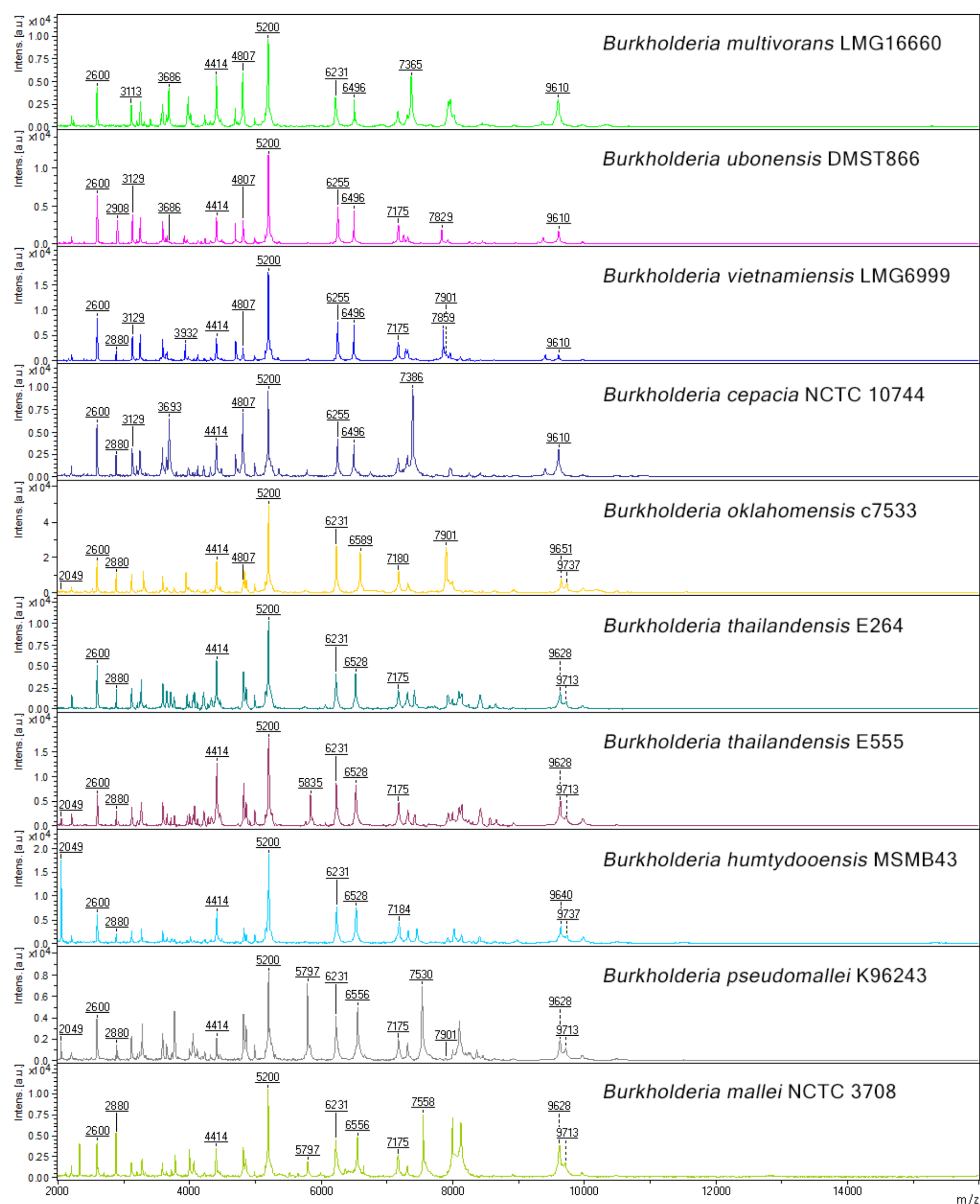


Figure 29- An example of MALDI-TOF MS results of *B. pseudomallei* and eight related *Burkholderia spp.* The vertical axis displays the relative intensities of ions and the horizontal axis displays mass to charge ratio (m/z) or masses of ions (Da) (Suttisunhakul et al. 2017).

Chapter Summary