Melioidosis Reference Manual

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Contents

Foreword: This manual was written to support clinical and laboratory colleagues. "Could my patient have melioidosis?" "What investigations can help with diagnosis?" "What clinical samples should I collect and how?" "How can the microbiology laboratory isolate, identify and confirm *Burkholderia pseudomallei?*" "How can we undertake environmental sampling?". Rapid advances are occurring in these areas and some content will become out of date and need future revisions. Readers are recommended to look at the most recent literature for new diagnostics and reviews.
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Chapter 1: Overview of Melioidosis

Chapter Overview and Introduction to Melioidosis

This chapter provides a brief overview of melioidosis, a neglected tropical diseases (NTD) endemic throughout many tropical and subtropical regions of the world. It was first described in a series of 38 patients in Myanmar in 1912. Preliminary autopsies of the patients showed a "peculiar cheesy consolidation" of the lungs, gross subcutaneous or splenic abscesses with "non-Gram-staining- [*Burkholderia mallei*]-like bacilli" (Whitmore & Krishnaswami 1912). The infection was termed melioidosis in 1921 (Stanton & Fletcher 1921). Recent modeling has since estimated that melioidosis kills more people every year globally than diseases that are considerably better recognized, including dengue and leptospirosis. The disease still frequently goes undiagnosed in resource-poor settings, meaning that the true burden of infection is likely greater than what is currently acknowledged (Limmathurotsakul et al. 2016).

Etiology

Melioidosis is caused by the environmental bacterium, *Burkholderia pseudomallei* (Cheng & Currie 2005). *B. pseudomallei* is a gram-negative, oxidase-positive, motile bacillus with a characteristic "safety pin" (bi-polar) appearance upon staining. The bacterium grows well under aerobic conditions in an optimal temperature of 37-42°C on routine culture media, though selective Ashdown or TBSS-C50 media is typically required to isolate the bacterium from sites with normal microbiota or from the environment (Wuthiekanun et al. 1990). Colonies have a characteristically wrinkled morphology and metallic appearance with a characteristic earthy odor (Foong et al. 2014; Wiersinga et al. 2018). *B. pseudomallei* is innately resistant to many antibiotics, including macrolides, narrow-spectrum cephalosporins, the majority of penicillins, polymyxins, and aminoglycosides (Currie 2015).

For environmental specimens, differentiation from the avirulent but closely related species, *B. thailandensis,* is done based on the ability to assimilate L-arabinose (Chaiyaroj et al. 1999).

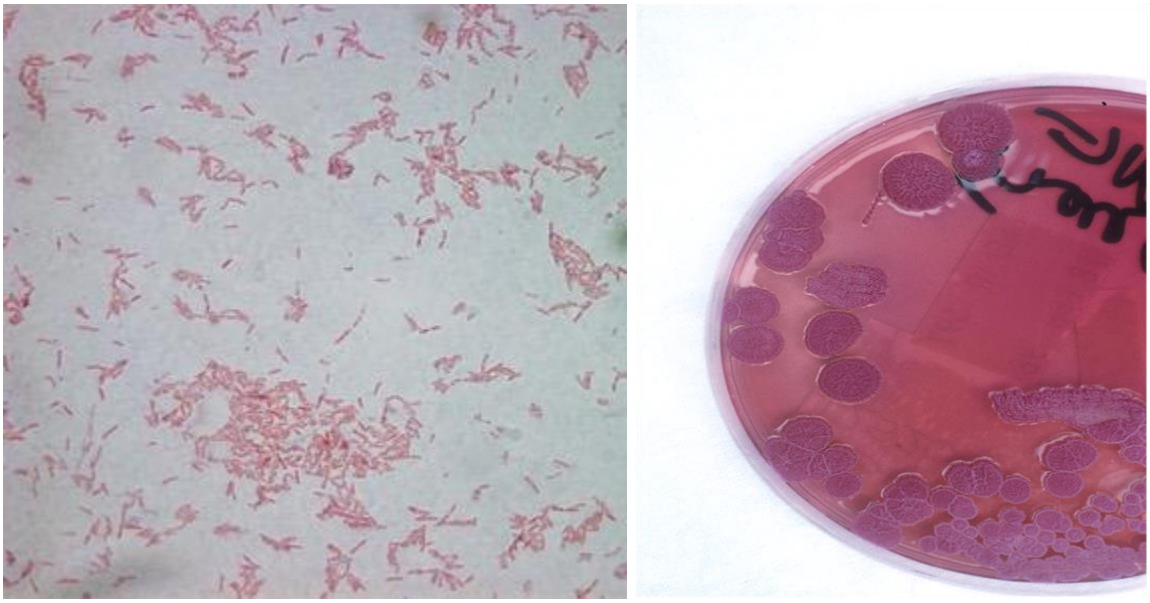


Figure 1- Typical Gram stain with Gram-negative bipolar appearance and characteristic dry, wrinkled morphology on Ashdown agar (Images courtesy of Menzies School of Health Research).

Epidemiology

Global Known and Predicted Occurrence

Melioidosis has recently been recognized as a significant source of global morbidity and mortality, with one recent model estimating 165,000 human cases and 89,000 deaths to occur annually worldwide (Limmathurotsakul et al. 2016). The vast majority of cases are diagnosed in Southeast Asia and Northern Australia, with these two regions representing hyper-endemic areas of the world (Currie et al. 2008). Thailand has by far the largest number of culture-confirmed cases of melioidosis, with 2,000 to 3,000 individuals diagnosed with the infection each year, most of which are reported in the northeastern provinces (Chaowagul et al. 1989; Limmathurotsakul et al. 2010). With recent advancements in diagnostic facilities

and better awareness of the disease, the *B. pseudomallei* endemic region has now been expanded to include parts of southern China, Taiwan, Malaysia, Indonesia, as well as a significant portion of the Indian subcontinent (Cheng & Currie 2005; Dance 1991). Here, recent modelling by Limmathurotsakul et al. (2016) projected more than 50,000 cases to occur per annum. Escalating numbers of endemic infections also continue to be reported from the Philippines, Papua New Guinea, and the Pacific Ocean islands of New Caledonia. Cases of melioidosis are increasingly being documented from elsewhere outside the classic endemic region as a result of improved clinical surveillance and laboratory diagnosis (Dance & Limmathurotsakul 2018), with occasional clinical cases and environmental isolates of *B. pseudomallei* reported in the Middle East, Africa, several Indian Ocean and Caribbean islands, as well as Central and South America (Benoit et al. 2015; Currie 2015; Hall et al. 2019; Sarovich et al. 2016). Sporadic cases from Europe and the USA are mostly considered to be travel-related, though recent human cases reported in Texas, USA suggest possible endemicity in the region (Birnie et al. 2019; Cossaboom et al. 2020). Tourists from nonendemic regions of the globe with diabetes and cystic fibrosis are at an increased at risk, particularly if exposed to rainy season soil and severe storms (Currie 2003; Geake et al. 2015; O'Sullivan et al. 2011).

Despite recent advancements, melioidosis still frequently goes undiagnosed in resource-poor settings, meaning that the true burden of disease is likely greater than what is currently acknowledged (Limmathurotsakul et al. 2016). Global climate change and extreme weather, combined with urbanization and alteration to soil composition, may cause an increase in the incidence and the geographical spread of melioidosis.

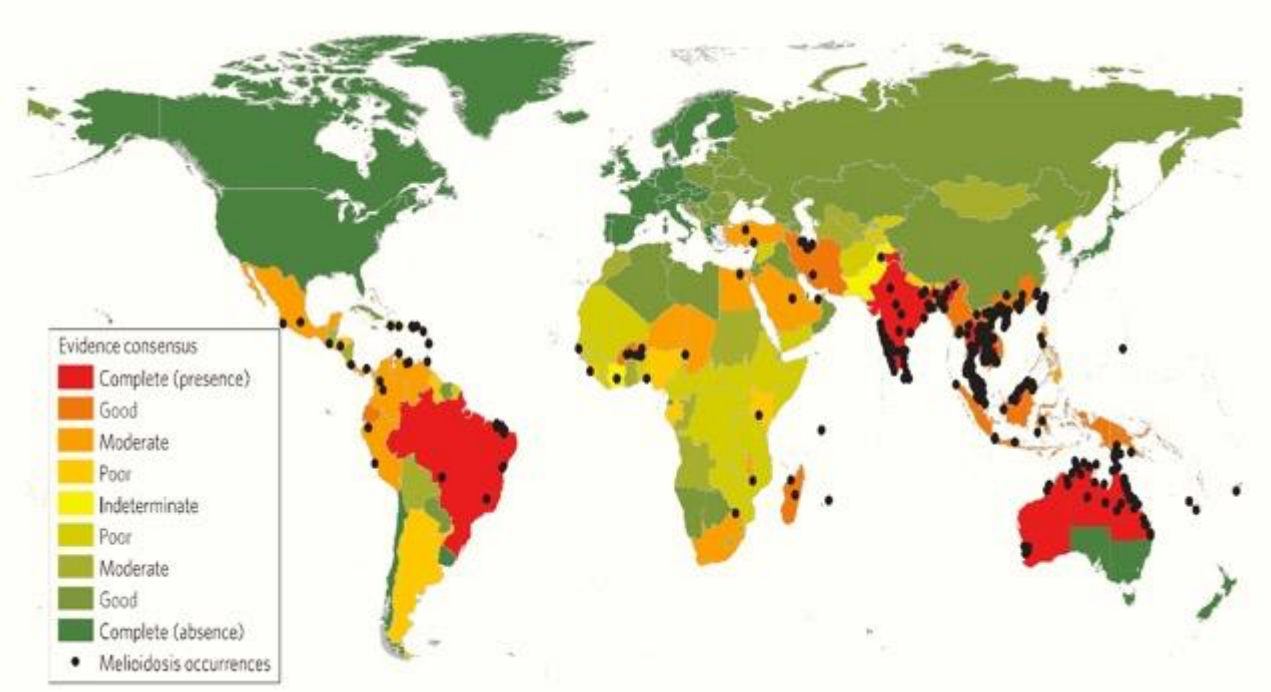


Figure 2- Evidence-based consensus for presence of *B. pseudomallei* and recorded geographic locations of melioidosis infection (Limmathurotsakul et al. 2016).

Routes of Exposure

Infection with *B. pseudomallei* typically occurs via percutaneous inoculation, inhalation, or ingestion of contaminated soil or water (Cheng & Currie 2005). In most regions, the disease is highly seasonal, with rainy season peaks corresponding with higher infection rates. Vertical and sexual transmission (Thatrimontrichai & Maneenil 2012), zoonotic transmission from animals with melioidosis (Low Choy et al. 2000) and transmission to laboratory staff are very uncommon but have been documented (Benoit et al. 2015; Green & Tuffnell 1968; Schlech et al. 1981).

Inoculation with *B. pseudomallei*-contaminated soil is considered to be the primary route of melioidosis infection in humans, though further habitats for the bacterium and other potential sources of infection have been revealed in recent years (Currie et al. 2010).

Ingestion is now recognized as being a common route of infection in animals and studies from Southeast Asia have suggested that ingestion of *B. pseudomallei* from unchlorinated drinking water supplies and other freshwater sources may be more common in humans than previously acknowledged (Currie et al 2015; Limmathurotsakul et al. 2013; Zimmermann et al. 2018). Outbreaks of melioidosis in Australia have been associated with *B. pseudomallei* contaminated drinking-water supplies, with the implicated water sources shown to be unchlorinated or containing inadequate levels of chlorine (Currie et al. 2001; Inglis et al. 2000; McRobb et al. 2015). Though human-to-human transmission is rare, a reported case of transmission to an infant from a mother with melioidosis mastitis via ingestion of breast milk has been described (Ralph et al. 2004), as has an instance of possible sexual transmission (McCormick et. al 1975).

Inhalation via contaminated aerosols has also been recognized as a significant source of infection (Currie et al. 2015). These cases typically follow severe weather events, such as cyclones and typhoons, and may be associated with increased disease severity (Currie & Jacups 2003; Parameswaran et al. 2012). During severe weather events such as tropical monsoonal storms or cyclones, inhalation may become a more prominent route of *B. pseudomallei* transmission and infection (Cheng et al. 2006; Mu et al. 2014). Studies from northern Australia and Taiwan have demonstrated an association between the occurrence of cases and the timing and severity of rainfall-related events (Kaestli et al. 2016; Lam et al. 2012). Melioidosis pneumonia was also documented in patients after the December 2004 Indian Ocean tsunami. While these cases were thought to be primarily associated with aspiration events, inoculation via lacerations in the skin may have also occurred (Allworth 2005; Chierakul et al. 2005). Collectively, this suggests that cases of inhalational melioidosis could increase if current predictions about the growing number and severity of weather-related events are realized (Chen et al. 2015; Liu et al. 2015).

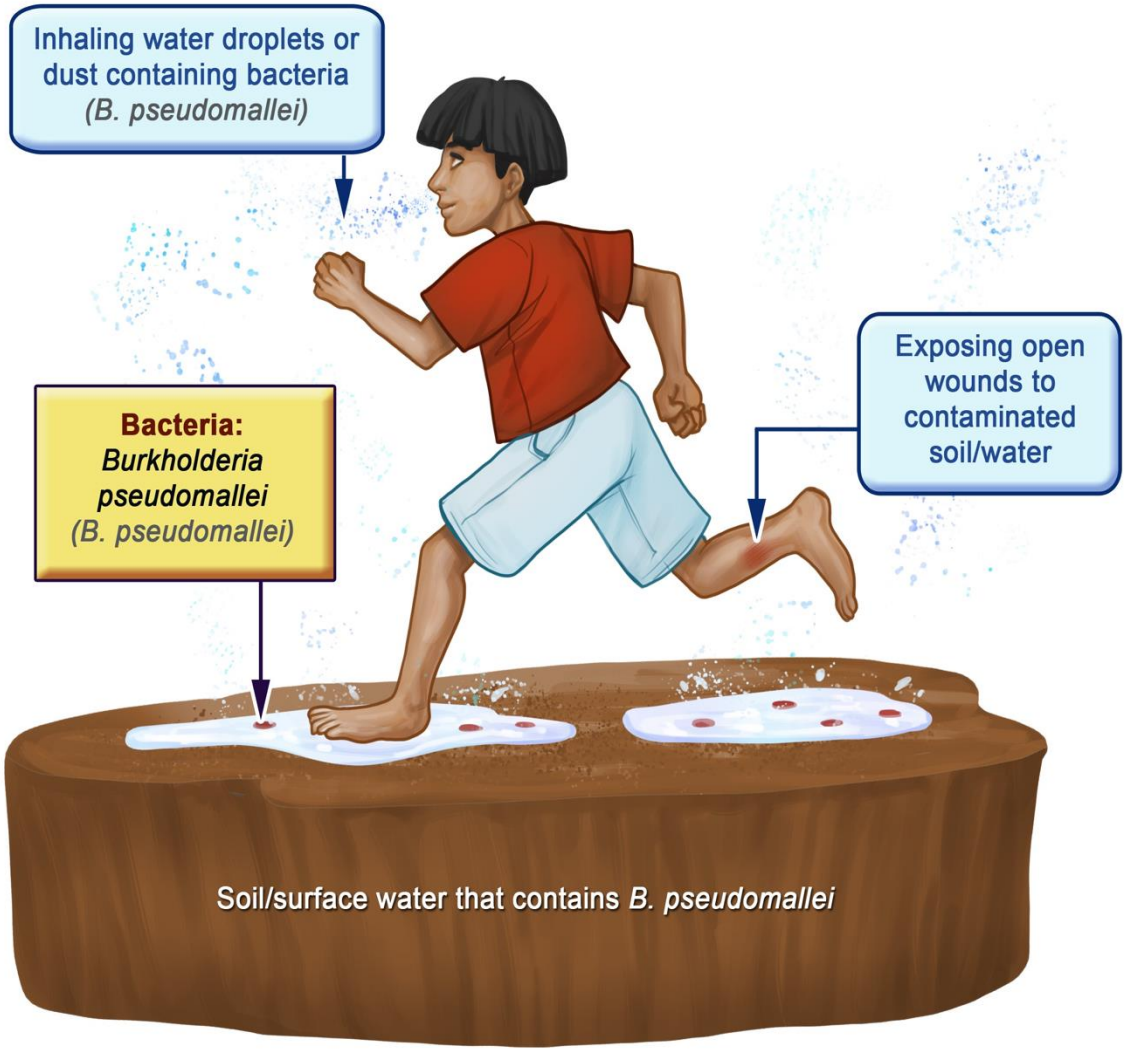


Figure 3- Routes of exposure to *B. pseudomallei* in the environment.

Risk Factors

Approximately 80% of patients diagnosed with melioidosis have one or more risk factor for the disease, however in children, who make up 5-15% of cases, identified risk factors are much less frequent (McLeod et al. 2014). Diabetes mellitus is the most common risk factor predisposing individuals to melioidosis and is present in >50% of all patients. Those with

diabetes mellitus are thought to have a 12-fold higher risk of melioidosis (after adjusting for age, sex and other disease-specific risk factors) (Currie et al. 2004; Limmathurotsakul et al. 2010; Wiersinga et al. 2018). Individuals with regular exposure to mud and surface water, such as rice farmers, are particularly susceptible, especially during the rainy season (Limmathurotsakul et al. 2013).

People with the following conditions are at increased risk for developing melioidosis (Currie 2008):

1. Diabetes (the strongest risk factor)
2. Excessive alcohol use
3. Chronic lung diseases, especially cystic fibrosis
4. Chronic liver disease
5. Chronic kidney disease
6. Malignancy, especially if on chemotherapy
7. Other forms of immunosuppression, especially long-term corticosteroid use
8. Thalassemia

Disease severity and clinical manifestations are influenced by the route of infection, virulence of the infecting *B. pseudomallei* strain, bacterial load, and most significantly, host risk factors. Infection with HIV (human immunodeficiency virus) does not appear to predispose an individual to development of the disease (Wiersinga et al. 2018). Death from melioidosis, particularly in those without known risk factors, is extremely uncommon with prompt diagnosis, appropriate antimicrobial therapy and effective sepsis management.

Chapter Summary

This preceding chapter gives a brief overview of melioidosis and its causative agent, *Burkholderia pseudomallei*. Melioidosis a still frequently underdiagnosed in many settings,

meaning that the true burden of disease is likely greater than what is currently acknowledged. The reasons for this are numerous, including the fact that it mainly affects the disadvantaged rural poor in areas without the necessary diagnostic facilities. The disease is likely to become more frequent in the years to come as risk factors, such as the prevalence of diabetes, as well the number and severity of weather-related events begin to increase.

Chapter 2: Clinical Presentations and Disease Progression

Chapter Overview

The following chapter discusses common clinical manifestations of melioidosis in humans and animals and the current recommended treatment guidelines. Control measures for the disease are also described. Since public awareness of melioidosis is limited, particularly in poorly-resourced countries, primary prevention should involve education about minimizing exposure to wet season soils, surface water, and aerosols generated during severe weather events. This is especially important for high-risk groups, such as diabetics, patients on immunosuppressive therapy and those on long-term steroid treatment.

Disease Progression in Humans

Infection occurs in all age groups, although only a small percentage of cases are diagnosed in children, many of whom have relatively mild, localized infections (McLeod et al. 2014). The majority of cases are diagnosed in adults aged 40 to 70 years, who tend to present with more severe infections (Currie et al. 2010; Suputtamongkol et al. 1994). The incubation period of acute infections is usually between 1 and 21 days, with a median of nine days (Currie et al. 2000). More severe disease often has shorter incubation times and is thought to occur following inhalation or aspiration of contaminated water (Chierakul et al. 2005). Melioidosis mortality rates have been demonstrated to be 10-50% with proper treatment (Wuthiekanun & Peacock 2006) and death can occur within the first 48 hours of hospital presentation as a result of septic shock (Limmathurotsakul et al. 2010).

While rare, it is possible for the disease to remain latent in some individuals, with clinical symptoms delayed by several months to years before melioidosis develops. Activation from latency has been estimated to account for <5% of all melioidosis cases (Currie et al. 2010).

Reported latency periods have ranged from 19 years to 29 years (Gee et al. 2017), suggesting *B. pseudomallei* may enter a dormant state to evade immune surveillance (Adler et al. 2009). The site of latency and mechanisms by which *B. pseudomallei* survives undetected remain uncertain, although there is evidence to suggest that some immunocompromising conditions may lead to activation of a latent infection (Gan 2005; Jabbar & Currie 2012).

Clinical Presentations in Humans

Disease manifestations vary from localized abscess or pneumonia to acute septicaemia, or may present as a chronic infection, though specific clinical presentations and severity may differ depending on the route of exposure, host immune system, as well as bacterial strain-specific virulence factors and inoculating dose (Wiersinga et al. 2018). While those infected frequently present with a septic illness, including pneumonia and disseminated hepatic, splenic and other organ abscesses, the disease can exhibit a diverse range of symptoms, giving rise to its nickname as the “great mimicker” (Yee et al. 1988). Initial bacterial multiplication at the site of entry may lead to a local lesion such as skin sore, abscess formation, or to pneumonia following inhalation. The progression of the disease is very variable, with some patients developing acute, fulminating infection and others developing chronic, indolent, granulomatous lesions, which may occasionally be asymptomatic (Kingsley et al. 2016).

Cutaneous melioidosis often presents with a solitary lesion at the site of inoculation and failure to respond to standard anti-staphylococcal/anti-streptococcal antibiotics (Currie et al. 2010; Suputtamongkol et al. 1999). Visceral abscesses are commonly documented in the spleen, liver, adrenals, parotids, kidneys, and the prostate. Skin involvement is seen more frequently in children (60%) than in adults (13%) (Currie et al. 2010; McLeod et al. 2014).

Cutaneous melioidosis may resolve spontaneously in some patients, but in others the organism may spread via the bloodstream or lymphatic system, leading to pneumonia and secondary abscesses (McLeod et al. 2014).

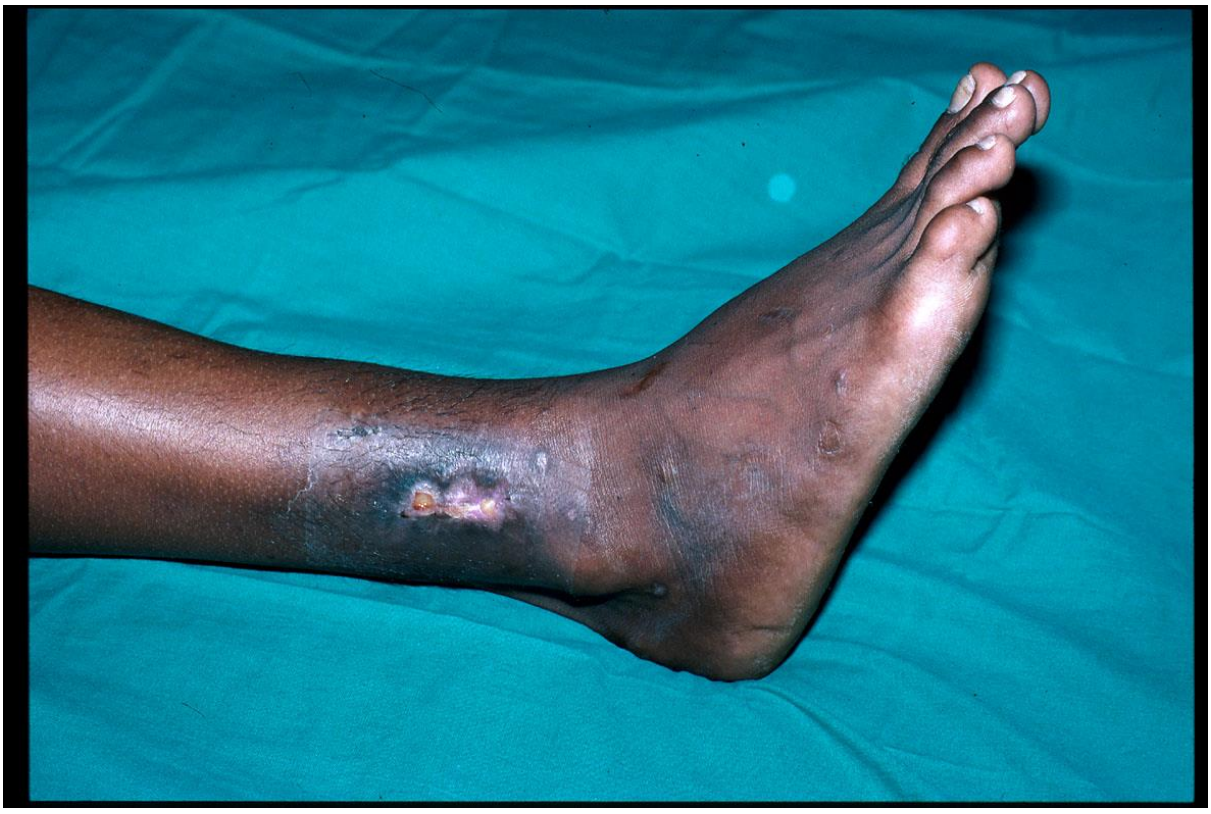


Figure 4- An ulcerated cutaneous lesion on the right ankle of a patient with melioidosis (Image courtesy of Menzies School of Health Research, Australia).

Most cases (85%) of melioidosis result in an acute infection. Apart from those with cutaneous-only melioidosis, the majority of these patients (regardless of the route of infection) present with bacteremia with or without pneumonia, and/or localized abscesses. Of these, approximately 20% develop septic shock (mortality up to 90%). In endemic regions acute pneumonia, particularly with upper lobe consolidation, may justify further investigation of melioidosis (Meumann et al. 2012; Wiersinga et al. 2018). While

rare, osteomyelitis and septic arthritis have been described resulting from penetrating injury or haematogenous dissemination. Additionally, mycotic vascular aneurysms, pericarditis, mediastinal masses, orchitis, and scrotal abscesses have been reported as clinical manifestations of the disease (Cheng & Currie 2005; Currie et al. 2010).

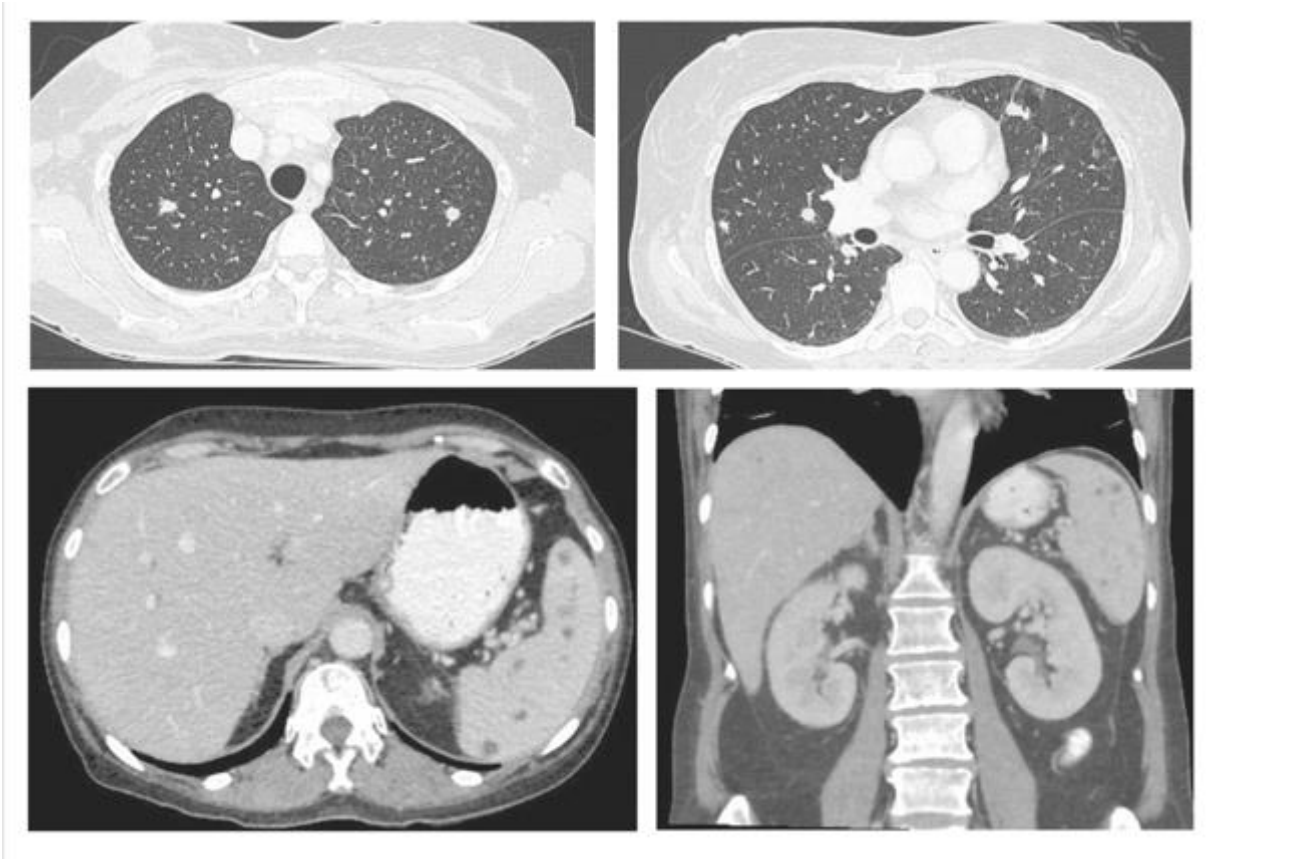


Figure 5- CT imaging showing multiple nodules in both lungs (top) and hypoechoic nodules in the liver and spleen (bottom) (Sukauichai & Pattarowas 2020).

There are several notable differences in presentation between patients in northern Australia and Southeast Asia. Suppurative parotitis, accounting for up to 40% of cases of melioidosis in children in Thailand and Cambodia, is very rare in Australia (Cheng & Currie 2005). In Australia, prostatic melioidosis is a more common clinical manifestation and is present in up to 20% of male patients. Neurologic melioidosis also occurs more frequently in Australian

patients and typically manifests as brainstem encephalitis, often with cranial-nerve palsies (especially cranial nerve VII), or as myelitis with peripheral motor weakness (Currie et al. 2010; McLeod et al. 2014). Current mortality rates for melioidosis are approximately 40% northeast Thailand (35% in children) (Limmathurotsakul et al. 2010) and less than 14% in Australia (Currie 2015). This difference is likely due to a combination of access to diagnostic facilities and melioidosis-specific antibiotics, as well as state-of-the-art intensive care and severe sepsis management of patients.

While most patients develop acute melioidosis following recent infection, some individuals can develop chronic melioidosis (symptoms >2 months). Chronic melioidosis can mimic pulmonary tuberculosis in that the infection may or may not be disseminated and cavitary lesions in the upper lobes of the lungs can be seen in disseminated infections. In a 20-year prospective Australian study, chronic infection occurred in 11% of cases (Currie 2016). Recurrent melioidosis can also occur, often in the first year after initial presentation (Currie 2015). Recurrent melioidosis following therapy can be defined either as relapse (with molecular typing showing the same strain) or re-infection with a new strain. Relapse correlates with duration of and compliance with the eradication therapy as well as severity of initial infection. In the past, recurrence was found to occur in as many as 13–23% of melioidosis patients. Improved antibiotic therapy and longer courses of treatment have significantly reduced the overall recurrence rate to as low as 1.2–7% (Chetchotisakd et al. 2014; Rachlin et al. 2016; Sarovich et al. 2014). Approximately a quarter of recurrences are due to reinfection while the remainder are due to relapse from a persistent focus of infection (Limmathurotsakul 2008).

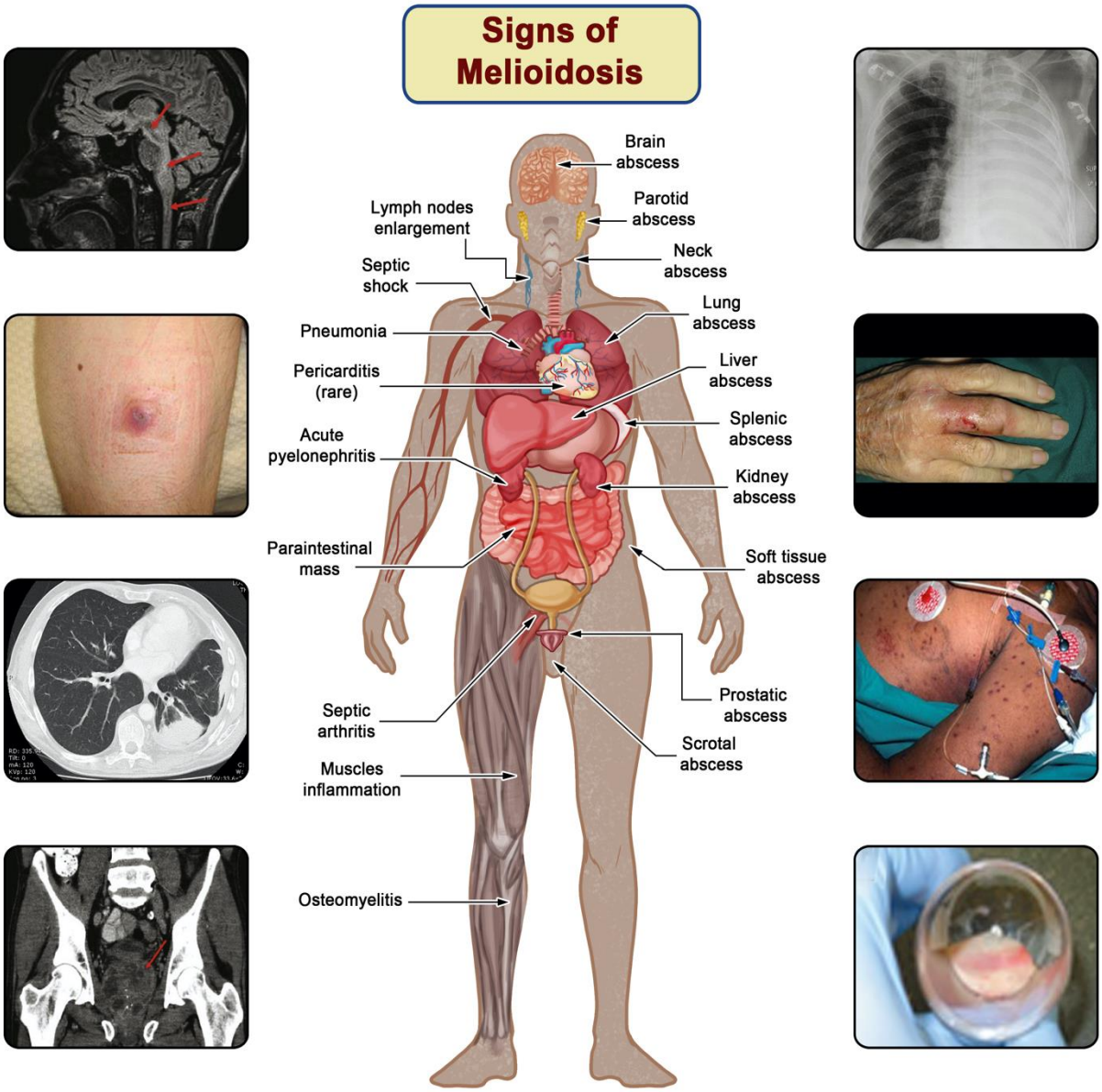


Figure 6- Possible clinical manifestations of melioidosis in humans (images courtesy of Bart Currie, Menzies School of Health Research, Darwin, Australia).

Disease Progression and Clinical Presentations in Animals

In melioidosis-endemic areas, the disease has been identified in a wide array of species including livestock, domestic pets, and exotic imported animals (Limmathurotsakul et al. 2012; Low Choy et al. 2000; Sprague & Neubauer 2004). While infection can occur in native wildlife, these animals are typically resistant to disease, with an underlying immunosuppressive condition usually accountable for infection (Asche 1991; Egerton 1963; Low Choy et al. 2000).

Certain species are acknowledged to be particularly susceptible to infection and disease, including goats (Tonpitak et al. 2014), sheep (Cottew 1950), camels (Forbes-Faulkner et al. 1992), alpacas (Low Choy et al. 2000), and marine mammals (Hicks et al. 2000). Exotic animals imported to endemic regions appear especially at risk, most notably non-human primates, including iconic species such as gorillas and meerkats (Rachlin et al. 2019; Sim et al. 2018). Several cases have also been documented in green iguanas imported to the USA and Europe from disease-endemic regions, highlighting the potential role the species may play as a reservoir for *B. pseudomallei* (Elschner et al. 2014; Hellebuyck et al. 2018; Zehnder et al. 2014). Although rare, outbreaks have also been described in endemic and non-endemic regions, including piggeries in Queensland, Australia (Ketterer et al. 1986), in imported sheep, goats and pigs from Aruba (Netherland Antilles) and in a Paris zoo (Dance et al. 1992). Regular outbreaks described in intensive livestock farms and in zoos indicate that stressful conditions may lead to the initiation of disease onset, especially in non-native species. While cases of animal melioidosis are frequently described throughout Northern Australia, cases from Southeast Asia and other endemic countries lacking contemporary diagnostic facilities are very rarely reported (Limmathurotsakul et al. 2012).

Similar to humans, melioidosis is most likely to occur after heavy rainfall or soil disturbance, resulting in exposure to the bacterium. Animals can also become infected by ingesting contaminated water or soil and inhaling contaminated aerosols during heavy rainfall or strong winds. Additionally, infected animals may further spread the bacterium through contact with wounds, nasal secretions, milk, faeces and urine (Sprague & Neubauer 2004).

Clinical presentations of melioidosis in animals varies based on species. Symptoms can include depression, fever, weight loss, respiratory symptoms such as heavy breathing and sneezing, lameness and swelling of the joints, and death. Symptoms of melioidosis in particular species include (Merck Vet Manual 2016):

Animal	Susceptibility	Common Symptoms
Sheep	Very susceptible	Respiratory disease is common. Signs include cough, respiratory distress and discharge from nose and eye. Some sheep become lame and have swollen joints.
Goats	Very susceptible	Pneumonia is common with symptoms including severe cough, respiratory distress and discharge from nose and eye. Lameness, anorexia, neurological symptoms and mastitis are also frequently reported.
Camelids (camels, llamas, alpacas):	Very susceptible	Respiratory disease with coughing, nasal discharge and difficulty breathing is common. Hindleg weakness, lack of coordination, wasting and acute disease with death have all been reported.
Pigs	Moderately susceptible	Adults typically develop few clinical symptoms. Wasting, nervous signs such as lack of coordination, skin ulcers and diarrhoea have been all been reported. Juvenile pigs may develop acute infection with fever, anorexia, coughing and discharge from the nose and eye.
Horses	Moderately susceptible	Symptoms may include weakness, wasting, swelling of the limbs, colic, diarrhoea, coughing and nasal discharge. Skin infections resembling fungal eczema can later become

		papular (blister-like lumps). Acute disease with high fever, limb swelling, diarrhoea and death have also been reported.
Cat	Less susceptible	Abscesses have been reported in various organs in cats. Jaundice, anemia, neurological disease and osteomyelitis have also been described.
Dog	Less susceptible	Curd-like nodules or abscesses in any organ. Blood infection, fever, diarrhea, and pneumonia have been described. Skin lesions and inflammation of the lymphatic system can occur, as well as loss of appetite, muscle pain, and swelling of the limbs.
Cattle	Less susceptible	Melioidosis is rarely reported in cattle. Signs in those reported include fever, difficulty breathing and nervous symptoms.
Birds	Less susceptible	Birds appear to be relatively resistant to infection. Cases with lethargy, anorexia and diarrhoea leading to death have been described.

Table 1- Clinical symptoms of melioidosis in common domesticated and farmed animals (Low Choy et al. 2000; Merck Vet Manual 2016; Sprague & Neubauer 2004).

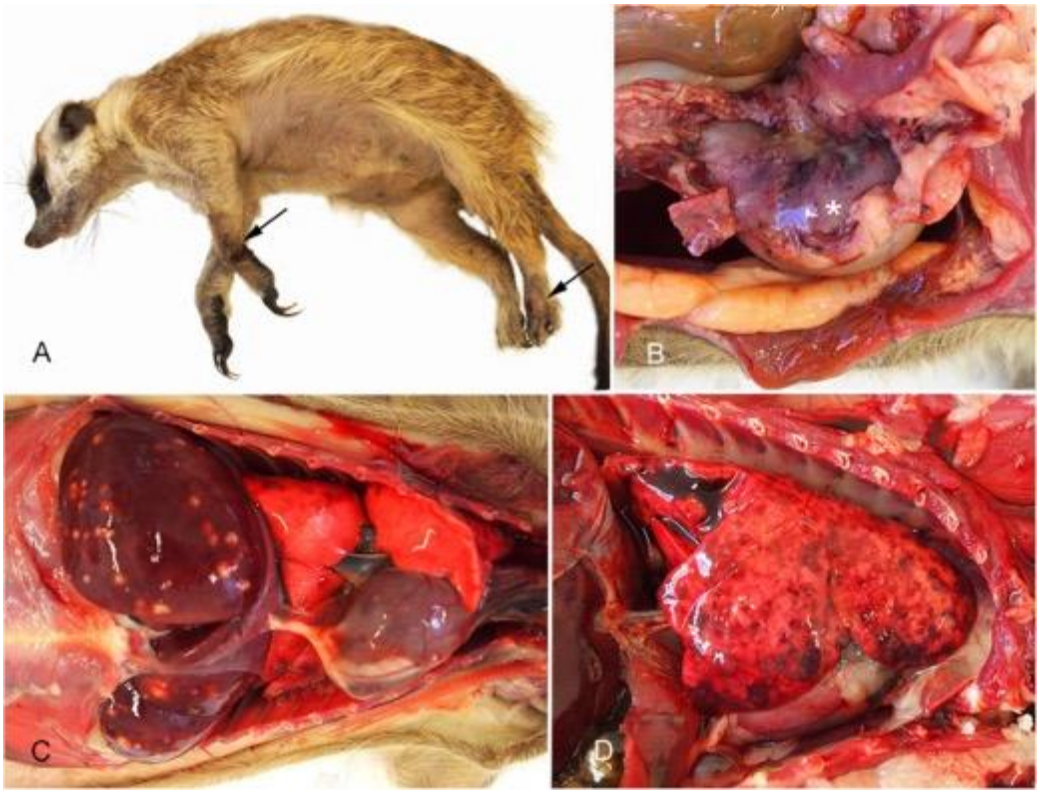


Figure 7- Gross pathology of melioidosis in meerkats. (a.) Typical ulcerative skin lesions (arrows). (b.) Stomach (asterisk) showing severe haemorrhage and purulent exudate in adjacent mesentery. (c.) Multifocal suppurative lesions throughout the liver. (d.) Lung with abundant coalescing red foci, representing severe, diffuse embolic showering of the lung (Rachlin et al 2019).

Treatment in Humans

Treatment for melioidosis consists of an initial intravenous intensive phase (Table 2) that lasts for approximately 14 days (though this can be longer when clinically indicated) and an oral eradication phase (Table 3). Appropriate oral eradication can dramatically decrease the risk of relapse, which can occur when intravenous antimicrobial drugs only are used.

Without eradication therapy, patients have a high risk of relapse and may develop serious disease with high mortality rates similar to those for primary infection. The relapse rate after completing the full eradication treatment is approximately 10%, but this can increase to 30% if oral treatment regimen is taken for less than eight weeks (Chaowagul et al. 1999; Lipsitz et al. 2012). Other studies have found that failure to complete at least 12 weeks of treatment is a primary determinant in disease relapse (Chetchotisakd et al. 2014). Recent studies from Australia support longer initial intravenous therapy (4 to 8 weeks or longer) for complicated pneumonia, deep-seated infection including prostatic abscesses, neurological melioidosis, osteomyelitis and septic arthritis (Pitman et al. 2015).

Intensive-Phase Antibiotic Treatment

Intravenous ceftazidime or meropenem are the preferred agents used during the intensive phase of treatment. Duration of therapy should be determined by the nature and severity of clinical presentations but should be given for a minimum of 10–14 days (Currie 2015). This should be increased for severely ill patients, including those with extensive pulmonary disease, deep-seated collections or organ abscesses, osteomyelitis, septic arthritis, or neurological melioidosis (Table 4) (Smith et al. 2018). In resource-poor settings it may not be affordable to extend intensive-phase treatment duration, but a minimum of 10 to 14 days is recommended (Dance 2014; Lipsitz et al. 2012). In this situation, completing a full course of oral eradication therapy is essential.

In patients with a collection (including skin ulcers/abscesses and abscesses in internal organs), and in bone/joint, genitourinary, or in CNS infections (but not for pneumonia), intravenous or oral trimethoprim/sulfamethoxazole may be added (Chierakul et al. 2005; Currie 2015). If trimethoprim/sulfamethoxazole is included, it should be continued for the entire duration of the intensive-phase treatment.

Repeat blood cultures in any bacteremic patient on day 7 of treatment and then weekly until negative. Patients with persistent bacteremia should have repeat blood cultures every 2 days after the start of antimicrobial therapy until negative. A repeat positive blood culture after >1 week of antimicrobial therapy typically indicates extensive bacterial burden or possible treatment failure, as does deterioration of clinical condition (worsening sepsis with organ dysfunction after 48 hours of therapy) (Lipsitz et al. 2012). Patients with persisting bacteremia or failure to respond to therapy should be examined for any undrained abscesses, and a switch from ceftazidime to meropenem should be considered for those on ceftazidime. Treatment failure is not normally related to the emergence of antimicrobial drug resistance (Lipsitz et al 2012). MICs should be performed where there is persistent or recurrent culture positivity (such as by E-test), in order to assess whether acquired resistance has developed.

Table 2- Recommended antimicrobials and dosages for initial intravenous intensive phase treatment of melioidosis. *Asterisk denotes agent of first choice. **NOTE-** Meropenem is often restricted to those requiring intensive care therapy, with ceftazidime used for all others. [1]In neurological melioidosis meropenem dose should be doubled to 40mg/kg/dose up to 2g 8-hourly.

Drug	Patient	Dosage/Route	Frequency
***Ceftazidime**	Adult	Up to 2 g/kg intravenous	Every 8 h, or 6 g/d by continuous infusion after a 2-g bolus for at least 14 days
	Child	50 mg/kg intravenous	
OR **[1]Meropenem**	Adult	Up to 1 g/kg intravenous	Every 8 h for at least 14 days
	Child	25 mg/kg intravenous	

For neurological melioidosis, osteomyelitis and septic arthritis, genitourinary infection including prostatic abscesses, and skin and soft tissue infections: **ADD** **Trimethoprim/Sulfamethoxazole** **AND** **Folate (0.1mg/kg up to 5mg) PER DAY**	Adult, >60 kg	320/1600 mg orally/intravenously	Twice daily
	Adult, 40–60 kg	240/1200 mg orally/intravenously	Three times daily
	Adult, <40 kg	160 /800 mg orally/intravenously	Twice daily
	Child	6 mg/30 mg/kg orally/intravenously	Twice daily

Eradication-Phase Antibiotic Treatment

Oral trimethoprim/sulfamethoxazole is the treatment of choice during the eradication phase of treatment (Chetchotisakd et al. 2014; Dance 2014; Lipsitz et al. 2012). Eradication therapy should be continued for a minimum of 3 months post cessation of IV therapy but can be extended to 6 months if neurological melioidosis, osteomyelitis or vascular mycotic aneurysms are present. Some patients with localized disease (should be confirmed by imaging to exclude other foci) who are systemically well may be safely treated with oral eradication therapy alone (Dance 2014; McLeod et al. 2014). Simultaneous administration of folic acid should be considered when giving trimethoprim/sulfamethoxazole in high doses for a long duration to reduce potential adverse effects associated with the drug's anti-folate effect (e.g., bone marrow toxicity). Alternative but inferior eradication therapy for these patients is doxycycline or amoxicillin/clavulanic acid (Cheng et al. 2008).

Table 3- Recommended antimicrobials and dosages for oral eradication phase treatment of melioidosis. *Asterisk denotes agent of first choice.

Drug	Patient	Recommended Dosage/Frequency
Trimethoprim/sulfamethoxazole* AND Folate (0.1mg/kg up to 5mg) PER DAY	Adult, >60 kg	160 mg/800 mg tablets: 2 tablets every 12 h
	Adult, 40–60 kg	80 mg/400 mg tablets: 3 tablets every 12 h
	Adult, <40 kg	160 mg/800 mg tablets: 1 tablet every 12 h **OR** 80 mg/400 mg tablets: 2 tablets every 12 h
	Child	6 mg/30 mg/kg; maximum dose 320 mg/1,600 mg every 12 h
OR Doxycycline adults & children 12 years or older 100mg every 12 hours		
OR Amoxicillin/clavulanic acid (co-amoxiclav)	Adult, ≥60 kg	500 mg/125 mg tablets: 3 tablets every 8 h
	Adult, <60 kg	500 mg/125 mg tablets: 2 tablets every 8 h
	Child	20 mg/5 mg/kg every 8 h; maximum dose 1,000 mg/250 mg every 8 h

Table 4- Recommended duration of antibiotic treatment of melioidosis. [a] Use clinical judgement to guide duration if improvement is slow or blood cultures are still positive after 7 days. [b] Enlargement of any hilar or mediastinal lymph node >10mm diameter. [c]Abscess anywhere other than skin, lungs, bone, CNS or vasculature. [d] Time is the date of most recent drainage of collection where culture of the drainage grew *B. pseudomallei* or where no specimen was sent for culture; time is not reset if drainage is culture-negative. [e] Where simultaneous oral therapy is not indicated during the intensive phase, oral eradication therapy should begin during the start of the last week of intensive intravenous therapy. [f] May require life-long suppressive antibiotic treatment if vascular prosthetic surgery is performed (Sullivan et al. 2020).

Site of Infection	Minimum Intensive Phase Duration (Weeks)[a]	Eradication Phase Duration (Months)[e]
Skin abscess	2	3
Bacteraemia without focus	2	3
Pneumonia ICU admit **OR** mediastinal lymphadenopathy[b] **OR** bacteremia and bilateral or unilateral multi-lobar pneumonia	4	3
Pneumonia with bacteremia and pneumonia but only a single lobe involved **OR** bilateral or unilateral multi-lobar pneumonia but not bacteremia	4	3
All other pneumonias	2	3
Deep seated collection[c]	4 [d]	3
Septic arthritis	4 [d]	3
Osteomyelitis	6	6
Central nervous system infection	8	6
Mycotic aneurysm or other arterial infection[f]	8	6[f]

Post-Exposure Prophylaxis

If high-risk laboratory exposure to *B. pseudomallei* occurs, post-exposure prophylaxis (PEP) is recommended. The first-line recommended PEP is oral trimethoprim–sulfamethoxazole (Table 5) (Peacock et al. 2008). Prophylaxis should last for 21 days. It is important that the potential benefits of PEP are considered before commencing, since trimethoprim–sulfamethoxazole can have severe adverse side effects. For low-risk exposure incidents, a decision to begin prophylaxis should be based on the presence of known risk factors for melioidosis in the exposed staff. Individuals with disease risk factors should be advised to start PEP. If staff have no known risk factors, monitoring for the development of symptoms or seroconversion is normally sufficient (Lipsitz et al. 2012; Peacock et al. 2008). See Chapter 4 for more detail about *B. pseudomallei* biosafety and high and low risk exposure incidents in the laboratory.

Table 5- Recommended *B. pseudomallei* post-exposure antibiotic prophylaxis. *Asterisk

Drug	Dosage	Frequency
Trimethoprim sulfamethoxazole*	TMP-SMX orally 8-40 mg/kg 2 x 160-800mg (960mg) tablets if more than 60kg, 3 x 80-400 (480mg) tablets if 40kg-60kg, and 1 x 160-800mg (960mg) OR 2 x 80-400 (480mg) tablets if adult less than 40kg **PLUS** Folate 0.1mg/kg up to 5mg PER DAY	Every 12 hours
OR Doxycycline	Adults & children 12 years and older 100mg	Every 12 hours
OR Amoxicillin clavulanic acid	20/5 mg/kg/dose (or 3 x 500/125 tabs) if >60 kg, and 2 x 500/125 tabs if <= 60kg	Every 8 hours

denotes first choice treatment agent (Peacock et al. 2008).

Antimicrobial Drug Resistance

B. pseudomallei is naturally resistant to many antimicrobial agents and should be considered when selecting an appropriate therapy. Antimicrobial agents that are considered unsuitable for treatment include penicillin, ampicillin, first and second generation cephalosporins, gentamicin, tobramycin, streptomycin, macrolides and polymyxins. Of note, clonal groups of isolates susceptible to gentamicin are common in Sarawak, Malaysia (Podin et al. 2014; Wiersinga et al. 2018). Primary ceftazidime resistance is thought to be rare but can occur naturally (<1%) (Lipsitz et al. 2012).

Prevention and Control in Humans

Public awareness of melioidosis, particularly in poorly-resourced countries with limited diagnostic capabilities, may be limited and preventive approaches are not always adopted. Primary prevention in endemic regions should involve education about minimizing exposure to wet season soils, surface water, and aerosols generated during severe weather events.

This is especially important for high-risk groups, such as diabetics, patients on immunosuppressive therapy and those on long-term steroid treatment. Patients with cystic fibrosis should also consider avoiding travel to areas of high endemicity during the rainy season (Boyd et al. 2016; Currie 2016). Closed footwear and gloves should be recommended to gardeners in highly endemic areas, and guidelines for the prevention of melioidosis in Thailand suggest that certain occupations such as rice farmers as well as travellers to the region should wear protective equipment if direct contact with soil or water is unavoidable (e.g., boots and gloves) (Suntornsut et al. 2020). Wounds or sores that have come into direct contact with soil or water should be thoroughly rinsed and cleaned to prevent infection. In higher risk individuals with underlying medical conditions, the cessation of smoking is advised, and the application of herbal remedies or organic substances to wounds is discouraged (Limmathurotsakul et al. 2013).

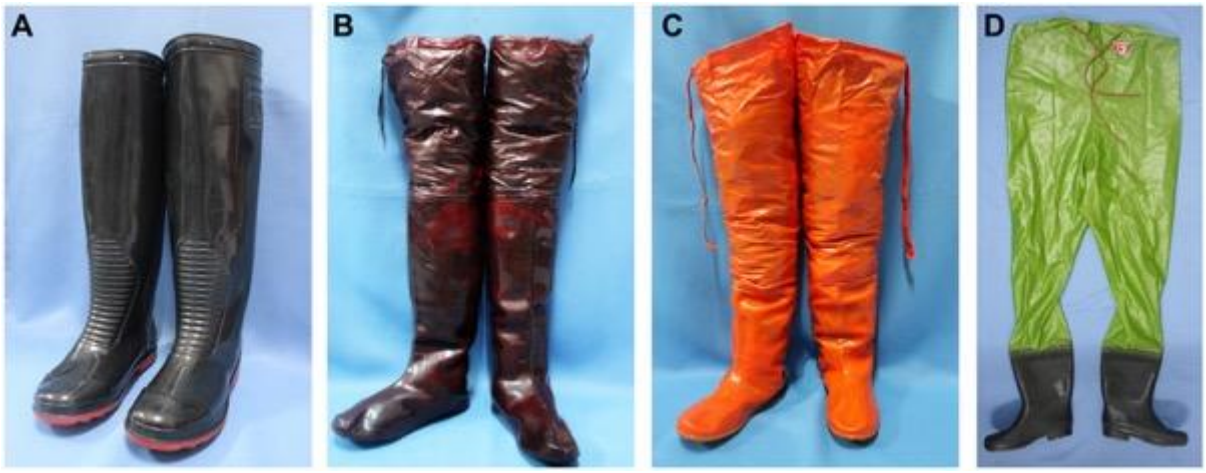


Figure 8- Various types of boots recommended for the prevention of melioidosis in rice farmers: Wellington boots (a), over-the-knee boots (b), hip boots (c) and half-body waders (d) (Suntornsut et al. 2016).

While appropriate chlorination of drinking water has been shown to be effective at preventing melioidosis outbreaks in Northern Australia, in resource-poor countries where water is untreated, drinking supplies should be boiled before consumption (Boyd et al. 2016; Howard & Inglis 2003). In high-income countries, ultraviolet light treatment can be implemented for the remediation of *B. pseudomallei*-contaminated private domestic water supplies, especially if household individuals are at an increased risk of infection (McRobb et al. 2013).

Prevention and Control in Animals

In animals, strategies to prevent potential infection with melioidosis are crucial for highly susceptible non-native species imported into endemic regions. This is particularly relevant for exotic species on display in zoos and wildlife parks. Adequate water hygiene is the predominant method of melioidosis prevention in endemic areas and animals should have their water supplies chlorinated (Low Choy et al. 2000) or sterilized using ultraviolet light filtration (McRobb et al. 2013). Additional strategies to prevent infection include limiting access to high-risk areas and providing adequate drainage of pens or enclosures to prevent surface water accumulation. Pens should preferably be concreted or made on dry solid ground while unpenned animals should be removed from the area of contamination (Fitzpatrick & Kearney 2008). Animals, such as non-human primates imported from melioidosis-endemic areas for laboratory purposes also present a potential occupational health and safety risk. Laboratory facilities importing animals from melioidosis-endemic regions should use precautionary measures to avoid potential exposures, including staff training in appropriate procedures for handling potentially infected animals, proper use of PPE and storage of baseline serum samples (Johnson et al. 2013).

Where animals are bred for human consumption, stringent control and disposal of sewage is necessary to prevent the spread of *B. pseudomallei* and the possibility of animal-to-human transmission (Inglis & Sousa 2009). Wearing gloves and disinfecting contaminated knives are recommended to prevent transmission during the processing of meat products (Ketterer et al. 1986). Since milk from goats and dairy cows may contain *B. pseudomallei,* pasteurization is recommended (Low Choy et al. 2000).

Case Definition Recommended by the U.S. Centers for Disease Control and Prevention

Clinical Description- Clinical presentation of melioidosis varies on a case-by-case basis. The following characteristics are typical of melioidosis.

a) An acute or chronic localized infection which may or may not include symptoms of fever and muscle aches. Such infection often results in ulcer, nodule, or skin abscess; rarely as parotid abscess.
b) An acute pulmonary infection with symptoms of high fever, headache, breathlessness, chest pain, anorexia, and general muscle soreness.
c) A bloodstream infection with symptoms of fever, headache, respiratory distress, abdominal discomfort, joint pain, muscle tenderness, and/or disorientation.
d) A disseminated infection with symptoms of fever, weight loss, stomach or chest pain, muscle or joint pain, and/or headache or seizure. Abscesses in the liver, lung, spleen, and prostate are often observed in patients diagnosed with disseminated infections; less frequently, brain abscesses may be seen.
e) A neurological infection with symptoms of severe and persistent headache and fever, with or without neck stiffness, confusion and altered sensorium.

Laboratory Criteria for Diagnosis

Confirmed cases:

a) Isolation of *B. pseudomallei* from a clinical specimen of a case of severe febrile illness: Culture of the bacterium may be done from various clinical samples such as blood, sputum or other respiratory secretions (like endotracheal aspirate), urine, pus, body fluids (like pericardial, pleural or peritoneal fluid), throat swab, or swabs/aspirates from organ infection (bone and joint) or abscesses or wounds.

Probable:

a) Evidence of a fourfold or greater rise in *B. pseudomallei* antibody titer between acute- and convalescent-phase serum specimens obtained greater than or equal to 2 weeks apart.

b) Evidence of *B. pseudomallei* DNA (for example, by validated polymerase chain reaction) in a clinical specimen collected from a normally sterile site (blood) or lesion of other affected tissue (abscesses, wound).

Case Classifications

Probable:

A case that meets the clinical case definition, one or more of the probable lab criteria, and one of the following epidemiologic findings:

a) History of travel to a melioidosis-endemic region, OR

b) Known exposure to *B. pseudomallei* as a result of intentional release or occupational risk (lab exposure).

Confirmed:

A case that is laboratory confirmed with clinical evidence.

Chapter Summary

The following chapter discusses common clinical presentations observed in human and animal cases of melioidosis. Current recommended treatment guidelines and recommendations for PEP are also described. Since public awareness of melioidosis is

limited, particularly in under-resourced countries with limited diagnostic capacity, primary prevention and control of the disease should aim to increase education about the infection. This includes information about minimizing exposure to wet season soil, surface water and aerosols generated during severe weather events.

Chapter 3: Handling in the Field and Personal Protective Equipment (PPE)

Chapter Overview

B. pseudomallei is considered a biothreat agent by the U.S. Centers for Disease Control and Prevention (CDC) due its high case fatality rate, difficulty in diagnosis and treatment in regions where it is not endemic, intrinsic antibiotic resistance, and potential for aerosol spread. Use of appropriate personal protective equipment (PPE) is essential to prevent accidental exposure, whether in the field, or handling culture specimens in the laboratory. The following chapter describes the minimum PPE recommended for handling potentially contaminated material or live cultures containing *B. pseudomallei*.

Minimum PPE for Specimen Collection in the Field

Personal protective equipment (PPE) should be used to protect yourself from accidental exposure to *B. pseudomallei* and is an essential part of safely working with the bacterium. Individuals should wear PPE when working with wet soil and muddy water. This includes gloves, protective clothing and enclosed footwear. A well-fitted mask should be worn if exposed to aerosols generated from muddy water and wet soil. Proper respiratory protection, such as an N95 respirator, should be considered for areas like water storage tanks that may contain biofilms or sludge. A surgical-type mask does not protect against infection. Prior to sample collection, any skin abrasions (e.g., cuts, scratches, lesions, etc.) present on skin should be covered using water resistant bandaging. Always avoid any skin contact with contaminated materials, soil or water. If available, wear long sleeves and long pants to cover exposed skin. Wash hands as soon as possible after handling or collecting specimens or samples and after removing gloves (Peacock et al. 2008).

PPE for melioidosis clinical and/or environmental specimen collection includes but is not limited to: long sleeved gowns or aprons, eye goggles and gloves, shoe covers/boots, and a fitted respirator. The minimal PPE necessary depends on the activity being performed (Table 6).

Table 6- Recommended minimum PPE for specimen collection.

Activity	Minimum PPE
Specimen collection from humans and animals	Use standard precautions as per local routine practice (e.g., disposable gloves).
Environmental sample collection (soil, water, air, plants, biofilms, sludge)	Disposable gloves, boots or closed shoes that can be disinfected.

PPE for Laboratory Diagnostics

Laboratory personnel, particularly those who may handle live cultures of *B. pseudomallei,* are at risk of laboratory-acquired melioidosis, since the procedures required to prepare specimens for culture may cause particles to aerosolize and release *B. pseudomallei* into the air.

Adequate PPE in the laboratory is important to avoid accidental exposure to *B. pseudomallei* and prevent contamination of the laboratory space. Lab coats and gloves should be worn at all times when handling live cultures to prevent any skin from becoming exposed. Lab coats with a cuff are preferred and should extend the full length of the arm to the wrist. This ensures the glove can be pulled past the wrist and over the cuff, covering all skin. If a cuffed lab coat is not available there are several other ways to ensure the glove extends over the wrist. Using longer wristed gloves or fastening the end of the lab coat to the wrist with a tie or tape are both options. Eye and respiratory protection should also be used if there is any possibility of aerosolization or splashing. Procedures that may generate aerosols include

centrifugation, shaking/vortexing, pouring liquid, opening tubes that contain liquid and streaking plates. Sealed cups should be used in all centrifuges, and these should be opened only in a biological safety cabinet (BSC). When working with larger quantities of sample or with liquid specimens, eye protection and respiratory protection should be considered. Respiratory protection is essential when centrifuging or performing work on *B. pseudomallei*-suspected or confirmed cultures outside of a BSC (WHO 2020; BMBL 6th ed. 2020). Good laboratory practices and use of appropriate PPE will prevent most laboratory accidents involving exposure to *B. pseudomallei*. See Chapter 4 for further detail on *B. pseudomallei* biosafety in the laboratory.

Putting on and Taking off PPE

It is important to use safe lab practices while wearing PPE to protect yourself and prevent contamination. These include keeping hands away from the face, restricting the number of surfaces touched and changing gloves regularly. Hand hygiene should be performed between steps if hands become contaminated and immediately after removing all PPE, including gloves. Using the correct techniques for putting on and taking off PPE is extremely important to ensure optimal protection. PPE should be put on and taken off in a certain sequence to limit the spread of contamination.

<u>Sequence for putting on PPE (see diagram below):</u>

1. Gown (or long sleeves and pants)
2. Boots or boot covers (if required-see guidelines above)
3. Mask or respirator (if required-see guidelines above)
4. Goggles or face shield (if required-see guidelines above)
5. Gloves

Sequence for taking off PPE (see diagram below):

1. Boots or boot covers (if wearing)
2. Gloves
3. Goggles or face shield (if wearing)
4. Gown
5. Mask or respirator (if wearing)
6. Wash hands or use an alcohol-based hand sanitizer immediately after removing all PPE

Additional Tips for Glove Use:

- Change gloves if torn or heavily soiled
- Discard gloves after use, never wash or re-use disposable gloves

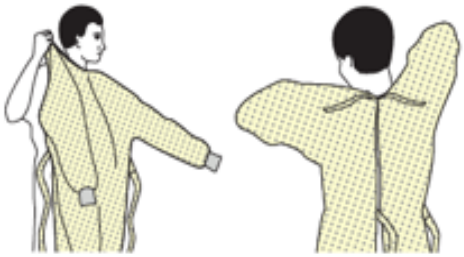
Disposable items should be placed in a biohazard bag and sealed for disposal, while reusable items should be decontaminated in a 10% bleach or 70% ethanol solution. Reusable items typically include rubber boots, face shields and eye protection. These items should be soaked for at least 15 minutes before being rinsed off with water and allowed to dry.

SEQUENCE FOR PUTTING ON PERSONAL PROTECTIVE EQUIPMENT (PPE)

The type of PPE used will vary based on the level of precautions required, such as standard and contact, droplet or airborne infection isolation precautions. The procedure for putting on and removing PPE should be tailored to the specific type of PPE.

1. GOWN

- Fully cover torso from neck to knees, arms to end of wrists, and wrap around the back
- Fasten in back of neck and waist



2. MASK OR RESPIRATOR

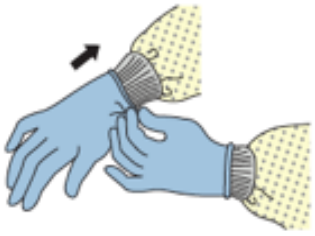
- Secure ties or elastic bands at middle of head and neck
- Fit flexible band to nose bridge
- Fit snug to face and below chin
- Fit-check respirator

3. GOGGLES OR FACE SHIELD

- Place over face and eyes and adjust to fit

4. GLOVES

- Extend to cover wrist of isolation gown



USE SAFE WORK PRACTICES TO PROTECT YOURSELF AND LIMIT THE SPREAD OF CONTAMINATION

- Keep hands away from face
- Limit surfaces touched
- Change gloves when torn or heavily contaminated
- Perform hand hygiene

CDC

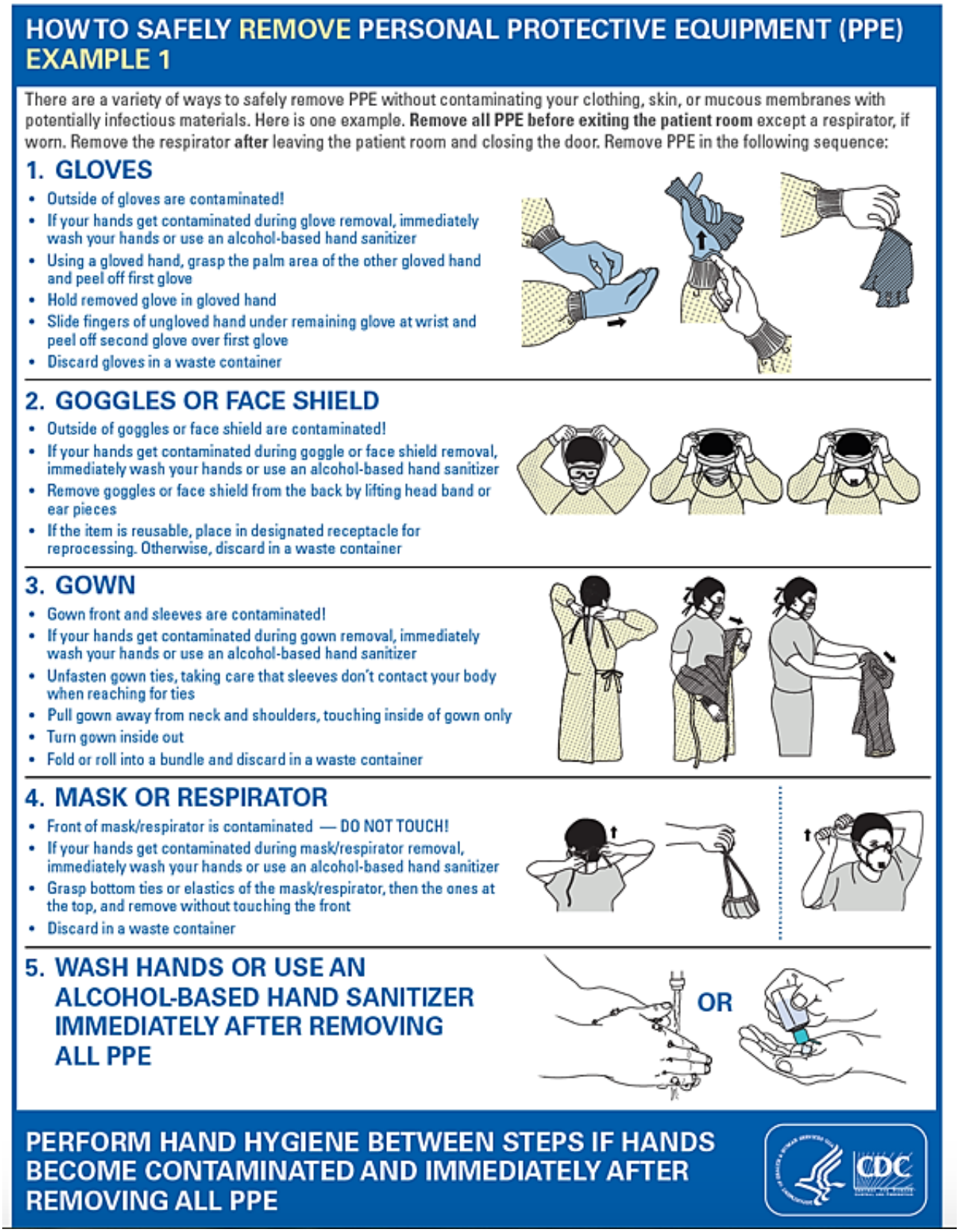


Figure 9- Instructional diagrams/sequences for putting on and taking off PPE (CDC 2014).

Chapter Summary

The preceding chapter describes the minimum recommended PPE when handling potentially infectious material or specimens contaminated with *B. pseudomallei*. Use of PPE is essential to prevent accidental exposure to *B. pseudomallei*, whether in the field or in the laboratory.

Chapter 4: *B. pseudomallei* Laboratory Biosafety

Chapter Overview and Introduction to Biosafety

Biosafety refers to the safe handling and containment of infectious microorganisms and hazardous biological materials (BMBL 6th ed. 2020). It involves the principles, technologies and practices that are necessary to prevent unintentional exposure to pathogens and toxins, or their accidental release. In the context of *B. pseudomallei*, biosafety typically relates to the safe handling of the organism and samples in order to minimize the risk of exposure and what to do should exposure occur.

Biosecurity is complementary to biosafety and refers to security measures that are designed to prevent the loss, theft, misuse, or release of pathogens and toxins. This includes measures that address access to facilities, storage and accountability of materials and data as well as publication policies (BMBL 6th ed. 2020). *B. pseudomallei* is currently listed as a Tier 1 select agent by the U.S. Centers for Disease Control and Prevention (CDC) due to the current lack of a vaccine, the high mortality rate, potential for aerosolization, and significant antibiotic resistance profile. Appropriate biosafety is thus a primary concern when handling *B. pseudomallei* in the laboratory.

Risk Categories and Biosafety Levels

There are four risk groups for microorganisms. Risk Group 1 includes microorganisms that do not or are unlikely to cause disease in humans or animals, while Risk Group 4 includes pathogens that usually cause serious disease in humans or animals and can be transmitted from person to person. These organisms pose a high risk for both the individual and community and usually do not have effective treatment or preventative measures. *B.*

pseudomallei is listed as a Risk Group 3 pathogen by the WHO, since it does not normally spread by casual contact from one individual to another but can still result in serious disease with an increased risk of aerosol spread.

