The Epidemiology of *Burkholderia pseudomallei* in Townsville Groundwater

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Abstract

Burkholderia pseudomallei is a saprophytic bacterium that is the causative agent of melioidosis, a serious opportunistic infection affecting humans and animals. It particularly affects individuals in tropical regions, making it a priority in Northern Australia. Melioidosis is acquired through environmental exposure, however the reservoir of infection and routes of transmission are rarely determined. This lack of information impairs an informed public health response. Groundwater has recently been implicated in disease. We investigated the environmental characteristics and genomic epidemiology of *B. pseudomallei* from a seasonal creek in Townsville to determine if surface groundwater is a clinically significant reservoir of infection.

Water samples were collected from a seasonal creek (Goondaloo Creek) weekly over one year, cultured using Ashdown media, and confirmed as *B. pseudomallei* using Type III Secretion System (TTSS) qPCR. Confirmed colonies were stored and analysed in a high containment facility. Of 59 water samples, 18 were culture and PCR positive for *B. pseudomallei* with organisms isolated exclusively from turbid flowing water after heavy rainfall. Approximately 30mm of rainfall or greater was sufficient to detect *B. pseudomallei* in these creek samples.

Multi-Locus Sequence Typing (MLST) was used for targeted allele sequencing of Goondaloo Creek *B. pseudomallei* isolates. The Oxford Nanopore Technology (ONT) MinION platform was established and validated in the high containment facility at James Cook University (JCU) and targeted ONT sequencing was compared to outsourced (AGRF) Illumina whole genome sequencing (WGS). ONT was found to be accurate, less expensive and time efficient. Of the 48 sequenced isolates, 18 were distinct Sequence Types (STs), with 12 novel STs and 6 previously recorded in pubMLST. All 6 previously recorded STs were linked to human and/or animal clinical isolates. ST283 was most common and had the most human/animal submission on pubMLST, followed by ST1966. ST1966, ST276, ST283, ST2070, ST2074, and ST2075 matched previous Townsville clinical case isolates. The isolates from Goondaloo Creek were closely related to previous Townsville and Australian isolates. This study revealed that surface groundwater may act as a mobile reservoir for clinically relevant strains of *B. pseudomallei* in the Townsville region.

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List of Abbreviations

ace	Acetyl Coenzyme A Reductase
AGRF	Australian Genome Research Facility
AI	Artificial Intelligence
ASH-A	Ashdown Agar
ASH-B	Ashdown Broth
$BimA_{Bm}$	*Burkholderia* Intracellular Motility Factor A Variant
BOM	Bureau of Meteorology
BOX-PCR	BOX-A1R-Based Repetitive Extragenic Palindromic-Base Pairs
bp	Base Pairs
Bps	*Burkholderia pseudomallei*
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
gltB	Glutamate Synthase
gmhD	ADP Glycerol-Mannoheptose Epimerase
GPS	Global Positioning System
JCU	James Cook University
KMA	k-mer Alignment
lepA	GTP-Binding Elongation Factor
lipA	Lipoic Acid Synthetase
MLST	Multi-Locus Sequence Typing
narK	Nitrite Extrusion Protein
ndh	NADH Dehydrogenase
ONT	Oxford Nanopore Technology
PCR	Polymerase Chain Reaction
qPCR	Quantitative Real-Time PCR
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
TTSS	Type III Secretion System
WGS	Whole Genome Sequencing
WHO	World Health Organisation

Chapter 1

General Introduction

Burkholderia pseudomallei, formerly known as *Pseudomonas pseudomallei*, is a saprophytic bacterium and the causative agent of melioidosis (Whitmore & Krishnaswami 1912). In 2015, melioidosis had an estimated global burden of 165,000 cases per year, with 89,000 resulting in death (Limmathurotsakul et al. 2016). The prevalence of *B. pseudomallei*, and thus melioidosis incidence, is typically associated with tropical and subtropical regions and communities (Limmathurotsakul et al. 2016; Meumann et al. 2023). However, evidence suggests that *B. pseudomallei* persists in an expanded endemic zone (Chapple et al. 2016; Yip et al. 2015). Melioidosis incidence usually increases during rainfall, but environmental changes also contribute to case clusters (Currie et al. 2021). *Burkholderia pseudomallei* is widespread throughout the environment in endemic areas and has been detected in soil, water and plant rhizospheres (Limmathurotsakul et al. 2016; Wiersinga, Currie & Peacock 2012).

Melioidosis is acquired from environmental exposure to *B. pseudomallei* through percutaneous inoculation, ingestion or inhalation (Currie et al. 2021; Webb et al. 2022). The incubation period for acute melioidosis is 1-21 days, with a median of 4 days (Currie et al. 2021). Most individuals will not develop the disease after exposure, but those with comorbidities that impair the innate or adaptive immune system are at a higher risk. Common comorbidities include diabetes, excessive alcohol consumption, and chronic renal and respiratory diseases (Chantratita et al. 2023; Currie et al. 2021; Gassiep et al. 2022). Melioidosis can be difficult to diagnose in regions where it is under-recognised due to a lack of distinctive disease characteristics.

Melioidosis can present with a variety of symptoms depending on the route of transmission, host response, and disease progression. Patients most commonly present with pneumonia, bacteraemia, and septic shock. Other symptoms include genitourinary infection, skin infection, soft tissue abscesses, osteomyelitis, septic arthritis, and neurological disease (Chantratita et al. 2023; Currie et al. 2021; Gassiep et al. 2022). The lack of distinctive clinical features compared to other community-acquired infections can lead to misdiagnosis. Therefore, further laboratory testing of the infectious agent, especially in endemic regions, is crucial for accurate diagnosis and effective treatment.

Directed treatment for diagnosed melioidosis is crucial for improving patient outcomes. *Burkholderia pseudomallei* is inherently resistant to various first-line antibiotics, including penicillin, ampicillin, first-generation and second-generation cephalosporins, gentamicin, tobramycin, streptomycin, macrolides, and polymyxins (Bugrysheva et al. 2017; Rhodes & Schweizer 2016). Laboratory confirmation of melioidosis informs the recommended treatment (Sullivan et al. 2020). Effective diagnosis and treatment are guided by community awareness and clinical suspicion. Although

clinicians in the Australian tropics have a reasonably good understanding of melioidosis, the source of infection is rarely investigated.

To inform community awareness and clinical suspicion, especially in the context of a changing climate, a thorough understanding of the epidemiology and geographical boundaries of *B. pseudomallei* is essential to understand and predict risk. The main niche traditionally investigated is soil (Limmathurotsakul, Dance, et al. 2013). Research has traditionally focused on the soil niche of *B. pseudomallei*, which prefers acidic soils with low salinity and high water availability (Baker et al. 2015; Inglis & Sagripanti 2006; Limmathurotsakul et al. 2016; Palasatien et al. 2008). Soils that are well irrigated and/or poorly draining due to farming practices or landscape topography and hydrology are associated with the presence of *B. pseudomallei* and melioidosis case clustering (Corkeron, Norton & Nelson 2010; Goodrick, Todd & Stewart 2018). Recently, water has also been increasingly implicated in the disease.

Melioidosis incidence has long been associated with the wet season (Currie & Jacups 2003; Currie et al. 2021; Ganeshalingam et al. 2023). Increased rainfall results in an abundance of surface groundwater, which can contain viable *B. pseudomallei* (Baker et al. 2011; Rachlin et al. 2021; Zimmermann et al. 2018). Groundwater from aquifers, such as bores and seeps, have also been identified as potential reservoirs of infection (Baker et al. 2011; Baker & Warner 2016; Draper et al. 2010; Tran et al. 2022). Additionally, artificially created groundwater from agricultural activities, such as farmlands in Australia, rice paddy fields in Thailand, and gardens, has been implicated (Kaestli et al. 2015; Kaestli et al. 2009; Ong et al. 2017). Survival and persistence of *B. pseudomallei* in groundwater varies depending on the type of groundwater. For example, the organism is only temporarily identified in surface runoff groundwater from rainfall or persistently identified in bore water year-round (Baker et al. 2011; Draper et al. 2010). Unlike soil, water also has a greater potential to spread. This mobility might contribute to an expanding biogeographical spread of *B. pseudomallei* within a region as seen with ST562 in Darwin (Meumann et al. 2021). Groundwater is an ecological niche of *B. pseudomallei*, the different characteristics of water likely influencing the survival, persistence, and movement of *B. pseudomallei* in the environment.

In the Townsville region, water plays a significant role in the epidemiology of *B. pseudomallei*. As in many other regions, melioidosis incidence increases during the wet season (Ganeshalingam et al. 2023). Groundwater seeps on Castle Hill and across greater Townsville have been shown to contain *B. pseudomallei* through culture or Polymerase Chain Reaction (PCR) methods (Baker et al. 2011; Baker & Warner 2016). Surface groundwater from Castle Hill, collected from drains in surrounding urban areas after heavy rainfall, has also been used to isolate and culture *B. pseudomallei*. Strains from Castle Hill were found to have sequences closely related to clinical case isolates from Townsville hospitals (Baker et al. 2011). Although annual cases of melioidosis in the Townsville region are often

attributed anecdotally to specific events or locations, evidence-based genomic epidemiological investigations have rarely been conducted for confirmation.

Without evidence-based genomic epidemiological investigation into *B. pseudomallei*, managing and preventing melioidosis cases becomes more challenging. Understanding infection reservoirs and transmission routes relies on such investigations. Knowledge of specific locations or events linked to disease can help prevent cases by informing at-risk groups to avoid these areas or discontinue certain activities. For example, a recent melioidosis outbreak at a Cairns school, which resulted in seven immunocompetent children becoming ill, was traced to a mud pit used in an obstacle course (Smith et al. 2023). Future cases can be prevented by discontinuing the use of mud pits in school events within melioidosis endemic regions. The identification and confirmation of the reservoir of infection was achieved through matching clinical and environmental isolates using Multi-Locus Sequence Typing (MLST), a common genomic epidemiological tool.

MLST is an unambiguous method for investigating strain diversity within a species. Each strain is assigned a Sequence Type (ST) (Godoy et al. 2003; Jolley, Bray & Maiden 2018). Two isolates with the same ST are assumed to be closely related if isolated from the same location. MLST can be used for environmental surveillance and matching clinical and environmental isolates to investigate reservoirs of infection and transmission routes for *B. pseudomallei* (Gassiep et al. 2024; Smith et al. 2023). It can also be used to assess the genetic diversity found within a region as recently done by Gassiep et al. (2024) who determined the genetic diversity of *B. pseudomallei* clinical isolates in Townsville over time. In MLST, sequencing is performed on *B. pseudomallei* isolates to determine the allele sequences of internal fragments of seven housekeeping genes, and then an overall ST is assigned (Godoy et al. 2003; Jolley, Bray & Maiden 2018). The availability of sequencing options significantly affects the time required for genomic epidemiological analysis. The lack of regular genomic epidemiological investigation for *B. pseudomallei* in Townsville is partly due to the absence of readily accessible in-house sequencing capacity with a fast turnaround time.

In Townsville, sequencing has been outsourced to third-party companies such as the Australian Genome Research Facility (AGRF) and Macrogen (Baker et al. 2011). However, the additional time needed to send samples for sequencing delays results beyond clinically relevant timeframes. Ideally, sequencing technology should be inexpensive, easily accessible, have high throughput capacity, and be rapid and reliable. These elements are essential for making sequencing accessible to less-resourced regions and ensuring timely outbreak investigations. Establishing in-house sequencing capacity in Townsville that meets these criteria is the first step toward standardising genomic epidemiological investigation for *B. pseudomallei*. This would enable more timely and accurate investigations into potential reservoirs of infection, particularly groundwater, to better understand the genomic epidemiology of *B. pseudomallei*.

Chapter 2

Literature Review

2.1 Melioidosis and *B. pseudomallei*

2.1.1 Clinical Epidemiology

Burkholderia pseudomallei, the causative agent of melioidosis, is an opportunistic pathogen of animals and humans (Whitmore & Krishnaswami 1912). It has been classified as a Tier One Biological Warfare agent due to its potential use as a biological weapon (CDC 2018). As a saprophyte, *B. pseudomallei* is found in soil, water, and the plant rhizosphere (Limmathurotsakul et al. 2016; Wiersinga, Currie & Peacock 2012). Humans and animals primarily acquire melioidosis through percutaneous inoculation, ingestion, or inhalation of *B. pseudomallei* from contaminated soil and water (Currie et al. 2021; Webb et al. 2022). Most melioidosis cases occur in individuals with clinical risk factors that impair the innate and adaptive immune systems.

Diabetes mellitus is the leading clinical risk factor for melioidosis in both Australia and Thailand. In Australia, 45%-48% of cases are associated with diabetes, while in Thailand, this figure rises to 70%. Other recognised risk factors for melioidosis include excessive alcohol consumption, chronic kidney and lung disease, malignancy, immunosuppression, immunosuppressive therapy, and heart disease (rheumatic heart disease and congestive heart failure) (Chantratita et al. 2023; Currie et al. 2021; Gassiep et al. 2022). A recent study conducted in Townsville, Queensland determined that excessive alcohol consumption was identified as a risk factor in 55% of patients, which is higher than the 48% observed for Diabetes mellitus (Gassiep et al. 2022). Although most patients have at least one risk factor for melioidosis, a minority do not. Observational studies in Australia and Thailand found that 11%-16% of patients had no evident risk factors (Chantratita et al. 2023; Currie et al. 2021; Gassiep et al. 2022).

Melioidosis cannot be distinguished from other community-acquired infections based on clinical and radiological characteristics. Patients can present with a variety of symptoms, including bacteraemia, pneumonia, genitourinary infection, skin infection, soft tissue abscesses, osteomyelitis, septic arthritis, and neurological disease. More than 50% of melioidosis patients present with pneumonia, approximately 50% have bacteriaemia, and 20% progress to septic shock (Chantratita et al. 2023; Currie et al. 2021). Other recent studies conducted in Queensland determined that bacteraemia was present in 70%-71% of melioidosis patients and pneumonia in 65%-69% (Gassiep et al. 2022; Gassiep et al. 2023). The diverse clinical presentations make it challenging to distinguish melioidosis from

other infections. Therefore, diagnostic tests are necessary to accurately diagnose melioidosis in patients.

The gold standard for testing *B. pseudomallei* is culture. This bacterium typically grows on routine agars such as Blood agar and MacConkey agar. Ashdown agar, a specialised medium, is both selective and differential for *B. pseudomallei*. The colonies are cream coloured with a metallic sheen, becoming dry (matte appearance) and wrinkled. On Ashdown agar, the colonies appear purple (mauve) (Hoffmaster et al. 2015; Wiersinga et al. 2018). Clinical specimens for diagnostic testing include blood, sputum, urine, throat and rectal swabs, pus, and bodily fluids from normally sterile sites. A positive culture from any site is diagnostic for melioidosis. However, *B. pseudomallei* colonies may not appear for 48 hours and can be overgrown by faster-growing bacteria. Without relevant microbiology expertise, *B. pseudomallei* may be misidentified or considered a contaminant (Hoffmaster et al. 2015).

Commercial identification systems, molecular methods, and antibiotic susceptibility testing can aid in confirming *B. pseudomallei*. Biochemical tests such as API 20NE and VITEK 2 can be used, but misidentification is common (Deepak, Crawley & Phang 2008; Hoffmaster et al. 2015; Lowe, Haswell & Lewis 2006). Latex agglutination assays offer a rapid diagnostic tool for screening suspect colonies (Duval et al. 2014). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is increasingly used, but its commercial databases currently lack *B. pseudomallei* (Gassiep, Armstrong & Norton 2019; Watthanaworawit et al. 2021). Published PCR methods, such as the Type III Secretion System (TTSS) Quantitative Real-Time PCR (qPCR), are available for identifying *B. pseudomallei* but are limited to laboratories with in-house PCR capabilities, often reference laboratories in lower resourced countries (Hoffmaster et al. 2015). Antibiotic susceptibility testing is a simple method for confirming *B. pseudomallei* in resource-limited settings. Resistance to gentamicin and colistin, along with susceptibility to amoxicillin–clavulanate for Gram-negative bacilli that produce cytochrome oxidase, indicates *B. pseudomallei* (Hoffmaster et al. 2015; Trinh et al. 2018). However, some isolates have shown susceptibility to gentamicin (Podin et al. 2014).

Melioidosis is rapidly progressive and can be fatal without appropriate diagnosis and treatment (Savelkoel et al. 2021). In Australia, a well-resourced country, strategies for early diagnosis, specific antimicrobial therapy, and advanced intensive care can reduce the mortality rate to below 10% (Currie et al. 2021; Stewart et al. 2017). However, in some regions the mortality rate can be higher as seen in Townsville with a fatality rate of 23%-25% (Gassiep et al. 2022; Gassiep et al. 2023). In contrast, resource-poor settings like Thailand have significantly higher mortality rates, with 25% of patients dying within one month and 34% within one year of infection (Chantratita et al. 2023).

Early diagnosis of melioidosis through clinical awareness and laboratory confirmation of *B. pseudomallei* is essential because empiric antibiotics are ineffective due to the bacterium’s inherent

drug resistance. *Burkholderia pseudomallei* is resistant to penicillin, ampicillin, first-generation and second-generation cephalosporins, gentamicin, tobramycin, streptomycin, macrolides, and polymyxins (Bugrysheva et al. 2017; Rhodes & Schweizer 2016). The 2020 Darwin melioidosis treatment guideline recommends a two-phase treatment: an intensive phase and an eradication phase. The intensive phase involves 10-14 days of intravenous ceftazidime, meropenem, or imipenem, with or without trimethoprim-sulfamethoxazole. This is followed by a minimum of three months of oral trimethoprim-sulfamethoxazole for the eradication phase (Sullivan et al. 2020). Clinical awareness of melioidosis is crucial for effective diagnosis and treatment. Understanding the biogeography of *B. pseudomallei* and its ecological niche is important for informing at-risk communities.

2.1.2 Microbial Biogeography

Biogeography is the study of species diversity across ecosystems and geographic boundaries. This concept, originally proposed by Alfred Wallace, was demonstrated by the contrasting animal and plant diversity on either side of the Wallace line shown in Figure 2.1 (Wallace 1863). Microbial life also exhibits biogeography.

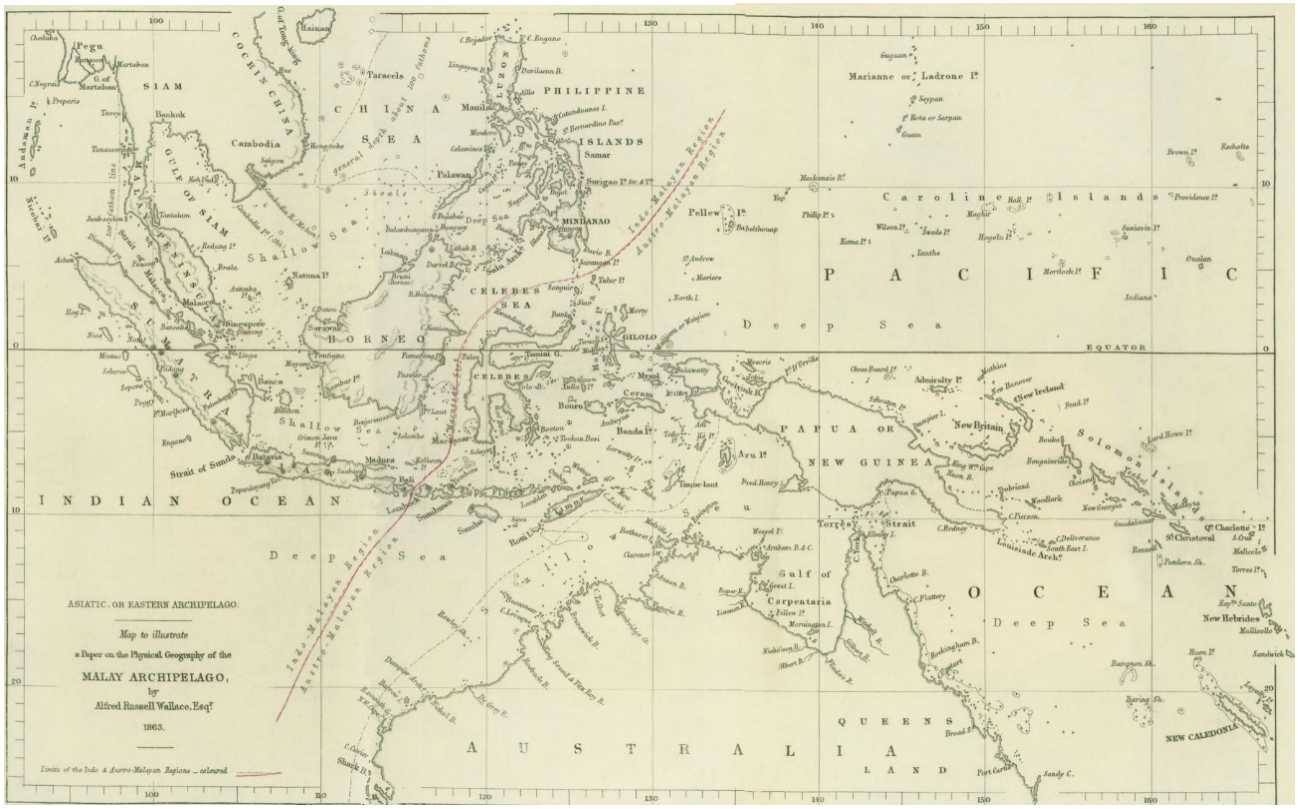

Figure 2.1 - Original map depicting the Wallace line drawn by Alfred Wallace in 1863 (Wallace 1863).

Environmental microorganisms are not evenly distributed throughout the environment. Factors such as environmental heterogeneity, microorganism characteristics, dispersal limitations, and ecological drift

contribute to significant microbial genomic clustering (Chu et al. 2020; Dickey et al. 2021; The Systematics Association 2011). Environmental heterogeneity, influenced by varying climates, altitudes, pH levels, precipitation, and organic matter, has been linked to global niche differentiation of fungi and bacteria (Bahram et al. 2018; Delgado-Baquerizo et al. 2016). Zinger et al. (2019) demonstrated that body size predicts microbial community assembly. Additionally, homogenising dispersal limitations and the stochasticity of ecological drift are important drivers of microbial community assembly on small geographical scales (Bahram et al. 2016; Powell et al. 2015). Overall, both niche-based (environmental heterogeneity and microorganism characteristics) and niche-neutral (dispersal limitations and ecological drift) processes drive microbial community assembly and influence microbial biogeographical boundaries (Bahram et al. 2016; Bahram et al. 2018; Chu et al. 2020; Delgado-Baquerizo et al. 2016; Dickey et al. 2021; Powell et al. 2015; The Systematics Association 2011; Zinger et al. 2019). Investigating these factors is essential for understanding the ecological niche and biogeographical boundaries of environmental organisms.

Clinical evidence of non-random clustering of environmentally acquired infectious agents supports the existence of microbial biogeographical boundaries. Pathogens that demonstrate preferred environmental niches, acting as reservoirs of infection, include but are not limited to *Vibrio cholerae*, *Mycobacterium ulcerans*, *Naegleria fowleri*, *Clostridium tetani*, *Clostridium botulinum*, *Bacillus anthracis*, and *B. pseudomallei*. *Vibrio cholerae*, *M. ulcerans* and *N. fowleri* are examples of organisms associated with the water niche. *Vibrio cholerae*, the causative agent of cholera, is found in brackish or marine water niches (Deen, Mengel & Clemens 2020). *Mycobacterium ulcerans*, responsible for Buruli ulcer, is typically associated with stagnant or slow-flowing aquatic environments (O'Brien et al. 2019). *Naegleria fowleri*, an amoeba causing meningoencephalitis, thrives in warm freshwater bodies (Visvesvara, Moura & Schuster 2007). *Clostridium tetani* and *C. botulinum*, causing tetanus and botulism respectively, are widespread in soil (Espelund & Klaveness 2014; George, De Jesus & Vivekanandan 2023). *Bacillus anthracis*, also associated with soil, has a nonuniform distribution with endemic regions in Africa, the Middle East, South America, and Central Asia experiencing continued anthrax outbreaks (Deka, Vieira & Bower 2022). *Burkholderia pseudomallei* (see below), also exhibits distinct global biogeographical boundaries.

2.1.3 Global Burden and Distribution of B. pseudomallei

In 2015, the global burden of melioidosis was estimated at 165,000 cases annually, resulting in approximately 89,000 deaths (Limmathurotsakul et al. 2016). Another study in 2019 assessed the

global burden of melioidosis in disability-adjusted life years (DALYs), estimating it to be 4.64 million DALYs (Birnie et al. 2019). Despite this high burden, melioidosis is not recognised as a neglected tropical disease (NTD) by the World Health Organisation (WHO). Its estimated global burden surpasses that of several WHO-declared NTDs, including leptospirosis (2.9 million DALYs), dengue (2.86 million DALYs), schistosomiasis (2.63 million DALYs), lymphatic filariasis (1.24 million DALYs), and leishmaniasis (1.06 million DALYs) (Birnie et al. 2019).

Globally, *B. pseudomallei* is primarily biogeographically linked to tropical and subtropical regions, with high endemicity in Northern Australia and Southeast Asia. Other endemic areas include South Asia, sub-Saharan Africa, Central and South America, the Caribbean, and Pacific and Indian Ocean Islands. Melioidosis has been identified as endemic in approximately 45 countries based on evidence from animal and human cases, as well as environmental isolations of *B. pseudomallei* from 1910 to 2014 (Limmathurotsakul et al. 2016). A more recent model, using a modified method from the 2015 study, extended the timeframe from 1910 to 2022, identifying 12 additional countries: Benin, Cameroon, Democratic Republic of Congo, Eritrea, Federal States of Micronesia, Ghana, Mali, Nepal, Nicaragua, Saint Kitts and Nevis, Trinidad and Tobago, and the Southern United States of America (Meumann et al. 2023). Countries determined to be endemic generally have documented isolation of *B. pseudomallei* from both clinical and environmental samples. The major limitation of this modelling technique is the lack of surveillance in countries with minimal microbiological surveillance and laboratories. Although the 2022 model incorporates more evidence and presents an updated distribution, it may not accurately reflect the true distribution of *B. pseudomallei* due to underreporting and insufficient surveillance in regions lacking the necessary expertise and microbiological tools.

2.2 The Environmental Microbiology of *B. pseudomallei*

Burkholderia pseudomallei displays non-random spatial and temporal distribution within regions of endemicity (Chapple et al. 2016; Limmathurotsakul et al. 2016; Rachlin et al. 2020). It is typically found in soil and freshwater environments, and demonstrates an uneven distribution. Investigation into the geographical spread of *B. pseudomallei* STs previously has determined that STs often demonstrate restricted distribution with localised spatial clustering in the environment (Chapple et al. 2016; Rachlin et al. 2020). The uneven distribution of *B. pseudomallei* in the environment leads to clustering of melioidosis cases within endemic regions (Corkeron, Norton & Nelson 2010; Palasatien et al. 2008; Rachlin et al. 2020). To determine the explanation for the specific clustering patterns of *B. pseudomallei*, the characteristics of the niches it inhabits have been investigated.

2.2.1 Soil Niche: Reservoir of Infection

Investigation into the ecological niche of *B. pseudomallei* has described both the microenvironments and types of soil associated with its presence, as well as larger scale topological features of the land. Variations in soil microenvironments, arising from its physicochemical properties, are hypothesised to influence the spatial distribution of *B. pseudomallei*. Commonly investigated soil factors include pH, nutrient availability, salinity, and water availability.

Burkholderia pseudomallei favours an acidic environment, with an optimal pH range of 4-8 (Chen et al. 2003; Kaestli et al. 2015; Wang-Ngarm, Chareonsudjai & Chareonsudjai 2014). Two studies have isolated the bacterium from soils with pH levels of 4-6 (Ngamsang et al. 2015; Palasatien et al. 2008). In Australia, *B. pseudomallei* was found in acidic soils with pH values between 5.2 and 5.8, while *B. pseudomallei* negative soils had pH levels of 6-7 (Kaestli et al. 2009). However, other studies in Thailand found no significant pH difference between positive and negative locations (Ngamsang et al. 2015; Sermswan et al. 2015; Suebrasri et al. 2013).

High salinity in brackish or marine water inhibits *B. pseudomallei*. For *B. pseudomallei* growth, salinity must be within freshwater ranges. *Burkholderia pseudomallei* grows comfortably within a salinity range of 0-0.5%, but values above 1% are inhibitory (Wang-Ngarm, Chareonsudjai & Chareonsudjai 2014). In environmental soil samples, lower salinity levels were reported. An Australian study determined that *B. pseudomallei* growth was inhibited in soils with an electrical conductivity (EC) of 0.7 ds/m (0.045% salinity) (Kaestli et al. 2015). A study in Thailand by Suebrasri et al. (2013) found that *B. pseudomallei* was isolated from soils with an EC of 0.03-0.93 ds/m (0.002-0.06% salinity) and reported no significant salinity difference between positive and negative locations.

Nutrient availability in soil typically includes investigations into nitrogen, carbon, and iron. Soils treated with nitrogen fertilisers have been shown to improve the growth of *B. pseudomallei*, supported by Palasatien et al. (2008), who found a correlation between positive *B. pseudomallei* samples and higher nitrogen content (Kaestli et al. 2015). Total organic carbon has also been positively correlated with *B. pseudomallei* presence in soil, although Baker et al. (2015) noted that *B. pseudomallei* can exist in soils with low total organic carbon (Palasatien et al. 2008; Wang-Ngarm, Chareonsudjai & Chareonsudjai 2014). Iron availability promotes *B. pseudomallei* growth, as demonstrated by soils treated with 75-150mg/kg of iron improving the growth of the bacterium (Wang-Ngarm, Chareonsudjai & Chareonsudjai 2014). Ngamsang et al. (2015) showed higher iron levels in positive

B. pseudomallei soil samples compared to negative ones (45.4±18.4mg/kg versus 17.9±7.2 mg/kg respectively). However, Suebrasri et al. (2013) reported a broad range of iron content in positive samples (6.17 mg/kg to 288.48 mg/kg). Finally, a study conducted in Australia by Baker et al. (2015) found that positive samples had lower iron content than negative samples (13 mg/kg versus 160mg/kg respectively).

Burkholderia pseudomallei persistence is correlated with higher water availability in soil. Kaestli et al. (2015) and Larsen et al. (2013) demonstrated that regular irrigation of soil increases *B. pseudomallei* growth and promotes its survival. Additionally, positive *B. pseudomallei* soil samples from the environment have been associated with higher moisture content (Kaestli et al. 2009; Larsen et al. 2013; Palasatien et al. 2008). However, *B. pseudomallei* can also persist in drought conditions within desert and temperate regions (Chapple et al. 2016; Yip et al. 2015). Soil type and land topology significantly impact water availability in soil.

Geospatial investigations of *B. pseudomallei* have shown that topological features of the land and soil type can affect the persistence of the bacterium. *Burkholderia pseudomallei* is associated with sand and clay soils (Corkeron, Norton & Nelson 2010; Goodrick, Todd & Stewart 2018; Kaestli et al. 2009; Palasatien et al. 2008; Pongmala et al. 2022). Corkeron, Norton and Nelson (2010) demonstrated that the spread of melioidosis cases is linked to a combination of soil type, geomorphic position, and drainage, resulting in areas that are well irrigated or poorly drained. Similarly, Goodrick, Todd and Stewart (2018) found that melioidosis cases in Cairns, Australia, are associated with areas of poor water drainage or constant irrigation. These factors create areas of higher water availability, favourable for sustaining *B. pseudomallei*.

2.2.2 Water Niche: Reservoir of Infection and Vehicle for Transmission

Underground water reservoirs, known as aquifers, are significant reservoirs of infection for *B. pseudomallei*. Bore water, commonly used in tropical regions for domestic and agricultural purposes, is a type of groundwater obtained from these aquifers. Studies conducted in Darwin, Australia, revealed that 20-26% of bore water samples tested during the dry season contained *B. pseudomallei;* this proportion increased to 33%-57% during the wet season (Draper et al. 2010; Mayo et al. 2011). Mayo et al. (2011) and McRobb et al. (2015) linked melioidosis cases to contaminated bore water, similar to a case study in Vietnam where contaminated borehole water resulted in the deaths of three children (Tran et al. 2022). Additionally, groundwater seeps from aquifers have been found to contain *B. pseudomallei* in them (Baker et al. 2011). Ingestion of untreated or poorly treated water, as well as inhalation aerosolised droplets, can lead to melioidosis outbreaks.

B. pseudomallei incidence is temporally linked to climatic factors, with a significant increase in melioidosis cases during the wet season. In Australia, 80% of melioidosis cases occur during the wet season (Currie et al. 2021). A study in Townsville, Australia, connected melioidosis cases to high humidity and rainfall (Ganeshalingam et al. 2023). Increases in rainfall, groundwater levels, dew point, cloud cover, and maximum temperature are all associated with a higher incidence of melioidosis, particularly during the La Niña phase of the El Niño– Southern Oscillation in Northern Australia (Kaestli et al. 2016). In Laos and Cambodia, melioidosis cases are linked to increased humidity and wind speed (Bulterys et al. 2018). In Darwin, Australia, for every 100mm increase in total monthly rainfall, there is a 14% average increase in melioidosis incidence (Currie et al. 2021). Similarly, in Townsville, a three-fold increase in melioidosis incidence occurs if more than 200mm of rain falls within a fortnight (Ganeshalingam et al. 2023). These temporal increases in melioidosis cases during extreme weather and heavy rainfall support the hypothesis that water acts as a vehicle for *B. pseudomallei* transmission.

During heavy rainfall and extreme weather events, it is hypothesised that *B. pseudomallei* is brought to the surface by the rising water table, where it then proliferates (Pongmala et al. 2022; Thomas, Forbes-Faulkner & Parker 1979). *B. pseudomallei* can be carried within surface groundwater as spillover from the rising water table and then washed away. *B. pseudomallei* has been isolated from drain water around Castle Hill in Townsville, Australia, after rainfall (Baker et al. 2011). Similarly, studies in Laos have demonstrated that *B. pseudomallei* can be isolated from rivers that act as sentinels for the organism after heavy rainfall (Zimmermann et al. 2018). In Vientiane, Laos, land runoff water from heavy rainfall was used to isolate *B. pseudomallei* (Rachlin et al. 2021). Temporally, the isolation of *B. pseudomallei* from surface groundwater is associated with heavy rainfall events. However, a fine time scale temporal analysis of the effects of rainfall and other climatic factors on the isolation and abundance of *B. pseudomallei* from surface groundwater remains to be investigated.

2.3 Genomic Epidemiology of *B. pseudomallei*

Investigating the environmental footprint and genomic epidemiology of *B. pseudomallei* is essential. Understanding the environmental reservoirs of *B. pseudomallei* and their future changes is crucial for informing at-risk communities. *B. pseudomallei* exhibits large strain diversity due to extensive horizontal gene transfer and recombination events (Pearson et al. 2009; Price, Currie & Sarovich 2017). Studying these diverse strains globally and locally can illuminate the geographic distribution, importation and spread of *B. pseudomallei*, identify virulent strains through genetic association with clinical manifestations, determine high-risk areas, and facilitate matching clinical isolates to their environmental reservoirs (Chapple et al. 2016; Gassiep et al. 2023; Pearson et al. 2009; McRobb et al.

2014; McRobb et al. 2015). It is vital to not only map the changing biogeographical boundaries of *B. pseudomallei* but also to thoroughly understand the mechanisms of its spread. Evidence supports the dissemination of *B. pseudomallei* through anthropogenic, animal, and environmental processes (Chewapreecha et al. 2017; Hampton et al. 2011; Zimmermann et al. 2018). This understanding is especially important for virulent strains that are more likely to cause severe disease. For example, strains containing the *Burkholderia* intracellular motility factor A variant (*BimA*$_{Bm}$) are associated with neurological melioidosis, leading to severe neurological damage or increased mortality (Burnard et al. 2022; Jitprasutwit et al. 2023; Sarovich et al. 2014). Matching environmental and clinical isolates can identify reservoirs of virulent strains and elucidate infection routes (Smith et al. 2023). Currently, environmental surveillance is not routinely performed in Townsville, and no clinical and environmental matches have been established there. The closest match was made by Baker et al. (2011) while investigating isolates from Castle Hill and comparing them to the Townsville Hospital's clinical database. A recent study investigated the genomic epidemiology of *B. pseudomallei* clinical isolates in Townsville 1997 to 2020, and identified a diversity of 59 STs within the region (Gassiep et al. 2024). Not only is there a genetically diverse range of STs within the Townsville region, but this study also identified a change in STs over time (Gassiep et al. 2024). Identification of STs associated with melioidosis patients within the region over time combined with environmental surveillance efforts will help inform environmental reservoirs of infection and how these may be changing into the future.

Environmental surveillance of *B. pseudomallei* and its genomic epidemiology is typically conducted using MLST. MLST is a precise method for characterising bacterial isolates through sequencing internal fragments of selected housekeeping genes. Each distinct sequence is assigned a unique allele number. The combination of allele numbers from the seven loci forms the ST of the isolate (Godoy et al. 2003; Jolley, Bray & Maiden 2018). For *B. pseudomallei*, seven housekeeping genes - Acetyl coenzyme A reductase (*ace*), Glutamate synthase (*gltB*), ADP glycerol-mannoheptose epimerase (*gmhD*), GTP-binding elongation factor (*lepA*), Lipoic acid synthetase (*lipA*), Nitrite extrusion protein (*nark*) and NADH dehydrogenase (*ndh*) - are used to determine the ST, each internal fragment from each gene approximately 500 bp in size (Godoy et al. 2003). Primers for these genes can be found on the pubMLST website (https://pubmlst.org/organisms/burkholderia-pseudomallei/primers).

The process for determining the MLST of *B. pseudomallei* involves several steps. First, a sample is collected and *B. pseudomallei* is isolated. After DNA extraction, PCR is performed to amplify the seven loci. The resulting amplicons are then cleaned and sequenced. Alternatively, whole genome sequencing (WGS) can be used to identify the alleles at each of the seven loci *in silico.* These gene fragments are then compared against the MLST database to assign allele numbers and determine the ST of the isolate. Differences between alleles often consist of single nucleotide polymorphisms (SNP) (Jolley, Bray & Maiden 2018). Therefore, high-quality sequencing is essential for accurately

identifying SNP differences and determining STs. When implementing MLST on a broad scale for environmental surveillance of *B. pseudomallei*, considerations such as sequencing quality, time, and availability are crucial.

2.3.1 Main Sequencing Technology Options

First-generation Sanger sequencing, also known as the chain termination method, remains in use still to this day. This technique synthesises multiple strands of the same DNA product while incorporating modified and labelled nucleotides that terminate the synthesis process. The strands are then separated by size through capillary electrophoresis, and the labelled nucleotide colour is detected by a laser beam and detector. Sanger sequencing is highly accurate for sequencing small products under 1000 bp (Heather & Chain 2016; Shendure et al. 2017). However, it can only sequence a single DNA sequence per sample, making it unsuitable for high-throughput applications. Compared to second-generation and third-generation sequencing technologies, which can sequence multiple samples in parallel, the cost of Sanger sequencing for a large number of samples or WGS is significantly higher.

Second-generation Illumina sequencing is currently the primary technology used. This method involves fragmenting a DNA library, binding the fragments to a flow cell, and amplifying them to form clusters. Individual DNA fragments are then sequenced simultaneously using fluorescent ‘reversible-terminator’ dNTPs. The incorporation of nucleotides is monitored by exciting the fluorophores with lasers. Millions of small DNA fragments can be sequenced simultaneously with high accuracy (Heather & Chain 2016; Shendure et al. 2017). The parallel sequencing capability makes this technology highly desirable for high-throughput applications.

Third-generation Oxford Nanopore Technology (ONT) sequencing allows for the parallel sequencing of single molecules in real-time. ONT uses nanoscale protein pores, termed ‘nanopores,’ embedded in an electrically resistant polymer membrane that act as biosensors. A constant voltage is applied to the nanopores to produce an ionic current. DNA or RNA passing through the nanopores causes changes in the ionic current corresponding to the sequence. A major advantage of ONT over Sanger and Illumina technologies is the ability to produce long-read sequences. The parallel nature of sequencing also supports high-throughput applications. Although the error rate of ONT sequencing is higher, recent advancements are continuously improving its accuracy (Wang et al. 2021).

2.3.2 Sequencing for MLST

Currently, Illumina sequencing is the primary technology for MLST determination, providing high-

quality reads suitable for MLST and SNP analysis. However, the high cost of Illumina sequencing devices limits their availability in many laboratories, necessitating outsourcing (Illumina 2024). The outsourcing process introduces a significant delay, with results taking approximately 2-6 weeks (AGRF 2024). As a result, determining the MLST of an isolate within a clinically relevant timeframe is challenging. During melioidosis outbreaks, rapid identification of the environmental reservoir is crucial to mitigate further cases. The lack of local Illumina sequencing facilities is particularly problematic for high biosecurity containment organisms like *B. pseudomallei*, which require handling in a PC3 facility (Peacock et al. 2008). Transporting DNA samples out of a PC3 facility adds time and risk to the process, contributing to the lack of routine clinical and environmental matching of *B. pseudomallei* isolates.

The introduction of ONT sequencing is set to change this landscape. Recent advancements have made ONT sequencing more reliable for SNP analysis. The older R9.4.1 chemistry was successfully used for accurate SNP analysis and MLST determination of *Salmonella enteritidis* (Xian et al. 2022). Promising results have also been achieved in human forensics (Tytgat et al. 2022). The newer R10.4.1 chemistry has further improved sequencing quality. Ritchie et al. (2023) utilised ONT R10.4.1 technology to investigate the core genome MLST of *Shigella sonnei*. With ONT sequencing, MLST determination can be completed in less than a week (Buytaers et al. 2021; Ferreira et al. 2021; Ritchie et al. 2023). Additionally, the low cost of MinION sequencing devices allows for local setup and immediate sequencing upon sample arrival. The capability is particularly advantageous for high containment organisms like *B. pseudomallei*, as a sequencer can be installed within PC3 facilities. ONT technology holds great promise for real-time environmental surveillance of *B. pseudomallei*, especially as it continues to improve.

2.4 Expanding Melioidosis Endemic Zones

2.4.1 Anthropogenic Changes and Melioidosis

Changing land use and expanding infrastructure will impact the prevalence of melioidosis within and outside endemic regions. Environmental alterations, such as land disturbance, affect the distribution of *B. pseudomallei*. For instance, farmland, which frequently involves surface soil disturbance and regular irrigation, tends to have higher *B. pseudomallei*-positive soil compared to undisturbed natural sites (Kaestli et al. 2009). As farmland areas expand with a growing population, more landscape changes that favour *B. pseudomallei* are expected. Gardens also harbour large quantities of *B. pseudomallei*, with regular irrigation being a consistent factor in its growth (Kaestli et al. 2015).

Additionally, expanding infrastructure into endemic areas is likely to increase melioidosis cases. For example, Vientiane Capital in Laos, which houses over 10% of the population, has been built in an endemic area, resulting in 54.6% of melioidosis cases in Laos from 1999 to 2017 (Rachlin et al. 2021). Similarly, as Townsville in Australia grows, more melioidosis cases are expected. A recent study investigated the genomic epidemiology of clinical *B. pseudomallei* isolates in Townsville over time (1997 to 2020) and identified a diversity of STs that changed over the duration of the study. The cause of changing STs within Townville melioidosis cases was not known and required further investigation, but could be the result of anthropogenic changes related to soil types, soil disturbance as a result of infrastructure development, and prevalence of risk factors within the population of residents dwelling in higher risk zones for *B. pseudomallei* (Gassiep et al. 2024). Infrastructure expansion necessitates landscape changes, potentially increasing *B. pseudomallei* exposure.

Heavy earthworks related to expanding infrastructure also pose a risk for melioidosis outbreaks. Pongmala et al. (2022) found that *B. pseudomallei* concentrations were highest at soil depths of 100cm to 200cm and groundwater persistence was a controlling factor for *B. pseudomallei* occurrence. Deep earthworks that bring soil from these depths to the surface can expose large concentrations of *B. pseudomallei*. This was observed during motorway construction in Cairns, Australia, from 2011 to 2017, where construction was associated with an increase in melioidosis cases (Smith et al. 2021). Earthworks in endemic regions will likely lead to more melioidosis cases in surrounding areas. Anthropogenic environmental changes will likely shift the biogeographical boundaries of *B. pseudomallei* in the future.

2.4.2 The Challenges of a Changing Climate

Climate change will affect the prevalence of *B. pseudomallei* in the environment, likely leading to an increase in melioidosis cases. Rising global temperatures are expected to result in more extreme weather events, including changes in precipitation patterns and droughts. Some tropical and subtropical areas will experience decreased precipitation, while high latitude regions, the equatorial Pacific, and monsoonal areas are projected to see more intense tropical storms (IPCC 2018; IPCC 2022). A climate report for Australia by the Bureau of Meteorology (BOM) and Commonwealth Scientific Industrial Research Organisation (CSIRO) predicts increased rainfall during the wet season in Northern Queensland, with more frequent heavy rainfall events leading to a higher risk of flash flooding (BOM & CSIRO 2022). Regions in South, East, and Southeast Asia and West Africa are also expected to see increased monsoon rainfall (IPCC 2018; IPCC 2022). Given the strong association between rainfall, extreme weather events, and melioidosis cases, the number of cases is likely to rise in the future.

Climate change-related outbreaks of melioidosis are beginning to occur in regions not traditionally considered endemic for *B. pseudomallei.* Southern Queensland, for example, was not regarded as an endemic zone until recently, when *B. pseudomallei* was isolated from both clinical and environmental samples from the Brisbane region. These outbreaks were linked to two successive La Niña events that caused increased rainfall and flooding (Gassiep et al. 2023). Regions previously considered latent for *B. pseudomallei,* which are projected to experience more heavy rainfall and extreme weather events, will likely see more melioidosis cases. For instance, Central Australia, a desert region typically unsuitable for *B. pseudomallei,* experienced heavy rainfall and flooding in 2011, resulting in six melioidosis cases (Yip et al. 2015). Similarly, a temperate region in southwest Western Australia saw sporadic cases between 1966 and 1992, and after higher-than-average rainfall in 2017, a melioidosis cluster in animals lead to over 20 deaths (Webb et al. 2020). Monitoring climate change-driven shifts in the biogeographical boundaries of *B. pseudomallei* and the appearance of melioidosis cases will be crucial to keeping at-risk communities informed.

2.5 Conclusions

Understanding the biogeographical boundaries and genomic epidemiology of *B. pseudomallei* is crucial for informing clinicians and communities about the changing risk of melioidosis. Therefore, environmental surveillance of *B. pseudomallei* is essential. Although local research has investigated soil as a niche for *B. pseudomallei* in Townsville, studies on groundwater are limited. Understanding the role of groundwater as a reservoir for *B. pseudomallei* is vital for effective melioidosis risk management. To investigate the groundwater niche effectively, using ONT sequencing for rapid environmental surveillance and genomic analysis of *B. pseudomallei* will be key. ONT sequencing enables real-time data collection and analysis locally, significantly enhancing the speed and accessibility of environmental surveillance efforts.

2.6 Objectives

The study investigates whether groundwater acts as a significant reservoir of infection for *B. pseudomallei* in Townsville, focusing on three key objectives:

1. Characterising the environmental and climatic factors influencing *B. pseudomallei* presence in surface groundwater, utilising a sentinel seasonal creek for longitudinal study. This involves site selection for longitudinal study, correlating rainfall patterns with *B. pseudomallei* concentration, and exploring specific groundwater characteristics linked to the bacterium's presence.

2. Establishing and validating rapid sequencing techniques, specifically ONT sequencing, in a high containment facility. This includes validating ONT for MLST of *B. pseudomallei*, and comparing its read accuracy, cost, and efficiency with Illumina WGS.
3. Investigating the genomic epidemiology of *B. pseudomallei* strains isolated from Townsville's groundwater. This encompasses assessing diversity of *B. pseudomallei* STs, identifying dominant STs, evaluating clinical relevance of sequenced STs, and exploring genetic relatedness to other sequences from Townsville and Australia.

These objectives aim to deepen understanding of *B. pseudomallei's* reservoirs in Townsville, enhance genomic epidemiology capabilities, and inform strategies for disease management and prevention.

Chapter 3

Environmental Characteristics Associated with *Burkholderia pseudomallei* Presence in Surface Groundwater in Townsville

3.1 Introduction

Burkholderia pseudomallei is a Gram-negative saprophytic bacterium that causes melioidosis, an opportunistic infection in humans. Infection occurs through exposure to environmental *B. pseudomallei* via inoculation, ingestion, or inhalation, although its epidemiology is still poorly understood (Limmathurotsakul, Kanoksil, et al. 2013; Yip et al. 2015). *B. pseudomallei* exhibits a non-random spatial and temporal distribution, favouring tropical and subtropical regions such as Northern Australia and Southeast Asia (Limmathurotsakul et al. 2016). It is typically found in soil and freshwater environments. The uneven distribution of *B. pseudomallei* in the environment leads to clustering of melioidosis cases within endemic regions (Corkeron, Norton & Nelson 2010; Palasatien et al. 2008; Rachlin et al. 2020). Melioidosis incidence increases during the wet season and is linked to extreme weather events (Chen et al. 2015; Cheng et al. 2006; Currie et al. 2021; Ganeshalingam et al. 2023). One hypothesis suggests that the rising water table during the wet season brings the bacteria to the surface, where they can multiply under favourable conditions (Thomas, Forbes-Faulkner & Parker 1979). Studies by Baker et al. (2011) and Baker and Warner (2016) have investigated groundwater in Townsville for the presence of *B. pseudomallei*.

Townsville, Northern Queensland, Australia, is an endemic region for *B. pseudomallei*. Investigations by Baker et al. (2011) and Baker and Warner (2016) isolated the bacterium from groundwater in Castle Hill, Mt Stuart, and Mt Louisa (Figure 1 in Baker and Warner (2016)). Viable *B. pseudomallei* was found in surface groundwater flowing into residential areas from Castle Hill after heavy rainfall (Baker et al. 2011). Surface groundwater likely acts as a conduit for the bacterium, contributing to more melioidosis cases as it spreads into densely populated regions. Despite these findings, detailed knowledge of the temporal presence of *B. pseudomallei* in surface groundwater, both short- and long-term, remains limited due to a lack of comprehensive studies on the relationship between surface groundwater and *B. pseudomallei* in Townsville.

This study aims to describe the environmental characteristics influencing *B. pseudomallei* presence in surface groundwater and suggest strategies for reducing exposure to high concentrations of the bacterium.

3.2 Materials and Methods

3.2.1 Biosafety and Risk Assessment

The biosafety approval number is JCU-IBC-20220412-25. The James Cook University (JCU) Risk Assessment number is 13567.

3.2.2 Isolation of B. pseudomallei

One litre of surface groundwater from study sites (Section 3.2.3) was collected into sterile glass bottles. Ten replicate 100 µL volumes of sample water was inoculated onto Ashdown agar (ASH-A), incubated for two days at 37^{o}C in ambient air, and analysed for typical colonial morphology of *B. pseudomallei* to determine CFU/mL (Ashdown 1979; Ashdown & Clarke 1992) (A Appendix Table A.1). The sum of the characteristic *B. pseudomallei* colonies from all ten replicate plates was used to determine CFU/mL. The remaining water was filtered through 0.22µm filters, and the paper incubated in Ashdown broth (ASH-B) using similar incubation conditions (A Appendix Table A.1). Approximately 10 µL of ASH-B was dilution streaked onto ASH-A from each sample after one, two and five days of incubation. Recipe for ASH-A and ASH-B included in Appendix A Table A.1.

Quantification of *B. pseudomallei* colonies was determined by recognition of characteristic colony morphology and standard plate count methods. A subset of 10% or the square root of total colonies if 10% was below 10 colonies was collected to represent diversity (Clesceri et al. 1996; FT-020 2021; FT-024 1991). A subset of suspect colonies were confirmed by TTSS qPCR modified from Baker et al. (2011). Briefly, PCR reactions consisted of 1x GoTaq Colourless Master Mix (Promega), 0.4 µM forward and reverse primer, 0.256µM probe, 50-100 ng DNA, and molecular-grade H_2O to 20 µL. Thermocycling conditions consisted of a 5 min initial denaturation at 95^{o}C, followed by 45 cycles of 15 sec at 95^{o}C and 15 sec at 59^{o}C. A single colony of bacteria was directly added to each PCR reaction (Colony PCR). A Mic PCR Biomolecular Systems machine (micPCR version 2.10.1) was used for all PCR reactions. Confirmed *B. pseudomallei* colonies were stored in 20% Glycerol Tryptic Soy Broth (GTSB) and frozen at -80^{o}C (A Appendix Table A.1 and Table A.10). Confirmed isolates were moved to a high containment (PC3) facility.

3.2.3 Selection of Location for Longitudinal Study

Previous studies conducted in Townsville showed *B. pseudomallei* was present at the following sites across the Townsville region: Mt Stuart, Mt Louisa, and Castle Hill (Baker et al. 2011; Baker & Warner 2016). Goondaloo Creek contains water that flows off Mt Stuart after rainfall and is located at JCU. These four endemic sites were selected and assessed on a single occasion after heavy rain, for use as a sentinel site location for a longitudinal study (Figure 3.1). Samples were collected at the following GPS points (determined by ArcGIS Field Maps) for each site: Mt Stuart (latitude 19.344022°S, longitude 146.781673°E), Mt Louisa (latitude 19.271080°S, longitude 146.724317°E), Castle Hill (latitude 19.259098°S, longitude 146.806614°E), and Goondaloo Creek (latitude 19.323687°S, longitude 146.762842°E). Exact locations were selected at time of sample collection based on presence of collectible flowing surface groundwater. The criteria for inclusion were presence of high concentrations (> 14 CFU/mL, mean concentration of *B. pseudomallei* in surface groundwater surrounding Castle Hill previously) of *B. pseudomallei*, proximity of sampling site to JCU (≤ 30min roundtrip, where a roundtrip is travel to and from the location), and broad area representation of collected water sample (multiple watercourses converging into single site) (Figure 3.2) (Baker et al. 2011). Samples were collected within 24 hours of significant rainfall and were collected, processed, and quantified as described in Section 3.2.2.

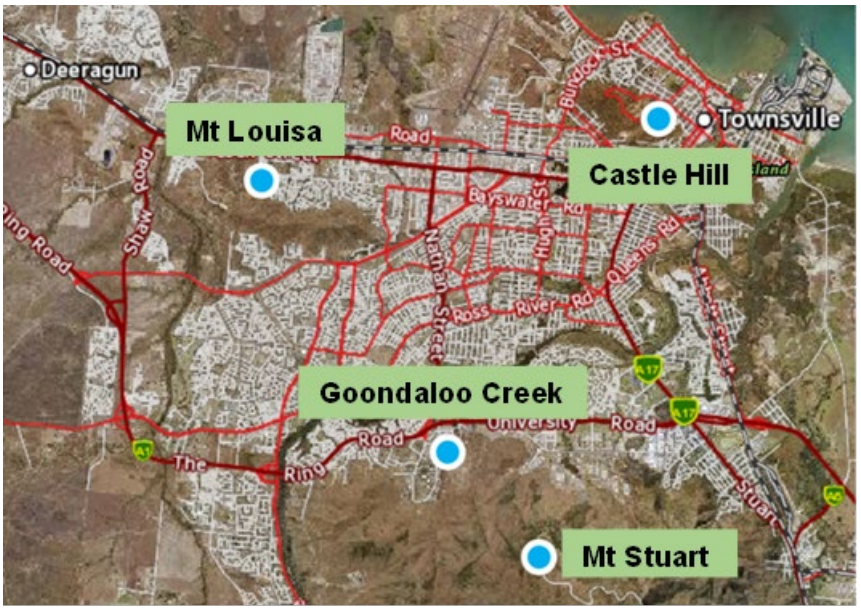

Figure 3.1 - Surface groundwater sampling locations across Townsville. Map modified from Queensland Globe.

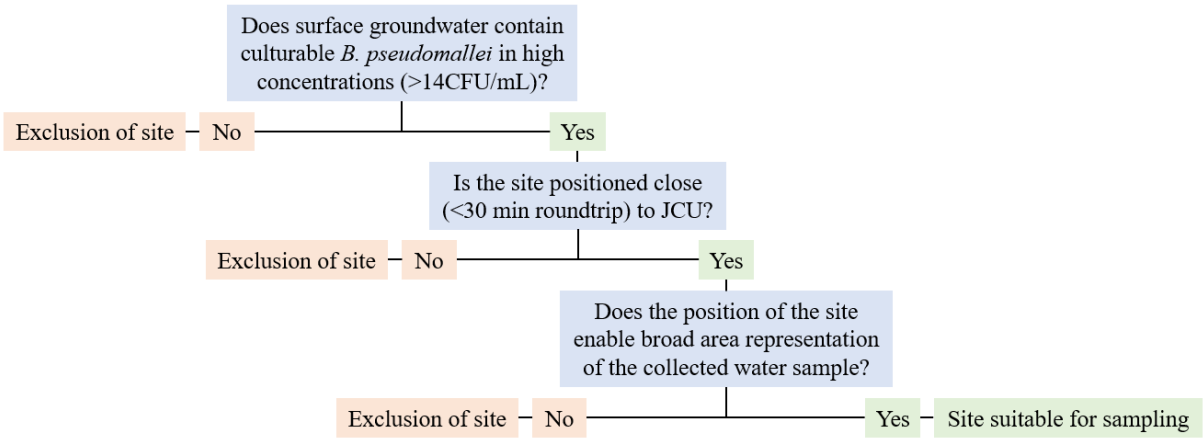

Figure 3.2 - Inclusion criteria applied to surface groundwater sampling sites for selection of a longitudinal sampling site as described in Section 3.2.3.