The design of the laboratory relates to the classification of microorganisms being brought into the space. There are four levels of laboratory design: Biosafety Levels (BSL) 1, 2, 3 and 4. The assignment of a microorganism to a biosafety level takes both the risk level and several other factors into consideration including:

1. Pathogenicity of the organism and infectious dose.
2. Mode of transmission and host range of the organism.
3. Immunity, density and standards of environmental hygiene of the local population.
4. Presence of appropriate vectors.
5. Reports of laboratory acquired infections.
6. Local availability of prophylaxis by immunization or effective treatment.

For example, a pathogen assigned to risk group 2 will usually require a BSL-2 facility. However, if the experiments being conducted generate a high-concentration of aerosols, then a BSL-3 facility would be more appropriate (WHO 2020; BMBL 6th ed. 2020). Where biosafety facilities are available, BSL-2 practices and containment facilities are recommended when handling potentially infectious clinical materials and primary cultures of *B. pseudomallei* (BMBL 6th ed. 2020). It is recommended that if handling large volumes of *B. pseudomallei* culture or there is the potential for aerosolization to occur (such as during centrifugation or vortexing) that the specimen or culture be transferred to a BSL-3 facility as soon as possible.

In the United States, researchers and facilities handling select agents (including *B. pseudomallei*) must be registered with the Federal Select Agent Program (FSAP), inspected and approved by the relevant federal agencies before they can obtain the microorganism**.** BSL-3 containment equipment and facilities are recommended for all manipulations of suspect cultures, animal necropsies, 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 (where possible). Elsewhere, practices should follow appropriate national biosafety guidelines (BMBL 6th ed. 2020). All laboratory workers, regardless of location, should undergo organism- and site-specific training. This should aim to include training for new workers and refresher training for all current workers done annually (Peacock et al. 2008).

***B. pseudomallei* Risk Assessment**

All handling of *B. pseudomallei* or specimens that may potentially contain it should be the subject of a risk assessment prior to starting any work with the organism. This should take into account the type of work, local circumstances, the nature of routine specimen handling, local melioidosis endemicity, and any predisposing risk factors for melioidosis in staff who may handle the organism. Laboratories should always take any local or national guidelines into account (Gassiep et al. 2021).

Staff with risk factors for the disease should be educated about their increased risk and alternative work arrangements should be discussed and provided if available. All laboratory staff working with *B. pseudomallei* with an identifiable risk factor should be examined for *B. pseudomallei* infection if they have any febrile illness symptoms, regardless of whether there is any history of laboratory exposure. Examples of high and lower risk work with *B. pseudomallei* are listed below and are shown in Table 7 (from Peacock et al. 2008).

High Risk:

- Predisposing condition without use of appropriate PPE, including diabetes mellitus, chronic liver or kidney disease, heavy alcohol use, chronic lung disease (including cystic fibrosis), thalassemia, immunosuppression or long-term steroid use.
- Needle stick or any other injury where skin is penetrated by an instrument contaminated with *B. pseudomallei.*
- Splash event resulting in contamination of mouth or eyes.
- Production of aerosols outside of a BSC through sonication, centrifugation, etc.

Low Risk:

- Unintentional opening of the lid of an agar plate growing *B. pseudomallei* outside a BSC.
- Unintentional sniffing of agar plate growing *B. pseudomallei*.
- Splash event amounting to contact of gloved hand/protected body part with *B. pseudomallei.*
- Spillage of small volume of liquid culture (<1mL) within a BSC.
- Contact of intact skin with bacterial culture.

LOW RISK	HIGH RISK
Accidental opening of agar plate lid growing *B. pseudomallei* outside a biological safety cabinet	The presence of any predisposing melioidosis risk factor without appropriate personal protective equipment (PPE)
Unintentional sniffing of agar plate growing *B. pseudomallei* in the absence of contact with bacterium	Needle prick or penetrating injury with any instrument contaminated with *B. pseudomallei*
Contact of *B. pseudomallei* with gloved hand or protected body following splash event, in the absence of any visible evidence of aerosol production	Bite or scratch by experimental animal infected with *B. pseudomallei*
Spillage of small volume of liquid culture (<1ml) in a biological safety cabinet	Splash event leading to contact with the mouth or eyes

Contamination of intact skin with culture	Production of aerosols outside biological safety cabinet (e.g. centrifuge incident)

Table 7- Risk assessment of laboratory incidents involving *B. pseudomallei* (Peacock et al. 2008).

Laboratory-Acquired Infection with *B. pseudomallei*

Laboratory-acquired infections (LAIs) occur when a laboratory worker becomes infected. This can happen when there are lapses in good laboratory practice (GLP), such as inappropriate specimen handling or waste decontamination. Poor laboratory staff training can also increase the risk of LAIs or other biological accidents in the laboratory, and may also contribute to improper pathogen storage, labelling and transportation (WHO 2020).

GLP will prevent most laboratory accidents involving exposure to *B. pseudomallei*. The organism has been routinely handled with minimal precautions in many laboratories in endemic areas without staff becoming infected, and while LAI with *B. pseudomallei* has been reported previously, these cases have typically followed lapses in GLP. This suggests that the risk of LAI with *B. pseudomallei* is lower than several other hazard group 3 pathogens, including *Brucella species* or *Francisella tularensis* (Dance et al. 2017; Gassiep et al. 2021; Green & Tufnell 1968; Schlech et al. 1981). This should be taken into account when conducting a risk assessment and when deciding on the need for post-exposure prophylaxis (PEP).

Laboratories should prepare a written protocol that describes how staff will be managed if an exposure incident occurs, including the arrangements available for immediate medical assessment. Where possible, a physician with experience in the treatment of melioidosis should be specified in advance. Where possible, baseline serum samples should also be

obtained from workers before starting work with *B. pseudomallei* and stored at –80°C. Testing of serum is only required if a suspected exposure incident occurs (Peacock et al. 2008). A well-documented log of these samples should be kept.

If accidental exposure to *B. pseudomallei* is believed to have occurred, the site of contamination should be washed with clean water immediately, followed by appropriate disinfection of the area. The specified laboratory safety officer should be informed as soon as possible. The specific bacterial strain, its antimicrobial drug susceptibility (if known) and other pertinent information should be documented. While high-risk exposure events warrant increased attention and monitoring, all exposure events should be taken seriously. A risk assessment should be carried out that examines whether the exposure incident poses a low or high risk and if PEP is required (see Chapter 2 for further guidance on PEP dosage). Due to the significant health risks associated with PEP, it is advised that only those who have underlying risk factors that predispose them to melioidosis and are involved in high-risk exposure events are given treatment if exposure to *B. pseudomallei* has occurred (Peacock et al. 2008).

B. pseudomallei Laboratory Biosafety

GLP will prevent most exposure to *B. pseudomallei* and suspected specimens should always be handled with appropriate Personal Protective Equipment (PPE), including gloves, gowns, closed shoes and goggles (see Chapter 3 for further detail regarding appropriate PPE).

Depending on the facilities available, handling of *B. pseudomallei* or suspected specimens should be done in a Biosafety Cabinet (BSC). BSCs are essential for safe laboratory work. BSCs are different from polymerase chain reaction (PCR) clean cabinets in that they provide protection both for the person performing the work and for the work being performed. The

risk for laboratory acquired infection is significantly decreased when a BSC used. However, work performed within a BSC should still be done carefully and safely. Initial primary culture inoculations do not require a BSC, however, where available, work should be transferred to one once *B. pseudomallei* is identified or is suspected or where there is the potential for aerosolization. If the bacterial identity is confirmed the risk of potential exposure to laboratory staff should be assessed.

All testing in the BSC should be done while wearing gloves to protect from infections through the skin (Peacock et al. 2008). Centrifugation and vortexing should be avoided outside of the BSC and plates and tubes should be taped shut. All suspected plates and tubes should be put inside of a well-marked sealable container and transferred to an incubator after appropriate disinfection once manipulations have been undertaken in a BSC. The

container should be transferred back to the BSC before it is opened to examine plates and bacterial cultures.

Checklist for Safe Use of Biological Safety Cabinets

This checklist is a template you can edit and modify as necessary to incorporate your laboratory-specific standard operating procedures (SOPs). Use this checklist as a daily reminder of the activities/tasks needed for safely working in a Biological Safety Cabinet (BSC), as a training tool, or for annual audit of operational protocols.

Biological Safety Cabinet (BSC) Checklist

Preparing for Work in a BSC:

- ☐ Put on PPE according to your laboratory SOP.
- ☐ Turn UV light OFF (if used).
- ☐ Turn fluorescent light ON.
- ☐ Turn the cabinet ON, allow it to run for 4 minutes (or Manufacturer's recommended time) to purge the BSC of particulates. Some cabinets may alarm until purge is complete.
- ☐ Verify proper sash height and that sash alarm is ON.
- ☐ Verify drain valve underneath the cabinet is closed (valve handle is perpendicular to valve body).
- ☐ Check cabinet's certification sticker expiration date is within 1 year.
- ☐ Record the pressure differential gauge reading (if present), compare it against the calibration set point.
- ☐ Verify inward airflow according to your laboratory SOP (e.g., tissue test, smoke test).
- ☐ Schedule uninterrupted work time, if possible.
- ☐ Decontaminate all surfaces of the cabinet, according to your laboratory SOP.
- ☐ Collect all materials needed for the work.

Safe Use of a BSC:

- ☐ Place absorbent plastic-backed material to protect the work surface, if required by your lab SOP.
- ☐ Wipe the external surfaces of any equipment or supplies that you will need to place in the BSC.
- ☐ Place materials as far back in the cabinet as practical without blocking front and rear grilles.
- ☐ Separate clean/sterile items from dirty/potential contaminated items inside the BSC.
- ☐ Work from clean to dirty to minimize cross-contamination.
    - One person at a time should work in a cabinet. If two people need to work in a BSC, use a 6' cabinet and document the protocol-driven need in a risk assessment completed by the Lab Supervisor prior to starting work.
- ☐ Move your arms slowly in and out of the BSC. Do not move arms in sweeping, sideways movements.
- ☐ If using the vacuum system, protect the vacuum line with a filter.
- ☐ Do not use open flame without approval from the Safety Office.
- ☐ Adjust the stool/bench height so your face is above the bottom of the sash and your arms enter the cabinet with elbows at 90° angles, armpits level with the bottom of the sash. Use a footrest if your feet do not touch the floor.
- ☐ Discard all waste in a biohazard bag **inside** of the BSC.

After Completing Work in a BSC:

- ☐ Do NOT turn cabinet OFF while removing items and decontaminating the cabinet.
- ☐ Close and surface decontaminate ALL containers and lab materials before removal.
- ☐ Surface decontaminate the exterior of the biohazard waste bag/container before removal and disposal.
- ☐ Decontaminate cabinet interior, including sidewalls, back wall, inside of sash and work surface.
- ☐ Remove the gloves you were using in the BSC and dispose of them according to your laboratory SOP.
- ☐ Turn fluorescent light and blower motor switches OFF.
- ☐ If required by your lab SOP, turn UV light ON. Keep the sash closed while the UV light is on.
- ☐ Wash your hands.

Working with *B. pseudomallei* Outside of a Biosafety Cabinet

In laboratories without access to a BSC and biosafety facilities, it is essential that appropriate GLPs are used, including training, competency assessments, and use of PPE (wearing lab gowns, gloves, goggles and closed footwear). Sniffing of open plates should be avoided. If *B. pseudomallei* is identified or is highly suspected in any specimen, staff should put on a well-fitted respirator (e.g. N95 mask) before continuing work. Respiratory protection is especially important when centrifuging or performing other procedures that may generate aerosols outside of a biological safety cabinet (BSC). Exposure to aerosols presents the greatest *B. pseudomallei* biohazard, potentially resulting in inhalation, ingestion, and mucous membrane contact. Staff should be trained in the appropriate use of equipment and equipment should be available at all times. PPE should be checked, maintained and restocked regularly. If working with confirmed of suspected *B. pseudomallei* outside of a BSC, laboratory staff in higher risk categories (e.g. members with diabetes, lung or kidney disease) should be identified and assigned to alternative tasks.

Laboratory and Biosafety Cabinet Decontamination

Steam autoclaving is the preferred method of sterilization. Materials for decontamination and disposal should be placed in containers such as autoclavable plastic bags that are color-coded according to whether the contents are to be autoclaved and/or incinerated. Work surfaces of the BSC and bench should be decontaminated after each diagnostic test and immediately following any spills of infectious material. All contaminated materials, such as gloves, paper products, etc., should be sterilized before disposal, for example by autoclaving (WHO 2020). Small spills within BSCs can be handled using a towel dampened with decontaminating solution. Large spills that result in liquids flowing through BSC grills require more extensive decontamination. All items inside the cabinet should be decontaminated and

then removed from the BSC. Once removed, decontaminating solution can be poured into the workspace, ensuring that the drain valve is closed. Thirty minutes is typically adequate for decontamination, but this may vary depending on the disinfectant used. Any spillage and disinfectant solution should be wiped up with paper towels and discarded into a biohazard bag (BMBL 6th ed. 2020; Wuthiekanun et al. 2011).

B. pseudomallei is susceptible to numerous disinfectants, including benzalkonium chloride, iodine, 10% bleach solution, 70% ethanol and 2% glutaraldehyde and is effectively killed by the commercial disinfectants, Perasafe and Virkon using a contact time of approximately 30 minutes (Wuthiekanun et al. 2011). The pathogen can also be destroyed by heating to above 74 °C for 10 min or by ultraviolet irradiation (Rose & O'Connell 2009).

Additional Technical Resources

General international biosafety guidelines are provided by the biosafety manual of the World Health Organization and can be found here: https://www.who.int/publications/i/item/9789240011311.

For additional information regarding standard practices, safety equipment and facility specifications recommended for biosafety laboratories in the USA, see the Biosafety in Microbiological and Biomedical Laboratories- 6th Edition (BMBL 6th ed. 2020) available from: https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf.

Chapter Summary

B. pseudomallei is currently listed as a Tier 1 select agent by the U.S. Centers for Disease Control and Prevention (CDC) due to the current lack of a vaccine, the high mortality rate,

potential for aerosolization, and significant antibiotic resistance profile. Appropriate biosafety is thus a primary concern when handling *B. pseudomallei* in the laboratory. Researchers and facilities handling *B. pseudomallei* should always follow appropriate national biosafety guidelines when handling the bacterium in the laboratory.

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).

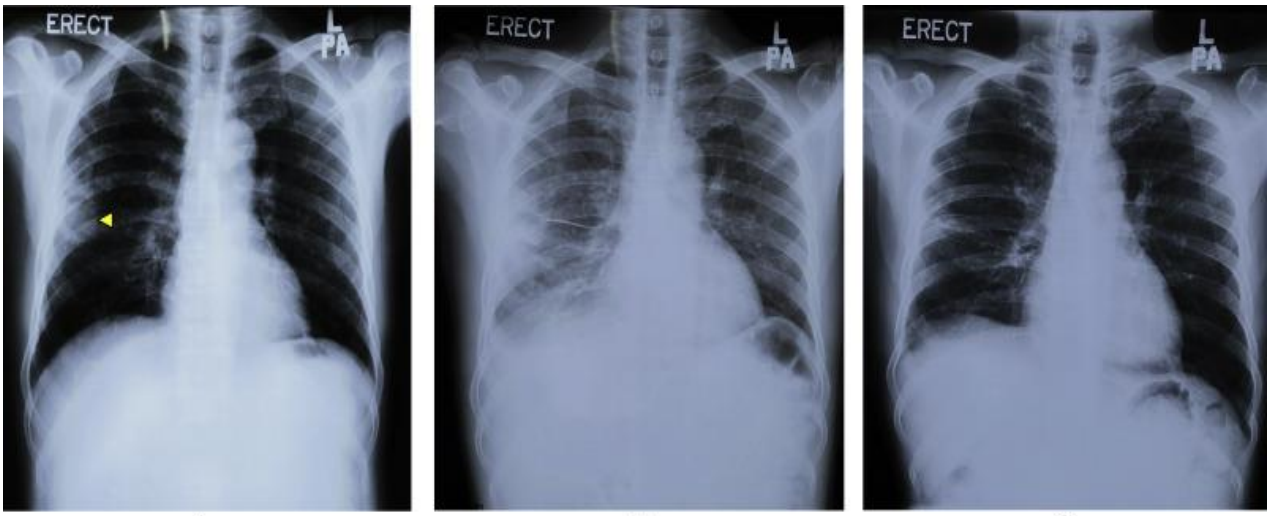


Figure 10- A) Right pulmonary opacity with cavity at right midzone (arrow) on first admission. B) Worsening of right pulmonary lesion compared to first admission with right-sided pleural effusion. C) Resolving right pulmonary lesion and resolved pleural effusion after 1 month of treatment (Chang et al.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

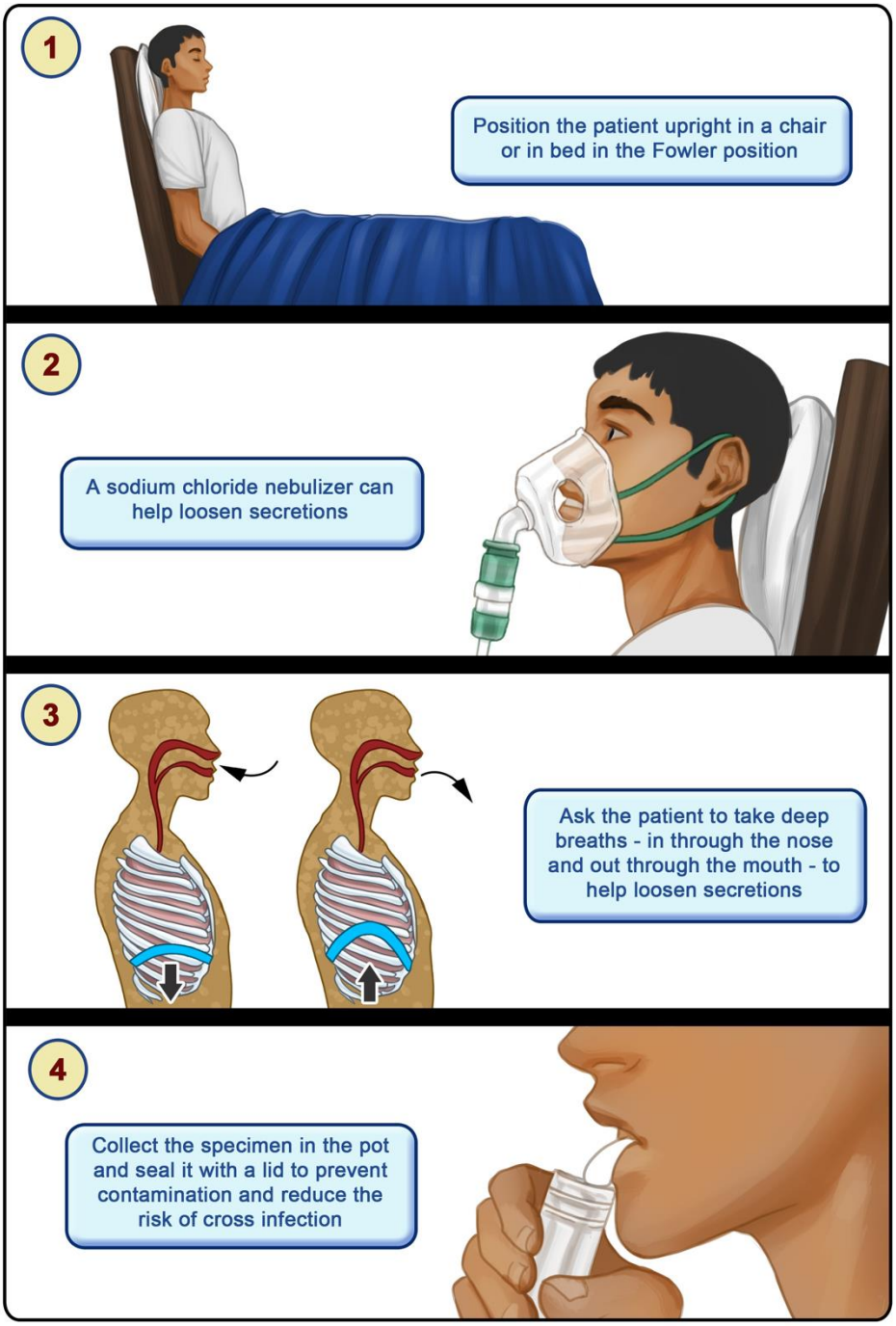


Figure 11- Procedure for obtaining a sputum specimen.

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.

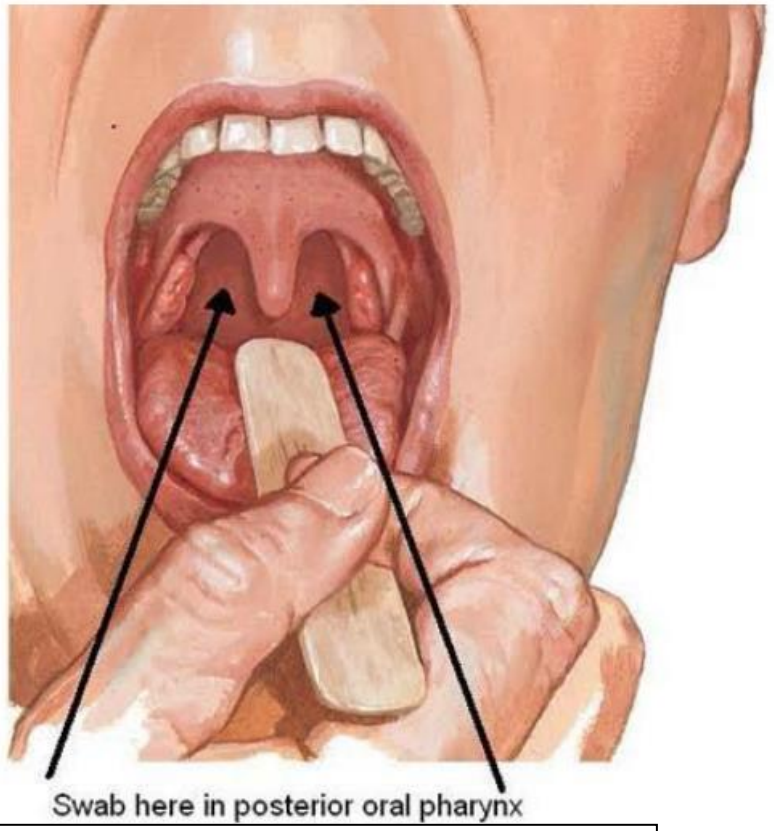


Figure 12- Appropriate throat swab specimen collection technique (APHL).

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

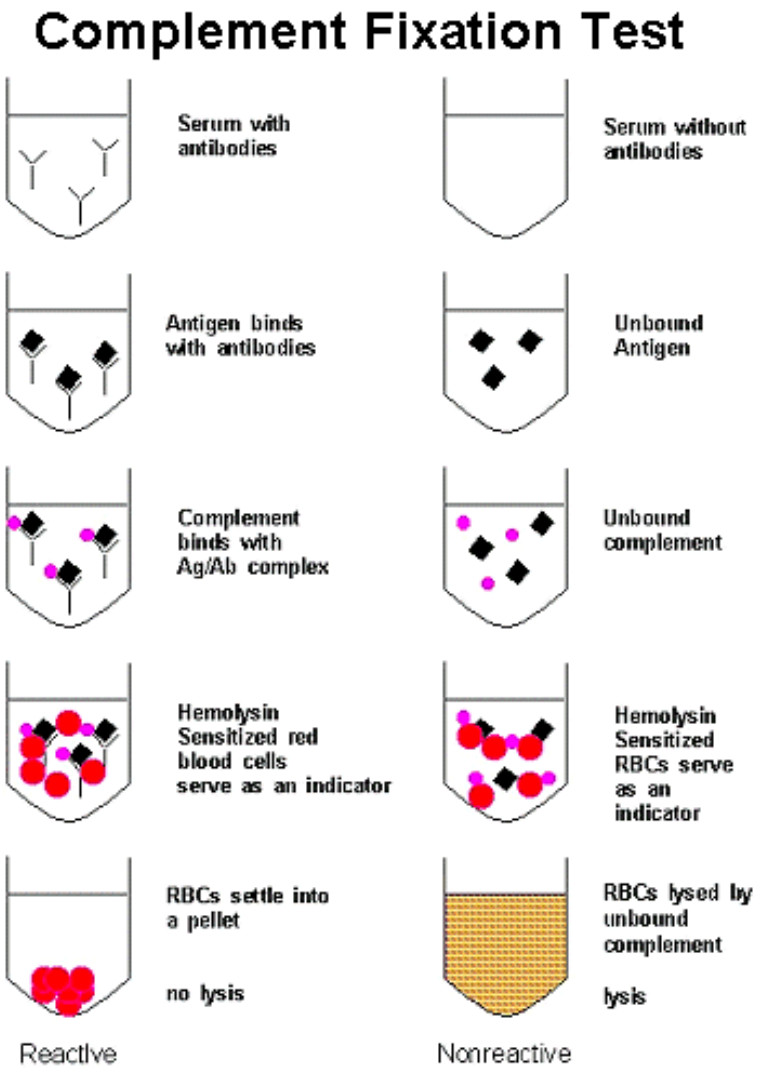


Figure 13- In a positive CFT test, the complement is bound to an antigen-antibody complex and will remain unlysed. These cells will settle to the bottom of the well and form a button. Likewise, in a negative test, there is no antigen-antibody complex so the complement will interact with the sensitized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

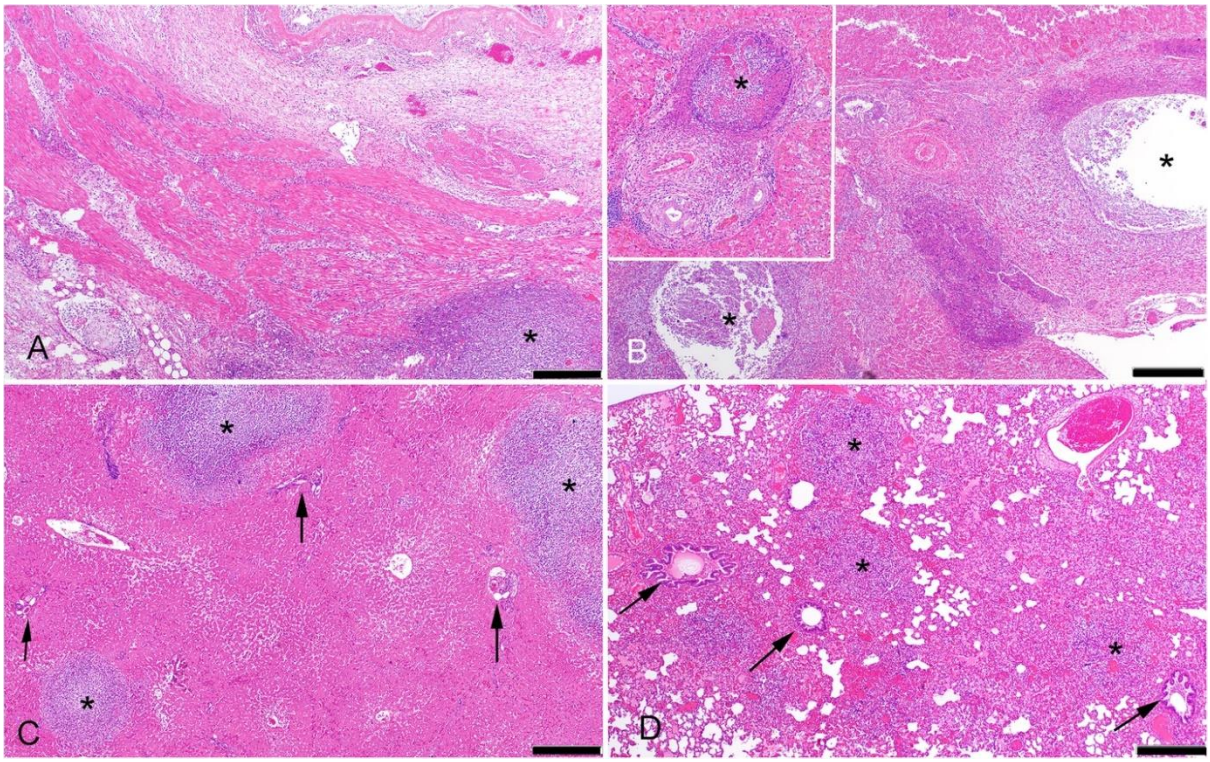


Figure 14- Formalin-fixed lesions taken for histopathology from meerkats with melioidosis showing (a.) The gastric wall with marked congestion, vascular thrombosis and foci of necrotic neutrophils (asterisk). (b.) Entrance of portal vein (asterisks indicate two sections of the lumen) at the hepatic hilus. 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).

- <u>Primary packaging should always consist of the following:</u>
    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).

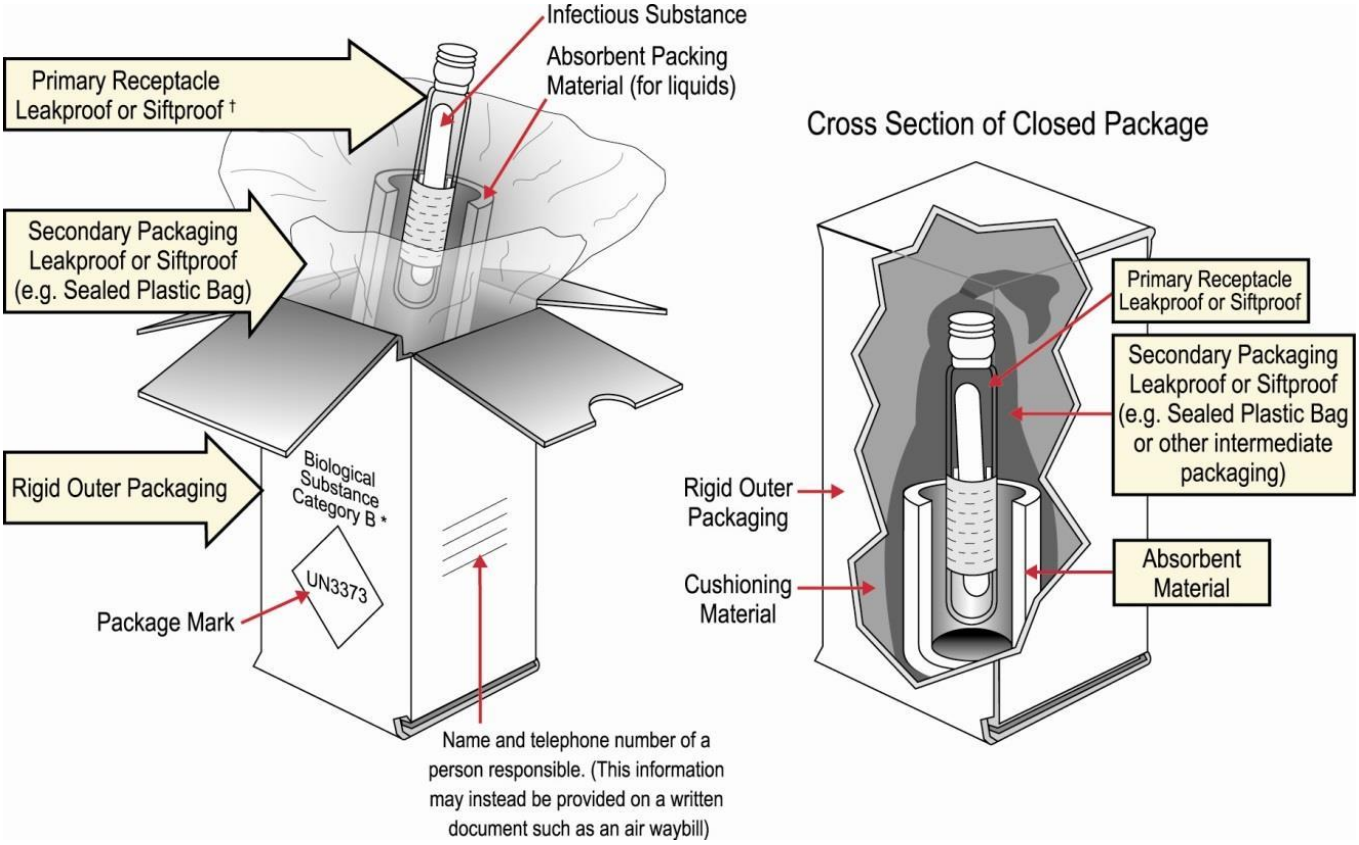


Figure 16- Category B packaging schematic demonstrating triple packaging of diagnostic specimens (CDC 2014).

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.

	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.
	This marking must appear on an overpack when the regulations require the use of packages bearing UN Specification Markings.
	This marking is required when shipping patient/diagnostic specimens.
	This label is required when shipping a substance or specimen on dry ice.
	This label is required when shipping infectious substances. Please note when shipping infectious substances you must use UN certified 6.2 Infectious Substance Packaging.
	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.

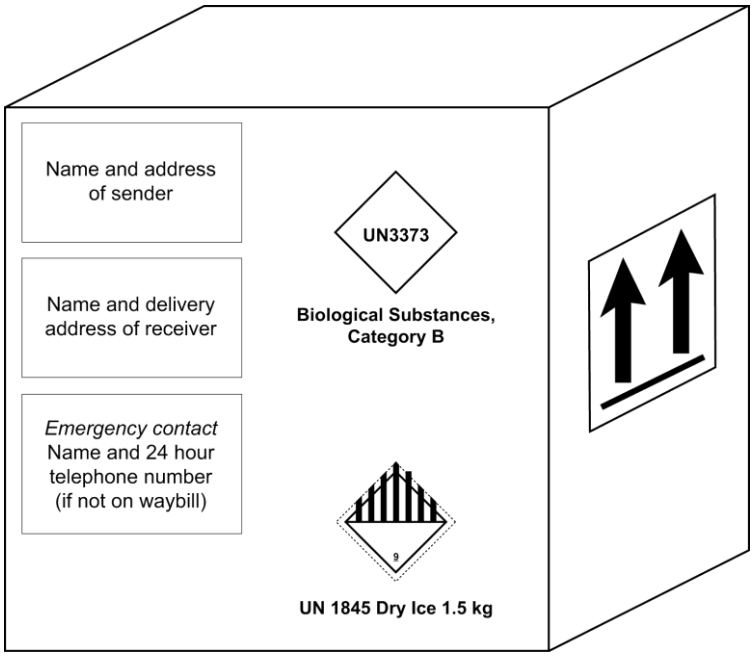


Figure 18- An example of appropriately labelled package for transporting Category B Biological Substances by air with dry ice.

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

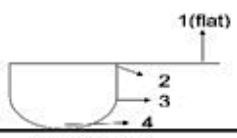
1. Select the location of the sampling site as described above. Ensure you have recorded the GIS location of each sampling site.
    a.) **NOTE- It is important that you obtain the appropriate permissions and clearances from relevant councils/governing bodies/landowners PRIOR to sampling commencement!**
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)			**Study No's** _____ to _____
	m m	y y	
General location parameters			**Area**
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? 			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

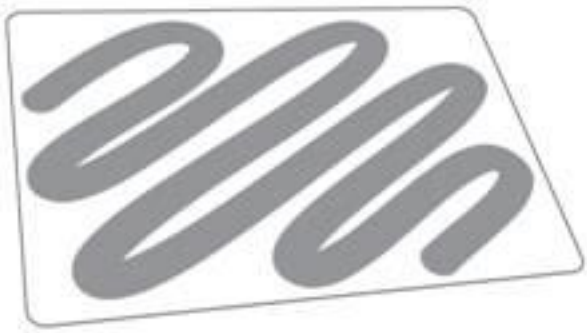
Equipment

- 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).

Chapter 10: *B. pseudomallei* Bacterial Culture Detection

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).

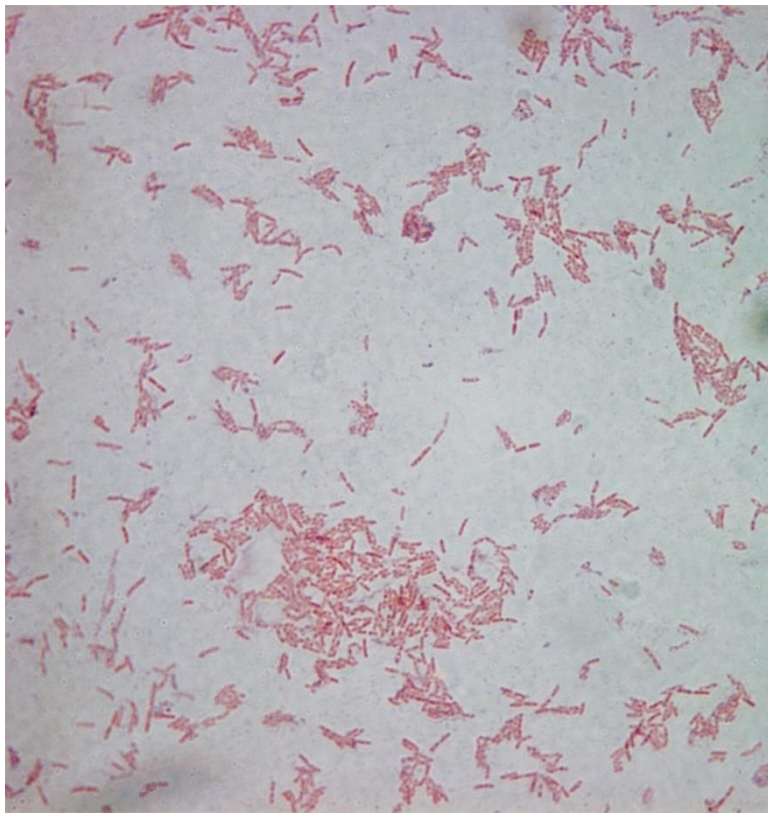


Figure 24- Typical Gram stain of *B. pseudomallei* under the microscope. *B. pseudomallei* is a gram-negative rod, often with a characteristic bipolar staining.

Gram Stain Protocol

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

Chapter 10: *B. pseudomallei* Bacterial Culture Detection

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).

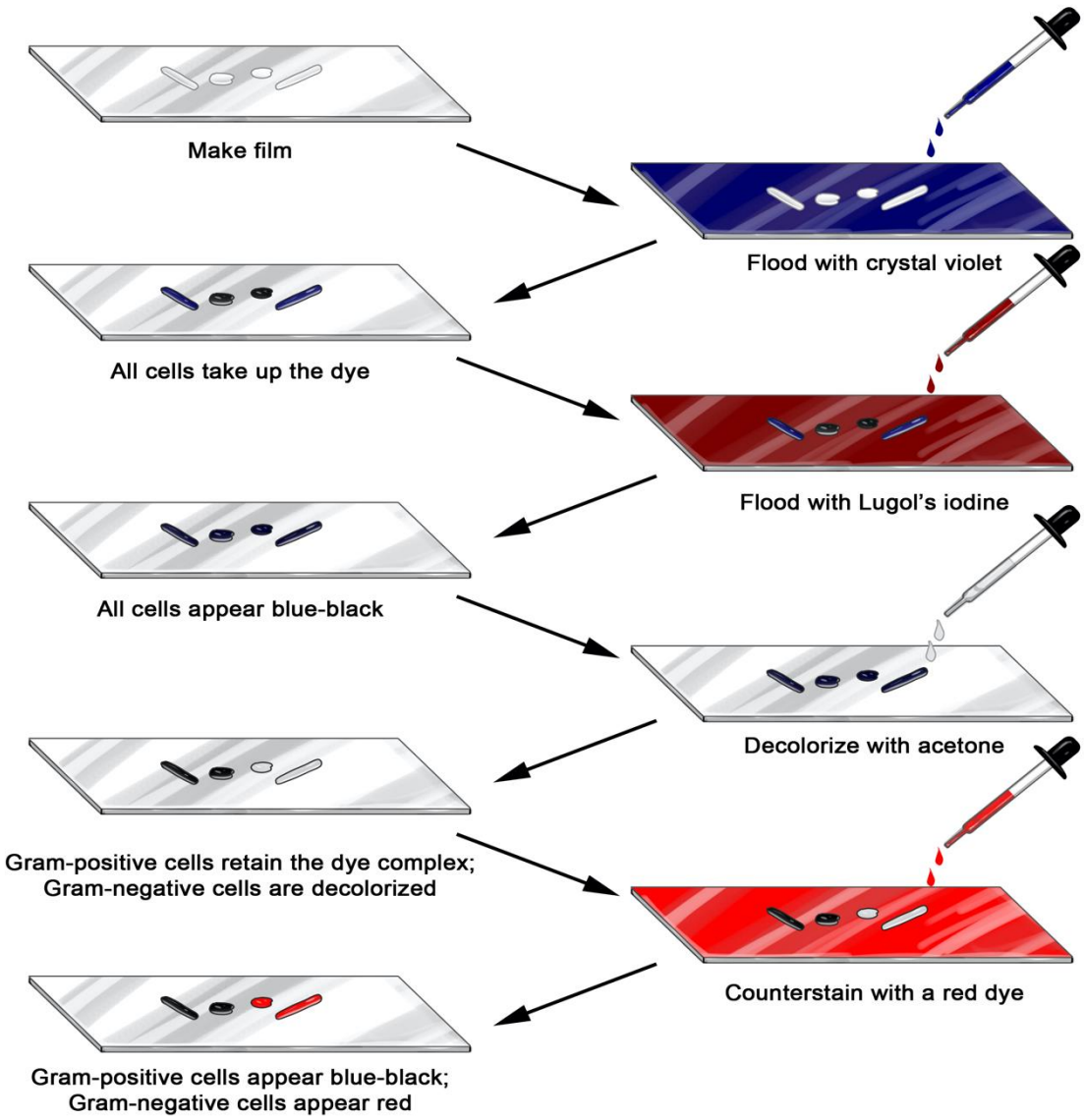


Figure 25- Example of a protocol for performing a Gram Stain in the microbiology lab.

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℃ 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.

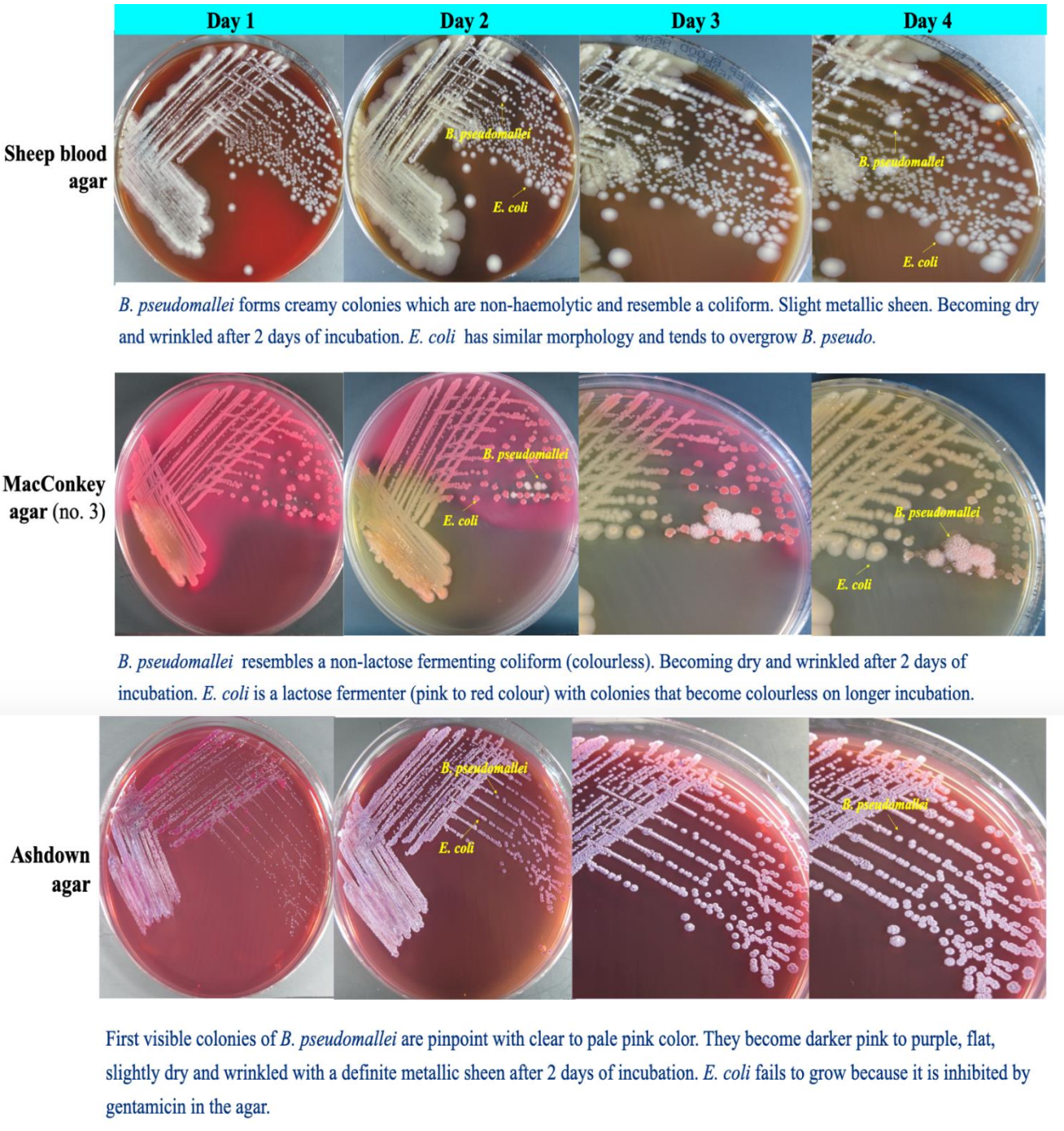


Figure 26- Colony morphology of *Burkholderia pseudomallei* on different culture media (image modified from Melioidosis.Info 2016).

3. Conventional Biochemical Tests

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).

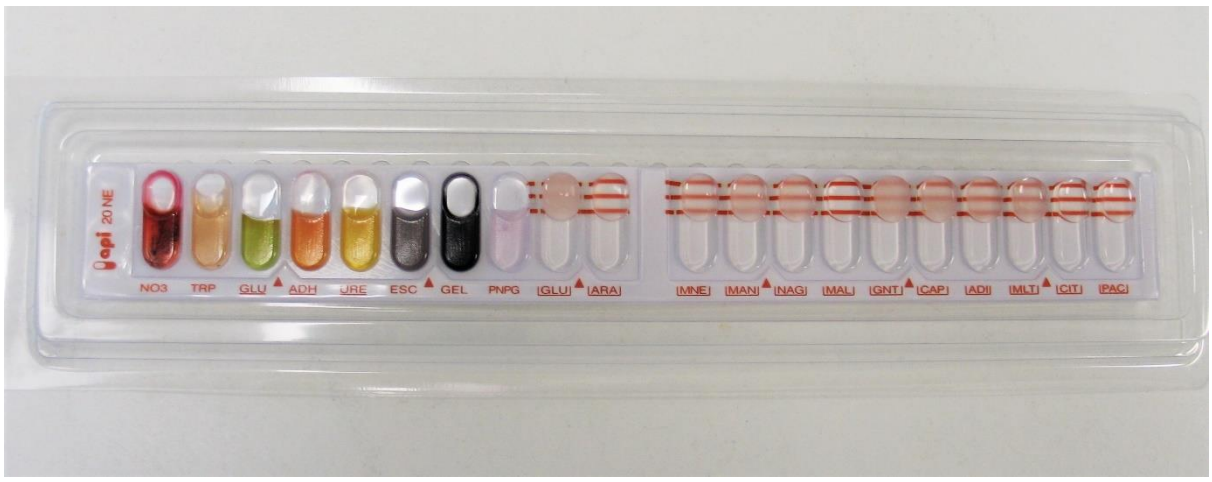


Figure 27- API 20NE commercial detection system showing characteristic profile of *B. pseudomallei.*

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 $1.5x10^8$ 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

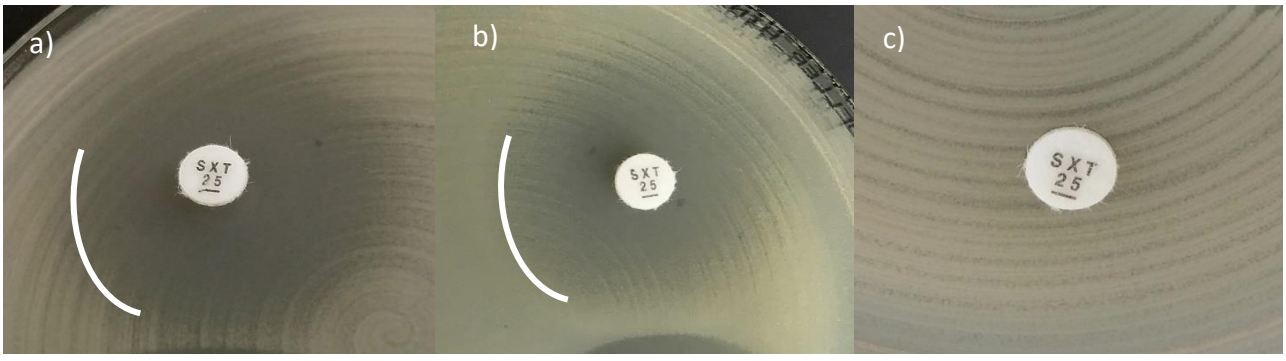


Figure 28- Examples of inhibition zones for *Burkholderia pseudomallei* with trimethoprim-sulfamethoxazole. a-b) An outer zone can be seen. Read the outer zone edge and interpret according to the breakpoints. c) Growth up to the disk and no sign of inhibition zone. Report resistant (EUCAST 2020).

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.

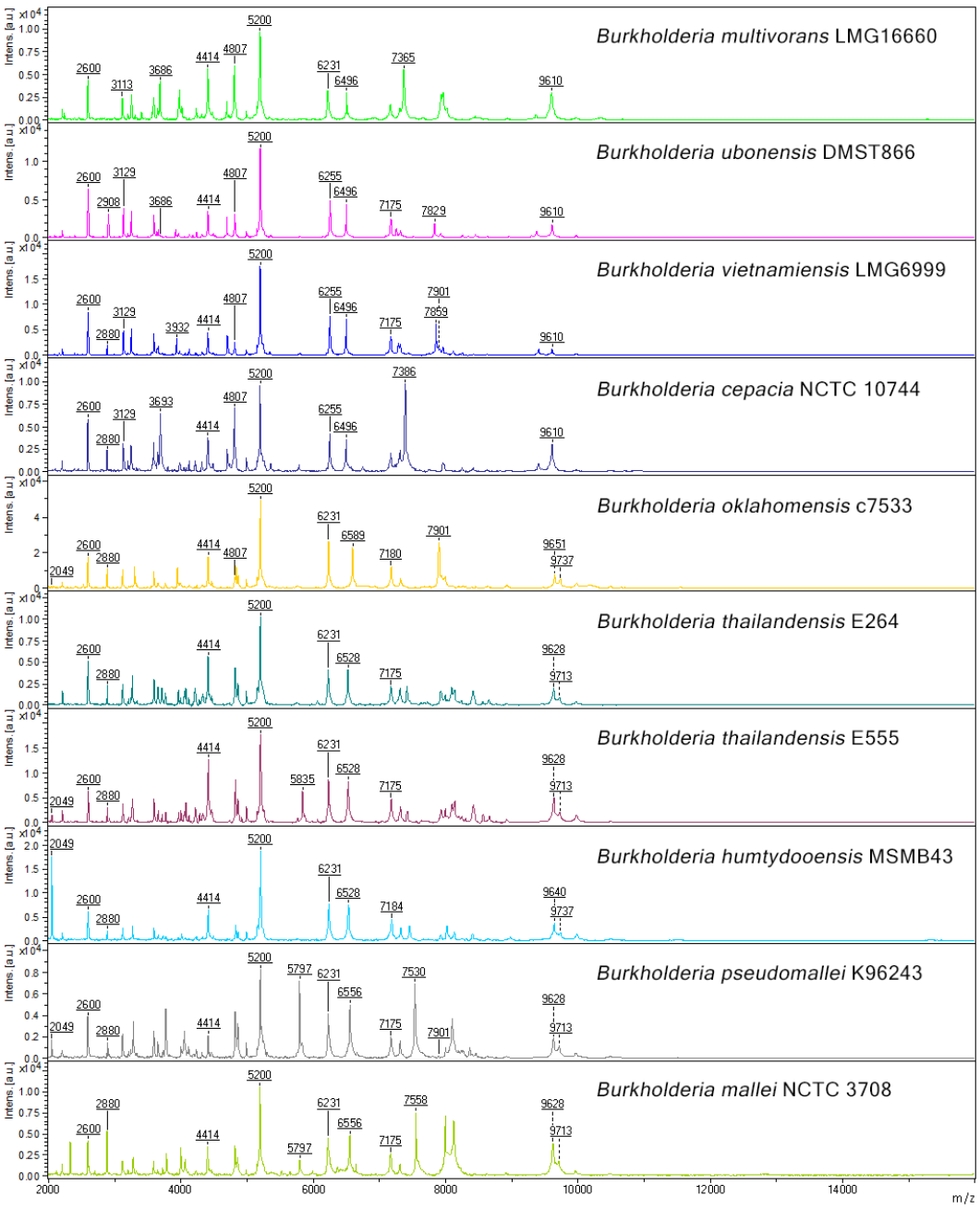


Figure 29- An example of MALDI-TOF MS results of *B. pseudomallei* and eight related *Burkholderia spp.* The vertical axis displays the relative intensities of ions and the horizontal axis displays mass to charge ratio (m/z) or masses of ions (Da) (Suttisunhakul et al. 2017).

Chapter Summary

Chapter 10: *B. pseudomallei* Bacterial Culture Detection

All specimens from patients and animals that meet the case definition for suspected melioidosis infection should be tested for *B. pseudomallei.* The preceding chapter describes the current routine methods of *B. pseudomallei* processing for the accurate diagnosis of melioidosis. Detailed protocols for the most commonly used methods are discussed further in subsequent chapters.

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

Chapter Overview

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

Testing Workflow 1 (APHL 2016):

Microscopy Characteristics

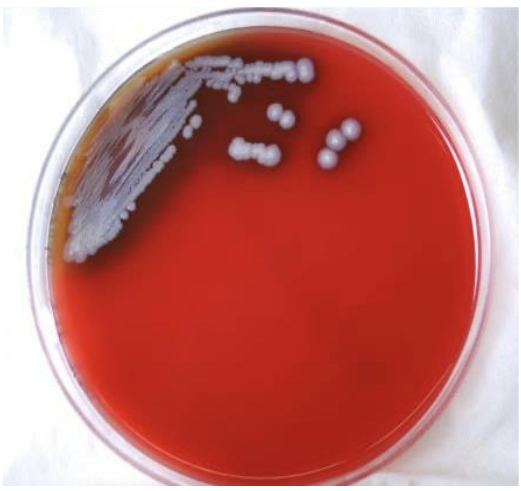
1. Gram Stain
    a) Small gram-negative rods (2-5 x 0.4-0.8 µm)
    b) Organized in long, parallel bundles (smooth form) or irregularly (rough form)
    c) Bipolar staining (not seen in mucoid strains)
2. Colony Morphology and Growth
    a) On Sheep Blood agar (BAP) and Chocolate agar (CHOC):
     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

c) Growth at 42°C

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

24 HOURS ON BAP

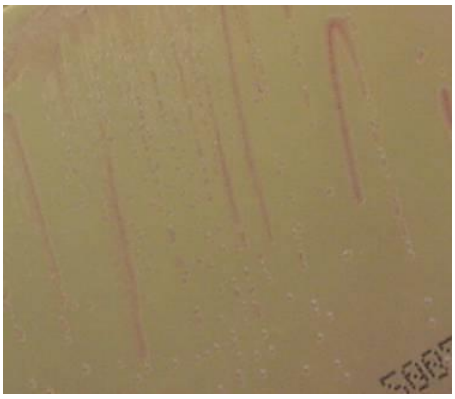
24 HOURS ON CHOC



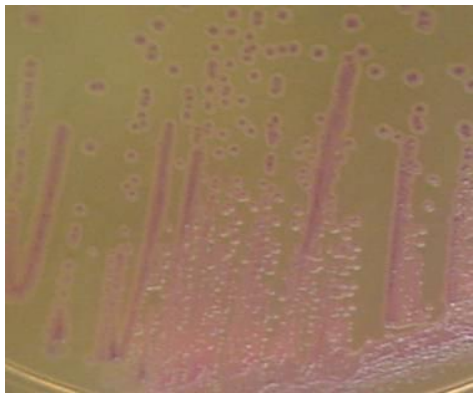
72 HOURS ON BAP

72 HOURS ON CHOC

24 HOURS ON MAC



48 HOURS ON MAC



3. Biochemical Testing

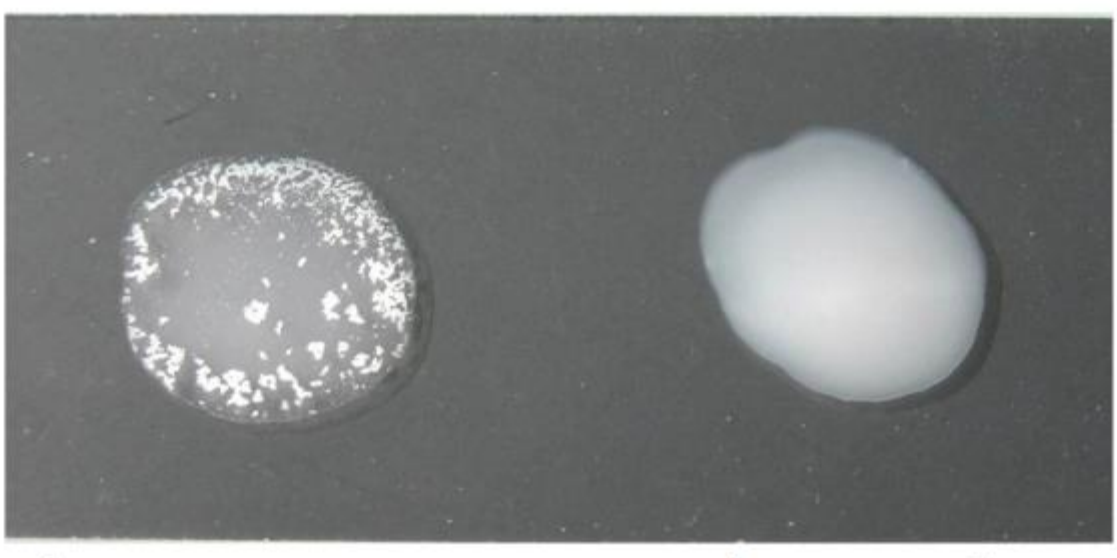
    a. Oxidase- *Burkholderia pseudomallei* is oxidase positive (+).

4. 3-Disk Diffusion Antimicrobial Susceptibly Testing

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

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

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



If you see the following characteristics:

• Gram-negative coccobacilli that may demonstrate bipolar staining

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

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

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

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

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

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

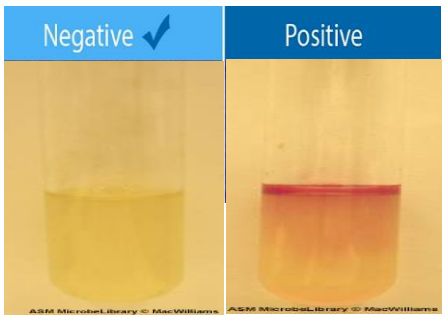
d. On Ashdown agar, colonies are typically pinpoint in size at 24 h and become purple, flat, and wrinkled at 48 h.

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

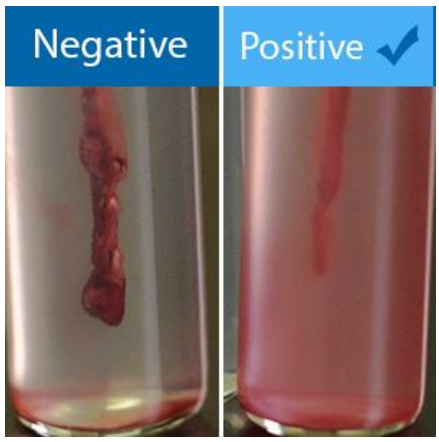
f. Growth at 42°C.

3. Biochemical Tests:

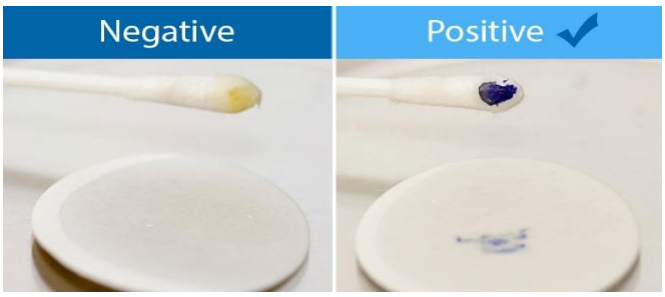
a. Indole- *Burkholderia pseudomallei* is indole negative (-).



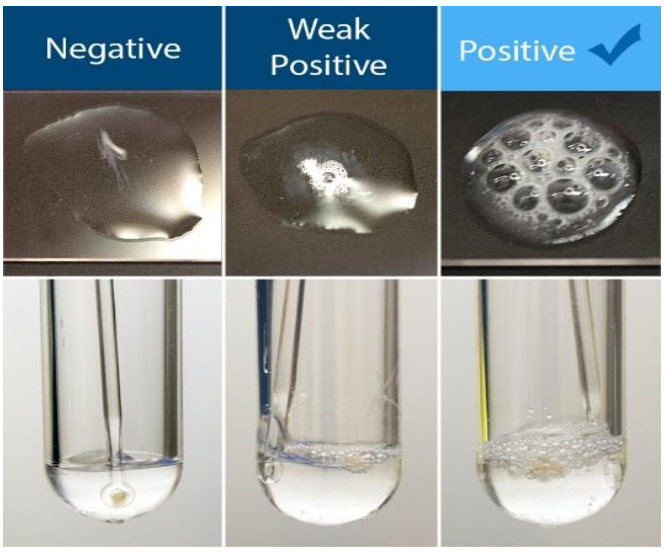
b. Motility- Use Motility Test Medium with 2,3,5-triphenyltetrazolium chloride (TTC). *Burkholderia pseudomallei* is motile (+).



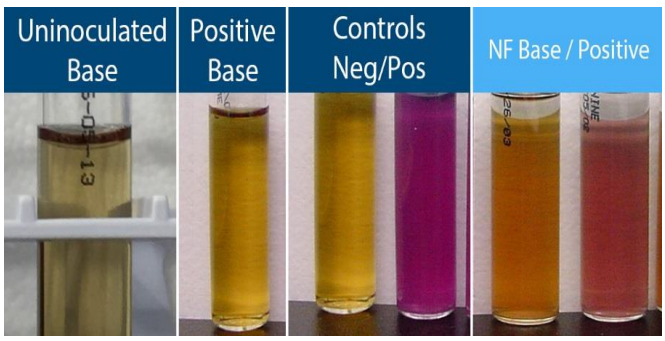
c. Oxidase- *Burkholderia pseudomallei* is oxidase positive (+).



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



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



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

5. Polymerase Chain Reaction (PCR)

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

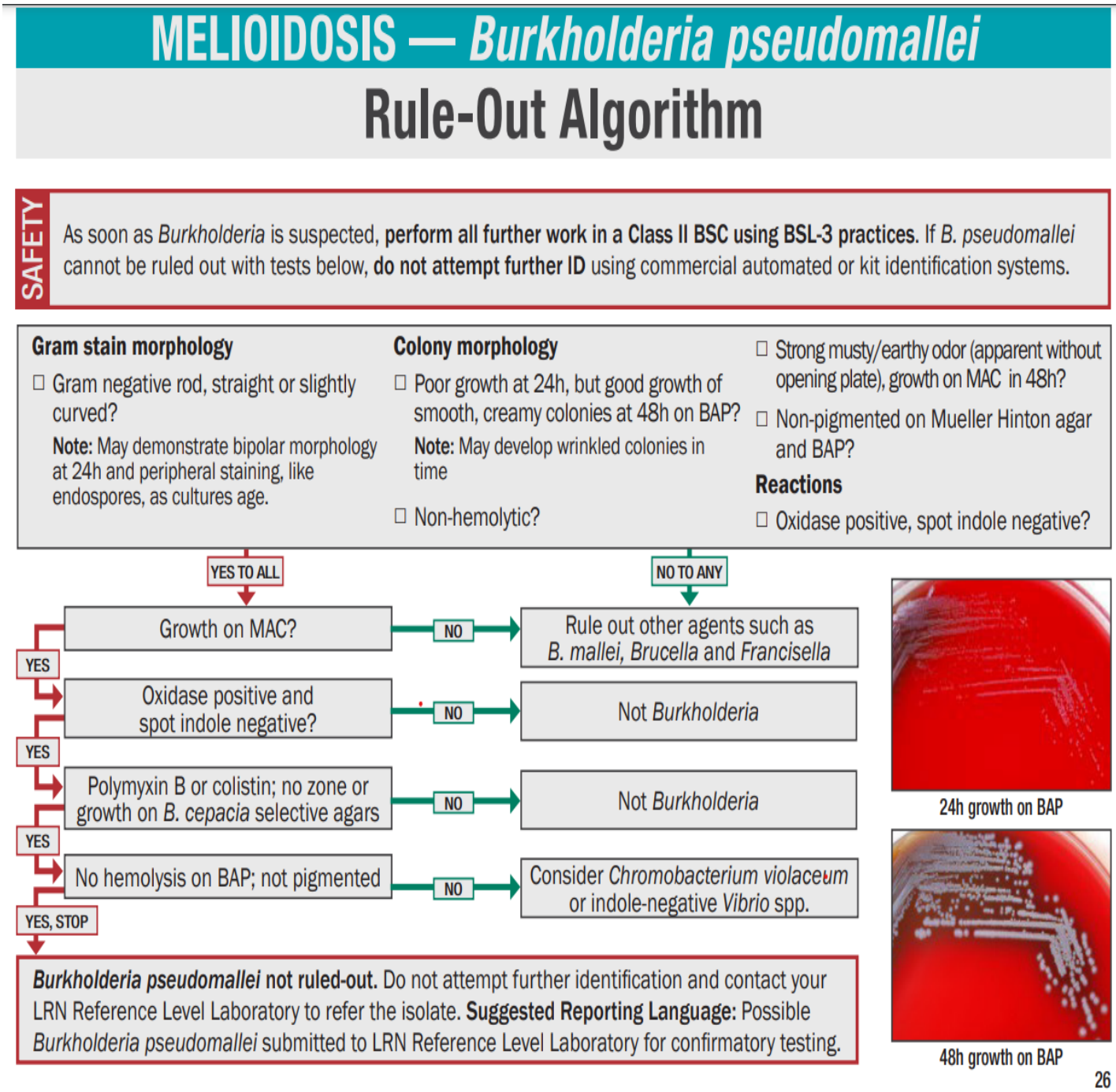


Figure 30- *B. pseudomallei* rule-out testing algorithm (APHL 2017).

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

Chapter Summary

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

Chapter 10: *B. pseudomallei* Bacterial Culture Detection

Chapter Overview

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

Laboratory Media used for *B. pseudomallei* Culture

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

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

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

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

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

Media Preparation

Ashdown's Selective Agar Plates (grams/liter)

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

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

Procedure

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

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

Ashdown's Selective Broth (grams/liter)

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

Procedure

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

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

Threonine Basal Salt Solution (TBSS-C50)

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

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

Procedure

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

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

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

2. Solution B- Base medium

Procedure

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

3. Solution C- L-Threonine Solution

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

Procedure

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

4. TBSS-C50 Solution Procedure

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

Media Quality Control (Wuthiekanun & Dance 2012)

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

Colony Morphology

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

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

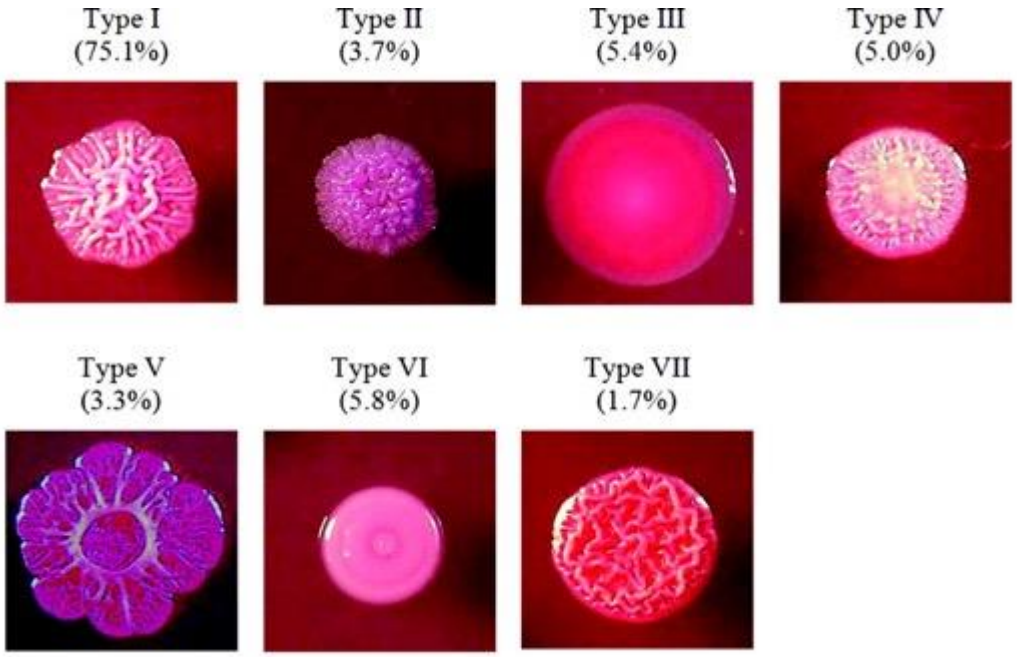


Figure 31- Morphological variants of *B. pseudomallei* colonies grown on Ashdown's agar for 4 days at 37°C in air (Chantratita et al. 2007).

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

Morphology on Different Culture Media

- Blood agar

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

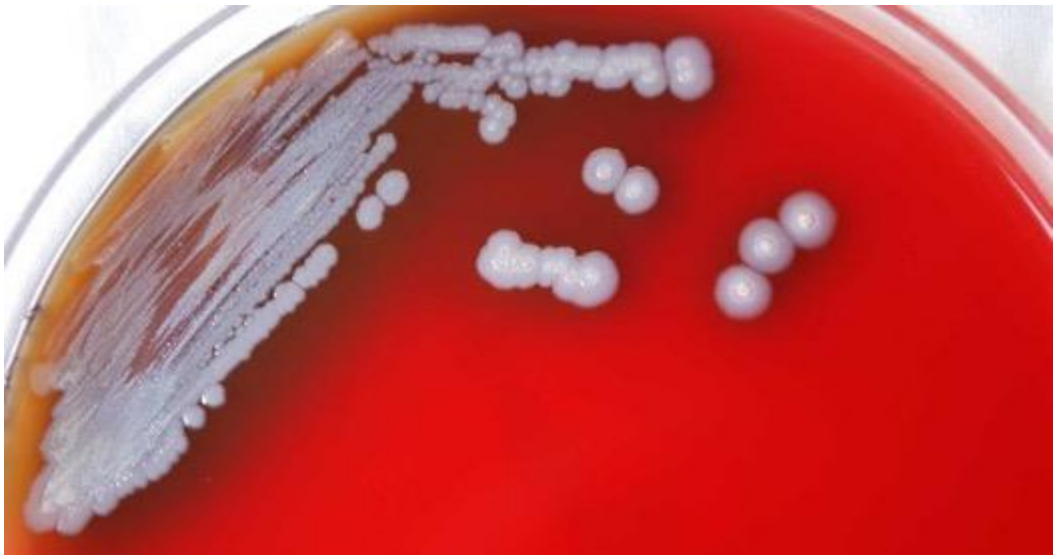


Figure 32- *Burkholderia pseudomallei* bacteria grown on a medium of sheep's blood agar (FDA 2019: CDC/Parker and Marsh 2019).

- MacConkey agar

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

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

- Ashdown's agar

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

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

Clinical Specimen Culturing

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

Clinical Culturing Protocols

Specimens

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

Equipment

- Incubator set at 37°C

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

Media

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

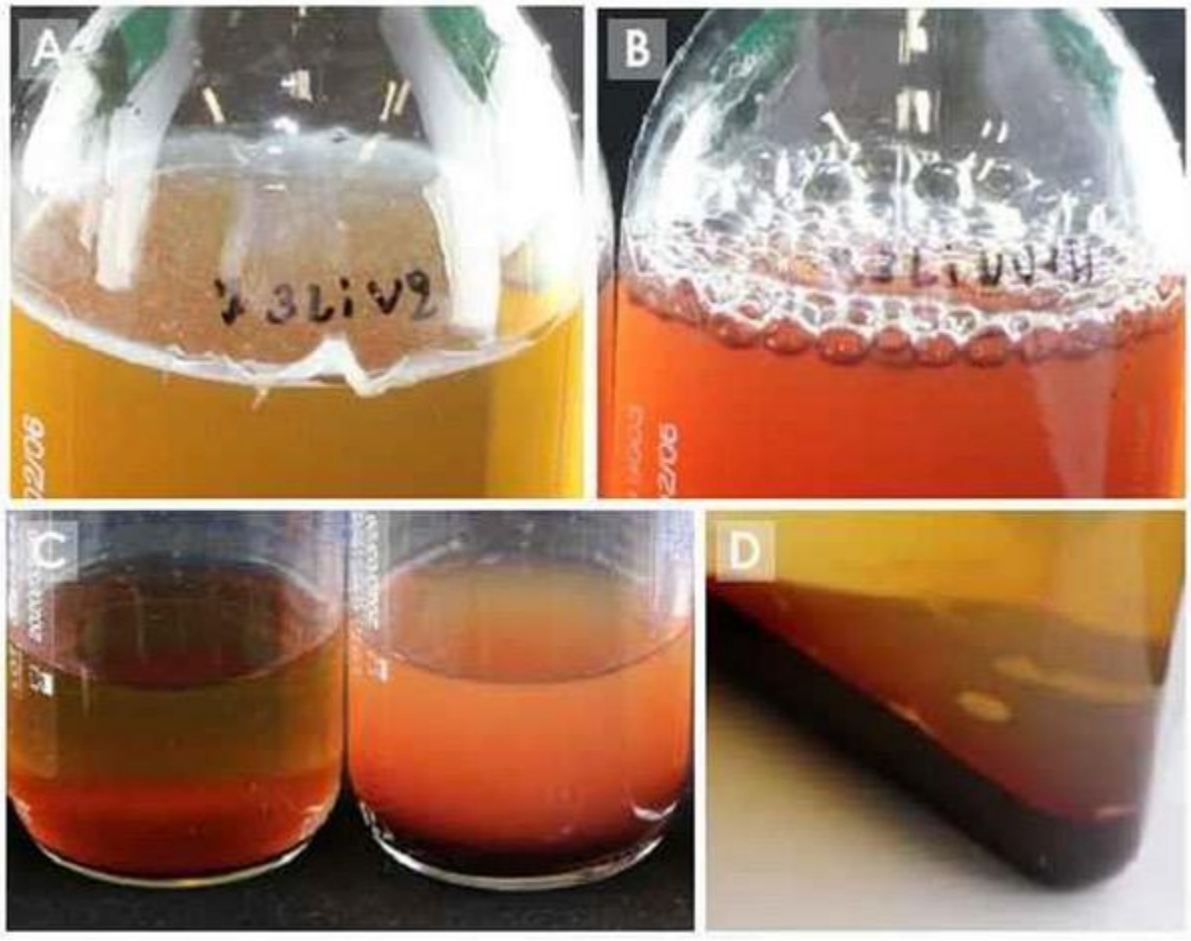


Figure 35- Signs of growth in blood culture bottles. (A) pellicle formation on surface; (B) gas production; (C) turbidity (left bottle: no growth; right bottle: turbidity); (D) puff balls. A and C are more typical observed with *B. pseudomallei* grown in blood culture (Ombelet et al. 2019).

B. pseudomallei **Environmental Sample Culturing**

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

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

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

General Safety Considerations

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

Equipment

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

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

Media

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

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

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

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

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

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

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

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

OR

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

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

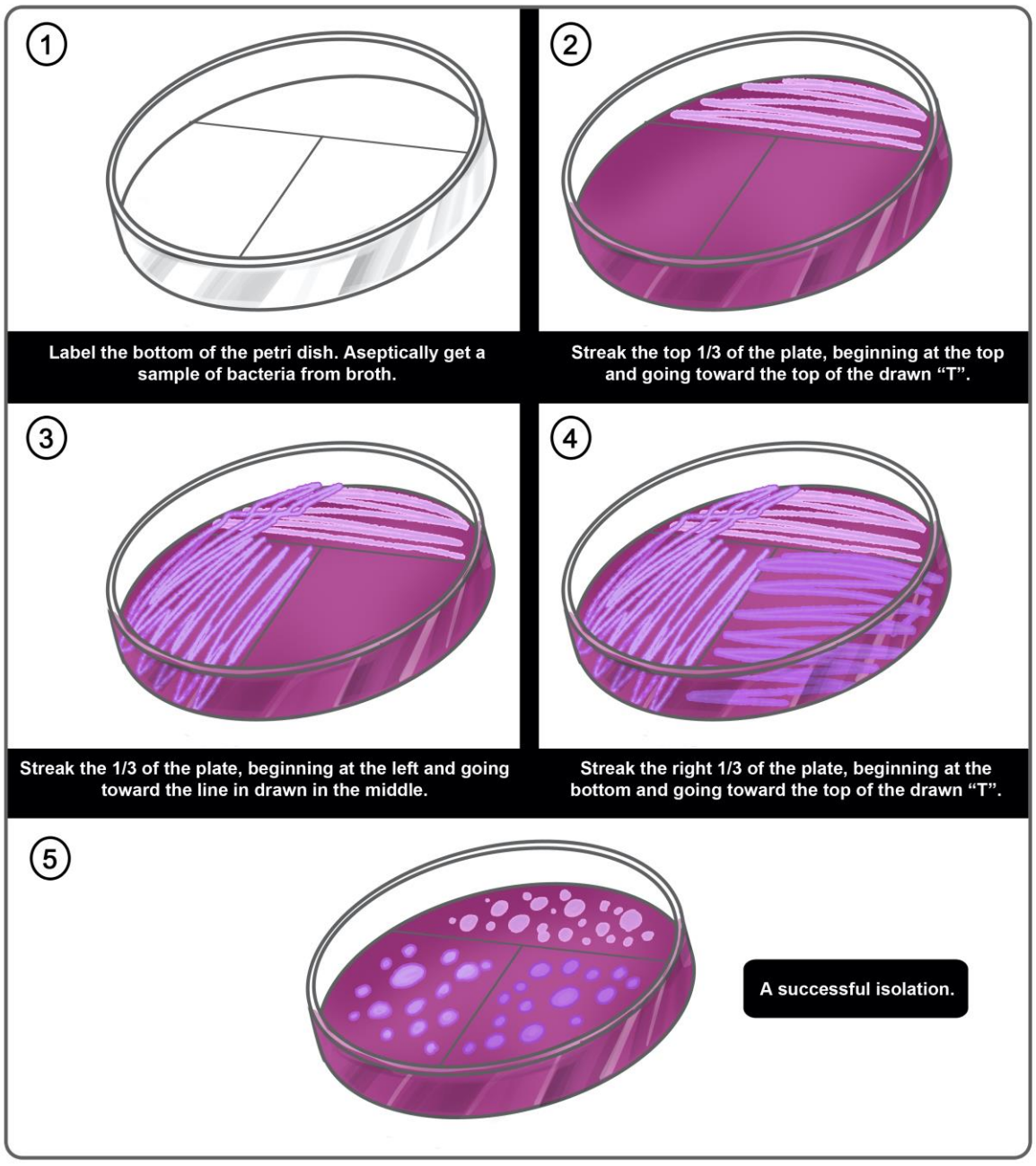


Figure 36- Subculture streaking technique for suspected *B. pseudomallei* colonies to Ashdown's agar to achieve single colonies.

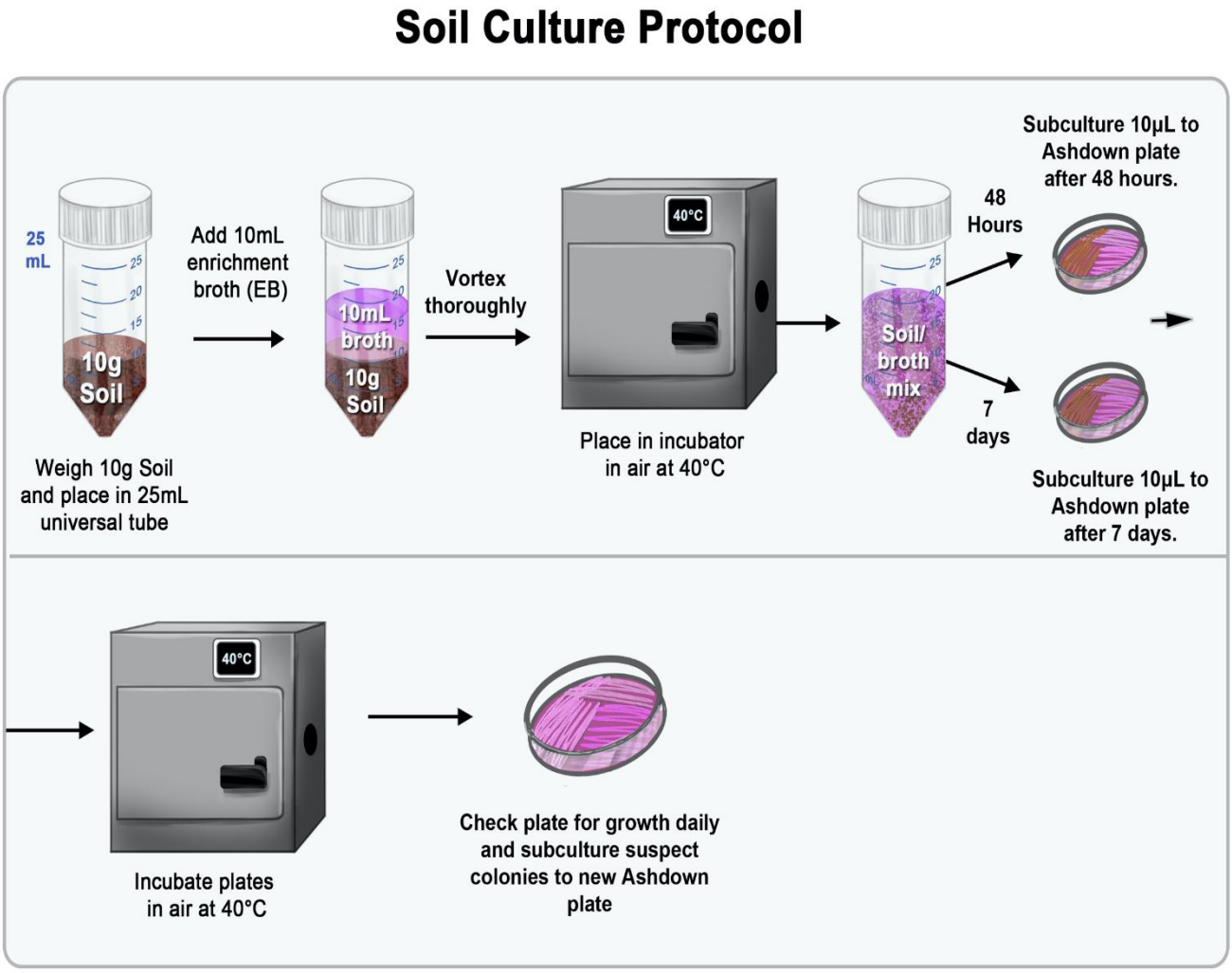


Figure 37- Diagram demonstrating the steps involved in culturing *B. pseudomallei* from soil specimens.

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

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

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

B. pseudomallei Culture Detection from Water Samples

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

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

Water Sample Filtration and Water Culture Protocol

Equipment (Additional to List Above)

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

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

Procedure (Figure 39)

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

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

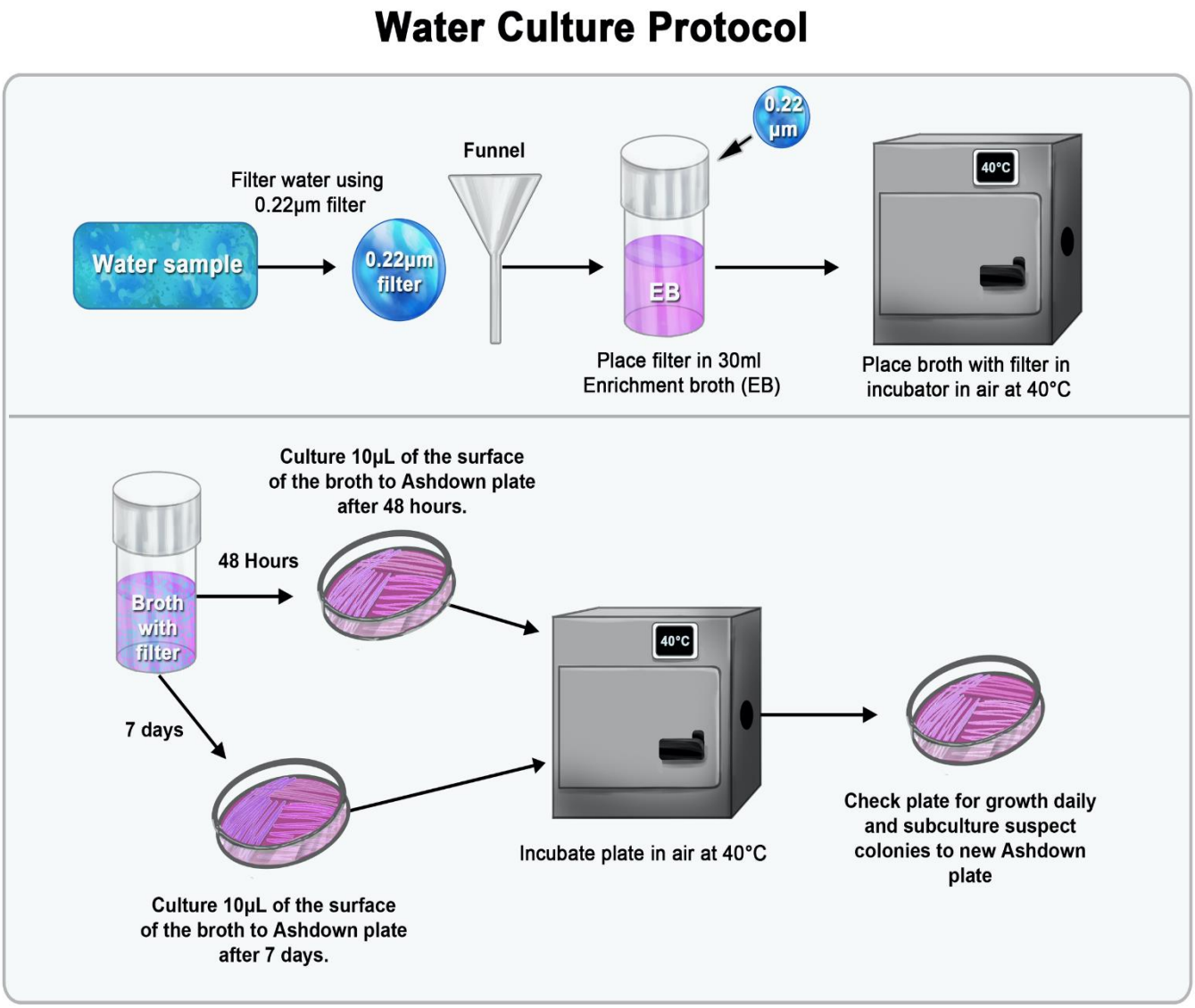


Figure 39- Diagram demonstrating the steps involved in culturing *B. pseudomallei* from water specimens.

B. pseudomallei Culture Detection from Air Samples

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

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

Air Sample Culturing Protocol

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

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

Identification of *Burkholderia pseudomallei*-Suspected Colonies

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

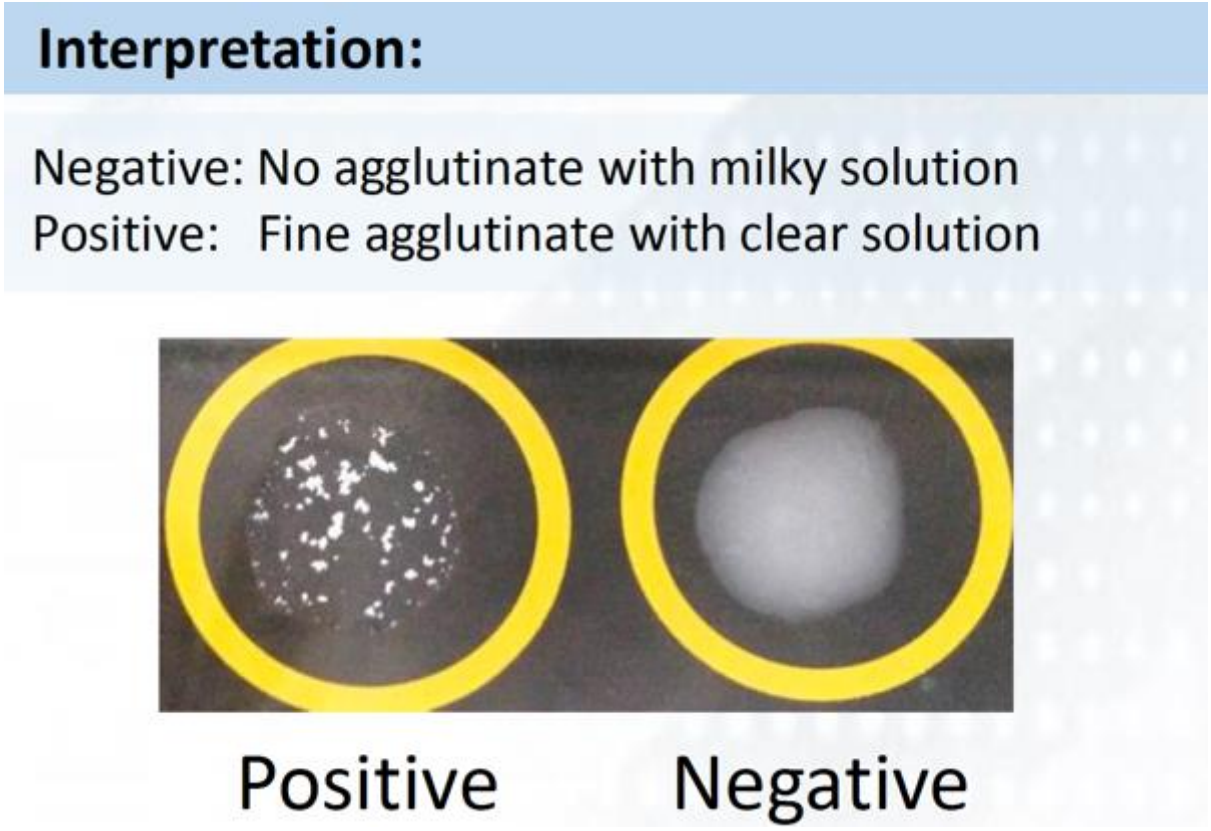


Figure 40- Interpretation of latex agglutination assay for suspected *B. pseudomallei* colonies (Melioidosis.Info 2016).

Chapter Summary

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

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

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

Chapter Overview

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

Antibody Serology Detection

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

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

Common Serological Assays for Melioidosis

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

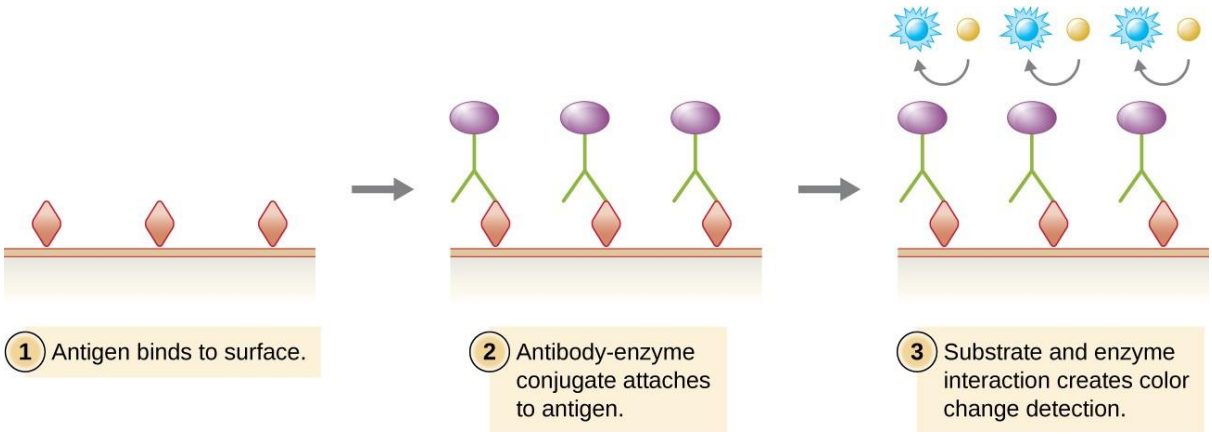


Figure 41- Direct ELISA, such as the Hcp1 ELISA, use an enzyme-antibody conjugate to deliver a detectable substrate to the site of an antigen. The substrate may be colorless and converted into a

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

Indirect Haemagglutination Assay

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

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

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

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

IHA Protocol

Materials

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

Controls

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

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

Determination of optimal *B. pseudomallei* antigen concentrations

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

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

Procedure (Melioidosis.Info 2011)

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

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

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

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

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

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

3. Preparation of 10% sensitized sheep red cells

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

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

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

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

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

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

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

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

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

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

6. Interpretation of results

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

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

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

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

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

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

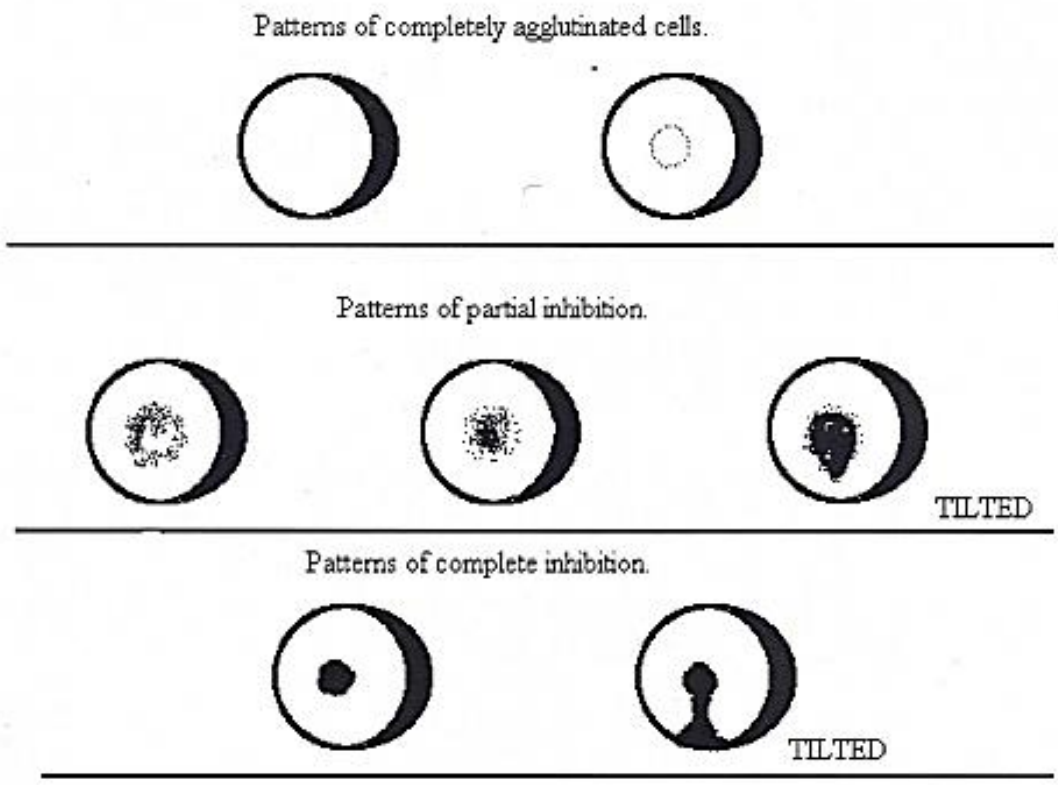


Figure 43- Examples of settled cell patterns showing full and partial agglutination, as well as a negative IHA result (complete inhibition) (APHA).

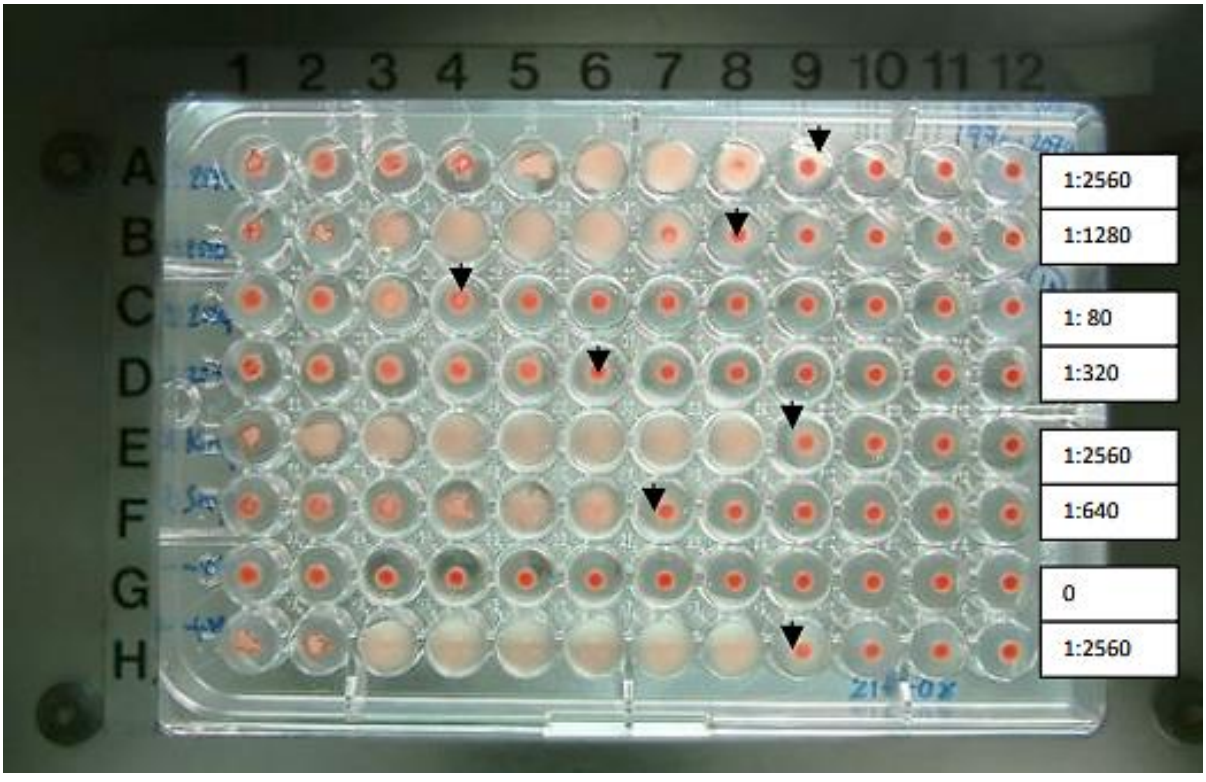


Figure 44- Example of an IHA plate setup and interpretation of agglutination results from different patient samples. Row G and column 12 both serve as negative controls, while Row H functions as the positive control (Melioidosis.Info 2011).

Alsever's solution

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

Alsever's solution Ingredients:

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

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

Direct Antigen Detection Methods

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

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

1. Lateral Flow Immunoassay

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

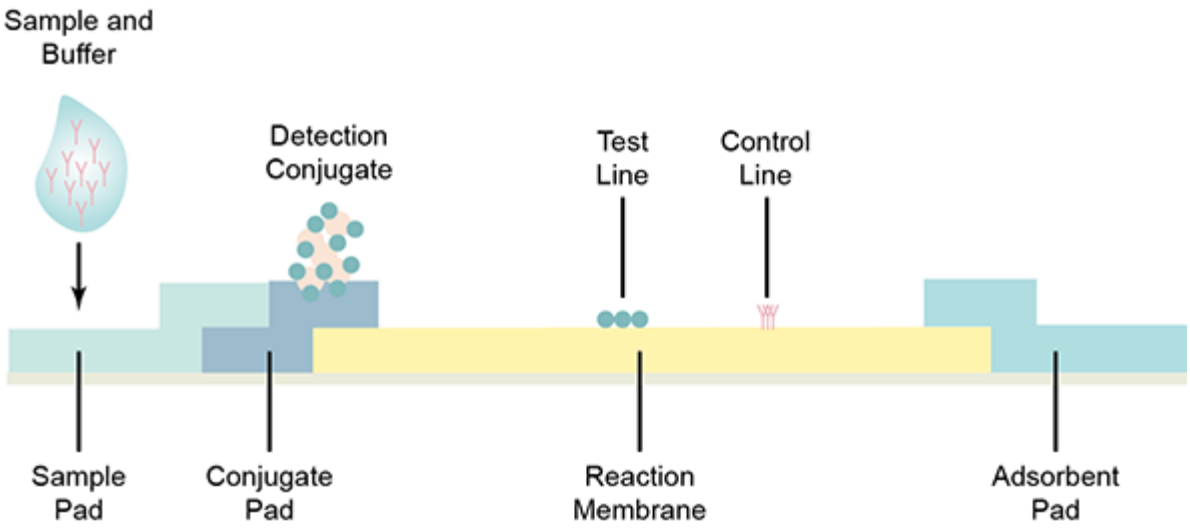


Figure 45- The basic structure of lateral flow assay. The sample pad is where sample is dropped; The conjugate pad which transfers the detector reagent and test sample onto the membrane; The reaction membrane contains the test line and control line demonstrating the antigen-antibody interaction; The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

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

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

Active Melioidosis Detect ™ LFI Protocol

Materials and equipment

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

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

Blood Specimens

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

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

Sputum Specimens

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

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

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

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

3. Results should be read after 15 minutes.

Pus Specimens

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

Urine Specimens

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

Culture specimens

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

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

From bacterial isolates:

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

Results Interpretation

Positive result

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

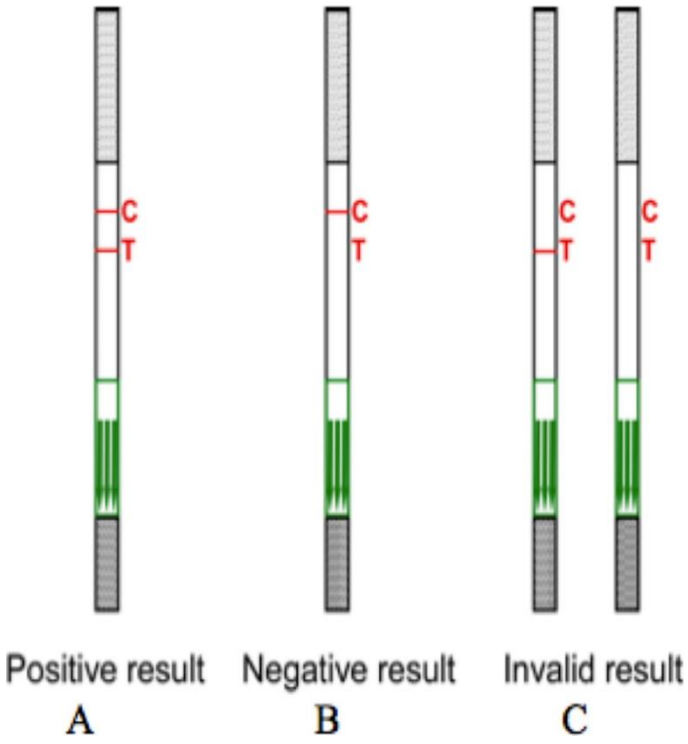


Figure 46- Interpretation according to the manufacturer instructions (InBios). C, control line; T, test line.

Negative result

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

An Invalid Result

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

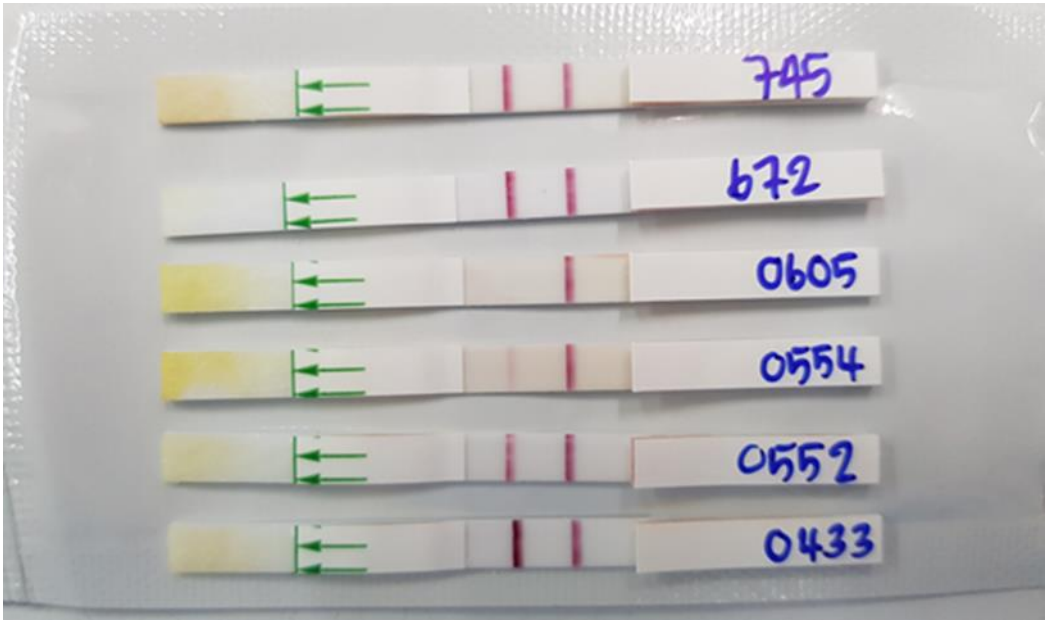


Figure 47- Positive, weak positive and negative LFI results. Test strips 745, 672, 0552, and 0433 are positive, test 0554 is a weak positive result and test 0605 shows a negative test result (Wongsuvan et al. 2018). Note: An updated cassette version of the AMD LFI is also now available through InBios.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

2. Latex Agglutination

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

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

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

Latex Agglutination Protocol

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

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

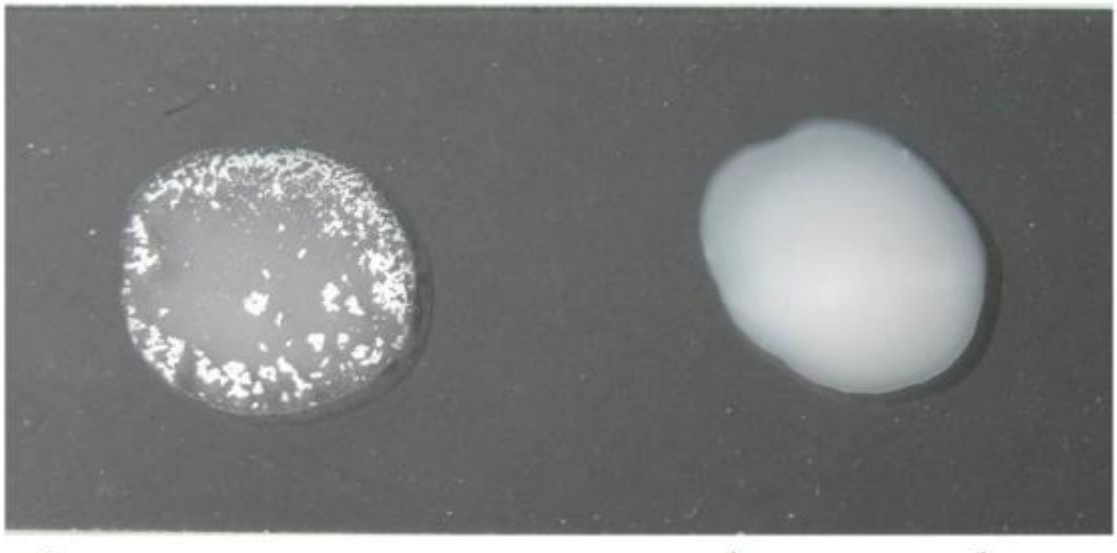


Figure 48- Positive latex agglutination test (left) shown after 10 µL of latex reagent is mixed with 10 µL of positive control reagent. A negative latex agglutination test is shown on the right after mixing 10 µL latex reagent with 10 µL negative control reagent (Melioidosis.Info 2012).

3. Immunofluorescence Assay (IFA)

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

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

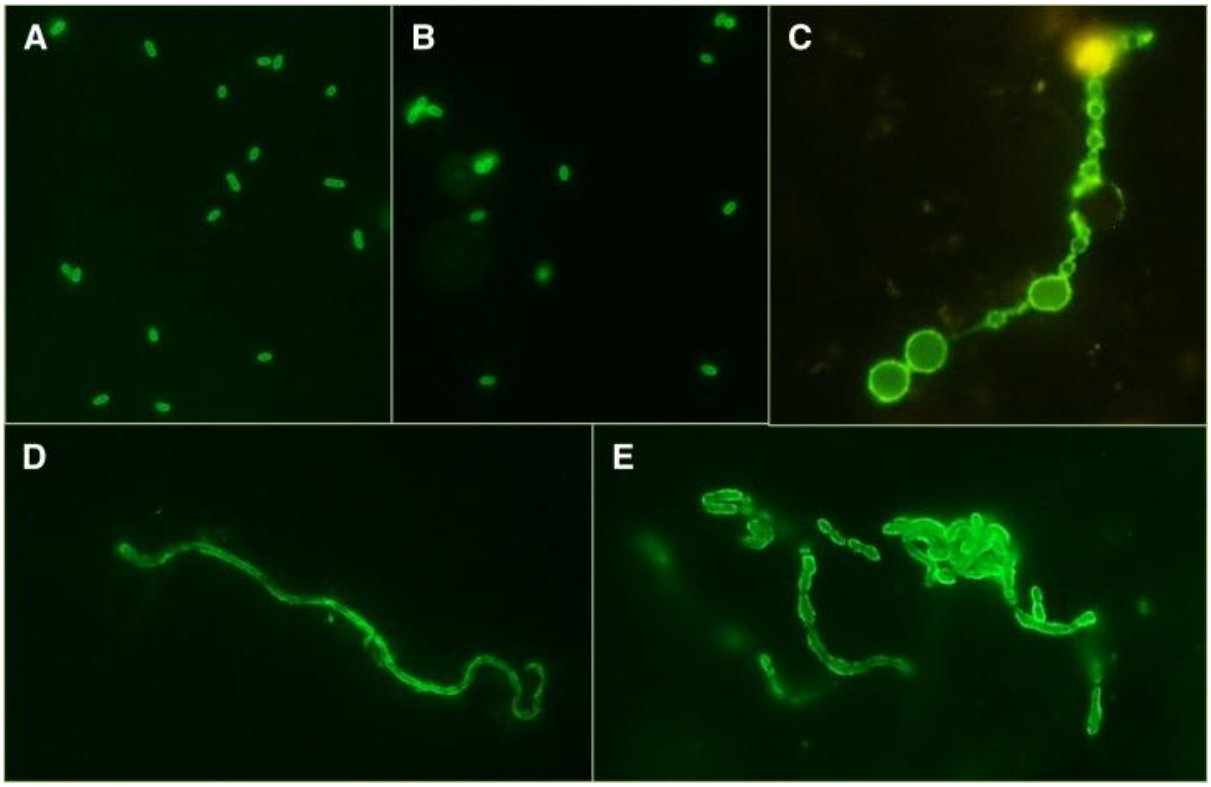


Figure 49- Fluorescent microscopy of *Burkholderia pseudomallei* stained with monoclonal antibody-IFA reagent. Bacterial cultures were grown on Columbia agar (A) LB broth (B), or from clinical samples (urine [C], pus [D], or sputum [E]) from patients with melioidosis (Tandhavanant et al. 2013).

Enzyme-Linked Immunosorbent Assays (ELISAs)

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

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

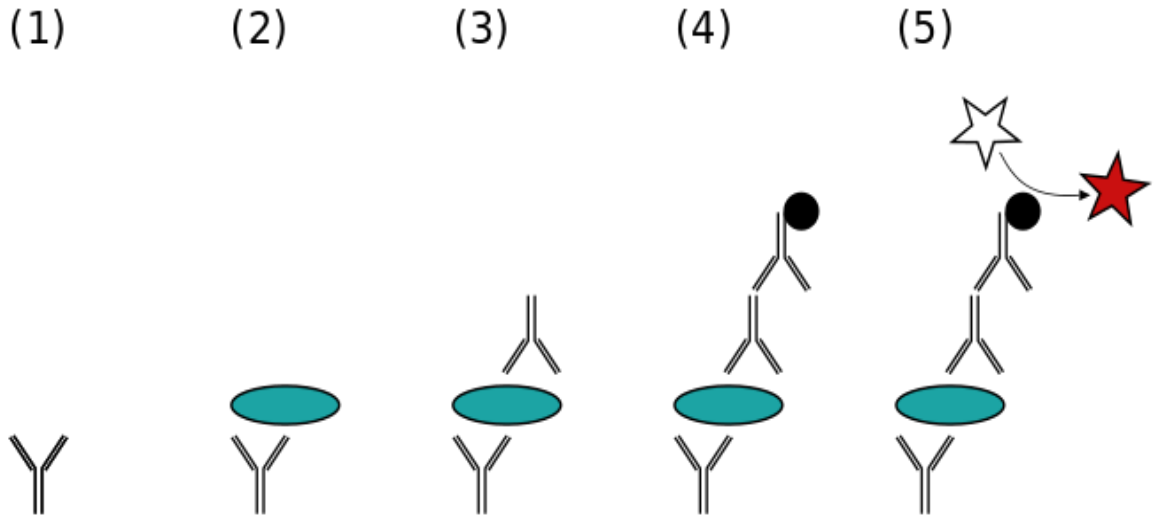


Figure 50- Example of a sandwich ELISA (1) the plate is coated with a capture antibody; (2) the sample is added and will bind to the capture antibody if the antigen is present; (3) a detection antibody is then added and will bind to the antigen; (4) a secondary antibody that is linked to an enzyme is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

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

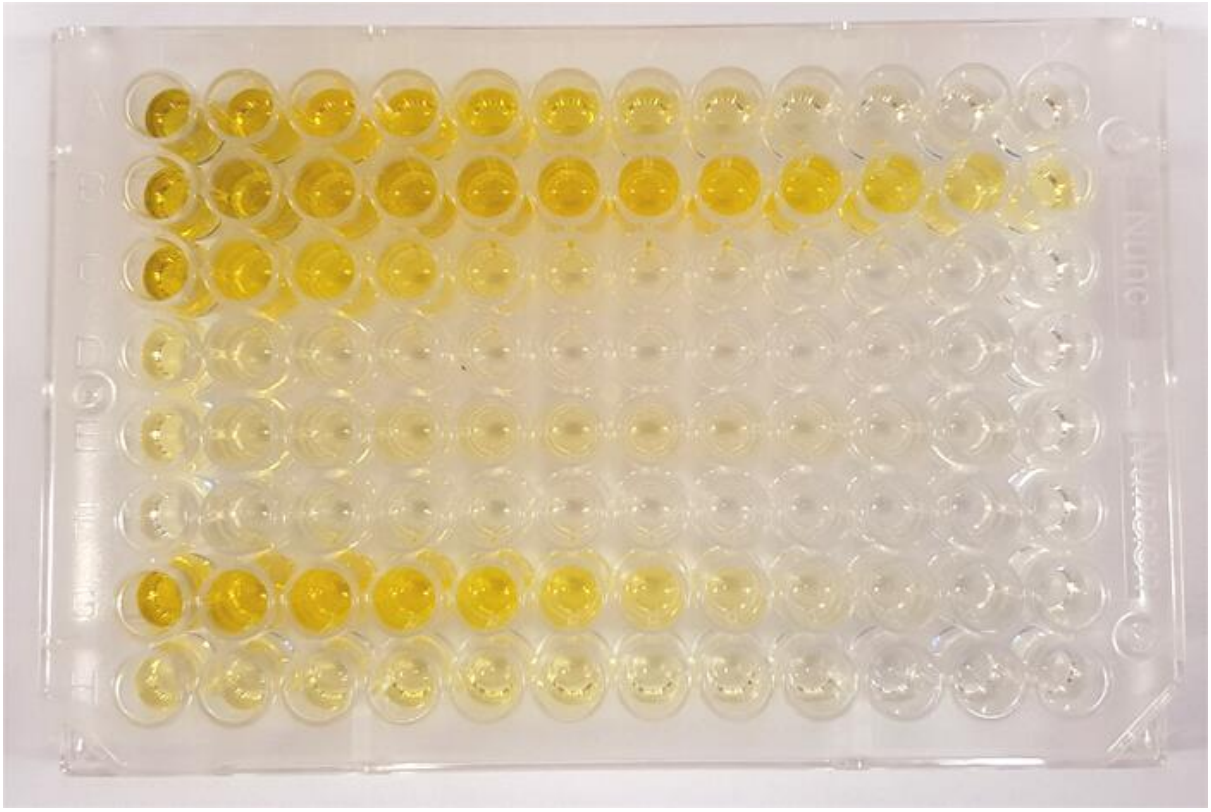


Figure 51- The yellow color indicates the target antigen is present. The higher the degree of color, the greater the concentration of the target antigen.

Chapter Summary

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

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

Chapter 12: Polymerase Chain Reaction (PCR) Overview

Chapter Overview

Polymerase chain reaction (PCR) is a laboratory technique used to amplify strands of DNA or RNA (ThermoFisher 2012). It is relatively simple to use and generates results rapidly. The technique is also highly sensitive and specific with results in just a few hours. PCR is increasingly used to detect bacterial DNA in clinical and environmental samples. In some melioidosis-endemic regions, PCR is routinely used to confirm or identify suspected *B. pseudomallei* colonies grown on selective agar that are also oxidase-positive, colistin resistant and co-amoxiclav susceptible. It is also commonly used to screen environmental samples and can help to determine which samples to attempt more extensive bacterial culturing.

The following chapter presents an overview of the PCR process, its essential components and briefly describes the main types of PCR used to detect *B. pseudomallei* in clinical and environmental specimens. Good laboratory practice guidelines and PCR laboratory setup are also explained. Additional details about DNA extraction and specific PCR protocols can be found in Chapters 13-15.

Introduction to PCR

Every human, animal, plant, parasite, bacterium and virus contains unique DNA or RNA sequences. Using PCR, these unique sequences can be copied or “amplified”. PCR amplification is achieved by mimicking the replication process that occurs naturally in cells, where DNA is duplicated using the enzyme DNA polymerase and free nucleotides (Figure xxx). Samples are exposed to cycles of heating and cooling (termed “thermal cycling”). During these cycles, DNA primers bind to the target DNA sequence, allowing the DNA polymerase to generate copies of the target sequence in large quantities (ThermoFisher 2012).

PCR is used to generate enough target DNA so that it can be further analyzed, including sequencing, visualization by gel electrophoresis, or cloning into a plasmid for further experiments. PCR is used in many areas of molecular biology, research, forensic science, medical diagnostics and evolutionary ecology. In clinical diagnostic labs, PCR enables rapid and highly specific diagnosis of infectious pathogens (Cai et al. 2014). PCR also allows for the identification of non-culturable or slower-growing microorganisms such as mycobacteria or anaerobic bacteria, discriminates between non-pathogenic and pathogenic strains through the identification of specific genes, and can confirm the purity of an isolated sample (Salis 2009).

Despite this, there are several limitations of PCR for clinical diagnostics that should be noted, including supply and equipment costs and initial staff training expenses. Additionally, previous information about the target sequence is necessary to create the appropriate primers. Here, the precise upstream sequence of the target region on each of the two single-stranded template strands must be known to ensure that the DNA polymerase can bind and add the correct complementary bases to DNA strands during DNA synthesis (Garibyan & Avashia 2013). Another limitation of PCR is that even a small DNA contaminant can be amplified, resulting in confusing or false-positive results. False-negatives due to too low concentration of target DNA can also occur (Podnecky et al. 2013). It is important to recognize that unlike bacterial culture detection, PCR can only detect the presence of DNA, not active growth of an organism.

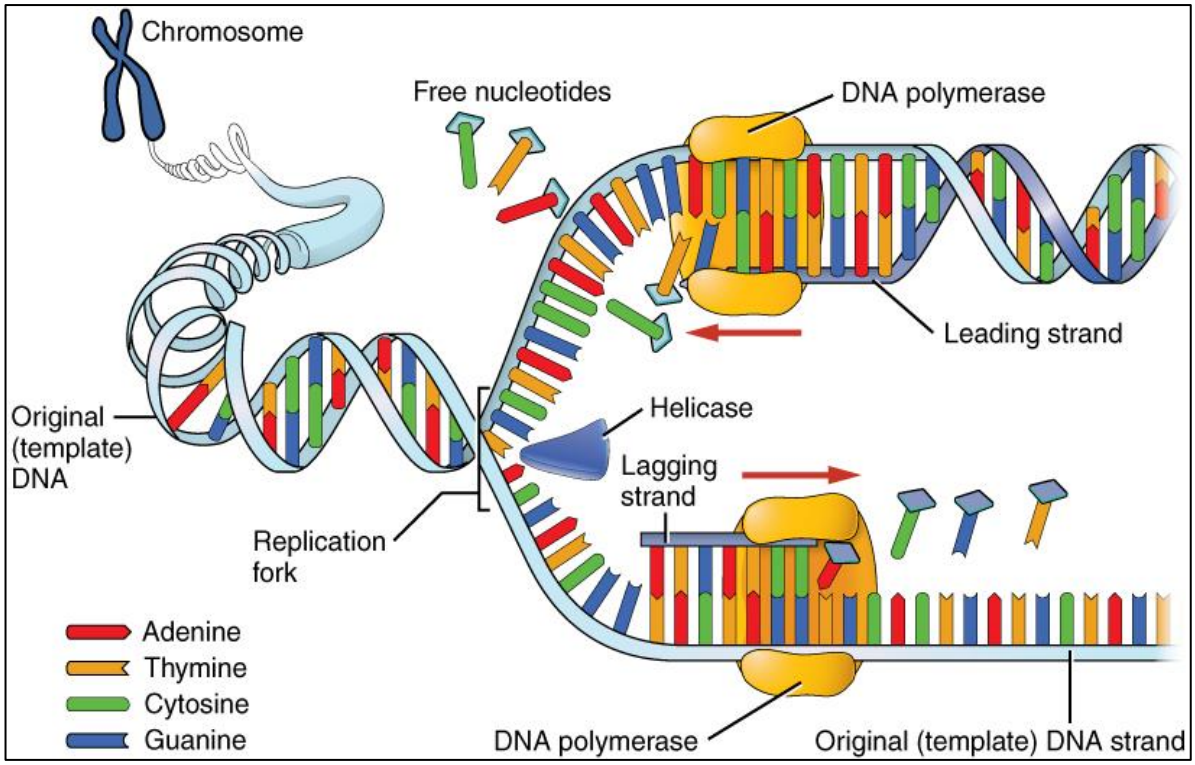


Figure 52- Typical DNA replication process. The enzyme DNA polymerase moves along the DNA template strand and adds free nucleotides to create a duplicate copy. This phenomenon is the basis for PCR, where specific DNA sequences are amplified (Samiksha 2016).

PCR Components

PCR mimics the DNA replication process that occurs naturally in cells. It uses a small quantity of DNA that serves as the initial template, a pair of primers that binds to each end of the target sequence, DNA polymerase, four deoxyribonucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), and a few essential ions (e.g. magnesium) and salts (ThermoFisher 2012). The DNA is then exposed to cycles of heating and cooling. Figures 53-54 give an overview of how these components work together to replicate the template DNA.

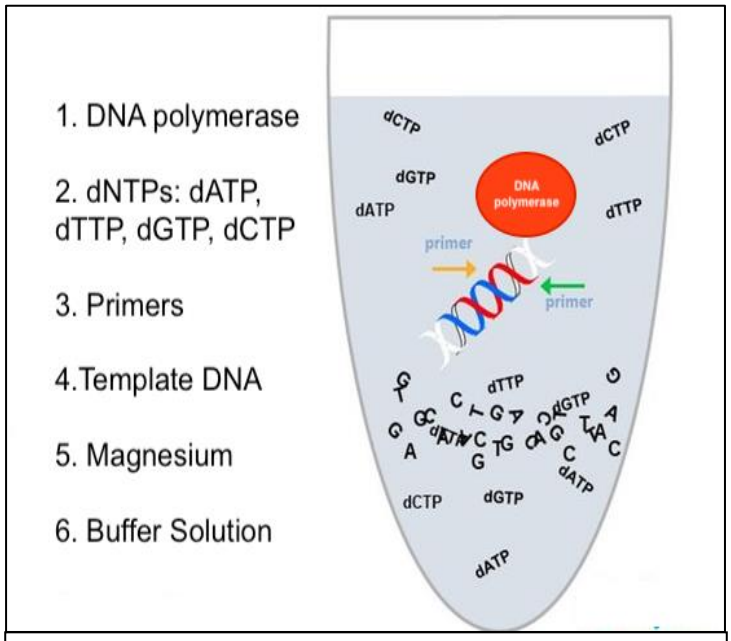


Figure 53- Solution of components making up a PCR reaction inside a test tube (Adapted from Christensen 2018).

1. DNA polymerase: An enzyme that synthesizes DNA molecules from dNTPs. DNA polymerase cannot initiate synthesis of a new strand of DNA, it can only add to an existing DNA strand. During replication, the polymerase "reads" the template DNA and creates a new strand to match it by adding new nucleotides, one by one.
2. Deoxyribonucleotide triphosphates (dNTPs): dNTP is a solution of nucleotides. These are used as the building blocks of DNA. The mixture contains equal amounts of the bases adenine (dATP), thymine (dTTP), guanine (dGTP) and cytosine (dCTP). These nucleotides are added by the DNA polymerase and bind to their corresponding nucleotides as in normal replication: adenine pairs with thymine (AT bond) and guanine pairs with cytosine (GC bond).
3. Primers: Primers are short sequences of single-stranded DNA that are complementary to the template DNA. Since DNA polymerase can only add to existing strands of DNA, primers must first bind to the template DNA first and initiate replication. There is a forward primer that complements the upstream end of the template DNA and a reverse primer that is the reverse complement of the downstream end. Primers should be 18-24 nucleotides in length and specific to the target sequence.
4. Template DNA: DNA that contains the region to be copied. Ideally, there should be 10-1,000 copies of the template DNA for each PCR reaction. Excess template can reduce the efficiency of the assay.

5. Magnesium: Magnesium is an essential cofactor for DNA polymerase. It is used to stabilize double stranded DNA and limit DNA denaturation during PCR. $MgCl_2$ typically comes in the prepared buffer solution with any PCR kit.
6. Buffer Solution: This is a solution that provides a stable chemical environment for the optimal activity of the DNA polymerase. It normally comes with the PCR kit being used (Bustin 2004; Hollis 2013).

PCR Process

PCR amplification relies on the concept of thermal cycling, where samples are exposed to cycles of heating and cooling to induce DNA replication (ThermoFisher 2012). To automate this process, a thermocycler machine is used to initiate each stage of the reaction by raising and lowering the temperature at specific times for a specified number of cycles. There are three major steps that make up each PCR cycle. Most protocols incorporate a total of 35-40 cycles, resulting in exponential replication of the target DNA (Figure 55) (Garibyan & Avashia 2013; Lorenz 2012).

1. Denaturation: During denaturation, the solution is first heated. This temperature is normally between 94° and 98°C. As the heat increases, it breaks apart the two double strands of DNA and separates into two single strands. The amount of time needed depends on the guanine and cytosine (GC) bond content of the DNA. GC bonds are much stronger than adenine and thymine (AT) bonds and therefore take more energy to break. The higher the GC content of the template DNA, the longer the denaturation step should be.
2. Annealing: During annealing the reaction is held for several minutes and is then cooled to between 30° and 65°C. This temperature is based on the melting temperatures of the primers being used. This mixture is held at this temperature for around one minute, which allows primers to bind, or anneal, to the complementary sequence.

3. Elongation (also called Extension): During the extension stage, the sample is heated again. This is typically between 60°and 75°C and the reaction is held at this temperature for less than one minute. The time of extension is dependent on the length of the target, with longer templates needing a longer elongation time. The DNA polymerase will then generate a new DNA strand by binding to the primers and adding dNTPs to the template strand, producing a complimentary strand of DNA. This step allows for the DNA polymerase to extend the primers that have hybridized by adding dNTPs (Lorenz 2012).

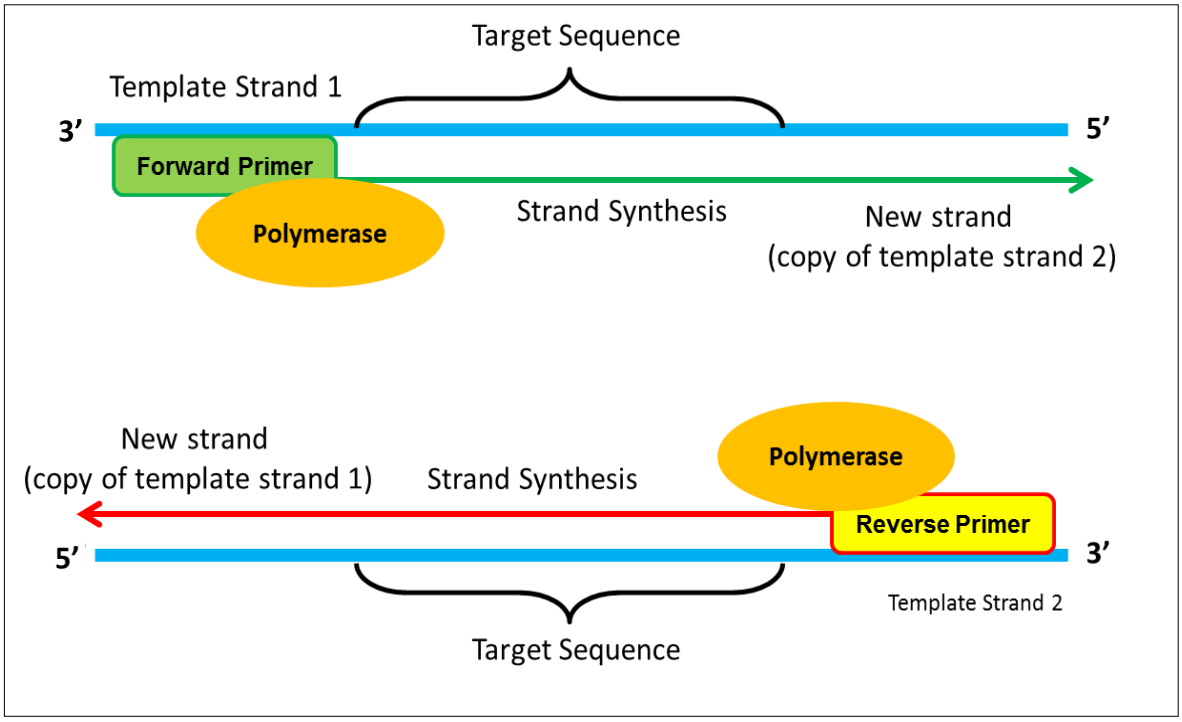


Figure 54- Overview of the components of PCR. First, primer pairs must bind to the template DNA strand. Then DNA polymerase can begin to replicate the DNA sequence by adding dNTPs to the primer. This results in a replicate copy of the template DNA sequence (Hollis 2013).

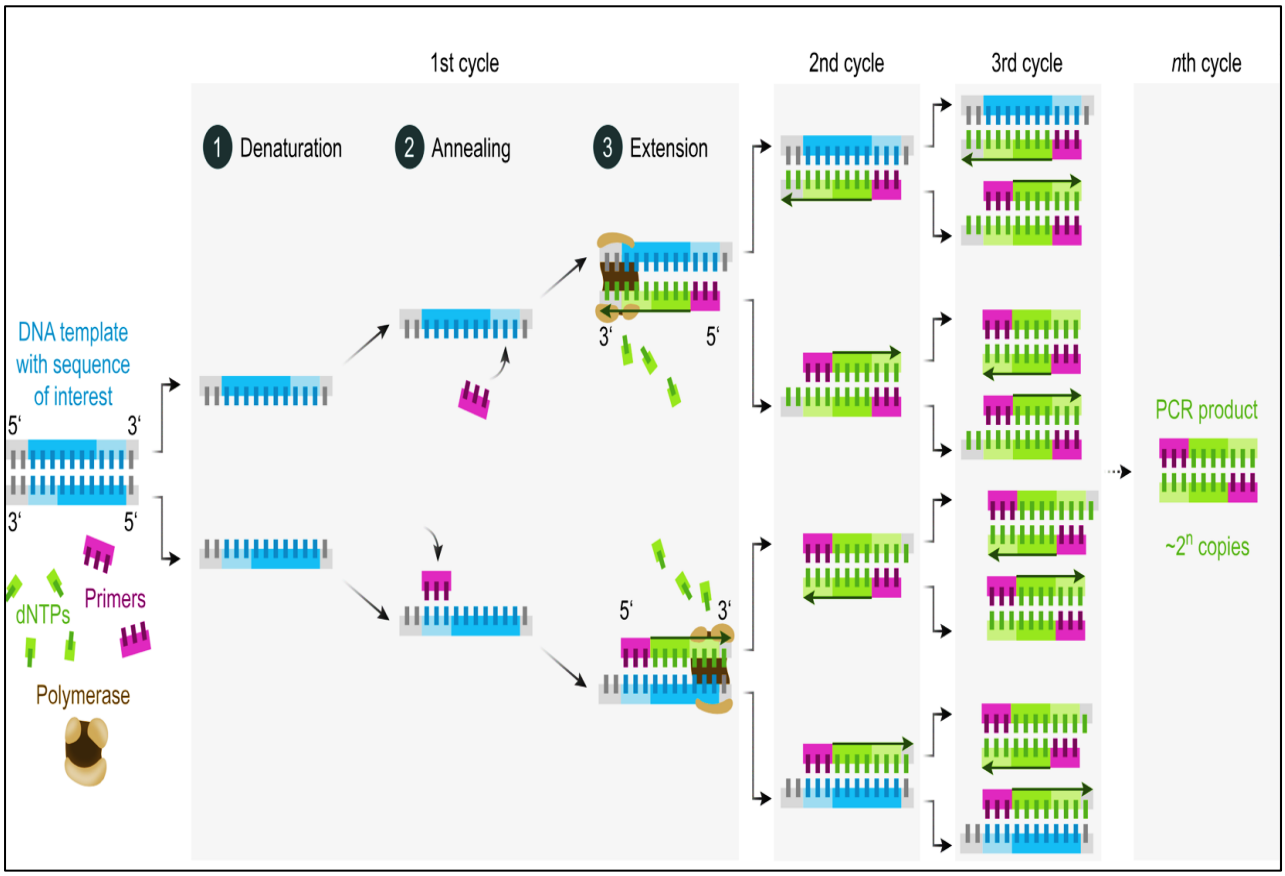


Figure 55- Exponential replication of target DNA. After several cycles of denaturation, annealing and elongation, the DNA template is amplified exponentially (Enzoklop, CC BY-SA 4.0 Wikimedia Commons).

PCR Types: Conventional vs Real-Time PCR

With conventional PCR, DNA sequences are amplified and analysis of the product is performed at the end of the PCR reaction. Amplified products need to be visualized with a secondary procedure after the PCR has finished, such as agarose gel electrophoresis or polyacrylamide gel electrophoresis (PAGE). A modification of conventional PCR detection, called quantitative PCR (qPCR) or real-time PCR enables researchers to amplify a particular DNA sequence and to quantify it simultaneously (Garibyan & Avashia 2013).

Real-time PCR enables the amount of a given sequence present in a sample to be analyzed by measuring the increase of DNA product after each round of PCR amplification. There are two main methods of DNA quantitation used during real-time PCR. These are shown in Figure 56 below. One uses DNA-targeted intercalating fluorescent dyes that show enhanced fluorescence upon binding. As the quantity of the template DNA increases with each amplification cycle, the number of bound fluorophores and intensity of the fluorescent signal increases. The second method uses fluorophore-labeled sequence-specific DNA probes. The most widely utilized type is the TaqMan probe, which uses the 5'-3' exonuclease activity of Taq polymerase to break apart the fluorophore from the quencher, producing fluorescence (Figure 56) (Bonetta 2005; Smith & Osborn 2009).

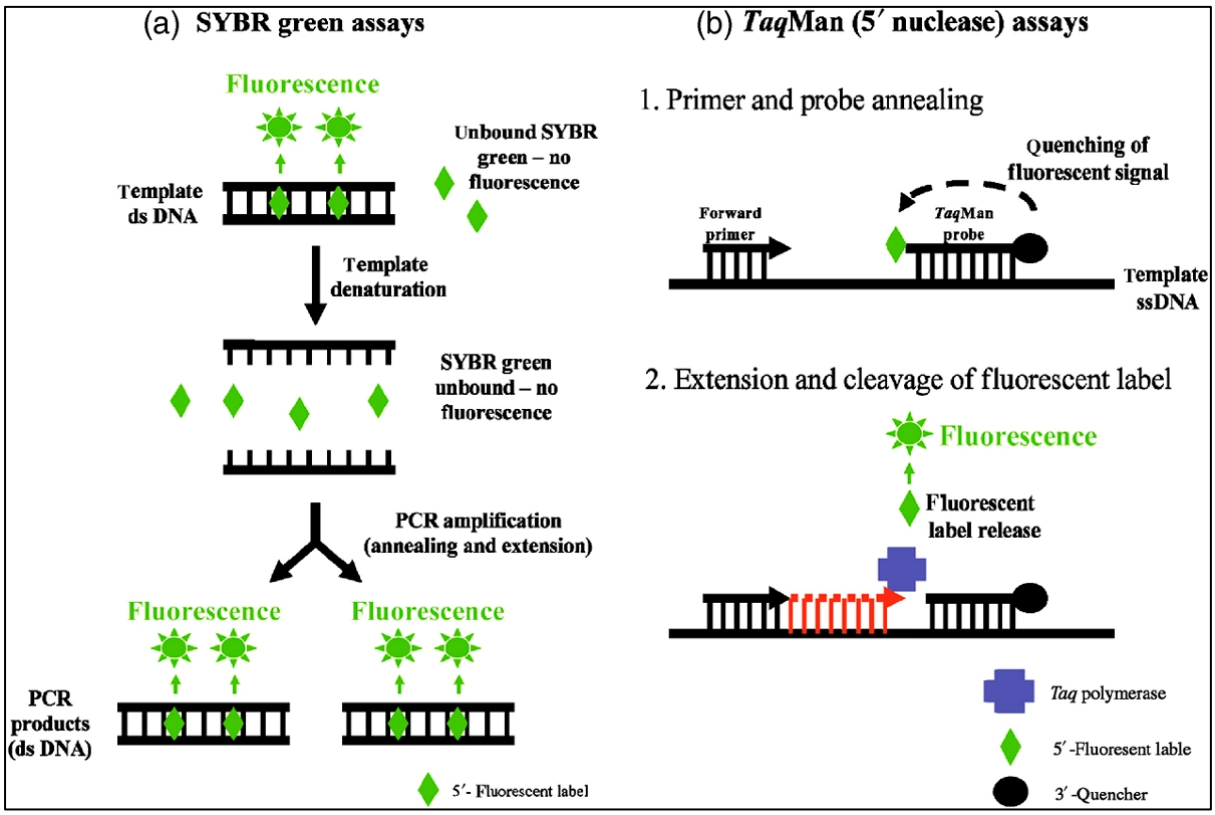


Figure 56- Detection processes for real-time PCR: (a) DNA-targeted intercalating fluorescent dyes, such as SYBR Green and (b) fluorophore-labeled sequence-specific DNA probes, such as TaqMan assay (Smith & Osborn 2009).

As the number of DNA copies increases, the fluorescent signal increases. By plotting fluorescence against cycle number and comparing the results against a standard curve, the user is able to determine the quantity of DNA at each step of the PCR reaction (Smith & Osborn 2009). Additional details and *B. pseudomallei*-specific protocols for conventional and real-time PCR assays can be found in Chapters 14 and 15.

Pros and Cons of Real-Time PCR (qPCR) Over Conventional PCR Assays

Advantages of real-time PCR

1. **Speed:** DNA is detected while the PCR reaction is occurring. Amplified products do not need to be visualized with a secondary procedure after the PCR has finished (e.g. on an agarose gel).
2. **Quantification:** Allows for quantification (measurement) at the exponential phase that depends on initial sample quantity.
3. **Sensitivity:** real-time PCR can distinguish as few as two-fold increases in the quantity of target DNA generated and can detect as little as a few molecules of staring DNA.
4. **Throughput:** real-time PCR can process a large number of samples quickly, particularly when using automated extraction systems.
5. **Replicability**: results are normally very reproducible (Čepin 2017; Smith & Osborn 2009).

Disadvantages of qPCR

1. **Cost:** the equipment required for fluorescence detection means that real-time PCR thermocyclers are five to ten times more expensive than conventional PCR thermocyclers. Highly specific reagents and consumables are also required because of the sensitive fluorescent detection method. qPCR kits often contain expensive master mix solutions (a premixed concentrated solution that includes all components for a real-time PCR reaction that are not sample-specific).

2. **Prone to contamination:** real-time PCR is an extremely sensitive method and very prone to error and contamination. Several controls, such as a no template control (a sample containing no DNA) and buffer control need to be included when performing the assay to assure quality control checks in every run.
3. **Complexity:** interpretation of results is more complex and requires further data analysis than with conventional PCR assays (Čepin 2017; Smith & Osborn 2009).

Table 16- Comparison of real-time versus conventional PCR (ThermoFisher 2012).

	Real-time PCR	Conventional PCR
Process	Measures PCR amplification as it occurs. Data is collected during the exponential growth phase of PCR when the quantity of the PCR product is directly proportional to the amount of template nucleic acid.	Measures the amount of accumulated PCR product at the end of the PCR cycles.
Applications	• Quantitation of gene expression • Quality control and assay validation • Pathogen detection • Copy number variation	Amplification of DNA for: • Sequencing • Genotyping • Cloning • Pathogen detection
Advantages	• Increased dynamic range of detection • No post-PCR processing • Higher sensitivity and specificity • Closed system reduces the risk of contamination • An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated • Shorter turnaround time • No post-PCR processing	• Less expensive reagents and equipment • Less complex results interpretation • Widely used molecular diagnostic format
Disadvantages	• Cost of equipment and consumables • Data analysis and interpretation of results are more complicated • More sensitive to errors and PCR inhibition	• Lower sensitivity and specificity • Lower resolution • Non-automated • Results are not expressed quantitatively • Post-PCR processing takes additional time

PCR Laboratory Setup and Good Laboratory Practice

The capacity for PCR to generate many copies of a target sequence from a small quantity of DNA has made PCR a useful and widely implemented diagnostic tool. This also means that certain precautionary measures must be used to prevent false positive results and contamination of the assay or laboratory space. False positive or misleading results can occur through cross-contamination of samples and different reactions prepared at the same time, or through contamination of stock solutions. Other microorganisms present within the laboratory environment are also a significant source of contamination. When conducting PCR experiments, measures can be undertaken to avoid the contamination of reagents, laboratory equipment and bench space. Good laboratory practices should be used to help reduce the likelihood of contamination (Mifflin 2007; Public Health England 2018; U.S. EPA 2004). The following general precautions will help to reduce the chance of contamination.

1. Organization of PCR Workspace and Equipment

The prevention of PCR contamination starts with the distribution of PCR laboratory workspaces. Workspaces should be organized to ensure that the flow of work occurs in one direction, from clean areas (pre-PCR) to dirty areas (post-PCR). Ideally, the air in clean areas should be positively pressurized (air forced out of the room) to ensure that airborne microorganisms or particles do not contaminate the samples or reagents in that room. Dirty areas should be negatively pressurized (air forced in) to help prevent the escape of amplified products (Figure 57) (Mifflin 2007; WHO 2018).