3.2.4 Longitudinal Study at Selected Location

Water samples were collected weekly for over a year. Opportunistic sampling was also conducted, where samples were collected on days of significant rainfall (> 20mm) and extreme weather events. Samples were not collected when the location had no water. Samples were processed as described in Section 3.2.2. Meteorological data, including temperature, rainfall, and solar exposure were collected from Climate Data Online (BOM). Data selected for inclusion had complete daily records for the duration of the longitudinal study. Temperature and rainfall were included due to previous investigations into their association with melioidosis incidence (Ganeshalingam et al. 2023; Kaestli et al. 2016). Solar exposure was included due to previous investigation into *B. pseudomallei* inactivation by sunlight (Sagripanti et al. 2009). The closest weather station to the study site, Townsville airport station (station number 032040, latitude 19.25° S, longitude 146.77° E), located approximately 8.2 km away, was selected. A visual turbidity scale, with four qualitative categories, was used to assess collected water samples (Figure 3.3). Zero was colourless and transparent water. One was minimally coloured and transparent water. Two was brown coloured and transparent water. Three was heavily brown coloured and translucent/opaque water. Presence or absence of flowing water was recorded. Absence of flowing water was characterised when there was no observable movement of water.

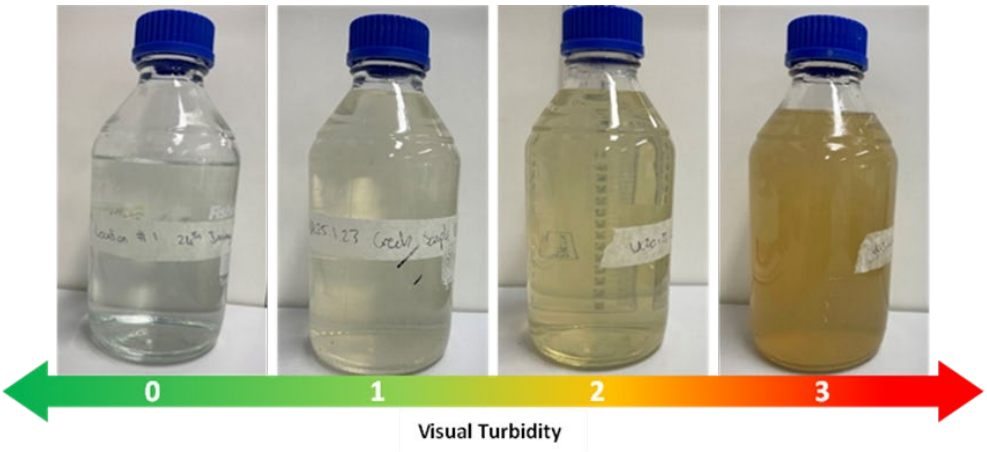

Figure 3.3 - Visual turbidity scale of surface groundwater as described in Section 3.2.4. Images taken on iPhone 12.

3.2.5 Data Analysis

All descriptive statistics, measures of normality, and graphs were generated in GraphPad Prism (Version 10.0.1). Nonparametric statistical tests were used for all analyses. Fraction of positive culture samples within total samples was determined using a Fraction of Total test. Statistical difference between *B. pseudomallei* concentrations in flowing versus non-flowing water groups was determined using a Mann-Whitney test. Statistical difference of *B. pseudomallei* concentration between visual turbidity groups was determined using a Kruskal-Wallis test. A Dunn's Multiple Comparisons test was used to determine which visual turbidity group relationships are significant. Significant relationships between climatic factors and *B. pseudomallei* concentration were determined using a Spearman Rank Correlation Coefficient test for correlation and simple linear regression.

3.3 Results

3.3.1 Longitudinal Study Location

Burkholderia pseudomallei was detected in three of four locations, including Mt Louisa, Mt Stuart, Castle Hill (Figure 3.4). Goondaloo Creek (Mt Stuart) was closest to JCU and most easily accessed. Goondaloo Creek was a drain catchment for surface groundwater that flowed off Mt Stuart, representing a broad area from which surface groundwater originated from (Figure 3.5). Goondaloo Creek fulfilled all inclusion criteria described in Section 3.2.3 and was selected for longitudinal sampling (Table 3.1).

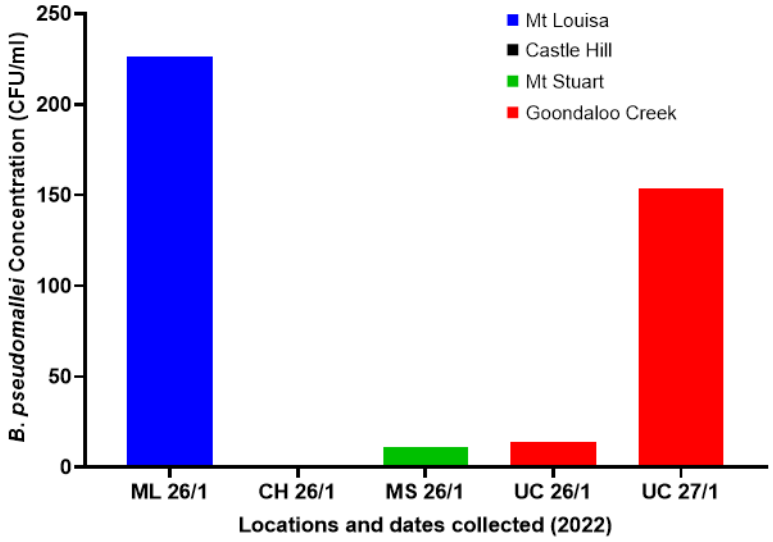

Figure 3.4 - Concentration of *B. pseudomallei* (CFU/mL) from each sampling location. Sampling locations were Mt Louisa (ML sampled on 26th January 2022), Castle Hill (CH sampled 26th January 2022), Mt Stuart (MS sampled on 26th January 2022), and Goondaloo Creek (UC sampled on 26th and 27th January 2022) (A Appendix Table A.2).

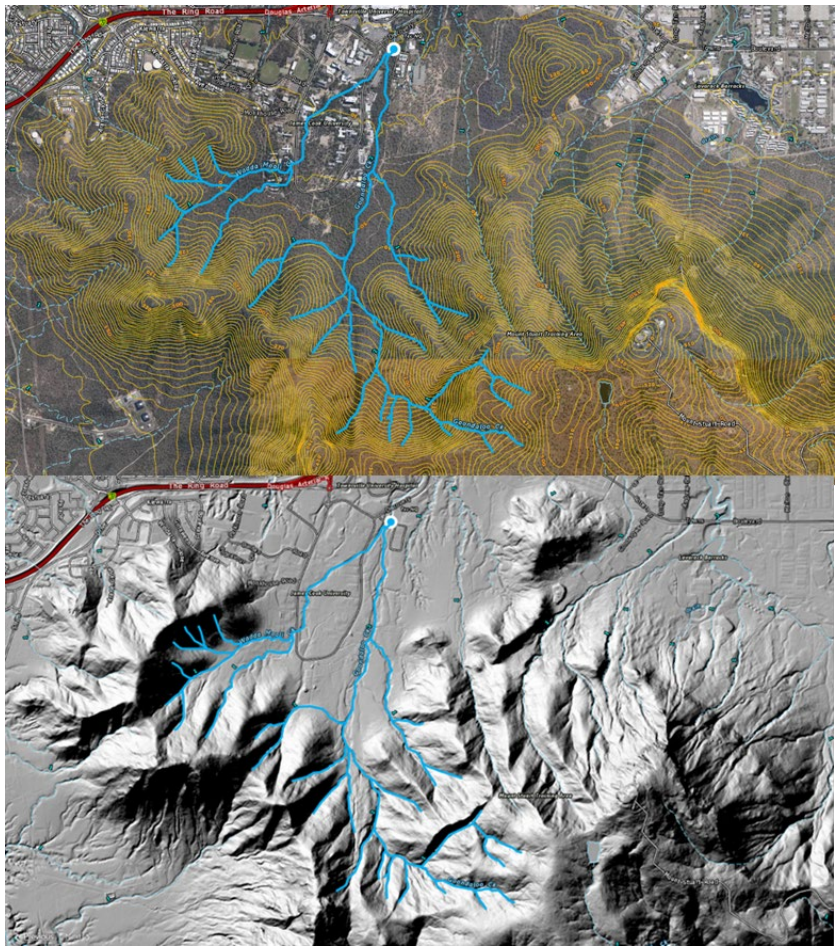

Figure 3.5 - Waterways flowing off Mt Stuart that converge into Goondaloo Creek drain catchment sampling site (◎). Waterways are indicated in blue. Top map shows elevation using contours. Bottom map shows elevation using hillshade traditional. Maps created in Queensland Globe.

Table 3.1 - Longitudinal study inclusion criteria by each sampling location.

Location	Inclusion Criteria (tick represents satisfied criteria)		
	High concentration of *B. pseudomallei* (>14 CFU/mL)	Close proximity to JCU (< 30min roundtrip)	Broad area representation of water sample
Mt Louisa	✓	✓	
Castle Hill			
Mt Stuart			
Goondaloo Creek	✓	✓	✓

*All criterion points were checked for each location (A Appendix: Table A.2, Table A.3, and Figure A.1).

3.3.2 Annual Prevalence of B. pseudomallei in Creek Water

Prevalence of *B. pseudomallei* in creek water collected over a year from Goondaloo Creek is presented in Figure 3.6. Water samples collected from both weekly and opportunistic sampling of Goondaloo Creek revealed 18 out of 59 samples (30.5% [95% confidence interval (CI), 20.25 to 43.15]) were culture positive for *B. pseudomallei* (A Appendix Table A.4).

3.3.3 Prevalence and Concentration of B. pseudomallei Correlates with Extent of Rainfall

Burkholderia pseudomallei was exclusively isolated from creek water up to two days after heavy rainfall, before dropping below the limit of detection of 1 CFU/mL (Figure 3.6). Continuous heavy rainfall prolonged the presence of *B. pseudomallei* in creek water. A Spearman Rank Correlation Coefficient test for nonparametric correlation analysis revealed a significant relationship between rainfall and *B. pseudomallei* concentration (P = <0.0001) with a strong positive correlation (r = 0.8141 [95% CI, 0.7010 to 0.8872]) (Figure 3.7). Simple linear regression determined a significant relationship where extent of rainfall weakly predicted *B. pseudomallei* concentration (R^2 = 0.4932, F (1, 57) = 55.47, P = <0.0001) with a fitted regression model of $y = 0.5810x - 3.631$. Approximately 30mm of rainfall was also needed to begin detecting *B. pseudomallei* in creek water (A Appendix Table A.4 and Table A.5).

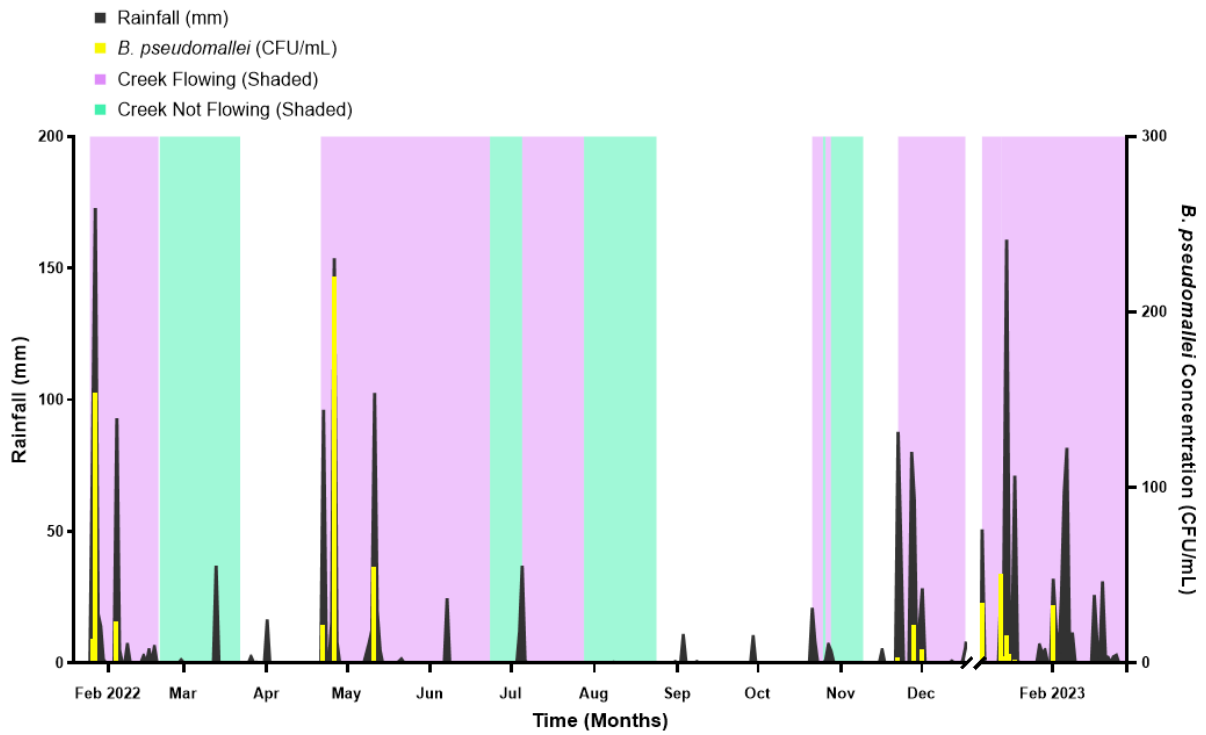

Figure 3.6 - Longitudinal weekly and opportunistic sampling for *B. pseudomallei* concentration (CFU/mL) over time (months) against rainfall (mm) for Goondaloo Creek. Rainfall (mm) (black) is shown on the left *y* axis (A Appendix Table A.5). *B. pseudomallei* concentration (CFU/mL) (yellow) is shown on the right *y* axis (A Appendix Table A.4). Time (months) is shown on the *x* axis. Purple shading represents periods when creek water was flowing and green shading represents non-flowing water periods (A Appendix Table A.9). No shading represents period when the creek was dry (no water).

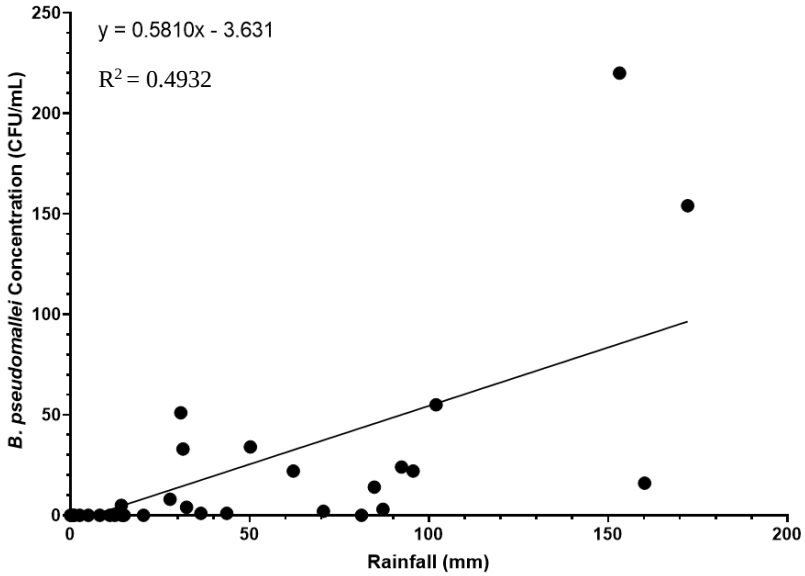

Figure 3.7 - Concentration of *Burkholderia pseudomallei* (CFU/mL) in creek water during different volumes of rainfall (mm). A simple linear regression model was fitted to the data; line represents best fit, equation of line and R^2 values included on graph (A Appendix Table A.4 and Table A.5).

3.3.4 Detection of B. pseudomallei Correlates with Water Flow and Water Turbidity

Burkholderia pseudomallei was exclusively isolated from flowing and turbid creek water (Figure 3.8 and Figure 3.9). A nonparametric Mann-Whitney test revealed a significant difference (P = 0.0233) in *B. pseudomallei* concentration between flowing and non-flowing water (A Appendix Table A.4 and Table A.9). *Burkholderia pseudomallei* was only detected in turbidity level two or three water (Figure 3.9). Turbidity level two water yielded concentrations below 10 CFU/mL while level three water yielded concentrations up to 220 CFU/mL. A nonparametric Kruskal-Wallis test determined a statistical difference (P = <0.0001) between turbidity level groups. Dunn's Multiple Comparisons test then identified level zero vs three (P = <0.0001), level one vs three (P = <0.0001), and level two vs three (P = 0.0011) as significant (A Appendix Table A.4 and Table A.8).

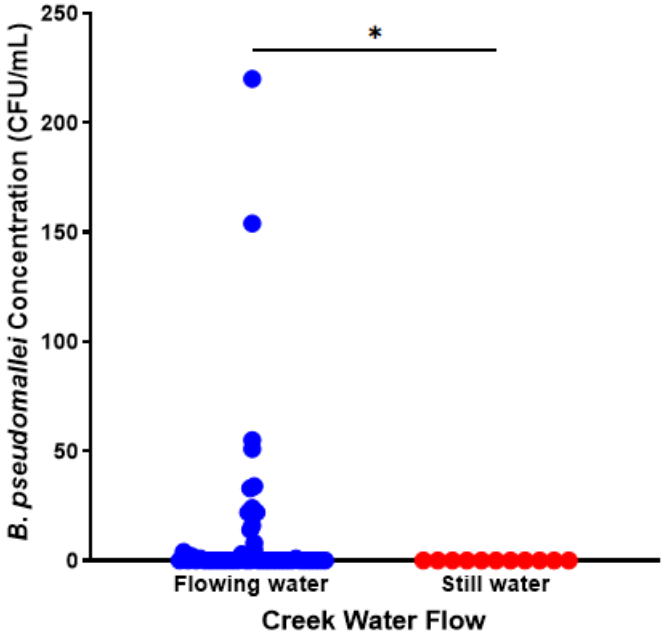

Figure 3.8 - *B. pseudomallei* is found at higher concentrations in flowing water rather than still water. Flowing water shown as blue (n=48) and still water shown as red (n=11). Statistically significant comparison is represented by * ($P \leq 0.05$) (A Appendix Table A.4 and Table A.9).

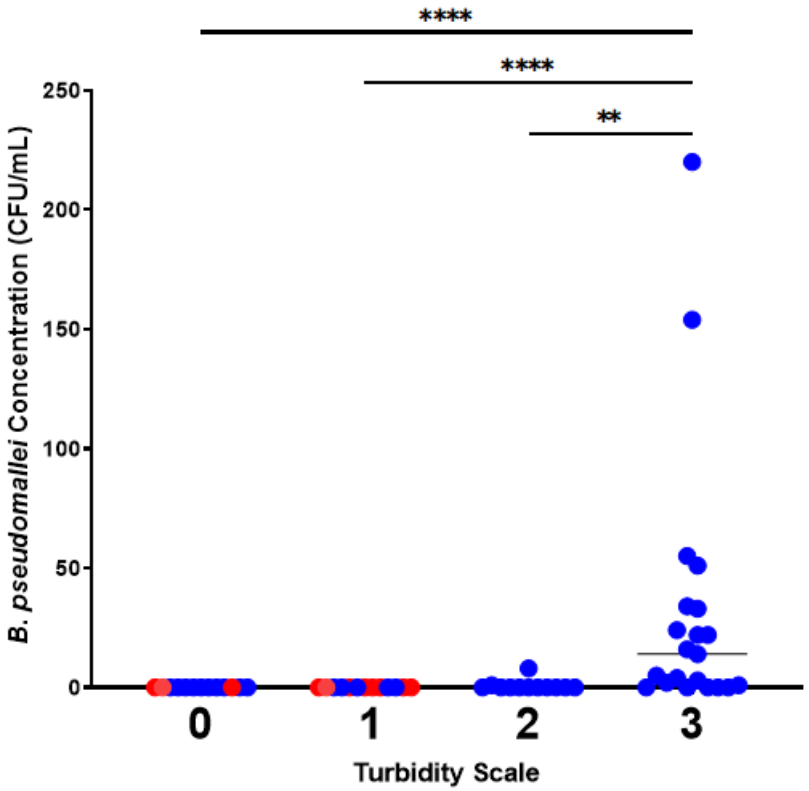

Figure 3.9 - Highest *B. pseudomallei* concentration (CFU/mL) is in turbid water. Data representing flowing water and still water are coloured blue (n = 48) and red (n = 11), respectively. Statistically significant comparisons are represented by * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), and **** ($P \leq 0.0001$) (A Appendix Table A.4 and Table A.8).

3.3.5 Association of B. pseudomallei in Water with Other Meteorological Parameters

In addition to rainfall, the relationship between *B. pseudomallei* concentrations in creek water and other meteorological factors such as Temperature and Solar Exposure were investigated. A nonparametric Spearman Rank Correlation Coefficient test for correlation determined there was no statistically significant relationship between daily temperature and *B. pseudomallei* concentration (P = 0.1297) (Figure 3.10) (A Appendix Table A.4 and Table A.7). Simple linear regression determined no significant relationship between daily temperature and *B. pseudomallei* concentration ($R^2 = 0.0144$, F (1, 57) = 0.8335, P = 0.3651). The data was fitted with a regression model of $y = -1.228x + 47.41$ (Figure 3.10). A Spearman Rank Correlation Coefficient test revealed a significant negative relationship (P = 0.0006) between solar exposure and *B. pseudomallei* concentration (r = -0.4371 [95% CI, -0.6296 to -0.1941]) (Figure 3.11) (A Appendix Table A.4 and Table A.6). Simple linear regression determined no significant relationship between solar exposure and *B. pseudomallei*

concentration (R^2 = 0.0573, F (1, 56) = 3.404, P = 0.0703). The data was fitted with a regression model of y = -1.230*x* + 34.48 (Figure 3.11).

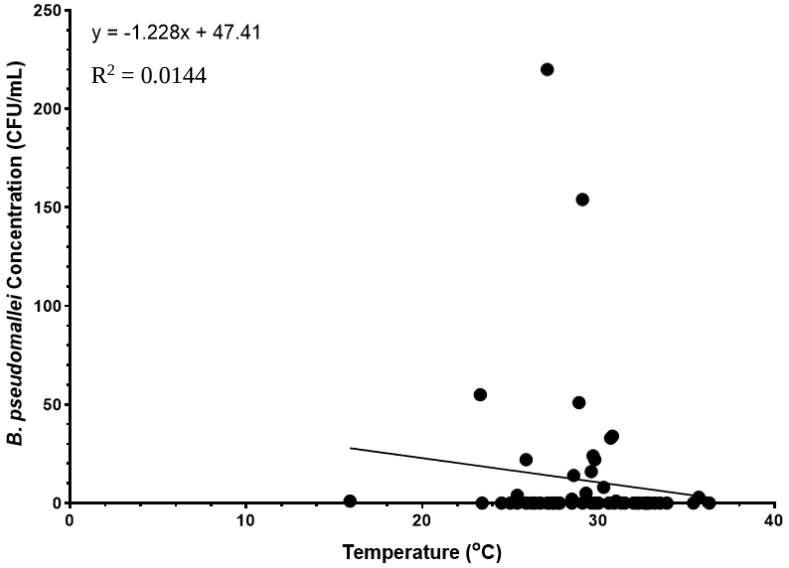

Figure 3.10 – Influence of daily temperature (°C) on *Burkholderia pseudomallei* concentration (CFU/mL). A simple linear regression model was fitted to the data; line represents best fit, equation of line and R^2 values included on graph (A Appendix Table A.4 and Table A.7).

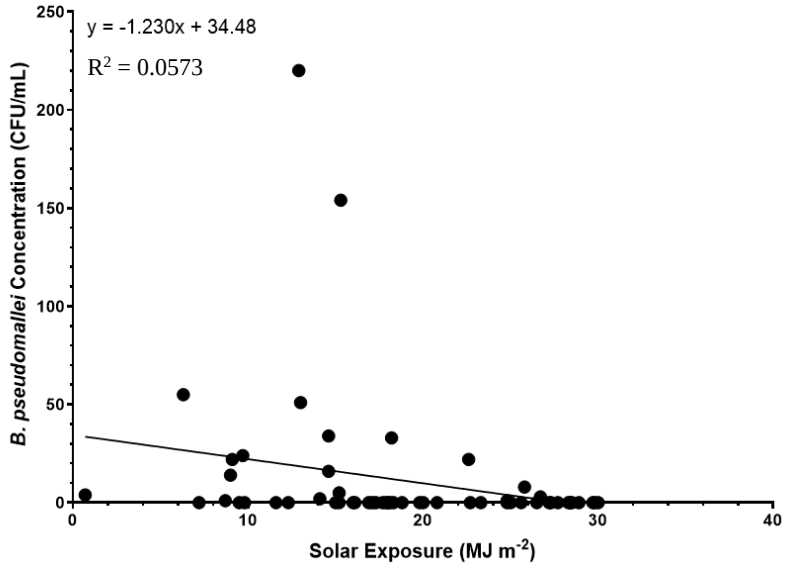

Figure 3.11 – Effect of solar exposure (MJ m^{-2}) on concentration of *B. pseudomallei* (CFU/mL). A simple linear regression model was fitted to the data; line represents best fit, equation of line and R^2 values included on graph (A Appendix Table A.4 and Table A.6).

3.4 Discussion

Surface groundwater has been implicated as a potential carrier of *B. pseudomallei* following rainfall, suggesting it may serve as a transmission vehicle. Previous studies have emphasised this phenomenon, particularly during the initial heavy rainfall of the wet season and extreme weather events (Baker et al. 2011; Zimmermann et al. 2018; Rachlin et al. 2020; Rachlin et al. 2021). However, recent research in Cairns challenges the notion that extreme weather alone significantly increases melioidosis incidence (Stewart, Smith & Hanson, 2017). Uncertainty remains regarding the temporal dynamics of *B. pseudomallei* in surface groundwater and the impact of meteorological factors on this relationship.

In selecting the study site, we confirmed that *B. pseudomallei* could be isolated from surface groundwater at multiple locations across Townsville. The types of groundwater initially sampled in this study varied between locations, where Goondaloo Creek reflected creek water and the Mt Stuart, Mt Louisa, and Castle Hill sites represent surface runoff water closer to what would be found in a drain. Water collected from the Mt Louisa and Mt Stuart sites corroborates earlier work by Baker et al. (2011) in Townsville where drain water contains *B. pseudomallei* after rainfall. Water seeps were not investigated in this study but were previously evidenced to contain *B. pseudomallei* both by culture and PCR (Baker et al. 2011; Baker et al. 2016). The presence of *B. pseudomallei* in river water has been determined previously and our data from sampling Goondaloo Creek water supports the results previously seen (Zimmermann et al. 2018). The analysis of creek water and surface runoff water in this study suggests that groundwater may influence melioidosis incidence through increased availability of the organism within the environment during and after rainfall. Easily detectable concentrations of the organism within groundwater makes it a viable strategy for determining *B. pseudomallei* environmental endemicity zones within Townsville. Especially since a similar strategy of sampling drain water from cities within Loas (Vientiane) and Australia (Darwin) has been used previously to investigate the genetic diversity and clustering of *B. pseudomallei* (Rachlin et al. 2020; Rachlin et al. 2021).

We demonstrated that surface groundwater can mobilise high concentrations of viable *B. pseudomallei* from its primary environmental reservoirs during significant rainfall events throughout the year, regardless of season. The dynamics influencing *B. pseudomallei* concentration in this seasonal creek are linked to extent of rainfall, duration of rainfall, and turbidity of the water. Our data show that heavy rainfall over short periods leads to high concentrations of *B. pseudomallei* in creek water. There is a significant relationship between increased turbidity of creek water and *B. pseudomallei* concentration. A similar relationship was seen in rivers after intense rainfall, where an increase in turbidity was associated with the detection of *B. pseudomallei* (Zimmerman et al. 2018). Intense rainfall, linked to high concentrations of *B. pseudomallei* in creek water, also mobilises particulate

matter and surrounding organic matter, and increases turbidity. This is particularly evident in areas with established lateral flow paths rather than vertical ones (Solano-Rivera et al. 2019; Zimmermann, Francke & Elsenbeer 2012). Thus, significant rainfall events that cause erosion may result in *B. pseudomallei* from soil and surrounding organic matter being picked up and carried in surface groundwater. This trend was observed in our study, where greater rainfall led to increased *B. pseudomallei* concentrations, although these concentrations dropped significantly after the first flush of rainfall and creek flow ceased.

Climate change reports by the BOM and CSIRO predict changes in rainfall patterns that will likely increase *B. pseudomallei* exposure in Australia. Northern Australia has already experienced increased rainfall during the wet season (BOM & CSIRO 2022). Additionally, there are predictions of more frequent heavy rainfall events and flash flooding across Australia, which will create ideal conditions for *B. pseudomallei* to be mobilised from its reservoirs and spread over larger areas (BOM & CSIRO 2022). This excessive water, especially during flash floods, significantly increases the risk of melioidosis for residents. In this study, the unusually wet sampling year resulted in 18 positive cultures of *B. pseudomallei* in creek water. The Queensland government reported 43 melioidosis cases in Townsville in 2022, a notable increase compared to previous drier years (17 in 2021, 9 in 2020, and 22 2019) (Queensland Health 2023). Therefore, increasing rainfall and its intensity in Far North Queensland will likely lead to more events that facilitate *B. pseudomallei* exposure as already seen in areas such as Brisbane, Central Australia, and southwest Western Australia that have had outbreaks associated with unusually heavy rainfall (Gassiep et al. 2023; Webb et al. 2020; Yip et al. 2015).