Where possible have separate designated rooms, or at minimum, physically separate areas for:

a. Sample/specimen preparation and nucleic acid extraction
b. Pre-PCR: Reagent/master mix preparation
c. Pre-PCR: Nucleic acid template addition
d. Post-PCR: PCR amplification, handling of the amplified product and product analysis e.g. gel electrophoresis.

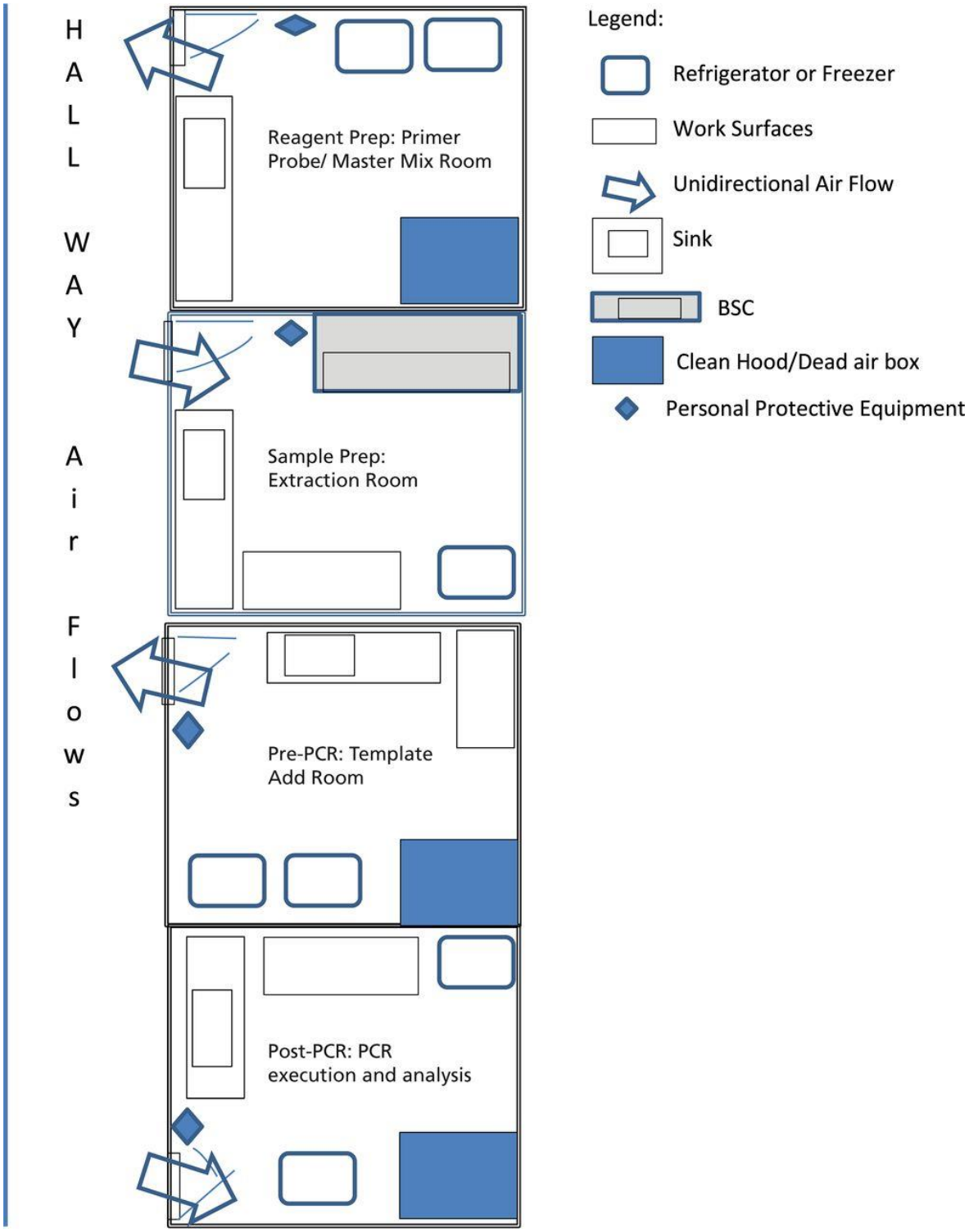


Figure 57- Sample floor plan for a molecular laboratory to ensure the segregation of specimens and nucleic acid template from clean reagents for PCR (Carey et al. 2018).

Sample/specimen preparation and nucleic acid extraction area

A sample preparation area should be used for sample processing and for preparing PCR controls. Laboratories can also include a designated space in this area for sample/specimen reception and storage. Clean gloves, laboratory coats and closed footwear should be worn at all times and separate pipettes and laboratory coats also should be designated for all work done here to control contamination from this room to any other location. This room should also be kept under negative pressure, which will help to prevent contamination outside of this space. Where possible, equipment from this room should not be removed and used in the reagent/master mix preparation area (U.S. EPA 2004).

Pre-PCR: Reagent/master mix preparation area

This area should be positively pressurized and should be the cleanest of all PCR preparation areas. Specimens, extracted DNA and amplified PCR products should not be handled in this area. Where possible, a laminar flow cabinet equipped with a UV light should be used in this area. A laminar flow cabinet is a hooded and enclosed bench that is used to prevent contamination of biological samples where air is drawn through a HEPA filter and blown towards the user. Due to the direction of air flow, the sample/reagents are protected from the user while the user is not protected from the sample/reagents (Figure 58) (U.S. EPA 2004).

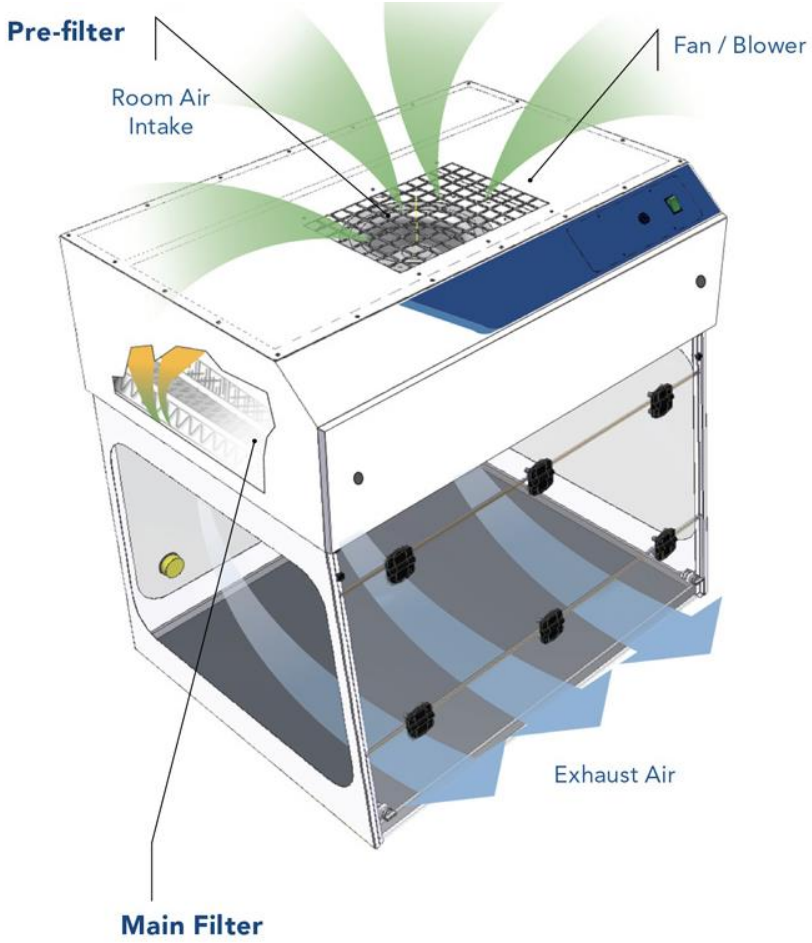


Figure 58- A laminar flow cabinet is a workspace enclosed on three sides that prevents cross contamination between samples and offers UV lights for sterilization (Air Science 2021).

PCR reagents should be kept in a freezer or refrigerator according to the manufacturer specifications. Fridges and freezers should not contain any template DNA or specimens, as this can lead to contamination of the reagents. Gloves should be changed regularly but especially when entering the pre-PCR area and laminar flow cabinet. The pre-PCR area and laminar flow cabinet should be cleaned before and after use as below:

1. Wipe down all items in the cabinet, e.g. pipettes, tip boxes, vortex, centrifuge, tube racks, pens, etc. with 10% bleach solution or a commercial DNA-destroying decontaminant (such as DNA-Erase), followed by 70% ethanol, and allow to dry.

2. If working in a laminar flow cabinet turn on the UV light for 30 minutes.

Pre-PCR: Nucleic acid (DNA/RNA) template addition area

This area should be used for the addition of extracted DNA/RNA and controls to master mix tubes or plates. Nucleic acid must be handled using a separate set of pipettes, filter tips, tube racks, fresh gloves, lab coats and other equipment. To prevent contamination of extracted DNA, laboratory staff should change gloves regularly and before handling controls. Ensure equipment has been wiped clean with 10% bleach or commercial DNA-destroying decontaminant before adding controls. Sample template nucleic acid and controls should be stored in separate designated fridges or freezers in this area. The sample workspace should be cleaned in the same way as the reagent/master mix preparation area (U.S. EPA 2004; Viana & Wallis 2011).

Post-PCR: PCR amplification, handling of the amplified product and product analysis

This space is used for post-amplification practices and typically contains thermocyclers and real-time PCR machines. The air should be negatively pressurized to help prevent the escape of amplified products and be separate from the pre-PCR areas. The risk of contamination is high in this area and PCR reagents and the extracted nucleic acid should not be handled here where possible. This area should also have a separate set of gloves, lab coats, PCR plate and tube racks, pipettes, filter tips, and other equipment to minimize contamination (WHO 2018).

Post-PCR product analysis can also be performed here, although where resources and space permit, this should preferably be done in a separate contained post-PCR area. Product detection equipment, e.g. gel electrophoresis tanks, power packs, gel documentation system and reagents (e.g., loading dyes, molecular marker, agarose gel, and buffer components) should be kept here. The area should also contain a separate set of gloves, lab coats, plate and tube racks, pipettes,

filter tips, bins and other equipment. Workspaces and equipment should be cleaned as described above (WHO 2018).

In resource or space-limited settings, having four separate rooms or spaces may not be possible. In this instance, PCR setup can include two work spaces: a pre-PCR clean room/area that contains no DNA (only master mix) and a post-PCR dirty room/area where extracted DNA is added to the master mix, such as in Figure 59.

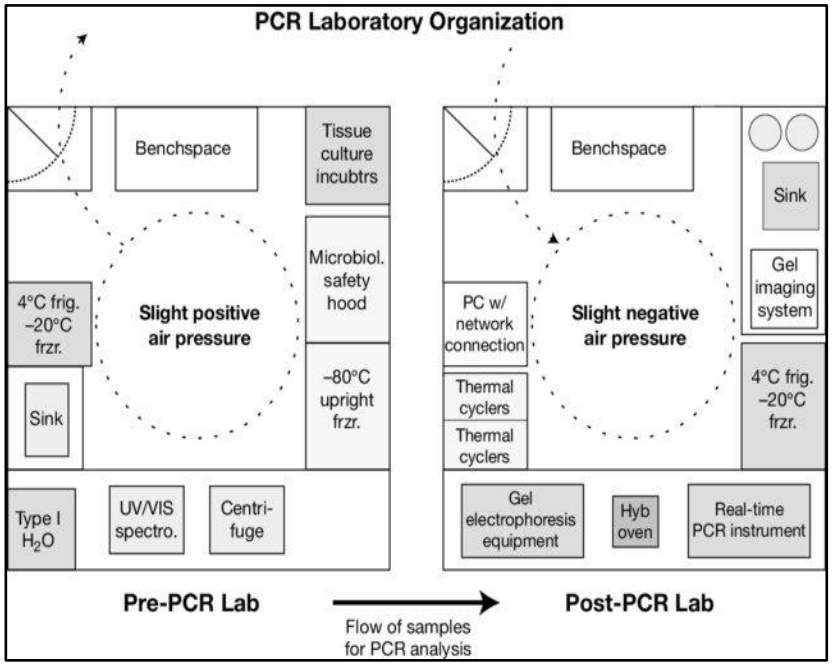


Figure 59- Organization of a PCR laboratory with two separate pre-and post-PCR rooms (Mifflin 2007).

If separation of workspaces cannot be achieved, extra care must be taken to avoid DNA contamination of primer and probe reagents. Here, a possible (though less desirable) option is to do the master mix and reagent preparation in a containment cabinet, such as laminar flow cabinet (Mifflin 2007; Viana & Wallis 2011).

2. Unidirectional Workflow

Ideally, laboratory staff should abide by a unidirectional workflow. Pre-PCR rooms, particularly those used to prepare/aliquot PCR stock reagents, should not be entered on the same day after working in rooms where products and clinical specimens are handled. If one must go against the unidirectional workflow, care should be taken as described below:

a. Hands should be washed and gloves and lab coats changed when moving between areas.

b. Reagents and equipment should not be moved from a dirty area to a clean area. If this is unavoidable, they should be cleaned with 10% bleach or a commercial DNA decontaminant, followed by sterile water or 70% ethanol (only on non-plastic surfaces). Ultra-violet (UV) light should be where if available.

c. Lab books and paperwork must not be taken into the pre-PCR rooms if they have been used in the post-PCR rooms. If necessary, make duplicate print-outs of protocols/sample IDs, etc. (Mifflin 2007; Viana & Wallis 2011; WHO 2018).

3. Good Laboratory Practices and Handling of Reagents

a. PCR reagents should be aliquoted as they are received to avoid unnecessary freeze-thawing and to prevent stock reagents from contamination as soon.

b. If contamination is detected, all work should be suspended until the source of contamination has been determined and eliminated. A full investigation should be carried out to determine why the contamination occurred. Occasionally, discarding all suspected reagents is necessary.

c. All reagents, stocks, tubes etc. should be clearly labelled. Do not keep reagents past the expiration date.

d. Briefly spin/centrifuge reagent and sample tubes before opening to avoid the generation of aerosols. Open and close all tubes and reaction plates carefully so samples do not splash. Close tubes immediately after use to avoid the introduction of contaminants.

e. Bench work areas in PCR laboratories should be cleaned with disinfectant solution before and after use.

f. Use powder-free gloves to avoid assay inhibition and change frequently.

g. Protect reagents containing fluorescent probes from light to avoid degradation (U.S EPA 2004; Viana & Wallis 2011; WHO 2018).

4. Pipetting Technique

Appropriate pipetting technique is important to ensure both the accuracy and quality of PCR results. Correct pipetting technique can also minimize contamination and help to prevent false positive or ambiguous results (see Figure 60 below). Pipette reagents and samples using filter tips, which will help to prevent cross-contamination of samples. Confirm with the manufacturer that the filter tips fit the brand of pipette to be used prior to purchase (U.S EPA 2004; WHO 2018). All equipment should also be calibrated regularly according to the manufacturer guidelines. If manufacturer instructions permit, pipettes should be sterilized by autoclave on a regular basis. If pipettes cannot be autoclaved or no autoclave is available, clean them with 10% bleach (followed by a thorough wipe down with sterile water) or with a commercial DNA-destroying decontaminant followed by UV exposure after use (Viana & Wallis 2011; WHO 2018). Good practice for accurate pipetting can be found at:

- Gilson guide to pipetting: https://www.gilson.com/pub/static/frontend/Gilson/customtheme/en_US/images/docs/2019_Guide_to_Pipetting_LT800550-F_compressed.pdf.

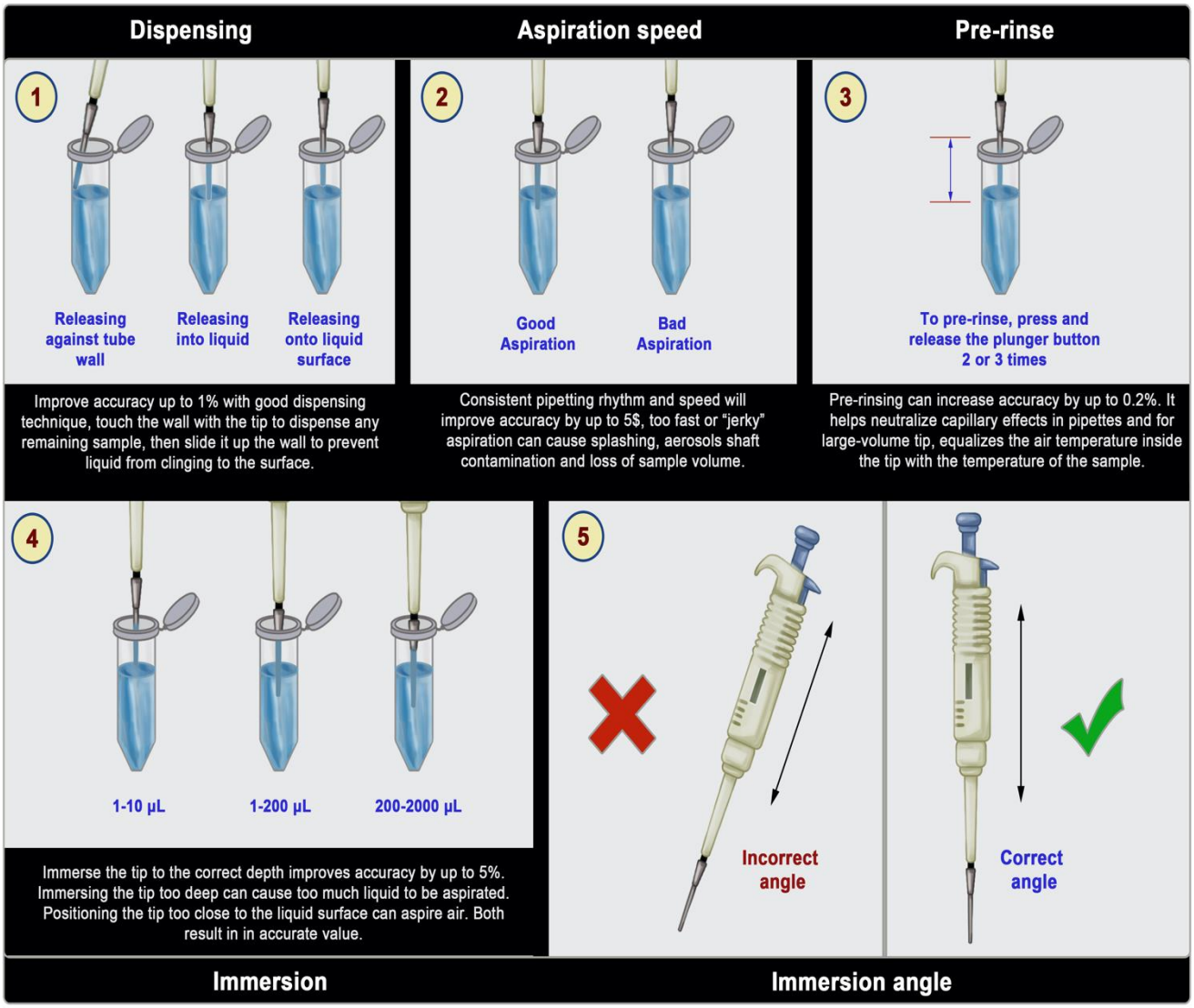


Figure 60- Good laboratory practice for accurate pipetting during PCR molecular reactions.

5. Quality Controls

Quality controls are important for ensuring that contamination of reagents or workspace has not occurred and that the assay is working as expected. Non-template controls (NTCs) contain all reagents except for template nucleic acid. Rather, PCR-grade water is normally added to these PCR wells. It is recommended that water used both in the clean and dirty area is added to at least two

NTC reaction wells. If any of these NTC well generates a positive PCR result, the water should be replaced and reactions should be run again using new NTCs.

PCR reaction positive controls should also be run using DNA from a known positive *B. pseudomallei* isolate or using non-*B. pseudomallei* template DNA (plasmid DNA) that contains the PCR target gene of interest, which will help to minimize cross-contamination between reaction wells. If using DNA from a known positive *B. pseudomallei* isolate, this should be diluted in PCR-grade water to decrease the likelihood of contamination of the PCR workspace or reaction wells, helping to avoid false positives (Viana & Wallis 2011; WHO 2018).

When extracting DNA from human clinical specimens RNAse P can be used as an internal positive extraction control to monitor sample quality and the presence of PCR reaction inhibitors (Esakova & Krasilnikov 2010). An additional control for contamination at the DNA extraction step is the extraction of water (see Chapter 13 for further detail on DNA extraction methods and controls). If the extracted water negative control generates an amplification curve that crosses the threshold, then contamination has occurred during the DNA extraction process. Reagents should be replaced and the extraction workspaces and equipment should by cleaned thoroughly.

Chapter Summary

Polymerase chain reaction (PCR) is a laboratory technique used to amplify strands of DNA or RNA (ThermoFisher 2012). It is a highly specific method of *B. pseudomallei* detection that can generate results rapidly. In some melioidosis-endemic regions, PCR is used to confirm or identify oxidase-positive colonies with a morphology typical of *B. pseudomallei* on selective agar that are also resistant to colistin and susceptible to co-amoxiclav. PCR is also frequently used for direct detection from environmental specimens. The preceding chapter presents a general overview of PCR, PCR laboratory setup and briefly describes the main types of assays used to detect *B. pseudomallei* in

clinical and environmental specimens. Additional details about DNA extraction and specific PCR protocols can be found in Chapters 13-15.

Chapter 13: *B. pseudomallei* DNA extraction from Clinical and Environmental Specimens

Chapter Overview

Extraction of nucleic acid (DNA or RNA) serves as a starting point for multiple downstream applications, including clinical diagnosis and experimental research. The following chapter presents an overview of the typical DNA extraction process, DNA extraction quality control, and protocols for DNA extraction from *B. pseudomallei* clinical and environmental specimens. DNA isolated from these protocols can be used for subsequent molecular PCR detection and diagnosis of *B. pseudomallei* infection. Conventional and real-time PCR procedures are discussed in Chapters 14 and 15.

DNA Extraction Introduction

DNA extraction is a technique used to isolate DNA from the inside of a cell. Once extracted, DNA can be used for downstream molecular analyses, including PCR and genetic sequencing. There are three primary steps involved: lysis, separation and purification (Elkins 2013).

1. Lysis- During lysis, the cell membrane walls and cell nucleus are broken open, releasing the DNA. This can be accomplished several different ways. It can be done enzymatically using lysozyme, which digests peptidoglycans in the cell wall, or using detergents like sodium dodecylsulfate (SDS) to dissolve the cellular proteins. It can also be achieved mechanically utilizing glass beads, which are vortexed with the sample. Mechanical lysis is generally faster than enzymatic methods.
2. Separation- During separation, DNA is separated from any cellular debris. This often involves the use of sodium (Na+) ions to neutralize the negative charge present in DNA molecules. This makes the DNA less water soluble and more stable.

3. Purification- After separation of DNA from the aqueous solution, alcohol (e.g. isopropanol or ethanol) is added. This leads to precipitation of DNA out of the solution as DNA does not dissolve in alcohol. It is then rinsed to remove cellular debris.

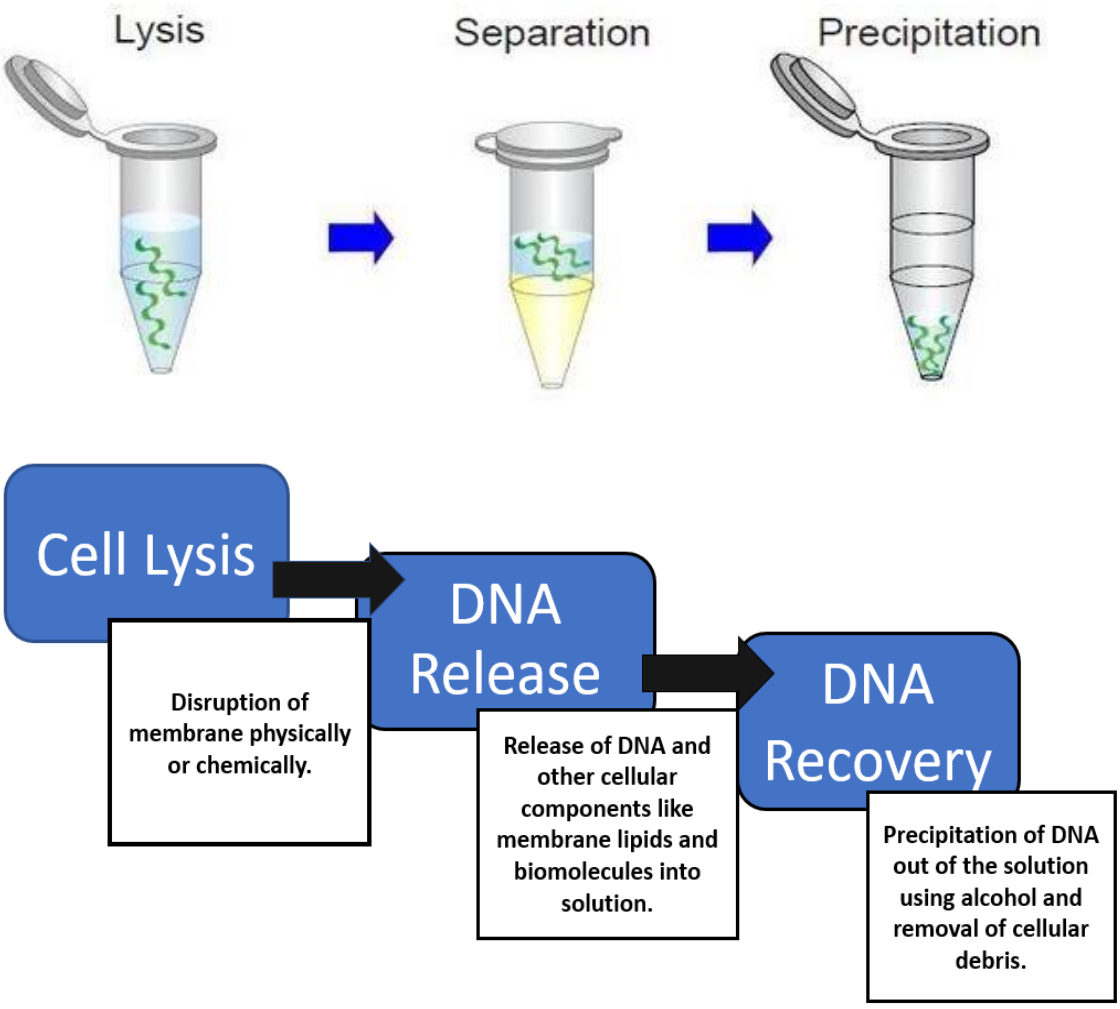


Figure 61- Steps involved in basic DNA extraction. Three major processes involved are cell lysis, separation of DNA from other cellular components and debris, followed by precipitation and purification of the DNA from the aqueous solution (Bio-Helix 2016).

Methods of DNA Extraction

Common DNA extraction methods include organic chemical extraction, Chelex® extraction and solid-phase extraction (Elkins 2013).

1. Organic chemical extraction- Organic chemical extraction involves the addition of several chemical solutions and nomally incorporates a lysis step, a phenol chloroform extraction, an ethanol precipitation, and washing steps (Elkins 2013). Organic extraction is inexpensive, simple and yields large quantities of pure DNA. However, there are still many steps involved and the procedure may still take longer than other extraction methods. It also uses toxic chemicals such as phenol and chloroform and there is an increased risk of contamination because multiple tubes are used to transfer the DNA (Tan & Yiap 2009).
2. Chelex® extraction- Here, Chelex® resin beads are added to the sample and this solution is then heated, vortexed and centrifuged. The cellular materials bind to the Chelex® beads, while the DNA is available in the supernatant (Singh et al. 2018). The Chelex® method is faster, simpler and relatively inexpensive compared to other extraction techniques, and it only requires one tube, which decreases the risk of DNA contamination. However, the DNA is lower quality and degrades quickly after being extracted. It is typically only used for more rapid PCR-based analyses (Butler 2005).
3. Solid-phase extraction- Solid-phase extraction methods work through the binding of nucleic acids to solid support membranes, such as magnetic beads coated with silica or other materials. These are washed with alcohol to remove contaminants and then with a liquid that makes the nucleic acids soluble again, freeing the DNA from the beads/support (termed “elution”). This produces high-quality, double-stranded DNA that can be used for PCR and additional downstream analyses such as genetic sequencing. Many solid-phase extraction commercial kits are available through different commercial companies. However, solid-phase extraction kits tend to be more expensive than organic chemical extraction or Chelex® extraction (Elkins 2013; Singh et al. 2018).

   Solid-phase extraction methods can also be fully automated using advanced laboratory instruments such as the QIAcube or EZ1 (QIAGEN) or MagNA Pure LC Instrument (Roche

Diagnostics), which typically come preinstalled with multiple protocols for purification of plasmid DNA, genomic DNA, RNA, viral proteins, as well as DNA and RNA clean-up (Butler 2005). Automated instruments often use the same kits as manual solid-phase nucleic acid extraction but can dramatically decrease the time and effort required to extract the DNA. Automated kits also require less time for set-up, increasing throughput. However, testing of only one or a few samples using automated systems can be costly. The initial cost of setting up automated systems is also more than with manual extraction kits since a robot system must be purchases to run the kits (Meumann et al. 2006; Podnecky et al. 2013). See more information about automated kits and the QIAcube or EZ1 at www.qiagen.com, or MagNA Pure LC (Roche Diagnostics) available at https://www.lifescience.roche.com/en_au/products/magna-pure-.

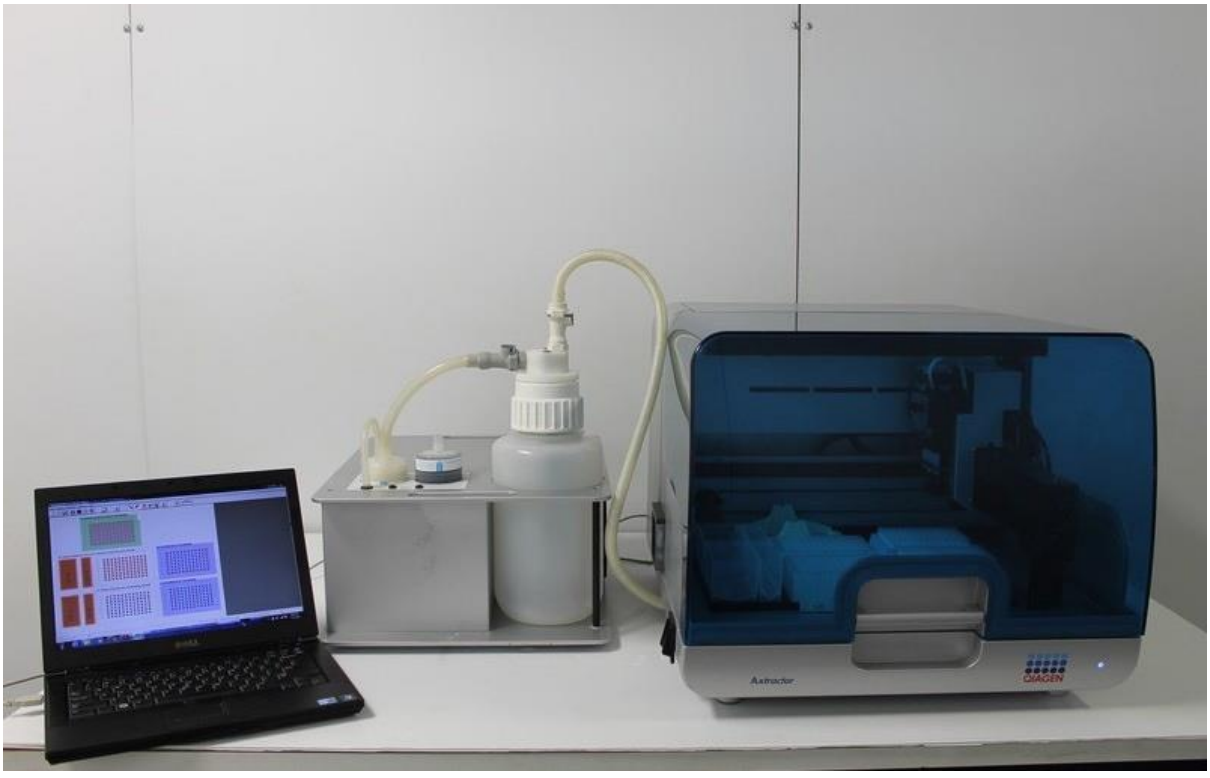


Figure 62- Automated QIAcube extraction robot and typical laboratory setup.

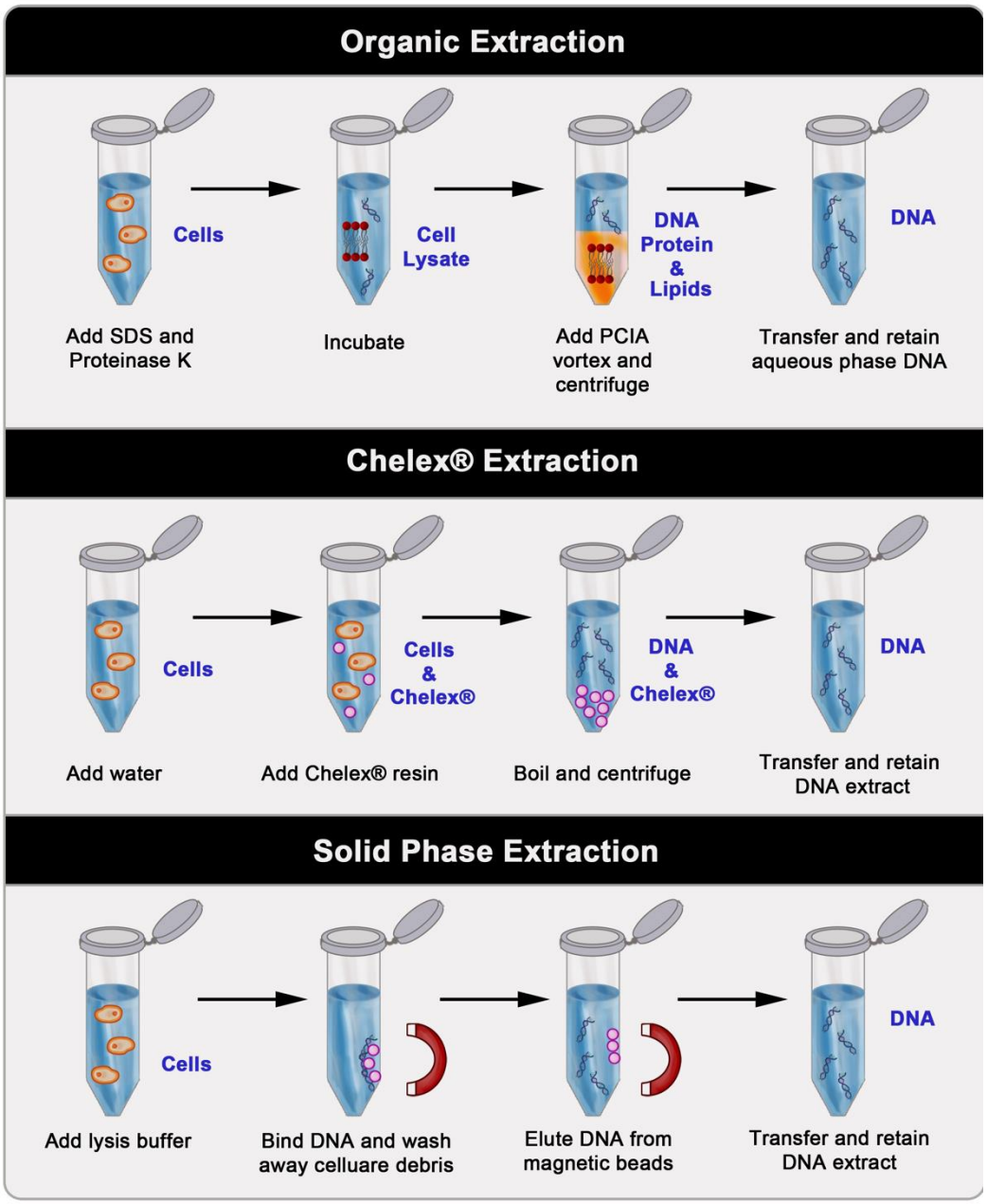


Figure 63- The three major methods of DNA extraction. Top: Organic extraction using sodium dodecylsulfate (SDS), Proteinase K and phenol:chloroform:isoamyl alcohol (PCIA) separates DNA from lipids and proteins. Middle: Extraction using Chelex® resin. This releases DNA by boiling and vortexing and inactivates degradative enzymes such as nucleases. Bottom: Solid phase extraction selectively binds DNA to a solid support such as silica in the presence of a high salt solution.

There are multiple factors to consider when selecting the most appropriate DNA extraction method, including (Dhaliwal 2013):

1. Sample type and storage method- DNA can be extracted from many sources, including human tissues, blood, bacteria, yeast, fungi, stool, body fluids, spores, soil, and other clinical samples (Thatcher 2015). Sample preparations may be fresh or previously frozen, paraffin-embedded or formalin-fixed tissue, ethanol-fixed, etc. (Mullegama et al. 2019).
2. Intended use- The quality and quantity of the extracted DNA should be adequate for the intended downstream purposes, e.g., sequencing, molecular fingerprinting, or PCR detection.
3. Humic content- Humic substances are naturally occurring organic compounds that arise from the decomposition of plant, animal, and microbial material. They are a natural component of soils. If the sample has humic content a kit/method that removes these substances should be used since they can inhibit downstream applications like PCR (Maccarthy 2001).
4. DNA yield- The amount of DNA extracted from a sample will vary depending upon the sample type, inhibitors, and the method of extraction.
5. Simplicity- The best extraction method may depend on the experience of the user and the amount of control the user desires during extraction.
6. Cost- Manual and automated commercial kits are often more expensive than organic or Chelex® extraction methods and may not be practical in poorly-resourced laboratories for routine specimen diagnostics. These systems also require access to technical help and should take maintenance and repair costs into consideration.

B. pseudomallei DNA Extraction

Solid-Phase Extraction: Commercial Kits for *B. pseudomallei* DNA Extraction

The use of solid-phase extraction commercial kits for the isolation of *B. pseudomallei* DNA from clinical and environmental samples has many advantages, including the wide availability of kits, ease

of use, comparatively low cost as well as the standardization of reagents and protocols (Podnecky et al. 2013). Commercial kits vary in a several ways, including components that make up the lysis buffer, the mechanisms used to separate DNA, the type of membrane used, the wash buffer used and the way the DNA is recovered. Different extraction methods can generate different yields and purity. While no DNA extraction method has been shown to be optimal for all specimen types (Rantakokko-Jalava 2002) many have been evaluated and validated for *B. pseudomallei*-specific applications such as soil and water DNA extraction (Draper et al. 2010; Kaestli et al. 2007; Knappik et al. 2015), whole blood (Podnecky et al. 2013) and extraction from other melioidosis clinical specimens (Meumann et al. 2006; Richardson et al. 2012). The ability to detect *B. pseudomallei* with PCR has been shown to vary for different DNA kits and is dependent on the bacterial load, the quantity and quality of the bacterial DNA extracted and the elimination of PCR inhibitors (Meumann et al. 2006; Podnecky et al. 2013). Therefore, selecting the best kit and protocols are crucial.

Common commercial kits used for the extraction of *B. pseudomallei* DNA from clinical and environmental specimens include:

For clinical specimen extraction-

1. QIAamp DNA Mini Kit (QIAGEN)- The QIAamp DNA Mini Kit is a widely used and well-validated kit used to extract *B. pseudomallei* DNA from clinical specimens (Richardson et al. 2012). It offers a fast and easy method for purification of total DNA for reliable PCR testing. Genomic DNA can be extracted from many sample types including whole blood, plasma, serum, buffy coat, bone marrow, other body fluids, lymphocytes, cultured cells, and tissue. The QIAamp procedure can be used on fresh or frozen whole blood or blood that has been treated with citrate, heparin, or EDTA. It also requires no phenol/chloroform extraction or alcohol precipitation and very little handling. The QIAamp procedures can also be automated on the QIAcube (Dhaliwal 2013; QIAGEN 2016).

2. High Pure PCR Template Preparation Kit (High Pure, Roche Diagnostics)- Here, cells are lysed by a short incubation with a lysis buffer and Proteinase K in the presence of a chaotropic salt (i.e. a molecule that can disrupt the hydrogen bonds between water molecules). DNA binds to specific glass fibers present in the filter tubes and a low salt elution releases the DNA from the glass fibers (Merk et al. 2006). This kit can be used on a wide variety of specimens, however *B. pseudomallei* DNA yields from whole blood have been low in previous studies, likely due to reduced concentrations of circulating bacteria in blood (Podnecky et al. 2013).
3. DNeasy UltraClean Microbial DNA (QIAGEN)- This kit allows for the extraction of high-quality genomic DNA from many different microorganisms, such as yeast, fungi, Gram-negative and Gram-positive bacteria and spores. Microorganisms are lysed through a combination of heat, detergents, and mechanical force. The freed DNA then binds to a silica filter, and the DNA is washed and eluted in a DNA-free Tris buffer (Dhaliwal 2013).
4. DNeasy Blood and Tissue (QIAGEN)- This kit can be used to extract genomic DNA from multiple sample types including fresh or frozen, formalin-fixed or paraffin-embedded tissues and cells, as well as blood, and bacteria cultures. A silica-based method is used to extract the DNA and can be completed in around 20 minutes following cell lysis (Boyle & Lew 1995; Merk et al. 2006).

For environmental sample extraction:

1. DNeasy PowerSoil (QIAGEN): This can isolate *B. pseudomallei* DNA from soil and other environmental samples. It uses a humic content removal step that eliminates PCR inhibitors and generates high-quality DNA that can be used for many downstream applications (Dhaliwal 2013; Kaestli et al. 2007).
2. QIAamp Fast Stool Kit (QIAGEN): This kit allows for rapid purification of high-quality bacterial genomic DNA from samples containing high levels of inhibitors, such as soil. InhibitEX Buffer is used to efficiently remove PCR inhibitors commonly present in environmental samples (Hall et al. 2019).

Specimen Types

Previous studies have demonstrated that certain specimens with higher bacterial loads (e.g. sputum, pus and urine) are particularly useful clinical specimens for diagnostic PCR testing in patients with melioidosis and that standard DNA extraction methods are robust for these samples (Meumann et al. 2006; Richardson et al. 2012). DNA extraction of *B. pseudomallei* from direct whole blood remains challenging due to the low bacterial concentration and increased presence of inhibitors (Lau et al. 2010; Podnecky et al. 2013), however DNA extracted from plasma, buffy coat blood fractions and from cultured blood specimens have shown enhanced proportional isolation of *B. pseudomallei* DNA (Richardson et al. 2012). Additional steps that degrade inhibitory human DNA and facilitate bacterial cell lysis may improve the quality and quantity of DNA extracted from whole blood and other clinical specimens.

B. pseudomallei Clinical Specimen Extraction Protocols Using Solid-Phase Commercial DNA Extraction Kits

Extraction Quality Controls

Quality extraction controls are important in ensuring that procedures are valid and the results are reliable. To control for false positive and false negative results both a negative and positive extraction control should be utilized (Hornung et al. 2019):

1. Negative Control (extraction blank/contamination control)- This sample contains no sample DNA, only water. This control will ensure that no contamination occurred during the extraction and that any DNA used in the PCR is from the specimen samples only.
2. Internal Positive Control (amplification control)- The presence of PCR inhibitors or errors during sample extraction or thermocycler malfunctions are the most common causes of false negative PCR results and can be controlled by including an Internal Positive Control (IPC). This is used to confirm that DNA amplification has occurred, detects false negatives, and

qualitatively detects the presence of amplification inhibitory substances in a sample. There are two main types of IPCs:

A. Endogenous IPCs- These occur naturally in the specimen, such as a sequence of the from normal microflora genomes (e.g., 16s) or RNAse P in the human genome.

B. Exogenous IPCs- These are external templates that are spiked into samples either during DNA extraction or before PCR amplification. They are simultaneously extracted and then amplified with the target nucleic acid and serve as sensitive indicator of loss or degradation of the specimen DNA either during sample processing or due to inhibition. There are many exogenous IPCs available commercially (e.g., TaqMan® Exogenous Internal Positive Control (IPC)) Reagents- See specific real-time PCR protocol in Chapter 15). If the IPC is negative, it indicates the specimen may contain PCR inhibitors and will need to be diluted until the inhibitor is no longer affecting the C_t values. It may also indicate a problem with specimen collection or the DNA extraction. If IPC is not available, RNase P (RP) can be used as an internal control for human clinical specimens. For further information about use of the RNase P internal control with human clinical specimens see: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2F4316848_TaqMan_RNaseP_Cntrl_PI.pdf&title=UHJvZHVjdCBJbmZvIFNoZWV0OiBUYXFNYW4gUk5hc2UgUCBDb250cm9sIFJlYWdlbnRzIEtpdA==.

General Safety and Handling Measures (BMBL 6th ed. 2020)

A. Patient specimens should be handled at the BSL-2 safety level.

B. Do not pipette by mouth.

C. Wear disposable powder-free gloves, lab coat and goggles while handling reagents or specimens. Wash hands thoroughly after performing the test.

D. All materials should be disposed so that infectious agents are inactivated:

   i. Solid wastes should be autoclaved or sterilized using a pressure cooker if an autoclave is not available.

   ii. To liquid wastes: Add 10% bleach solution. Allow 20-30 minutes for decontamination before disposal.

   iii. All wipes/cleaning material should be treated as hazardous waste.

E. Spills should be wiped thoroughly with a 10% bleach solution. The wiped area should be covered with absorbent material, saturated with the 10% bleach solution and allowed to stand for 20-30 minutes.

F. Do not use the reagents beyond the expiration date.

G. The DNA extraction procedure and Quality Control protocols must be followed closely to obtain reliable test results.

H. It is important to pipette the exact reagent volume and to mix thoroughly after the reagents have been added. Failure to do so could result in inaccurate results.

I. Do not interchange equipment or reagents from different kits.

Equipment and Reagents

- Commercial DNA extraction kit (e.g. QIAamp DNA Mini Kit) containing Buffers and Protease or Proteinase K
- Centrifuge filter columns (such as Microcon YM-100 Columns or Millipore Ultrafree-MC Centrifugal Filter Devices, 0.1 µm)
- 2.0 mL microcentrifuge tubes with O-rings
- Capped 1.5 mL microcentrifuge tubes (e.g., Cat. No. T6050G, Marsh Biomedical Products)
- Tube racks for microcentrifuge tubes

- Biosafety cabinet (BSC)
- Pipettes and barrier/filter pipette tips for volumes 50–1000 µL
- Vortex mixer
- Microcentrifuge with aerosol-tight, autoclavable motor (or similar centrifuge capable of 20,000 ×g/14,000 rpm)
- Personal Protective Equipment (lab coat, eye protection and gloves)
- Water bath or heat block set to 56°C
- Waterproof markers
- KayDry or Kimwipe tissues or similar lab towels
- Biohazard waste disposal (bags and pans)
- Forceps/tweezers
- Distilled water
- 10% bleach solution, made fresh daily (be sure to check the concentration of hypochlorite to determine the correct dilution to use)
- Ethanol, 100%
- Ethanol, 70%
- Phosphate Buffered Saline (PBS)
- Specimens

1. Before Starting the Extraction

Before beginning with the DNA extraction be sure to do the following:

1. Bring specimens to room temperature.
2. Heat a water bath or heat block to 56°C.
3. Ensure that all reagents provided in the extraction kit (e.g. buffer AW1, buffer AW2, Proteinase K) have been prepared according to the manufacturer instructions and are within the expiration date.

4. Prior to beginning the extraction protocol, turn on BSC. Disinfect before use by spraying with a 10% bleach solution and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.
5. Label all tubes with the appropriate unique specimen/sample identifier.
6. Disinfect all equipment before use by spraying with a 10% bleach solution and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.
7. Place the following items in the cabinet: a plastic bag-lined discard waste pan or bucket, pipettes and pipette tips, PBS, Buffers AL and Proteinase K or Protease from the extraction kit, vortex mixer, appropriate number of 2 mL O-ring tubes and labeled 1.5 mL centrifuge tubes (including positive and negative extraction controls) placed in microcentrifuge rack.
8. Prepare enzymatic lysis buffer detergent with lysozyme as described below (if using).
9. Prepare specimens as described below before beginning the extraction. Specimen preparation will vary depending on the source material.

<u>Enzymatic lysis buffer detergent</u>

While not required, using an enzymatic lysis buffer detergent with lysozyme pre-treatment is recommended when performing DNA extractions from *B. pseudomallei* specimens. It may be particularly useful for specimens with lower *B. pseudomallei* bacterial loads, such as whole blood. Lysozyme is an enzyme used to break down the bacterial cell wall and increase protein or nucleic acid extraction concentation. Enzymatic lysis buffer (without lysozyme) can be made up in large batches and stored at 4°C. Lysozyme should be added immediately before use.

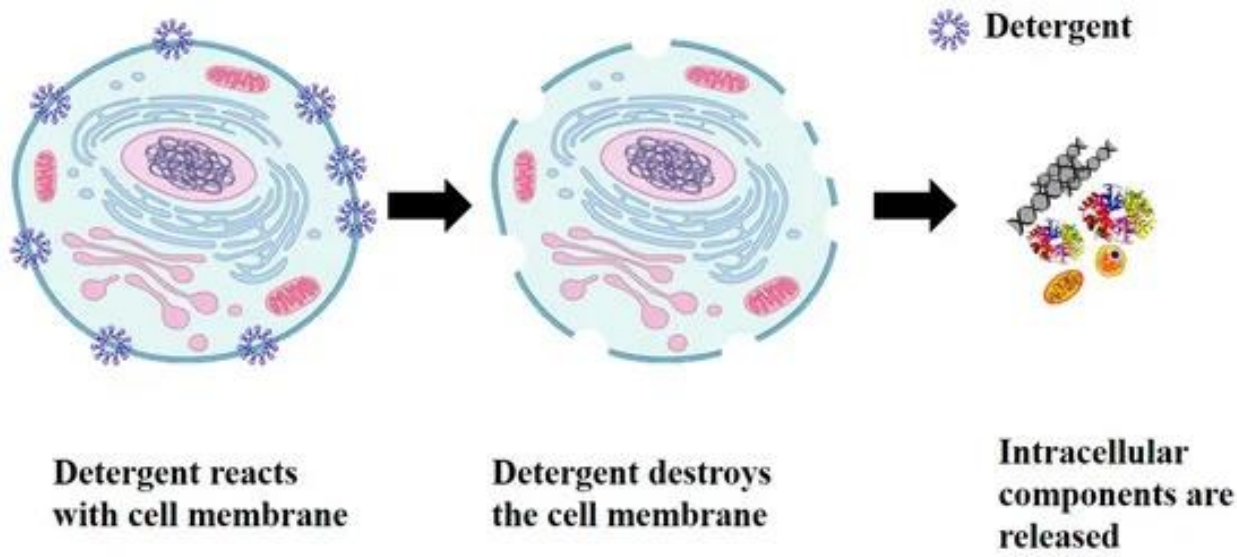


Figure 64- Cell lysis is an important step of the DNA extraction process that works to open the cell membrane and release the intracellular components. Enzymatic lysis buffer can help to increase the bacterial DNA concentration that is extracted (Shehadul Islam et al. 2017).

Lysis buffer detergent ingredients:

- 0.5M Tris–HCl, pH 8.0
- 0.5M EDTA
- 1.2% Triton
- Distilled water
- 20 mg/mL lysozyme powder

For 1 liter of lysis buffer:

1. Add 40 mL 0.5M Tris-HCL and 2 mL 0.5M EDTA and 946 mL of distilled water to a sealable glass bottle.
2. Mix well and autoclave or use a pressure cooker to sterilize.
3. Allow to cool to room temperature after autoclaving or pressure cooking. **Add triton only after completely cooled** (this is done to prevent a foam explosion from occurring!).
4. Store at 4°C until ready to use.
5. Aliquot/measure out enough enzymatic lysis buffer for the extraction batch prior to use (each sample requires 360 µL of lysis buffer) and add it to the correct measure of lysozyme (20 mg/mL powder). **This solution must be made fresh on the day.**
    a. Lysozyme (mg) = (number of samples to be extracted + 4) x (360/1000) x 20. NOTE- it is easier to add the liquid buffer to the small quantity of powder and mix than the other way around. Vortex to mix thoroughly.

2. Specimen Preparation

Preparation of Whole Blood and Plasma and Buffy Coat from Whole Blood

This method is based on whole blood collected in standard EDTA tubes (such as Vacutainer® tubes). Since the concentration of *B. pseudomallei* circulating in whole blood is relatively low, preparation of plasma and/or buffy coat from whole blood is recommended for DNA extraction from *B. pseudomallei*.

Whole blood

1. Mix the blood collection tube by inverting immediately after collection. Inadequate mixing of tubes with anticoagulants may allow micro-clots to form and interfere with extraction. Specimens intended for whole blood analyses should not be centrifuged.

Whole blood specimens stored in Vacutainer® EDTA tubes.

2. Transfer 200 µL of patient whole blood stored in EDTA tubes to a clean, labelled 1.5 mL microcentrifuge tube.

<u>For Plasma and Buffy Coat</u>

1. Centrifuge EDTA blood tubes containing whole blood for 15 minutes at 800xg (2200-2500 rpm). The centrifuged blood should appear similar to the Figure below (Figure 65):

   NOTE- Most blood samples require separation of plasma and buffy coat from cells within two hours of collection.

2. While blood is spinning, clean cabinet and set up workspace in cabinet as described above.

3. Carefully remove the collection tube from centrifuge.
4. Using a disposable transfer pipette, remove most of the plasma (top layer above the buffy coat and red blood cells), taking care not to disturb the buffy coat layer below. Leave approximately 1 cm of plasma above the buffy layer. If the buffy coat layer is disturbed, spin the cells again at 800xg for 2-3 minutes. Store plasma in labelled 2 mL microcentrifuge tubes.

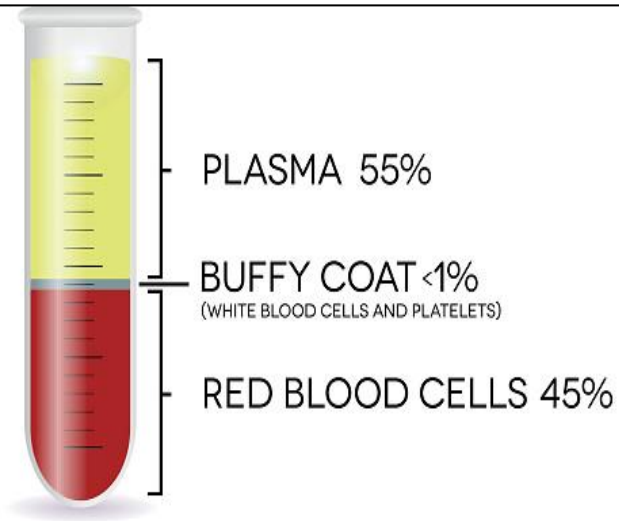


Figure 65- Schematic diagram of a fractioned whole blood sample. The buffy coat layer contains most of the white blood cells and platelets and is located between the plasma and red cells (SP&A Application Laboratory 2014).

5. Using a disposable transfer pipette, remove the buffy coat layer gently and place into a labelled 2 mL microcentrifuge tube. Try not to touch any of the red blood cells. If the buffy coat sample contains visible red cells, spin the 2 mL tubes in a centrifuge at 10,000xg for 30 seconds. Transfer the buffy coat layer into a clean 2 mL tube.
6. Transfer 200 µL of the buffy coat for each patient specimen being extracted into a 2 mL sterile screw cap O-ring microtube.

Body Fluids (e.g., wound abscess, pus)

1. Mix sample by inverting.
2. Transfer 200 µL of sample to sterile 2 mL microcentrifuge tube.

Urine

1. Transfer 200 µL of the sample to a clean 2 mL microcentrifuge tube.

2. Centrifuge the urine sample at 20,000xg/14,000 rpm for 10 minutes.
3. Discard the supernatant.
4. If the urine has already been pelleted than retain the whole pellet for DNA extraction.

Sputum

An initial step containing a sputolysin (such as Calbiochem CAS 578517) pre-treatment is recommended for DNA extractions from sputum specimens. This helps to breakdown the mucin matrix, releasing bacteria that may be trapped within the sputum (Nielsen et al. 2004).

1. Make up sputolysin solution as per manufacturer's instructions.
2. Add equal volume of sputolysin solution to the specimen.
3. Place in a shaking incubator at 37°C and 150 rpm for 20 minutes. If a shaking incubator is not available vortex the solution for 30 seconds to one minute until mixed thoroughly and place in a 37°C incubator for 20 minutes.
4. Centrifuge at 8,000xg for 20 minutes.
5. Discard the clear supernatant.
6. Calculate the volume of the resulting pellet and add 5x the volume of PBS.
7. Vortex to mix thoroughly.
8. Transfer 400 µL of the pellet/PBS solution to a sterile 2 mL microcentrifuge tube.

Rectal Swab

Stool samples and rectal swabs typically contain many compounds that can degrade DNA and inhibit downstream reactions. To ensure removal of these substances, reagents, such as InhibitEX Buffer (QIAGEN Cat No./ID: 19593) are recommended prior to DNA extraction. These work by adhering to inhibitory substances so that they can be removed early in the extraction process (QIAGEN 2010).

1. Add 1 mL InhibitEX Buffer to a 2 mL microcentrifuge tube with O-ring.
2. Using sterile forceps/tweezers and scissors, cut the shaft of the swab ~1.0 cm above the absorbent material. It is good practice to change gloves between samples after this step.
3. Place the cut swab in the 2 mL microcentrifuge tube containing the InhibitEX Buffer.
4. Vortex on high for 1 minute and leave for 10 minutes at room temperature, making sure the entire swab is within the InhibitEX Buffer. Centrifuge for 1 minute at 10,000xg.
5. Transfer 400 µL of the supernatant to clean 2 mL microcentrifuge tube.

All Other Swab Specimens (E.g., Nose, Throat, Wound, etc.)

1. Add 400 µL saline (0.9% NaCl) or PBS solution to a 2 mL microcentrifuge tube.
2. Using sterile forceps/tweezers and scissors, cut the shaft of the swab ~1.0 cm above the absorbent material. It is good practice to change gloves between samples after this step.
3. Place the cut swab in the 2 mL microcentrifuge tube containing the saline or PBS.
4. Vortex on high for 1 minute and leave for 10 minutes at room temperature, making sure the entire swab is within the saline or PBS. Centrifuge for 1 minute at 10,000xg.
5. Transfer 400 µL of the supernatant to clean 2 mL microcentrifuge tube.

Bacterial Cultures-

1. Culture the specimen as described previously in Chapter 10 on Ashdown agar or other *B. pseudomallei*-selective agar and incubate at 37^{o}C for 48 hours.
2. After 48 hours, transfer a small amount of the culture (approximately half (1/2) of a 10 µL disposable loop) to a 2 mL sterile screw-cap tube.
3. Add 360 µL enzymatic lysis buffer containing lysozyme (If using- see components above) to the 2 mL sterile screw-cap tube. Vortex for 30 sec.
4. Place into a 37^{o}C incubator; leave overnight, but not longer than 24 hours.

DNA extracted from bacterial cultures can also be used for downstream genetic typing or whole genome sequencing. However, it is important to ensure that the culture is pure and contains only a single *B. pseudomallei* isolate. To ensure it is a pure culture:

- From the initial culture plate, use a sterile loop to select a single colony suspected (or already confirmed) to be *B. pseudomallei*.
- Ensure that only the bacteria colony of interest is touched.
- Subculture the single colony to Chocolate or Ashdown agar and incubate at 37°C for 48 hours.
- After 48 hours, transfer a small amount of the culture (approximately half (1/2) of a 10 µL disposable loop) to a 2 mL sterile screw-cap (O-ring) tube.
- Add 360 µL enzymatic lysis buffer containing lysozyme to the 2 mL sterile screw-cap tube. Vortex for 30 sec.
- Place into the 37°C incubator; leave overnight, but not longer than 24 hours.

NOTE- DNA extracted from bacterial cultures can be from clinical or environmental specimens. **However, because of the increased volume/concentration of bacteria present when culturing specimens, DNA extraction from cultures that are already confirmed or suspected to be *B. pseudomallei* should be handled in a BSL-3 laboratory until the bacteria has been effectively lysed and is no longer viable/infectious (usually after incubation with Buffer AL).**

See more about appropriate biosafety techniques for handling *B. pseudomallei* culture specimens and additional information about BSL-3 laboratory specifications available from the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition available at: https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2020-P.pdf.

3. *B. pseudomallei* Clinical Specimen DNA Extraction- Manual Kit Procedure

NOTE- The following protocol is based on the QIAamp DNA Mini Kit. It is a summary of the procedures included in the kit manual provided by the manufacturer. Please refer to the manual for detailed product information and protocols: https://www.qiagen.com/us/resources/resourcedetail?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en

Similar well-validated kits that have been shown to yield a comparable concentration of high-quality genomic DNA (as described at the beginning of the chapter) from the same volume of specimen can be used if QIAamp is not available. Likewise, in resource-poor laboratories, DNA from clinical specimens can be extracted using Chelex®100 resin beads. Chelex® extraction can be done directly on bacterial cultures grown using selective enrichment media (Ashdown or TBSS-C50 as described in Chapter 10) or from bacterial growth on agar plates. See below for Chelex® 100 extraction details and protocols (de Lamballerie et al. 1992).

Manual Kit Extraction Protocol

1. Place 360 µL of enzymatic lysis buffer (if using; see above) into a 2 mL microcentrifuge tube.
2. Add specimen (amount depends on specimen type; see specifications above). **For culture specimens, the buffer should be added the day before (as above) and incubated overnight.**
3. Vortex samples for 30 seconds.
4. Incubate tubes in a heat block at 37°C for 30-60 mins (this does not need to be done for culture specimens as they will have been in the incubator overnight).
5. Briefly centrifuge tubes at 10,000xg for 30 seconds.
6. Add 20 µL Proteinase K and then 200 µL AL Buffer.

7. Vortex for 30 seconds and briefly centrifuge at 10,000xg for 30 seconds.
8. Incubate tubes using a preheated heat block or water bath at 56°C for 1-2 hours.
9. Briefly centrifuge tubes at 10,000xg for 30 seconds.
10. Add 200 µL of 100% ethanol.
11. Vortex for 30 seconds and briefly centrifuge at 6,000xg for 30 seconds.
12. Load 550 µL of the sample onto the spin column.
13. Centrifuge column at 20,000xg (~14,000rpm) for 1 minute. If liquid has not entirely gone through the column then spin again.
14. Place the spin column in a clean 2 mL collection tube.
15. Repeat steps 12-14 until the entire sample has been loaded into the column.
16. Add 500 µL of Buffer AW1 to the center of the column filter. Try not to wet the rim of the tube.
17. Centrifuge column at 20,000xg (~14,000 rpm) for 1 minute. If liquid has not entirely gone through the column then spin again.
18. Place the spin column in a clean 2 mL collection tube.
19. Add 500 µL of Buffer AW2 to the center of the column filter. Try not to wet the rim of the tube.
20. Centrifuge column at 20,000xg (~14,000 rpm) for 1 minute. If liquid has not entirely gone through the column then spin again.
21. Place the spin column in a clean 2 mL collection tube.
22. Centrifuge column at max speed (20,000xg/~14,000 rpm) for 3 mins to dry the column.
23. Place the spin column in a clean 2 mL collection tube.

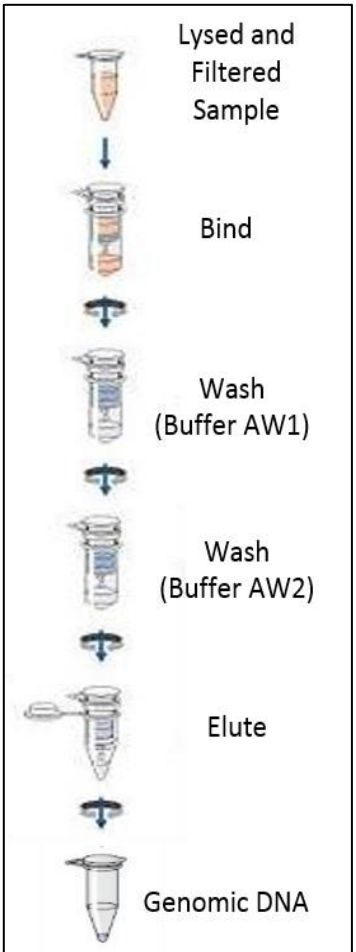


Figure 66- Flow chart of DNA extraction procedure (QIAGEN 2016).

24. Add 200 µL AE Buffer or distilled water to the spin column.
25. Leave at room temperature for 10 minutes.
26. Centrifuge at 6,000xg for 1 minute.
27. Re-load the 200 µL of eluate onto the same column to maximize the concentration of DNA recovered. Leave at room temperature for 10 minutes.
28. Centrifuge at 6,000xg for 1 minute. Retain collection tube and discard column.
29. Transfer DNA solution to a new labelled 1.5 mL screw cap tube and store at 4°C.
30. Disinfect all bench tops, centrifuge, vortex, pipettes and cabinet with a 10% bleach solution or DNAerase and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.

B. pseudomallei Environmental Sample DNA Extraction Protocols Using Commercial DNA Extraction Kits

Direct DNA extraction from soil and sediment is especially challenging as particles can reduce the extraction efficiency of commercial kits (Kaestli et al. 2007; Young et al. 2014). The quality and quantity of DNA extracted is decreased by incomplete cell lysis in the soil mixture and also DNA adherence to soil particles (Krsek & Wellington 1999). Additionally, humic substances present in most organic material are strong inhibitors of PCR (Tsai & Olson 1992). A detection method that combines bacterial culture using selective enrichment broth, followed by DNA extraction and real-time PCR has been shown to be the most robust method of detection for *B. pseudomallei* in environmental specimens (Dance et al. 2018; Kaestli et al. 2007). Protocols combining bacterial culture and commercial kit-based DNA extraction methods for the detection of *B. pseudomallei* in soil, water and air specimens are described below. The protocols are based on the DNeasy PowerSoil kit (QIAGEN Cat# 12888-100). Please refer to the manual below for detailed product information and protocols:

https://www.qiagen.com/au/resources/resourcedetail?id=5a0517a7-711d-4085-8a28-2bb25fab828a&lang=en

Figure 67- QIAGEN DNeasy PowerSoil Kit with kit components and reagents.

Similar well-validated kits that have been shown to yield a comparable concentration of high-quality genomic DNA from the same volume of specimen can be used if PowerSoil kit is not available. Likewise, in poorly-resourced laboratories, DNA from environmental samples can be extracted using Chelex® 100 resin beads. This can be done directly from bacterial cultures using selective enrichment media (Ashdown or TBSS-C50 as described in Chapter 10) or from bacterial growth on agar plates. See below for details and protocols (de Lamballerie et al. 1992).

Solid-phase *B. pseudomallei* DNA Extraction from Environmental Samples

Equipment and Reagents-

- Vortex Adapter for 24 tubes (1.5-2 mL) (cat. no. 13000-V1-24)
- Vortex-Genie® 2 Vortex
- Centrifuge for 50 mL conical centrifuge tubes as well as 2mL tubes
- BSC
- Heat block or water bath set to 70°C
- Shaking incubator or standing/benchtop incubator set to 37°C
- DNeasy PowerSoil Kit (QIAGEN Cat# 12888-100)
- 50 mL conical centrifuge tube (e.g., Falcon tube)
- 2 mL Centrifuge filter columns (such as Microcon YM-100 Columns or Millipore Ultrafree-MC Centrifugal Filter Devices, 0.1 µm)
- 1.5 mL microcentrifuge tubes (e.g. Axygen™ MCT200LC or Eppendorf™ Snap-Cap Microcentrifuge Safe-Lock™ Tubes)
- Pipette and pipette tips
- Disposable culture loops
- Ashdown or TBSS-C50 Broth (see Chapter 10 for components)
- Aurintricarboxylic acid (ATA- see below for components)
- Proteinase K (20 mg/mL- e.g., ThermoFisher Cat# AM2546)
- KayDry or Kimwipe tissues or similar lab towels
- Biohazard waste disposal (bags and pans)
- Tape or lab Parafilm®
- Waterproof labelling marker
- Distilled water
- 10% bleach, made fresh daily (this is a dilution of household bleach and water, be sure to check the concentration of hypochlorite to determine the correct dilution to use)

- Ethanol, 70%
- PPE (gloves, lab coat, goggles)

Aurintricarboxylic acid (ATA)

While not required, the addition of ATA to soil has been shown to significantly increase DNA yield. ATA is an inhibitor of nucleases and has been successfully used for *B. pseudomallei* DNA extraction from soil (Kaestli et al. 2007).

ATA components-

- ATA (Sigma-Aldrich A1895-5G)
- Distilled water

Protocol-

1. To 500 mL of distilled water: add 1.75 grams (3.5 mg/mL) ATA.
2. Adjust pH to 8 using sodium hydroxide (NaOH).
3. Autoclave or sterilize using a pressure cooker.
4. Aliquot/divide into 50 mL measurements.
5. Freeze at -20°C until ready to use. Ensure the solution is protected from light.

Extraction Controls

Quality extraction controls are important to ensure the procedure is valid and the results are reliable. DNA extraction protocols must include a negative control. Using a known *B. pseudomallei* positive control or an internal extraction positive controls is recommended:

1. Negative control (extraction blank)- This sample contains no sample DNA, only water. This control will ensure that no contamination occurred during the extraction and that any DNA used in the PCR is from the specimen samples only.

2. Positive control- A known *B. pseudomallei* positive control can be run concurrently with samples each time an extraction is performed. Care should be taken to ensure no cross-contamination occurs between the positive controls and the samples or negative controls. Likewise, a standardized internal extraction positive control may also be used (such as BIOLINE #BIO-35028 or Primerdesign™ Precision). When performing DNA extraction, it can be helpful to include an external source of DNA template that is added to the lysis buffer. This is purified alongside the sample DNA and can then be detected as a positive control for the extraction during the PCR run. Its successful amplification can be used to show that PCR inhibitors are not present or are not there in a high concentration.

General Safety and Handling Measures (BMBL 6th ed. 2020)

1. Samples should be handled at the BSL-2 safety level.
2. Do not pipette by mouth.
3. Wear disposable powder-free gloves, lab coat and goggles while handling reagents or specimens. Wash hands thoroughly after performing the test.
4. Before start of sample processing, prepare aliquots of all buffers and solutions to use per batch of samples.
5. All materials used should be disposed of so that infectious agents are appropriately inactivated:
    a. Solid wastes should be autoclaved or sterilized using a pressure cooker if an autoclave is not available.
    b. All wipes/cleaning material should be treated as hazardous waste.
    c. Spills should be wiped with a 10% bleach solution. The area should be covered with absorbent material, soaked with a 10% bleach solution and allowed to stand for at least 20-30 minutes.
6. Do not use the reagents beyond the expiration date.

7. Extraction procedure and Quality Control protocols should be followed closely to achieve reliable results.
8. It is important to pipette the exact reagent volume written in the protocol and to mix thoroughly after each reagent is added. Failure to do so could result in inaccurate results.
9. Do not interchange equipment or reagents from different kits.

B. pseudomallei **DNA Extraction Protocol from Soil Samples**

A. Preparation of Soil Samples for Bacterial Culture in Selective Enrichment Broth

1. Prepare and label for each soil sample:
    A. 3x 50mL labelled conical centrifuge tubes
    B. 1x power bead tube
    C. 7x 2mL collection tubes
    D. 1x spin filter
2. Weigh 20 grams of soil into a 50 mL labelled conical centrifuge tube (e.g., Falcon tube).
3. Add 20 mL of Ashdown or TBSS-C50 selective enrichment broth to each falcon tube.
4. Mix thoroughly using a vortex for 30 seconds.

5. Place 50 mL tubes in a shaking incubator and shake for a minimum of 24-48 hours (samples may be left in the incubator up to 7 days before being processed) at 37˚C at 220 rpm. If a shaking incubator is not available vortex tubes thoroughly and place in a 37°C standing or bench top incubator. Try to keep the time in the incubator consistent for all samples.

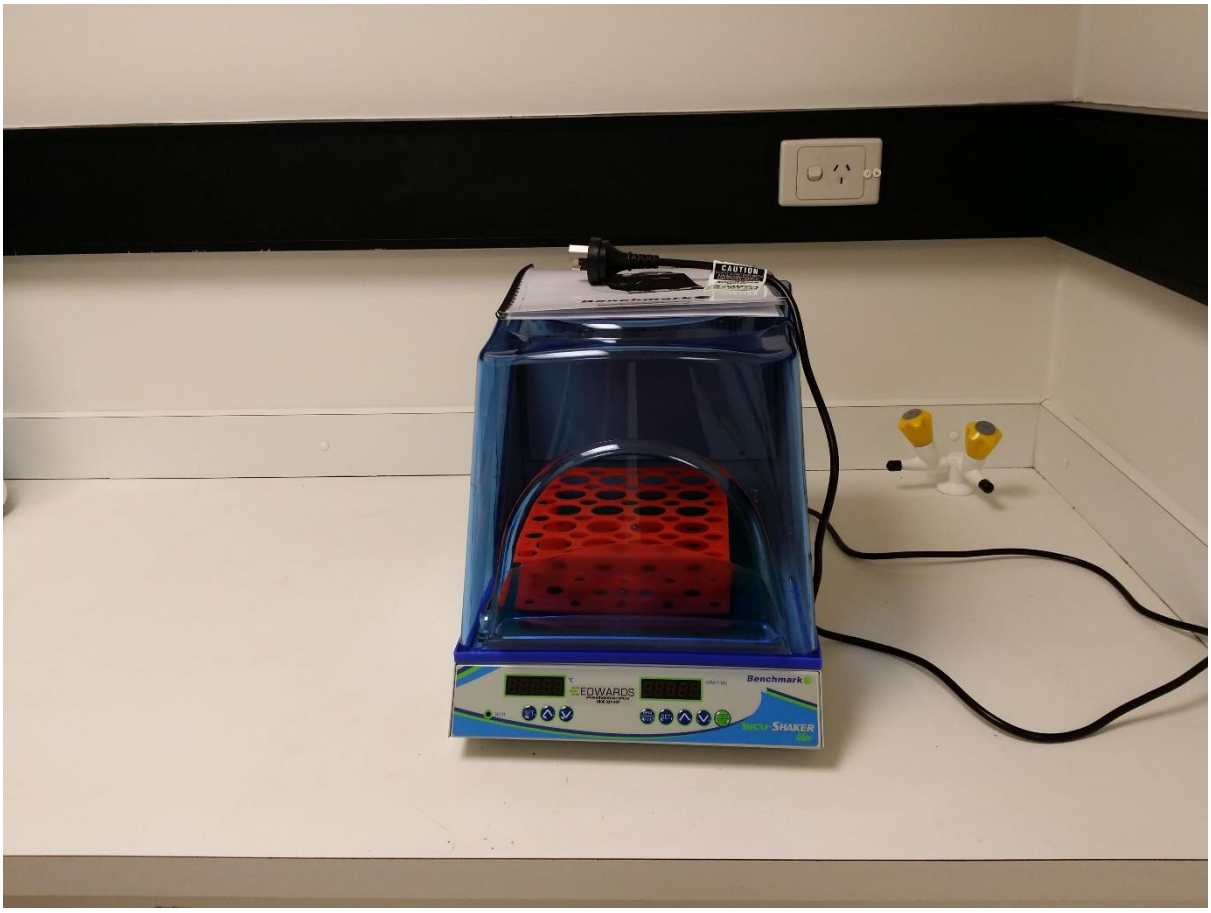


Figure 68- Example of a shaking incubator containing a fitted tube rack for 50 mL conical centrifuge tubes.

B. Soil Sample Processing

1. Remove soil samples from the incubator.
2. Pour the supernatant into a 50 mL labelled conical centrifuge tube.
3. Centrifuge the tube at 4,300xg for 30 seconds.
4. Transfer the supernatant into a clean 50mL tube containing 150 µL of ATA (if using; see above).

5. Seal the lid with tape or laboratory Parafilm®.
6. Centrifuge at 4,300xg for 45 minutes.
7. Discard the supernatant being careful not to disturb the pellet - if there is only a small soil pellet, leave a thin layer of supernatant on top of the pellet.
8. Pour contents of the PowerSoil PowerBead tube into the 50 mL conical centrifuge tube containing the soil pellet. Resuspend pellet with a clean loop and carefully transfer all back into the PowerBead tube. Discard the empty 50 mL tube.
9. Add 60 µL of Solution C1 to the sample and vortex briefly. If Solution C1 has precipitated, heat at 60°C until the precipitate dissolves.
10. Incubate the sample for 10 min at 70˚C in a heat block or water bath and transfer to ice for 2 minutes.
11. Add 20 µL of proteinase K (20 mg/mL) and incubate for 30 minutes in a heat block or water bath at 56˚ C.
12. Secure PowerBead Tubes horizontally using

Figure 69- Vortex-Genie 2 Vortex and the fitted vortex adaptor. The platform has been designed specifically for vigorous mixing and homogenization of multiple samples simultaneously.

a Vortex Adapter for 24 (1.5–2.0 mL) tubes (cat. no. 13000-V1-24). See Figure 69 above for an example the fitted vortex adapter and tube positioning.

13. Vortex at maximum speed for 20 min. Vortexing is critical for complete homogenization and cell lysis. Use of the Vortex Adapter will maximize sediment homogenization, which can lead to higher DNA yields. If this platform is not available, vortex EACH specimen vigorously for 1-2 minutes.
14. Centrifuge tubes at 10,000xg for 30 seconds.
15. Transfer the supernatant to a clean labelled 2 mL collection tube.
16. Add 250 µL of Solution C2 and vortex for 5 seconds. Incubate at 2–8°C for 5 minutes.
17. Centrifuge the tubes at 10,000 x g for 1 minute.
18. Avoiding the pellet, transfer up to 600 µL of supernatant to a clean 2 mL collection tube.
    Note: The pellet contains non-DNA organic and inorganic material including humic acid, cell debris and proteins. To improve DNA quality, avoid transferring any of the pellet.
19. Add 200 µL of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
20. Centrifuge the tubes for 1 minute at 10,000 x g.
21. Transfer up to 750 µL of supernatant to a clean 2 mL collection tube, ensuring the pellet is not touched.
22. Mix Solution C4 by shaking thoroughly and add 1200 µL to the supernatant. Use a larger 50 mL tube to combine solution C4 with the supernatant from the previous step if necessary. Vortex for 5 seconds.
23. Load 675 µL onto the Spin Column and centrifuge at 10,000xg for 1 min. Discard the flow-through.
24. Repeat Step 23 until all of the sample has been processed.
25. Add 500 µL of Solution C5. Centrifuge for 30 seconds at 10,000xg.
26. Discard the-flow through. Centrifuge again for 1 min at 10,000xg.

NOTE- It is important that all traces of wash solution are removed as the ethanol in Solution C5 can inhibit downstream applications such as PCR and gel electrophoresis.

27. Carefully place the Spin Column into a clean 2 mL Collection Tube.
28. Add 100 µL of Solution C6 to the center of the white filter membrane to wet the membrane.
29. Centrifuge at room temperature for 30 seconds at 10,000xg. Discard the Spin Column ensuring the eluate (flow-through) is retained (this is the DNA).
30. The DNA is now ready for downstream applications or can be stored at 4˚C or frozen (-20°C to -80°C) for longer-term storage.

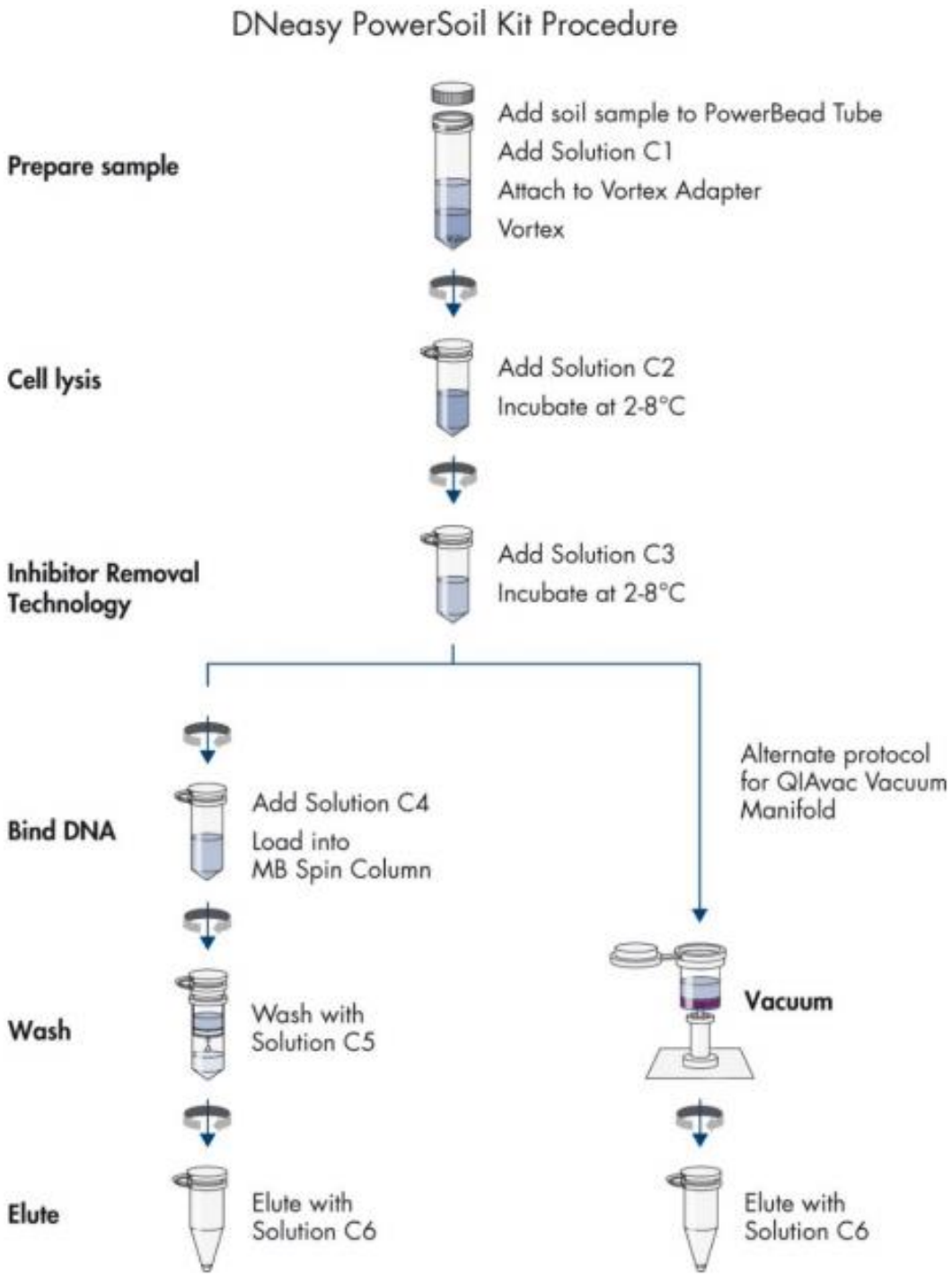


Figure 70- QIAGEN DNeasy PowerSoil protocol using spin columns (left; described above) and QIAvac Vacuum Manifold tubes (right; not described) (QIAGEN 2017).

***B. pseudomallei* DNA Extraction Protocol from Water Samples**

A. Preparation of Water Samples for Bacterial Culture in Selective Enrichment Broth

1. Prepare and label for each water sample:
    A. 3x 50mL labelled conical centrifuge tubes
    B. 1x power bead tube
    C. 7x 2mL collection tubes
    D. 1x spin filter
2. Filter 500 mL to 1L of water collected for each sample through 0.2-µm-pore-size, 47-mm-diameter cellulose acetate filters using an electrical, vacuum, or manual hand pump as described in Chapter 10.
3. Place the filter in 30 mL of selective enrichment broth (Ashdown or TBSS-C50) in a sterile screw-top container.
6. Vortex for 15 seconds with the lid on and place the container in a shaking incubator for 24-48 hours (up to 7 days) at 37˚C at 220 rpm. If a shaking incubator is not available vortex and place containers containing filters in a 37°C standing or bench top incubator for 24-48 hours. Try to keep the time in the incubator consistent for all samples.

B. Water Sample Processing

1. After 48 hours transfer the samples to a BSC. Carefully pour the enrichment broth into to a sterile labelled 50 mL conical centrifuge tube and spin at 4,300×*g* for 30 seconds.
2. Transfer the supernatant to a clean labelled 50 mL conical centrifuge tube and spin at 4,300x*g* for 45 minutes.
3. Discard the supernatant.
4. Transfer the pellet to a clean labelled 2 mL screw-cap centrifuge tube.
5. Follow steps 8-30 of the DNA extraction protocol for soil samples as above.

***B. pseudomallei* DNA Extraction Protocol from Air Samples**

A. Preparation of Air Samples for Bacterial Culture in Selective Enrichment Broth

1. Prepare and label for each water sample:
    a. 3x 50mL labelled conical centrifuge tubes
    b. 1x power bead tube
    c. 7x 2mL collection tubes
    d. 1x spin filter
2. Place the air filter in 30 mL of selective enrichment broth (Ashdown or TBSS-C50) in a sterile screw-top container as described in Chapter 10.
3. Vortex for 15 seconds with the lid on and place the container in a shaking incubator for 24-48 hours (up to 7 days) at 37˚C at 220 rpm. If a shaking incubator is not available vortex and place containers containing filters in a 37°C standing or bench top incubator for 24-48 hours. Try to keep the time in the incubator consistent for all samples.

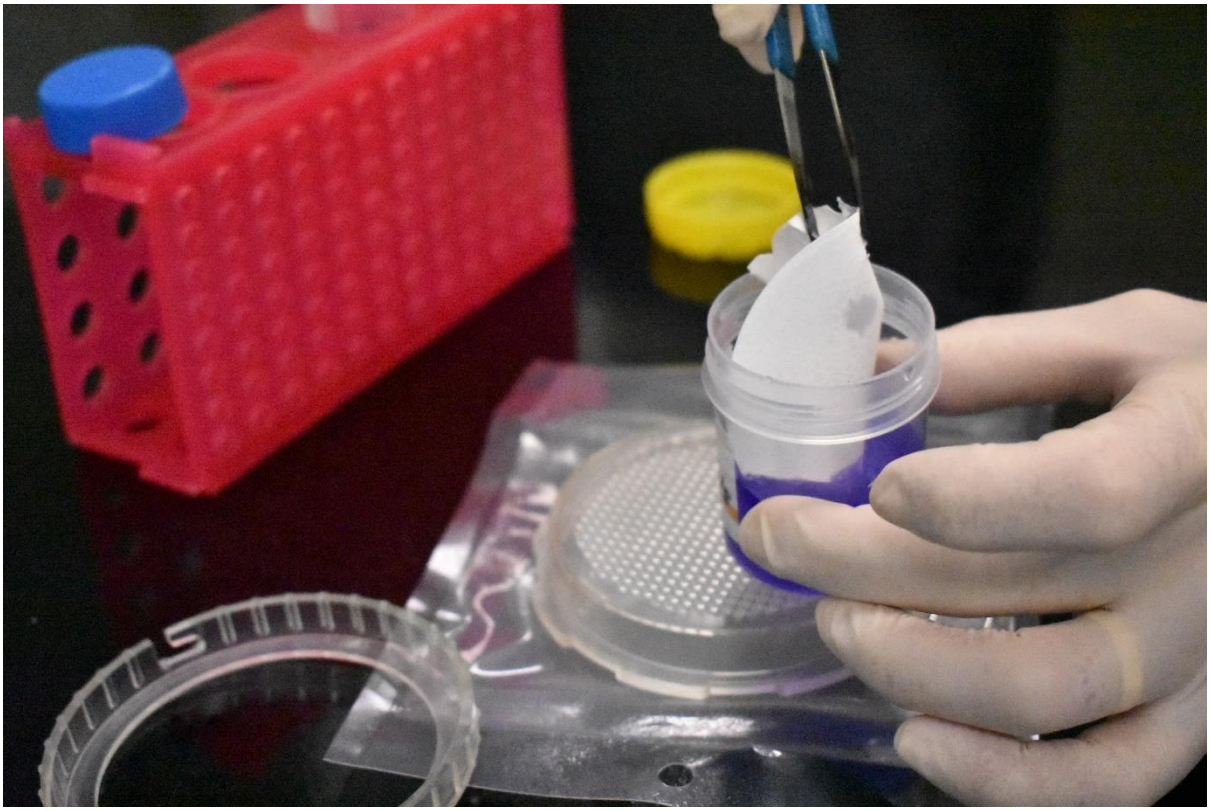


Figure 71- The air filter should be incubated in 30 mL of selective enrichment broth (Ashdown or TBSS-C50) in a sterile screw-top container prior to beginning the DNA extraction protocol.

B. Air Sample Processing

4. After 48 hours transfer the samples to a biosafety cabinet. Carefully pour the enrichment broth into to a sterile labelled 50 mL conical centrifuge tube and spin at 4,300×*g* for 30 seconds.
5. Transfer the supernatant to a clean labelled 50 mL conical centrifuge tube and spin at 4,300x*g* for 45 minutes.
6. Discard the supernatant.
4. Transfer the pellet to a clean labelled 2 mL screw-cap centrifuge tube.
5. Follow steps 8-30 of the DNA extraction protocol for soil samples as above.

Chelex® 100 DNA Extraction from *B. pseudomallei* Bacterial Cultures

Chelex® 100 is a chelating material (a compound that binds tightly to metal ions) that can be used as a method of DNA extraction for downstream PCR analysis. Chelex® extraction works by adding Chelex® resin beads to a specimen, heating the solution, then vortexing and centrifuging the solution. The cellular materials bind to the Chelex® beads, while the DNA is available in the supernatant. DNA extraction procedures utilizing Chelex® 100 are simple, rapid, involve no toxic organic solvents, and do not require large amounts of reagents or expensive equipment. As a result, it is a more cost-effective approach to DNA extraction compared to commercial silica-membrane based kits (e.g. QIAamp DNA Mini Kit) (de Lamballerie et al. 1992; Walsh et al. 1991).

There are several drawbacks of Chelex® DNA extraction that should be noted. The high heat steps involved in Chelex® extraction denature the DNA double helix, resulting in single-stranded DNA that is less stable and degrades quickly. As a result, Chelex®-extracted DNA should only be used for PCR-based analyses and not for other applications such as genetic sequencing. It should also not be stored long-term (no longer than 1-2 months is recommended). Additionally, the concentration of

DNA generated by this method is often lower than for other extraction methods and normally contains suspended impurities that inhibit downstream PCR (Butler 2005; de Lamballerie 1992). Consequently, the method is best suited to sample types containing comparatively high concentrations of *B. pseudomallei*, such as bacterial agar and broth culture samples.

Despite this, Chelex® 100 remains a simple and inexpensive method of bacterial DNA extraction, particularly when used for rapid PCR diagnosis of bacterial culture samples. It may be particularly well-suited to resource-poor diagnostic laboratories that do not have access to more expensive commercial kit-based DNA extraction reagents and equipment. **However, if a lab is going to conduct Chelex® extraction, they must follow standard protocols and adhere to the laboratory guidelines and reagents detailed below.**

See the Chelex® 100 instruction manual for further detail: https://www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT200.pdf.

Chelex® 100 DNA Extraction Procedures for Bacterial Cultures and Enrichment Culture Broth

Equipment and Reagents

- Chelex® 100 Resin- Mesh size 100-200 (Bio-Rad #1422825) (Figure 72)
- 150 mL glass beaker
- Magnetic stir bar
- Stir plate
- Distilled water
- Pipettes and pipette tips

- Heat block or water bath set to 95°C
- 2 mL flat bottom screw-cap microtubes (e.g., Fisherbrand™ Free-Standing Microcentrifuge Tubes with Screw Caps)
- 1.5 mL closable microtubes (e.g., Axygen™ MaxyClear Snaplock Microtubes or Eppendorf™ Snap-Cap Microcentrifuge Safe-Lock™ Tubes)
- Waterproof labelling pen
- Vortex mixer
- Microcentrifuge
- PPE (lab gloves, lab coat, googles)
- BSC
- 10% bleach solution or DNAerase
- 70% ethanol

Figure 72- Chelex® 100 Resin- Mesh size 100-200 from Bio-Rad.

1. Preparing Chelex® 100 Resin solution:

a. To a sterile beaker containing a clean magnetic stir bar on top of a stir plate:
   - Add 5 grams of Chelex® 100 resin to 50 mL of sterile water.
   - NOTE– Ensure the Chelex® mesh size is 100-200 and not 200-400 as specified on the bottle.

b. Aliquot/measure 250 µL into 2 mL flat-bottom screw-cap tubes. Ensure the stir plate is turned so there is adequate mixing and dispersal of resin beads as they are dispensed into each tube.

c. Store tubes at 4°C until needed.

2. Chelex® 100 DNA Extraction Protocol

From Enrichment Broth

1. Culture the clinical or environmental specimen in *B. pseudomallei*-selective enrichment broth (Ashdown or TBSS-C50 media) as described in Chapter 10.
2. Incubate the enrichment broth for 48 hours at 37°C.
3. In a BSC, pipette 1 mL of the broth and place it in a labelled 2 mL microcentrifuge tube.
4. Centrifuge the tube at max speed (20,000xg/~14,000rpm) for 5 minutes.
5. Remove 900 µL of the supernatant.
6. Resuspend the pellet and add it to the previously prepared 250 µL Chelex resin in labelled screw-cap tubes.
7. Vortex the tubes for 15 seconds.
8. Incubate the tubes at 95°C for 20 minutes using a heat block or water bath.
9. Vortex samples for 15 seconds.
10. Centrifuge the tubes at max speed (20,000xg/~14,000rpm) for 10 minutes.
11. Transfer 100 µL DNA solution to a new labelled 1.5 mL closable microtube (such as an Eppendorf tube) and store at 4°C.

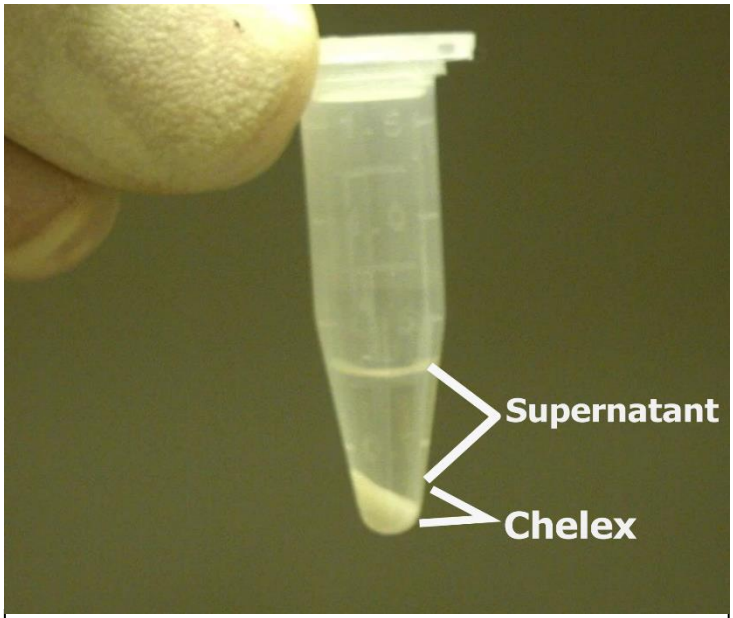


Figure 73- After heating and centrifugation, the DNA is available in the supernatant while Chelex® beads retain the cellular debris.

12. Disinfect all bench tops, centrifuge, vortex, pipettes and Biosafety cabinet with a 10% bleach solution (or DNAerase) and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.

The extracted Chelex® 100 DNA is not high quality and PCR screening should be performed within a month. Ensure no Chelex bead resin is present in the final DNA solution, as these can inhibit PCR reactions.

From Agar Culture Plates-

1. Culture the specimen on *B. pseudomallei* selective agar as described in Chapter 10 to achieve single colonies. Incubate plate for 48 hours at 37°C.
2. After 48 hours incubation, transfer some of the agar plate culture (approximately ½ of a loop of a 10 µL disposable loop) to the previously prepared 250 µL Chelex® 100 slurry in 2 mL screw-cap flat bottom tube in a Biosafety cabinet. Use the same loop to mix culture.
3. Vortex the tubes for 15 seconds.
4. Incubate the tubes at 95°C for 20 minutes using a heat block or water bath.
5. Vortex samples for 15 seconds.
6. Centrifuge the tubes at max speed (20,000xg/~14,000rpm) for 10 minutes.
7. Transfer 100 µL DNA solution to a new labelled 1.5 mL closable microtube (such as an Eppendorf tube) and store at 4°C.

8. Disinfect all bench tops, centrifuge, vortex, pipettes and Biosafety cabinet with a 10% bleach solution (or DNAerase) and wipe dry with a lab tissue. Repeat by spraying working surface with 70% ethanol and wipe dry.

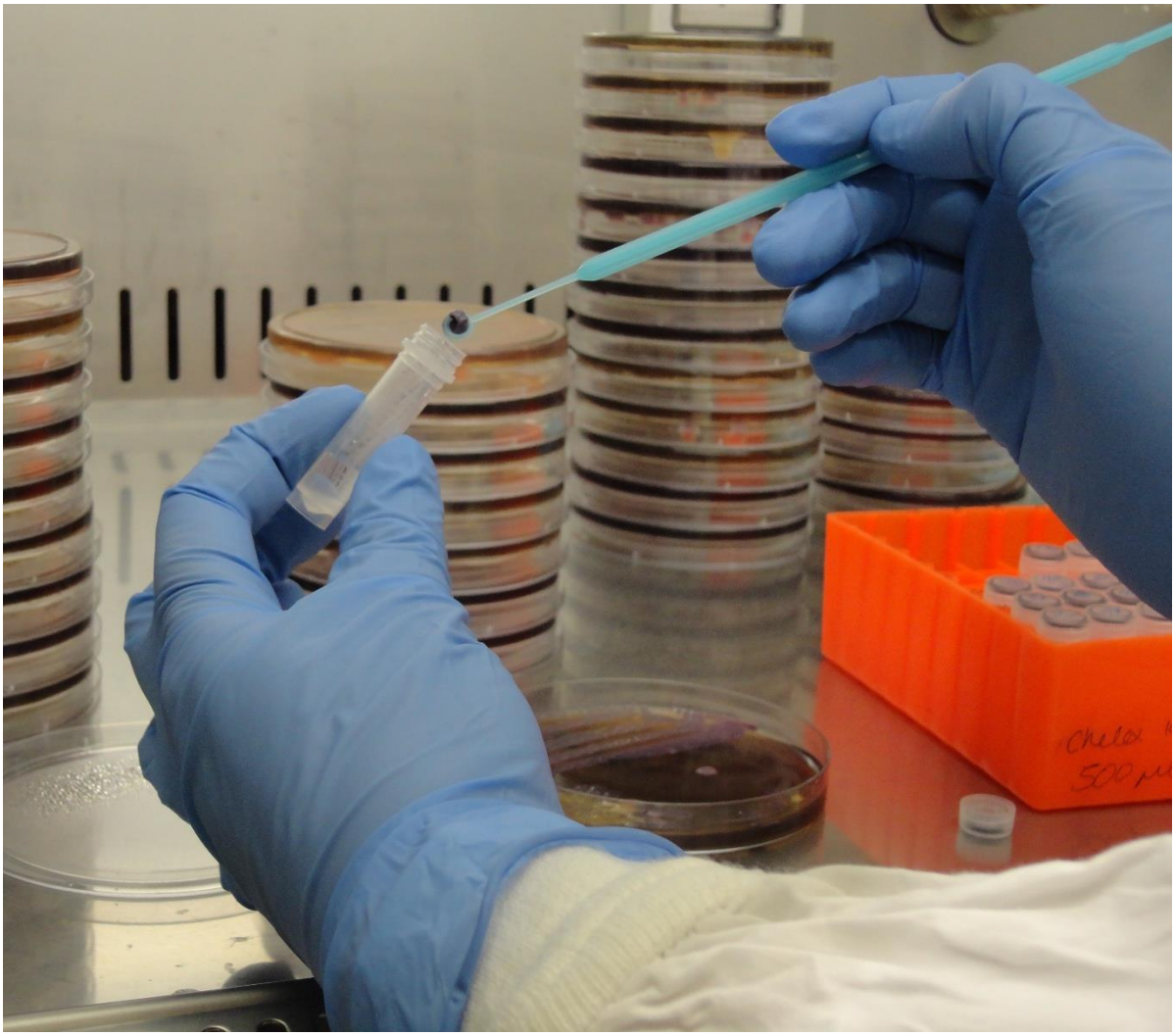


Figure 74- Add a small loopful of culture aseptically to a 2 mL screw-cap tube containing the Chelex® resin bead solution before vortexing and heating the mixture (Image courtesy of Menzies School of Health Research, Australia).

Assessing DNA Sample Purity and Quality/Quantity Control

Examining DNA sample purity, quality, and concentration after it has been extracted is essential to obtaining accurate and reliable results in many downstream molecular biology applications, such as PCR and genetic sequencing. Overestimating the DNA concentration in a sample can lead to too

little input DNA in a molecular reaction or for preparation of a “library” which is DNA prepared in a manner appropriate for whole genome sequencing (WGS). In the case of PCR, too little input DNA can result in weak or no amplification. PCR yield is also reduced if large amounts of RNA are left in the DNA sample, or by inhibitors such as residual EDTA or other negatively charged ions that chelate Mg^{2+}. DNA quality control (QC) usually includes quantitative and qualitative aspects (Bustin 2012; Wahlberg et al. 2012). Parameters monitored typically include:

- Nucleic acid concentration
- Detection of chemical contaminants and other impurities
- Nucleic acid intactness

Measuring DNA Quantity

DNA quantity is normally measured using UV spectroscopy or fluorescence (Brennan et al. 2009). To determine DNA concentrations using the UV-absorbance method, the absorbance of the DNA sample at 260 nm and 280 nm is typically measured using a spectrophotometer. By measuring the amount of light absorbed at a defined wavelength, the concentration of the molecules of interest can be calculated. (Glasel 1995; Olson & Morrow 2012; Wahlberg et al. 2012).

Fluorescence methods determine DNA concentration by using selective DNA binding dyes, such as SYBR Green I (SG), AccuBlue™ or PicoGreen that fluoresce when bound to double-stranded DNA (dsDNA). The fluorescence intensity can then be measured using a fluorimeter or fluorometer (such as the Qubit) (Singer et al. 1997).

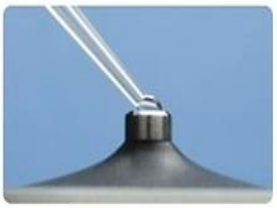
Figure 75- Example of a fluorescence detection methods used to quantify DNA concentration following extraction. The dyes can be used to easily quantify a large number of dsDNA samples (AmiScience 2018).

Measuring DNA Quality (Purity and Intactness)

DNA quality can be assessed in terms of purity, presence of inhibitors and intactness. Purity can be measured using UV spectroscopy, the presence of inhibitors using a PCR inhibition assay, and intactness using gel electrophoresis. UV spectroscopy is used to gauge DNA purity by measuring a sample's absorbance spectrum at 230nm, 260nm and 280 nm, and by measuring the A_{260}/A_{280} and A_{260}/A_{230} ratios (Glasel 1995). These absorbency ratios specify different types of contaminants and the suitability of the DNA for different downstream applications. The A_{260}/A_{280} ratio is a good indicator for PCR suitability. Ratios between 1.8 and 2.0 for A_{260}/A_{280} are used to indicate pure DNA. RNA and protein contamination are denoted by A_{260}/A_{280} ratios that are either above or below 1.8

and 2.0. For the A_{260}/A_{230} ratio the most commonly accepted range is 1.8 to 2.2 and values below this range may be indicative of phenol, salt, protein or polysaccharide contamination (Olson & Morrow 2012). To measure the purity, a specific spectrophotometer, called a NanoDrop, is typically used. These allow scientists to quickly quantify and assess the purity of a small amount (0.5 µL to 2 µL) of DNA or RNA sample (Desjardins et al. 2009).

With the arm open, a sample is pipetted directly onto the pedestal.



After the arm is closed, a sample column is formed.

The pedestal then moves to automatically adjust for an optimal path length (0.05–1mm).

When the measurement is complete, the surfaces are simply wiped with a lint-free lab wipe before going on to the next sample.

Figure 76- A NanoDrop machine is typically used to assess the purity and concentration of extracted nucleic acid. Very small quantities of a sample (0.5-2 µL) are added to the NanoDrop pedestal as shown above, rapidly measured by spectrophotometry and then wiped clean before adding the next sample (ThermoFisher 2021).

PCR inhibitors or impurities can decrease PCR efficiency. These can be examined using an internal inhibition control where known DNA plasmids are added to the extracted DNA and examined using qPCR. An increase in the threshold cycle (C_t) value compared to control reactions typically indicates that PCR inhibitors are present. DNA fragment size and intactness can also negatively affect detection assay results, since the efficiency is reduced if the qPCR target is fragmented, which can prevent amplification from occurring. Gel electrophoresis can be used to evaluate the size of extracted DNA fragments (Olson & Morrow 2012).

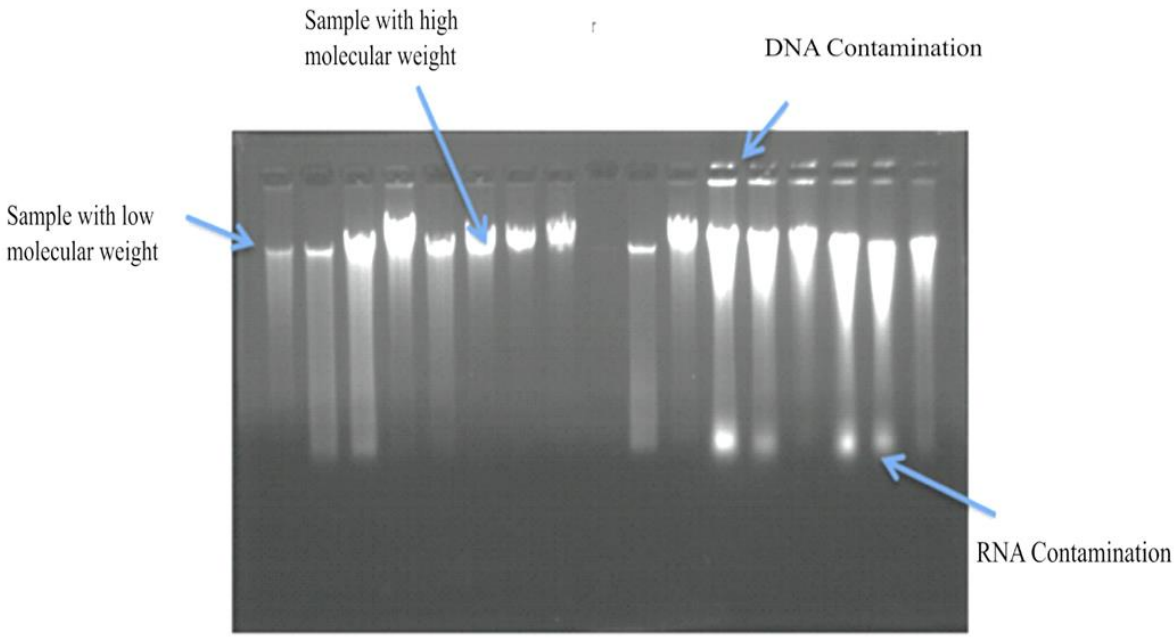


Figure 77- Gel electrophoresis can be used to examine the size of a DNA product and whether there are any PCR inhibitors caused by RNA or DNA contamination.

Chapter Summary

The preceding chapter presents an overview of various DNA extraction processes, DNA extraction quality control, and describes several protocols for DNA extraction from *B. pseudomallei* clinical and environmental specimens. DNA extraction is required for a variety of molecular biology applications and serves as a starting point for PCR diagnosis and detection. Specific protocols describing

conventional and real-time PCR detection of *B. pseudomallei* are discussed in the subsequent chapters.

Chapter 14: Conventional Polymerase Chain Reaction (PCR)

Chapter Overview and Introduction to Conventional PCR

PCR is frequently used in the diagnosis of bacterial pathogens because of its improved sensitivity and its high throughput. A range of PCR assay types exist, each with its own advantages and limitations. Conventional PCR is the most basic type of PCR assay that requires a post-PCR step for detection and visualization of the DNA product (Gal et al. 2005).

Several conventional PCR assays have been developed for the detection and sequencing of bacterial pathogens, including *B. pseudomallei*. Most diagnostic laboratories have now replaced routine conventional PCR testing for real-time PCR methods. However, conventional assays may still be particularly useful in resource-limited settings since the cost is reduced compared to real-time PCR machines and reagents (Garibyan & Avashia 2013). Additionally, conventional PCR still plays an essential role in bacterial sequencing and strain typing. Sequencing and strain typing of *B. pseudomallei* are discussed further in Chapter 16.

Conventional PCR Limitations

With conventional PCR, DNA sequences are amplified and analysis of the product is performed at the end of the PCR reaction. Amplified products need to be visualized with a secondary procedure after the PCR has finished, such as agarose gel electrophoresis or polyacrylamide gel electrophoresis (PAGE). Electrophoresis requires that reaction tubes are opened and handled, increasing the risk of contamination. Conventional PCR is also more time consuming and less sensitive and specific than real-time PCR (ThermoFisher 2012; Smith & Osborn 2009).

Additionally, as with other types of PCR assays, the components and procedures needed to effectively perform conventional PCR testing are very specific and cannot be altered. **If a lab is going to conduct conventional PCR testing, they must follow standard protocols without substitution and adhere to the appropriate laboratory guidelines detailed below.**

B. pseudomallei Conventional PCR Detection

A number of conventional PCR assays have been developed for the identification and detection of *B. pseudomallei* (Table 17). Specific PCR targets have included 23S ribosomal RNA (rRNA) (Lew & Desmarchelier 1994), 16S rRNA (Dharakul et al. 1996), lipopolysaccharide (LPS) (Rattanathongkom et al. 1997), flagellin C (*fliC*) (Sonthayanon et al. 2002), metalloprotease gene (*mprA*) (Neubauer et al. 2007), and the type three secretion (TTS1) system gene cluster of *B. pseudomallei* (Gal et al. 2005; Winstanley & Hart 2000).

Table 17- Conventional PCR targets and primers used for the detection of *B. pseudomallei*.

Target	Primers	Reference
mprA	Bpm-f: 5'-ACTGCTTCGTTCAAGGCGACCGTC-3' Bpm-r: 5'-TGACGGCCTGAACGTCCGCGC-3'	Neubauer et al. 2007
16-23s rDNA spacer (Semi-Nested PCR)	Bp1- 5'-CGATGATCGTTGGCGCTT-3' Bp2- 5'-ACTTACGGGCATCTCA-3' Bp3- 5'-ATTAGAGTCGAACAAT-3' Bp4- 5'-CGTTGTGCCGTATTCCAAT-3'	Inglis et al. 2005
TTS1 (*orf2)*	BPTTSF- 5'-CTTCAATCTGCTCTTTCCGTT-3' BPTTSR- 5'-CAGGACGGTTTCGGACGAA-3'	Winstanley & Hart 2000
fliC	PMA-1- 5'CTGTCGTCGACGGCCGT-3' PMA-2- 5'GGTTCGAGACCGTTTGCG-3'	Sonthayanon et al. 2002
pKKU-S23 LPS	LPS1- 5'-CTCTCAGATTGCTGACAAACCC-3' LPS2- 5'-CGGATGAACTCGAAATCCACCG-3'	Rattanathongkom et al. 1997

16s rDNA	PPM3- 5' AATCATTCTGGCTAATACCCG-3' PPM4- 5' CGGTTCTCTTTCGAGCTCG-3'	Brook et al. 1997
16s rDNA (Nested PCR)	U33- 5'-AAGTCGAACGGCAGCACGG-3' OL731- 5'-TTTGCTCCCCACGCTTTCG-3' BS3L- 5'-ACGGGCTTCGGCTGGTG-3' BS4R- 5'-CACTCCGGGTATTAGCCAG-3'	Dharakul et al. 1996
23s rRNA	PPMA- 5'-GGTAGCCTGCGAAAAGCTACGGGGGAG-3' PPMB2- 5'-CCTGCGCGGAACATGTAACGGGGCT-3' PPMC- 5'-CCACCTGCGTCGGTTTGCGGTACGG-3' PPM2- 5'-CTCTCCTACCATCGAGAC-3'	Lew & Desmarchelier 1994

The current "gold standard" species-specific PCR assay for *B. pseudomallei* is based on the amplification of *orf2* in the type three secretion system 1 (TTS1) cluster. Type three secretion (TTS) systems have been identified in several gram-negative pathogens such as *Shigella* and *Salmonella spp.* as important virulence determinants that allow for bacterial invasion and escape from host cells (Stevens et al. 2003; Winstanley et al. 1999). Several studies have shown that a 548-base pair (bp)-long TTS segment that is part of *orf2* is invariably present in *B. pseudomallei* but not closely related species such as *Burkholderia thailandensis* or *Burkholderia mallei* (Novak et al. 2006; Rainbow et al. 2002; Smith-Vaughan et al. 2003).

Conventional PCR targeting the TTS1 gene cluster is performed using oligonucleotide primers BPTTSF and BPTTSR (see Table 17 above), which exhibit high sensitivity and specificity for *B. pseudomallei* (Gal et al. 2005; Winstanley & Hart 2000). The assay has been shown to be particularly robust for sputum, wound and culture specimens, reflecting the high bacterial load. However, the sensitivity of the assay from blood samples is reduced compared to other clinical specimen types, most likely due to the low concentration of *B. pseudomallei* circulating in whole blood (Wuthiekanun

et al. 2007). Improved sensitivity from blood has been shown when sample DNA is increased from 1 µL to 5 µL (Gal et al. 2005; Tellapragada et al. 2017).

B. pseudomallei TTS1 Conventional PCR Procedures

General Comments Before Starting

PCR is a very sensitive method that that is also very prone to contamination. Precautions should be taken to minimize cross-contamination between samples:

- It is recommended that separate spaces or areas are used where pre- and post-PCR steps are performed (see Chapter 12 for further detail).
- If individual rooms are not possible, separate bench spaces should be used. Separate pipettes, laboratory coats and gloves should be used in each of these areas.
- Working in a PCR laminar flow hood or PCR box is also recommended to minimize cross-contamination.
- Decontamination of surfaces and equipment with 10% bleach solution or commercial DNA decontaminant followed by 70% ethanol should be performed after any handling of DNA.
- Always use filter-barrier pipette tips and change and discard gloves frequently.
- Remove DNA templates, controls and other reagents from the freezer and allow them to thaw completely before use. Briefly vortex and centrifuge tubes for 30 seconds at 1000 rpm before using.
- It is good practice to keep reagents on ice while setting up the reaction plate and PCR master mix to prevent reagent degradation.
- Prior to beginning the PCR, plan the experiment by filling out and printing a plate template worksheet. Ensure PCR reagents are available and have been correctly calculated.

- Primer working stock solutions should be made up to 20 µM before starting. This will help to prevent primer contamination and limit repeat freeze-thaw cycles every time the reaction is performed.

Quality Controls (CLIA 2010; U.S. EPA 2004)

One of the most important aspects of any PCR method is the use of the quality controls to ensure that the procedure has worked correctly, and the results are reliable. Laboratories should analyze positive and negative controls on a routine basis to demonstrate adequate performance of the PCR. Routine PCR controls should include:

1. Negative control- PCR negative controls are used to demonstrate that contamination has not occurred during sample processing. No amplification of DNA should be detected in these controls. Two types of PCR negative controls that should be used include:
    a. Non-template control- PCR grade water should be used as a non-template control (NTC). NTCs should be included in each PCR run. It is advisable to include one NTC for work performed in the clean PCR set-up room/area and one for work performed in the dirty PCR set-up area/room.
    b. DNA extraction blank- This is used to examine if contamination has occurred during sample processing or PCR analysis. DNA extraction is done on sterile water and this is processed alongside the test samples using the same preparation and extraction procedures as the test samples.
2. Positive control- PCR positive controls are used to check that the reagents have been prepared appropriately. They should be run concurrently with each PCR batch. Positive controls are prepared by the addition of an external control to the master mix. An exogenous control can be:
    a. Purified DNA from a known isolate of *B. pseudomallei* containing the sequence of interest. Care must be taken to ensure no cross-contamination occurs between the

positive controls and the samples or negative controls. *B. pseudomallei* control DNA should be diluted in sterile PCR-grade water to minimize the risk of cross-contamination.

b. A specific template that contains the gene sequence of interest.

c. A related template sequence that is amplified with an efficiency that is comparable to the target sequence.

3. Internal positive control (IPC)- The presence of PCR inhibitors or errors during DNA extraction is a common cause of false-negative PCR results and can be easily controlled for by including an Internal Positive Control (IPC). This is used to confirm DNA amplification, detect false negatives, and examine the presence of amplification inhibitors. See Chapters 13 and 15 for further details and protocols.

Equipment and Reagents

- Primers (see below)
- Appropriate positive and negative controls

- 10x PCR Buffer
- 10mM dNTPs Mix
- HotStarTaq DNA Polymerase (QIAGEN)
- PCR-grade water
- 10% bleach or commercial DNA decontaminant
- 70% ethanol
- PPE (gloves, lab coats and safety glasses)
- Laboratory pens/markers
- Racks for 0.2 mL, 1.5mL microcentrifuge tubes and 96-well plate
- Pipettes and filter/barrier pipette tips
- Sterile PCR microcentrifuge tubes
- Benchtop microcentrifuge
- 96-well polypropylene PCR plates (e.g. Thermo Scientific™ AB0600) or PCR strips (e.g. Axygen™ 0.2 mL Polypropylene PCR Tube Strips #16272591)
- PCR strip caps to cover wells
- Vortex
- Thermocycler (such as Eppendorf Mastercycler® or T100™ Bio-Rad)

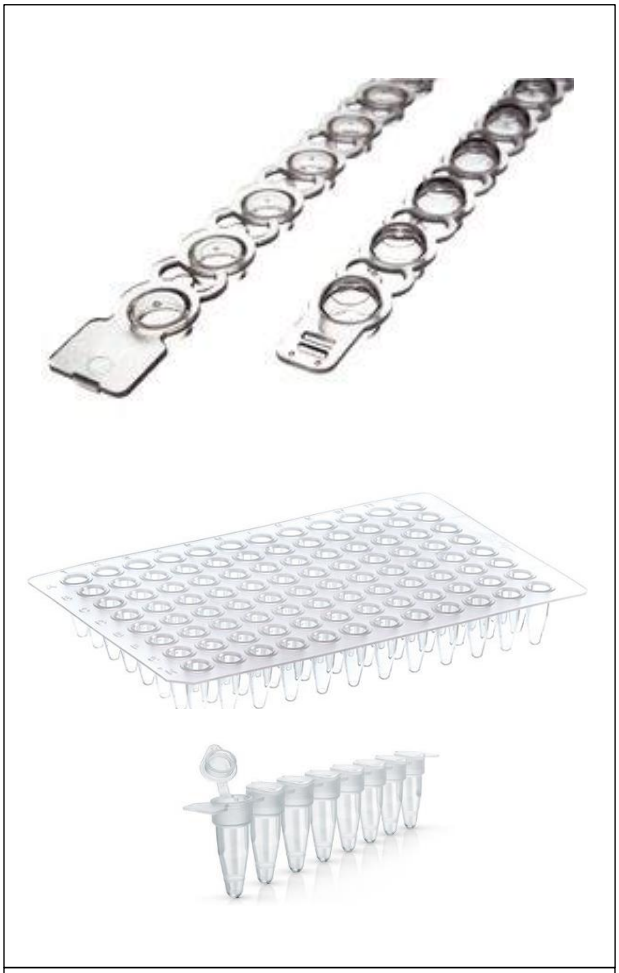


Figure 78- 96-well PCR strip caps, plate and PCR strips (Thermo-Scientific ™).

Primer Sequences-

TTS1 (*orf2*)- 548 base pair length (GenBank AF074878):

1. Forward- BPTTSF- 5'-CTTCAAT CTGCTCTTTCCGTT-3'
2. Reverse- BPTTSR- 5' -CAGGACG GTTTCGGACGAA-3'

Protocol for 25 µL Reaction

Each PCR reaction should contain:

- Deoxynucleoside triphosphates (dNTPs)
- PCR buffer containing $MgCl_2$
- HotStarTaq DNA polymerase (QIAGEN)
- Forward primer
- Reverse primer
- Extracted DNA template from specimen
- PCR-grade water

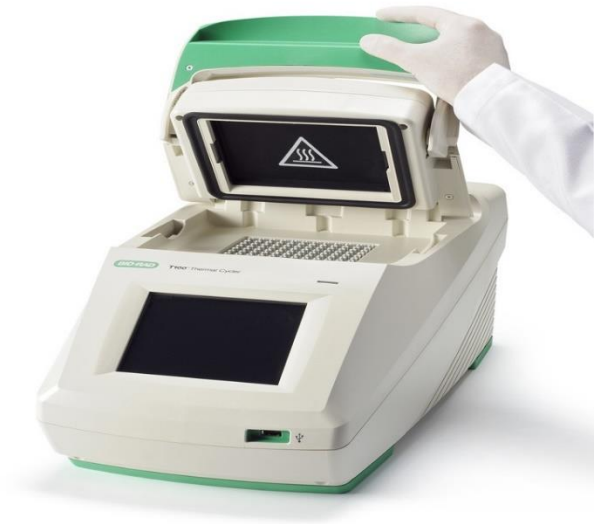


Figure 79- A conventional PCR machine and editable template for a PCR protocol available on the Bio-Rad T100™. The protocol editor displays the protocol in a graphical format using editable boxes (Bio-Rad).

A master mix should be prepared that includes all components listed above **except the DNA template. Prepare two more reactions than the number of specimens to be tested to ensure that adequate master mix is available**, as some will be lost during pipetting.

1. Combine the reagents for the master mix as shown below. Each reagent volume should be multiplied by the number of reactions being run (+2 extra reactions to account for pipetting loss) (QIAGEN HotStarTaq PCR Handbook).

Component	For 25 µL Reaction	Final Concentration
10X PCR Buffer	2.5 µL	1X
10 mM dNTP Mix	0.5 µL	200 µM of each dNTP
20 µM Forward Primer	0.625 µL	0.5 µM
20 µM Reverse Primer	0.625 µL	0.5 µM
HotStarTaq DNA Polymerase	0.125 µL	2.5 Units/reaction
PCR-grade Water	15.625µL	
DNA Template	5 µL	

2. Gently mix the master mix then briefly spin in a centrifuge.
3. Pipette 20 µL of the master mix into each well of a 96-well plate or PCR strips based on the template worksheet.
4. Cover the wells using cap strips or a plate seal. Spray down the clean workspace with 10% bleach or DNA decontaminant and repeat with 70% ethanol. Remove lab coat and gloves and put on a new pair of gloves. Take the 96-well plate or PCR strips to the DNA template addition area.
5. Put on new laboratory coat. Very carefully remove the cover from the 96-well plate or PCR strips. Open these slowly to prevent aerosolization and spraying of any liquid in the wells.
6. Add 5 µL of template DNA and extraction controls to the appropriate well of the 96-well plate or PCR strip based on the template worksheet.
7. Add 5 µL water to the “NTC” well.
8. Add 5 µL of the positive control template and IPC to the correct well.
9. Cover the wells of each column of the PCR plate or each PCR strip using strip caps as you go and secure caps tightly.

10. Wipe down the work area with bleach or DNAerase, followed by 70% ethanol. Where possible, quickly spin the plate at 1000 rpm to remove droplets that may have formed. Transport the plate/strips to the PCR thermocycler area and place directly in the PCR thermocycler.

PCR Thermocycler Set-Up

Set the following PCR cycle on the thermocycler:

Stage	Temperature	Time	# of Cycles
Initial Denaturation	95°C	15 minutes	1 cycle
Amplification	95°C	30 seconds	35 cycles
	55°C	30 seconds	
	72°C	30 seconds	
Final Extension	72°C	5 minutes	1 cycle
Storage	4°C	hold	

1. Double check all temperature and times are correct and press start.
2. Once the reaction has finished, store the PCR products at 4°C until running on a gel.

Gel Electrophoresis

With conventional PCR, PCR products must be further examined after the reaction has run to ensure the correct product has been amplified after completing the reaction cycle. This is usually done by analyzing the size of the DNA product by means of gel electrophoresis. Gel electrophoresis is used to separate DNA fragments based on size and charge allowing for their visualization and purification. Electrophoresis uses an electrical current to move the

DNA through a gel toward a positive electrode (Aaij & Borst 1972). The DNA molecules travel through the gel at different speeds based on size, allowing them to be separated from one another. This means it is possible to see how many different DNA fragments are present in a sample and their relative sizes. Since the gel is composed of small pores, smaller fragments of DNA will move faster through the gel than the larger ones. After the electrophoresis is complete, a dye is applied to the gel which binds to the DNA. Under UV light, this dye will fluoresce, allowing for visualization of the PCR product. By running a DNA ladder (a standard containing DNA fragments of known sizes) alongside the PCR products, the size can be determined and compared to the expected result (Brody & Kern 2004; Lee et al. 2012).

Gel electrophoresis consists of three main steps (Lee et al. 2012):

1. Pouring the Gel- Electrophoresis gels are normally composed of agarose, a polysaccharide that is typically purchased in a dry powder form. Gels can be made at different percentages but 1 or 2% are used most frequently. The mixture is heated until dissolved and a DNA intercalating dye, typically ethidium bromide (EtBr) is added. The solution is poured into a gel cast with combs (Figure 80). After cooling, this hardens into a solid gel, with small indentations at one end. These are termed “wells”, which are where the DNA samples are loaded.

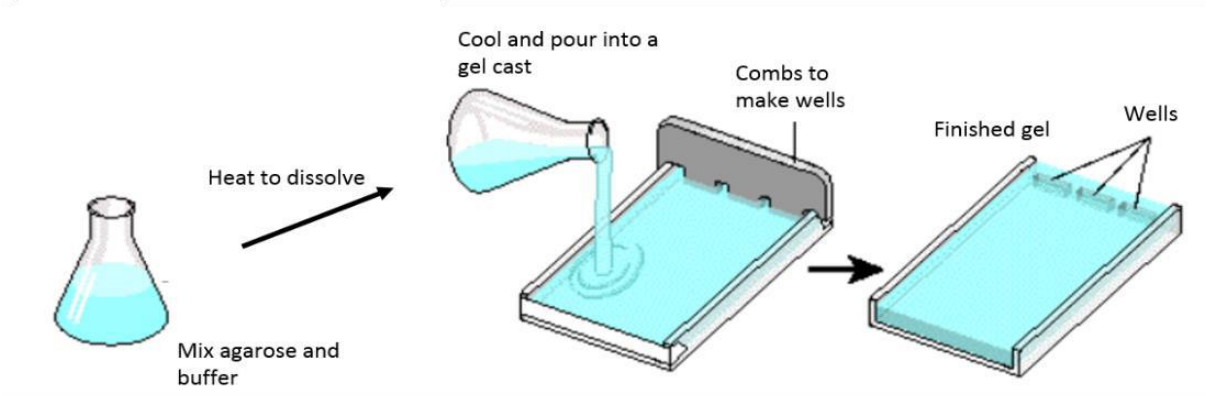


Figure 80- Pouring a gel for electrophoresis. After boiling and dissolving the solution of agarose, the solution is poured and allowed to cool, forming a solid gel. The combs are removed, leaving indentations where the samples are loaded (WUAS 2007).

2. Running the Gel- After the gel has cooled and is solid, it is placed into an electrophoresis gel box/tank. One side of the tank is connected to a positive electrode, while the other is connected to a negative electrode. The end of the gel that contains the wells should be placed at the end with the negative electrode. This is the end the DNA will migrate towards. The box is then filled with buffer which will help to conduct current through the gel. A loading dye is added to each DNA sample before being loaded into the wells. This is done to help track the DNA samples through the gel (to prevent DNA from running off the gel) and to help the samples sink into the well and not float out into the buffer (Armstrong & Schulz 2015). Alongside the samples, a DNA ladder is loaded to help analyze the DNA sample sizes. Commercial DNA ladders come in different size ranges, but one that is reflective of the size range of the expected DNA fragments should be used. Once the current is turned on, the samples migrate down the gel toward the positive end (Figure 81).

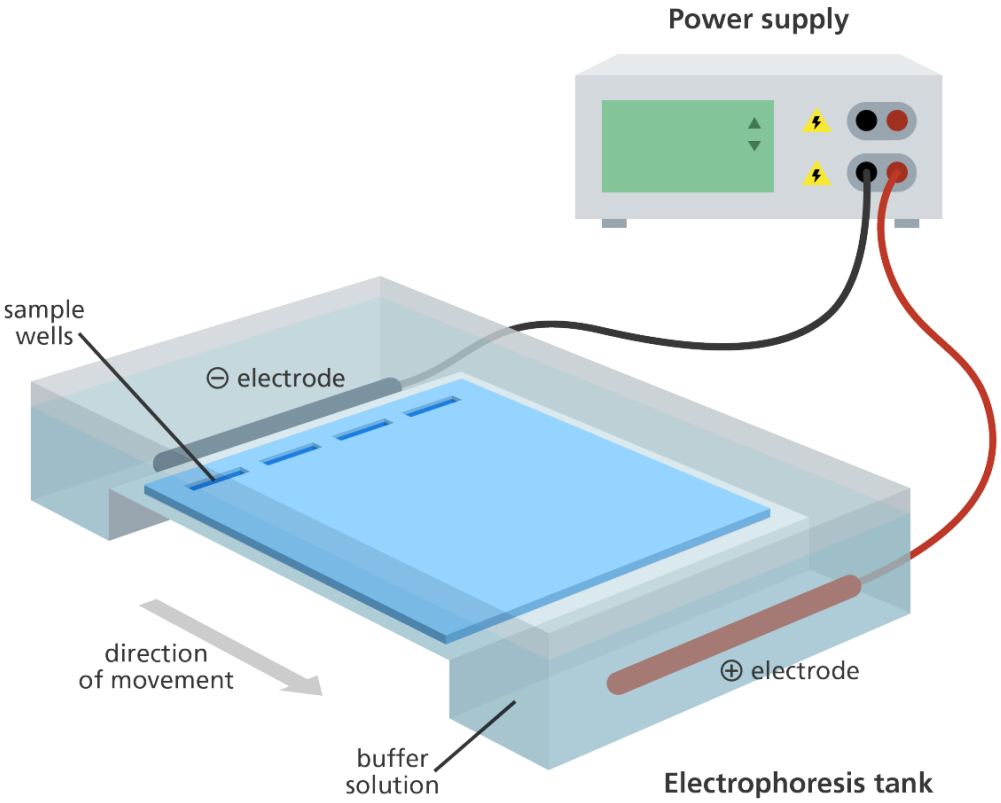


Figure 81- DNA electrophoresis equipment. A gel is placed inside an electrophoresis tank containing running buffer. The DNA samples are then loaded into the sample wells at one end of the gel and an electrical current is passed across the gel. The negatively-charged DNA moves towards the positive end of the gel (Genome Research Limited- yourgenome CC BY 4.0 2017).

3. Visualizing the DNA fragments- After the gel has finished running, the DNA bands are visualized by exposing the gel to UV light as shown in Figure 82. A distinct line that contains DNA fragments of the same size is called a band. Their approximate sizes can then be determined using the DNA ladder, which is used to indicate how many base pairs (bp) long

the DNA fragment is. If the band size is correct, the target sequence can be confirmed in the sample.

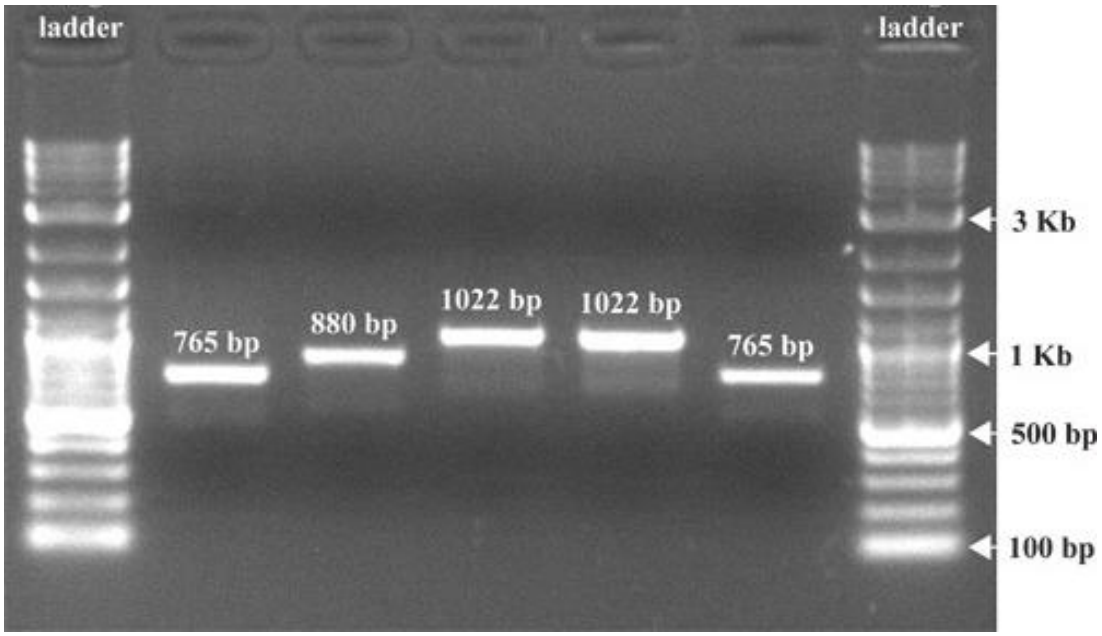


Figure 82- An image of a gel post electrophoresis using an EtBr stain. The gel was exposed to UV light and visualized with a gel documentation system (Lee et al. 2012).

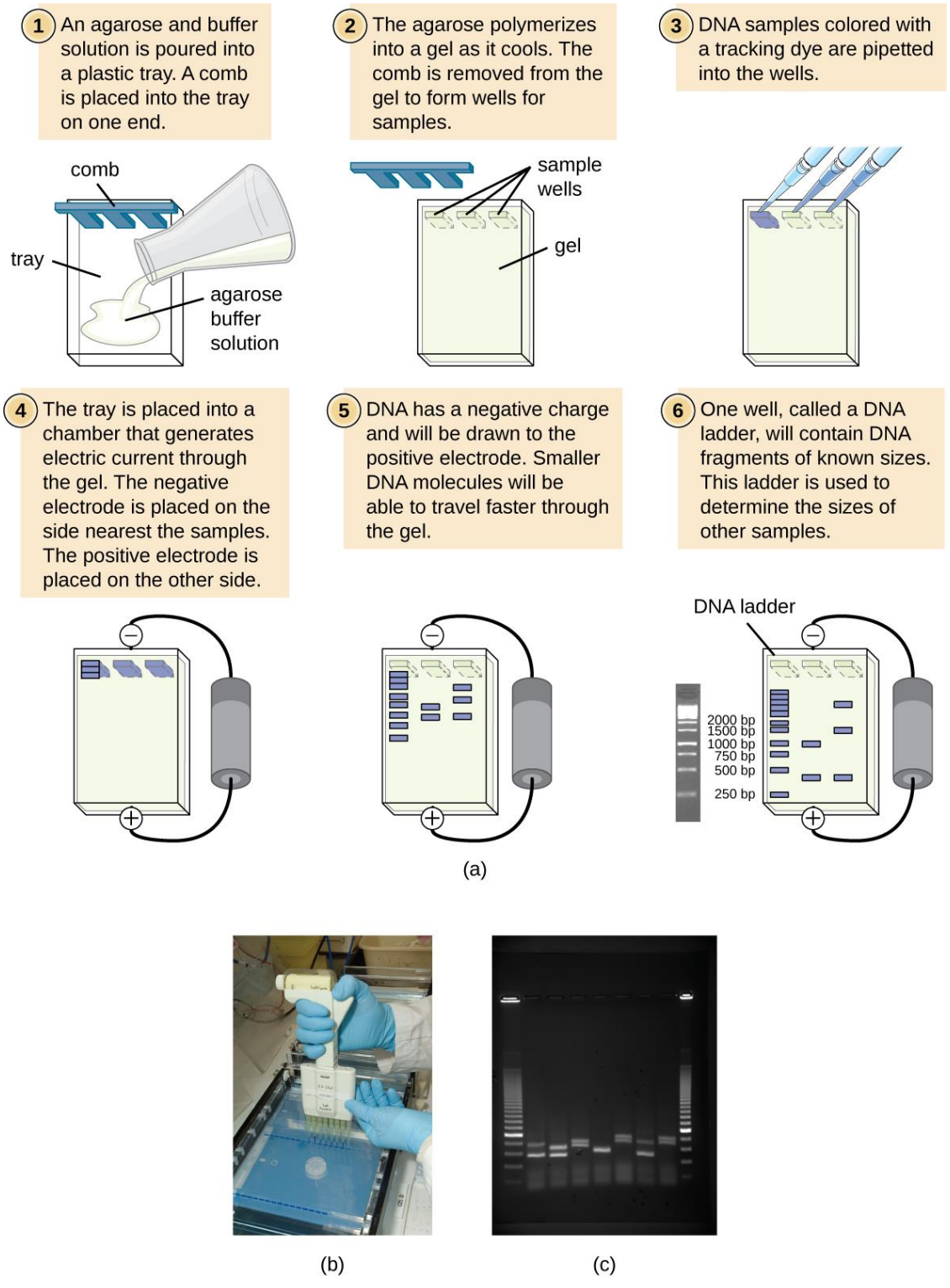


Figure 83- (a) The steps involved in preparing and running agarose gel electrophoresis. (b) DNA samples mixed with loading dye are loaded into the gel. (c) The gel is run then exposed to UV light and visualized with a gel documentation system (Parker et al. OpenStax CC BY 4.0 2016).

Gel Electrophoresis Protocol

Equipment:

- Microwavable glass flask or beaker
- Electrophoresis tank
- Power supply
- Stir plate
- Gel casting tray
- Fixed-wall gel caster
- Well combs
- Gel box
- Gel documentation system (e.g. Gel Doc XR Bio-Rad Laboratories)
- UV light source
- Microwave

Figure 84- A gel documentation system required to visualize DNA fragments (Bio-Rad 2017).

Consumables and Reagents

- 10% bleach solution or commercial DNA-removing decontaminant (e.g. DNAerase)
- 70% ethanol
- 1.5 mL sterile microcentrifuge tubes
- Agarose powder (molecular biology grade; e.g. Fisher BioReagents BP160100)
- 0.5X Tris Borate EDTA Buffer (TBE; Thermo Scientific™ #B52) OR 1X Tris Acetate EDTA Buffer (TAE; Thermo Scientific™ #B49)
    - OR if making up buffer solutions from scratch:

        - EDTA- 0.5 M, pH 8.0
        - NaOH (sodium hydroxide)
        - Tris base (Sigma-Aldrich #10708976001)
        - Boric acid (for TBE; Sigma-Aldrich #B0394) or acetic acid (for TAE; Sigma-Aldrich #A6283)
    - NOTE- TBE buffer is normally used for extended/long runs or repeated runs in the same buffer. TAE has better conductivity than TBE and DNA fragments will migrate faster through the gel.
- DNA intercalating dye (Ethidium Bromide- powdered or aqueous form; e.g. Sigma-Aldrich #E7637)
- 6X DNA Loading Dye (containing bromophenol blue, e.g. Thermo Scientific™ #R0611)
- 1kb DNA ladder (Promega #G7541)

Preparing Reagent Stock Solutions (WHO & CDC 2011)

<u>EDTA; 0.5 M, pH 8.0 (100 ml):</u>

1. Dissolve 18.6 g EDTA in 100 mL distilled H_2O.
2. Adjust pH to 8.0 with 10 M sodium hydroxide (NaOH) (~5 mL).
3. Store at room temperature.

<u>TAE (Tris/acetate/EDTA) electrophoresis buffer, 50X stock solution*</u> (if making up solution from scratch)

1. To 750 mL of distilled H_2O add:
2. 242 g Tris base
3. 57.1 mL of acetic acid

4. 100 mL of 0.5 M EDTA pH 8.0
5. Add distilled H_2O to 1000 mL and mix well on a stir plate.
6. Store at room temperature.

*TAE stock solution should be diluted to 1X in H_2O before use.

TBE (Tris/borate/EDTA) electrophoresis buffer, 10X stock solution* (if making up solution from scratch)

1. To 900 mL of distilled H_2O add:
    a. 108 g Tris base (890 mM)
    b. 55 g boric acid (890 mM)
    c. 40 mL 0.5 M EDTA, pH 8.0 (20 mM)
2. Add distilled H_2O to 1000 mL and mix well on a stir plate.
3. Store at room temperature.

*TBE stock solution should be diluted to 0.5X in H_2O before use.

Ethidium bromide (EtBr), 10mg/mL (if using powdered form)

1. Dissolve 0.2g EtBr in 20 mL distilled H_2O
2. Mix well and store in the dark in 1 mL aliquots at 4°C.

Gel Electrophoresis Protocol

Making a Standard 1% Agarose Gel (Addgene 2018; Kasibhatla et al. 2006; Lee et al. 2012; Sambrook & Russell 2001)

1. Weigh out 1 gram of agarose powder.

2. Mix agarose powder with 100 mL x1 TAE or 0.5X TBE in a microwavable flask. Be sure to use the same buffer as the one in the gel box. Different buffers should not be mixed.
3. Microwave until the agarose is completely dissolved (approximately 1-3 minutes). Take caution not to over boil the solution.

NOTE: **The flask can get very hot and the solution has a tendency to boil over. Stop and gently mix the flask periodically while heating to prevent this from occurring.**

4. Let the agarose solution cool to approximately 50-60 °C (approx. 3-5 minutes).
5. Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (2-3 µL per 100 mL gel). EtBr binds to the DNA and allows for visualization of the DNA using UV light.

NOTE: **EtBr has potential carcinogenic or teratogenic effects. Gloves, lab coat, and eye protection should be worn at all times. Nitrile gloves are more efficient than latex gloves at protecting against EtBr. Make sure to let the agarose cool to between 50°C and 60°C before adding the dye in order to minimize inhalational exposure (the compounds can produce a gas if the gel is too hot). Stock solutions of 10 mg/mL EtBr are commercially available. This should be kept in the dark at room temperature and can be re-used up to 10 times within 1 month. This should be discarded using institutional and local guidelines for hazardous waste. Do not pour the solution directly down the drain. EtBr can also be treated using de-staining bags (e.g. Amresco E732-25) or charcoal filtration. The treated solutions can then be discarded down the drain. Do not place gels in the normal waste disposal. Gels containing EtBr should be placed in sturdy plastic bags or containers to prevent leakage and disposed of as hazardous chemical waste (CDC 2017).**

6. Secure the gel tray in the fixed-wall gel caster with the well comb in place. Pour the agarose into the casting tray. Be sure to pour the solution slowly to prevent any bubbles from forming. If necessary, these can be pushed to the sides of the gel or popped using a pipette tip.

7. Let the gel sit at room temperature for 30-45 mins until it has completely solidified. Likewise, the gel can also be placed at 4 °C for 10-15 minutes.

Loading DNA Samples and Running the Gel

1. Once solid, place the agarose gel into the gel box and carefully remove the comb.
2. Fill gel box with 1xTAE or 0.5XTBE so the gel is completely covered.
3. Load 5 µL of the DNA ladder into the first lane of the gel.
4. Dispense 1-2 volumes (5-10 µL) of 6X DNA loading dye per 5µl sample onto a square of wax paper (i.e. Parafilm). This will produce a dot/bead.
5. Add each of the DNA PCR products to be tested to a single drop/bead of loading dye.
6. Mix the DNA samples and loading dye by pipetting up and down, then carefully load 5 µL of the mixed sample into a well of the gel. Repeat this for all DNA PCR products. **Note-** When loading the sample into the well, place the pipette into the buffer just above the well. Carefully push the sample out to fill the well. Be sure all of the sample is unloaded, before raising the pipette out of the buffer.