Understanding the temporal patterns of *B. pseudomallei* in surface groundwater can inform strategies to limit exposure. This study showed that rainfall of approximately 30mm or more consistently lead to the isolation of *B. pseudomallei* from creek water collected from Goondaloo Creek. Additionally, *B. pseudomallei* was detectable in water for up to two days after significant rainfall, with this period extending with continuous heavy rainfall. At-risk individuals should exercise caution around surface groundwater resulting from heavy rainfall for up to two days, or longer if rainfall continues. Using a visual turbidity scale, avoiding surface groundwater that appears to have brown colouration and is translucent or opaque is advisable. Although these results were measured from creek water, the mobility and potential for spread of groundwater, means these results likely speak broadly to the characteristics of when *B. pseudomallei* is most available in the surrounding environment. This result is supported from previous investigations into different forms of groundwater, where culturable *B. pseudomallei* was most readily detected in groundwater after rainfall or during the wet season (Baker et al. 2011; Rachlin et al. 2020; Rachlin et al. 2021; Zimmerman et al. 2018). This study has revealed that *B. pseudomallei* mobilisation in creek water is not random and is instead closely associated with rainfall, specifically rainfall amount and duration, and how this influences the runoff of surface groundwater. The potential exposure to *B. pseudomallei* from surface groundwater is a year-round

concern, influenced by rainfall patterns. Other meteorological factors like solar exposure and temperature did not appear to have an influential relationship on *B. pseudomallei* in groundwater. However, investigating other meteorological factors like cloud cover and humidity in the future may identify more factors that have an influential relationship on *B. pseudomallei* presence in groundwater, especially since these factors have been associated with an increase in melioidosis incidence in Townsville previously (Ganeshalingam et al. 2023).

This study's findings on rainfall and *B. pseudomallei* presence in groundwater can help predict melioidosis incidence. Increased availability of the organism in the surrounding environment by means of surface groundwater as a vehicle may lead to higher incidence of melioidosis. In Darwin, heavy rainfall significantly impacts melioidosis incidence (Kaestli et al. 2016). A study in Townsville that investigated climactic factors associated with melioidosis incidence also concluded that humidity and rainfall are important predictors of increased melioidosis cases, with prolonged rainfall having a greater impact than heavy downpours (Ganeshalingam et al. 2023). Although our study found that *B. pseudomallei* was typically isolated shortly after heavy rainfall rather than over prolonged periods, possibly due to the sampling site favouring flowing over stationary water as a creek site, washed out *B. pseudomallei* from various environments could be disseminated downstream where it could persist in the environment for a longer period of time. Studies investigating drain water in Darwin (Australia) and Vientiane (Loas) collected during the wet season, but not necessarily after heavy rainfall, detected *B. pseudomallei* (Rachlin et al. 2020; Rachlin et al. 2021). Weekly and convenience sampling data for investigating the temporal dynamics of *B. pseudomallei* in creek water presents the opportunity to enhance current public health prevention strategies.

Current public health prevention strategies in Australia emphasise avoiding contact with environmental soil and water. Strategies include wearing rubber boots and gloves to prevent skin contact with muddy water and soil, and wearing masks to avoid inhaling aerosolised bacteria. Additionally, avoiding outdoor activities during storms with heavy rainfall is recommended (Boyd et al. 2016; NT Health 2021; Queensland Health 2010). Similar strategies are employed in Thailand (Hinjoy et al. 2018; Suntornsut et al. 2016). However, there are no specific guidelines on the appearance of water to be avoided, apart from describing it as "muddy," nor on the timespans associated with high-risk periods for melioidosis. This study provides a basis to begin describing high-risk surface groundwater, which is water that appears turbid and brown coloured, occurring after approximately 30mm of rainfall or more. To best avoid contact with contaminated water it is recommended from this study's findings to avoid high risk surface groundwater for up to two days after the rainfall event or longer if there is a prolonged period of rainfall.

Urban expansion into areas populated with *B. pseudomallei* will increase melioidosis cases due to higher exposure risks. For instance, in Vientiane Capital, Laos, over 10% of the population resides in

an endemic area for *B. pseudomallei*, resulting in 54.6% of all document melioidosis cases in Laos between 1999 and 2017 being reported from this city (Rachlin et al. 2021). Similarly, in Townsville, the area around Castle Hill, which has previously tested positive *B. pseudomallei* in surface groundwater, shows a clinical cluster of melioidosis cases (Corkeron, Norton & Nelson 2010). Future development plans in Townsville, including expansion into the Mt Louisa area, which has also shown high concentrations of *B. pseudomallei*, may result in a higher burden of melioidosis among residents, similar to the situation in Castle Hill (Baker et al. 2011; Corkeron, Norton & Nelson 2010).

The sampling strategy used in this study may provide an efficient method for monitoring large areas of land that are endemic or suspected to be endemic for *B. pseudomallei*. Previous studies have shown that *B. pseudomallei* is detectable in surface groundwater and river water after heavy rainfall or during the wet season (Baker et al. 2011; Rachlin et al. 2020; Rachlin et al. 2021; Zimmermann et al. 2018). This study supports these observations, suggesting that surface groundwater mobilises *B. pseudomallei* from its terrestrial reservoirs and acts as a vehicle. Sampling surface groundwater provides a simpler method for detecting *B. pseudomallei* compared to soil sampling, which can require extensive efforts due to the bacterium's deep and localised presence in soil (Kaestli et al. 2009; Pongmala et al. 2022). Additionally, sampling of water has been shown to identify a higher diversity of *B. pseudomallei* strains compared to soil sampling (Rachlin et al. 2020). Our study demonstrated that strategic sampling of surface groundwater can effectively screen a large area for *B. pseudomallei* presence. This technique has been used previously in Townsville (Australia), Darwin (Australia), and Laos, where water from drains and rivers has been used to detect *B. pseudomallei* (Baker et al. 2011; Rachlin et al. 2020; Rachlin et al. 2021; Zimmermann et al. 2018). Collecting surface groundwater at key geographical locations may represent a more efficient method for environmental sampling of *B. pseudomallei* and identifying at-risk areas for melioidosis.

There are several limitations within this study. Only a single millilitre of water from each sample was assessed using the direct plate counting method to determine the concentration of *B. pseudomallei*, assuming uniform distribution in the collected water. Additionally, while most samples were processed on the same day as collection, three were not, potentially affecting the accuracy of their *B. pseudomallei* concentrations. The study did not convenience sample all rainfall events throughout the year, possibly missing instances where surface groundwater contained *B. pseudomallei*. Only one significant rainfall event was sampled daily during the sampling period due to resource constraints. Meteorological conditions were determined using a single weather station approximately nine kilometres from the sample site, which may not accurately reflect the conditions at the sampling site. The study also lacked a clear assessment of the water flow from Mt Stuart to the collection site and did not analyse the turbidity components of the samples to determine the soil type or organic matter. Future studies should include on-site meteorological measurements and should analyse both turbidity components of water and soil from suspected erosion zones on Mt Stuart to better understand the

origins of mobilised *B. pseudomallei* in surface groundwater using similar approaches by Zimmerman et al. (2012) and Zimmerman et al. (2018) to assess the physicochemical properties of water, erosion patterns, and suspended sediment in water.

In conclusion, surface groundwater resulting from significant rainfall may serve as a vehicle for carrying *B. pseudomallei*, implicating it in disease transmission. The interplay of geography, surface hydrology, and meteorological factors such as rainfall is crucial when modelling at-risk areas for melioidosis in endemic regions. Enhancing public awareness and implementing strategies to help high-risk individuals avoid surface groundwater when it is most likely to carry *B. pseudomallei* can reduce melioidosis incidence in these regions. Finally, implementing surface groundwater sampling in locations that collect water from large areas of land offers a more efficient method for environmental *B. pseudomallei* sampling.

Chapter 4

Oxford Nanopore: A Fast, Accurate, and Less Expensive Tool for Multi-Locus Sequence Typing of *Burkholderia pseudomallei*

4.1 Introduction

Genomic epidemiology is a powerful public health tool, essential for surveillance, outbreak investigation and management. MLST is a common method for genomic epidemiological investigation of *B. pseudomallei*. This method categorises the genetic diversity of a species unambiguously. *Burkholderia pseudomallei* exhibits significant strain diversity due to extensive horizontal gene transfer and recombination events (Jolley, Bray & Maiden 2018). MLST assesses internal fragments from seven housekeeping genes, assigning each an allele number. The combination of these seven allele numbers determines the ST (Jolley, Bray & Maiden 2018). To determine a strain's ST, the internal fragments from the seven housekeeping genes are sequenced either by targeted sequencing or WGS. MLST is valuable for matching clinical and environmental isolates, performing an investigation into the genetic diversity of *B. pseudomallei* isolates within a region, and for environmental surveillance of *B. pseudomallei*, informing reservoirs of infection and transmission routes (Gassiep et al. 2024; Rachlin et al. 2021; Webb et al. 2022). In Townsville, a large investigation into the genomic epidemiology of *B. pseudomallei* clinical isolates collected from 1997 to 2020 was performed using MLST, and identified 59 distinct STs that changed during the study (Gassiep et al. 2024).

Currently, common sequencing methods for pathogen genomics is targeted or WGS using Sanger, Illumina, MGI, ONT, and PacBio platforms. To access these platforms, especially in lower resourced and rural locations, samples are sent to third-party companies such as the AGRF in Australia (Rachlin et al. 2021; Smith et al. 2023). Outsourcing samples increases the time required to investigate the genomic epidemiology of a pathogen, impacting the ability to respond rapidly to outbreaks. Recently, ONT sequencing has gained popularity for its portability and rapid turnaround time of results (Ferreira et al. 2021; Ritchie et al. 2023). However, the quality of ONT reads has historically had high error rates (Chen, Erickson & Meng 2021; Wang et al. 2021). With ongoing technological advancements, ONT sequencing quality has significantly improved, making it viable for SNP determination within alleles for MLST (Greig et al. 2019; Ritchie et al. 2023; Tytgat et al. 2022; Xian et al. 2022). ONT's robustness and portability allows it to be set up in rural or high-containment facilities, making it especially suitable for *B. pseudomallei* (CDC 2018). Establishing in-house ONT sequencing in Townsville would reduce the time needed to determine an ST of *B. pseudomallei* and improve the response time to a melioidosis outbreak.

This study aims to establish in-house sequencing capacity using ONT in a high-containment facility and validate its use compared to Illumina sequencing in terms of read accuracy, turnaround time and cost.

4.2 Materials and Methods

4.2.1 MLST PCR of B. pseudomallei Isolates

A total of 50 *B. pseudomallei* isolates were sequenced. Two *B. pseudomallei* controls and 48 Goondaloo Creek *B. pseudomallei* isolates previously collected and stored (Section 3.2.2), were selected for sequencing. Selection was based on the following criteria:

1. Two controls, ST276 and ST814, were chosen. These two isolates were isolated and stored from previous sampling in Townsville (2010) conducted by Baker et al. (2011). These local isolates were available for immediate use and their STs had previously been determined. These two isolates were suitable to use as sequencing controls to ensure the ST produced by ONT is a match for their known STs.
2. The total number of available Goondaloo Creek *B. pseudomallei* isolates collected throughout the longitudinal study in Chapter 3 were separated into groups representing the dates they were collected.
3. A percentage was calculated based on the total number of 48 positions available for sequencing and total number of isolates within each group to determine how many isolates would be sequenced from each group. A minimum of one isolate was sequenced from each group. Isolates were randomly selected within groups. Isolates were selected to be representative of STs present in water samples for the duration of the longitudinal study conducted in Chapter 3.

In the JCU PC3 facility, isolates were cultured on ASH-A, using a single pure colony from each for DNA extraction. The Roche High Pure PCR Template kit (Roche Diagnostics, Germany) was used for DNA extraction following manufacturer's instructions for a Gram-negative bacterium (Roche Life Science 2023). The internal fragments from the seven housekeeping genes for *B. pseudomallei* MLST, *ace, gltB, gmhD, lepA, lipA, narK,* and *ndh*, were amplified with PCR. Housekeeping gene internal fragment primers and PCR conditions were referenced from PubMLST (Godoy et al. 2003). MLST PCR reactions consisted of 1x GoTaq Colourless Master Mix (Promega), 0.4 μM forward and reverse primer, 50-100 ng DNA and molecular-grade H_2O to 30 μL. Thermocycling conditions for *ace, gltB, lipA, narK,* and *ndh* consisted of a 3 min initial denaturation at 95^{o}C, followed by 40 cycles of 30 sec at 95^{o}C, 30 sec at 62^{o}C, and 30 sec at 72^{o}C, ending with a 10 min final elongation step at 72^{o}C.

Thermocycling conditions for *gmhD* and *lepA* were optimised using a PCR temperature gradient (B Appendix Figure B.1). The final thermocycling conditions for *gmhD* and *lep A* consisted of a 3 min initial denaturation at 95^{o}C, followed by 40 cycles of 30 sec at 95^{o}C, 30 sec at 59^{o}C, and 30 sec at 72^{o}C, ending with a 10 min final elongation step at 72^{o}C. A BioRad CFX96 Real-Time System C1000 Touch Thermal Cycler (BioRad CFX Manager 3.1, version 3.1.1517.0823) was used for all PCR reactions. Suitability of PCR amplicons was determined by gel electrophoresis, using a 1.5% agarose gel and 100 bp Hyperladder (Bioline) to verify product size.

4.2.2 Use of MLST PCR Products for Targeted ONT Sequencing in a High Containment Facility (PC3)

All seven MLST amplicons from each isolate were pooled together and cleaned using the Wizard SV Gel and PCR Clean-up System kit and protocol (Promega 2024). DNA samples were quantified using a Qubit 3.0 Fluorometer (version APPv1.02 and MCUv0.21) and Qubit dsDNA HS Assay kit. DNA samples were diluted to 62 ng or 200 fmol of DNA. Diluted DNA samples were prepared for sequencing using the Oxford Nanopore Native Barcoding kit (SQK-NBD114-24) and protocol (ONT 2024, *Ligation sequencing amplicons - Native Barcoding Kit 24 V14 (SQK_NBD114.24)*). Up to 24 barcoded DNA samples were pooled together. Pooled DNA libraries were loaded onto a R10.4.1 FLO-MIN114 MinION flow cell and sequenced using the Oxford Nanopore Native Barcoding kit (SQK-NBD114-24), with the addition of UltraPure Bovine Serum Albumin (50mg/ml) (Invitrogen) as specified in the Native Barcoding kit protocol (ONT 2024, *Ligation sequencing amplicons - Native Barcoding Kit 24 V14 (SQK_NBD114.24)*).

The Firmware version of the Mk1C device was MinION FPGA 2.4.3. Software versions for the Mk1C device were: MinKNOW 23.07.12, Bream 7.7.6, Configuration 5.7.11, Guppy 7.1.4, MinKNOW Core 5.7.5. Hardware checks and flow cell checks were performed as described in the Mk1C device manual (ONT 2024, *MinION Mk1C manual*). A sequencing run was set up and started as described in the MinKNOW protocol. High accuracy model basecalling was used with barcode splitting active during basecalling. The high accuracy model (default recommended) was selected due to its higher raw read accuracy compared to the fast model and less computationally intensive requirements compared to the super accurate model (ONT 2024, *MinKNOW protocol*). Sequencing data was stored in FASTQ files, with 4000 reads per file. FASTQ files were stored in a gzip compressed archive folder (ONT 2024, *MinKNOW protocol*). Raw FASTQ files were transferred from the Mk1C computer to a computer outside the PC3 laboratory via wireless connection. The Mk1C device and external computer were connected to the same ethernet, using FileZilla to setup a remote connection and perform a data transfer as described in the MinION Mk1C manual (ONT 2024, *MinION Mk1C manual*).

Targeted ONT sequencing data was first processed using NanoPlot (version 1.42.0) to generate reports on quality, abundance, and size of sequenced reads (De Coster et al. 2018). Raw data files from ONT sequencing were uploaded to the Center for Genomic Epidemiology webservice for analysis (https://cge.food.dtu.dk/services/MLST/) using software version 2.0.9 (2022-05-11). Sequences were mapped using k-mer alignment (KMA) with a minimum depth of 80×. A total of 8000 reads per isolate were uploaded with an average read depth of 1000× per MLST PCR product per isolate. KMA mapped raw reads to a current version of the *B. pseudomallei* pubMLST database (2023-06-19) to identify matches or close matches (Clausen, Aarestrup & Lund 2018; Larsen et al. 2012). KMA also generated a consensus sequence for each MLST PCR product per isolate. Consensus sequences were then searched on the pubMLST database (https://pubmlst.org/organisms/burkholderia-pseudomallei) for matches and close matches as described by Jolley, Bray & Maiden (2018).

4.2.3 Validation of ONT Sequencing using Illumina WGS

From the 48 Goondaloo Creek *B. pseudomallei* ONT sequenced isolates, 12 were selected for Illumina WGS (AGRF). Selection criteria included isolates that had novel profiles or alleles identified from ONT sequencing. DNA samples prepared in Section 4.2.1 for the 12 isolates were checked for integrity with gel electrophoresis, using a 0.5 % agarose gel and 1 Kb ladder (Sharp DNA Ladder, Real Biotech Corporation). The quantity of DNA was determined using a Qubit 3.0 Fluorometer (version APPv1.02 and MCUv0.21) and Qubit dsDNA HS Assay kit. Quality of DNA samples was evaluated using a NanoDrop 2000c spectrophotometer (NanoDrop 2000/2000c version 1.6.198). A total of ≥100 ng of DNA per isolate was sent for Illumina WGS at AGRF. The sequencing package was a 1 Gbp Bundle (Prep M) (NGS-DNB-324) per sample with a total sequencing depth of approximately 138×.

Raw FASTQ.gz files were transferred from the AGRF server to a local computer using FileZilla. WGS data was first processed using FastQC (version 0.12.1) to generate reports on quality, abundance, and size of sequenced reads (Andrews 2023; Rachlin et al. 2021; Smith et al. 2023). MultiQC (version 1.11) was used to aggregate the FastQC reports (Ewels et al. 2016; Smith et al. 2023). Illumina sequences were trimmed using Trimmomatic (version 0.36.6) and genomes were constructed using SPAdes (version 3.15.5) (Bankevich et al. 2012; Bolger Lohse & Usadel 2014; Rachlin et al. 2021; Smith et al. 2023). Contigs were then queried on the pubMLST database for matches and close matches (Jolley, Bray & Maiden 2018; Rachlin et al. 2021).

4.2.4 Comparison of Targeted ONT and Illumina WGS

Accuracy of reads was compared between targeted ONT and allele sequences from Illumina WGS similar to the webservice MLST tool on the Center for Genomic Epidemiology, where two sequences would be aligned and the percentage similarity determined using the number of matching aligned base pairs divided by the total number of base pairs (Center for Genomic Epidemiology 2011). MAAFT (version 7.505) was used to pairwise align corresponding targeted ONT and Illumina MLST DNA sequences (Katoh et al. 2005; Katoh et al. 2002; Katoh & Standley 2013).

Time was compared between targeted ONT and Illumina WGS workflows modified from Ferreira et al. (2021). Workflows were broken down into major steps starting from culture, and the estimated time for each step calculated using the assumption that a single person working an average eight-hour day was completing the workflow for a single sample. Time per step was estimated in days or weeks.

Cost was compared between targeted ONT and Illumina WGS workflows. All kits, reagents, consumables, and/or service charges were collected for targeted ONT and outsourced AGRF Illumina WGS. Complete costs for all materials and/or services for each workflow was calculated for 50 isolates assuming the user did not have access to previously available resources. Number of preparations per kit/reagent/consumable/service were calculated and total cost of the item divided by number of preparations. Cost of a single preparation from each kit/reagent/consumable/service were added together for each workflow and the cost per sample calculated. Machines and equipment were assumed to be available with no extra cost. Initial acquisition cost for an ONT MinION device and benchtop Illumina iSeq 100 device was also investigated.

4.3 Results

4.3.1 Targeted ONT Sequencing ST Determination of B. pseudomallei Isolates Compared to Illumina WGS

Two known ST controls, ST276 and ST814, were included in ONT sequencing, and their expected STs were confirmed (C Appendix Table C.1). Validation using Illumina WGS revealed complete consensus between the two sequencing methods (Table 4.1, Figure 4.1, and B Appendix Figure B.4-Figure B.15). All MLST sequences for each isolate and overall ST profiles were 100% matched for all 12 isolates between targeted ONT and Illumina WGS (B Appendix Figure B.4-Figure B.15).

Table 4.1 - Table listing sequence similarity between targeted ONT sequencing and extracted alleles from Illumina WGS (B Appendix Figure B.4-Figure B.15).

Isolate	ONT ST	Illumina ST	Percentage Similarity of Sequences
27.1.22-15	2070	2070	100%
27.1.22-17	2080	2080	100%
27.1.22-19	2071	2071	100%
4.2.22-9	2072	2072	100%
4.2.22-10	NA*	NA*	100%
22.11.22-2	2073	2073	100%
6.1.23-3	2074	2074	100%
13.1.23-1	2075	2075	100%
1.2.23-4	2076	2076	100%
27.1.22-14	2077	2077	100%
14.1.23-2	2078	2078	100%
16.1.23-1	2079	2079	100%

*Not applicable (NA) as ST cannot be assigned due to incomplete profile from novel *lepA* allele.

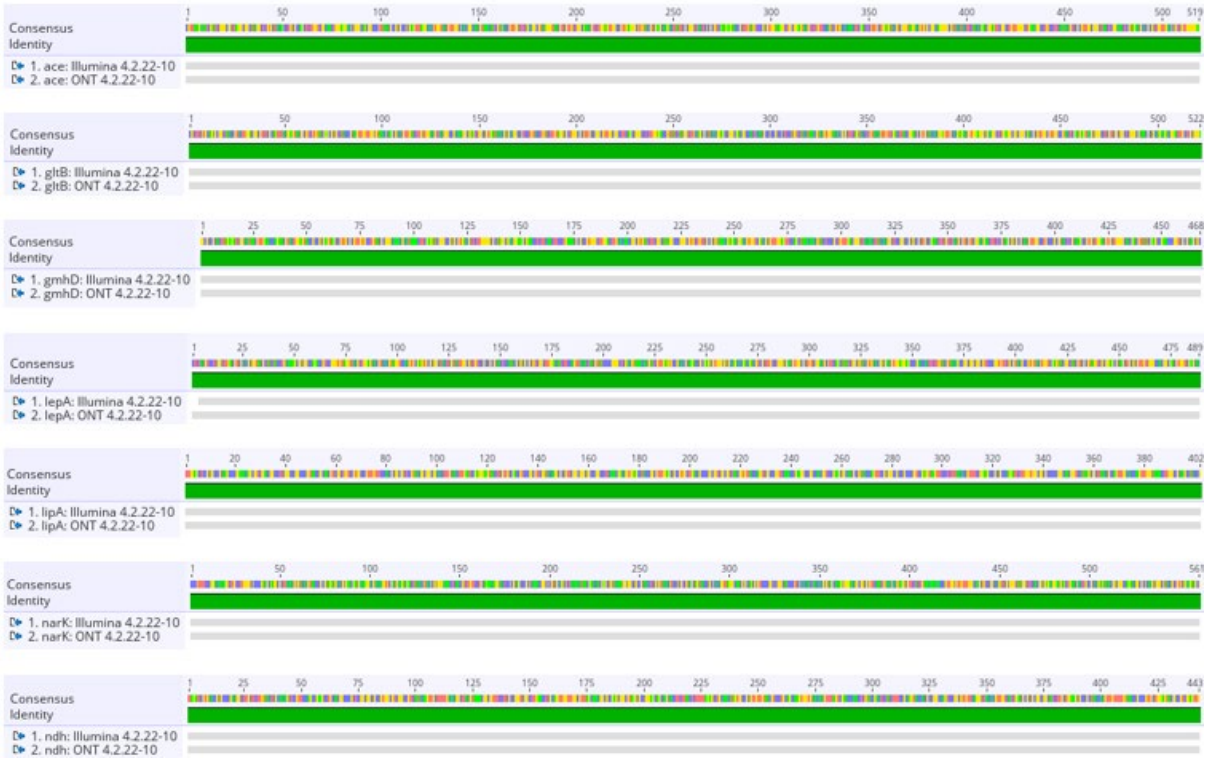

Figure 4.1 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 4.2.22-10 (B Appendix Figure B.4-Figure B.15).

4.3.2 Targeted ONT Sequencing Turnaround Time Compared to Illumina WGS

A time comparison between targeted ONT and Illumina WGS revealed targeted ONT sequencing as faster (Figure 4.2). Workflow steps analysed included culture, DNA extraction, PCR amplification

(ONT only), quality and quantity assessment, sample preparation (ONT only), shipment (AGRF Illumina only), sequencing, and data analysis. Targeted ONT sequencing was fastest, with approximately a one-week turnaround time. Illumina (AGRF) WGS had an approximately seven-week turnaround time. Overall, targeted ONT sequencing was completed seven times faster than outsourced Illumina WGS.

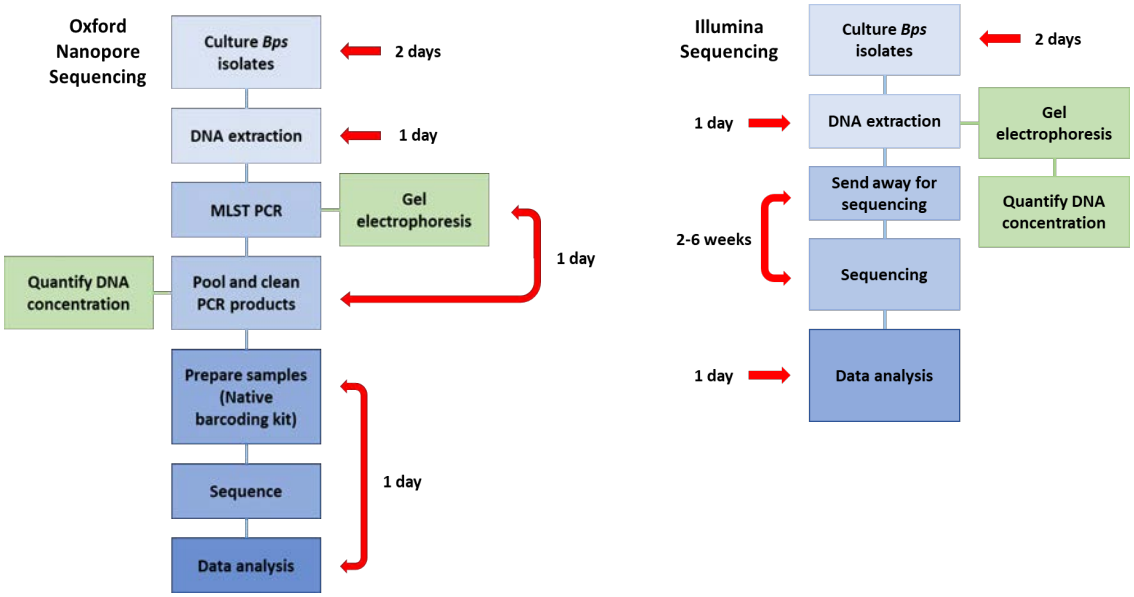

Figure 4.2 - Time comparison of targeted ONT versus Illumina (AGRF) WGS.

4.3.3 Targeted ONT Sequencing Expense Compared to Illumina WGS

A cost comparison between targeted ONT and outsourced (AGRF) Illumina WGS determined targeted ONT sequencing as less expensive (Table 4.2) (B Appendix Table B.1 and Table B.2). Targeted ONT sequencing was approximately $80 per sample, whereas AGRF Illumina WGS was $156 per sample. Total workflow cost for reagents/consumables/services for sequencing 50 isolates was approximately $8546 for Targeted ONT sequencing and $8877 for AGRF Illumina WGS. Targeted ONT sequencing was less expensive for both total workflow cost and cost per sample compared to AGRF Illumina WGS.

Table 4.2 - Cost comparison of targeted ONT versus Illumina (AGRF) WGS (B Appendix Table B.1 and Table B.2).

Sequencing Technology Workflow	**Total Workflow Cost for Reagents/Consumables/Services for 50 isolates**	**Cost per Sample Preparation**
Targeted ONT	$8546	$80
Outsourced (AGRF) Illumina WGS	$8877	$156

4.4 Discussion

Melioidosis is a disease that primarily affects regional Australians living in tropical areas. Cases are rarely investigated to determine the source of infection, leaving the precise epidemiology unclear. Genomic epidemiology, a tool increasingly embraced by public health authorities, has limited accessibility. For Townsville, the closest major sequencing facility is located approximately 1100km away in Brisbane. To improve access in Townsville, the establishment and validation of ONT sequencing for MLST of *B. pseudomallei* was investigated. In-house ONT sequencing, compared to outsourcing to companies like AGRF, is a fast, accurate, and lower cost option for genomic epidemiological investigation.