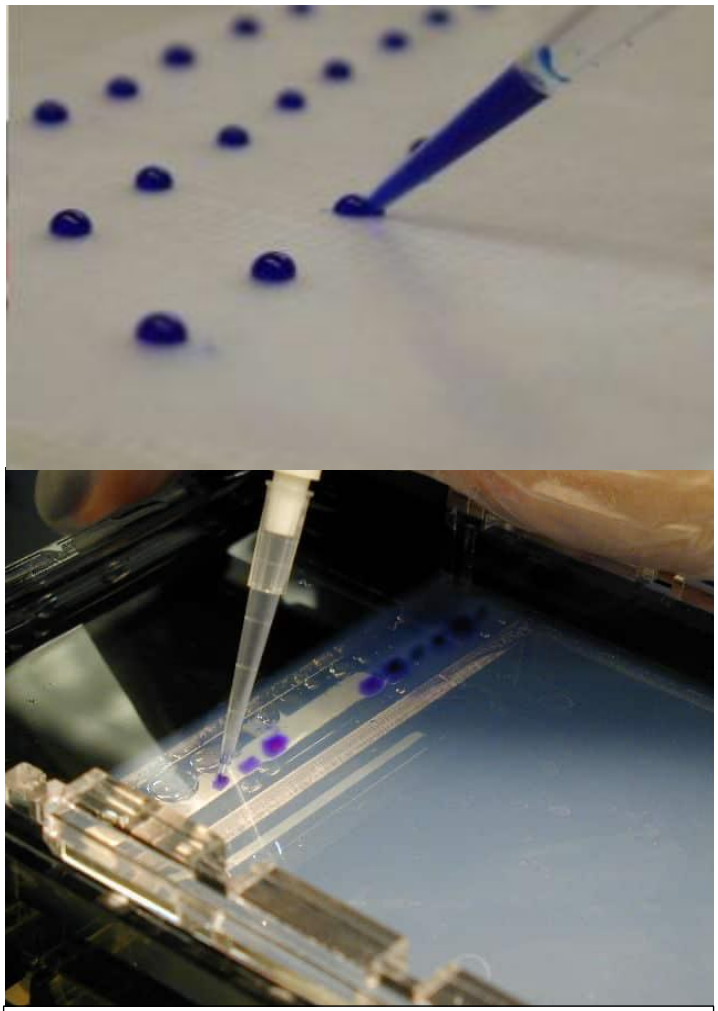


Figure 85- Loading dye (purple) is added to the DNA samples before being carefully loaded into the wells of the gel (Anderson 2019).

7. Connect the terminals from the power source to the appropriate terminals of the Gel Tank. The DNA is negatively charged and will run towards the positive (red) electrode.
8. Turn on the power source and adjust to an appropriate voltage (this varies based on many factors, but 80-120 V is recommended). Run the gel until the dye line is approximately 75-80% of the way down the gel. The time depends on the product size. Larger bands will need to run longer while smaller bands will need to run for less time. A typical run time is about 40 minutes to 1.5 hours, depending on the gel concentration, voltage and product size. Track the samples using the loading buffer to ensure the samples do not run off the gel.
9. Turn off the power, disconnect the electrodes and remove the gel from the gel box. Be sure to thoroughly rinse and clean the gel box with distilled water and allow to dry.

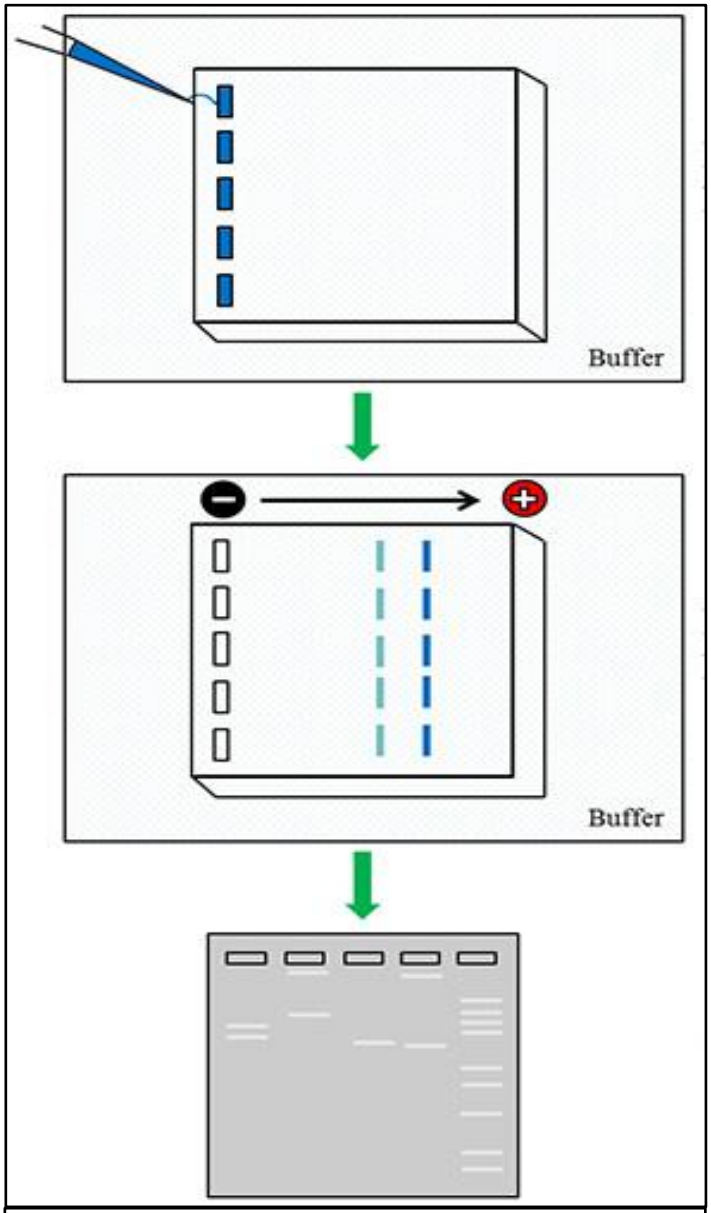


Figure 86- Agarose gel electrophoresis. DNA samples are loaded into the gel with a pipette. An electrical field is applied to separate samples. The stained gel is then visualized using a UV light source (Sambrook et al. 1989).

Analysis of PCR Products on an Agarose Gel

1. Visualize or photograph DNA fragments using any gel documentation imaging system with UV light (e.g. Gel Doc XR Bio-Rad Laboratories).
2. The negative control should not be amplified. The negative and positive control should have a banding pattern as expected.

Note- When using UV light be sure to wear safety goggles or a face shield, as well as gloves and a lab coat.

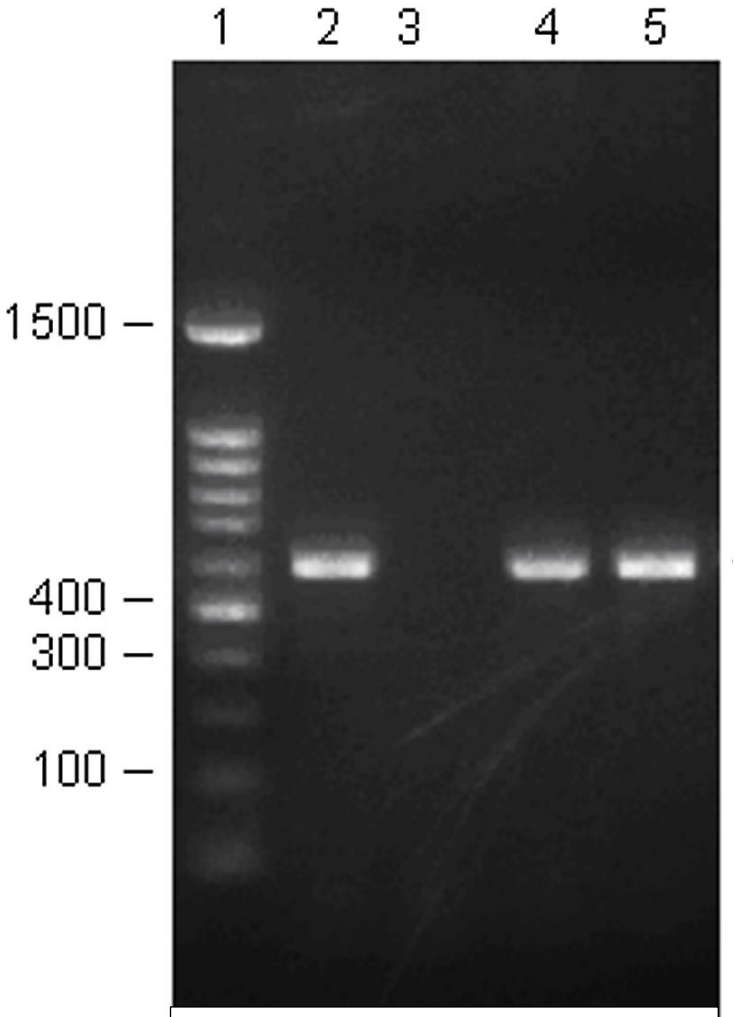


Figure 87- Gel electrophoresis PCR analysis of *B. pseudomallei* 550bp 16S gene. Lane 1- DNA ladder, 2- Positive control, 3- Negative control, 4-5- Sample DNA (Jilani et al. 2016).

Troubleshooting and Modifications to Conventional PCR and Gel Electrophoresis

There are several methods that can help to increase the resolution of DNA bands, such as running the gel at a lower voltage for longer or loading less DNA into the wells. If bands are running too close together, the agarose percentage of the gel can also be adjusted so they have better separation. A higher percentage agarose gel can be used to distinguish smaller bands while a lower percentage gel can be used to help separate larger bands. Using too much running

buffer may also distort bands, while using too little will cause the gel to partially dry out. An uneven gel can also cause issues with the electrical current and rapid migration of DNA at edges, which can lead to bands having a smiling or sad (frowning) shape (Blair 1991; Voytas 2000). See examples of problems associated with gel electrophoresis in Figure 88 below.

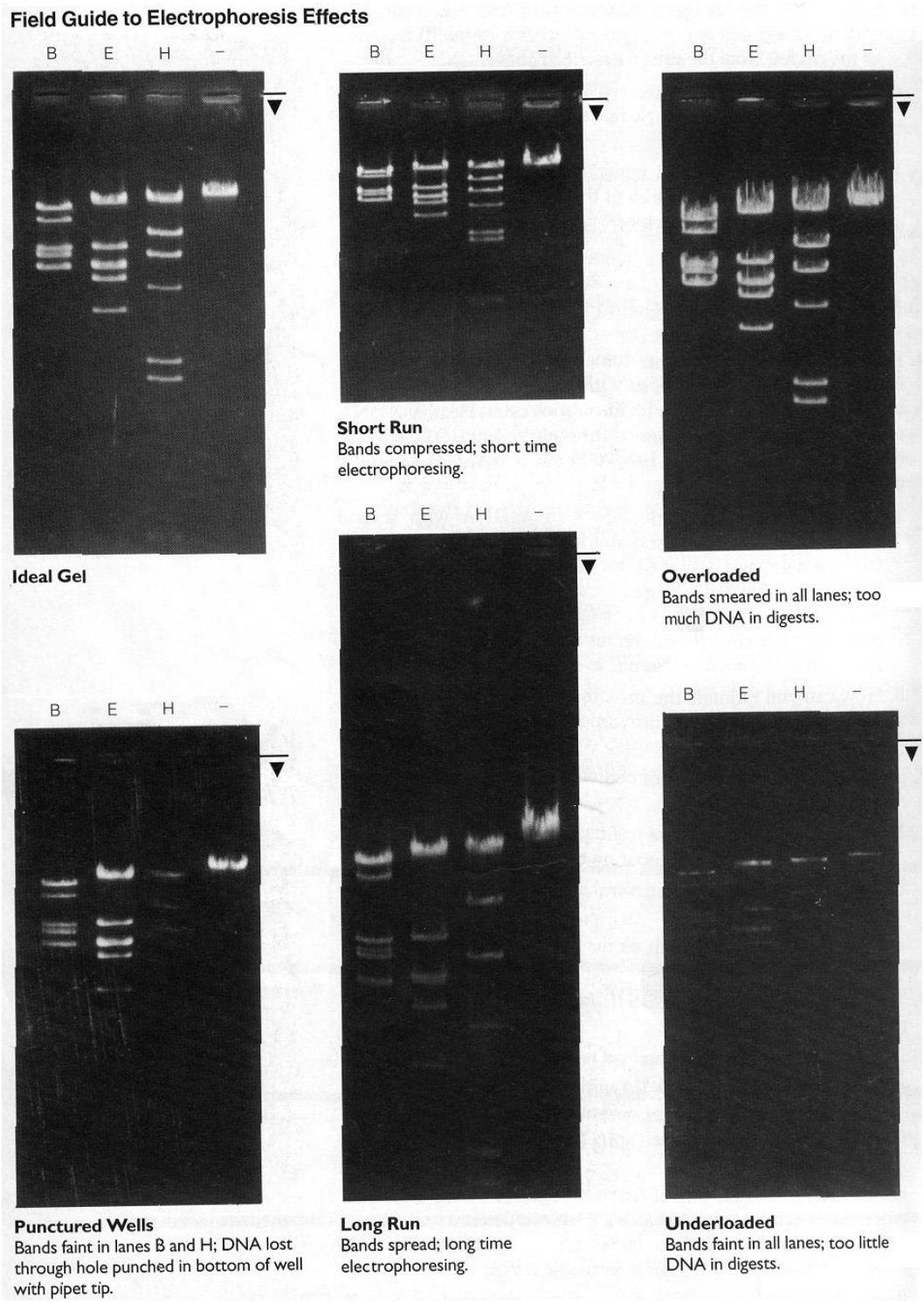


Figure 88- Several unexpected band effects may be seen when visualizing the finished gel product. These issues are often remedied by rerunning the gel again at a different voltage or using more or less of the DNA product (University of San Diego 2018). See additional conventional PCR and electrophoresis troubleshooting methods in Table 18 below.

Table 18- Possible causes and recommendations for common problems associated with conventional PCR and gel electrophoresis (Bio-Rad 2021).

Observation	Possible Cause	Recommendation
No bands or faint bands	Extension time is too short	Double check the cycle profile- generally use 1min/kb.
	Annealing temperature is too high	Double check the primers for proper T_m (melting temperature). Optimize temperature as needed.
	Template is degraded	Degraded template will usually have a smear in the lane. Re-extract DNA and repeat.
	PCR contains inhibitors	Dilute existing template DNA and repeat.
	Component was left out of master mix	Ensure that all reagents were added and repeat. If this still fails, throw out reagents and make fresh stock solutions.
Nonspecific bands	Too many cycles	Use fewer cycles if the template concentration is high.
	Thermocycler ramping speed is too low	The ramp speed should always be set to the maximum.
	Too much primer	Double check dilutions or use a lower concentration.
	Contamination of the master mix	Make new master mix and repeat.
	Contaminants of the PCR water	If bands appear in negative control, toss out water and repeat with fresh stocks.
	Too much template	Dilute existing template and repeat.

Smeared bands	Template is degraded	Re-extract DNA and repeat.
	Contamination	Use fresh reagents and repeat.

Chapter Summary

Conventional PCR is a sensitive and specific method used for *B. pseudomallei* detection. With conventional PCR, PCR products must be further examined after the reaction has run to ensure the correct product has been amplified after completing the reaction cycle. This is usually done by analyzing the size of the DNA product by means of gel electrophoresis. The preceding chapter describes a method for conventional TTS1-PCR detection of *B. pseudomallei* and agarose gel electrophoresis for DNA product confirmation. *B. pseudomallei* strain typing and bacterial species identification using conventional PCR techniques are described in Chapter 16.

Chapter 15: Real-Time Polymerase Chain Reaction (PCR) Detection of *B. pseudomallei*

Chapter Overview and Introduction to Real-Time PCR

Real-time PCR (quantitative PCR, qPCR) is a popular method used for the detection of microbial pathogens. Fluorescence is measured after each cycle, allowing the user to monitor the progress of the PCR as it occurs (i.e., in real time). The increase in fluorescence is proportional to the amount of amplified product and is quantitatively measured throughout the PCR run. Specialized thermal cyclers that are able to detect fluorescence are used to detect a signal while amplification occurs in "real-time" (Bustin 2000; Kubista et al. 2006).

The main advantages of real-time PCR are that it provides a fast and high-throughput (large-scale) means of detection through simultaneous amplification and visualization of newly formed DNA products, eliminating the need for a post-processing gel electrophoresis step. Real-time PCR also helps to prevent contamination of samples downstream, since no further manipulation is required to visualize the products after amplification. Determination of the bacterial load by real-time PCR can also be used to indicate a patient's response to antimicrobial treatment in real-time (Kralik & Ricchi 2017; Kubista et al. 2006).

Since most clinical and environmental samples contain low numbers of bacteria, a pre-enrichment step using an enrichment culture media (such as Ashdown media in the case of *B. pseudomallei*) is recommended prior to DNA extraction to promote the growth of the bacteria for downstream real-time PCR detection. This also helps to eliminate possible PCR inhibitors or competing microbiota that may be present in the sample (see Chapter 10 and Chapter 13 for further detail) (Kralik & Ricchi 2017).

Types of Real-Time PCR Assays

There are two primary methods used for the visualization of amplified DNA during real-time PCR: 1) Non-specific fluorescent DNA dyes such as SYBR® Green I, which use DNA-targeted intercalating fluorescent dyes that show enhanced fluorescence upon binding and 2) Fluorescently-labelled sequence-specific DNA probes, which use a sequence-specific fluorescent probe designed to bind to the target DNA between the two PCR primers (Figure 89) (Holland et al. 1991; Kubista et al. 2006; ThermoFisher 2014).

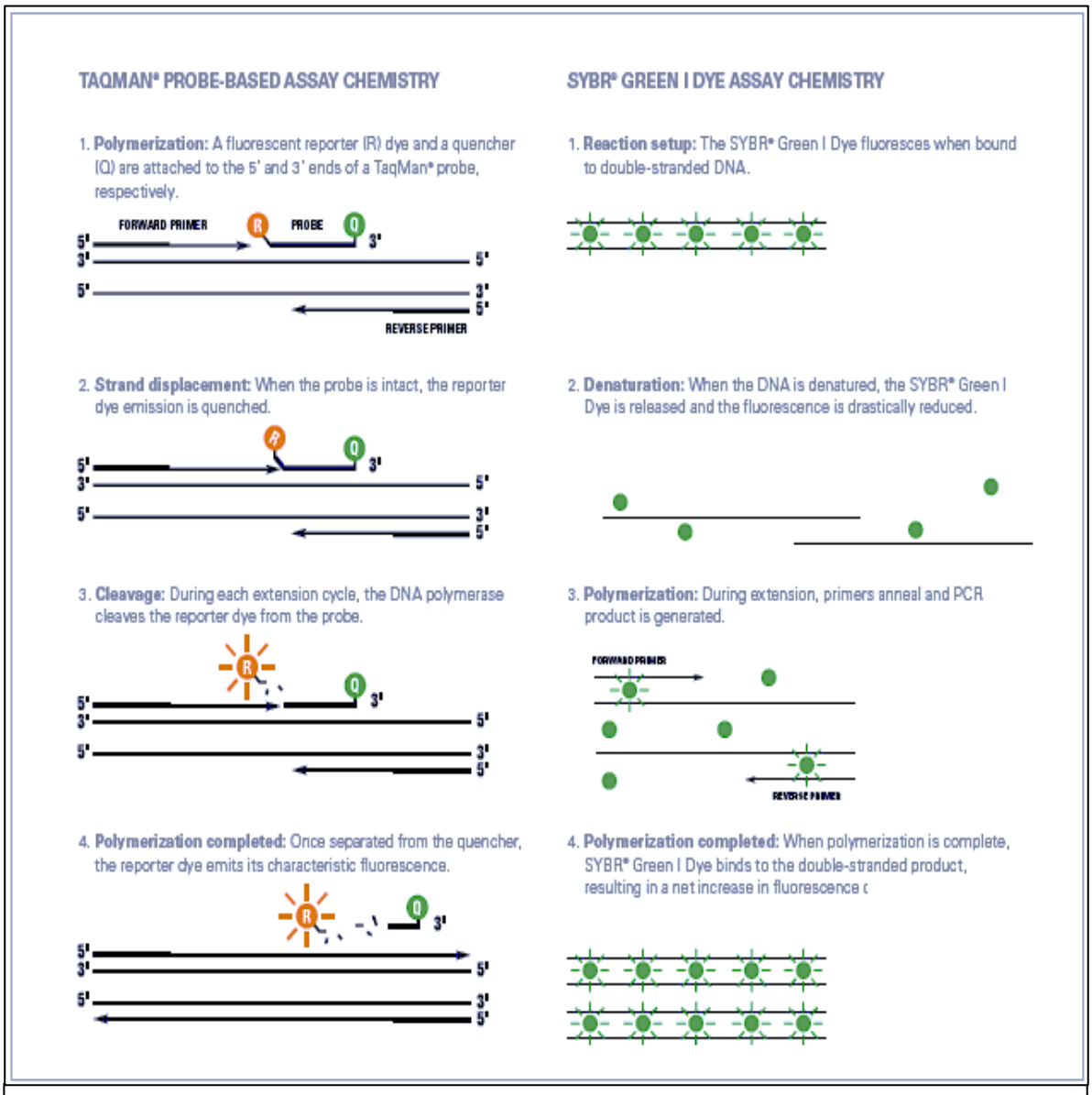


Figure 89- Comparison of TaqMan® and SYBR® Green real-time PCR processes (ThermoFisher 2014).

1. SYBR® Green I PCR: SYBR® Green I is a fluorescent DNA binding dye that binds to any double-stranded DNA. Once incorporated into double stranded DNA, the dye emits a stronger signal then when in solution alone (Figure 89). As the amount of the template DNA increases with each amplification cycle, the number of bound fluorophores and intensity of the fluorescent signal increases. A SYBR® Green real-time PCR protocol follows the conventional PCR amplification profile. This type of real-time PCR lacks in specificity as the dye will incorporate into any double stranded DNA, whether it is target or non-target product.

2. Fluorescently Labelled Sequence-Specific Probes: Fluorescently labeled probes, such as TaqMan® or fluorescence resonance energy transfer (FRET) probes, provide a highly sensitive and specific method of detection. A probe is a short, gene-specific sequence that is designed to bind the target DNA between the two PCR primers. With TaqMan® assays, a reporter dye is attached to the 5' end of the probe. On the 3' end is a quencher dye, which stops fluorescence of the reporter dye when the probe is intact. During PCR, the primers and probe anneal to the target DNA. DNA polymerase extends the primer upstream of the probe and eventually the 5' exonuclease activity will cleave it (break it apart). Once the probe is cleaved, this causes the reporter dye to fluoresce. This signals that amplification has occurred and allows for accumulated PCR product to be visible over time. (Figure 89).

   PCR with fluorescence resonance energy transfer (FRET) probes utilize two labelled probes that bind to the PCR product (Figure 90). When the two probes bind to the DNA, the fluorophores come into close proximity, enabling energy to transfer from a donor fluorophore to an acceptor fluorophore. This results in a fluorescent signal that is proportional to the amount of target sequence (QIAGEN 2010).

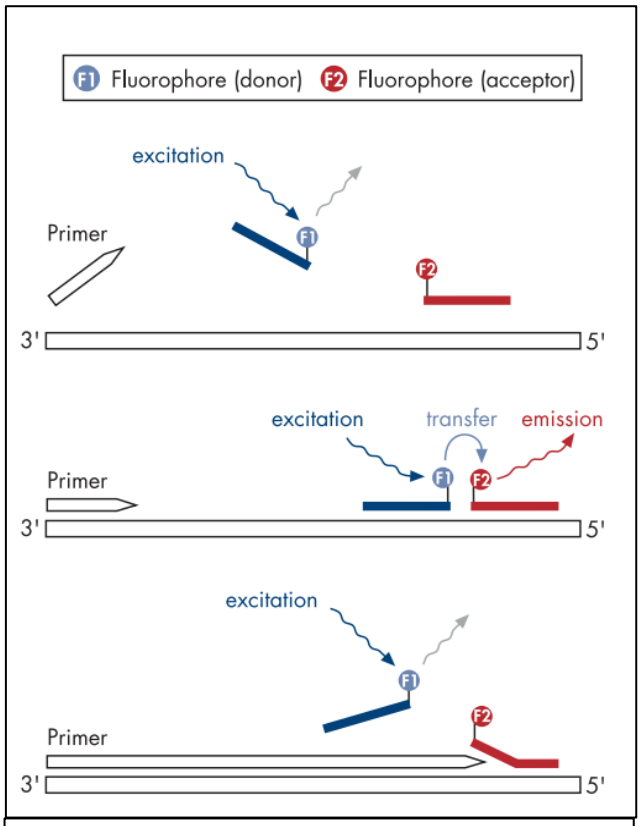


Figure 90- FRET probe method: No fluorescence is detected when not bound to the target sequence. During the PCR annealing step, both probes hybridize to the target sequence, bringing the donor and acceptor fluorophores into close proximity. This

Real-Time PCR Data Analysis

At the start of the qPCR reaction, fluorescence remains at a low level and is not detectable. Eventually, enough amplified product will accumulate and will give off a detectable signal. The cycle number at which this occurs is called the cycle threshold, or C_t, or the number of cycles required for the fluorescent signal to cross the threshold level (ThermoFisher 2014). The C_t value is proportional to the quantity of template present at the start of amplification and is inversely proportional to the amount of target DNA. This means that the lower the C_t value, the greater the amount of target DNA present (Figure 91). If a large amount of template is present at the start of the reaction, fewer amplification cycles are required to produce a fluorescent signal above the threshold and the reaction will have a low, or early C_t value. Likewise, if less template is present at the beginning of the reaction, more cycles are necessary for the fluorescence signal to rise above background and the

reaction will have a higher/later C_t value. Generally, C_t values of ≤ 29 indicate an abundant amount of the DNA target. Values between 30-37 indicate a moderate amount of target, and 38-40 indicate minimal amounts of target (ThermoFisher 2014).

The change in fluorescence over the course of the reaction is measured by a thermocycler machine that combines thermal cycling with fluorescent dye visualization. By plotting fluorescence against the cycle number, the thermocycler produces an amplification plot that displays the amount of amplified product over the duration of the entire PCR run. The amplification plot portrays the exponential phase and a plateau phase of the reaction. During the exponential phase, the amount of PCR product increases with every cycle. However, PCR reagents are consumed as the reaction progresses and will enter the plateau phase (Figure 91).

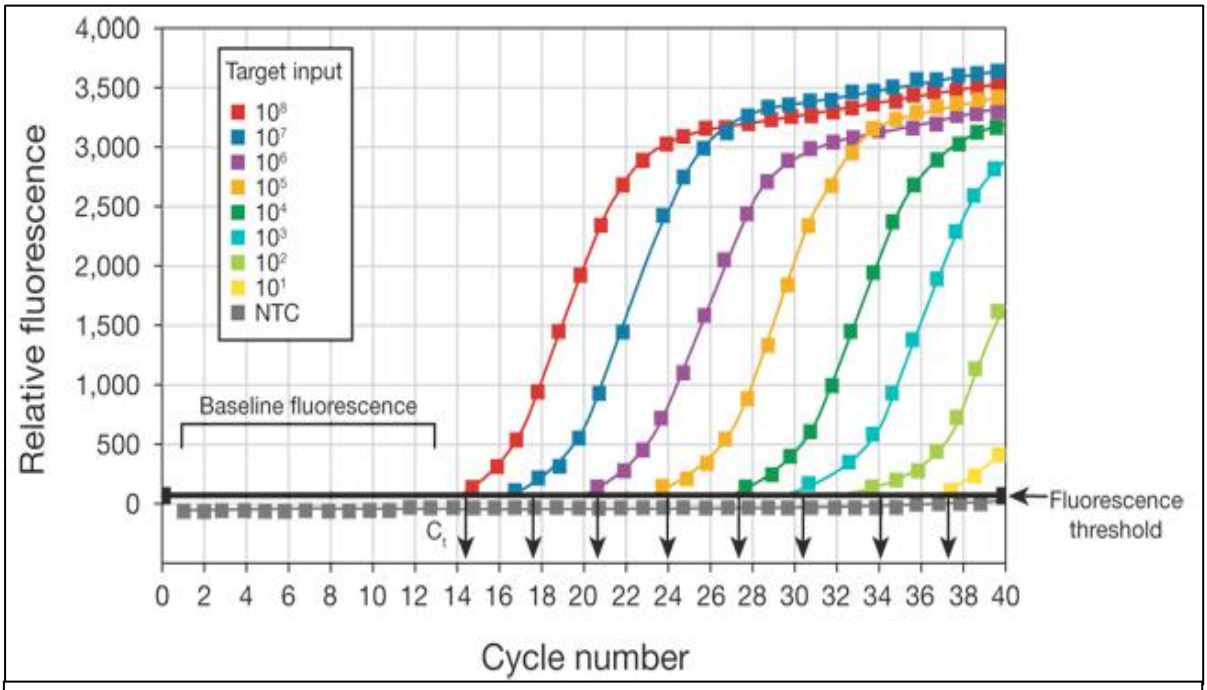


Figure 91- Amplification plot showing relative fluorescence vs cycle number. In this plot, the number of PCR cycles is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the reaction tube, is shown on the y-axis (ThermoFisher 2014).

There are two approaches that can be used for data analysis of real-time PCR: absolute and relative. Absolute quantification is used when the quantity of nucleic acid, called the "copy number", per amount of sample is required (ThermoFisher 2014). To perform this type quantification, a template of known concentration is serially diluted and amplified alongside the unknown samples. The data is then graphed to generate a standard curve (Figure 92). The standard curve is plotted as target concentration vs resulting C_t value. The sample C_t values can then be used to determine the DNA product copy number (i.e. amount of DNA product).

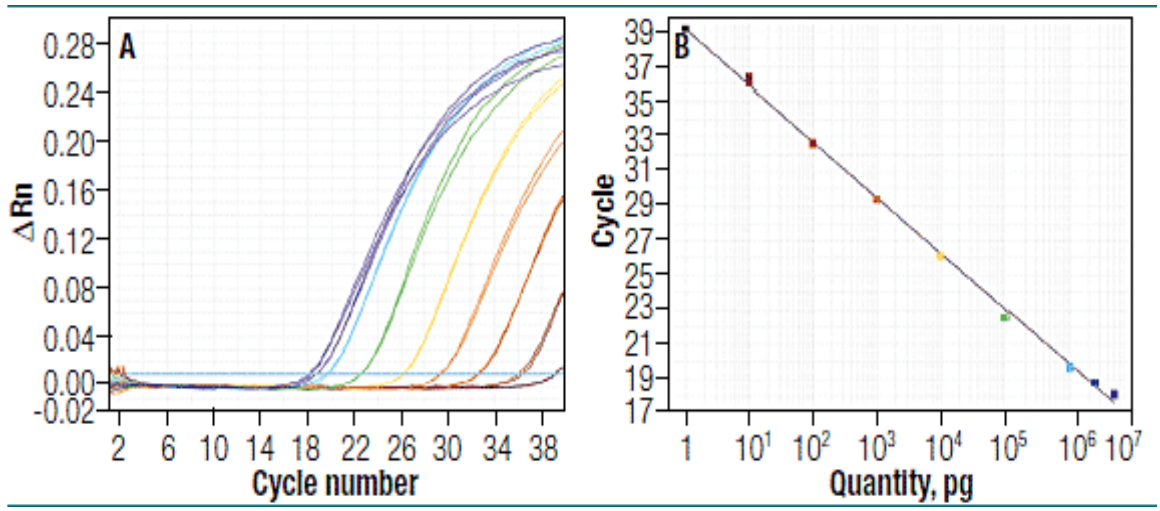


Figure 92- Absolute quantification analysis: Amplification plot showing a dilution series (A) used to generate a standard curve based on C_t values (B) (ThermoFisher 2014).

Relative quantification is used when the relative expression level of a gene of interest is wanted. With relative quantification, changes in gene expression are measured against another reference sample (such as an untreated control sample). In this type of analysis, a standard curve is not generated as the change can be calculated from the C_t values of the unknown and reference samples. Instead, the result output is a ratio: the relative amount (difference) of a target nucleic acid versus equivalent amount of test and control sample. An example of when relative quantification would be used is when measuring gene expression levels in response to a drug. Here, the level of

gene expression in a particular gene of interest in a treated sample is compared to the level of gene expression in an untreated sample (Figure 93) (ThermoFisher 2020a).

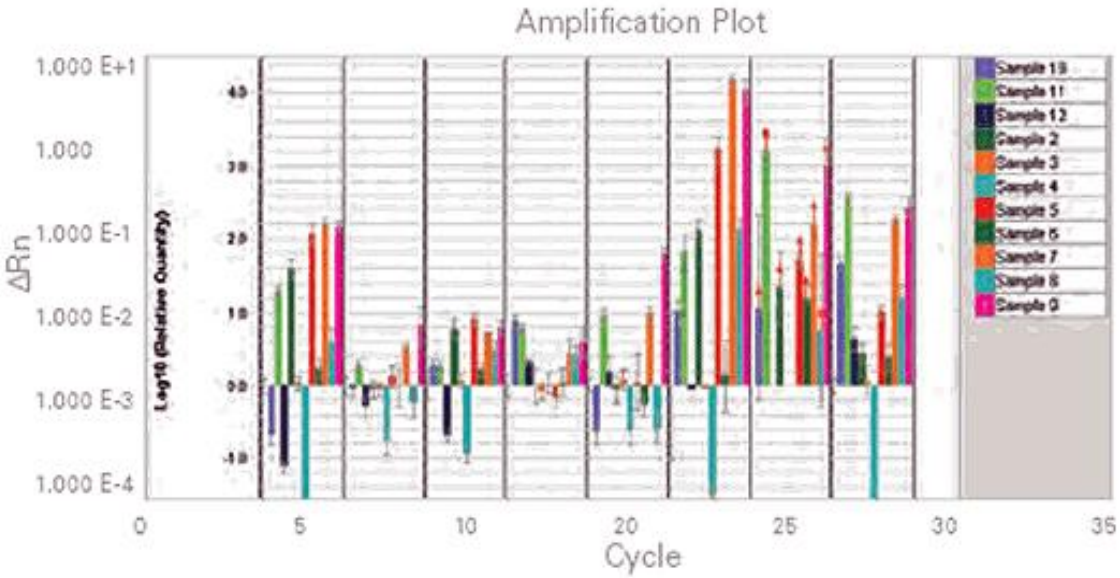


Figure 93- Relative quantification analysis where changes in gene expression level in a given sample are measured against a reference sample (ThermoFisher 2020a).

For further information about real-time PCR set-up and data analysis:

1. ThermoFisher Real-Time PCR Handbook available at: https://www.thermofisher.com/au/en/home/global/forms/life-science/digital-experience.html
2. QIAGEN: Critical Factors for Successful Real-Time PCR available at: https://www.qiagen.com/au/resources/resourcedetail?id=f7efb4f4-fbcf-4b25-9315-c4702414e8d6&lang=en.

NOTE- As a result of the very high specificity and sensitivity of real-time PCR method, protocols described here must be followed as written. Reagents and procedures should not be altered without prior testing and appropriate assay validation. There are also high initial setup and

training costs associated with the procedure that labs should account for beforehand. If a lab is going to conduct real-time PCR testing, they must be able to follow standard protocols without substitution and adhere to the appropriate laboratory guidelines detailed below.

Specifics of the *Burkholderia pseudomallei* Real-Time PCR

A number of PCR assays have been developed and evaluated for the detection of *B. pseudomallei,* including several conventional PCR methods (Gal et al. 2005; Lew & Desmarchelier 1994; Winstanley & Hart 2000). These are discussed in further detail in Chapter 14. The most widely used real-time PCR assay utilizes primers and probes that are specific to a 115-base-pair region within *orf2* (open reading frame 2) of a *B. pseudomallei* type III secretion system (TTS) gene cluster, called TTS1. This has been demonstrated to be a highly sensitive and specific gene target that is able to distinguish *B. pseudomallei* from other closely related *Burkholderia spp.* in both clinical and environmental samples (Kaestli et al. 2007; Novak et al. 2006).

Primer and Probe Sequences-

- BPTTS4176F Forward: 5'- CGTCTCTATACTGTCGAGCAATCG -3'
- BPTTS4290R Reverse: 5'- CGTGCACACCGGTCAGTATC -3'
- BPTTS4208P Probe: FAM-CCGGAATCTGGATCACCACCACTTTCC-BHQ

Quality Controls (CLIA 2010; U.S. EPA 2004)

One of the most important aspects of any laboratory method is the use of the quality controls to ensure that the procedure has worked correctly and the results are reliable. Laboratories should test positive and negative controls on a regular basis to check that the PCR is working as expected. Routine PCR controls should include:

4. Negative control- PCR negative controls are used to show that no contamination has been introduced into the master mix or during PCR analysis. No amplification of nucleic acids should be detected in these wells. Two types of PCR negative controls that should be used include:
    a. Non-template control- PCR grade water should be used as a non-template control (NTC). NTCs should be included in each PCR run. It is also advisable to include one NTC for work done in both the clean PCR room/area and dirty PCR room/area.
    b. DNA extraction blank- This is used to examine if there is any contamination during sample processing and PCR analysis. Extraction is performed using sterile water and this is processed alongside the test samples. The same extraction and PCR procedures should be used as the test samples.
5. Positive control- PCR positive controls are used to show that PCR reagents have been prepared appropriately. These should be run with each PCR batch. Positive controls are typically performed through adding an external control to the master mix. This can be:
    a. Purified DNA from a known isolate of *B. pseudomallei* containing the sequence of interest. Care must be taken to ensure no cross-contamination occurs between the positive controls and the samples or negative controls. *B. pseudomallei* control DNA should be diluted in sterile PCR-grade water to minimize the risk of cross-contamination.
    b. A specific template containing the gene sequence to be amplified, including primer binding sites.
    c. A related sequence that has been shown to amplify with an efficiency that is comparable to the target sequence.

A protocol for pLepBaBp+, a plasmid which contains DNA sequences that act as a positive control for PCR detection of several bacterial species, including *Bacillus anthracis*, pathogenic *Leptospira spp.* and *Burkholderia pseudomallei*, is described below. Specifics on pLepBaBp+ plasmid propagation can also be found at the end of the chapter.

6. Internal positive control (IPC)- The presence of PCR inhibitors or errors during sample extraction are common causes of false negative PCR results. These can be easily examined using an Internal Positive Control (IPC), which is used to confirm DNA amplification, detect false negative results and if there are any inhibitory substances in a sample. IPC should be added to each reaction mix and should always show a positive result. This is particularly important if testing DNA from human clinical specimens. If the IPC is negative, it indicates the specimen may contain PCR inhibitors and will need to be diluted until the inhibitor is no longer affecting the C_t values. It may also indicate a problem with specimen collection or the DNA extraction. PCR assays often utilize a DNA dilution factor of 1 in 10 (1:10) or 1 in 100 (1:100), however this may vary depending on the level of inhibition.
    a. A protocol using the TaqMan® Exogenous Internal Positive Control (IPC) is described in the real-time PCR protocol below. If an exogenous IPC is not available, RNase P can be used as an internal control for human clinical specimens. For further information about RNase P and use of the RNase P internal control with human clinical specimens see: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2F4316848_TaqMan_RNaseP_Cntrl_PI.pdf&title=UHJvZHVjdCBJbmZvIFNoZWV0OiBUYXFNYW4gUk5hc2UgUCBDb250cm9sIFJlYWdlbnRzIEtpdA==.

General Considerations and Safety Precautions

The high sensitivity of real-time PCR significantly increases the risk for cross-contamination. False positive results may occur if the specimen or reagents become contaminated. Good laboratory practice should be used during preparation of samples to minimize the risk of cross-contamination. The following precautions are recommended:

1. Maintain separate areas, equipment and supplies for pre- and post- PCR processes. Workflow in the laboratory should occur in a unidirectional manner. Never bring amplified PCR products into the master mix set up area.
2. Change gloves regularly, especially if contamination is suspected to have occurred.
3. Open and close sample tubes and plates carefully. Tubes should be centrifuged before opening. Keep tubes closed when possible.
4. Use positive-displacement pipettes or aerosol-barrier filter pipette tips.
5. Clean lab benches and equipment with 10% bleach solution or commercial DNA decontaminant, followed by 70% ethanol after every set up. UV light may also be used with proper precautions.
6. All patient specimens and positive controls should be considered potentially infectious and handled with appropriate safety precautions. Perform all manipulations of samples within a biological safety cabinet (BSC) where available.
7. Use appropriate PPE including gloves, eye protection, and lab coats while handling samples, reagents, pipettes, and other equipment and reagents.
8. PCR reagents should be kept on a cold block or on ice during use to ensure stability.

Reagent Storage and Handling

- Store all dried primers and probes and the positive control at 2-8°C until re-hydrated for use.
- Once the primers and probes have been diluted to the correct working concentration for PCR, they should be stored at -20°C.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes and master mix enzyme must be thawed before use and kept on a cold block or ice.

Consumables and Reagents

- 10% bleach (10:1 water: concentrated bleach- prepare fresh weekly) or commercial DNA-removing surface decontaminant liquid (such as DNase or RNase AWAY™)
- 70% ethanol
- 1.5 mL microcentrifuge tubes (PCR grade)
- 96 well polypropylene plates, tube strips or individual PCR tubes
- Pre-sterilized aerosol-barrier filter tips (2-20 µL, 10 µL, 200 µL, and 1000 µL)
- PCR cap strips or adhesive plate seals (e.g. MicroAmp® Optical Adhesive Film)
- Adhesive plate seals applicator (e.g. MicroAmp® Adhesive Film Applicator)
- Commercial real-time PCR master mix (e.g. LightCycler® FastStart DNA Master HybProbe Kit)
- Probe (see sequence listed above)
- Forward and Reverse Primers (see sequences listed above)
- DNA extracted from samples to be tested
- TaqMan® Exogenous Internal Positive Control (IPC) Reagents (Applied Biosystems ™)
- Positive control pLepBaBp+ plasmid DNA at a concentration of 10-12g/µL or other known positive control
- NTC and negative DNA extraction “blank” control
- PCR grade water
- Laboratory pens/markers
- Kimwipes or other laboratory tissue
- Personal Protective Equipment (lab coat, gloves, eye protection, shoe covers, hair net)

Equipment

- Vortex
- Racks/holders for 1.5mL microcentrifuge tubes, tube strips or PCR plates

- Microcentrifuge
- Real-time PCR thermocycler machine (e.g. Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument or Applied Biosystems 7500 Fast Real-Time PCR System)
- Pipettes (1-10 µL, 2-20 µL, 20-200 µL, and 100-1000 µL)

NOTE- The primary type of equipment is the real-time PCR thermocycler machine. When selecting an appropriate machine, be sure to consider the fluorescent filters used. These filters should be able to detect the wavelengths of light that are emitted by the fluorophores coupled to the probes. A desktop or laptop computer and the relevant real-time PCR thermocycler software are also necessary to view and analyze results. It is recommended that real-time PCR machines and computer used for data analysis are plugged into a backup battery with surge protection. This will protect data and keep PCR reactions running in the event power is lost (Csako 2006).

Preparation of DNA Template for Real-Time PCR

Commercial kits such as the QIAamp DNA Mini Kit (QIAGEN), Chelex©, or crude boil-prep extraction methods can be used for the extraction of genomic DNA for real-time PCR detection (See Chapter 13 for further detail and protocols). However, it is important that DNA extraction from clinical specimens is performed in a separate room or area than the PCR reaction assembly area. If separate rooms are not possible, separate laboratory benches should be used, along with separate pipettes, laboratory coats, and gloves. Decontamination of surfaces and equipment with 10% bleach followed by 70% ethanol should be done after handling any DNA template.

Extracted DNA is typically very concentrated, which may lead to PCR inhibition and non-specific background signal. DNA quality control should be performed after extraction to check for purity and concentration as described in Chapter 13. An Internal Positive Control (IPC) can be added to each reaction mix to indicate possible assay inhibition. The IPC should always have a positive PCR result. If

the IPC is negative, it typically indicates that the DNA may contain PCR inhibitors and will need to be diluted. PCR assays often utilize a DNA dilution factor of 1 in 10 (1:10) or 1 in 100 (1:100), however this may vary depending on the level of inhibition.

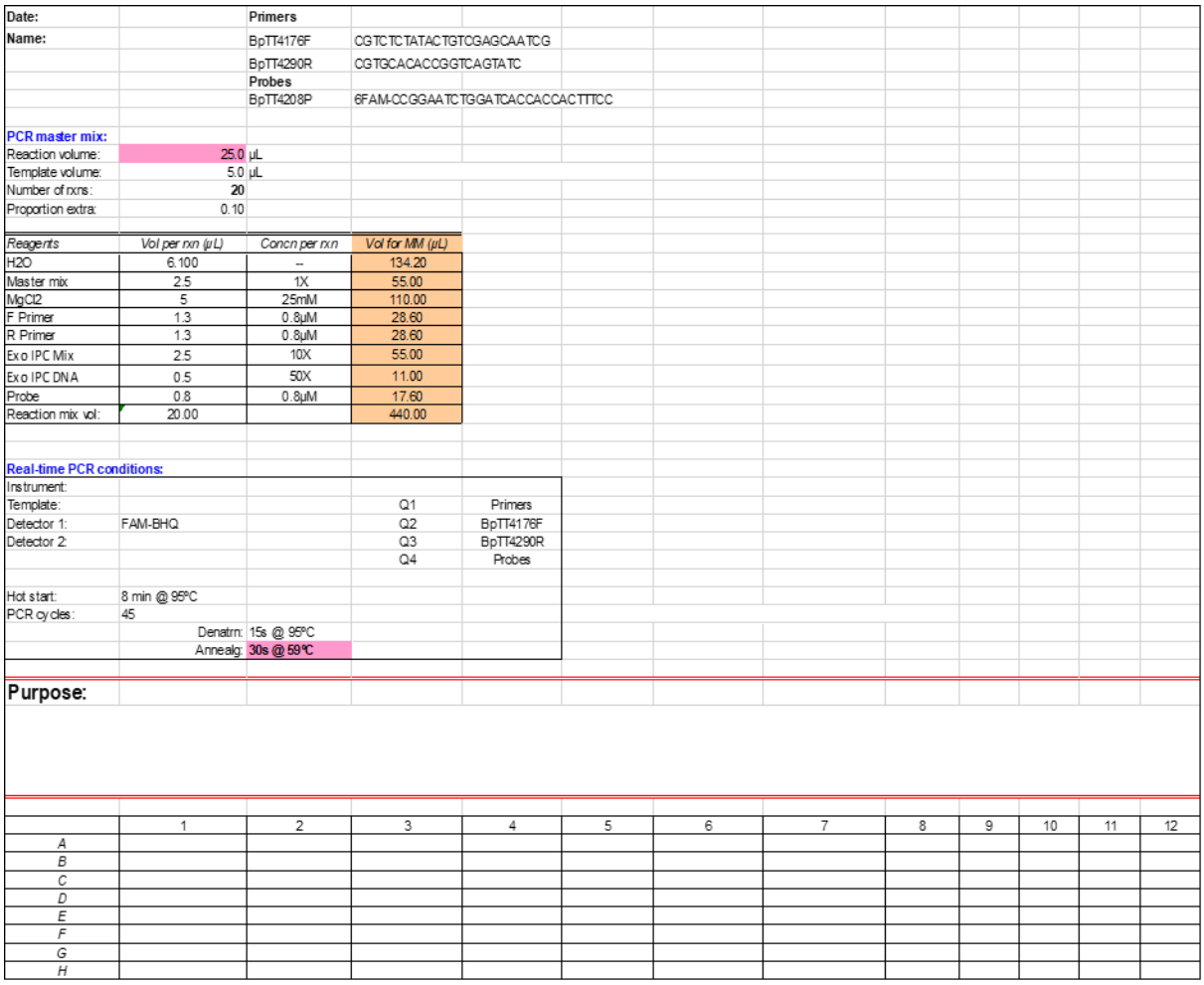
Technical Resources

- LightCycler® FastStart DNA Master HybProbe Kit Manual https://lifescience.roche.com/en_us/products/lightcycler-faststart-dna-master-hybprobe.html
- TaqMan® Exogenous Internal Positive Control (IPC) Manual http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041040.pdf

***B. pseudomallei* TTS1 Real-Time PCR Protocol** (adapted from Novak et al. 2006)

Before Starting the Procedure

A. Prior to starting, plan the experiment by filling out and printing a PCR template worksheet (see Figure 94 below for an example template).
B. Be sure sufficient quantities of working stocks of primers and probes to be used are available and ensure primers and probes have been diluted to the appropriate working concentration.



Date:		**Primers**		
Name:		BpTT4176F	CGTCTCTATACTGTCGAGCAATCG	
		BpTT4290R	CGTGCACACCGGTCAGTATC	
		Probes		
		BpTT4208P	6FAM-CCGGAATCTGGATCACCACCACTTTCC	
PCR master mix:				
Reaction volume:	25.0	µL		
Template volume:	5.0	µL		
Number of rxns:	20			
Proportion extra:	0.10			

Reagents	*Vol per rxn (µL)*	*Concn per rxn*	*Vol for MM (µL)*
H2O	6.100	--	134.20
Master mix	2.5	1X	55.00
MgCl2	5	25mM	110.00
F Primer	1.3	0.8µM	28.60
R Primer	1.3	0.8µM	28.60
Exo IPC Mix	2.5	10X	55.00
Exo IPC DNA	0.5	50X	11.00
Probe	0.8	0.8µM	17.60
Reaction mix vol:	20.00		440.00

Real-time PCR conditions:				
Instrument:				
Template:			Q1	Primers
Detector 1:	FAM-BHQ		Q2	BpTT4176F
Detector 2:			Q3	BpTT4290R
			Q4	Probes
Hot start:	8 min @ 95ºC			
PCR cycles:	45			
	Denatrn:	15s @ 95ºC		
	Annealg:	30s @ 59ºC		

Purpose:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 94- Example of a PCR template worksheet. This should be filled out prior to starting the protocol.

Primer and Probe Dilutions

Primers and probes must be diluted from the concentrated stocks into working stocks. When diluting primers and probes, use sterile filter tips and be sure to work in a sterile area that is free from possible extraneous template DNA in order to avoid cross-contamination. It is best to work in a clean PCR cabinet or hood if this is available.

Concentrated stocks of primers and probes should be stored at -20°C. Working stocks can be stored at 4°C if used regularly. Ensure probes are stored in the dark as they are light-sensitive. Primer and

probe stocks should be aliquoted when they first arrive in the laboratory before freezing for longer-term storage. The length of time that primers and probes should be stored will vary. Using positive controls with each reaction will help to determine when primers and probes should be replaced (QIAGEN 2010).

For the real-time *B. pseudomallei* TTS1 PCR assay, primer and probes should be diluted to 8 µM before being used in the reaction. To do this:

From Concentrated Liquid Stock:

This is done is two steps:

1. Find the volume of primer stock needed:

   (Concentration of Stock)*(Volume of Stock) = (Concentration Wanted)*(Volume Wanted)

2. Find the volume of nuclease-free water to add:

   (Volume Wanted) – (Volume of Stock)

Example: Want a primer concentration of 8 µM in 200 µL. The given primer concentration is 400 µM.

1. Find the volume of primer stock needed:
   A. Concentration Wanted: 8 µM
   B. Volume Wanted: 200 µL
   C. Concentration of Stock: 400 µM
   D. Volume of Stock (µL) =

      (400 µM)*(x µL) = (8 µM)*(200 µL)

      (400 µM)*(x µL) = 1600 µM*µL

      x µL = (1600/400) µL

      x = 4 µL of stock primer

2. Find the volume of nuclease free water to add:

200 µL - 4 µL = 196 µL of nuclease-free water

Answer: Adding 4 µL of stock primer to 196 µL of nuclease-free water will yield a solution of 200 µL at 8 µM concentration.

From Lyophilized Stock (Freeze-Dried Precipitate Powder)**:**

Lyophilized primers and probes must be resuspended in solution before they can be used. The lyophilized primers will come with a technical data sheet that will list the quantity of primer in nanomoles (nmol). This quantity should be used to determine the concentration of the primer stock wanted. This concentration can be used to create a 100µM suspension.

*This calculation works for a 100µM final concentration. *

Example: The primer quantity given is 40 nmol.

1. Multiply the nmol concentration of the lyophilized primer or probe by 10 to get the resuspension volume in microliters:

   40 x 10 =400 µL

   Answer: Adding 400 µL of either nuclease-free water or TE buffer (see note below) to the lyophilized primers will give a concentration of 100µM.

2. Add the water or buffer directly to the tube of lyophilized primers. Ensure that all the material is rehydrated by pipetting up and down or vortexing. Look for any faint pellets or smears still at the bottom of the tube. Once resuspended, allow the primers to sit for a few minutes at room temperature, then centrifuge to the bottom and dilute to 8µM (the working stock required for the TTS1 PCR- See Dilutions from Concentrated Liquid Stock above).

NOTE: Weak buffers such as TE (10mM Tris pH 7.5-8.0, 1mM EDTA) or Tris (10mM Tris-HCl pH 8.0) are usually preferred with TE as the best choice. These buffers give a more stable environment for the primers/probes. If these options are not available, sterile, nuclease-free water can also be used.

Real-Time TTS1 PCR Protocol

Set-Up Procedure:

1. Map out where the samples will be placed in the 96-well plate using a template worksheet. Label the plate accordingly. There should be one reaction well per sample, plus the negative DNA extraction "blank" control, two no template controls (NTC) (one for both the clean and dirty PCR set-up rooms/areas) and the PCR positive control.
2. Enter the DNA clean room or designated area. Gather PCR reagents including the PCR master mix, primers, probes, and PCR grade water. Allow working stocks of primers and probes to thaw completely before use If they are stored at -20°C. Briefly vortex each tube before use to bring down any droplets.
3. Prepare master mix according to the table below. Combine Vials 1a and 1b to make the final Vial 1 mix. Make enough mix for all samples, plus two extra samples (for example, if you have 12 samples to test including all controls, make enough master mix for 14 samples).

Master Mix Reagent	µL Per Reaction
Water	6.1
Vial 1 Mix (Vials 1a and 1b combined from Lightcycler® FastStart DNA Master Hybprobe kit (Roche))	2.5
$MgCl_2$ 25 mM (Vial 2 from Lightcycler FastStart DNA Master Hybprobe kit)	5.0
Forward Primer [8 µM]	1.3
Reverse Primer [8 µM]	1.3
Probe [8 µM]	0.8
10X Exo IPC Mix (from TaqMan® Exogenous IPC Kit)	2.5
50x Exo IPC DNA (from TaqMan® Exogenous IPC kit)	0.5
Total	**20**

4. Gently mix the master mix and briefly spin in a microcentrifuge.
5. Load 20 µL of master mix into the appropriate well, according to the worksheet created in Step 1. Do this for each reaction.
6. Add 5 µL sterile PCR-grade water to the “clean room” NTC well. Use the adhesive plate film or caps to cover the wells.
7. Return all reagents to the appropriate storage area and wipe working surfaces with 10% bleach or DNA decontaminant, followed by 70% ethanol.
8. Take the plate to the dirty room/area. Put on a new laboratory coat and keep the same pair of gloves on. Carefully remove the adhesive cover or caps from plate to prevent splashing or spraying of samples.
9. Add 5 µL to the appropriate well of the following in this order:
    - Extracted sample DNA
    - Extracted negative “blank” control
    - Dirty room no template control (NTC)
    - pLepBaBp+ plasmid DNA or other known positive control template
10. Cap the columns of wells as you go, covering the wells to protect them from the positive control. Use the roller tool to secure caps or plate seal applicator to secure plate seal film. Ensure caps or film are tightly fitted to avoid evaporation from wells during PCR.
    - NOTE- Incorrectly capped or uncapped wells may cause a reaction to fail and contaminate the real-time PCR machine. If this happens, wipe down the interior of the machine with 70% ethanol.
11. Wipe down the workspace with 10% bleach or DNA decontaminant, followed by 70% ethanol and turn on the UV light for 1 hour, if it is available. Remove laboratory coat and gloves and discard the gloves.

12. If possible, spin the plate at 500xg for a few seconds to bring down any droplets and collect reagents to the bottom of the wells.
13. Transport the plate directly to the real-time PCR machine and place in the machine.

Software Procedure:

The following instructions are detailed for the Applied Biosystems (ABI) 7500 instrument. If another instrument is being used, follow the manufacturer's instructions for setting up the software and for data analysis. The thermal cycler conditions will stay the same regardless of the machine being used.

1. Turn on ABI 7500 Real Time PCR System instrument.
2. Open the plate tray and place test plate into instrument. Close the tray.
3. Launch the Applied Biosystems 7500 System Software.
4. Click "Create New Document".
5. Change "Run Mode" to "Standard 7500".
6. Next to "Plate Name", enter the experiment number. Click Next.
7. Select appropriate detectors for FAM-BHQ (for TTS1) and VIC (for the ABI IPC). Add them to the Detection in the Document window. NOTE: Refer to the 7500-programming document to create the FAM-BHQ detector.
8. Next to "Passive Reference", select "None". Click Next.
9. Using the mouse, click and drag to select boxes corresponding to the wells containing samples.
10. Next to the detector box, check the "Use" box for both FAM-BHQ and VIC ABI IPC.
11. Change the task menu to mark "U" for unknown, "S" for positive "standard" controls or "NTC" for no template controls. Enter sample names into the appropriate wells. Click Finish.
12. Select the Instrument tab. Modify the thermal cycler conditions according to the adjacent table.
13. Other settings:

	Cycles	Temp	Time
Initial denaturation	1	95°C	8 min
Amplification	45	95°C 59°C	15 sec 30 sec

a. Verify that the number of cycles is 45.

b. Change the sample volume from 20 µL to 25 µL.

c. Verify that "Run Mode" is "Standard 7500"

14. Click File→Save As, and name and save the file in an appropriate project folder.
15. Enter information about the run in the "Reason for Change Entry" screen and click OK.
16. Click Start.

Data Analysis

1. When the run is complete, click on the Results tab.
2. Click on the Amplification Plot tab to view the raw data.
3. Highlight all the samples so that all the curves appear on the graph.
4. Click the radio button next to "Manual C_t" and enter a number corresponding to the scale on the left (19200, for example).
5. Using the mouse, click and drag the red threshold line until it lies above any background noise and within the exponential phase of the fluorescence curves.
6. Click "Analyze". The red threshold line will turn green, indicating the data has been analyzed.
7. Click File → Save to save the file again. Enter information about the run in the "Reason for Change Entry" screen.
8. Click the Report tab to display the C_t values. Record or print results according to your institution's reporting process.

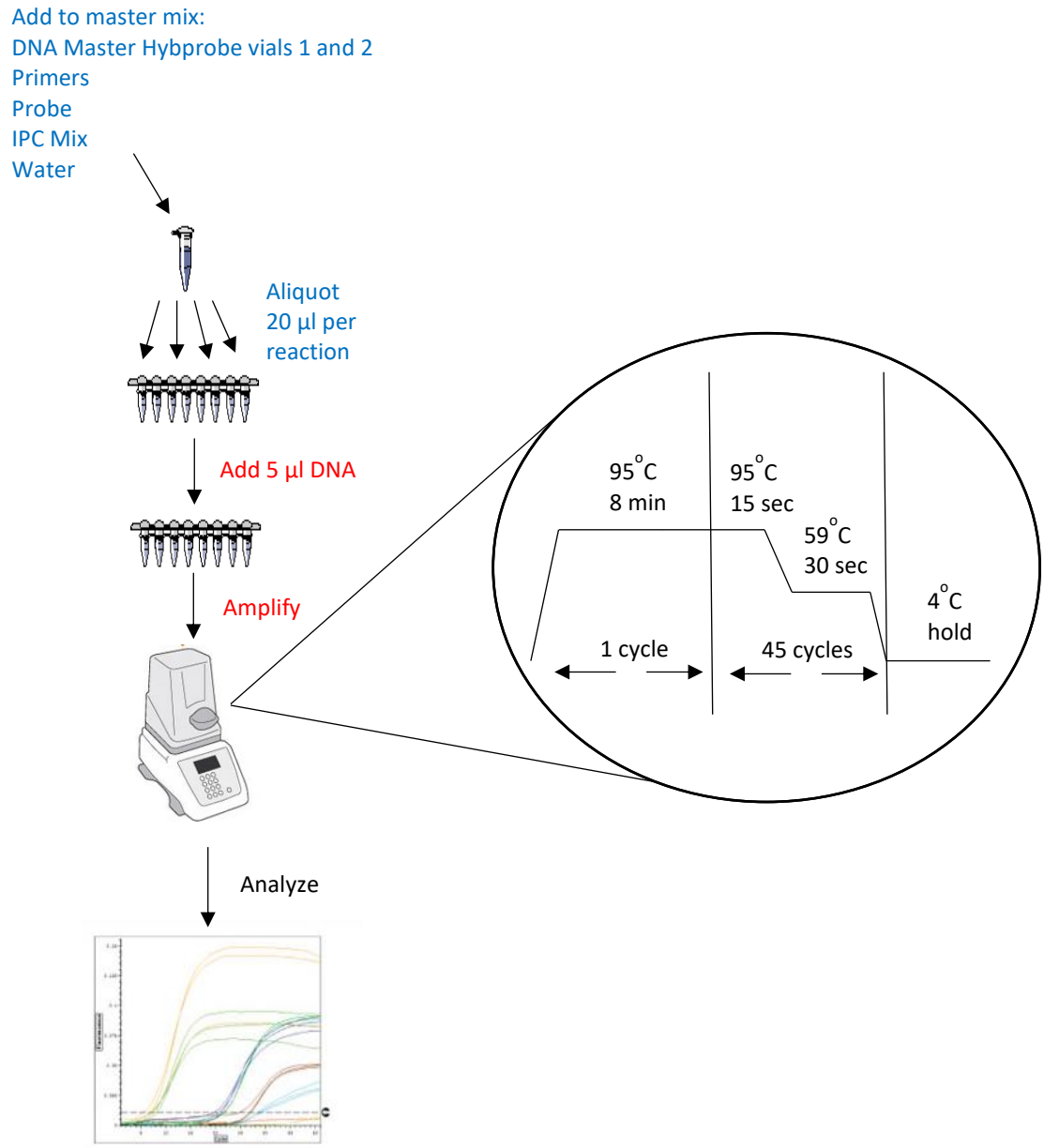


Figure 95- Work flow chart for Real-Time PCR Procedure: All steps in blue are completed in a "clean room" while steps in red are completed in a "dirty room"

Interpretation of Results and DNA Sample Storage

The readout of the data generated by real-time PCR machines typically generates an amplification plot as well as PCR C_t sample values (Figure 96). The curves that are generated in the amplification plot should be sigmoidal (characteristic "S"-shaped), ideally leveling out and reaching a plateau as the last cycle is approached. This indicates that all the reagents have been used. If a plot is lacking

these characteristics, it should be considered negative or retested. Each curve should be carefully examined, and all C_t values should be documented. The electronic data files created for each run should be saved for future reference (Nolan et al. 2013).

The cycle number at which the fluorescence curve for each sample crosses the fluorescence threshold (this can be manually set or generated automatically by the data analysis software) is called the cycle threshold value (C_t value). The plate readout shows the C_t value generated by each reaction. NTCs and negative controls should produce straight lines close to zero and give a "No C_t" result. Positive controls should have C_t values that are less than 35 and the curves generated should all be sigmoidal (WHO & CDC 2011).

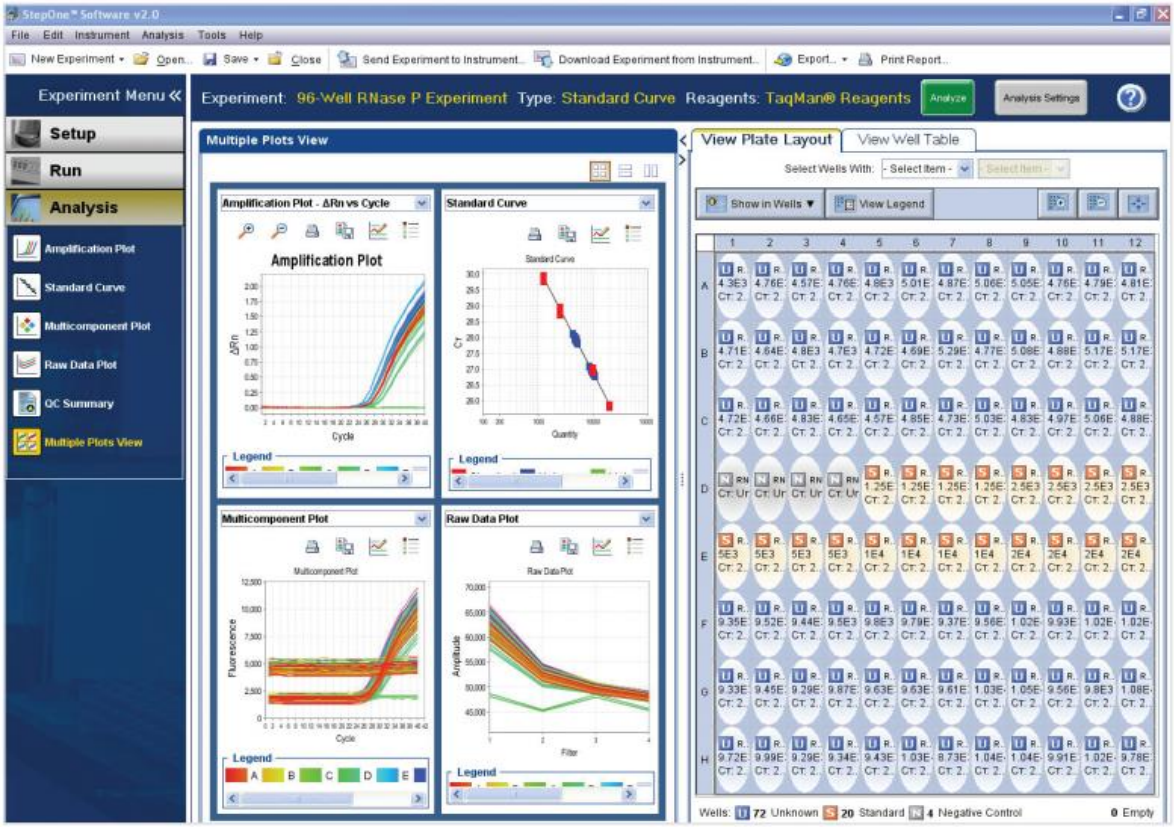


Figure 96- Typical real-time PCR readout depicting an amplification plot and plate layout of tested samples.

For the *B. pseudomallei* TTS1 assay:

1. Positive: C_t values ≤ 35 for TTS1 and IPC and appropriate results for positive and negative controls in the run.
2. Negative: C_t value negative or > 40 for TTS1 and IPC, and appropriate results for positive and negative controls in the run.
3. C_t values between 36 and 40 are considered equivocal. These samples should be diluted 1:10 using PCR-grade water to reduce the presence of inhibitors and retested. Other troubleshooting suggestions are described below.

Extracted DNA should be stored at ≤ -20°C for short-term storage and at ≤ -70°C for long-term storage.

NOTE- Labs that are setting up real-time PCR detection assays for the first time should consider pairing with a reference laboratory to help with results interpretation and ensure appropriate Quality Control.

Real-Time PCR Troubleshooting

If any NTC wells generates a C_t result or a curve that crosses the threshold, the water from that room should be thrown out. If the extracted water negative control generates an amplification curve that crosses the threshold, contamination has likely occurred during DNA extraction. Reagents used for DNA extraction should be replaced and the extraction space should be thoroughly cleaned (ThermoFisher 2014).

Additional factors to consider when troubleshooting real-time PCR reactions:

1. Ensure separate rooms, laboratory coats, gloves, pipettes and sterile barrier filter tips are being used for master mix preparation and template addition.

2. Ensure all work areas are appropriately cleaned and decontaminated.
3. Reaction plates should be covered to protect during transport from the clean room to the dirty room.
4. Consumable reagents (e.g. pipette tips, plates, caps, adhesive films, etc.) should not be reused.
5. Ensure the DNA extraction process was performed correctly.
6. If unsure of a result, the specimen or extraction can be transferred to a reference laboratory for confirmation.

Table 19- Real-time PCR troubleshooting (ThermoFisher 2014).

Observation	Possible Cause	Recommendation
Amplification curve shows abnormal values (the increment change of fluorescent signal at each time point, "ΔR_n", is abnormal)	The baseline was set improperly	Refer to the manual baseline set previously and ensure that all sample C_t values are higher than the baseline
	An amplification signal is detected too early	Dilute the DNA sample and re-run
Amplification curve shows no amplification across all samples	Degraded reagents and/or probe	Dispose of all reagents and start again with fresh stocks. Make sure to store and handle reagents properly to avoid early expiration.
	Degraded or contaminated template	Re-extract DNA and repeat PCR.
	Inhibitors present in reaction	If the TaqMan® IPC control is negative, repeat using diluted DNA to ensure no inhibitors are present.
	One or more of the reaction components were not added	Verify that all components were added.
Duplicate samples show different amplification	Inaccurate pipetting	Follow proper pipetting practices and repeat.
Negative controls show amplification*	Contaminated reagents	Repeat PCR with new reagents. Use proper handling to ensure no further contamination. Never use DNA in a clean room.
	Contamination through pipetting	Re-run and be careful to not contaminate negative wells with any DNA
Positive controls show no amplification*	Degraded or contaminated template	Re-extract control DNA and repeat PCR.
	One or more of the reaction components were not added	Verify that all components were added.
Noisy signal above the threshold	Evaporation	Check the seal of the optical adhesive cover for leaks.
	Inaccurate pipetting	Check calibration of pipettes. Follow proper pipetting techniques.

*If any of the control samples result in curves that are not expected (e.g. positive control shows no amplification or negative control shows amplification) the sample results cannot be confirmed and the entire PCR should be repeated before results can be reported.

Propagation of Plasmid pLepBaBp+

Background

Plasmid pLepBaBp+ contains short target sequences that act as positive controls for certain real-time PCR assays. This plasmid was developed using a pUC plasmid transformed into an *Escherichia coli* strain named TOP10 (Invitrogen – catalogue number C4040) (Figure 97). pUC is a series of plasmid cloning vectors that is a circular double stranded DNA with 2686 base pairs. It is a widely used vector since the recombinant plasmid-containing cells (cells with DNA that have been modified to include genes from multiple sources) can be distinguished from the non-recombinant cells based on the color differences of bacterial colonies on selective culture agar (see below for detail) (Yanisch-Perron et al. 1985).

The plasmid contains short target sequences for PCR based detection of a few bacterial species: *B. anthracis*, pathogenic *Leptospira spp.* and *B. pseudomallei*. The plasmid also contains a sequence for RNase P which serves as an internal positive control for human DNA. To produce more plasmid, grow the *E. coli* in selective media (Luria Burtani) containing ampicillin. DNA can then be extracted from a colony using any plasmid DNA extraction method (CDC 2020).

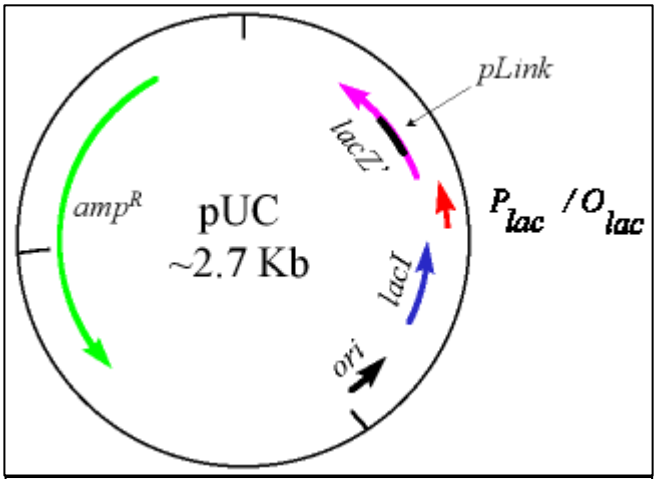


Figure 97- Map of the pUC plasmid used to create the pLepBaBp+ control in *E. coli*. This plasmid contains an ampicillin resistance cassette allowing it to be grown in selective media (Blaber 1998).

pLepBaBp+ Plasmid Propagation Protocol

For the real-time PCR procedure above, the plasmid DNA should be used at a concentration of 10^{-12} g/µL (i.e. 1 picogram/µL). Interference of signal can occur if used at too high of a concentration.

Equipment and Reagents

- Sterile inoculating loops or sterile toothpicks
- Erlenmeyer Flask (50 mL)
- Luria Burtani (LB) AMP100 Broth (i.e. with 100µg/mL ampicillin)
- LB Agar AMP100 plates (i.e. with 100µg/mL ampicillin)
- *Escherichia coli* strain TOP10 (Invitrogen – catalog number C4040)
- Incubator set to 37°C
- Shaking Incubator
- Spectrophotometer
- Plasmid Extraction Kit (various brands available)
- Parafilm
- Personal Protective Equipment (lab coat, gloves, eye protection, shoe covers)

Protocol

The plasmid is in an *E. coli* TOP10 15% glycerol stock that is best stored at -80°C. For maximum preservation, do not thaw out the tube at any time.

1. Warm LB agar AMP100 plate to 37°C prior to streaking.
2. Open the tube of *E. coli* TOP10 and scrape the surface of the frozen broth with either a microbiological loop or toothpick.
3. Streak LB agar AMP100 plate with the bacteria.
4. Incubate LB agar AMP100 plate overnight (12 to 18 hours) at 37°C.
5. Examine plate to verify colonies present.

6. Select a single colony using a microbiological loop or toothpick.
7. Inoculate Erlenmeyer Flask containing at least 5 mL of LB AMP100 broth.
8. Incubate at 37°C in shaking incubator at 200 rpm for 12 to 18 hours or mix thoroughly and place in a standing incubator if not available.
9. Prepare plasmid using any preferred miniprep DNA extraction kit.

NOTE- Plate may be preserved up to one week. Use parafilm to seal plate and leave at 4°C. The *E. coli* with plasmid stock tube should be stored in 15% glycerol at -80°C. For maximum preservation, do not fully thaw out the tube at any time.

Chapter Summary

Real-time PCR is a highly a sensitive and specific method used for the detection, quantification and typing of multiple pathogens, including *B. pseudomallei*. The main advantages of real-time PCR are that it provides a fast and high-throughput method of detection through simultaneous amplification and visualization of newly formed DNA products. The preceding chapter describes a method for real-time PCR detection using the *B. pseudomallei* type III secretion system (TTS1) gene cluster. As a result of the very high specificity and sensitivity of the detection assay, real-time PCR protocols must be followed exactly as described. If a lab is going to conduct real-time PCR testing, they should be able to follow standard protocols without substitution and adhere to the appropriate laboratory guidelines detailed above. Labs that are setting up real-time PCR detection assays for the first time should consider pairing with a reference laboratory to help with results interpretation and ensure appropriate Quality Control.

Chapter 16: Molecular Methods for *B. pseudomallei* Bacterial Strain Typing

Chapter Overview and Introduction to *B. pseudomallei* Strain Typing

Bacterial strain typing, or the classification of different genetic variants of bacterial species, is important for disease diagnosis, treatment, and epidemiological surveillance. This is particularly relevant for bacteria exhibiting high levels of pathogenicity and virulence, including *B. pseudomallei*. *B*. *pseudomallei* is classified as a Tier 1 select agent by the US Centers for Disease Control and Prevention (https://www.selectagents.gov/). Potential outbreak scenarios thus warrant rapid public health response and high-resolution tools to quickly investigate source etiology.

In endemic regions, individual cases of melioidosis are typically the result of infection by a single environmental isolate of *B. pseudomallei*. Despite this, clonal or "point-source" outbreaks have been described on several occasions, including contamination of drinking water supplies in two remote Indigenous communities from Northern Australia (Currie et al. 2001; Sarovich et al. 2017), contaminated hand-washing detergent (Gal et al. 2004), as well as cases traced to domestic water wells/bores (McRobb et al. 2015). A handful of outbreaks have also been described in non-endemic settings. One of the most notorious of these outbreaks took place at a Paris zoo between 1975 and 1979. Aside from infecting numerous animals at the zoo, several horse and human melioidosis cases elsewhere in France also occurred as a result (Mollaret 1988). Outbreaks are also frequently reported in non-native animal species in endemic regions, such as in slender-tailed meerkats from Thailand and north Australian zoos (P. Kongmakee 2015; Rachlin et al. 2019), marine mammals from a Hong Kong Ocean Park (Hicks et al. 2000), exotic birds (Hampton et al., 2011), as well as numerous non-human primate species (Sim et al. 2018).

The following chapter discusses several of the more widely used methods for *B. pseudomallei* molecular strain typing, with specific protocols provided for multi-locus sequence typing (MLST), multilocus variable number of tandem repeats analysis (MLVA) and BOX-PCR. An overview of whole-genome sequencing (WGS) technology and its future implications for bacterial source-tracing are also presented at the end of the chapter.

General Approaches to Bacterial Strain Typing

A variety of molecular typing methods have been designed for epidemiological investigations of bacteria and for examining bacterial population genetics. Some methods are more useful for examining localized outbreaks, while others are more suitable for determining the global or long-term evolutionary relatedness of strains (Chan et al. 2001; Maiden et al. 1998). Several current approaches to *B. pseudomallei* strain typing include:

1. Multilocus sequence typing (MLST) – MLST involves sequencing 400-600 base pair fragments of DNA at seven different conserved genes. It enables small variations within a species to be detected. The method is highly discriminatory and can be used to compare the relatedness of isolates globally, but can be both time consuming and expensive, making it impractical for rapid outbreak scenarios (Maiden et al. 1998).
2. Multilocus variable number of tandem repeats analysis (MLVA) – MLVA is a molecular fingerprinting method based on PCR amplification and sequencing of repetitive DNA sequences termed “variable number tandem repeats” (VNTRs). It generates a specific MLVA pattern that is unique to the bacterial strain being investigated. MLVA is quicker to obtain results than MLST, however there are issues with reproducibility and inter-laboratory comparison (van Belkum et al. 1998).
3. Repetitive sequence-based PCR (rep-PCR) – Bacterial genomes contain multiple non-coding, repetitive regions of DNA. Rep-PCR uses these repetitive sequences to produce fragments of variable sizes. These can then be separated using gel electrophoresis, producing a

characteristic pattern of bands, sometimes called a genetic “fingerprint”. One type of rep-PCR frequently used for *B. pseudomallei* is the BOX-PCR (Versalovic et al. 1991).

For local outbreak investigations, molecular typing and fingerprinting methods that are highly resolving are necessary to distinguish small differences in genetically related strains. These types of assays are also useful for distinguishing small genetic changes during sustained outbreaks. The methods that have been used for this purpose include BOX-PCR and multiple-locus variable-number tandem repeat analysis (MLVA) (Maiden et al. 1998). During an outbreak or a cluster of cases, rapid high-resolution fingerprinting methods can be used alongside epidemiological data and more conserved typing techniques, such as MLST, to help discriminate and determine the relationship between isolates.

B. pseudomallei Multilocus Sequence Typing

Multilocus sequence typing (MLST) has become the most widely used method of *B. pseudomallei* strain typing globally, as it indexes variations within fragments of seven housekeeping genes that are selectively neutral. This allows for easy interlaboratory comparison and simple, unambiguous strain characterization (Maiden et al. 1998). Different allele numbers are assigned to each of the seven housekeeping alleles based upon point mutations that occur within each gene. Once assigned, the allele numbers of each of the housekeeping genes are arranged into a string of seven numbers to produce an allele profile. The allele profile corresponds directly to a specific sequence type (ST). An online MLST database has also allowed for standardization of strain types and has conferred the ability to analyze evolutionary relatedness between isolates (Maiden et al. 2013) (https://pubmlst.org/organisms/burkholderia-pseudomallei). Typing of *B. pseudomallei* using MLST scheme is useful to explore sequence type diversity in a particular geographical area (Cheng et al. 2008), track the source of melioidosis outbreaks (Inglis et al. 2000) and define whether recurrent

melioidosis is due to a relapse of the same bacterial ST or reinfection with a different ST (Maharjan et al. 2005; Rachlin et al. 2016).

The MLST scheme specific for *B. pseudomallei* was originally developed by Godoy *et al.* in 2003 and employs seven house-keeping alleles 400bp-600bp in length (Godoy et al. 2003; McCombie et al. 2006). The scheme has now been used to identify more than 1900 *B. pseudomallei* MLST sequence types (as of March 2021). In recent years, whole-genome sequencing (WGS) has provided a rapid alternative for extracting MLST profiles; however, the PCR-based Sanger sequencing method for MLST is still used by many researchers across the globe.

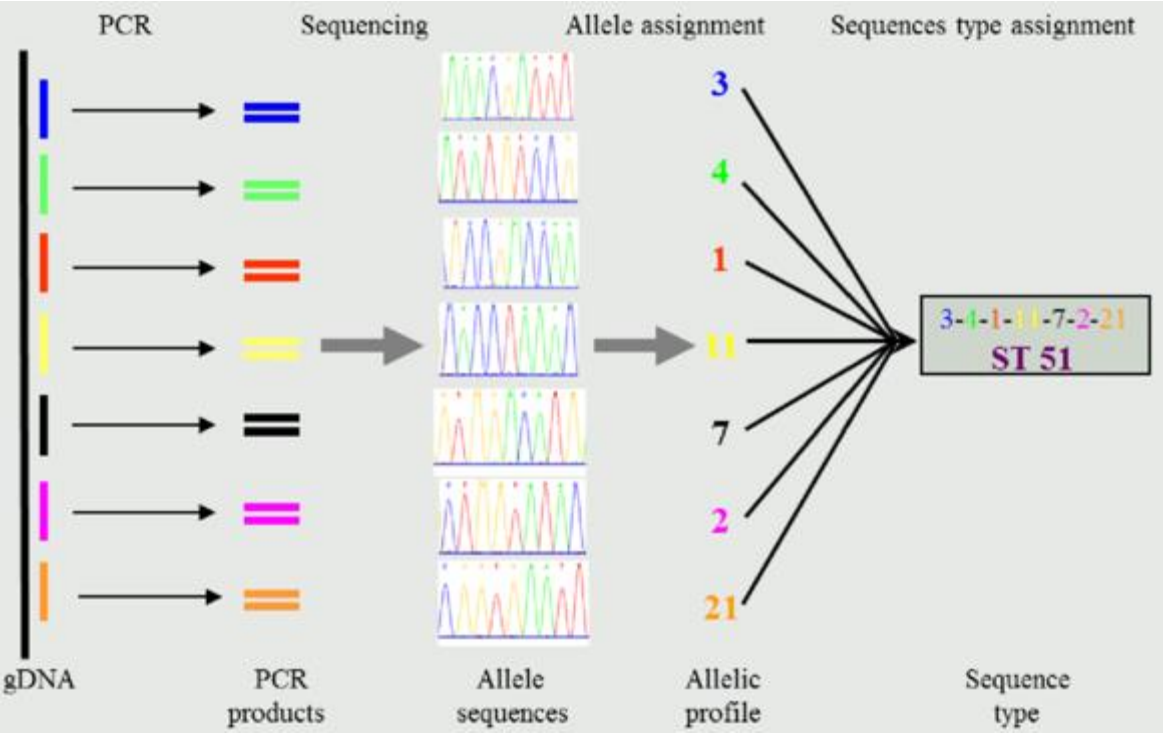


Figure 98- Schematic diagram of the typical bacterial MLST protocol. Allele numbers are assigned to each of the seven PCR amplified housekeeping gene fragments giving an allele profile for a bacterial isolate. Examination of the online MLST database using the allele profile results in ST assignment (Ruppitsch 2016).

B. pseudomallei MLST Protocol

General Comments Before Starting

PCR is very susceptible to contamination and additional precautions should be taken to minimize cross-contamination:

- Where possible, separate the areas where different PCR steps are performed, including PCR reagent assembly, addition of the DNA template, and detection of PCR products (see Chapter 12 for further detail).
- If individual rooms are not possible, separate laboratory bench spaces should be used for these steps. Separate pipettes, laboratory coats and gloves should be used in each of these areas.
- Working in a PCR laminar flow hood is also recommended to minimize cross-contamination.
- Decontamination of surfaces and equipment with a 10% bleach solution or DNAerase/commercial decontaminant solution followed by 70% ethanol should be performed after any handling of nucleic acids at the laboratory bench.
- Always use filter-barrier pipette tips and change and discard gloves frequently.
- Remove DNA templates, controls and other reagents from the freezer and allow them to thaw completely before use. Briefly vortex and centrifuge tubes for 30 seconds at 1000 rpm before using.
- It is good practice to keep reagents on ice while setting up the reaction plate and PCR master mix to prevent reagent degradation.
- Prior to beginning the PCR, plan the experiment by filling out and printing a plate template worksheet (see example below). Ensure sufficient quantities of primer working solutions and other PCR reagents are available and have been correctly calculated.
- Primer working stock solutions should be made up to 20 µM before starting. This will help to prevent primer contamination and limit repeat freeze-thaw cycles every time the reaction is performed.

- Care must be taken when interpretating results, particularly during inter-laboratory studies. Minor differences in electrophoresis conditions can change the distance bands migrate through the gel, confusing comparison between isolates.

Equipment and Reagents

- Primers (see Table 20 below)
- PCR positive and negative controls
- 10x PCR Buffer
- 10mM dNTP Mix
- Taq DNA Polymerase
- Q solution (QIAGEN) or Betaine solution (e.g. Sigma-Aldrich B0300)

NOTE- While not required, these additives are commonly used to enhance amplification of GC-rich sequences. 5% Dimethyl sulfoxide (DMSO) may also be used if these solutions are not available.

- $MgCl_2$
- PCR-grade water
- 10% bleach solution or commercial DNA decontaminant (e.g., DNAerase)
- 70% ethanol
- PPE (gloves, lab coats and safety glasses)
- Laboratory pens/markers
- Racks for 1.5mL microcentrifuge tubes
- Pipettes and filter/barrier pipette tips
- Sterile PCR microcentrifuge tubes
- Benchtop microcentrifuge
- 96-well polypropylene PCR plates (e.g. Thermo Scientific™ AB0600) or PCR strips (e.g. Axygen™ 0.2 mL Polypropylene PCR Tube Strips #16272591)
- PCR strip caps to cover wells

- Vortex
- Thermocycler (such as Eppendorf Mastercycler® or T100™ Bio-Rad)

Primers and PCR conditions for MLST of *B. pseudomallei*

The following primers should be used for the amplification of the seven house-keeping gene fragments:

Locus	Gene function	Product Size (bp)	Forward Primer (5'-3')	Reverse Primer (5'-3')
ace	Acetyl coenzyme A reductase	519	5'-CGGCGCTTCTCAAAACGATA-3'	5'-GAATCGCCTTCACCATGTC-3'
gltB	Glutamate synthase	522	5'-ACGCTCGCGATCGCGATGAA-3'	5'- TTCAGCACGAGCGTCTGCTG-3'
gmhD	ADP glycerol-mannoheptose epimerase	468	5'-GCAGTTCCTGTATGCGTC-3'	5'-GAAGCACTGGTACTTGCC-3'
lepA	GTP-binding elongation factor	486	5'-CATATTCGCAATTTCTCGATC-3'	5'-CACGAGCATCACGACGCCG-3'
lipA	Lipoic acid synthase	402	5'-GGCACCGCGACGTTCATG-3'	5'-GACCATCAGGCCCGATTTCG-3'
narK	Nitrate extrusion protein	561	5'-CTACTCGTGCGCTGGGAT-3'	5'-GACGATGAACGGCACCCAC-3'
ndh	NADH dehydrogenase	443	5'- AGTCGCGACGTTCTACAC-3'	5'- CGAGTTGCAGACGAGATA-3'

Table 20- House-keeping alleles used for *B. pseudomallei* MLST. Gene functions, primers and product sizes are also shown (https://pubmlst.org/organisms/burkholderia-pseudomallei) (Godoy et al. 2003).

NOTE- Alternative primers for the *B. pseudomallei* MLST assay are also described by Price et al. (2016) *J Med Microbiol* 65:992–7.

A. Preparation of DNA Template

For each of the PCR assays described in this chapter, DNA must be extracted from the specimen before it can be tested. **Molecular typing requires isolation of DNA from a pure bacterial culture**. This is done to ensure there is only one bacterial isolate extracted in the event there is a mixed or

"polyclonal" infection and ensures that there is sufficient quantity of bacterial DNA for typing. See Chapter 13 for specific protocols on DNA extraction from bacterial culture.

B. MLST PCR Set-Up

1. Create a plate map to keep track the samples being tested. An example of an appropriate plate map is shown below (Here, K96243 is used for the positive control).

	1	2	3	4	5	6	7	8	9	10	11	12	
A	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*ace* locus
B	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*gltB* locus
C	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*gmhD* locus
D	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*lepA* locus
E	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*lipA* locus
F	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*narK* locus
G	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*ndh* locus
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	

2. Prepare the PCR master mix reagents in the designated PCR clean room/area. The master mix should include all reagents **except the template DNA**. This should be done for all 7 MLST alleles/primer sets to be tested. Ensure two additional reactions are prepared when calculating master mix quantities to account for reagents lost during pipetting. Reactions should be performed in 50 µL volumes either in 8-well tube strips or in 96-well plates. When setting up the PCR reactions keep reagents on ice to prevent degradation.

Reagent	Volume (µL) per Reaction	Final Concentration
PCR grade water	27.75	
10X buffer	5.0	1X final concentration
25mM $MgCl_2$	3	1.5 mM final concentration
10 mM dNTPs	1	200 µM final concentration
20 mM forward primer	1	0.4 µM final concentration
20 mM reverse primer	1	0.4 µM final concentration
Q Solution/Betaine Solution	10	1X final concentration
Taq DNA polymerase	0.25	2.5U final concentration
Template DNA	1.0	
Final volume	50.0	

3. Gently mix the master mix then briefly spin in a microcentrifuge.
4. Pipette 49 µL of this master mix (for each MLST allele/primer set) into the appropriate well of a 96-well plate or PCR strips, according to the plate template worksheet.
5. Cover the wells using cap strip covers. Spray and wipe down the clean workspace with bleach or DNAerase and repeat this with 70% ethanol. Remove laboratory coat and gloves and put on a new pair of gloves. Carry the 96-well plate or PCR strips to the DNA template addition area.
6. Put on a new laboratory coat while keeping the same pair of gloves on. Carefully remove the cap strip covers from the 96-well plate or PCR strips. Open these slowly to prevent aerosolization and spraying of any liquid in the wells.
7. Add 1 µL of template DNA to each appropriate well of 96-well plate or PCR strip using the plate template worksheet for reference.
8. Add 1 µL water to the 'NTC' well.
9. Add 1 µL of the positive control DNA to the correct well. One positive control should be used for each master mix.
10. Cover the wells of each column of the PCR plate or each PCR strip using strip cap covers as you go and secure caps tightly.
11. Spray and wipe down the area with bleach or DNAerase and repeat with 70% ethanol. Remove laboratory coat and gloves. If possible, briefly spin the plate at 1000 rpm to bring down any liquid droplets. Transport the plate directly to the PCR thermocycler room/area and place it in the PCR thermocycler.

C. PCR Thermocycler Set-Up

1. Set the following PCR cycle on the thermocycler:

Stage	Time	Temperature	# Cycles
Initial denaturation	4min	95°C	1
Amplification	30sec	95°C	35
	30sec	62°C	

	30sec	72°C	
Final Extension	10min	72°C	1
Hold	--	4°C	--

2. Double check all temperature and times are correct and press start.
3. Once the reaction has finished, store the PCR products at 4°C until ready for subsequent product analysis or store at -20°C long-term.

D. Analysis of PCR Products by Gel Electrophoresis

It is useful to check for a successful PCR amplification using gel electrophoresis before beginning PCR product purification and sequencing.

1. While the MLST PCR is running, make up an agarose gel **(refer to Chapter 14- Conventional PCR for the full gel electrophoresis protocol).**
2. Once the PCR has finished running, turn off the PCR machine, briefly centrifuge the tubes and take the tubes into the post-PCR processing gel electrophoresis room.
3. Carefully load 5 μL of each DNA product (mixed with loading dye- 1-2 drops or approx. 2 uL) and the DNA ladder into the gel.
4. Run the gel for approximately 45-60 minutes at 100V (check frequently to ensure the products do not run off the gel).
5. Remove the gel from the tank and place on the gel doc to visualize the products.
6. A strong band should appear for each sample to be sequenced. Ensure these bands are the correct size. Only a single DNA fragment should have amplified. This may involve some optimization of the PCR annealing temperature. Since B*. pseudomallei* has a high G+C content, the use of additives such as DMSO with betaine "Q-Solution" can help to improve the amplification of PCR fragments (https://pubmlst.org/organisms/burkholderia-pseudomallei).

Refer to the troubleshooting guide in Chapter 14 if any of the reactions do not display bands on the gel, if there are multiple bands present in one lane, or if bands are very faint. An example of how PCR products should look is shown in the figure below (Figure 99) (Price et al. 2016).

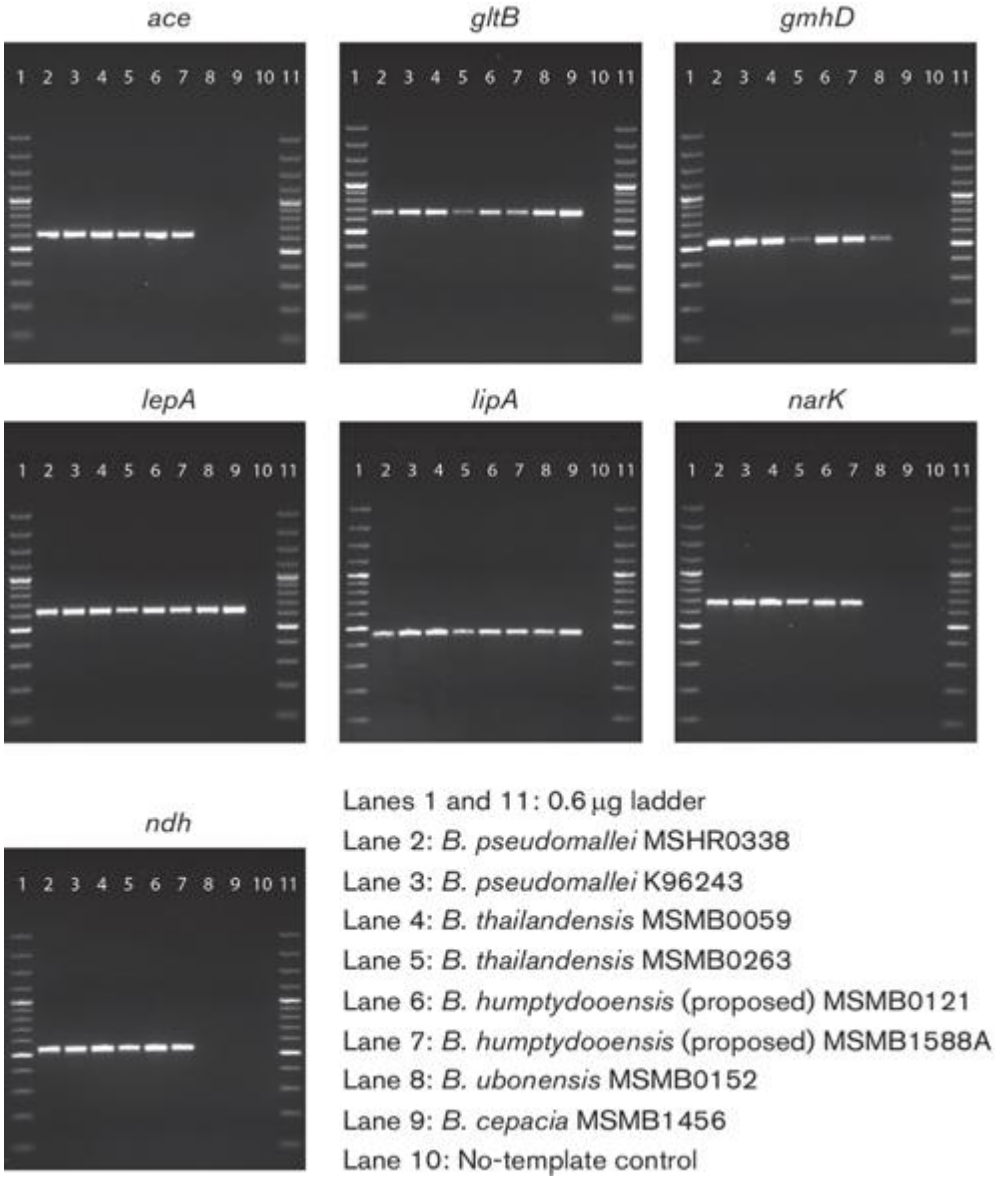


Figure 99- Example of successful amplification of the seven MLST gene alleles. Here, an alternative set of *B. pseudomallei* MLST primers (see the reference for the alternative primers above) are used to amplify members of the *Burkholderia pseudomallei* complex (*Burkholderia pseudomallei, Burkholderia humptydooensis, Burkholderia thailandensis*). Members of the *Burkholderia cepacia*

complex (*Burkholderia cepacia* and *Burkholderia ubonenesis)* do not amplify across all seven alleles (Price et. al 2016).

E. Purification of PCR Products

To perform MLST Sanger sequencing, the amplified products must be purified before sequencing reactions can be performed. This is done to remove any unincorporated PCR reagents and dyes. Purification can be carried out using one of several commercially available kits, such as QIAquick (QIAGEN), which utilize spin columns to purify the products. Likewise, the reaction products can be purified by ethanol precipitation methods or PEG_{8000}/2.5M NaCl precipitation. The inexpensive cost of reagents may make ethanol or PEG_{8000}/2.5M NaCl precipitation a more practical option in resource-poor labs. See below for a simple protocol for PEG_{8000}/2.5M NaCl purification.

NOTE- Sequencing generally requires about 10ng of purified PCR product per 100 bp product size (e.g., for a 500 bp PCR product, about 50 ng of template is required for sequencing) (QIAGEN 1998).

A protocol for QIAquick (QIAGEN) purification, can be accessed at:

https://www.qiagen.com/au/resources/resourcedetail?id=e0fab087-ea52-4c16-b79f-c224bf760c39&lang=en

For PEG_{8000}/2.5M NaCl Precipitation (Glenn 2021; Kusukawa et al. 1990):

Materials-

- 20% PEG (Polyethylene glycol), 2.5 M NaCl –
  - For 50mL, mix the following in a 50 mL conical flask:
    1. 10.0 g Polyethylene Glycol 8000
    2. 7.3 g NaCl
    3. Distilled H_2O up to 50 mL –
  - Place into an incubator set to 37°C for 20 minutes.

- 80% ethanol
- TLE = 10 mM Tris-HCl, 0.1 mM EDTA

Procedure

1. Add 50 µL of PEG to a sterile Eppendorf tube. Transfer the PCR product to the tube and mix thoroughly by pipetting up and down.
2. Incubate the mixture at 37°C for 15 min.
3. While the mixture is incubating, place the 80% ethanol on ice.
4. Once the mixture has been incubated, centrifuge tubes at high speed (~15,000 x g) for 15 minutes.
5. Use a sterile pipette to remove the supernatant and discard it.
6. Add 125 µL of cold 80% ethanol to the tube and centrifuge for two minutes. Carefully remove the supernatant with a pipette and discard.
7. Repeat step 6.
8. Evaporate residual ethanol using a speed-vac for 5-10 minutes or by opening the tube and leaving in a 37°C heat block. There should be no trace of the ethanol remaining.
9. Resuspend the pellet in 25 µL of distilled water or buffer of choice (e.g TEL buffer- Tris-HCl/EDTA) or make up to the appropriate concentration required for sequencing. Pipette up and down enough so that the DNA has fully gone into solution.
10. Laboratories should ensure they follow the protocols described by the sequencing platform being employed to ensure the correct concentration of product is sent.

F. Sanger Sequencing of Purified MLST PCR Products

The purified PCR gene fragments should be sequenced in both forward and reverse directions and then aligned using sequence alignment software. Sequencing each strand provides more confidence than if only a single strand is used to determine the base calls. The aligned sequences can then be trimmed to the correct length for comparison and examined for miscalled bases, insertions, gaps,

overlapping nucleotide signals and any sequence differences. High-quality sequencing is essential for MLST, as a single base pair change can result in a new allele number being designated (Maiden et al. 2013).

For *B. pseudomallei* MLST, the same 7 MLST primers are also used for sequencing that are used for PCR amplification. Primers should be diluted in PCR-grade water to a concentration of 10pmol/μL before sending for sequencing.

Preparing primer dilutions

- The easiest way to prepare primer dilutions is to make a 1 in 2 (1:2) dilution of a 20 μM primer stock.

  NOTE: 20 μM = (20mol/L) x (1L/1000,000 μL) x (1000,000 pmol/1μmol) = 20pmol/μL
- Send 20 μL of 10pmol/μL for every 5 samples to be sequenced.

Once the PCR products have been purified and primers have been appropriately diluted, they can be shipped to any commercial sequencing laboratory for standard Sanger sequencing (e.g. Macrogen, Seoul, Korea: https://dna.macrogen.com/pageLinkDnaSys.do?menuCd=SUP100200&layout=page_sub&link=%2Fsupport%2FretrieveGuideCes#none.

Ensure samples are appropriately sealed to prevent spillage or evaporation during transport and that sample information is filled out correctly based on the specific commercial sequencing laboratory being used. Sequencing can also be done in-house if available using commercially available kits (e.g. Applied Biosystems BigDye™ Terminator chemistry).

G. Analysis of Sequence Data and Allele Determination

- After the data has been obtained from the sequencer, the sequencing files (also termed trace files) must be examined for incorrect base calls. The complementary strands must also be aligned and trimmed so they are the appropriate length. These aligned and trimmed sequences are called “DNA consensus sequences”- or a sequence of DNA that represents the most commonly encountered nucleotides found at a specific location.
- Various computer programs can be used to assemble, align and edit the trace files from the DNA sequencer and can be used to create consensus sequences, such as CLC Genomics Workbench (QIAGEN) or ChromasPro (Technelysium- free online download).
- Follow the specific software protocols to assemble and align the sequences.
- Sequences that are inadequate in length (too short) or poor in quality (displaying two peaks at the same nucleotide position, or sequences exhibiting an inadequate concentration of DNA) should be discarded and the samples should be re-amplified and sequenced again.
- Forward and reverse sequences that are high in quality can be aligned to create a consensus sequence for the seven housekeeping genes for each *B. pseudomallei* isolate.

H. MLST Analysis

Once consensus sequences have been created, they can be copied into the MLST website for allelic analysis and sequence type (ST) determination (https://pubmlst.org/organisms/burkholderia-pseudomallei). The software examines if the sequences are the correct length and do not contain any unspecified characters. The user is then able to check the allele at a single locus, to enter an allele profile, to search for isolates in the database that match a specific allele profile, or simply browse the database. Sequences not already present in the database can be submitted to the database curator as a new allele. The database curator will then examine the sequence trace files and assign a number to the new allele profile to add to the database.

To check individual alleles:

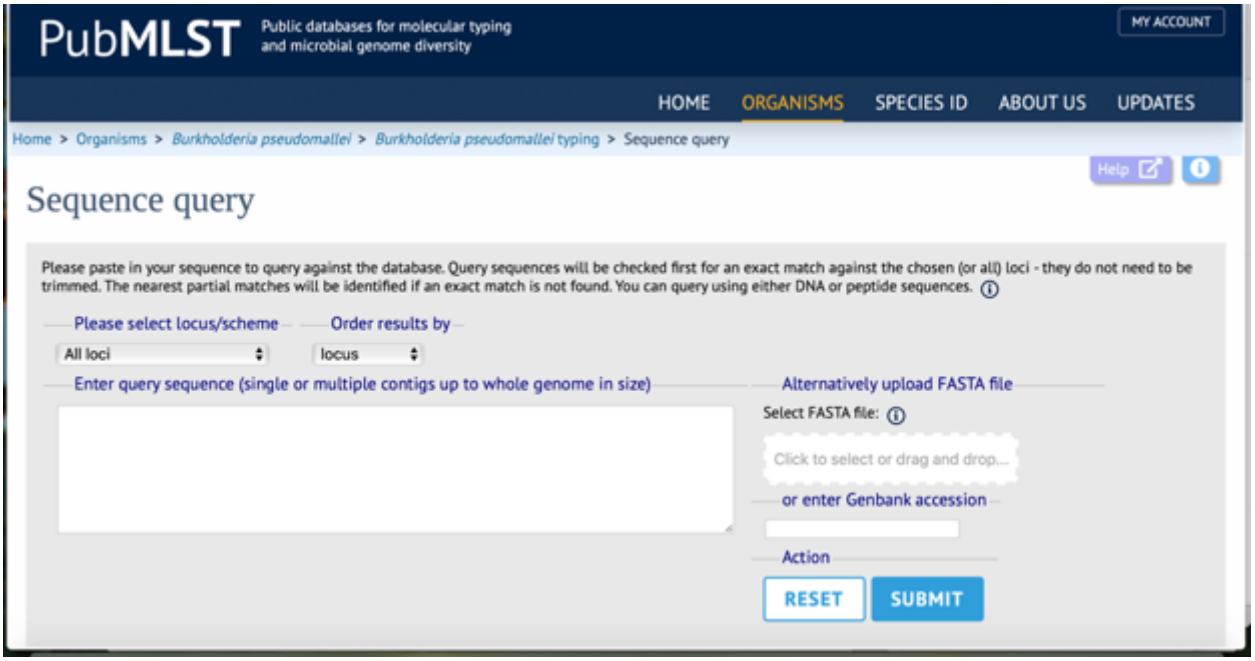
1. On the MLST website select “Single Sequence Query”

   (https://pubmlst.org/bigsdb?db=pubmlst_bpseudomallei_seqdef&page=sequenceQuery).

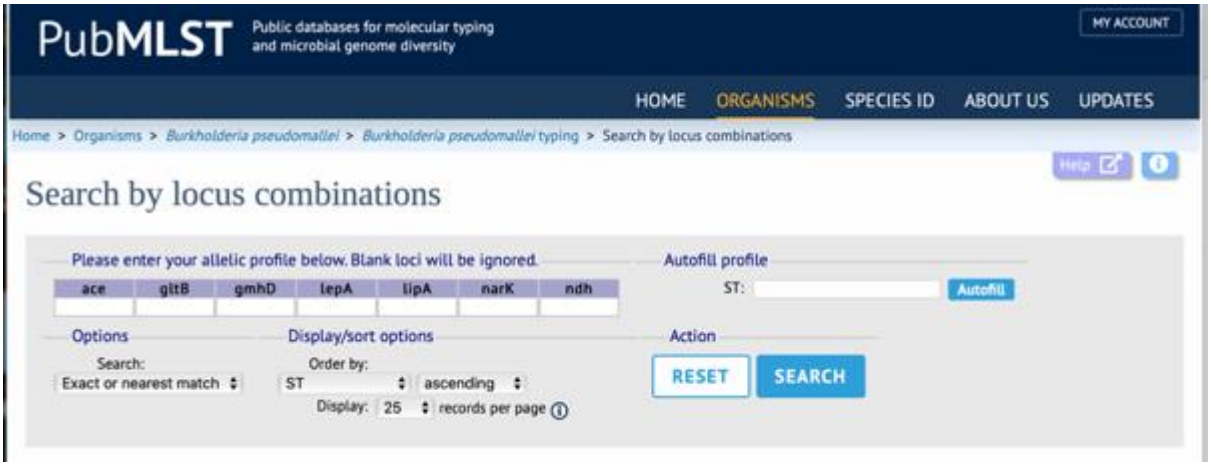
2. Choose the specific allele to be checked and paste your allele sequence into the box.

   Alternatively, you can upload individual sequence FASTA files.

3. Press submit. The number for your allele will be displayed at the top.

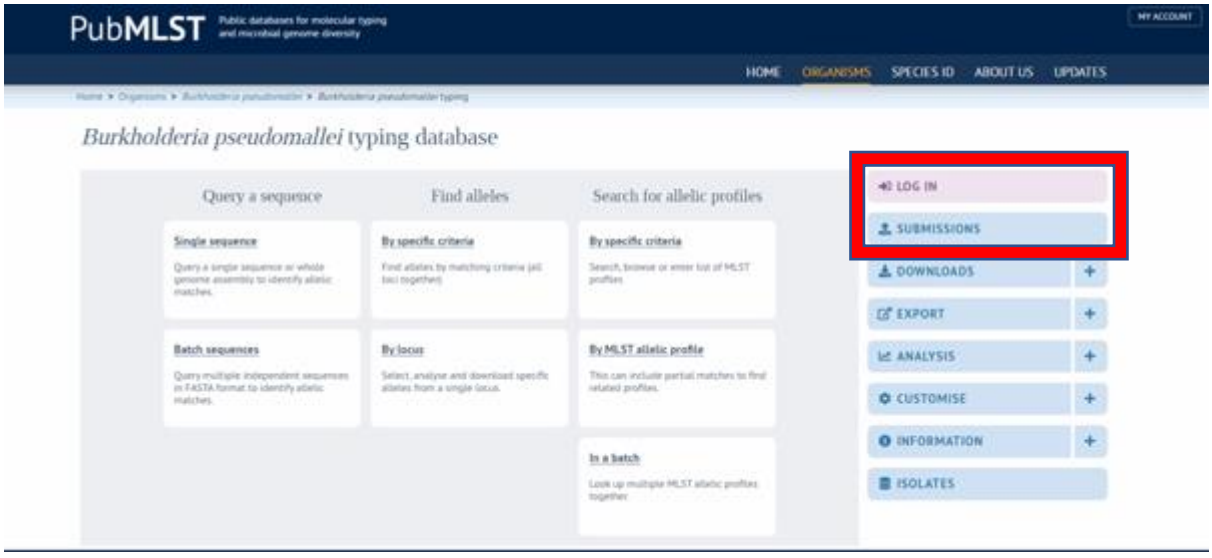


4. Write down the number that is assigned to each allele.
5. Go to “Search for Allelic Profiles by Specific Criteria”: https://pubmlst.org/bigsdb?db=pubmlst_bpseudomallei_seqdef&page=profiles&scheme_id=1.
6. Type in the number for each allele and press submit to view the designated ST type.

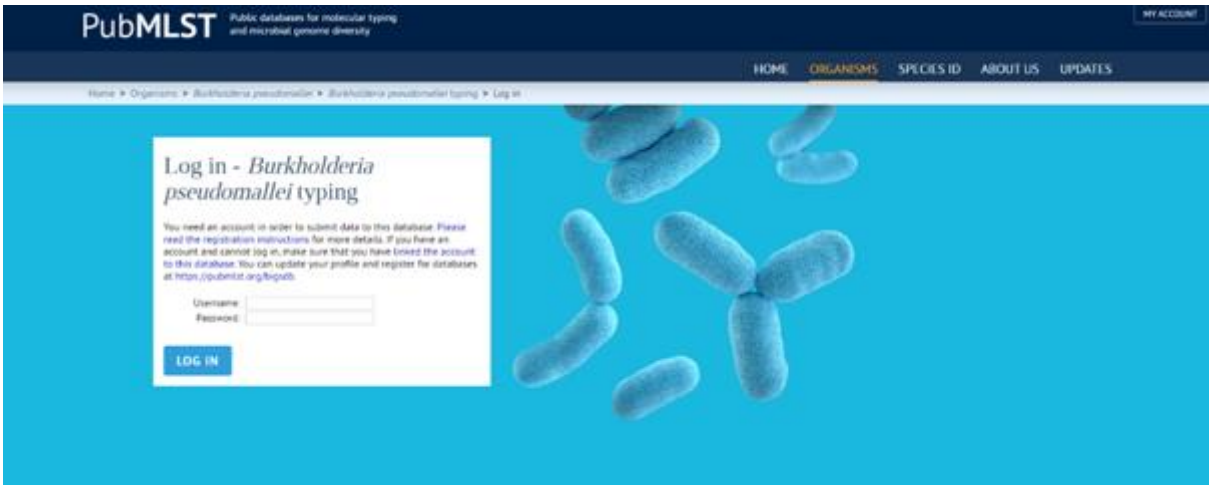


I. Submitting Data Using the Online Submission System

The automated submission system allows users to submit data (new alleles, ST profiles, or isolates) to the database. The MLST curator will then assign new alleles or STs and upload them to the database. New submissions can be made by clicking the ‘Typing” followed by “Submissions” tab on the database front page.



You must have an account in order to use the submission system. Once logged in, follow the instructions provided to submit MLST data.



NOTE- For any questions concerning how to use the *B. pseudomallei* MLST database or submitting isolate profiles, email the database curator (email found on the *B. pseudomallei* PubMLST homepage https://pubmlst.org/organisms/burkholderia-pseudomallei).

J. Phylogenetic Analysis Using MLST Data

The MLST online database has several links to various programs for further analyses. MLST data are frequently represented by a dendrogram (a tree diagram used to visualize evolutionary relationships and show the arrangement of clusters) using programs such as molecular evolutionary genetics analysis version-6 (MEGA 6) software (Tamura et al. 2013) (Figure 100) and eBURST analysis, which can be used to determine the evolutionary relatedness between isolates (Feil et al. 2004; Spratt et al. 2004) (Figure 101). A plugin for MLST eBURST analysis is available at https://pubmlst.org/bigsdb?db=pubmlst_bpseudomallei_seqdef&page=plugin&name=BURST&scheme_id=1&l=1). The global optimal based upon related sequence types (goeBURST) program, part of PHYLOViZ software, can also be used to examine *B. pseudomallei* isolate relatedness using MLST

data. geoBURST allows for higher discerning clustering based on double and triple locus variants (e.g. the number of alleles that differ from the ancestral genotype) and incorporates global MLST data (Francisco et al. 2009). See http://www.phyloviz.net/goeburst/ for more information and download options.

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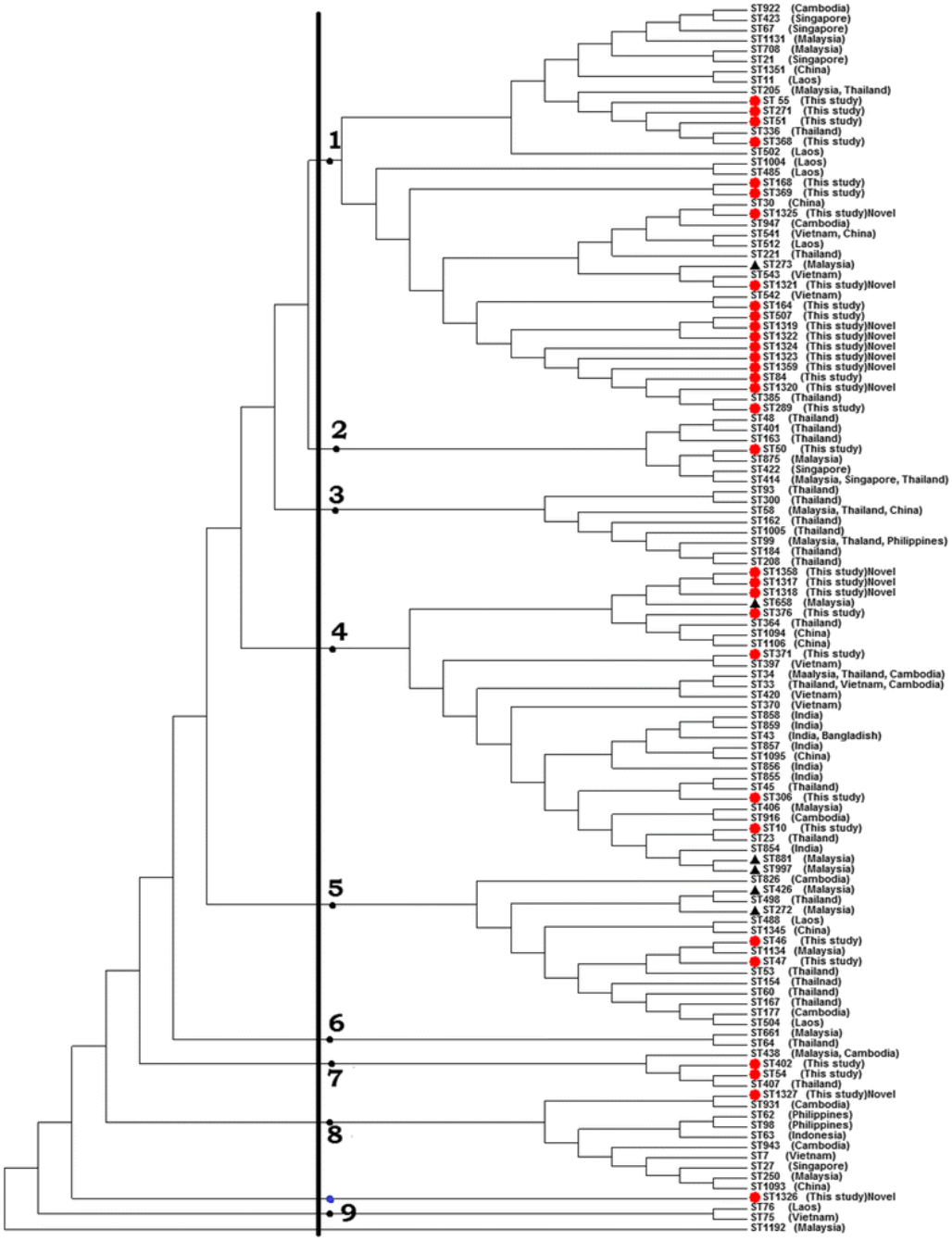


Figure 100- Here, the evolutionary relatedness of *B. pseudomallei* clinical isolates from Malaysia is compared to India, China and Southeast Asian isolates using a dendrogram based on an Unweighted

Pair Group Method with Arithmetic average (UPGMA) method in (MEGA 6) software (Zueter et al. 2018).

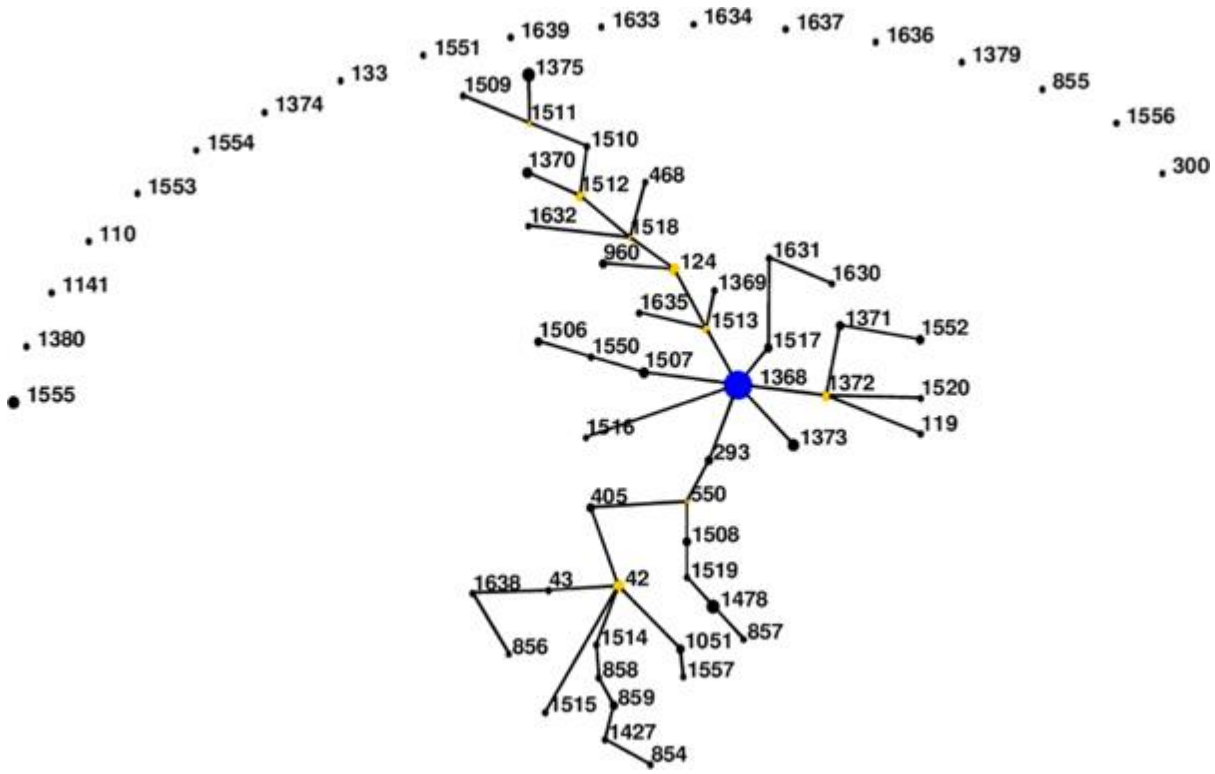


Figure 101- Genetic relationship of 130 Indian *B*. *pseudomallei* isolates using eBURST analysis. The blue dot demonstrates the founder (most ancestral genotype) and yellow dots refer to sub-group (secondary) founders. Each black dot represents single genotype (Kamthan et al. 2018).

Limitations of MLST

Although MLST is an effective and widely applicable scheme for examining *B. pseudomallei* strain relatedness and epidemiological surveillance, there are several limitations associated with its use. The scheme is incapable of determining minor variations amongst closely related isolates. MLST is not suitable to characterize small differences in strains from a potential point-source outbreak. Moreover, MLST can also take several days to weeks to obtain results, and is therefore unable to quickly distinguish the occurrence of an outbreak (Currie et al. 2007). In the event of a potential outbreak or case cluster of melioidosis, rapid and highly discriminatory fingerprinting methods, such as multiple-locus VNTR analysis (MLVA) or BOX-PCR, could be used to show how closely related isolates are. MLST may then be used for sequence typing and examining isolate divergence.

Methods for Rapid Strain Typing of *B. pseudomallei*

1. MLVA-4

   PCR-based variable-number tandem repeat (VNTR) loci is a widely utilized tool used for rapid strain typing of pathogens (Currie et al. 2009; Van Belkum 2007). Multiple-locus VNTR analysis (MLVA) is comprised of several tandemly repeated sequences of DNA that vary in copy number, producing different PCR-amplicons that can be visualised on a gel. MLVA allows for superior discrimination between closely related isolates (Liu et al. 2006; Van Belkum 2007). A 4-locus MLVA platform has more recently been developed for *B. pseudomallei.* This rapid and highly discriminatory typing tool can produce results within eight hours of receiving *B. pseudomallei* isolates. It has also been used on multiple occasions to identify point-source outbreaks of melioidosis (Currie et al. 2009).

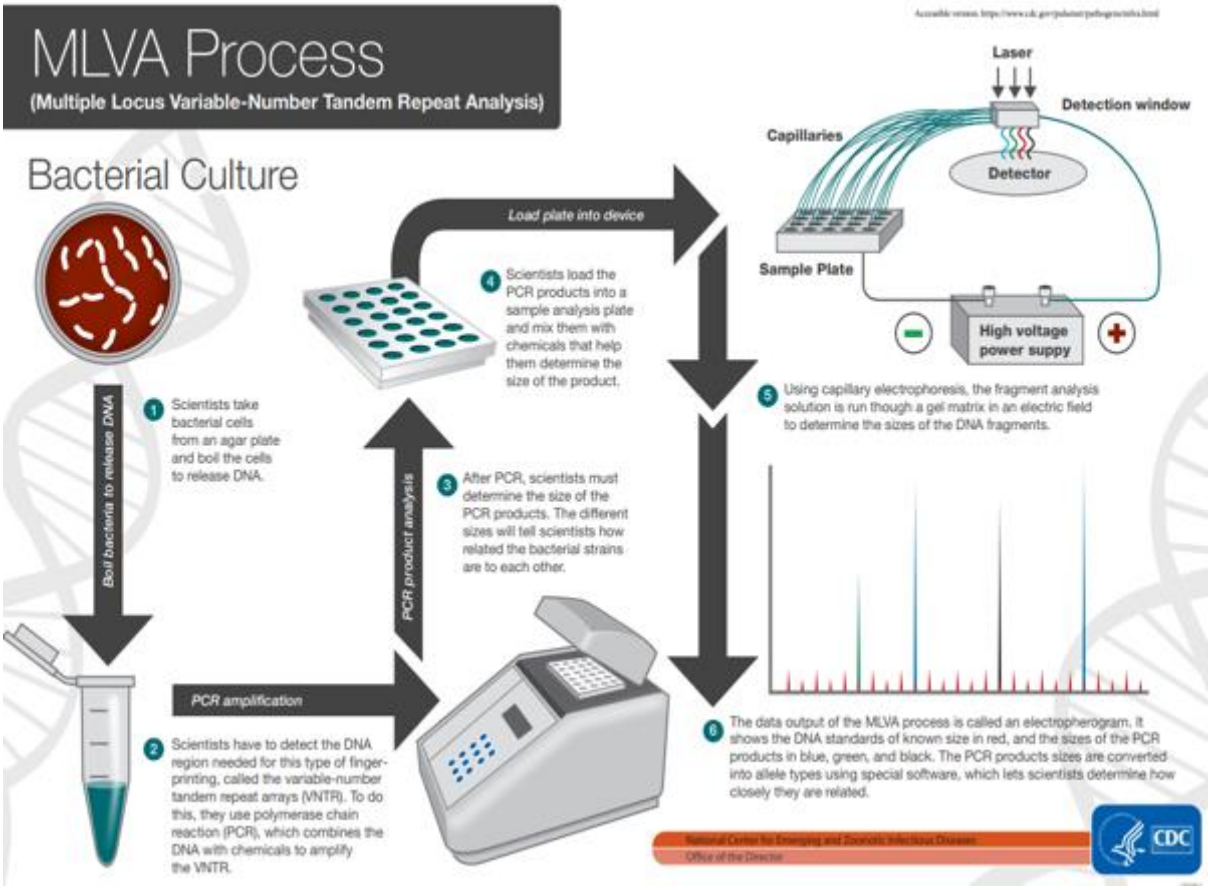


Figure 102- The MLVA process typically involves the detection of variable-number tandem repeats (VNTR) using PCR. Different sized products are used to determine how closely related bacterial strains are to one another (CDC 2016).

2. <u>BOX-PCR</u>

BOX-PCR was conceived as a simple and rapid tool to discriminate between *B. pseudomallei* isolates. It was initially adapted from methods used to differentiate *Burkholderia cenocepacia* isolates in cystic fibrosis patients but has since been demonstrated to be equally discriminatory in *B. pseudomallei* (Coenye et al. 2002; Currie et al. 2007). BOX-PCR can provide results within 10 hours of receipt of bacterial isolates, meaning it is a valuable tool for rapidly determining the relatedness of isolates during a possible outbreak scenario.

***B. pseudomallei* MLVA-4 Protocol**

The following protocol was provided by Menzies School of Health Research, Darwin, Australia. It has been adapted from Currie et al. *Emerg Infect Dis.* 2009; 15 (2):169-74. Contact mark.mayo@menzies.edu.au for further questions concerning the protocols described here.

NOTE- The protocols for MLVA-4 typing require specific data analysis software and thorough training of laboratory staff. This may make the technique impractical for many laboratories. In this instance, labs can also conduct BOX-PCR typing for rapid *B. pseudomallei* strain comparison (see the protocol below), as this method requires fewer technical equipment and training.

Technical Guides:

- GeneMapper Software- **http://tools.thermofisher.com/content/sfs/manuals/cms_042039.pdf**
- BioNumerics Software- **https://www.applied-maths.com/applications/mlva**

Primers:

LOCUS	2341	1788	933	389
Color-labeled forward primer sequence (5′ → 3′)	FAMGGCTTCGCACCC GCCCCATTTCAGC	PETGCGCGGCGAGAAC GGCAAGAACGAA	NEDATGGTGGCGGCC GTCGGCGAAAACC	VICGTTACAAGCGCGGG TCGGCAAGAGGCTGAA A
Reverse primer sequence (5′ → 3′)	GCACCGGGCGCGGC GCACTCG	GAGCATCGGGTGGGCG GCGCGTATTGAT	GCTCGAATGGGTGTA CGAAGGGCCACGCTG ATTC	GCCGGTGTTGAACGAG TGGGTGGCGTAAGC
Repeat sequence (5′ → 3′)	TTCGTGCGC	GTCGTGCGATCCTGCT	CGGCGAGGGAAA	GACGAACC
Minimum size (bp†)	111 (2)	235 (4)	171 (3)	221 (1)
Maximum size (bp)	243 (17)	382 (13)	337 (17)	292 (10)

†Error range in fragment sizing is ± 3 bp.

1. Create a list of isolates to be tested and set up a plate template

For each sample, 4 individual PCR reactions must be done (hence MLVA-4).

A. Create a sample list with the sample names/IDs and a plate template to keep track of sample locations (see example below).

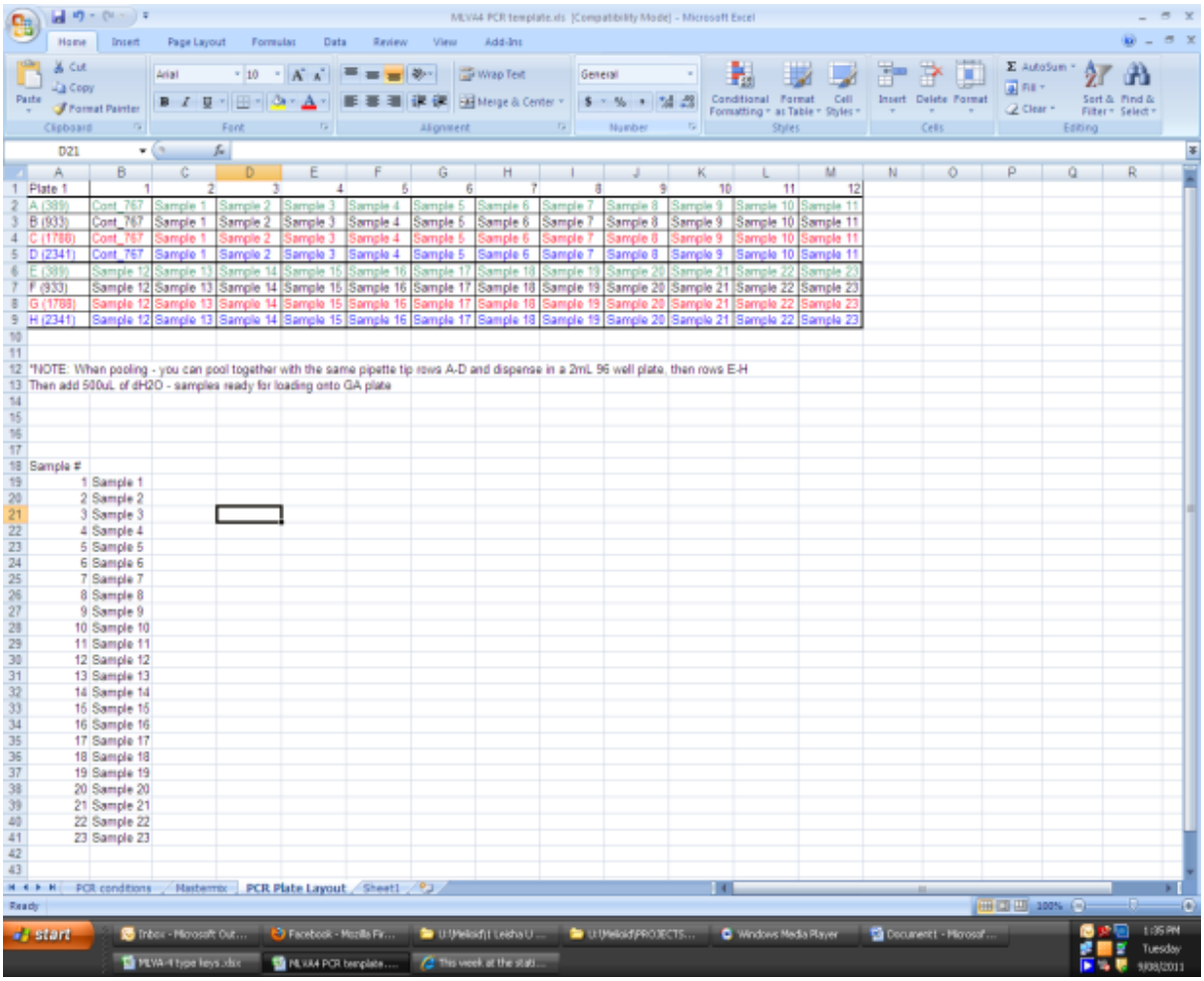


Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A (389)	Cont_767	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
B (933)	Cont_767	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
C (1788)	Cont_767	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
D (2341)	Cont_767	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
E (389)	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23
F (933)	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23
G (1788)	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23
H (2341)	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23

*NOTE: When pooling - you can pool together with the same pipette tip rows A-D and dispense in a 2mL 96 well plate, then rows E-H
Then add 500uL of dH2O - samples ready for loading onto GA plate

Sample #	
1	Sample 1
2	Sample 2
3	Sample 3
4	Sample 4
5	Sample 5
6	Sample 6
7	Sample 7
8	Sample 8
9	Sample 9
10	Sample 10
11	Sample 11
12	Sample 12
13	Sample 13
14	Sample 14
15	Sample 15
16	Sample 16
17	Sample 17
18	Sample 18
19	Sample 19
20	Sample 20
21	Sample 21
22	Sample 22
23	Sample 23

B. Calculate the amount of master mix required based on the table below. Each reaction should contain 10µL of master mix and 1µL DNA template. Be sure to include a negative control, positive control and at least 2 additional samples to allow for reagents lost during pipetting.

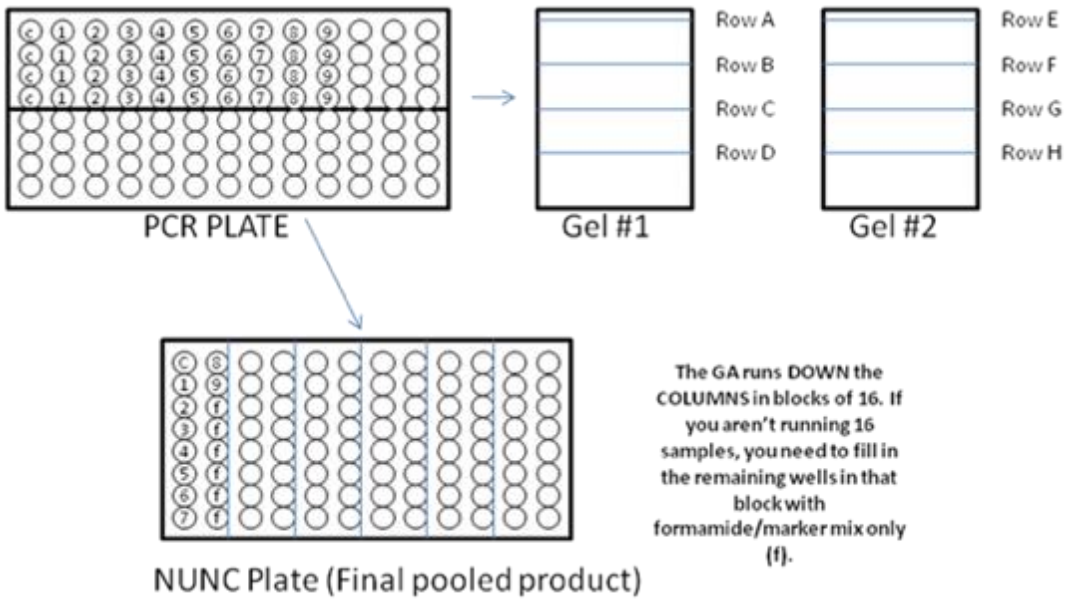
C. This will give the amount of master mix required for ONE SET OF PRIMERS. For MLVA 4, there are four primer sets: 389, 933, 1788 and 2341. Four master mix mixes should be created for each set of samples.

For Each MLVA Primer:					
	Start Concentration		Final Concentration.		Volume per reaction (µL)
PCR Buffer	10	X	1	X	1.1
$MgCl_2$	25	mM	3	mM	1.32
dNTPs	2	mM	0.2	mM	1.1
Betaine (Q solution)	5	M	1.2	M	2.64

Hot Star Taq	5	u/ul	0.08	u/ul	0.18
Forward Primer	20	uM	0.2	uM	0.11
Reverse Primer	20	uM	0.2	uM	0.11
PCR Water					3.4
DNA template					1
Total					11

2. Setting up the PCR

A. Prepare the four master mixes in the PCR clean room.
B. Ensure all surfaces and pipettes are cleaned before and after preparing the master mix with 10% bleach or DNA decontaminant, followed by 70% ethanol.
C. Take your master mixes to the DNA template addition area.
D. Draw a line across the 96-well PCR plate between rows D and E. Aliquot 10µL of master mix across the PCR plate in rows A-D, and then again in rows E-H (as shown below).



E. Add 1µL of the DNA control in column 1, only in rows A-D.
F. Continue adding samples across the plate (1-12) in rows A-D. Sample 13 -24 will be in rows E-H.
G. Seal PCR plate with strip cap covers or with PCR film (e.g. BIO-RAD Microseal 'B' film). If using film be sure to use a plate sealer/roller to completely seal and prevent evaporation during thermal cycling.
H. Briefly centrifuge the plate to remove bubbles.

3. Running the PCR

A. The PCR thermal cycling conditions are as follows:

Step	Temp. (°C)	Time
1	94	5 minutes
2	94	30 seconds

	68	30 seconds
	72	30 seconds
	Cycle 34 times	
3	72	5 minutes
	15	hold

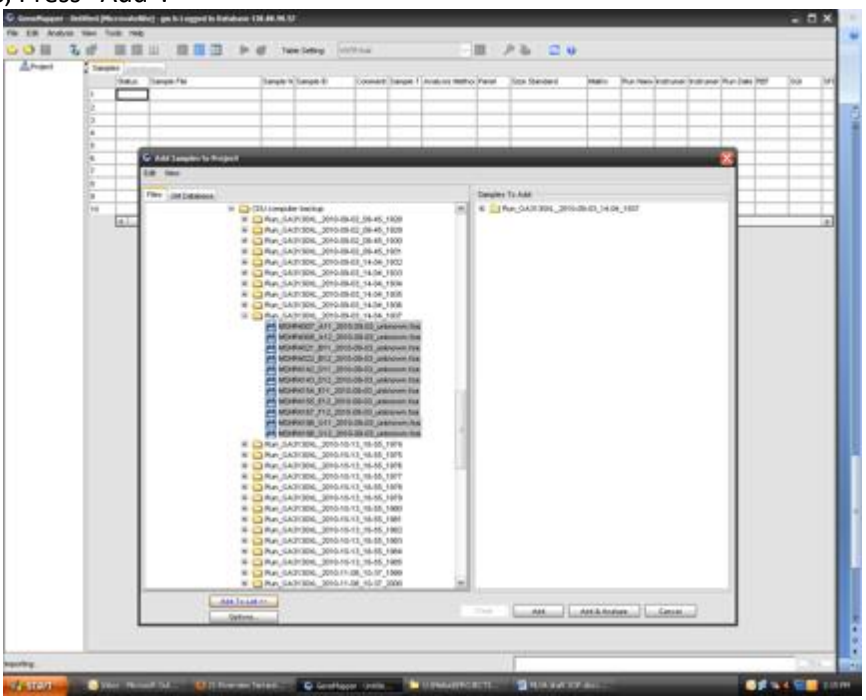
B. Briefly centrifuge the plate after the PCR has finished running.

4. **Running a gel to confirm PCR products**
   A. Make up a 1% 200 mL agarose gel for every 12 samples tested (e.g., if you have 23+control (24 total) samples in a 96 well plate, you will need 2 x 200mL gels).
   B. Follow the instructions found in "Chapter 14- Convention PCR" covering how to prepare an agarose gel for electrophoresis.
   C. In the first well of each row in the gel, add 6 µL of 100bp marker.
   D. Mix 2 µL loading dye with 5 µL of PCR products from row A and add the gel. Repeat this for the first 12 samples in rows A-D of the PCR plate (As shown in the image above).
   E. Be sure to mark the running tank with "samples A-D".
   F. With the second 200mL gel, repeat steps C-E using the DNA from rows E-H of the PCR plate.
   G. Run the gels at 100mV for ~30 minutes. It is important to keep checking the gel and where the dye front is. These are small PCR products; it is important make sure the products don't run off the gel.
   H. Visualize the gel using a gel documentation system.
   I. Check that all 4 products have amplified for each sample.
      a. If there are no bands at all on the gel (i.e. only a marker), the PCR has failed – repeat the PCR reaction again.
      b. If you have bands in 3 of the regions for a sample but one hasn't worked, repeat the PCR on the single region that has not work.
      c. If all 4 regions have failed (i.e. no bands) for a sample (but other samples are ok), the DNA extraction may not have worked for that sample and needs re-extraction.

5. Pooling the PCR Products for each sample
   A. PCR products must be pooled together before being sent for DNA sequencing.
   B. Pool products in a 96-well round-bottom plate (such as a 2mL NUNC plate) or into sterile closable microcentrifuge tubes (e.g. 1.7 mL Eppendorf tubes) if there are only a few samples.
   C. Take 3.5uL from row A of the PCR products. Transfer to column A of the round-bottom plate where the products will be pooled (as shown in the image above).
   D. Take 3.5uL of row B, C and D of the PCR products. Transfer to column A of the round-bottom plate. All 4 amplified regions for each sample will now be pooled.
   E. Take 3.5uL each from Row E, F, G and H of the PCR products and transfer to column B of the round-bottom plate.
   F. Carefully document and label which samples are in which wells of the round-bottom plate or in which tubes they have been placed.

6. Dilution of the pooled product
   A. To each of the pooled products add 500 µL of distilled H_2O. Pipette up and down to ensure it is well mixed.
   B. The samples can now be sent to any commercial sequencing laboratory (e.g. Macrogen, Seoul, South Korea).

7. Analyzing the sequencing results

A. Sequenced product sizes must now be analyzed. The following describes a protocol using "GeneMapper", which is a genotyping software package that provides DNA sizing for all Applied Biosystems Genetic Analyzers.
B. Go to File→ New Project. Select "Microsatellite".
C. Go to File→ Add samples to project.
D. Browse to find where the sample list created at the beginning of the procedure is stored.
E. Select/highlight the sample files (.fsa files) and Press "Add to list". Once you have all of your samples, Press "Add".
    a. 
F. You now need to tell the program which analysis methods you wish to use. In the first cell of each column that needs changing, select the required method. Then click at the top of the column (to highlight the whole column). Then press CONTROL D to auto fill for all samples. Select the appropriate analysis method, panel and size standard (these will need to be manually set when running the protocol for the first time).
G. The fragments are now ready to analyze: Click the Green Play button at the top of the screen.
H. Review the panel of indicators. The panels should appear green if the protocol has worked. If there are any yellow triangles or stop signs, this needs to be checked.
    a. Usually a yellow triangle or stop sign indicates there is a problem with the size standard and the program has been unable to call the size properly. See below on how to fix a size standard.

Fixing a size standard:

a. Select the sample that is red or yellow.
b. Click on the "Size Match Editor" icon.
c. Look at the plot – are there smooth, well defined peaks there? Are all the numbers on the peaks correct?
    a. If there are no numbers, you will need to add in the numbers by clicking on the peak and then right clicking – Add – then select the number (use the chart to guide you on which peak represents the right number). Click "Apply" and "Override SQ".
    b. If there are numbers, double check that they are in the right space.
    c. If there are no numbers and there are no nice peaks, the reaction has failed and will need to be repeated.