Reliable sequencing technology must produce high-quality reads, especially for MLST, which requires high-resolution sequencing to detect SNPs. ONT previously faced issues with high error rates for base pair calling (Chen, Erickson & Meng 2021; Wang et al. 2021). However, recent advancements in reagents, flow cells, basecalling software and bioinformatics tools have significantly improved sequencing accuracy (Greig et al. 2019; Ritchie et al. 2023; Sereika et al. 2022; Tytgat et al. 2022; Wang et al. 2021; Xian et al. 2022; Yan et al. 2021). Previously, the sequencing accuracy of ONT was not reliable enough to build genomes without short read or reference polishing, but with improvements to ONT, it is being increasingly used to produce accurate results for genomics and metagenomics (Linde et al. 2023; Sereika et al. 2022; Xian et al. 2022; Zhao et al. 2023). The utilisation of ONT for SNP analysis is also increasingly being supported, where studies have shown comparable results between ONT sequencing and Illumina sequencing for SNP analysis (Greig et al. 2019; Ritchie et al. 2023; Tytgat et al. 2022; Xian et al. 2022). As such, ONT is being tested and used to conduct genomic epidemiological investigation into outbreaks of various infectious agents such as SARS-CoV-2, Canine Rabies, Shiga toxin-producing *Escherichia coli*, *Shigella sonnei*, *Salmonella enteritidis* (Buytaers et al. 2021; Gigante et al. 2020; Ritchie et al. 2023; Xian et al. 2022; Yan et al. 2021). Our study found that in-house ONT sequencing was as accurate as outsourced AGRF Illumina WGS, with

a 100% consensus and no discrepancies in results. This high accuracy result for ONT is likely a direct result of the ultra-deep sequencing performed on the housekeeping gene internal fragment amplicons in the study to improve read quality, which has previously been shown to greatly reduce the ONT read error rate (Yan et al. 2021). Our study supports the use of ONT sequencing for accurate SNP analysis and MLST of *B. pseudomallei* housekeeping gene internal fragment amplicons. Thus, ONT is a viable tool to use for future MLST investigation of *B. pseudomallei*.

In our study, ONT sequencing offered a significantly faster turnaround time than outsourcing Illumina WGS. Other sequencing technologies that similarly rely on sending samples away for sequencing, such as PacBio WGS, are also likely to have a slower turnaround time compared to ONT sequencing with the additional step of transporting samples to external facilities. In our study, it was estimated that ONT can generate results in less than a week, whereas AGRF's Illumina WGS requires a minimum of three weeks. The use of third-party companies such as AGRF and Macrogen for any kind of sequencing, including Sanger sequencing and PacBio WGS, will likely introduce delays to turnaround time due to the need to send samples away. However, these time estimates specifically applies to our situation, given that we are a small laboratory with no in-house Illumina capability and no funding to establish in-house Illumina (which is costly, as discussed in the next paragraph), where we rely on sending samples away for Illumina sequencing. For large pathology laboratories that are well funded and already have Illumina or MGI setup in-house, the turnaround time is likely to be significantly shorter. Another potential alternative to Illumina for setup of in-house short read next generation sequencing is MGI, which is comparable in terms of quality to Illumina and overall has a lower cost (Kim et al. 2021; Korostin et al. 2020). Given the 1-21 day incubation period of *B. pseudomallei* before onset of melioidosis symptoms, with a median of four days, the quick results from in-house ONT can facilitate timely investigation and intervention during outbreaks (Buytaers et al. 2021; Gigante et al. 2020; Ritchie et al. 2023; Xian et al. 2022; Yan et al. 2021). Other studies that compared ONT and Illumina WGS have also concluded that ONT is faster and useful for pathogen outbreak investigation (Buytaers et al. 2021; Ferreira et al. 2021; Ritchie et al. 2023). ONT's portability and robustness make it suitable for in-field sequencing and setups in resource-limited areas, increasing accessibility and reducing turnaround times (Gigante et al. 2020).

For our study, comparing the costs of ONT and outsourced Illumina WGS shows that targeted ONT is generally more economical. ONT's overall workflow costs, including kits, reagents, and consumables, are lower. The cost per sample for targeted ONT is $80 compared to $156 for Illumina WGS, making ONT more sustainable for ongoing genomic investigations of *B. pseudomallei* in Townsville if done locally and in-house. The caveat to this analysis is that employment cost and profit margin were not factored into local ONT sequencing since it was done in-house, unlike AGRF which would have these factored into the commercial cost. Additionally, the information return of WGS versus targeted sequencing is much higher since further analyses into genes within the genome can determine

virulence factors like antimicrobial resistance genes, biosynthetic gene clusters, and virulence genes like $BimA_{Bm}$ (Alam et al. 2022; Burnard et al. 2022; Jitprasutwit et al. 2023; Sarovich et al. 2014; Gassiep et al. 2024). For small isolated genomic investigations, utilising third party companies and eliminating the need to acquire and establish in-house sequencing resources, is likely cheaper. Additionally, this offers the user variety in the sequencing services they can choose, improving access to Sanger, Illumina, and PacBio sequencing services. The user simply needs to DNA extract the appropriate samples and send them away for preparation and sequencing. However, for large or longitudinal genomic investigations involving numerous isolates for sequencing, the high cost per sample for third-party company sequencing is not sustainable. Establishing in-house sequencing will reduce cost per sample as seen with this study, especially for ONT sequencing since it has relatively lower setting up costs compared to other sequencing technologies such as Illumina. An ONT MinION device, including a sequencing kit, control expansion kit, wash kit, and two flow cells, costs $3,025 AUD (ONT 2024, *MinION Mk1B starter pack*). In contrast, an Illumina iSeq100 device alone costs $30,442 AUD (Illumina 2024). The lower cost of ONT MinION devices is appealing for resource-limited regions, and can allow the establishment of multiple sequencing locations into the future.

This study has several limitations. Only 12 out of 50 ONT-sequenced isolates could undergo Illumina WGS due to resource constraints. The calculated pricing compares targeted ONT sequencing with Illumina AGRF WGS; however, it does not account for other sequencing services offered by ONT. The cost of Illumina sequencing includes personnel wages and profit margins of AGRF, which may not reflect the base sequencing cost. Additionally, establishing ONT locations requires access to the internet for software updates and essential equipment such as refrigerators and freezers for sample preparation.

The successful establishment of in-house ONT sequencing in Townsville offers opportunities for in-depth genomic epidemiological investigation into *B. pseudomallei*. Increased local access to sequencing will encourage environmental and clinical isolate matching and enhance environmental surveillance. Improved understanding of the reservoirs of infection and transmission routes for *B. pseudomallei* will inform melioidosis epidemiology and public health messages. Widespread environmental surveillance will help delineate the biogeographical boundaries of *B. pseudomallei* in Townsville and track changes over time. Furthermore, this technology's utility extends beyond *B. pseudomallei*, providing opportunities to investigate other infectious agents. Expanding in-house sequencing capacity by developing the established ONT workflow and resources will be crucial for ongoing and future investigations into *B. pseudomallei* and other pathogens.

Chapter 5

The Genomic Epidemiology of *Burkholderia pseudomallei* Isolated from Townsville Groundwater

5.1 Introduction

Awareness of melioidosis in endemic regions is crucial for improving patient outcomes. Recognising the combination of patients' clinical history, recent exposure-related activities, and regions of endemicity helps inform melioidosis diagnosis. Community awareness can also improve behaviours associated with environmental exposure to *B. pseudomallei,* reducing melioidosis incidence, especially among at-risk groups. To better inform communities about the melioidosis threat, genomic epidemiological investigation of *B. pseudomallei* is necessary.

MLST is a widely used method for determining the genomic epidemiology of *B. pseudomallei* (Baker et al. 2011; Rachlin et al. 2021; Smith et al. 2023). MLST sequences internal fragments from seven housekeeping genes, assigning each a unique allele number; the combination of these numbers determines the ST (Jolley, Bray & Maiden 2018). MLST is an unambiguous method for assessing genetic diversity and is used for matching environmental and clinical isolates, environmental surveillance, determining transmission routes, and identifying reservoirs of infection. WGS can further investigate genomic features associated with higher virulence, such as the $BimA_{Bm}$ variant linked to severe neurological illness (Burnard et al. 2022; Jitprasutwit et al. 2023; Sarovich et al. 2014; Gassiep et al. 2024). Genomic epidemiological investigation of Townsville *B. pseudomallei* isolates will enhance our understanding of the organism in the environment and how melioidosis is acquired.

Understanding the reservoirs of infection, transmission routes, and high-risk areas for melioidosis in Townsville will aid in managing and preventing outbreaks (Corkeron, Norton & Nelson 2010; Gassiep et al. 2024; Smith et al. 2023; Tran et al. 2022). For example, genomic investigation into a melioidosis outbreak after a Cairns school sporting event, linked a mud pit as the reservoir of infection to clinical case isolates. Identification of the reservoir of infection was then used to justify the discontinuation of mud pits from future sporting events (Smith et al. 2023). Another recent example is the retrospective study that characterised the genetic diversity and virulence factors present within the *B. pseudomallei* clinical isolates collected in Townsville from 1997 to 2020 (Gassiep et al. 2024). The establishment of in-house ONT sequencing in the previous chapter (Chapter 4) offers a potential tool for genomic epidemiological investigation of *B. pseudomallei* in Townsville, with long term applications due to its affordability, portability, rapid turnaround time, and high throughput capacity (Buytaers et al. 2021; Ferreira et al. 2021; Ritchie et al. 2023). Utilising ONT sequencing to investigate the environmental

reservoirs of *B. pseudomallei* across Townsville, and further linking these reservoirs to melioidosis cases, will inform the epidemiology of melioidosis. This will provide opportunities to develop more targeted strategies to help at-risk communities living in high-risk areas for melioidosis.

This study aims to investigate the genomic epidemiology of *B. pseudomallei* isolates obtained in Chapter 3 using the previously validated ONT-derived MLST (Chapter 4).

5.2 Materials and Methods

5.2.1 Targeted ONT Sequencing of B. pseudomallei Isolates

From all *B. pseudomallei* isolates collected in Chapter 3, 48 were selected and prepared for targeted ONT sequencing as described in Section 4.2.1. Raw sequence data was retrieved and the MLST of each isolate determined as described in Section 4.2.2.

5.2.2 PubMLST Sequence Submission

Novel allele sequences were aligned to example pubMLST database allele sequences using MAAFT (version 7.505) and trimmed to correct length as described on PubMLST (https://pubmlst.org/organisms/burkholderia-pseudomallei) for submission (Jolley, Bray & Maiden 2018; Katoh et al. 2005; Katoh et al. 2002; Katoh & Standley 2013). Novel allele sequences were submitted to the typing pubMLST MLST database for assessment and curation (C Appendix Table C.2). Once assessed by the database curator, novel ST profiles were submitted to the typing pubMLST MLST database for assessment and curation. All complete *B. pseudomallei* ST profiles were submitted to the isolate pubMLST MLST database for curation. STs and PubMLST ID numbers have been listed in C Appendix Table C.1 for each sequenced Goondaloo Creek *B. pseudomallei* isolate.

5.2.3 Data Analysis

Descriptive statistics, measures of normality, and graphs were generated in GraphPad Prism (Version 10.0.1). A Chi Square Test of Good Fitness (Outcome vs Expected) was used to compare observed and expected distributions of Goondaloo Creek sequenced STs.

Phylogenetic trees were created to determine relatedness within Goondaloo Creek STs, and how closely related these are to Townsville and Australian representative STs. All Australian and

Townsville *B. pseudomallei* ST sequences and metadata were exported from pubMLST as a fasta file and excel file respectively (Jolley, Bray & Maiden 2018). ST sequences were downloaded as concatenated sequences. Description fields included in the metadata for each ST are: entry identification number, isolate, aliases, country, continent, region, travel, history, travel region, year, species, source, animal source, source material, disease, detailed disease, age year, age month, sex, comments, sender, curator, date entered, datestamp, housekeeping gene internal fragment allele numbers (*ace, gltB, gmhD, lepA, lipA, narK, ndh*), and sequence type. All sequences within the Townsville dataset were retained. Duplicate values within the Australian dataset identified using ST, region, and source were removed. Goondaloo Creek ST sequences were concatenated and added to both Australian and Townsville datasets. A Thailand ST10 sequence was included in all datasets to root the phylogenetic trees (Rachlin et al. 2021). MAAFT (version 7.505) was used to create sequence alignments of the concatenated sequences (Katoh et al. 2005; Katoh et al. 2002; Katoh & Standley 2013). IQ-TREE (version 2.2.2.2) was used to determine the best fit model for each phylogenetic tree using ModelFinder and then generate the phylogenetic trees with 1000 nonparametric bootstrapping (≥80 indicated on phylogenetic tree) (Burnard et al. 2022; Gassiep et al. 2024; Kalyaanamoorthy et al. 2017; Nguyen et al. 2014; Rachlin et al. 2021). Tree of Life program (iTOL) (version 6.9.1) was used to annotate and edit phylogenetic trees (Letunic & Bork 2024; Rachlin et al. 2021).

5.3 Results

5.3.1 Genomic Diversity of Goondaloo Creek B. pseudomallei Isolates

From 48 targeted ONT sequenced isolates, a total of 18 different STs were identified, with 12 novel STs and six previously described on PubMLST. Two novel alleles were also identified, with a new allele for *lipA* (SNP difference) and *lepA* (three base pair insertion) (C Appendix Table C.2). The *lipA* allele was accepted by PubMLST but the *lepA* allele was rejected due to the three base pair insertion. From 48 isolates, 45 were accepted by PubMLST, with the remaining three (clonal) left with an incomplete ST profile due to the novel *lepA* allele. Novel alleles for *lipA* and *lepA* were confirmed by Illumina WGS (B Appendix Figure B.4-Figure B.15). ST283 (31.25%), ST1966 (10.42%), and ST2072 (10.42%) were the most common STs found (Figure 5.1) (C Appendix Table C.3). A Chi Square Test of Good Fitness (Outcome vs Expected), where expected distribution for each ST was 5.556%, determined that STs were significantly unequally distributed (P = <0.0001).

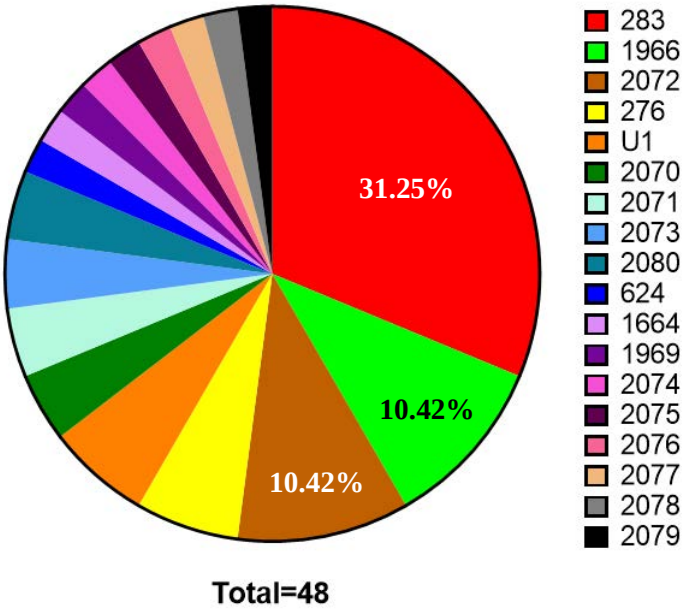

Figure 5.1 - Diversity and abundance of Goondaloo Creek STs. Identified STs are included in the key, listed from most to least abundant. Refer to C Appendix Table C.3 for all complete number and percentage values for each ST.

5.3.2 Relationship Between Goondaloo Creek B. pseudomallei Isolates and Previous Clinical Isolates

Nine STs (ST276, ST283, ST624, ST1664, ST1966, ST1969, ST2070, ST2074, and ST2075) were matched to STs previously isolated from animal and/or human cases described on PubMLST (Figure 5.2 and Table 5.1). ST283 had the most clinically related matches, with five animal submissions and five human submissions (Table 5.1). This was the first environmental isolation of ST283, ST624, ST1664, ST1966, ST1969, ST2070, ST2074, and ST2075 from previously documented STs on PubMLST.

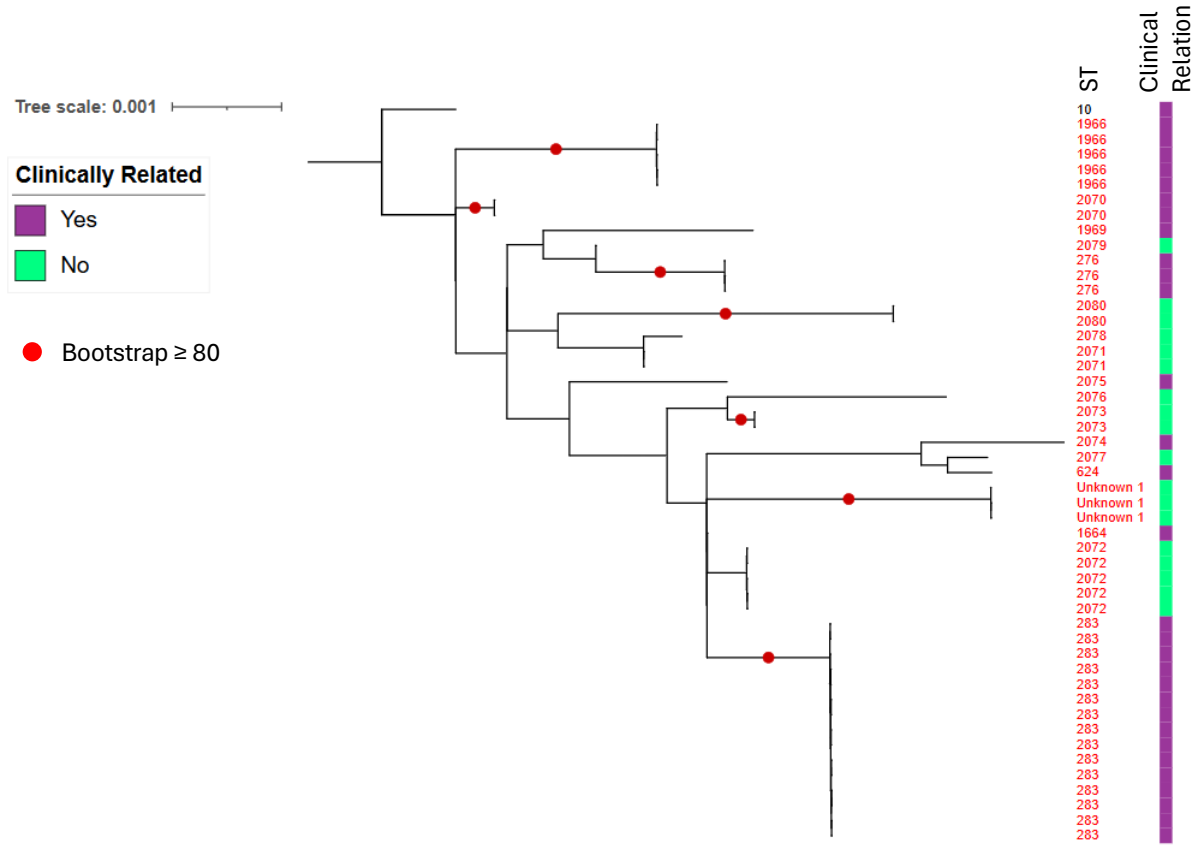

Figure 5.2 - Phylogenetic tree of Goondaloo Creek *B. pseudomallei* STs. Goondaloo Creek *B. pseudomallei* STs (red numbers), PubMLST matches to clinical human and animal case STs (purple) and lack of matches (green) is shown to right of phylogenetic tree. 'Unknown 1' refers to three of the sequenced Goondaloo Creek isolates that have a novel *lepA* and cannot be assigned an ST. A bootstrapping value greater than 80% is depicted by a red circle on the phylogenetic tree. Tree is rooted at ST10 (black number).

Table 5.1 - Goondaloo Creek STs, their novelty status at time of sequencing, and number of sample uploads from study and total submissions on PubMLST. Table was created after Goondaloo Creek STs were uploaded to PubMLST.

ST	**Novel to pubMLST at Time of Sequencing**	**Submissions Uploaded to pubMLST from this Study (All Environmental Source)**	**Identified Source/s of ST (Total Number of Submissions on pubMLST)**
276	No	3	Human (2) Animal (1) Environmental (10)
283	No	15	Human (5) Animal (5) Environmental (15)
624	No	1	Animal (1) Environmental (1)
1664	No	1	Human (1) Environmental (1)
1966	No	5	Human (3) Animal (2) Environmental (5)
1969	No	1	Animal (2) Environmental (1)
2070	Yes	2	Human (1) Environmental (2)
2071	Yes	2	Environmental (2)
2072	Yes	5	Environmental (5)
2073	Yes	2	Environmental (2)
2074	Yes	1	Human (1) Environmental (1)
2075	Yes	1	Human (1) Environmental (1)
2076	Yes	1	Environmental (1)
2077	Yes	1	Environmental (1)
2078	Yes	1	Environmental (1)
2079	Yes	1	Environmental (1)
2080	Yes	2	Environmental (2)
Unknown 1	Yes	0*	Environmental (0)*

∗ Three isolates sequenced in study with novel *lepA* allele but due to rejection of allele they had incomplete profiles and were not submitted.

5.3.3 Relationship Between Goondaloo Creek B. pseudomallei Isolates and Previous Clinical Townsville Isolates

Goondaloo Creek isolates are closely related to past human clinical case isolates from Townsville. ST1966, ST276, ST283, ST2070, ST2074, and ST2075 from Goondaloo Creek were matched with past Townsville human clinical case STs from PubMLST (Figure 5.3). ST276 was a match for previous environmentally isolated ST276 isolates in Townsville. Other Goondaloo Creek STs, such as ST276, ST624, ST1969, ST2071, ST2072, ST2073, ST2076, ST2077, ST2078, ST2079, ST2080, and Unknown 1, are grouped into clades containing Townsville clinical case STs (Figure 5.3). However, these groupings are not supported by bootstrapping.

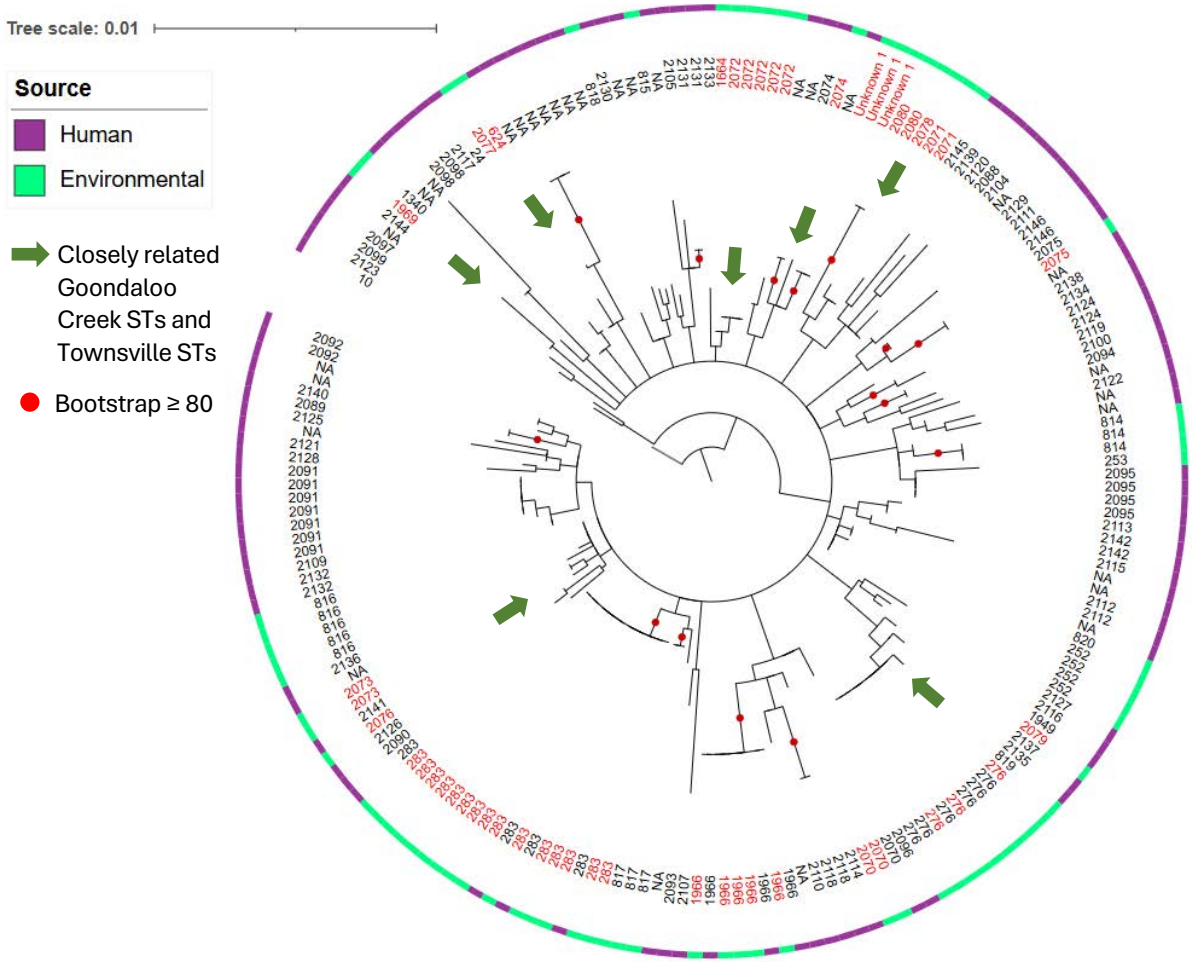

Figure 5.3 - Phylogenetic tree of Townsville STs and Goondaloo Creek STs. From outside in, PubMLST matches to clinical human and animal case STs (purple) and lack of matches (green), Townsville STs (black numbers) and Goondaloo Creek STs (red numbers), and clades containing both Goondaloo Creek STs and Townsville clinical STs (green arrow). A bootstrapping value greater than 80% is depicted by a red circle. Tree is rooted at ST10 (black number).

5.3.4 Relationship Between Goondaloo Creek B. pseudomallei Isolates and Australian Isolates

A phylogenetic tree containing all unique Australian STs by region and source demonstrated a wide spread distribution of Goondaloo Creek isolates throughout the tree (Figure 5.4). Goondaloo Creek STs formed clades throughout the tree with Australian STs. Goondaloo Creek STs were closely related to Australian STs as indicated by their wide spread distribution, rather than formation of separate distinct clades grouped away from Australian sequences.

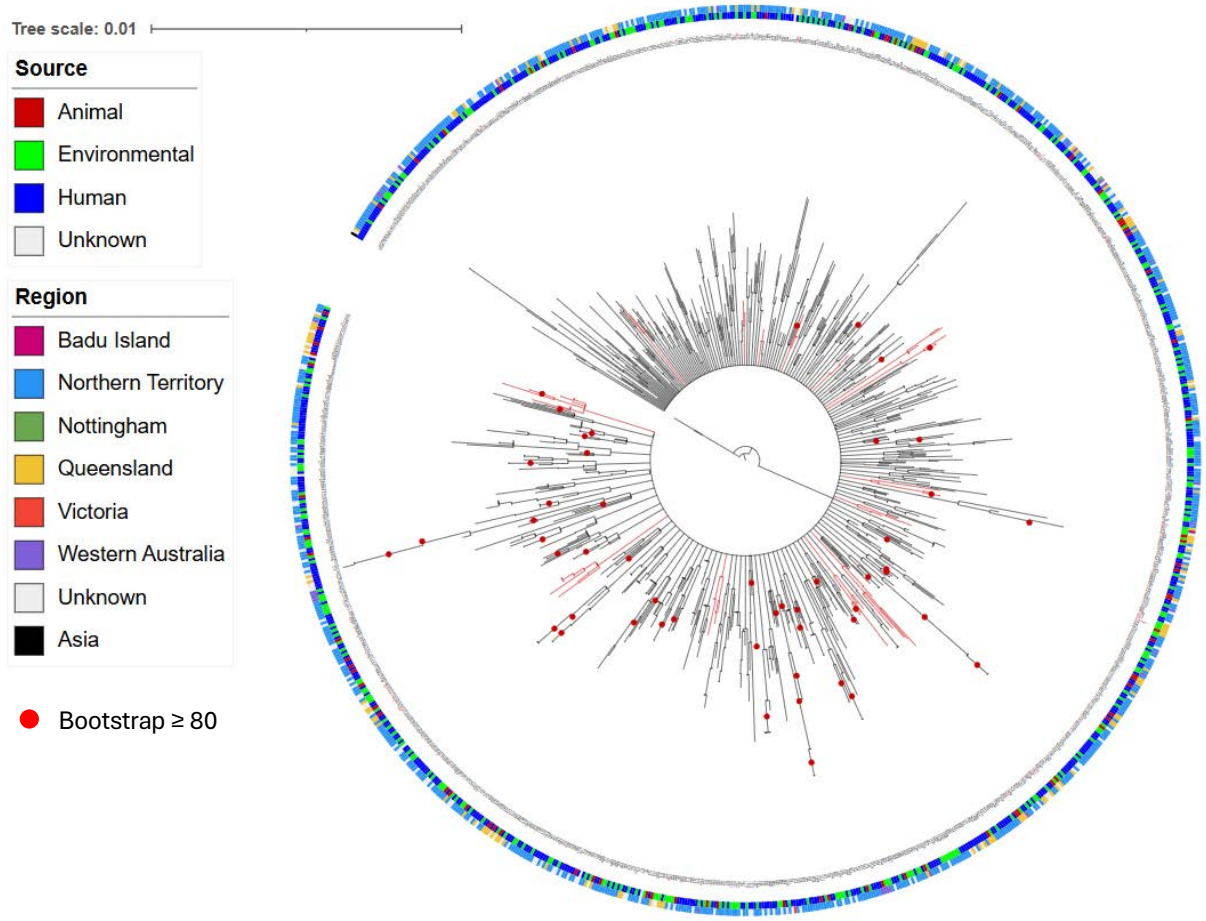

Figure 5.4 - Phylogenetic tree of Australian representative STs and Goondaloo Creek STs. From outside in, the first ring represents ST isolate region, the second ring represents ST isolate source, and Australian STs (black numbers) and Goondaloo Creek STs (red numbers) are shown innermost. Phylogenetic tree clades that contain a Goondaloo Creek ST are red. A bootstrapping value greater than 80% is depicted by a red circle. Tree is rooted at ST10 (black number).

5.4 Discussion

An investigation into the genomic epidemiology of *B. pseudomallei* collected from a seasonal creek in Townsville determined that surface groundwater, linked to rainfall events (Chapter 3), serves as an ecological niche of clinically relevant *B. pseudomallei*, as evidenced by MLST phylogeny (this Chapter). Sequencing revealed a diversity of 18 distinct STs, with nine of these previously associated with human and/or animal cases. Among the isolates, the most dominant STs were identified as ST283 and ST1966, both associated with human cases, including previous Townsville cases. ST276, ST2070, ST2074, and ST2075 from Goondaloo Creek were also matched with past Townsville human clinical case STs from PubMLST and Gassiep et al. (2024). In a recent study that investigated the genomic epidemiology of clinical *B. pseudomallei* in Townsville over a time period of 1997 to 2020, ST283 and ST276 accounted for 12% (15/128) and 6% (8/128) of melioidosis cases respectively (Gassiep et al. 2024). Unsurprisingly, the isolated *B. pseudomallei* strains from Goondaloo Creek appeared to be biogeographically linked to Australian and Townsville clades, indicating that these strains are likely local residents rather than introduced. However, the low bootstrapping values for closely related STs that were not a direct match for previous STs necessitates further genomic investigation to validate these close relationships. The close relatedness of these Goondaloo Creek isolates to other Townsville-specific isolates collected over decades supports the longstanding local establishment of these strains. Notably, two isolates have been associated with the Townsville region for at least two decades, with the earliest ST283 and ST1966 sequenced isolates from clinical cases originating from a time period of 1997 to 2004 (Gassiep et al. 2024).

ONT sequencing was successfully used for environmental surveillance of *B. pseudomallei* in Townsville, identifying surface groundwater as a potential reservoir of infection. Surface groundwater waterways similar to Goondaloo Creek are widespread throughout the Townsville region (ArcGIS Online 2024). These waterways, which can flow from hills and mountains known to harbor *B. pseudomallei*, may carry the pathogen into urban areas (Baker & Warner 2016). Previous studies have confirmed the presence of culturable *B. pseudomallei* in drain water around Castle Hill and similar findings have been reported in Vientiane, Laos (Baker et al. 2011; Rachlin et al. 2021). In Northern Australia, surface groundwater was hypothesised to have spread ST562, an introduced ST likely with an Asian origin, and expand its geographic area from a single introduction point to across Darwin resulting from increased connectivity of waterways and wetness after a La Niña period (Meumann et al. 2021). For the Townsville region, the distribution of clinical STs was shown to have changed over the duration of the study from 1997 to 2020 (Gassiep et al. 2024). Although the mechanisms of the change in ST distribution were not investigated, similar to ST562, this may have been influenced in part to the spread of groundwater (Gassiep et al. 2024; Meumann et al. 2021). The combined results of this study and previous investigations indicate that surface groundwater mobilises and spreads

clinically relevant *B. pseudomallei,* potentially contributing to the epidemiology of melioidosis in Townsville. Furthermore, surface groundwater might contribute to expanding the biogeographical boundaries of *B. pseudomallei*. Utilising ONT sequencing for ongoing environmental surveillance will be advantageous in managing melioidosis outbreaks by identifying and monitoring high-risk areas.

To efficiently investigate surface groundwater throughout Townsville, utilising the hydrology of the land for sentinel sampling should be employed. Leveraging the land's hydrology by using drain catchments to sample water collected from large surface areas may provide a more efficient strategy for sampling, especially since a similar strategy has been used within Loas (Vientiane) and Australia (Darwin) to investigate the genetic diversity and clustering of *B. pseudomallei* (Rachlin et al. 2020; Rachlin et al. 2021). Traditional environmental sampling of *B. pseudomallei* involves sampling multiple sites spaced 2.5 to 5 metres within a desired region, depending on the size of the region, this can result in a large number of sampling sites and resources required to conduct the investigation (Limmathurotsakul, Dance, et al. 2013). In this study, we were able to hypothetically sample a large area of land from which the surface groundwater originated from, in single water samples. This sampling method not only detects *B. pseudomallei* in generalised areas but also assesses a diversity of STs representative of those areas. In this study, sequencing of 48 Goondaloo Creek isolates revealed a large diversity of 18 *B. pseudomallei* STs, including both novel and previously identified STs. A similar sampling strategy was used previously in Australia to conduct an investigation of ST diversity and distribution throughout Darwin. This study collected environmental soil and water samples and determined water to contain a higher diversity of STs (Rachlin et al. 2020). Rachlin et al. (2021) also benefitted from using this sampling strategy, effectively sampling a large area of the city using 40 sites and identifying areas where *B. pseudomallei* positive samples clustered, indicating potential environmental reservoirs of infection. Conducting a large-scale investigation in other areas with similar hydrological features to Goondaloo Creek across Townsville may provide further insights into surface groundwater as a potential mobile reservoir of infection and help define the biogeographical boundaries of *B. pseudomallei* in the region.

Furthermore, linking the reservoir of infection to melioidosis cases, by clinical and environmental matching of *B. pseudomallei* isolates, will reveal how surface groundwater is contributing to the epidemiology of melioidosis in Townsville. A recent study in Cairns identified a mud pit used in a school obstacle course event as a reservoir of infection for *B. pseudomallei,* by matching clinical and environmental isolates, subsequently leading to its discontinuation in future events (Smith et al. 2023). Previous investigation into melioidosis case clustering in Townsville and how these are linked to the environment, particularly soil, identified high-risk areas for the community (Corkeron, Norton & Nelson 2010). Furthermore, how *B. pseudomallei* ST patterns change over time and how these affect melioidosis cases is also important to consider (Gassiep et al. 2024). Identification of events and locations linked to melioidosis incidence and further management of associated risks can help to

prevent melioidosis cases. Understanding the epidemiology of *B. pseudomallei*, by investigating the reservoirs of infection, routes of transmission, and case clustering provides a basis to create public health strategies that mitigate melioidosis incidence.

Several limitations of this study apply to the sequenced STs and the sequences used for phylogenetic analysis. The selection criteria for isolates may not fully represent the diversity of STs in Goondaloo Creek, as the number of isolates that could be sequenced was restricted to 48, limiting the representation of true diversity. Furthermore, the use of targeted sequencing for phylogenetic analysis, while reliable for determining STs, is less ideal compared to WGS. Concatenated STs reduce the number of informative sites available for analysis, resulting in lower bootstrapping values. Future studies using ONT for environmental surveillance should aim to address these limitations.

Future research should focus on increasing environmental surveillance of *B. pseudomallei* in surface groundwater throughout Townsville and beyond. Sequencing more creeks as sentinel sampling sites will further investigate the diversity of *B. pseudomallei* present. Expanding the number of study sites and correlating findings with melioidosis cases, both recent and past, will help elucidate the role of surface groundwater as a mobile reservoir of infection. A comprehensive environmental surveillance effort across Townsville will identify high-risk areas and populations, informing public health initiatives and alerting clinicians to monitor predisposed patients from high-risk areas after rainfall or significant weather events. Establishing routine matching of clinical and environmental isolates within Townsville, alongside regular environmental surveillance, will help track how STs spread in response to changing climate and environmental conditions.

Chapter 6

General Discussion

Surface groundwater in Townsville appears to act as an ecological niche for clinically relevant *B. pseudomallei* strains, impacting the mobilisation of the bacterium and epidemiology of melioidosis. The concentration and therefore availability of the organism during climatic events is not random. After rainfall of >30mm, surface groundwater mobilises *B. pseudomallei* and carries it along its flow path. *Burkholderia pseudomallei* concentrations decrease significantly after two days following heavy rainfall and is highest immediately after the first flush of rain. The degree of turbidity in the water appears to indicate the presence of high concentrations of *B. pseudomallei*. With further investigation across a broader geography, these characteristics could predict periods of high environmental risk, informing public health messaging to reduce exposure and melioidosis rates in this endemic region.

Similar phenomena may occur in other waterways in Townsville. Other waterways, such as those shown on Mt Stuart in Figure 6.1 and the greater Townsville region in Figure 6.2 and Figure 6.3, display similar hydrology to Goondaloo Creek. Various regions, including Mt Stuart, Castle Hill, Mt Louisa, Hervey Range, and Bluewater Reserve, have been identified as reservoirs of *B. pseudomallei* via PCR. Drain water around Castle Hill has contained culturable *B. pseudomallei* (Baker et al. 2011; Rachlin et al. 2021). Surface groundwater samples from Mt Louisa and Mt Stuart also contained the bacterium (Section 3.3.1). Similar results have been observed outside Australia, such as in Vientiane, Loas, where surface groundwater and rivers contained culturable *B. pseudomallei* following rainfall (Rachlin et al. 2021; Zimmermann et al. 2018). The exact mechanism by which *B. pseudomallei* is mobilised into surface groundwater remains unclear.

Several hypotheses may explain how *B. pseudomallei* appears in surface groundwater following rainfall. First, the rising water table may carry bacteria to the surface, allowing them to multiply in favourable conditions (Thomas, Forbes-Faulkner & Parker 1979). Second, rainfall may cause existing groundwater seeps, identified as reservoirs of *B. pseudomallei*, to overflow (Baker et al. 2011; Baker & Warner 2016). Third, water mixing with soil during rainfall, facilitating erosion processes, may leach *B. pseudomallei* into the water (Zimmermann et al. 2018). These hypotheses likely all contribute to the presence of *B. pseudomallei* in surface groundwater. The bacterium may be leached from soil reservoirs or groundwater seeps before being mobilised in surface groundwater and transported along waterways. Its presence is primarily noted in highly turbid water rich in particulate debris. Significant erosion during heavy rainfall can introduce large amounts of particulate matter in water, even in forested regions (Zimmermann, Francke & Elsenbeer 2012). Similar erosion processes in Loas rivers result in particle-rich water following rainfall that contains *B. pseudomallei* (Zimmermann et al. 2018).

Our study showed comparable results, with *B. pseudomallei* presence strongly associated with highly turbid water. Zimmermann et al. (2018) ruled out riverbed sediment as a reservoir for *B. pseudomallei,* suggesting that water carries the bacterium from another location. Although surface groundwater is a temporary reservoir, it may increase melioidosis cases during the wet season.

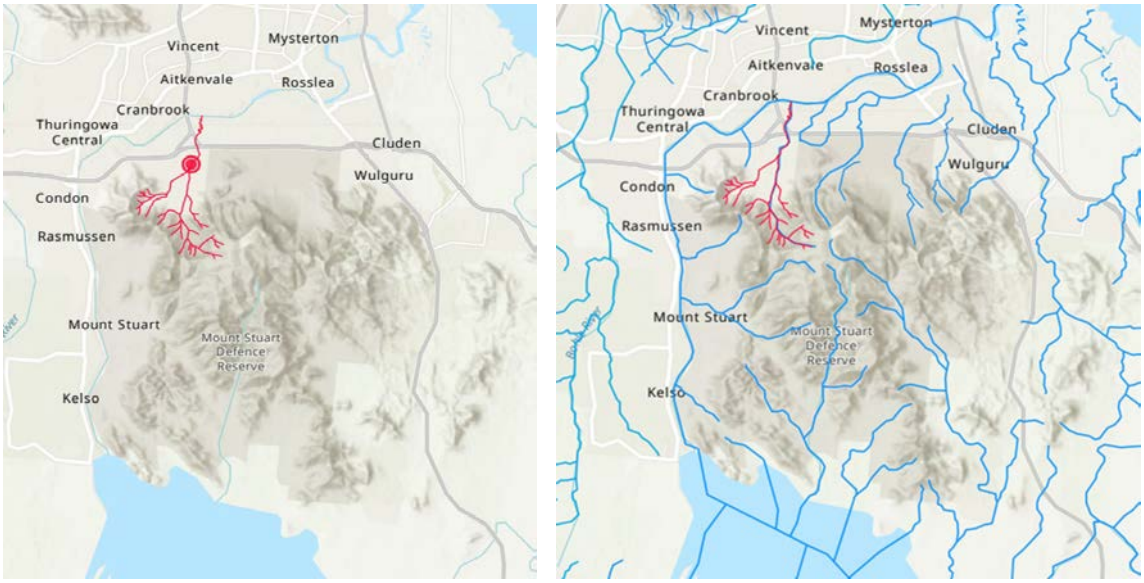

Figure 6.1 - Waterways that flow off Mt Stuart. Goondaloo Creek waterways are coloured red and all other waterways are coloured blue. Maps created in ArcGIS Online

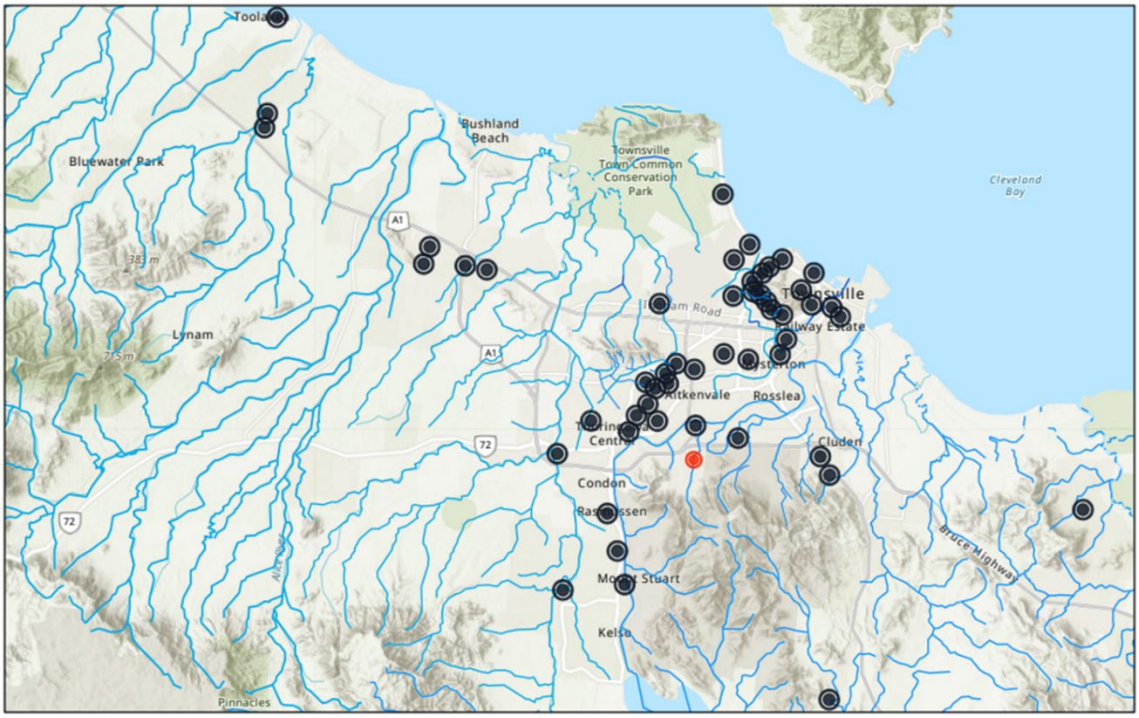

Figure 6.2 - Melioidosis cases from 1996 to 2008 and current waterways across the Townsville region. Melioidosis cases are shown as black circles, Goondaloo Creek sample site is depicted as a red circle, and waterways are shown in blue. Image created by overlaying map from Corkeron, Norton and Nelson (2010) onto waterway map created in ArcGIS Online.

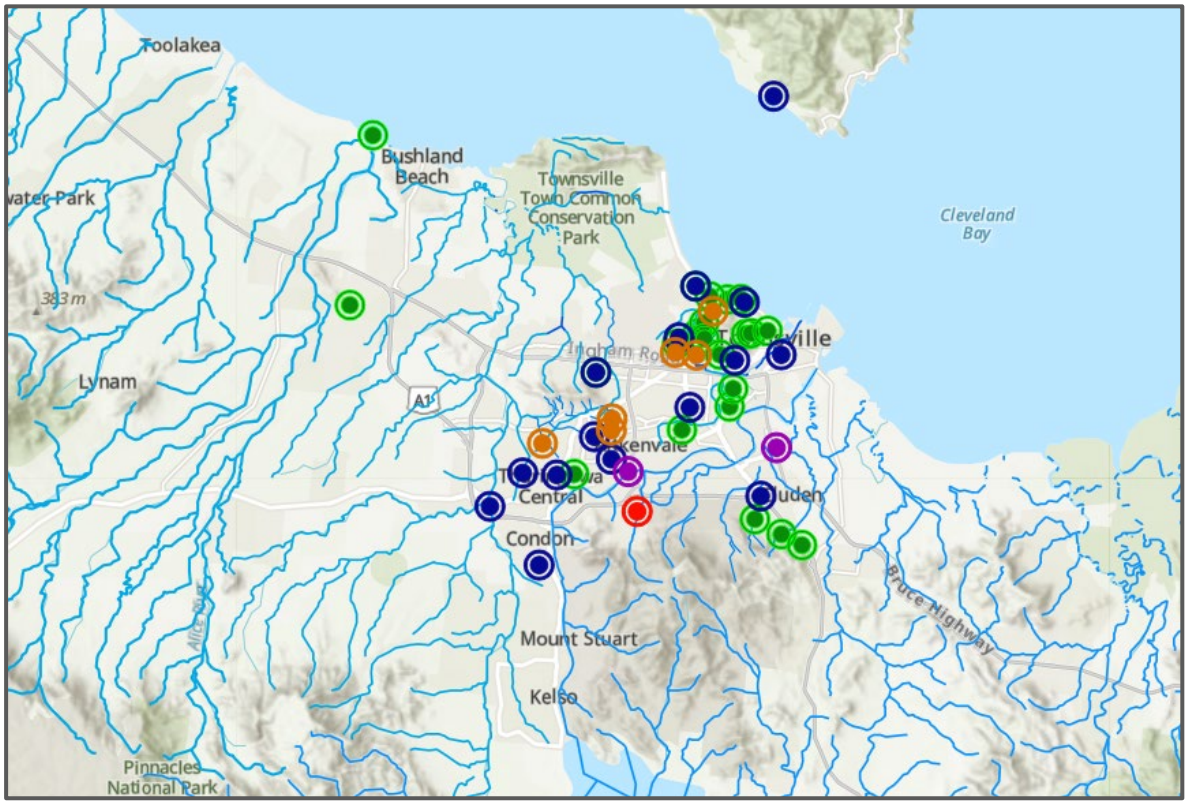

Figure 6.3 - Melioidosis cases from 1997 to 2020 and current waterways across the Townsville region. Melioidosis cases are shown as circles, representing ST252 (Green), ST283 (Blue), ST276 (Orange), and ST TSV-13 (Purple). Goondaloo Creek sample site is depicted as a red circle and waterways are shown in blue. Image created by overlaying maps from Gassiep et al. (2024) onto waterway map created in ArcGIS Online.

Surface groundwater flowing from areas that are reservoirs of *B. pseudomallei* might act as a mobile reservoir of infection, contributing to the increase in melioidosis incidence during the wet season. Increased rainfall during the wet season may wash *B. pseudomallei* into surrounding urban areas, leading to more cases of melioidosis. In Townsville and Darwin, Australia, rainfall and associated indicators such as cloud cover and humidity strongly correlate with increased melioidosis incidence (Ganeshalingam et al. 2023; Kaestli et al. 2016). Figure 6.2 shows melioidosis cases in Townsville by residential address from 1996-2008 overlaid on a current map highlighting the waterways (Corkeron, Norton & Nelson 2010). Despite being over a decade old, the data reveals a large cluster near Castle Hill, a smaller cluster near Mt Louisa, and sporadic cases around Mt Stuart. Figure 6.3 shows cases from 1997-2020 in Townsville by residential address and was created similarly by overlaying published maps onto a current map with waterways highlighted (Gassiep et al. 2024). Similar to Figure 6.2, Figure 6.3 also shows a large cluster around Castle Hill, a smaller cluster at the base of Mt Louisa, and sporadic cases at the base of Mt Stuart. The Castle Hill cluster is located close to the base where water runoff is likely. Mt Stuart's sporadic cases, potentially due to lower population density and less infrastructure compared to Castle Hill, appear near waterways that could carry contaminated

water off Mt Stuart similar to Goondaloo Creek (Corkeron, Norton & Nelson 2010; Gassiep et al. 2024). A melioidosis case downstream of the Goondaloo Creek study site shown in Figure 6.2 further supports this hypothesis (Corkeron, Norton & Nelson 2010). Additional investigation into similar sites as Goondaloo Creek near previous melioidosis cases is needed to provide more reliable evidence of surface groundwater as a reservoir of infection in Townsville.

Future sampling strategies should consider water sampling, hydrology, and land topology to determine the persistence of *B. pseudomallei* and potential melioidosis endemicity. Sentinel sampling of single sites known to collect water in drain catchments from large land surface areas would be more efficient than traditional soil sampling (Limmathurotsakul, Dance, et al. 2013). This strategy allows for the sampling of extensive land areas, reducing the need for resources such as time and cost, while still identifying general areas containing *B. pseudomallei*. Although, this sampling strategy has the limitation of requiring heavy rainfall before sampling can be conducted. Figure 6.4 illustrates the hydrology of Goondaloo Creek acting as a drain catchment for multiple waterways flowing off Mt Stuart. These sites can serve as sentinel sites for environmental screening before deploying resource-intensive fine-scale investigations. Increased environmental surveillance using this strategy will help delineate the biogeographical boundaries of *B. pseudomallei* within the Townsville region, informing high-risk areas for melioidosis.

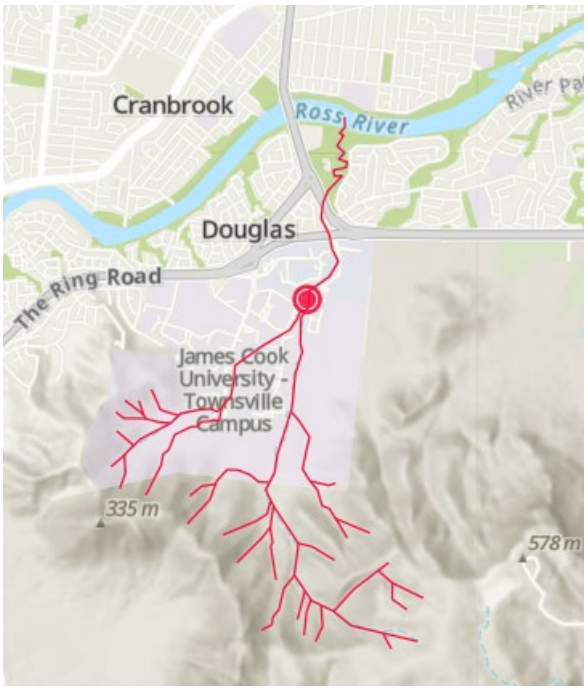

Figure 6.4 - Waterways that flowed off Mt Stuart and converged into Goondaloo Creek drain catchment sampling point. Waterways are displayed in red.

Enhanced surveillance of *B. pseudomallei* will provide opportunities to investigate its genomic epidemiology more thoroughly. Understanding not only the presence of *B. pseudomallei* in the Townsville region but also the distribution of specific strains through genomic investigation can

inform reservoirs of infection and transmission routes. For example, a recent study conducted by Gassiep et al. (2024) investigated the genetic diversity of clinical isolates of *B. pseudomallei* in Townsville over a 23-year period and identified a diversity of STs, including novel STs, with six that were associated with 50% of melioidosis cases in the region. This study has strengthened the understanding of STs that are associated with infection in the region, as well as identified how dominant STs may change over time (Gassiep et al. 2024). Establishing accurate, lower cost, and rapid turnaround time in-house sequencing via ONT provides the opportunity for sustainable genomic investigation of *B. pseudomallei* in Townsville. The addition of BOX-A1R-Based Repetitive Extragenic Palindromic-Base Pairs (BOX-PCR) within the ONT sequencing workflow can also decrease costs. BOX-PCR is a type of fingerprinting analysis that can identify clonal isolates and was adapted for *B. pseudomallei* clone exclusion by Currie et al. (2007). The addition of a BOX-PCR step can be used to screen for clonal isolates and remove duplicates, saving on resources needed for sequencing, as 30 of the 48 Goondaloo Creek *B. pseudomallei* isolates sequenced were duplicates in this study. Increased genomic surveillance will help better understand the reservoirs of infection and transmission routes, leading to informed strategies to reduce melioidosis incidence. This surveillance will become increasingly important as environmental conditions continue to change due to climate change.

Changing climatic conditions and land use may alter the biogeographical boundaries of *B. pseudomallei* and increase melioidosis incidence. Rising global temperatures are forecasted to increase precipitation and extreme weather events in Northern Australia (BOM & CSIRO 2022; Dey et al. 2019). An increase in heavy rainfall events, leading to flash flooding, is also predicted (BOM & CSIRO 2022; Dey et al. 2019). Already, Northern Australia has experienced an increase in rainfall over the last two decades compared to the last two centuries as well as an increasing wet season rainfall trend since the 1970s (Freund et al. 2017; O'Donnell et al. 2015). More frequent and intense rainfall events will likely mobilise *B. pseudomallei* throughout local environments, potentially leading to higher melioidosis incidence. Additionally, land use changes in response to climate change and population growth may further increase melioidosis cases.

Changing weather patterns and a growing population are likely to impact agriculture and infrastructure development. More fertile regions may be increasingly used to maximise agricultural yields. In Australia, tropical areas endemic for *B. pseudomallei* are likely to be developed. Agricultural soils that are regularly disturbed or well-irrigated soils are associated with the presence of *B. pseudomallei* (Corkeron, Norton & Nelson 2010; Goodrick, Todd & Stewart 2018; Kaestli et al. 2015; Kaestli et al. 2009*).* Infrastructure development, such as housing and roads, in endemic regions will likely facilitate more exposure to *B. pseudomallei*. Displacement of large amounts of soil can unearth significant concentrations of *B. pseudomallei*. For instance, a study found that the highest concentrations of *B. pseudomallei* are deeper in the soil, corresponding with the water table (Pongmala et al. 2022).

Upgrading a highway in Cairns was recently associated with an increase in melioidosis cases in the surrounding area due to soil disturbance (Smith et al. 2021). Long-term housing establishment in endemic regions will also likely increase melioidosis cases due to higher population density.

Changing climate conditions may also expand the biogeographical boundaries of *B. pseudomallei.* The organism's spread can be facilitated by animals, anthropogenic processes, and environmental factors (Chewapreecha et al. 2017; Hampton et al. 2011; Zimmermann et al. 2018). For example, Brisbane, previously considered non-endemic for *B. pseudomallei,* recently reported its first environmental isolation of the organism. This is hypothesised to be associated with two successive La Niña events, resulting in above-average rainfall and flooding (Gassiep et al. 2023). In Central Australia and southwest Western Australia, *B. pseudomallei* lies dormant most of the time due to unfavourable environmental conditions but revitalises in response to heavy rainfall (Chapple et al. 2016; Yip et al. 2015). These temperate and desert regions experience sporadic melioidosis outbreaks following above-average rainfall (Chapple et al. 2016; Yip et al. 2015). Increasing precipitation and heavy rainfall events will likely lead to more melioidosis outbreaks in these areas. Further investigation into the epidemiology of *B. pseudomallei* is needed to understand its current and future distribution both spatially and temporally.

A thorough understanding of the epidemiology of *B. pseudomallei* will enable the development of accurate predictive models of its environmental distribution. These models can forecast the organism's spatial and temporal distribution, changes in response to various variables, and identify high-risk communities based on socio-economic status and comorbidity burden. In 2016, an attempt was made to create a global predictive model for *B. pseudomallei* (Limmathurotsakul et al. 2016). This model estimated the global burden and mortality of melioidosis, mapped environmental suitability for *B. pseudomallei,* and identified both known and predicted endemic countries. However, it was never validated with environmental sampling. The model used multivariable analysis incorporating soil characteristics, precipitation/climate data, land surface temperature, and vegetation/moisture index characteristics on a global scale at a single point in time (Limmathurotsakul et al. 2016). For improved accuracy, future models should incorporate dynamic environmental suitability changes in response to seasonal variations, climate change, and other environmental factors such as hydrology and topology. Additionally, fine-scale modelling should be conducted to provide targeted strategies for individual communities. Although the initial model was a significant step forward, the need for improvement and validation is evident. An accurate predictive model will be essential for monitoring current and future biogeographical boundaries of *B. pseudomallei*, informing at-risk communities, and implementing strategies to reduce melioidosis incidence.