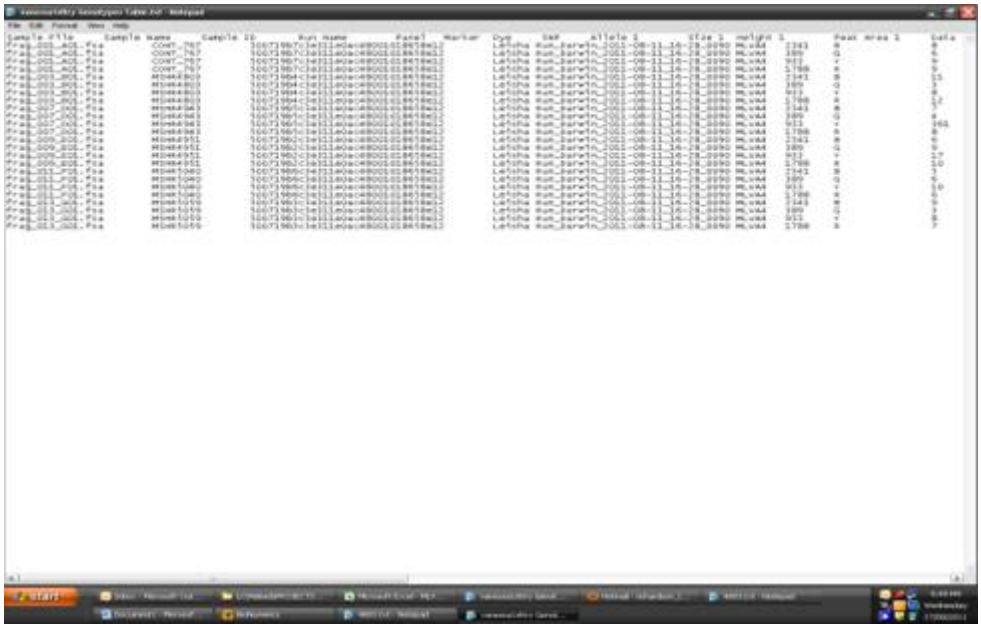
d. Once everything has been checked and all the samples display a green sign, proceed with the panel binning:

8. Panel Binning

A. Select the "Genotypes" tab. Check that all samples display the appropriately matching alleles.
B. Edit→ Select All.
C. Tools→ Panel Manager. Press the "+" next to MLVA 4. Select the MLVA 4 tab that appears.
D. Select→ "Apply" then "OK".
E. Go to Tools→ Table Editor settings. Click on the "Genetypes" tab then select "OK".
F. Click the "Display Plots" Icon – (multi coloured peaks).
G. You will now check all 4 markers for each isolate.
    a. If all 4 have a peak that falls in the grey areas (assigned "Bins"), they should have a box under the peak with the peak details.
    b. If the peak trace is messy, the reaction (PCR) will need to be repeated.
H. Click File→ "Save Panel" then "Exit".
I. Click File→ "Export table". The table should be saved as a .txt file for upload into BioNumerics Software.
J. Ensure that all the raw data files and the GeneMapper files/exported table are saved in an appropriate folder.

9. Preparing the GeneMapper export file for import into BioNumerics

A. Open the GeneMapper export .txt file in the BioNumerics software.



B. Select all (CTRL – A).
C. Open an excel worksheet and paste (CTRL –V) the GeneMapper export table.
D. Remove all columns except for:

Sample Name	Marker	Dye	Allele 1	Size 1	Height 1	Peak Area 1	Data Point 1

E. Remove the letters from the Sample Name for all isolates. BioNumerics can only import sample names based off the "Key" identifier. This means you will need to change the Sample name of all samples to include only the # (e.g. MSHR1234 should become 1234).
F. Assign the "MLVA number" (this number won't be uploaded into BioNumerics but will be manually added later).

a. Open your saved document that contains information about the key and the "Bin" ranges.
b. To determine the MLVA number, click on the "MLVA 4 type key" tab. Using the filter function in the spreadsheet, enter in the allele combinations given for each sample. A MLVA4 number for the sample will be given based on the combination.
c. If the combination does not exist, add it to the end of the list and give it the next "MLVA number" in sequence.
d. Write down this number separately as you will be manually adding this info in later.

G. Now delete the allele columns from the spreadsheet. The file cannot be uploaded into BioNumerics with the allele column still present.
H. The file should look like this:

Sample File Name	Marker	Dye	Size	Height	Peak Area	Data Point
4803	2341	B	188.61	375	4883	2780
4803	389	G	235.6	548	6599	3576
4803	933	Y	230.45	508	5660	3487
4803	1788	R	364.47	200	5035	5853
4943	2341	B	153.14	498	5883	2093
4943	389	G	243.29	653	7794	3549
4943	933	Y	361.04	292	3974	5542

I. Open up a new Excel document. Copy the results for one sample into the document:

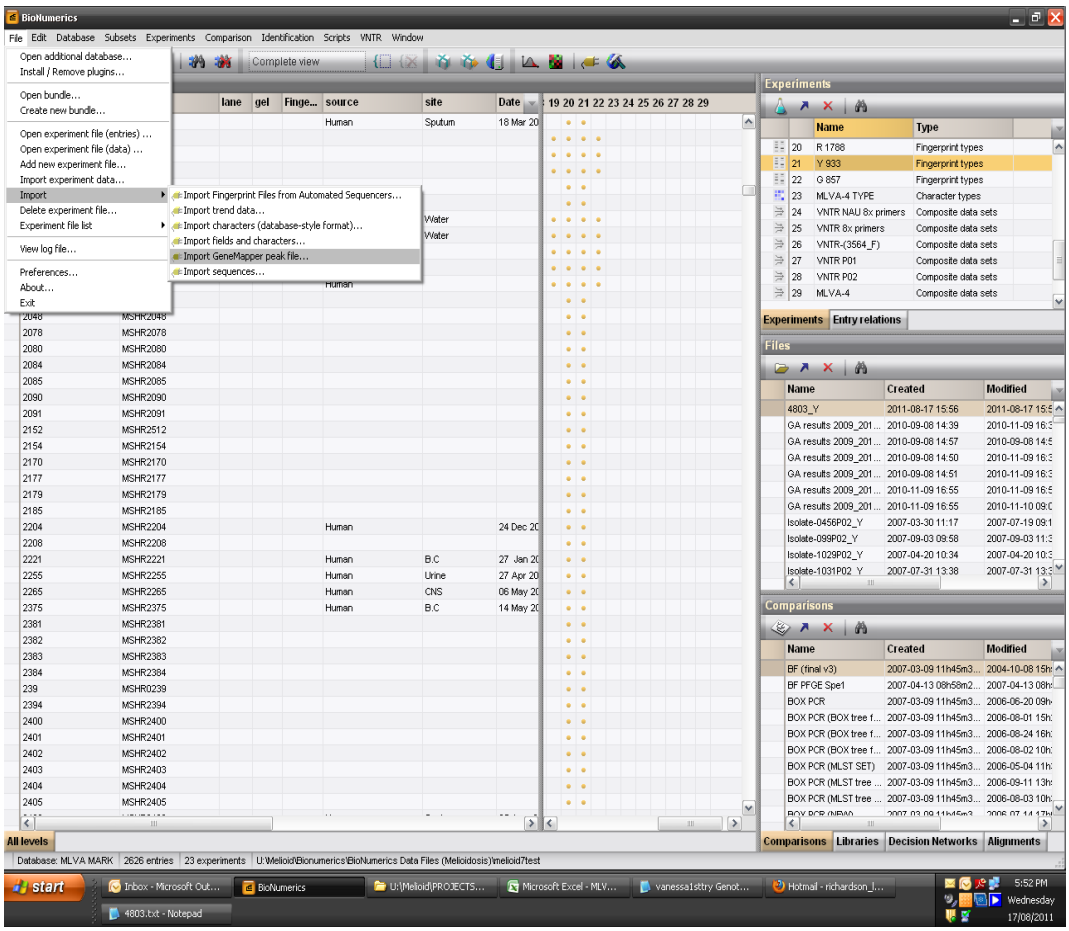
Sample File Name	Marker	Dye	Size	Height	Peak Area	Data Point
4803	2341	B	188.61	375	4883	2780
4803	389	G	235.6	548	6599	3576
4803	933	Y	230.45	508	5660	3487
4803	1788	R	364.47	200	5035	5853

J. Save the document as a tab delimited file with the sample name as the file name (i.e. MSHR4803.txt)
K. Open up another new Excel document. Paste in the next sample (with the headings). Save the document as a tab delimited file with the sample name.

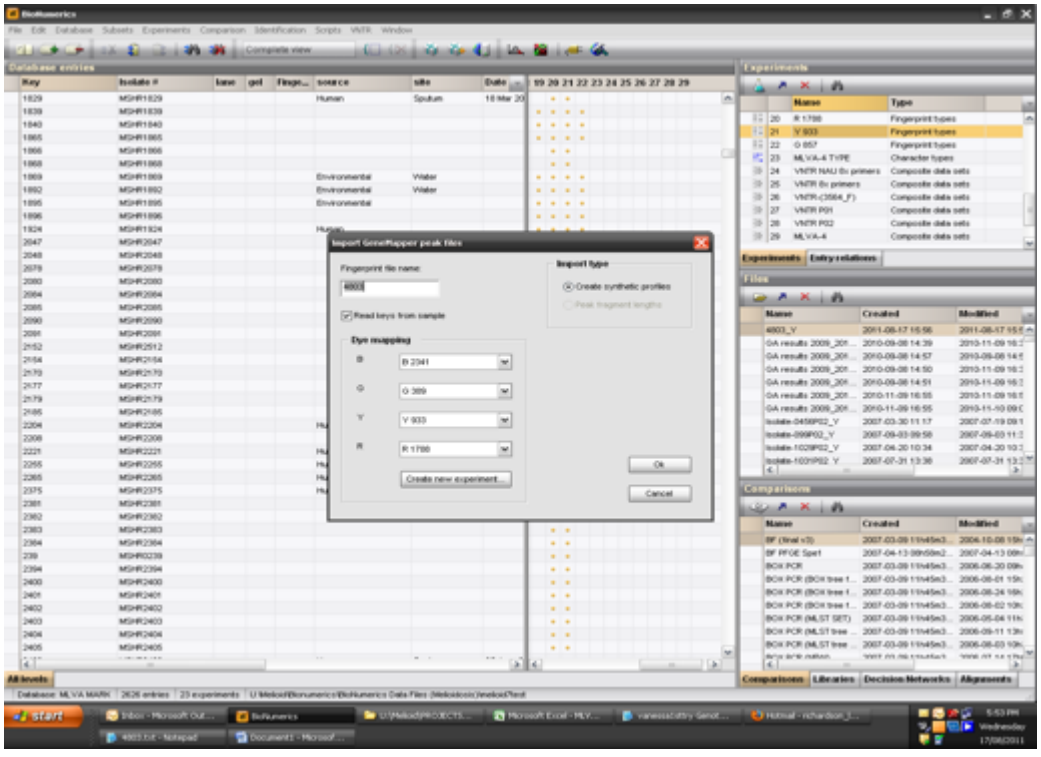
Sample Name	Marker	Dye	Size	Height	Peak Area	Data Point
4943	2341	B	153.14	498	5883	2093
4943	389	G	243.29	653	7794	3549
4943	933	Y	361.04	292	3974	5542
4943	1788	R	298.83	295	6824	4482

L. Do this for every isolate (saving each isolate/sample as its own file. Be sure to include the heading for each file). This may seem unnecessary, but it is difficult to remove a result from BioNumerics if a mistake is made unless each sample has its own file.
M. Open up the BioNumerics software.
N. Import the GeneMapper trace/peak file: File→ Import→ Import GeneMapper Peak File.

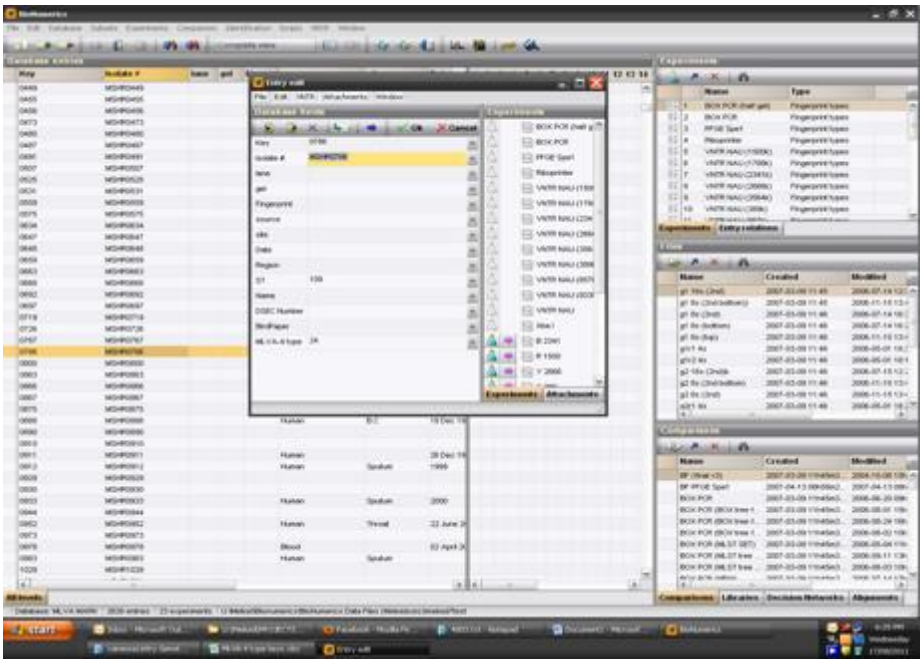
O. Select the tab delimited .txt file just created and open the file.



P. The next screen should appear automatically.



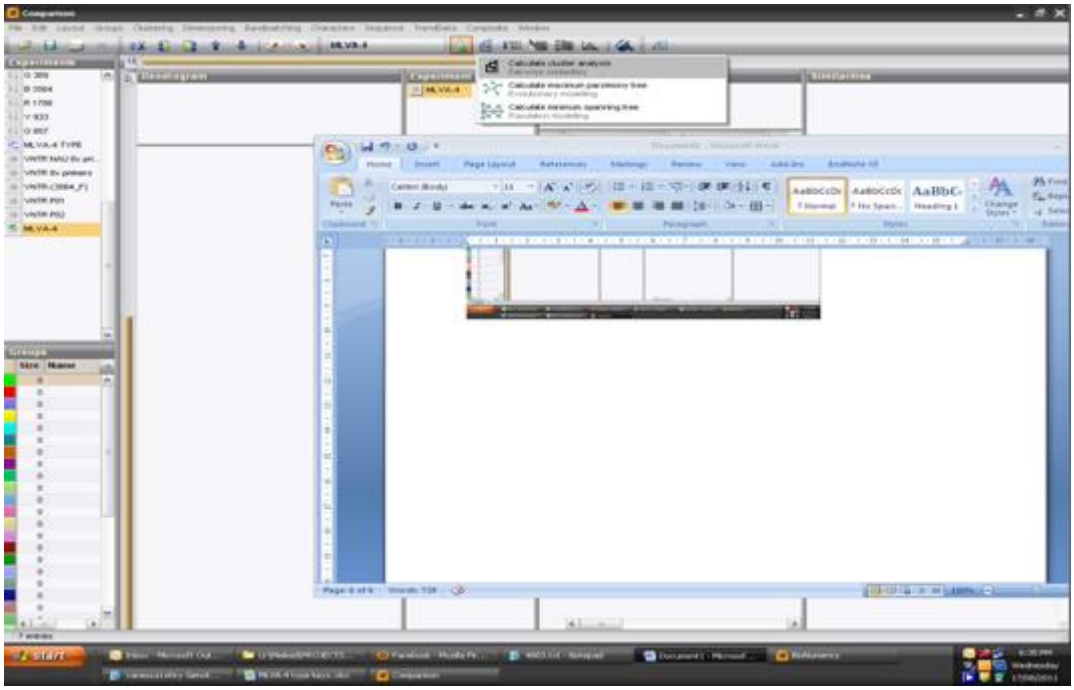
Q. Leave the boxes as they are. Click OK. The file will now download.
R. Repeat this for every sample you are uploading.
S. Find the uploaded isolates by locating their “Key” numbers on the left-hand side of the page.
T. Double click on the isolate. Enter the information you have about the isolate including the newly assigned MLVA4 number.



U. Click “OK” when finished.

To create a dendrogram:

a. Hold down the “CTRL” key and select (using the mouse) the isolates you wish to use in the dendrogram. A small orange arrow will appear next to the selected isolates. The tree will need to be rooted using an appropriate *B. pseudomallei* isolate.
b. Once all the isolates have been selected, click the “Comparisons” tab in the bottom right hand corner.
c. Select the “Create new comparison” icon.
d. The isolates will appear in a new window.
e. On the left side of the screen, in the Experiments box, scroll down to the bottom to MLVA4. Click this experiment (a green tick should appear over the MLVA4 icon).
f. Select the “Calculate cluster analysis” icon.
g. Choose “Calculate cluster analysis – Pairwise similarities”.



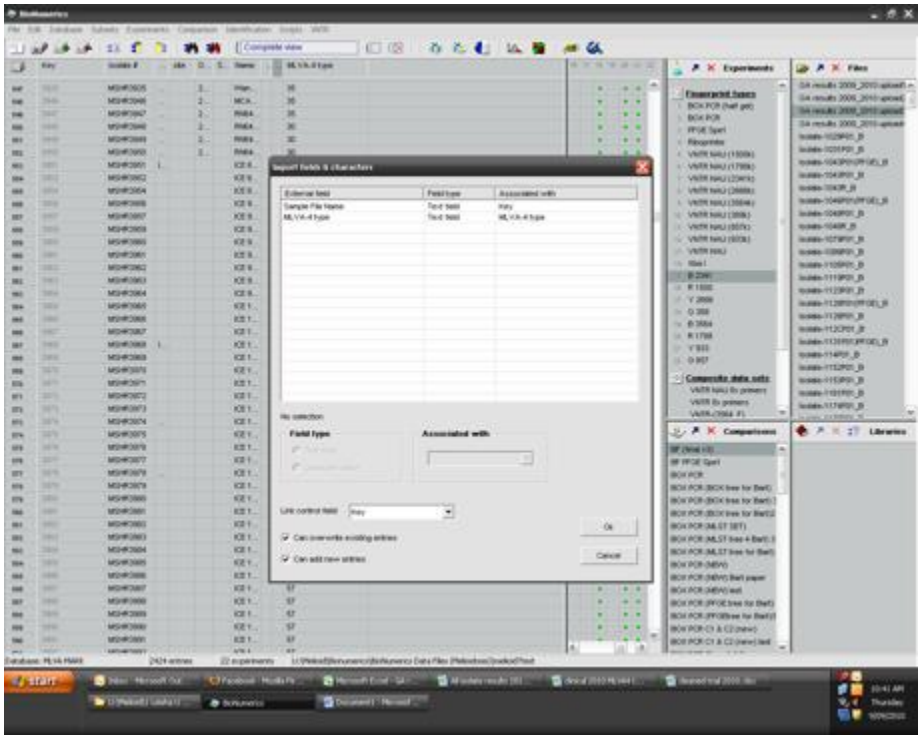
h. Use the default settings: Average from experiments and UPGMA for the "dendrogram type". Click OK.
i. To print: Click print preview.
j. Move the fields to suit what you wish to print.

Uploading sample information into BioNumerics:

A. Create a .txt file (start in excel and then save as .txt tab delimited file) that has the Sample names as the Key (e.g.MSHR4145 has a key of 4145) with the information in the following columns that you wish to import.

Sample File Name	MLVA-4 type
4018	15
4021	110
4022	44
4023	16
4024	16
4025	16
4026	16
4027	16
4028	16

B. In BioNumerics, open File→ Import→ Import fields and characters.
C. Locate the .txt file to import via the browse function. Click OK.
D. The wizard identifies the columns present in the file. You now need to link them to a field in BioNumerics.



E. Click on the first row. Make sure the “Field Type” function has “text” selected. You now link it with “Key” by selecting it from the drop-down box list.
F. Do the same for all other external fields until all are completed
G. “Link control field” should be set to “Key”
H. Click OK to upload.

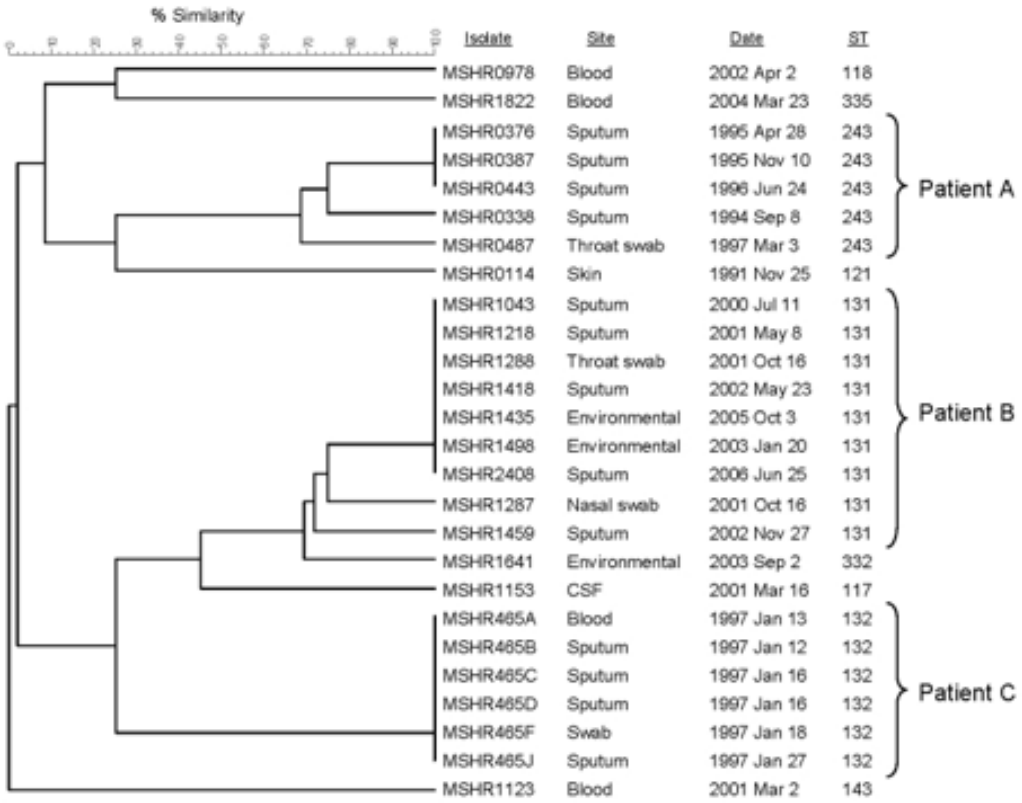


Figure 102- Dendrogram showing MLVA-4 profiles for isolates from three patients with melioidosis, along with corresponding isolate numbers and multilocus sequence types (Currie et al. 2009).

***B. pseudomallei* BOX-PCR Procedure** (adapted from Currie et al. 2007)

Primer:

BOX-A1R: (5'-CTACGGCAAGGCGACGCTGACG-3')

A. PCR Set-Up

1. Create a PCR plate template to keep track of where samples will be placed.
2. Calculate the master mix volumes needed for the number of samples to be tested based on the table below. Remember to include 2 additional samples to account for reagent lost during pipetting.

Reagent	Concentration	Volume per reaction (µL)
10X PCR Buffer	1X	2.5
$MgCl_2$ (25mM)	4.5 mM	4.5
dNTPs (2mM)	200µM	2.5
Q or Betaine Solution	1X	5
Hot Star Taq (QIAGEN)	5U	0.25
BOX-A1R Primer (30µM)	1.05µM	0.875
PCR Water		7.375
DNA template		2
Total		25

3. Prepare the master mix in the PCR clean room/area.
4. Gently mix the master mix then briefly spin in a microcentrifuge.
5. Pipette 23 µL of the master mix into the appropriate well of a 96-well plate or PCR strips according to the plate template worksheet.
6. Cover the wells using cap strip covers. Spray down the clean workspace with bleach or DNAerase and follow with 70% ethanol. Remove laboratory coat and gloves and put on a clean pair of gloves. Take the 96-well plate or PCR strips to the DNA template addition area.
7. Put on a new laboratory coat, keeping the same pair of gloves on. Very carefully, take off the cap strip covers from the 96-well plate or PCR strips. Open these slowly to prevent aerosolization and spraying of any liquid in the wells.
8. Add 2 µL of template DNA to each appropriate well of 96-well plate or PCR strip according to the plate template worksheet.
9. Add 1 µL water to the 'NTC' well.
10. Add 1 µL of the positive control DNA to the correct well.
11. Cover the wells of each column of the PCR plate or each PCR strip using strip caps as you go and secure caps tightly.
12. Wipe down the workbench area with bleach or DNAerase, followed by 70% ethanol. Remove laboratory coat and gloves. If possible, briefly spin the plate at 1000 rpm to remove any

droplets. Transport plate to the PCR thermocycler room/area and place it in the PCR thermocycler.

13. Place tubes/plates in the conventional PCR machine.
14. Set the following thermal cycling conditions and press start:

Stage	Time	Temperature	# Cycles
Initial denaturation	5min	95°C	1
Amplification	1min	95°C	35
	1min	40°C	
	2min	72°C	
Final Extension	10min	72°C	1
Hold	--	4°C	--

B. Running the Gel

1. Make a 2% agarose gels for the required number of samples to be run (follow the procedures outlined in Chapter 14- Conventional PCR).
2. Load the gel using a 100bp ladder and 2 µL DNA loading dye mixed with 10µL PCR product.
3. Place the lid on the gel tank, plug electrodes into a power pack setting it to run at 100mV.
4. Once ready lift gel from the tank and visualize results on Gel Doc machine.

C. Results Interpretation

BOX-PCR gel results should always be interpreted in the proper epidemiological context and the gels alone should not be used to confirm an epidemiological connection or outbreak. It is always important to confirm the link with another more robust typing method, such as MLST or whole-genome sequencing (see below). The quality of the gel electrophoresis results and the variability and the prevalence of the pattern in question should always be considered.

Only isolates displaying identical banding patterns should be considered as potentially being the same isolate/strain and the possible cause of an infection cluster, patient relapse, or point-source

outbreak. If the samples in question display different BOX-PCR banding patterns they should be considered unique, unrelated isolates (e.g., not part of the same cluster or a patient reinfection rather than relapse). An example is shown in Figure 103 below. Here, BOX-PCR has been used to demonstrate that a melioidosis patient has been reinfected with a new strain of *B. pseudomallei* and is unlikely to have had a relapse in their primary infection, as two distinct banding patterns are present.

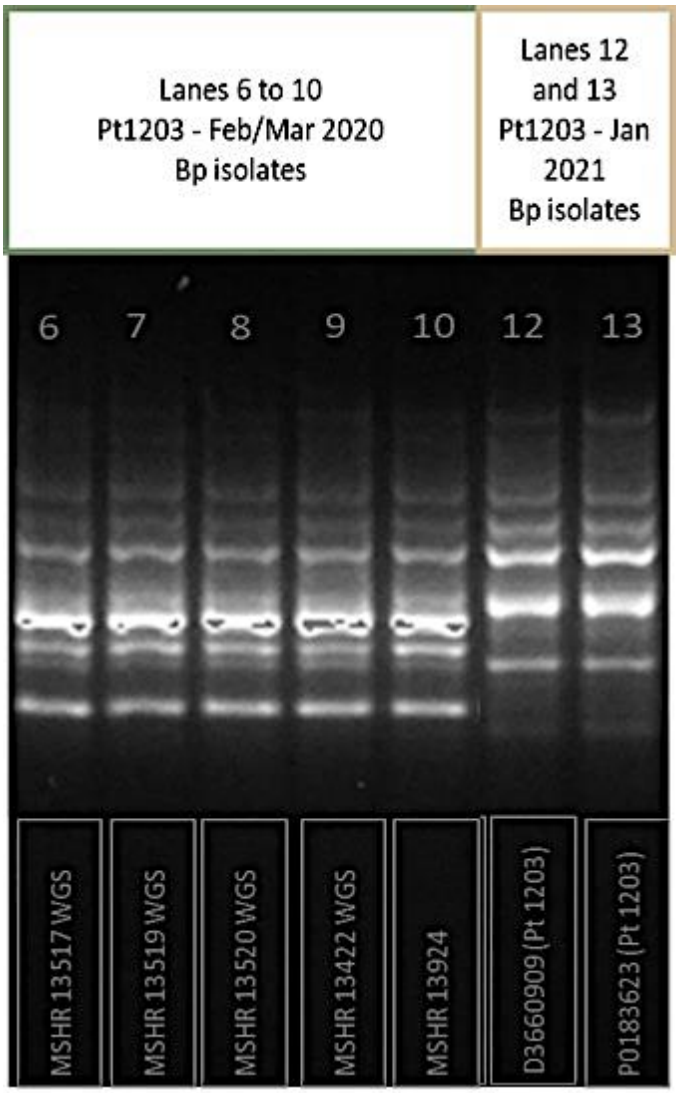


Figure 103- The BOX-PCR banding patterns in lanes 6-10 of the gel, which are isolates cultured from the patient's initial infection, have a unique banding pattern and are different than isolates cultured from the same patient's subsequent infection nearly one year later. This likely indicates that the patient was infected by a different strain of the bacteria and has not been reinfected by the same strain (e.g. from a single point-source) or have had a relapse in infection due to inadequate treatment (image courtesy of Menzies School of Health Research, Darwin Australia).

MLVA-4 and BOX-PCR Limitations

While both BOX-PCR and MLVA-4 have proved useful for smaller regional surveys of *B. pseudomallei*, a serious hindrance to their widespread practical application thus far has been the difficulty involved in data sharing, limiting inter-laboratory isolate evaluation (Cheng et al. 2005). These rapid strain typing methods can lack reproducibility and robustness and variations in results have been shown to occur between different labs or PCR machines (Currie et al. 2007). MLVA also requires trained and skilled laboratory technicians. It is recommended that these schemes be used in conjunction with a more robust and reproducible typing method, such as MLST, to confirm the event of an outbreak or case cluster and to verify the relatedness or divergence of strains.

Next Generation Sequencing for Bacterial Strain Typing

The arrival of the first high-throughput, rapid next-generation sequencing (NGS) technology has signalled an important shift in microbial genetics research. Since then, sequencing platforms have become more advanced in their data quality, cost, and accessibility, with whole-genome sequencing (WGS) platforms providing a comprehensive method for analyzing entire bacterial genomes (Edwards & Holt 2013; Köser et al. 2012). In addition to providing a much wealthier source of genetic information, WGS now also supersedes many genotyping methods in pricing. For example, WGS now costs less to carry out than conventional Sanger sequencing-based MLST.

Current WGS platforms are comparatively fast and can be generated in-house in some laboratories. Much of the data generated is also available through online public databases, including the National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih.gov/genbank/), allowing for extensive comparative analysis (Edwards & Holt 2013). The high-resolution data generated from WGS can be utilized to investigate various aspects of *B. pseudomallei* genetics, including the spread of drug resistance (Webb et al. 2017), within-host evolution (Price et al. 2010; Köser et al. 2012), as well as outbreak source attribution through the examination of isolate genome-content variability (McRobb et al. 2015; Sarovich, D. S. et al. 2017). Moreover, WGS has the

ability to extract MLST sequence types directly from reads, permitting a highly precise method of MLST typing of *B. pseudomallei* (Edwards & Holt 2013; Lam et al. 2012). The Bacterial Isolate Genome Sequence database (Jolley & Maiden 2010) now allows for the PubMLST website to use all levels of sequence data, including complete, finished genomes. Where resources permit, WGS and *in silico* MLST could be used after rapid fingerprinting of isolates to confirm an outbreak or case cluster and to further examine the relatedness of strains.

***B. pseudomallei* WGS Data Analysis**

When applying WGS to study the molecular epidemiology of bacterial pathogens such as *B. pseudomallei*, it is useful to examine variations in a genetic sequence, or set of sequences (Schürch et al. 2018). Genome variants can take several forms, including nucleotide insertions, when additional nucleotides are found in a sequence, nucleotide deletions within a sequence, or a single base change, referred to as a single nucleotide polymorphism (SNP) (Schürch et al. 2018). These variations can arise through point mutations, homologous recombination or discrepancies in genome content (Bryant et al. 2012). Most studies rely on SNP data for population and epidemiological analysis since variability between bacterial strains can be translated into measures of distance in core genome alignments, or can be indexed to assign unique allelic profiles (Schürch et al. 2018). Additionally, SNPs can be found even in regions of high homology, or regions of shared ancestry not recently affected by recombination, and thus can more accurately reflect evolutionary time (Pearson et al. 2009). SNPs are also more informative than other currently used molecular markers due to intrinsically slow mutation rates and extensive distribution across the entire genome. Large numbers of shared SNP loci can thus facilitate robust characterization amongst closely related isolates (Pearson et al. 2009).

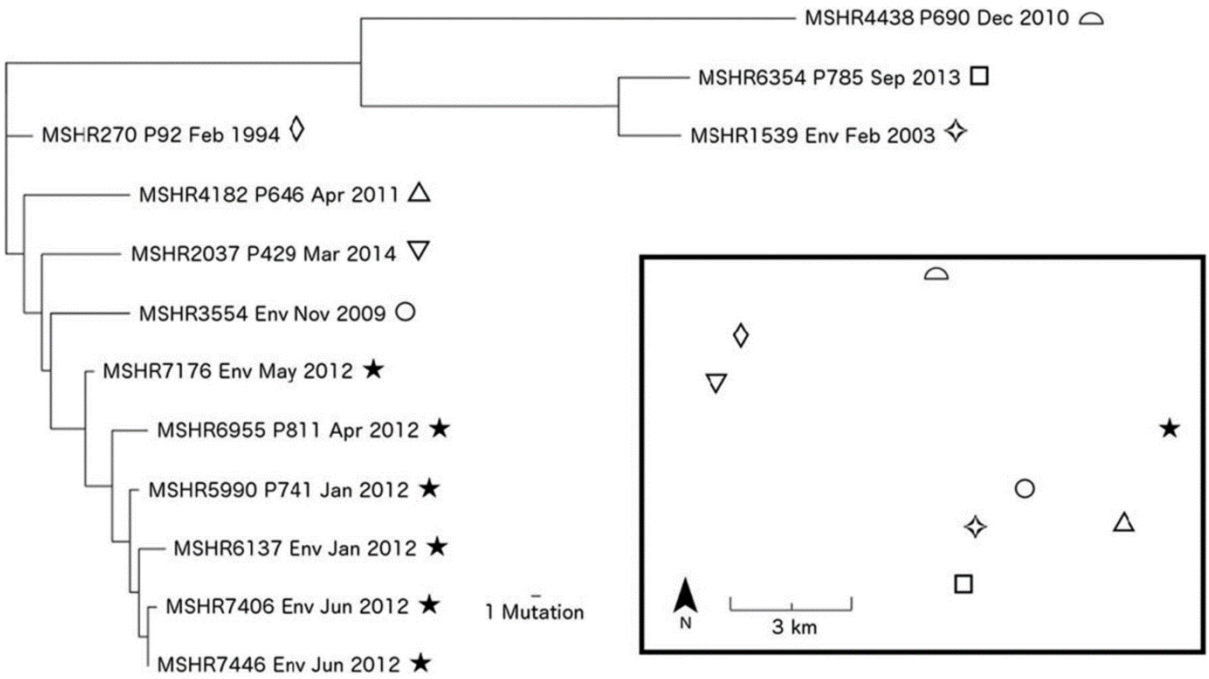


Figure 104- Whole-genome sequencing data from a melioidosis outbreak in the Darwin region of northern Australia. Here, a phylogenetic tree was constructed using combined SNP and indel data from *B. pseudomallei* environmental and clinical isolates to demonstrate that the source of a melioidosis outbreak was from a contaminated domestic water supply (McRobb et al. 2015).

Identification of insertions, deletions, or SNPs begins with mapping and aligning sequence reads to a closely related reference genome (Li & Homer 2010). Variant determining algorithms have quality filters that then attempt to define variants, versus sequencing errors. Alignments are normally performed using a microbial SNP pipeline, such as Snippy, NASP, or SPANDx (Sahl et al. 2016; Sarovich & Price 2014; Schürch et al. 2018). The resulting alignment can then be used for phylogenetic analysis. To increase resolution, genomes chosen for the reconstructed phylogeny are normally closely related to one another. This allows for discrimination between isolates that differ by only a few SNPs, which is especially important in facilitating investigations into outbreak source attribution (Pearson et al. 2009).

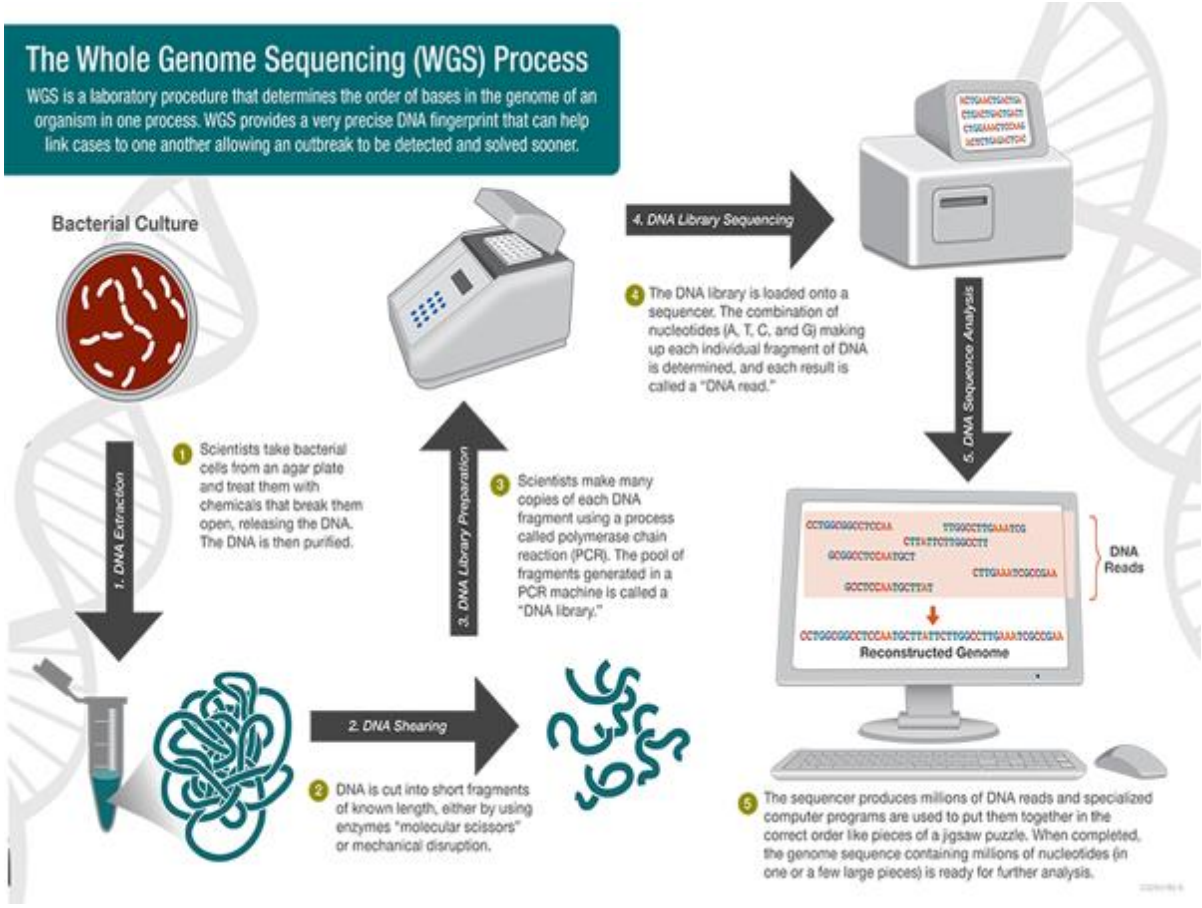


Figure 105- Whole-genome sequencing normally consists of DNA extraction, shearing the extracted DNA into small fragments, creating a “DNA library” that includes many copies of each DNA fragment. Each fragment is then sequenced to create a read, and finally, analysis of the sequenced products occurs (CDC 2013).

Most commercial sequencing laboratories now offer fast, well-priced WGS sequencing facilities. **However, due to the complexity of WGS and the extensive bioinformatics training required to properly analyze and interpret WGS data, it is best to contact a diagnostic reference laboratory or research laboratory with WGS capabilities and expertise if considering implementing the technique for the first time.**

Chapter Summary

The preceding chapter presents an overview of bacterial molecular characterization and several common methods used for *B. pseudomallei* strain typing. Bacterial strain typing is important for disease diagnosis, treatment, and epidemiological surveillance, particularly for highly pathogenic species such as *B. pseudomallei*. Potential outbreaks of melioidosis warrant rapid public health response and high-resolution molecular typing tools, such as those described above, are needed to quickly investigate the infection etiology.

Chapter 17: *B. pseudomallei* Suspected Isolate Reporting, Storage and Data Management

Chapter Overview

While melioidosis is not a notifiable disease in all countries, it is recommended that suspected cases be reported to local or national sentinel laboratories that can assist with diagnostic confirmation, epidemiological investigation, and provide important biosafety guidance. The U.S. Centers for Disease Control (CDC) and U.S. Department of Agriculture (USDA) have identified biological agents and toxins that they consider to be a threat to public health because of their potential use as bioterrorism agents. These are referred to as "select agents". Their transfer, possession, use, and disposal are strictly regulated in the United States. *B. pseudomallei* is classified as Tier 1 select agent (https://www.selectagents.gov) and its identification should be reported to a Laboratory Response Network (LRN) laboratory or sentinel laboratory as soon as possible upon identification. The following chapter describes the appropriate protocols for reporting and transporting *B. pseudomallei* to reference laboratories.

Reporting a Suspected or Confirmed *B. pseudomallei* Isolate

Any oxidase-positive, indole-negative, gram-negative rod that is resistant to gentamicin and colistin but sensitive to amoxicillin/clavulanic acid should be suspected as *B. pseudomallei* until this identification is ruled out (Trinh et al. 2018). For suspected or confirmed cases, clinical laboratories should refer to local and national disease authorities and comply with the specific reporting requirements. This typically involves reporting cases and laboratory results and may also mean that the isolate concerned is subject to specific biosafety and biosecurity measures. Laboratories that typically perform bacterial work on an open bench should transfer all suspected cultures to a class II BSC or higher where possible, especially when performing potentially aerosol-generating

procedures. See Chapter 4 for further detail regarding *B. pseudomallei* biosafety (ASM 2016; BMBL 6th ed. 2020; Peacock et al. 2008).

Although the risk of laboratory acquired infection with *B. pseudomallei* is relatively low compared with some other bacterial select agents, clinical laboratories should perform a risk assessment and develop protocols to prevent accidental exposure. This includes strictly enforcing standard laboratory procedures and GLP, conducting culture work for suspected isolates inside of a BSC, and wearing appropriate personal protective equipment (PPE), as described in Chapter 4 (BMBL 6th ed. 2020). If an accidental exposure occurs, the risk of exposure should be determined based on the guidelines described in Chapter 4 (APHL 2017; Peacock et al. 2008).

Data Management and Record Keeping

A data management system should be used to keep track of specimens and isolates processed by the clinical laboratory and those that are being stored long-term. A computerized record keeping system is recommended, but handwritten laboratory logbooks may be used where these are unavailable. Small or resource-poor laboratories may find that paper records are sufficient. However, computerized methods are recommended for laboratories that handle larger quantities of clinical specimens and isolates. Commercial laboratory information management systems (LIMS), such as Freezerworks, may also be used where resources permit.

Regardless of whether the data management and recordkeeping system is computerized or paper-based, all data must be accurately and reliably recorded and easily accessed. Human errors in record keeping are very common and great care should be taken to avoid errors. It is best to use a single unique specimen or isolate reference along with at least two other data items such as patient name, date of birth or hospital number. Bar code printers and scanners reduce the risk of human

transcription errors. It is also critical that laboratory data is linked with epidemiological data to ensure the accuracy.

It is recommended that individual laboratories maintain several records, including:

- Detailed records of every isolate.
- Secured access to isolates.
- Safety, security, and emergency response plans.
- Training records.
- Reference lab transfer forms (if used): The designated reference laboratory should advise on any required transfer forms. Always ensure the latest versions of these forms are being used.
- Safety and security incident reports.

Storage of Isolates

If isolates need to be sent to a reference laboratory for confirmation or further testing, they should be stored appropriately to ensure viability. Isolates should always be confirmed as pure cultures before they are stored. Fresh cultures (e.g., ~48 hours growth) should always be used when storing isolates. Aseptic techniques should be used when preparing isolates for storage to prevent contamination.

Short-term storage of *B. pseudomallei* can be carried out on agar slants/slopes (e.g. on tryptone soya agar slants) or in other liquid broth medium containing glycerol for 1-2 weeks. Instructions for preparation of agar slants and glycerol broth are described below. Slants can be produced as a 4 mL slant in a 7 mL screw-cap tube or 1 mL slant in a 2 mL screw-cap tube. They should be stored at 4°C until transporting. Broth should be frozen at -20°C (or -70°C for longer-term storage).

Tryptone Soya Agar Slope Protocol

1. Weigh out 15 grams of TSB powder (Tryptone Soya Broth – Oxoid CM0131 or locally available equivalent).
2. Put the TSB powder in a 1 L autoclavable glass beaker or flask containing a magnetic stirrer on top of a magnetic hot plate.
3. Add 500 mL of distilled water to the bottle and turn the stirrer on.
4. Allow the solution to mix thoroughly until all the powder is dissolved.
5. Add 5 grams of Bacteriological Agarose and stir for 5 minutes.
6. Autoclave for 30 minutes at 121°C or sterilize in a pressure cooker if an autoclave is not available.
7. Take the bottles out of the autoclave/pressure cooker once finished and allow to cool slightly (until the agar is at ~60°C).
8. Make 1 mL aliquots of the agar into 2 mL screw-cap tubes, or 4 mL slants in 7 mL screw-cap tubes. If the agar starts to solidify before finishing, sit the bottle in the water bath set at 60°C for 5 minutes and continue once it is liquid again.

Tryptone Soya Broth Containing 15% Glycerol

1. Weigh out 30 grams of TSB powder.
2. Add the TSB powder to a large 2 L glass beaker or flask containing a magnetic stirrer on top of a magnetic hot plate.
3. Add 850 mL of distilled water to the beaker and turn the stir plate on.
4. Allow to mix until all the powder is dissolved.
5. Add 150 mL of glycerol and stir for a further 5 minutes (or until all glycerol is mixed).
6. Divide the broth into 2 x 500mL glass bottles.
7. Autoclave for 30 minutes at 121°C or sterilize in a pressure cooker if an autoclave is not available.

8. Take the bottles out of the autoclave/pressure cooker once finished and allow to cool.
9. Once cool, aliquot 1 mL in to 2 mL screw cap tubes. Store at 4°C until needed.

Isolate Shipping and Transfer

Upon notification to the local health department of suspected *B. pseudomallei*, laboratories should be prepared to appropriately package and ship specimens or isolates. See the packaging and shipping section in Chapter 6 for additional information about appropriate packaging and shipping procedures.

Transport of diagnostic specimens and infectious substances should be done so that it minimizes any potential risks and also protects the viability of the isolate or specimen. The shipment of infectious substances or diagnostic specimens by air should comply with local, national, and international guidelines. International air transport regulations may be found in the IATA publication *Dangerous Goods Regulations*. https://www.iata.org/en/publications/dgr/. See Chapter 6 for more detail.

Agar slopes/slants in screw-cap tubes can be shipped at room temperature (25°C) (IATA 2019; WHO 2020).

***B. pseudomallei* Isolate Security, Destruction and Decontamination**

If suspected specimens and/or isolate cultures are to be transferred to a reference laboratory, they should be transported within 7 days of confirmed identification where possible. During this time, specimens and isolates should be secured against theft, loss, or release until they have been transferred. If a laboratory is unable to transfer isolates or store them appropriately long-term at -70°C, isolates should be inactivated or destroyed. Suspected or known loss of a specimen or associated materials must be reported immediately. Individuals not associated with the diagnostic laboratory should not have access to specimens or isolates and should be secured in locked cabinets,

rooms, or other containers. Rooms should also be secure against entry by unauthorized personnel (ASM 2016).

If a lab decides to transfer any specimens or cultures, staff should contact the local reference laboratory and work to ensure that the correct paperwork and protocols are followed. Copies of sample destruction logs, and documented reports should be maintained.

If a laboratory chooses to destroy any specimens or isolates, it is also critical that these are properly inactivated prior to disposal. Isolates should not be directly discarded into the biohazardous waste stream like other infectious medical waste materials. Inactivation using an autoclave/pressure-cooker or chemical decontamination should be done before disposal.

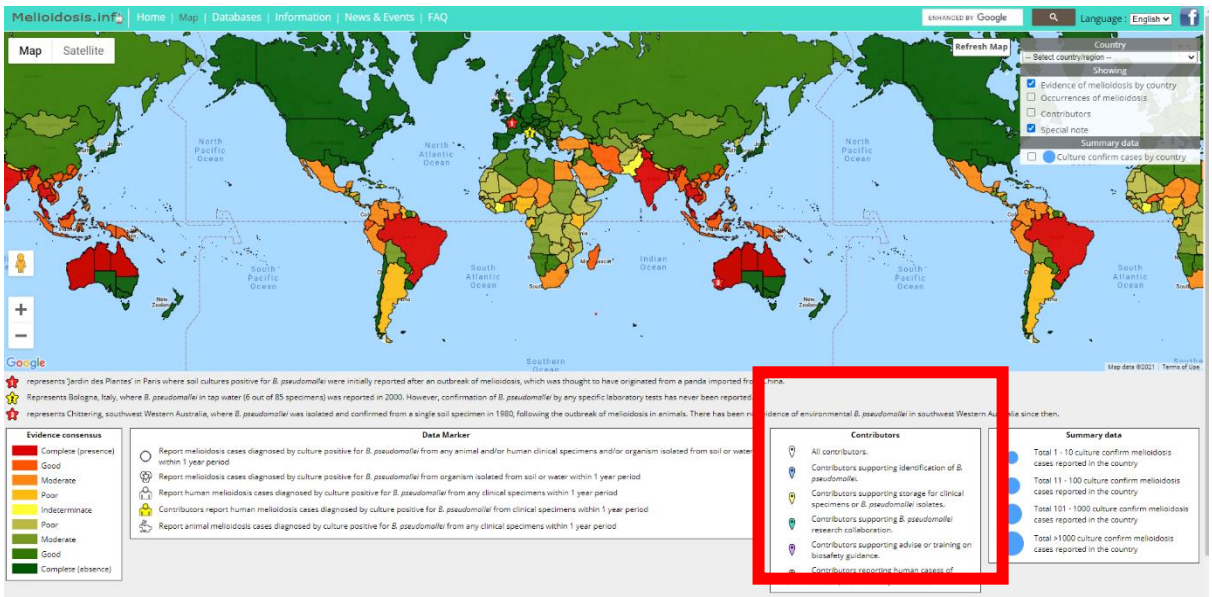
Chemical Decontamination Process (APHL 2016)

1. Prepare a fresh 1:10 bleach solution in any receptacle large enough to submerge all containers and plates suspected to contain *B. pseudomallei*.
2. Working inside of a BSC, place culture containers in the bleach solution so they are fully submerged and leave in the bleach solution. This should be done overnight.
3. Once overnight inactivation has occurred, discard the bleach solution down the drain with tap water running.
4. Package all inactivated culture plates and containers with other biohazardous waste for final treatment and disposal.

Checklist of appropriate steps to take if *B. pseudomallei* is to be transferred to a local or national reference laboratory for confirmation or storage:

____ 1. Contact the laboratory where the specimen/isolate will be transferred.
____ 2. Package the suspected isolate(s) and other clinical specimens (aspirates, biopsies, sputum specimens) according to local and national regulations and send to laboratory.
____ 3. Note who performed worked on cultures and if work was done inside of a BSC. Write down if any additional clinicians were involved in specimen collection (Peacock et al. 2018).
____4. Notify public health authorities such as the local health department epidemiologist or health officer as required by the local or national disease reporting regulations.
____5. Notify the infection control/disease team so the patient can be given appropriate treatment and an epidemiological investigation can be done.
____6. Discuss with the laboratory about whether any further specimens should be submitted for testing.
_____7. If *Burkholderia pseudomallei* is ruled out, continue with identification using local protocols.

In countries where cases of melioidosis have not previously been reported or where there are no regulatory authorities in place to report cases, the website www.melioidosis.info also offers methods to report culture-confirmed cases of melioidosis, provides support for *Burkholderia species*



identification, submitting unusual isolates, and advice or training on biosafety techniques. It can also help a laboratory to locate nearby Biosafety level 3 facilities to store *B. pseudomallei* clinical isolates. Contributors can be found on the main webpage map (https://www.melioidosis.info/map.aspx- red box).

Laboratories are also able to report cases by clicking on "Report cases" on the primary tab on the website (shown below- red box). If the email application does not open, please copy the form, fill out the requested information and email it to melioidosis.info@tropmedres.ac.

Chapter Summary

B. pseudomallei is classified as a Tier 1 select agent by the U.S. CDC because of its high virulence and potential use as a bioterrorist weapon. Accordingly, many countries strictly regulate the transfer, possession, use, and disposal of *B. pseudomallei* isolates and contaminated specimens. The preceding chapter describes the appropriate protocols for reporting, transporting and documenting suspected *B. pseudomallei* isolates or specimens. While melioidosis is not a notifiable disease in all countries, it is recommended that suspected cases be reported to local or national sentinel laboratories or public health authorities who can assist with diagnostic confirmation and provide

biosafety guidance. The website www.melioidosis.info also offers methods to report culture-confirmed cases of melioidosis, provides support for *Burkholderia species* identification and advice or training on biosafety techniques.

Chapter 18: Melioidosis Surveillance and Filing a Clinical Case Report Form

Chapter Overview and Introduction to Disease Surveillance

Surveillance encompasses the collection, classification, analysis, interpretation, and reporting of health-associated data that enables public health decision making and action. Disease surveillance is accomplished through the ongiong collection, evaluation and dissemination of morbidity, mortality, and health data. Effective surveillance is essential to any prevention and control program and for effective public health decision making (CDC 2012).

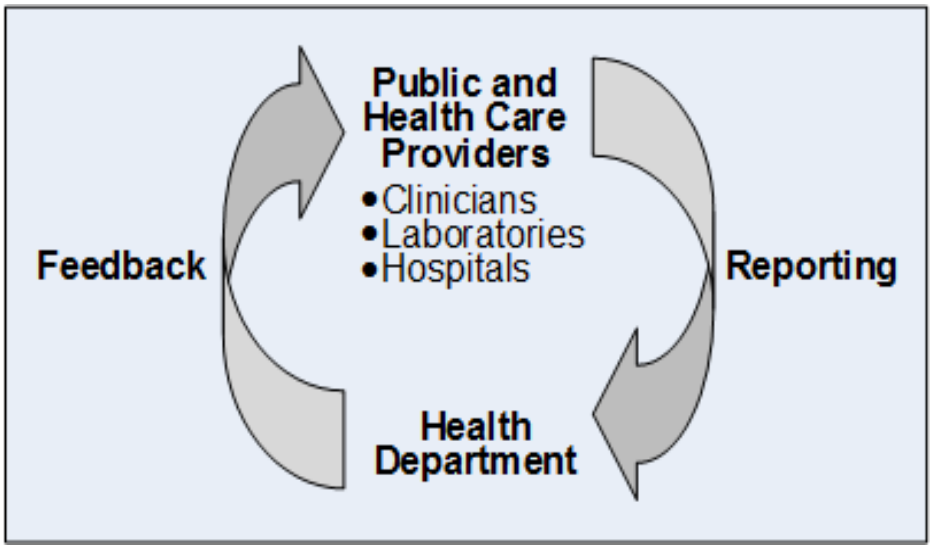


Figure 106- Surveillance starts with local healthcare providers like clinicians, laboratories, and hospitals and health departments. Feedback is provided by health departments back to local public and healthcare providers (CDC 2012).

Regulations often exist for reporting certain diseases. These diseases are usually of high epidemic potential or with high morbidity or mortality rates and are known as “notifiable” diseases. Due to the high morbidity and associated case fatality rates, melioidosis is included on several national

notifiable diseases lists, particularly in endemic regions of the world including Thailand and Australia (Hinjoy et al. 2018).

A case of melioidosis in a human should result in a report and investigation where possible. As the disease is rare or not well known in many countries, continuing education programs for healthcare workers and laboratory staff should include information on recognition and appropriate diagnostic tools and techniques. Certain at-risk populations, such as diabetics and those with underlying immunosuppressive conditions, may warrant more proactive surveillance, especially during periods of heavy rainfall or other adverse weather events (Suputtamongkol et al. 1999).

Surveillance Levels and Feedback

Infectious disease surveillance is conducted across multiple levels of government, enabling integrated and enhanced monitoring and feedback. Within a country, these levels can be broken down into: 1) local (e.g. clinician, hospital, laboratory), 2) intermediate (e.g. city, district, county, state), and 3) central/national surveillance (WHO 1999; WHO 2008).

1. Local Level Surveillance

The first effort to identify cases is normally focused at the local level (e.g., healthcare practitioners, clinics, hospitals, and laboratories) where an initial diagnosis is likely to be made. The primary responsibilities at this level include diagnosis and case management, reporting of cases and case and outbreak investigation (where resources permit). This is also the point at which surveillance data should first be collected and simple tabulation and graphing of data is performed (WHO 2008).

Local healthcare staff should complete a case-report form on every suspected or confirmed case of melioidosis (see an example case report in Figure 107 below). The form may be completed by a local healthcare professional or by health authorities at the local level. This should be done to establish

any possible sources of infection, including recreational or occupational hazards that may have led to exposure to contaminated soil or water.

Healthcare staff may develop a data collection form tailored to the specific details of that disease or a specific outbreak cluster. They may also use a generic case report form. Regardless of the case form that is used, it should include the following information (CDC 2012):

a. Identifying information- The patient's name, address, and telephone number are important in case physicians or investigators need to contact them for additional questions and to notify them of laboratory results. This information also helps to verigy if there are any duplicate records, while addresses allow the dispersal of infections to be mapped and visualized.
b. Demographic information- Information such as age, sex, race, occupation, etc. will help to characterize the populations at risk in that area (e.g., rice farmer, construction worker).
c. Clinical information- Clinical data will allow health investigators to establish whether the case definition for melioidosis has been met. Date of symptom onset is needed to chart the time course of the disease and possible time of exposure. Additional information, including length of illness, hospitalization or death, will help aid in the characterization of the infection.
d. Risk factor information- This information should be specifically tailored to melioidosis. For example, determining whether the patient is diabetic or has an immunosuppressive condition, or has had any recent notable exposure to soil or water.
e. Reporter information- The case report should include the name or information about reporter (typically a physician, clinic, hospital, or laboratory). Investigators will sometimes need to contact this individual for additional clinical information or to feedback any results.

Melioidosis Clinical Case Report Form

Patient ID: _____

Date of Birth (DoB): ___ / ___ / _____
(mm / dd / yyyy)

Name: _____
(given) (surname)

Residential Address: _____

Contact Phone #: _____

Gender: M / F

Race: 1 = White 2 = Asian 3 = Black or African American
4 = American Indian or Alaska Native
5 = Native Hawaiian or Other Pacific Islander 6 = Other

Occupation: _____

Recent Travel History: _____

Risk Factors

Diabetic:	Y / N	Smoking:	Y / N	Excessive Alcohol Use:	Y / N	Renal Disease:	Y / N
Chronic Lung Dis:	Y / N	Occ. Exp:	Y / N	Recreational Exposure:	Y / N	Inoculation Event:	Y / N
Malignancy:	Y / N	Immunosuppression:	Y / N	Cystic Fibrosis:	Y / N	Oral Steroid	Y / N
		Other:	_____	_____			

Admission No: __ Date: ___ / ___ / _____	Diagnosis Date ___ / ___ / _____	Discharge Date ___ / ___ / _____

Blood culture:
0 = -ve
1 = +ve
2 = not done

Died: 0 = no
1 = yes- initial illness
2 = yes- relapse
3 = yes- unrelated

Date: ___ / ___ / _____

	Treatment

Septic shock: Y / N	**ICU admission:**	**Y / N**	**Inotropes:**	**Y / N**
	Ventilated:	**Y / N**	**Renal Replacement Therapy:**	**Y / N**

Diagnoses: 1=Primary, 2=Secondary		
__ pneumonia	__ genito/urinary	__ bacteremic no focus
__ skin abscess(es)	__ soft tissue abscess(es)	__ CNS
__ osteomyelitis	__ septic arthritis	__ other clinical focus

Abscesses	
Prostate:Y / N	Spleen:Y / N
Liver:Y / N	Kidney:Y / N
Other: _____ _____ _____	

Chest XRay		
Left	**Right**	**Coding**
Upper: __	Upper: __	1 = consolidation lobar
Lingula: __	Middle: __	2 = other discrete cons.
Lower: __	Lower: __	3 = extensive patchy
		4 = patchy localised

Treatment	**Antibiotic**	**Dose**	**Duration**	**Compliance**
Intensive 1				
Intensive 2				
Eradication 1				
Eradication 2				

Blood			
WCC: _ _ _ _	Neutrophil: _ _ _	Lymphocytes: _ _ _ _	Sodium: _ _ _ _
Potassium: _ _ _ _	CO2: _ _ _	Urea: _ _ _ _	Creatinine: _ _ _ _
ALT: _ _ _ _	Bilirubin: _ _ _	GGT: _ _ _ _	Protein: _ _ _ _
	Albumin: _ _ _	Platelets: _ _ _ _	CRP: _ _ _ _
HbA1c (closest to admission) _ _ _ Date __/__/__			

Serology: Indirect Hemagglutination Assay (IHA)						
Date	**Titer**	**Date**	**Titer**	**Date**	**Titer**	**Coding**
__ / __ / __		__ / __ / __		__ / __ / __		1 = <1/20, 2 = 1/20,
__ / __ / __		__ / __ / __		__ / __ / __		3 = 1/40, 4 = 1/80,
__ / __ / __		__ / __ / __		__ / __ / __		5 = 1/160, 6 = 1/320,
__ / __ / __		__ / __ / __		__ / __ / __		7 = 1/640, 8 = 1/1280,
__ / __ / __		__ / __ / __		__ / __ / __		9 = >1/1280, 10 = not done

Figure 107- Example of a patient case report form that may be used if a case of melioidosis infection is suspected or confirmed in an individual. The form is adapted from Menzies School of Health Research and Royal Darwin Hospital, Darwin, Australia and can be tailored based on individual data and epidemiological reporting requirements.

If there is any apparent temporal and/or geographical clustering of melioidosis cases based on the data collected, additional investigations may need to be occur. These can include:

a. Collecting related soil and/or water samples for the detection of *B. pseudomallei* in the environment.
b. Investigating the genetic relatedness of isolates using molecular methods where resources allow.
c. Notifying local clinicians and residents about the cluster and about preventative measures that can be taken (e.g., chlorination or UV sterilization of domestic water supplies).

Local health authorities are normally expected to gather primary data and organize initial epidemiological investigations targeted at identifying and controlling the source of infection (including identification and investigation of additional cases and tracing possible sources of infection) as well as initiating control measures. They are also responsible for reporting to the intermediate level (e.g. state) or to the central/national level (e.g. federal), depending on the government structure (WHO 1999).

2. <u>Intermediate Level Surveillance</u>

Depending on the political structure of a country, data from local surveillance and local case reports may be managed at an intermediate level, such as a state health department. The intermediate level often collates and analyzes data from local levels to examine epidemiological links and trends. The intermediate level may also help with epidemiological investigations and monitoring of control measures and typically reports data to the central/national level.

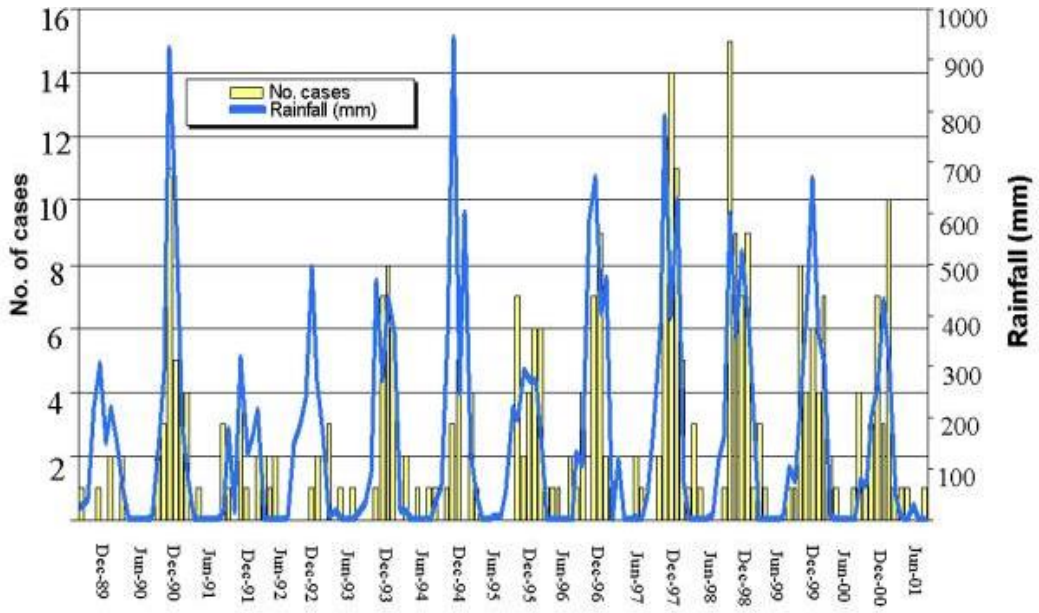


Figure 108- Surveillance of melioidosis cases; monthly rainfall vs. the number of melioidosis cases during a 12-year period in the Northern Territory, Australia. Here, the data is used to show that rainfall is associated with an increase of melioidosis cases (Currie & Jacups 2003).

3. Central/National Level Surveillance

Cases of melioidosis cases should be reported to the central/national level. This level will typically analyze and report data back to intermediate and local levels, devise national policies and allot resources based on the reported data. The central level may also offer practical and technical support to intermediate and/or local level laboratory or epidemiological staff and report summary surveillance data to international health authorities (WHO 2008).

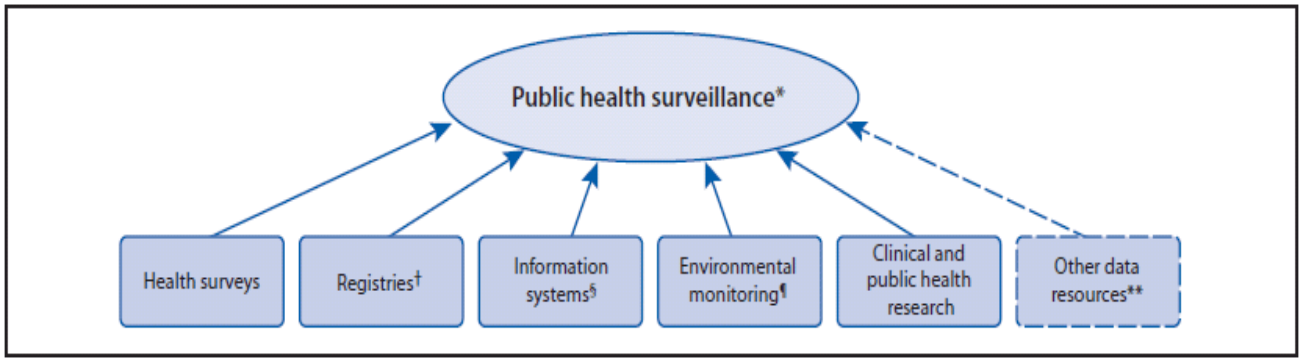


Figure 109- Conceptual framework for public health surveillance. Effective surveillance requires continuous collection, analysis, interpretation, and distribution of results so that effective action can be taken (Porta 2008).

Chapter Summary

Effective surveillance is essential to any disease prevention and control program. It is accomplished through the collection, analysis, and interpretation of case reports and associated health data, and the dissemination of data to those involved in disease control and public health decision making. A single case of melioidosis in a human should always result in a patient case report and investigation where possible. This information should be communicated to intermediate and central levels of surveillance, enabling integrated and enhanced monitoring and feedback of melioidosis infection.

Glossary

Aerobic bacteria- Bacteria that utilize oxygen for growth and oxygen-based metabolism.

Agglutinate- the formation of clumps of cells or particles by specific antibodies to surface antigenic components.

Algorithm- A procedure or formula for solving a problem, based on conducting a sequence of specified actions.

Aliquot- To divide a portion of the whole, especially one of two or more samples of something that have the same volume or weight**.**

Ashdown media- A selective culture medium for the isolation and characterization of *Burkholderia pseudomallei.*

Augur- A drilling device used for making holes in wood or in the ground that typically has a rotating blade. The rotation of the blade causes the material to move out of the hole being drilled.

Autoclave- A machine that uses steam under pressure to kill harmful bacteria, viruses, fungi, and spores.

Broth microdilution- A method used to test the susceptibility of microorganisms to antibiotics where wells are filled with broth containing different concentrations of the antibiotic.

Chaotropic- a substance that disrupts the structure and denatures molecules such as proteins and nucleic acids (e.g. DNA and RNA).

Chelating- A chemical compound that binds tightly to metal ions.

Electrophoresis- A laboratory method used to separate DNA, RNA, or protein based on size and electrical charge. An electric current is used to separate molecules through a gel.

Eluate- A solution that is available for analysis after an elution is performed.

Elution- A process in which particles are separated and extracted based upon size, shape and density by washing in a solvent (liquid substance capable of dissolving other substances).

Endemic- Natural to, native, confined to, or widespread within a place or population of people.

Enrichment broth- A liquid medium that contains different nutrients and is used to culture bacteria and other microorganisms.

Exonuclease- Enzymes that work to remove nucleotides from linear single-stranded DNA in the 3' to 5' direction.

Fluorophore- A fluorescent chemical compound that is able to re-emit light when energy is applied.

Geographic Information System (GIS)- A system designed to capture, store, analyze, and display geographical data that can be referenced to locations on the earth.

Gold standard- Something that serves as a reference that others can be measured or judged against.

Gram-positive- Bacteria that have a thick peptidoglycan layer, no outer lipid membrane and retain the color of the crystal violet stain in the Gram stain.

Gram-negative- Bacteria that have a cell wall composed of a thin layer of peptidoglycan and have an outer lipid membrane. The structure of their cell wall is unable to retain the crystal violet stain so they are colored by the counterstain.

Hemolysis- The rupturing or breakdown of red blood cells that leads to the release of their contents into the surrounding fluid.

Heterogeneous- Populations, samples or results that are different or mixed.

Homogenize- To blend different elements into a mixture that is the same throughout.

Inoculate- A process where a small streaking loop is dipped into a solution containing bacterial cells and is used to streak an agar plate with the bacteria.

Inoculum- A small amount of material containing bacteria, viruses, or other microorganisms that is used to start a culture.

Intercalating- A substance that inserts itself into the DNA structure of a cell and binds to the DNA.

Latent infection- An infection that typically does not cause noticeable symptoms and can last long periods of time without becoming active and causing symptoms.

Latex agglutination- A laboratory test used to check for specific antibodies or antigens in different body fluids such as saliva, urine, cerebrospinal fluid, or blood.

Lysis- Breaking down the membrane of a cell by viral, chemical, or physical means.

MacConkey agar- A selective culture medium used for the isolation and differentiation of Gram-negative enteric bacilli.

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)- A rapid and high-throughput method for microbial identification using specific protein signatures.

McFarland Standard- References used to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range. This is done to standardize microbial testing.

Minimum Inhibitory Concentration Testing (MIC)- A test used to determine the lowest concentration of an antimicrobial agent that will prevent visible growth of a microorganism.

Oligonucleotide- Short single strands of synthetic DNA or RNA that serve as the starting point for many molecular biology applications.

Oxidase test- A diagnostic test used to identify bacteria that produce cytochrome c oxidase, an enzyme that is part of the bacterial electron transport chain.

Pathogenic- Capable of causing disease.

Personal Protective Equipment (PPE)- PPE is clothing or equipment designed to be worn to protect a person from the risk of injury or illness.

Polymerase Chain Reaction (PCR)- A technique used to amplify small segments of DNA into many copies.

Post-Exposure Prophylaxis (PEP)- Treatment used to reduce the likelihood of acquiring an infection after potential exposure.

Quantify- To count or express something using numbers.

Quencher- Substances capable of absorbing energy from a fluorophore (such as a fluorescent dye) and re-emitting that energy as either heat or visible light.

Sapronotic- An infection that is a sapronosis, or a disease transmissible from the environment (e.g. soil, water, decaying plants). The organism must be able to grow and replicate in the environment.

Seropositive- Showing a significant level of serum antibodies, or other immunologic marker in the serum, indicating previous exposure to the infectious agent of interest.

Spatial autocorrelation- The degree to which one object is similar to other nearby objects. It is the tendency for samples or sites that are close together to have similar values.

Supernatant- The fluid lying above a precipitate/pellet following the centrifugation of a suspension.

TBSS-C50- An enrichment broth often used for soil culture that contains a threonine-basal salt solution plus colistin at 50 mg/liter.

Titer- A measurement of the amount or concentration of a substance in a solution. It usually refers to the amount of antibodies found in blood.

Vortex- To mix (as the contents of a test tube) by rapid spinning or circular motion.

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