Surface groundwater is likely playing a crucial role as a mobile reservoir of infection in Townsville. Water collected from Goondaloo Creek contained clinically relevant *B. pseudomallei,* supporting the

hypothesis that it is an important reservoir and vehicle for dissemination of the pathogen. Further investigation into how surface groundwater mobilises *B. pseudomallei* and facilitates exposure to high-risk individuals, as well as identifying high-risk areas across Townsville, is needed. Utilising sentinel sampling sites will provide a more efficient strategy for environmental surveillance of *B. pseudomallei*. The establishment of ONT in Townsville is a significant step toward increasing in-house sequencing capacity and improving genomic epidemiological investigation of *B. pseudomallei*. Enhancing environmental and genomic surveillance will be crucial for monitoring the effects of climate change and land use on the epidemiology of *B. pseudomallei*.

References

AGRF 2024, *Service guide Illumina SNP service*, AGRF, Melbourne, viewed 13 September 2023, <https://static1.squarespace.com/static/5c6a2bfa11f7845bc7a99405/t/6646c3fe29bc722f75ae36c6/1715913729173/SVG2404SRL_ServiceGuide_Illumina_SNP+%281%29.pdf>.

Alam, K, Islam, MM, Gong, K, Abbasi, MN, Li, RJ, Zhang, YM & Li, AY 2022, 'In silico genome mining of potential novel biosynthetic gene clusters for drug discovery from *Burkholderia* bacteria', *Computers in Biology and Medicine*, vol. 140, DOI: 10.1016/j.compbiomed.2021.105046.

Andrews, S 2023, *FastQC*, Babraham Bioinformatics, viewed 17 June 2024, <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.

ArcGIS Online 2024, *Interactive map*, ArcGIS Online, viewed 21 June 2024, <https://www.arcgis.com/apps/mapviewer/index.html>.

Ashdown, LR 1979, 'An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens', *Pathology*, vol. 11, no. 2, pp. 293-7, DOI: 10.3109/00313027909061954.

Ashdown, LR & Clarke, SG 1992, 'Evaluation of culture techniques for isolation of *Pseudomonas pseudomallei* from soil', *Applied and Environmental Microbiology*, vol. 58, no. 12, pp. 4011-5, DOI: 10.1128/aem.58.12.4011-4015.1992.

Bahram, M, Hildebrand, F, Forslund, SK, Anderson, JL, Soudzilovskaia, NA, Bodegom, PM, Bengtsson-Palme, J, Anslan, S, Coelho, LP, Harend, H, Huerta-Cepas, J, Medema, MH, Maltz, MR, Mundra, S, Olsson, PA, Pent, M, Polme, S, Sunagawa, S, Ryberg, M, Tedersoo, L & Bork, P 2018, 'Structure and function of the global topsoil microbiome', *Nature*, vol. 560, no. 7717, p. 233, DOI: 10.1038/s41586-018-0386-6.

Bahram, M, Kohout, P, Anslan, S, Harend, H, Abarenkov, K & Tedersoo, L 2016, 'Stochastic distribution of small soil eukaryotes resulting from high dispersal and drift in a local environment', *ISME Journal*, vol. 10, no. 4, pp. 885-96, DOI: 10.1038/ismej.2015.164.

Baker, A, Ezzahir, J, Gardiner, C, Shipton, W & Warner, J 2015, 'Environmental attributes influencing the distribution of *Burkholderia pseudomallei* in Northern Australia', *PLoS ONE*, vol. 10, no. 9, p. e0138953, viewed 29 July 2021, DOI: 10.1371/journal.pone.0138953.

Baker, A, Tahani, D, Gardiner, C, Bristow, KL, Greenhill, AR & Warner, J 2011, 'Groundwater seeps facilitate exposure to *Burkholderia pseudomallei*', *Applied Environmental Microbiology*, vol. 77, no. 20, pp. 7243-6, viewed 27 July 2021, DOI: 10.1128/AEM.05048-11.

Baker, AL & Warner, JM 2016, '*Burkholderia pseudomallei* is frequently detected in groundwater that discharges to major watercourses in northern Australia', *Folia Microbiologica*, vol. 61, no. 4, pp. 301-5, viewed 27 January 2022, DOI: 10.1007/s12223-015-0438-3.

Bankevich, A, Nurk, S, Antipov, D, Gurevich, AA, Dvorkin, M, Kulikov, AS, Lesin, VM, Nikolenko, SI, Pham, S, Prjibelski, AD, Pyshkin, AV, Sirotkin, AV, Vyahhi, N, Tesler, G, Alekseyev, MA & Pevzner, PA 2012, 'SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing', *Journal of Computational Biology*, vol. 19, no. 5, pp. 455-77, viewed 17 June 2024, DOI: 10.1089/cmb.2012.0021.

Birnie, E, Virk, HS, Savelkoel, J, Spijker, R, Bertherat, E, Dance, DAB, Limmathurotsakul, D, Devleesschauwer, B, Haagsma, JA & Wiersinga, WJ 2019, 'Global burden of melioidosis in 2015: a systematic review and data synthesis', *Lancet Infectious Diseases*, vol. 19, no. 8, pp. 892-902, DOI: 10.1016/S1473-3099(19)30157-4.

Bolger, AM, Lohse, M & Usadel, B 2014, 'Trimmomatic: a flexible trimmer for Illumina sequence data', *Bioinformatics*, vol. 30, no. 15, pp. 2114-20, viewed 17 June 2024, DOI: 10.1093/bioinformatics/btu170.

BOM & CSIRO 2022, *State of the climate 2022*, Commonwealth of Australia, Australia, viewed 26 July 2023, <https://www.csiro.au/en/research/environmental-impacts/climate-change/State-of-the-Climate>.

Bugrysheva, JV, Sue, D, Gee, JE, Elrod, MG, Hoffmaster, AR, Randall, LB, Chirakul, S, Tuanyok, A, Schweizer, HP & Weigel, LM 2017, 'Antibiotic resistance markers in *Burkholderia pseudomallei* strain Bp1651 identified by genome sequence analysis', *Antimicrobial Agents and Chemotherapy*, vol. 61, no. 6, DOI: 10.1128/aac.00010-17.

Bulterys, PL, Bulterys, MA, Phommasone, K, Luangraj, M, Mayxay, M, Kloprogge, S, Miliya, T, Vongsouvath, M, Newton, PN, Phetsouvanh, R, French, CT, Miller, JF, Turner, P & Dance, DAB 2018, 'Climatic drivers of melioidosis in Laos and Cambodia: a 16-year case series analysis', *Lancet Planetary Health*, vol. 2, no. 8, pp. E334-E43, DOI: Doi 10.1016/S2542-5196(18)30172-4.

Burnard, DM, Bauer, M, Falconer, CM, Gassiep, I, Norton, RM, Paterson, D & Harris, PNM 2022, 'Clinical *Burkholderia pseudomallei* isolates from north Queensland carry diverse bimA_{Bm} genes that are associated with central nervous system disease and are phylogenomically distinct from other Australian strains', *PLoS Neglected Tropical Diseases*, vol. 16, no. 6, DOI: 10.1371/journal.pntd.0009482.

Buytaers, FE, Saltykova, A, Denayer, S, Verhaegen, B, Vanneste, K, Roosens, NHC, Piérard, D, Marchal, K & De Keersmaecker, SCJ 2021, 'Towards real-time and affordable strain-sevel metagenomics-based foodborne outbreak investigations using Oxford Nanopore sequencing technologies', *Frontiers in Microbiology*, vol. 12, DOI: 10.3389/fmicb.2021.738284.

CDC 2018, *Bioterrorism agents/diseases*, viewed 18 August 2022, <https://emergency.cdc.gov/agent/agentlist-category.asp>.

Center for Genomic Epidemiology 2011, *MLST*, viewed 9 September 2023, <https://cge.food.dtu.dk/services/MLST/>.

Centre for Disease Control Northern Territory 2016, *Melioidosis awareness campaign - Don't get melioidosis*, vol. 23, no. 1, pp. 1-6, Northern Territory Government, Casuarina, viewed 10 July 2023,

< https://digitallibrary.health.nt.gov.au/entities/publication/e28c05f5-7ca5-458c-b976-fd13eb50f7ee/details>.

Chantratita, N, Phunpang, R, Yarasai, A, Dulsuk, A, Yimthin, T, Onofrey, LA, Coston, TD, Thiansukhon, E, Chaisuksant, S, Tanwisaid, K, Chuananont, S, Morakot, C, Sangsa, N, Chayangsu, S, Silakun, W, Buasi, N, Chetchotisakd, P, Day, NPJ, Lertmemongkolchai, G & West, TE 2023, 'Characteristics and one year outcomes of melioidosis patients in Northeastern Thailand: a prospective, multicenter cohort study', *Lancet Regional Health - Southeast Asia*, vol. 9, DOI: 10.1016/j.lansea.2022.100118.

Chapple, SNJ, Price, EP, Sarovich, DS, McRobb, E, Mayo, M, Kaestli, M, Spratt, BG & Currie, BJ 2016, '*Burkholderia pseudomallei* genotype distribution in the Northern Territory, Australia', *The American Society of Tropical Medicine and Hygiene*, vol. 94, no. 1, pp. 68-72, DOI: 10.4269/ajtmh.15-0627.

Chapple, SNJ, Sarovich, DS, Holden, MTG, Peacock, SJ, Buller, N, Golledge, C, Mayo, M, Currie, BJ & Price, EP 2016, 'Whole-genome sequencing of a quarter-century melioidosis outbreak in temperate Australia uncovers a region of low-prevalence endemicity', *Microbial Genomics*, vol. 2, no. 7, p. e000067, DOI: 10.1099/mgen.0.000067.

Chen, PS, Chen, YS, Lin, HH, Liu, PJ, Ni, WF, Hsueh, PT, Liang, SH, Chen, CL & Chen, YL 2015, 'Airborne transmission of melioidosis to humans from environmental aerosols contaminated with *B. pseudomallei*', *PLoS Neglected Tropical Diseases*, vol. 9, no. 6, DOI: 10.1371/journal.pntd.0003834.

Chen, Y, Chen, S, Kao, C & Chen, Y 2003, 'Effects of soil pH, temperature and water content on the growth of *Burkholderia pseudomallei*', *Folia Microbiologica*, vol. 48, no. 2, pp. 253-6, DOI: 10.1007/Bf02930965.

Chen, Z, Erickson, DL & Meng, JH 2021, 'Polishing the Oxford Nanopore long-read assemblies of bacterial pathogens with Illumina short reads to improve genomic analyses', *Genomics*, vol. 113, no. 3, pp. 1366-77, DOI: 10.1016/j.ygeno.2021.03.018.

Cheng, A, Jacups, S, Gal, D, Mayo, M & Currie, B 2006, 'Extreme weather events and environmental contamination are associated with case-clusters of melioidosis in the Northern Territory of Australia', *International Journal of Epidemiology*, vol. 35, no. 2, pp. 323-9, viewed 2 September 2021, DOI: 10.1093/ije/dyi271.

Chewapreecha, C, Holden, MTG, Vehkala, M, Valimaki, N, Yang, ZR, Harris, SR, Mather, AE, Tuanyok, A, De Smet, B, Le Hello, S, Bizet, C, Mayo, M, Wuthiekanun, V, Limmathurotsakul, D, Phetsouvanh, R, Spratt, BG, Corander, J, Keim, P, Dougan, G, Dance, DAB, Currie, BJ, Parkhill, J & Peacock, SJ 2017, 'Global and regional dissemination and evolution of *Burkholderia pseudomallei*', *Nature Microbiology*, vol. 2, no. 4, DOI: 10.1038/nmicrobiol.2016.263.

Chu, HY, Gao, GF, Ma, YY, Fan, KK & Delgado-Baquerizo, M 2020, 'Soil microbial biogeography in a changing world: recent advances and future perspectives', *mSystems*, vol. 5, no. 2, viewed 17 July 2022, DOI: 10.1128/mSystems.00803-19.

Clausen, PTLC, Aarestrup, FM & Lund, O 2018, 'Rapid and precise alignment of raw reads against redundant databases with KMA', *BMC Bioinformatics*, vol. 19, no. 1, p. 307, DOI: 10.1186/s12859-018-2336-6.

Clesceri, L, Eaton, A, Greenberg, A & Franson, MA 1996, *Standard methods for the examination of water and wastewater*, 19 edn, American Public Health Association, Washington DC.

Corkeron, ML, Norton, R & Nelson, PN 2010, 'Spatial analysis of melioidosis distribution in a suburban area', *Epidemiology and Infection*, vol. 138, no. 9, pp. 1346-52, DOI: 10.1017/S0950268809991634.

Currie, B & Jacups, S 2003, 'Intensity of rainfall and severity of melioidosis, Australia', *Emerging Infectious Diseases*, vol. 9, no. 12, pp. 1538–42, viewed 1 September 2021, DOI: 10.3201/eid0912.020750.

Currie, BJ, Gal, D, Mayo, M, Ward, L, Godoy, D, Spratt, BG & LiPuma, JJ 2007, 'Using BOX-PCR to exclude a clonal outbreak of melioidosis', *BMC Infectious Diseases*, vol. 7, no. 1, p. 68, DOI: 10.1186/1471-2334-7-68.

Currie, BJ, Mayo, M, Ward, LM, Kaestli, M, Meumann, EM, Webb, JR, Woerle, C, Baird, RW, Price, RN, Marshall, CS, Ralph, AP, Spencer, E, Davies, J, Huffam, SE, Janson, S, Lynar, S, Markey, P, Krause, VL & Anstey, NM 2021, 'The Darwin prospective melioidosis study: a 30-year prospective, observational investigation', *Lancet Infectious Diseases*, vol. 21, no. 12, pp. 1737-46, DOI: 10.1016/S1473-3099(21)00022-0.

De Coster, W, D'Hert, S, Schultz, DT, Cruts, M & Van Broeckhoven, C 2018, 'NanoPack: visualizing and processing long-read sequencing data', *Bioinformatics*, vol. 34, no. 15, pp. 2666-9, viewed 17 June 2024, DOI: 10.1093/bioinformatics/bty149.

Deen, J, Mengel, MA & Clemens, JD 2020, 'Epidemiology of cholera', *Vaccine*, vol. 38, pp. A31-A40, DOI: 10.1016/j.vaccine.2019.07.078.

Deepak, RN, Crawley, B & Phang, E 2008, '*Burkholderia pseudomallei* identification: a comparison between the API 20NE and VITEK2GN systems', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 102 Suppl 1, pp. S42-4, DOI: 10.1016/s0035-9203(08)70012-9.

Deka, MA, Vieira, AR & Bower, WA 2022, 'Modelling the ecological niche of naturally occurring anthrax at global and circumpolar extents using an ensemble modelling framework', *Transboundary and Emerging Diseases*, vol. 69, no. 5, pp. e2563-e77, DOI: 10.1111/tbed.14602.

Delgado-Baquerizo, M, Maestre, FT, Reich, PB, Trivedi, P, Osanai, Y, Liu, YR, Hamonts, K, Jeffries, TC & Singh, BK 2016, 'Carbon content and climate variability drive global soil bacterial diversity patterns', *Ecological Monographs*, vol. 86, no. 3, pp. 373-90, DOI: 10.1002/ecm.1216.

Dey, R, Lewis, SC, Arblaster, JM & Abram, NJ 2019, 'A review of past and projected changes in Australia's rainfall', *Wiley Interdisciplinary Reviews* vol. 10, no. 3, pp. e577-n/a, DOI: 10.1002/wcc.577.

Dickey, JR, Swenie, RA, Turner, SC, Winfrey, CC, Yaffar, D, Padukone, A, Beals, KK, Sheldon, KS & Kivlin, SN 2021, 'The utility of macroecological rules for microbial biogeography', *Frontiers in Ecology and Evolution*, vol. 9, viewed 20 July 2022, DOI: 10.3389/fevo.2021.633155.

Draper, A, Mayo, M, Harrington, G, Karp, D, Yinfoo, D, Ward, L, Haslem, A, Currie, B & Kaestli, M 2010, 'Association of the melioidosis agent *Burkholderia pseudomallei* with water parameters in rural water supplies in Northern Australia', *Applied and Environmental Microbiology*, vol. 76, no. 15, pp. 5305-7, viewed 27 January 2022, DOI: 10.1128/AEM.00287-10.

Duval, BD, Elrod, MG, Gee, JE, Chantratita, N, Tandhavanant, S, Limmathurotsakul, D & Hoffmaster, AR 2014, 'Evaluation of a latex agglutination assay for the identification of *Burkholderia pseudomallei* and *Burkholderia mallei*', *American Journal of Tropical Medicine and Hygiene*, vol. 90, no. 6, pp. 1043-6, DOI: 10.4269/ajtmh.14-0025.

Espelund, M & Klaveness, D 2014, 'Botulism outbreaks in natural environments – an update', *Frontiers in Microbiology*, vol. 5, DOI: 10.3389/fmicb.2014.00287.

Ewels, P, Magnusson, M, Lundin, S & Käller, M 2016, 'MultiQC: summarize analysis results for multiple tools and samples in a single report', *Bioinformatics*, vol. 32, no. 19, pp. 3047-8, viewed 17 June 2024, DOI: 10.1093/bioinformatics/btw354.

Ferreira, FA, Helmersen, K, Visnovska, T, Jørgensen, SB & Aamot, HV 2021, 'Rapid nanopore-based DNA sequencing protocol of antibiotic-resistant bacteria for use in surveillance and outbreak investigation', *Microbial Genomics*, vol. 7, no. 4, DOI: 10.1099/mgen.0.000557.

Freund, M, Henley, BJ, Karoly, DJ, Allen, KJ & Baker, PJ 2017, 'Multi-century cool- and warm-season rainfall reconstructions for Australia's major climatic regions', *Climate of the Past*, vol. 13, no. 12, pp. 1751-70, DOI: 10.5194/cp-13-1751-2017.

FT-020 2021, *Water microbiology, method 1: water quality - general requirements and guidance for microbiological examinations by culture (AS 4276.1:2021)*, 3 edn, Standards Australia, Australia.

FT-024 1991, *Food microbiology general procedures and techniques (AS 1766.1:1991)*, Standards Australia, Australia.

Ganeshalingam, V, Kaestli, M, Norton, RE & Gassiep, I 2023, 'The effect of climate on melioidosis incidence in Townsville, Australia: a dry tropical region', *Environmental Health and Preventive Medicine*, vol. 28, p. 33, DOI: 10.1265/ehpm.22-00177.

Gassiep, I, Armstrong, M & Norton, RE 2019, 'Identification of *Burkholderia pseudomallei* by use of the Vitek Mass Spectrometer', *Journal of Clinical Microbiology*, vol. 57, no. 5, DOI: 10.1128/jcm.00081-19.

Gassiep, I, Burnard, D, Permana, B, Bauer, MJ, Cuddihy, T, Forde, BM, Chatfield, MD, Ling, W, Norton, R & Harris, PNA 2023, 'Melioidosis Queensland: an analysis of clinical outcomes and genomic factors', *PLoS Neglected Tropical Diseases*, vol. 17, no. 10, p. e0011697, DOI: 10.1371/journal.pntd.0011697.

Gassiep, I, Chatfield, MD, Permana, B, Burnard, D, Bauer, MJ, Cuddihy, T, Forde, BM, Mayer-Coverdale, J, Norton, RE & Harris, PNA 2024, 'The genomic epidemiology of clinical *Burkholderia pseudomallei* isolates in North Queensland, Australia', *Pathogens*, vol. 13, no. 7, DOI: 10.3390/pathogens13070584.

Gassiep, I, Ganeshalingam, V, Chatfield, MD, Harris, PNA & Norton, RE 2022, 'The epidemiology of melioidosis in Townsville, Australia', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 116, no. 4, pp. 328-35, DOI: 10.1093/trstmh/trab125.

Gassiep, I, Grey, V, Thean, LJ, Farquhar, D, Clark, JE, Ariotti, L, Graham, R, Jennison, AV, Bergh, H, Anuradha, S, Dyer, W, James, C, Huang, A, Putt, E, Pakeerathan, V, Griffin, PM & Harris, PNA 2023, 'Expanding the geographic boundaries of melioidosis in Queensland, Australia', *American Journal of Tropical Medicine and Hygiene*, vol. 108, no. 6, pp. 1215-9, DOI: 10.4269/ajtmh.23-0002.

George, E, De Jesus, O & Vivekanandan, R 2023, *Clostridium tetani Infection*, StatPearls, StatPearls Publishing, <https://www.ncbi.nlm.nih.gov/books/NBK482484/>.

Gigante, CM, Yale, G, Condori, RE, Costa, NC, Long, NV, Minh, PQ, Chuong, VD, Tho, ND, Thanh, NT, Thin, NX, Hanh, NTH, Wambura, G, Ade, F, Mito, O, Chuchu, V, Muturi, M, Mwatondo, A, Hampson, K, Thumbi, SM, Thomae, BG, de Paz, VH, Meneses, S, Munyua, P, Moran, D, Cadena, L, Gibson, A, Wallace, RM, Pieracci, EG & Li, Y 2020, 'Portable Rabies Virus sequencing in Canine Rabies endemic countries using the Oxford Nanopore MinION', *Viruses*, vol. 12, no. 11, p. 1255, DOI: 10.3390/v12111255.

Godoy, D, Randle, G, Simpson, AJ, Aanensen, DM, Pitt, TL, Kinoshita, R & Spratt, BG 2003, 'Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*', *Journal of Clinical Microbiology*, vol. 41, no. 5, pp. 2068-79, DOI: 10.1128/jcm.41.5.2068-2079.2003.

Goodrick, I, Todd, G & Stewart, J 2018, 'Soil characteristics influencing the spatial distribution of melioidosis in far North Queensland, Australia', *Epidemiology and Infection*, vol. 146, no. 12, pp. 1602-7, DOI: 10.1017/S0950268818001188.

Greig, DR, Jenkins, C, Gharbia, S & Dallman, TJ 2019, 'Comparison of single-nucleotide variants identified by Illumina and Oxford Nanopore technologies in the context of a potential outbreak of Shiga toxin-producing *Escherichia coli*', *GigaScience*, vol. 8, no. 8, DOI: 10.1093/gigascience/giz104.

Hampton, V, Kaestli, M, Mayo, M, Choy, JL, Harrington, G, Richardson, L, Benedict, S, Noske, R, Garnett, ST, Godoy, D, Spratt, BG & Currie, BJ 2011, 'Melioidosis in birds and *Burkholderia pseudomallei* dispersal, Australia', *Emerging Infectious Diseases*, vol. 17, no. 7, pp. 1310-2, DOI: 10.3201/eid1707.100707.

Heather, JM & Chain, B 2016, 'The sequence of sequencers: The history of sequencing DNA', *Genomics*, vol. 107, no. 1, pp. 1-8, DOI: 10.1016/j.ygeno.2015.11.003.

Hinjoy, S, Hantrakun, V, Kongyu, S, Kaewrakmuk, J, Wangrangsimakul, T, Jitsuronk, S, Saengchun, W, Bhengsri, S, Akarachotpong, T, Thamthitiwat, S, Sangwichian, O, Anunnatsiri, S, Sermswan, RW, Lertmemongkolchai, G, Tharinjaroen, CS, Preechasuth, K, Udpaun, R, Chuensombut, P,

Waranyasirikul, N, Anudit, C, Narenpitak, S, Jutrakul, Y, Teparrukkul, P, Teerawattanasook, N, Thanvisej, K, Suphan, A, Sukbut, P, Ploddi, K, Sirichotirat, P, Chiewchanyon, B, Rukseree, K, Hongsuwan, M, Wongsuwan, G, Sunthornsut, P, Wuthiekanun, V, Sachaphimukh, S, Wannapinij, P, Chierakul, W, Chewapreecha, C, Thaipadungpanit, J, Chantratita, N, Korbsrisate, S, Taunyok, A, Dunachie, S, Palittapongarnpim, P, Sirisinha, S, Kitphati, R, Iamsirithaworn, S, Chaowagul, W, Chetchotisak, P, Whistler, T, Wongratanacheewin, S, Limmathurotsakul, D & Network, TM 2018, 'Melioidosis in Thailand: present and future', *Tropical Medicine and Infectious Disease*, vol. 3, no. 2, DOI: 10.3390/tropicalmed3020038.

Hoffmaster, A, AuCoin, D, Baccam, P, Baggett, H, Baird, R, Bhengsri, S, Blaney, D, Brett, P, Brooks, TJG, Brown, K, Chantratita, N, Cheng, A, Dance, DAB, Decuypere, S, Defenbaugh, D, Gee, J, Houghton, R, Jorakate, P, Lertmemongkolchai, G, Limmathurotsakul, D, Merlin, T, Mukhopadhyay, C, Norton, R, Peacock, S, Rolim, D, Simpson, A, Steinmetz, I, Stoddard, R, Stokes, M, Sue, D, Tuanyok, A, Whistler, T, Wuthiekanun, V & Walke, H 2015, 'Melioidosis diagnostic workshop, 2013', *Emerging Infectious Disease Journal*, vol. 21, no. 2, DOI: 10.3201/eid2102.141045.

Illumina 2024, *iSeq 100 sequencing system*, Illumina, viewed 9 July 2024, <https://www.illumina.com/systems/sequencing-platforms/iseq.html>.

Inglis, TJJ & Sagripanti, JL 2006, 'Environmental factors that affect the survival and persistence of *Burkholderia pseudomallei*', *Applied and Environmental Microbiology*, vol. 72, no. 11, pp. 6865-75, DOI: 10.1128/Aem.01036-06.

IPCC 2018, *Summary for policymakers: 2018*, Cambridge, UK and New York, NY, USA, DOI 10.1017/9781009157940.001., <https://www.ipcc.ch/sr15/chapter/spm/>.

IPCC 2022, *Summary for policymakers: 2022*, Cambridge, UK and New York, NY, USA, DOI 10.1017/9781009325844.001.

Jitprasutwit, N, Rungruengkitkun, A, Lohitthai, S, Reamtong, O, Indrawattana, N, Sookrung, N, Sricharunrat, T, Sukphopetch, P, Chatratita, N & Pumirat, P 2023, '*In vitro* roles of *Burkholderia* intracellular motility A (BimA) in infection of human neuroblastoma cell line', *Microbiology Spectrum*, vol. 11, no. 4, DOI: 10.1128/spectrum.01320-23.

Jolley, KA, Bray, JE & Maiden, MCJ 2018, 'Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications', *Wellcome Open Research*, vol. 3, p. 124, DOI: 10.12688/wellcomeopenres.14826.1.

Kaestli, M, Grist, EPM, Ward, L, Hill, A, Mayo, M & Currie, BJ 2016, 'The association of melioidosis with climatic factors in Darwin, Australia: a 23-year time-series analysis', *Journal of Infection*, vol. 72, no. 6, pp. 687-97, DOI: 10.1016/j.jinf.2016.02.015.

Kaestli, M, Harrington, G, Mayo, M, Chatfield, M, Harrington, I, Hill, A, Munksgaard, N, Gibb, K & Currie, B 2015, 'What drives the occurrence of the melioidosis bacterium *Burkholderia pseudomallei* in domestic gardens?', *PLOS Neglected Tropical Diseases*, vol. 9, no. 3, p. e0003635, viewed 1 September 2021, DOI: 10.1371/journal.pntd.0003635.

Kaestli, M, Mayo, M, Harrington, G, Ward, L, Watt, F, Hill, J, Cheng, A & Currie, B 2009, 'Landscape changes influence the occurrence of the melioidosis bacterium *Burkholderia pseudomallei*

in soil in Northern Australia', *PLoS Neglected Tropical Diseases*, vol. 3, no. 1, p. 364, viewed 1 September 2021, DOI: 10.1371/journal.pntd.0000364.

Kalyaanamoorthy, S, Minh, BQ, Wong, TKF, Von Haeseler, A & Jermiin, LS 2017, 'ModelFinder: fast model selection for accurate phylogenetic estimates', *Nature Methods*, vol. 14, no. 6, pp. 587-9, DOI: 10.1038/nmeth.4285.

Katoh, K, Kuma, KI, Toh, H & Miyata, T 2005, 'MAFFT version 5: improvement in accuracy of multiple sequence alignment', *Nucleic Acids Research*, vol. 33, no. 2, pp. 511-8, viewed 17 June 2024, DOI: 10.1093/nar/gki198.

Katoh, K, Misawa, K, Kuma, KI & Miyata, T 2002, 'MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform', *Nucleic Acids Research*, vol. 30, no. 14, pp. 3059-66, viewed 17 June 2024, DOI: 10.1093/nar/gkf436.

Katoh, K & Standley, DM 2013, 'MAFFT multiple sequence alignment software version 7: improvements in performance and usability', *Molecular Biology and Evolution*, vol. 30, no. 4, pp. 772-80, viewed 17 June 2024, DOI: 10.1093/molbev/mst010.

Kim, H-M, Jeon, S, Chung, O, Jun, JH, Kim, H-S, Blazyte, A, Lee, H-Y, Yu, Y, Cho, YS, Bolser, DM & Bhak, J 2021, 'Comparative analysis of 7 short-read sequencing platforms using the Korean Reference Genome: MGI and Illumina sequencing benchmark for whole-genome sequencing', *GigaScience*, vol. 10, no. 3, viewed 10/10/2024, DOI: 10.1093/gigascience/giab014.

Korostin, D, Kulemin, N, Naumov, V, Belova, V, Kwon, D & Gorbachev, A 2020, 'Comparative analysis of novel MGISEQ-2000 sequencing platform vs Illumina HiSeq 2500 for whole-genome sequencing', *PLoS ONE*, vol. 15, no. 3, p. e0230301, DOI: 10.1371/journal.pone.0230301.

Larsen, E, Smith, JJ, Norton, R & Corkeron, M 2013, 'Survival, sublethal injury, and recovery of environmental *Burkholderia pseudomallei* in soil subjected to desiccation', *Applied and Environmental Microbiology*, vol. 79, no. 7, pp. 2424-7, DOI: 10.1128/Aem.03168-12.

Larsen, MV, Cosentino, S, Rasmussen, S, Friis, C, Hasman, H, Marvig, RL, Jelsbak, L, Sicheritz-Pontén, T, Ussery, DW, Aarestrup, FM & Lund, O 2012, 'Multilocus sequence typing of total-genome-sequenced bacteria', *Journal of Clinical Microbiology*, vol. 50, no. 4, pp. 1355-61, DOI: 10.1128/jcm.06094-11.

Limmathurotsakul, D, Dance, DA, Wuthiekanun, V, Kaestli, M, Mayo, M, Warner, J, Wagner, DM, Tuanyok, A, Wertheim, H, Yoke Cheng, T, Mukhopadhyay, C, Puthucheary, S, Day, NP, Steinmetz, I, Currie, BJ & Peacock, SJ 2013, 'Systematic review and consensus guidelines for environmental sampling of *Burkholderia pseudomallei*', *PLoS Neglected Tropical Diseases*, vol. 7, no. 3, p. e2105, DOI: 10.1371/journal.pntd.0002105.

Limmathurotsakul, D, Golding, N, Dance, D, Messina, J, Pigott, D, Moyes, C, Rolim, D, Bertherat, E, Day, N, Peacock, S & Hay, S 2016, 'Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis', *Nature Microbiology*, vol. 1, viewed 1 September 2021, DOI: 10.1038/nmicrobiol.2015.8.

Limmathurotsakul, D, Kanoksil, M, Wuthiekanun, V, Kitphati, R, deStavola, B, Day, NP & Peacock, SJ 2013, 'Activities of daily living associated with acquisition of melioidosis in Northeast Thailand: a matched case-control study', *PLoS Neglected Tropical Diseases*, vol. 7, no. 2, p. e2072, DOI: 10.1371/journal.pntd.0002072.

Linde, J, Brangsch, H, Hölzer, M, Thomas, C, Elschner, MC, Melzer, F & Tomaso, H 2023, 'Comparison of Illumina and Oxford Nanopore Technology for genome analysis of *Francisella tularensis*, *Bacillus anthracis*, and *Brucella suis*', *BMC Genomics*, vol. 24, no. 1, DOI: 10.1186/s12864-023-09343-z.

Lowe, P, Haswell, H & Lewis, K 2006, 'Use of various common isolation media to evaluate the new VITEK 2 colorimetric GN Card for identification of *Burkholderia pseudomallei*', *Journal of Clinical Microbiology*, vol. 44, no. 3, pp. 854-6, DOI: 10.1128/jcm.44.3.854-856.2006.

Mayo, M, Kaesti, M, Harrington, G, Cheng, A, Ward, L, Karp, D, Jolly, P, Godoy, D, Spratt, B & Currie, B 2011, '*Burkholderia pseudomallei* in unchlorinated domestic bore water, tropical Northern Australia', *Emerging Infectious Diseases*, vol. 17, no. 7, pp. 1283-5, viewed 27 January 2022, DOI: 10.3201/eid1707.100614.

McRobb, E, Kaestli, M, Price, EP, Sarovich, DS, Mayo, M, Warner, J, Spratt, BG & Currie, BJ 2014, 'Distribution of *Burkholderia pseudomallei* in Northern Australia, a land of diversity', *Applied and Environmental Microbiology*, vol. 80, no. 11, pp. 3463-8, DOI: doi:10.1128/AEM.00128-14.

McRobb, E, Sarovich, DS, Price, EP, Kaestli, M, Mayo, M, Keim, P & Currie, BJ 2015, 'Tracing melioidosis back to the source: using whole-genome sequencing to investigate an outbreak originating from a contaminated domestic water supply', *Journal of Clinical Microbiology*, vol. 53, no. 4, pp. 1144-8, DOI: 10.1128/jcm.03453-14.

Meumann, EM, Kaestli, M, Mayo, M, Ward, L, Rachlin, A, Webb, JR, Kleinecke, M, Price, EP & Currie, BJ 2021, 'Emergence of *Burkholderia pseudomallei* sequence type 562, Northern Australia', *Emerging Infectious Diseases*, vol. 27, no. 4, pp. 1057-67, DOI: 10.3201/eid2704.202716.

Meumann, EM, Limmathurotsakul, D, Dunachie, SJ, Wiersinga, WJ & Currie, BJ 2023, '*Burkholderia pseudomallei* and melioidosis', *Nature Reviews Microbiology*, DOI: 10.1038/s41579-023-00972-5.

Ngamsang, R, Potisap, C, Boonmee, A, Lawongsa, P, Chaianunporn, T, Wongratanacheewin, S, Rodrigues, JLM & Sermswan, RW 2015, 'The contribution of soil physicochemical properties to the presence and genetic diversity of *Burkholderia pseudomallei*', *Southeast Asian Journal of Tropical Medicine and Public Health*, vol. 46, no. 1, pp. 38-50, <https://escholarship.org/content/qt5j08r2zn/qt5j08r2zn.pdf?t=p7pf22>.

Nguyen, L-T, Schmidt, HA, Von Haeseler, A & Minh, BQ 2014, 'IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies', *Molecular Biology and Evolution*, vol. 32, no. 1, pp. 268-74, viewed 19 June 2024, DOI: 10.1093/molbev/msu300.

Northern Territory Health 2021, *Melioidosis*, Northern Territory Government, viewed 6 August 2023, <https://health.nt.gov.au/__data/assets/pdf_file/0006/1303386/melioidosis-nt-health-factsheet.pdf>.

O'Brien, DP, Jeanne, I, Blasdell, K, Avumegah, M & Athan, E 2019, 'The changing epidemiology worldwide of *Mycobacterium ulcerans*', *Epidemiology and Infection*, vol. 147, DOI: 10.1017/S0950268818002662.

O'Donnell, AJ, Cook, ER, Palmer, JG, Turney, CSM, Page, GFM & Grierson, PF 2015, 'Tree rings show recent high Summer-Autumn precipitation in Northwest Australia is unprecedented within the last two centuries', *PLoS ONE*, vol. 10, no. 6, p. e0128533, DOI: 10.1371/journal.pone.0128533.

Ong, CEL, Wongsuvan, G, Chew, JSW, Kim, TY, Teng, LH, Amornchai, P, Wuthiekanun, V, Day, NPJ, Peacock, SJ, Cheng, TY, Yap, EPH & Limmathurotsakul, D 2017, 'Presence of *Burkholderia pseudomallei* in soil and paddy rice water in a rice field in Northeast Thailand, but not in air and rainwater', *Am J Trop Med Hyg*, vol. 97, no. 6, pp. 1702-5, DOI: 10.4269/ajtmh.17-0515.

ONT 2024, *Ligation sequencing amplicons - Native Barcoding Kit 24 V14 (SQK_NBD114.24)*, viewed 19 September 2023, <https://nanoporetech.com/document/ligation-sequencing-amplicons-native-barcoding-v14-sqk-nbd114-24#overview-of-the-protocol>.

ONT 2024, *MinION Mk1B starter pack*, viewed 12 February 2024, <https://store.nanoporetech.com/minion.html>.

ONT 2024, *MinION Mk1C manual*, viewed 9 September 2023, <https://nanoporetech.com/document/minion-mk1c-user-manual#minion-mk-1c-hardware-check>.

ONT 2024, *MinKNOW protocol*, viewed 9 September 2023, <https://nanoporetech.com/document/experiment-companion-minknow#introduction-to-local-bas>.

Palasatien, S, Lertsirivorakul, R, Royros, P, Wongratanacheewin, S & Sermswan, RW 2008, 'Soil physicochemical properties related to the presence of *Burkholderia pseudomallei*', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 102 Suppl 1, pp. S5-9, DOI: 10.1016/S0035-9203(08)70003-8.

Peacock, SJ, Schweizer, HP, Dance, DA, Smith, TL, Gee, JE, Wuthiekanun, V, DeShazer, D, Steinmetz, I, Tan, P & Currie, BJ 2008, 'Management of accidental laboratory exposure to *Burkholderia pseudomallei* and *B. mallei*', *Emerging Infectious Diseases*, vol. 14, no. 7, p. e2, DOI: 10.3201/eid1407.071501.

Pearson, T, Giffard, P, Beckstrom-Sternberg, S, Auerbach, R, Hornstra, H, Tuanyok, A, Price, EP, Glass, MB, Leadem, B, Beckstrom-Sternberg, JS, Allan, GJ, Foster, JT, Wagner, DM, Okinaka, RT, Sim, SH, Pearson, O, Wu, Z, Chang, J, Kaul, R, Hoffmaster, AR, Brettin, TS, Robison, RA, Mayo, M, Gee, JE, Tan, P, Currie, BJ & Keim, P 2009, 'Phylogeographic reconstruction of a bacterial species with high levels of lateral gene transfer', *BMC Biology*, vol. 7, no. 1, p. 78, DOI: 10.1186/1741-7007-7-78.

Podin, Y, Sarovich, DS, Price, EP, Kaestli, M, Mayo, M, Hii, K, Ngian, H, Wong, S, Wong, I, Wong, J, Mohan, A, Ooi, M, Fam, T, Wong, J, Tuanyok, A, Keim, P, Giffard, PM & Currie, BJ 2014, '*Burkholderia pseudomallei* isolates from Sarawak, Malaysian Borneo, are predominantly susceptible to aminoglycosides and macrolides', *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 1, pp. 162-6, DOI: 10.1128/aac.01842-13.

Pongmala, K, Pierret, A, Oliva, P, Pando, A, Davong, V, Rattanavong, S, Silvera, N, Luangraj, M, Boithias, L, Xayyathip, K, Menjot, L, Macouin, M, Rochelle-Newall, E, Robain, H, Vongvixay, A, Simpson, AJH, Dance, DAB & Ribolzi, O 2022, 'Distribution of *Burkholderia pseudomallei* within a 300-cm deep soil profile: implications for environmental sampling', *Scientific Reports*, vol. 12, no. 1, DOI: 10.1038/s41598-022-12795-0.

Powell, JR, Karunaratne, S, Campbell, CD, Yao, HY, Robinson, L & Singh, BK 2015, 'Deterministic processes vary during community assembly for ecologically dissimilar taxa', *Nature Communications*, vol. 6, DOI: 10.1038/ncomms9444.

Price, EP, Currie, BJ & Sarovich, DS 2017, 'Genomic insights into the melioidosis pathogen, *Burkholderia pseudomallei*', *Current Tropical Medicine Reports*, vol. 4, no. 3, pp. 95-102, DOI: 10.1007/s40475-017-0111-9.

Promega 2024, *Wizard® SV Gel and PCR Clean-Up system*, viewed 19 September 2023, <https://www.promega.com.au/products/nucleic-acid-extraction/clean-up-and-concentration/wizard-sv-gel-and-pcr-clean-up-system/?catNum=A9281#protocols>.

Queensland Health 2010, *Melioidosis*, Queensland Government viewed 10 July 2023, <https://www.health.qld.gov.au/cdcg/index/melioidosis>.

Queensland Health 2023, *Notifiable conditions annual reporting*, Queensland Government, viewed 7 August 2023, <https://www.health.qld.gov.au/clinical-practice/guidelines-procedures/diseases-infection/surveillance/reports/notifiable/annual>.

Rachlin, A, Luangraj, M, Kaestli, M, Rattanavong, S, Phoumin, P, Webb, JR, Mayo, M, Currie, B & Dance, DAB 2021, 'Using land runoff to survey the distribution and genetic diversity of *Burkholderia pseudomallei* strains in Vientiane, Laos', *Applied and Environmental Microbiology*, vol. 87, no. 4, DOI: 10.1128/AEM.02112-20.

Rachlin, A, Mayo, M, Webb, JR, Kleinecke, M, Rigas, V, Harrington, G, Currie, BJ & Kaestli, M 2020, 'Whole-genome sequencing of *Burkholderia pseudomallei* from an urban melioidosis hot spot reveals a fine-scale population structure and localised spatial clustering in the environment', *Scientific Reports*, vol. 10, no. 1, DOI: 10.1038/s41598-020-62300-8.

Rhodes, KA & Schweizer, HP 2016, 'Antibiotic resistance in *Burkholderia* species', *Drug Resistance Updates*, vol. 28, pp. 82-90, DOI: 10.1016/j.drup.2016.07.003.

Ritchie, G, Chorlton, SD, Matic, N, Bilawka, J, Gowland, L, Leung, V, Stefanovic, A, Romney, MG & Lowe, CF 2023, 'WGS of a cluster of MDR *Shigella sonnei* utilizing Oxford Nanopore R10.4.1 long-read sequencing', *Journal of Antimicrobial Chemotherapy*, DOI: 10.1093/jac/dkad346.

Roche Life Science 2023, *High pure PCR template preparation kit*, viewed 23 November 2023, <https://elabdoc-prod.roche.com/eLD/api/downloads/0d2a87a7-1e7a-ee11-2291-005056a71a5d?countryIsoCode=XG>.

Sagripanti, JL, Levy, A, Robertson, J, Merritt, A & Inglis, TJJ 2009, 'Inactivation of virulent *Burkholderia pseudomallei* by sunlight', *Photochemistry and Photobiology*, vol. 85, no. 4, pp. 978-86, DOI: 10.1111/j.1751-1097.2008.00518.x.

Sarovich, DS, Price, EP, Webb, JR, Ward, LM, Voutsinos, MY, Tuanyok, A, Mayo, M, Kaestli, M & Currie, BJ 2014, 'Variable virulence factors in *Burkholderia pseudomallei* (Melioidosis) associated with human disease', *PLoS ONE*, vol. 9, no. 3, DOI: 10.1371/journal.pone.0091682.

Savelkoel, J, Dance, D, Currie, B, Limmathurotsakul, D & Wiersinga, J 2021, 'A call to action: time to recognise melioidosis as a neglected tropical disease', *Lancet Infectious Diseases*, pp. 1-7, viewed 7 January 2022, DOI: 10.1016/S1473-3099(21)00394-7.

Sermswan, RW, Royrosa, P, Khakhum, N, Wongratanacheewin, S & Tuanyok, A 2015, 'Direct detection of *Burkholderia pseudomallei* and biological factors in soil', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 109, no. 7, pp. 462-8, DOI: 10.1093/trstmh/trv040.

Shendure, J, Balasubramanian, S, Church, GM, Gilbert, W, Rogers, J, Schloss, JA & Waterston, RH 2017, 'DNA sequencing at 40: past, present and future', *Nature*, vol. 550, no. 7676, pp. 345-53, DOI: 10.1038/nature24286.

Smith, S, Hanson, J & Currie, B 2018, 'Melioidosis: an Australian perspective', *Tropical Medicine and Infectious Disease*, vol. 3, no. 1, p. 27, viewed 1 September 2021, DOI: 10.3390/tropicalmed3010027.

Smith, S, Horne, P, Rubenach, S, Gair, R, Stewart, J, Fairhead, L & Hanson, J 2021, 'Increased incidence of melioidosis in far North Queensland, Queensland, Australia, 1998-2019', *Emerging Infectious Diseases*, vol. 27, no. 12, pp. 3119-23, DOI: 10.3201/eid2712.211302.

Smith, S, Marquardt, T, Jennison, AV, D'Addona, A, Stewart, J, Yarwood, T, Ho, J, Binotto, E, Harris, J, Fahmy, M, Esmonde, J, Richardson, M, Graham, RMA, Gair, R, Ariotti, L, Preston-Thomas, A, Rubenach, S, O'Sullivan, S, Allen, D, Ragh, T, Grayson, S, Manoy, S, Warner, JM, Meumann, EM, Robson, JM & Hanson, J 2023, 'Clinical manifestations and genomic evaluation of melioidosis outbreak among children after sporting event, Australia', *Emerging Infectious Diseases*, vol. 29, no. 11, pp. 2218-28, DOI: 10.3201/eid2911.230951.

Solano-Rivera, V, Geris, J, Granados-Bolanos, S, Brenes-Cambronero, L, Artavia-Rodriguez, G, Sanchez-Murillo, R & Birkel, C 2019, 'Exploring extreme rainfall impacts on flow and turbidity dynamics in a steep, pristine and tropical volcanic catchment', *Catena*, vol. 182, DOI: 10.1016/j.catena.2019.104118.

Stewart, JD, Smith, S, Binotto, E, McBride, WJ, Currie, BJ & Hanson, J 2017, 'The epidemiology and clinical features of melioidosis in Far North Queensland: Implications for patient management', *PLoS Neglected Tropical Diseases*, vol. 11, no. 3, p. e0005411, DOI: 10.1371/journal.pntd.0005411.

Suebrasri, T, Wang-ngarm, S, Chareonsudjai, P, Sermswan, R & Chareonsudjai, S 2013, 'Seasonal variation of soil environmental characteristics affect the presence of *Burkholderia pseudomallei* in Khon Kaen, Thailand', *African Journal of Microbiology Research*, vol. 7, pp. 1940-5, DOI: 10.5897/AJMR2012.2335.

Sullivan, RP, Marshall, CS, Anstey, NM, Ward, L & Currie, BJ 2020, '2020 Review and revision of the 2015 Darwin melioidosis treatment guideline; paradigm drift not shift', *PLoS Neglected Tropical Diseases*, vol. 14, no. 9, p. e0008659, DOI: 10.1371/journal.pntd.0008659.

Suntornsut, P, Wongsuwan, N, Malasit, M, Kitphati, R, Michie, S, Peacock, SJ & Limmathurotsakul, D 2016, 'Barriers and recommended interventions to prevent melioidosis in Northeast Thailand: a focus group study using the behaviour change wheel', *PLoS Neglected Tropical Diseases*, vol. 10, no. 7, DOI: 10.1371/journal.pntd.0004823.

The Systematics Association 2011, *Biogeography of microscopic organisms: is everything small everywhere?*, ed. D Fontaneto, vol. 79, Cambridge University Press, America, <https://books.google.com.au/books?hl=en&lr=&id=QdcLHCPgG-wC&oi=fnd&pg=PR1&dq=prokaryote+biogeography+theory&ots=Qo2NAOvAUW&sig=-UrgOiWhhWyuFb8qnYQE3MWqzq0#v=onepage&q&f=false>

Thomas, A, Forbes-Faulkner, J & Parker, M 1979, 'Isolation of *Pseudomonas pseudomallei* from clay layers at defined depths', *American Journal of Epidemiology*, vol. 110, no. 4, pp. 515-21, viewed 22 May 2023, DOI: 10.1093/oxfordjournals.aje.a112832.

Tran, QTL, Phan, PH, Bui, LNH, Bui, HTV, Hoang, NTB, Tran, DM & Trinh, TT 2022, 'Child melioidosis deaths caused by *Burkholderia pseudomallei* - contaminated borehole water, Vietnam, 2019', *Emerging Infectious Diseases*, vol. 28, no. 8, DOI: 10.3201/eid2808.220113.

Trinh, TT, Hoang, TS, Tran, DA, Trinh, VT, Göhler, A, Nguyen, TT, Hoang, SN, Krumkamp, R, Nguyen, LTN, May, J, Doan, PM, Do, CD, Que, TA & Steinmetz, I 2018, 'A simple laboratory algorithm for diagnosis of melioidosis in resource-constrained areas: a study from North-Central Vietnam', *Clinical Microbiology and Infection*, vol. 24, no. 1, pp. 84.e1-.e4, DOI: 10.1016/j.cmi.2017.07.029.

Tytgat, O, Skevin, S, Deforce, D & Van Nieuwerburgh, F 2022, 'Nanopore sequencing of a forensic combined STR and SNP multiplex', *Forensic Science International-Genetics*, vol. 56, DOI: 10.1016/j.fsigen.2021.102621.

Visvesvara, GS, Moura, H & Schuster, FL 2007, 'Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*', *FEMS Immunology and Medical Microbiology*, vol. 50, no. 1, pp. 1-26, DOI: 10.1111/j.1574-695X.2007.00232.x.

Wallace, AR 1863, 'On the physical geography of the Malay Archipelago', *Journal of the Royal Geographical Society of London*, vol. 33, pp. 217-34, viewed 16 August 2022, DOI: 10.2307/1798448.

Wang-Ngarm, S, Chareonsudjai, S & Chareonsudjai, P 2014, 'Physicochemical factors affecting the growth of *Burkholderia pseudomallei* in soil microcosm', *American Journal of Tropical Medicine and Hygiene*, vol. 90, no. 3, pp. 480-5, DOI: 10.4269/ajtmh.13-0446.

Wang, Y, Zhao, Y, Bollas, A, Wang, Y & Au, KF 2021, 'Nanopore sequencing technology, bioinformatics and applications', *Nature Biotechnology*, vol. 39, no. 11, pp. 1348-65, DOI: 10.1038/s41587-021-01108-x.

Watthanaworawit, W, Roberts, T, Hopkins, J, Gassiep, I, Norton, R, Robinson, MT, Silisouk, J, Sar, P, Sao, S, Amornchai, P, Limmathurotsakul, D, Wuthiekanun, V, Nosten, F, Simpson, AJH, Turner, P & Ling, CL 2021, 'A multi-country study using MALDI-TOF mass spectrometry for rapid identification of *Burkholderia pseudomallei*', *BMC Microbiology*, vol. 21, no. 1, p. 213, DOI: 10.1186/s12866-021-02276-1.

Webb, JR, Buller, N, Rachlin, A, Golledge, C, Sarovich, DS, Price, EP, Mayo, M & Currie, BJ 2020, 'A persisting nontropical focus of *Burkholderia pseudomallei* with limited genome evolution over five decades', *mSystems*, vol. 5, no. 6, DOI: 10.1128/mSystems.00726-20.

Webb, JR, Mayo, M, Rachlin, A, Woerle, C, Meumann, E, Rigas, V, Harrington, G, Kaestli, M & Currie, BJ 2022, 'Genomic epidemiology links *Burkholderia pseudomalle*i from individual human cases to *B. pseudomallei* from targeted environmental sampling in Northern Australia', *Journal of Clinical Microbiology*, vol. 60, no. 3, DOI: 10.1128/JCM.01648-21.

Whitmore, A & Krishnaswami, CS 1912, 'A hitherto undescribed infective disease in Rangoon', *Indian Medical Gazette*, vol. 47, no. 7, pp. 262-7, <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5168169/>.

Wiersinga, J, Virk, H, Torres, A, Currie, B, Peacock, S, Dance, D & Limmathurotsakul, D 2018, 'Melioidosis', *Nature Review Disease Primers*, vol. 4, viewed 28 October 2021, DOI: 10.1038/nrdp.2017.107.

Wiersinga, JW, Currie, BJ & Peacock, SJ 2012, 'Melioidosis', *New England Journal of Medicine*, vol. 367, no. 11, pp. 1035-44, DOI: 10.1056/NEJMra1204699.

Xian, Z, Li, S, Mann, DA, Huang, Y, Xu, F, Wu, X, Tang, S, Zhang, G, Stevenson, A, Ge, C & Deng, X 2022, 'Subtyping evaluation of *Salmonella Enteritidis* using SNP and core genome MLST with nanopore reads', *BioRxiv*, p. 2022.05.03.490560, DOI: 10.1101/2022.05.03.490560.

Xian, ZH, Li, ST, Mann, DA, Huang, YX, Xu, F, Wu, XW, Tang, SL, Zhang, GT, Stevenson, A, Ge, CT & Deng, XY 2022, 'Subtyping evaluation of *Salmonella enteritidis* using single nucleotide polymorphism and core genome multilocus sequence typing with nanopore reads', *Applied and Environmental Microbiology*, vol. 88, no. 15, DOI: 10.1128/aem.00785-22.

Yan, Y, Wu, K, Chen, J, Liu, HZ, Huang, Y, Zhang, Y, Xiong, J, Quan, WP, Wu, X, Liang, Y, He, KL, Jia, ZL, Wang, DP, Liu, D, Wei, HP & Chen, JJ 2021, 'Rapid acquisition of high-quality SARS-CoV-2 genome via amplicon-Oxford Nanopore sequencing', *Virologica Sinica*, vol. 36, no. 5, pp. 901-12, DOI: 10.1007/s12250-021-00378-8.

Yip, TW, Hewagama, S, Mayo, M, Price, EP, Sarovich, DS, Bastian, I, Baird, RW, Spratt, BG & Currie, BJ 2015, 'Endemic melioidosis in residents of desert region after atypically intense rainfall in Central Australia, 2011', *Emerging Infectious Diseases*, vol. 21, no. 6, pp. 1038-40, DOI: 10.3201/eid2106.141908.

Zhao, W, Zeng, W, Pang, B, Luo, M, Peng, Y, Xu, J, Kan, B, Li, Z & Lu, X 2023, 'Oxford nanopore long-read sequencing enables the generation of complete bacterial and plasmid genomes without short-read sequencing', *Frontiers in Microbiology*, vol. 14, DOI: 10.3389/fmicb.2023.1179966.

Zimmermann, A, Francke, T & Elsenbeer, H 2012, 'Forests and erosion: insights from a study of suspended-sediment dynamics in an overland flow-prone rainforest catchment', *Journal of Hydrology*, vol. 428, pp. 170-81, DOI: 10.1016/j.jhydrol.2012.01.039.

Zimmermann, RE, Ribolzi, O, Pierret, A, Rattanavong, S, Robinson, MT, Newton, PN, Davong, V, Auda, Y, Zopfi, J & Dance, DAB 2018, 'Rivers as carriers and potential sentinels for *Burkholderia pseudomallei* in Laos', *Scientific Reports*, vol. 8, DOI: 10.1038/s41598-018-26684-y.

Zinger, L, Taberlet, P, Schimann, H, Bonin, A, Boyer, F, De Barba, M, Gaucher, P, Gielly, L, Giguet-Covex, C, Iribar, A, Rejou-Mechain, M, Raye, G, Rioux, D, Schilling, V, Tymen, B, Viers, J, Zouiten, C, Thuiller, W, Coissac, E & Chave, J 2019, 'Body size determines soil community assembly in a tropical forest', *Molecular Ecology*, vol. 28, no. 3, pp. 528-43, DOI: 10.1111/mec.14919.

A Appendix

Table A.1 - Recipes for Ashdown Agar, Ashdown Broth, and Tryptic Soy Broth.

Ashdown Agar (8mg/L Gentamicin) (500 mL)	
Tryptone	6g
Technical Agar	6g
1% Aqueous Neutral Red	2mL
0.1% Crystal Violet	2mL
Glycerol	16mL
Distilled Water	495mL
Gentamicin Sulphate (8mg/mL)	0.5mL
Dissolve Tryptone and Technical Agar into Distilled Water. Add Neutral Red, Crystal Violet and Glycerol. Autoclave at 121^{o}C for 15min. Cool bottle in 56^{o}C dry water bath. Add Gentamicin Sulphate. Pour into sterile petri dishes.	
Ashdown Broth (50mg/L Colistin) (500mL)	
Tryptone	7.5g
0.1% Crystal Violet	2.5mL
Glycerol	20mL
Distilled Water	497.5mL
Colistin Sulfomethate (50mg/mL)	0.5mL
Dissolve Tryptone into Distilled Water. Add Crystal Violet and Glycerol. Autoclave at 121^{o}C for 15min. Cool bottle on bench at room temperature. Add Colistin Sulfomethate. Pour into sterile urine collection bottles.	
Tryptic Soy Broth and 20% Glycerol (500mL)	
Tryptic Soy Broth Powder	12g
Distilled Water	800mL
Glycerol	200mL
Dissolve Tryptic Soy Broth into Distilled Water. Add Glycerol. Autoclave at 121oC for 15min. Cool bottle on bench at room temperature. Aliquot 1mL volumes into 1mL sterile tubes.	

Table A.2 – Concentration of *B. pseudomallei* (CFU/mL) for each initial sampling location.

Initial Sampling Locations	***B. pseudomallei* Concentration (CFU/mL)**
Mt Stuart 26 Jan 2022	11
Mt Louisa 26 Jan 2022	226
Castle Hill 26 Jan 2022	0
Goondaloo Creek 26 Jan 2022	14
Goondaloo Creek 27 Jan 2022	154

Table A.3 – Roundtrip travel time to each of the initial sampling locations.

Initial Sampling Locations	**Roundtrip Travel Time by Car (minutes)**	**Roundtrip Travel Time by Walking (minutes)**
Mt Stuart	56	NA
Mt Louisa	30	NA
Castle Hill	50	NA
Goondaloo Creek	4	12

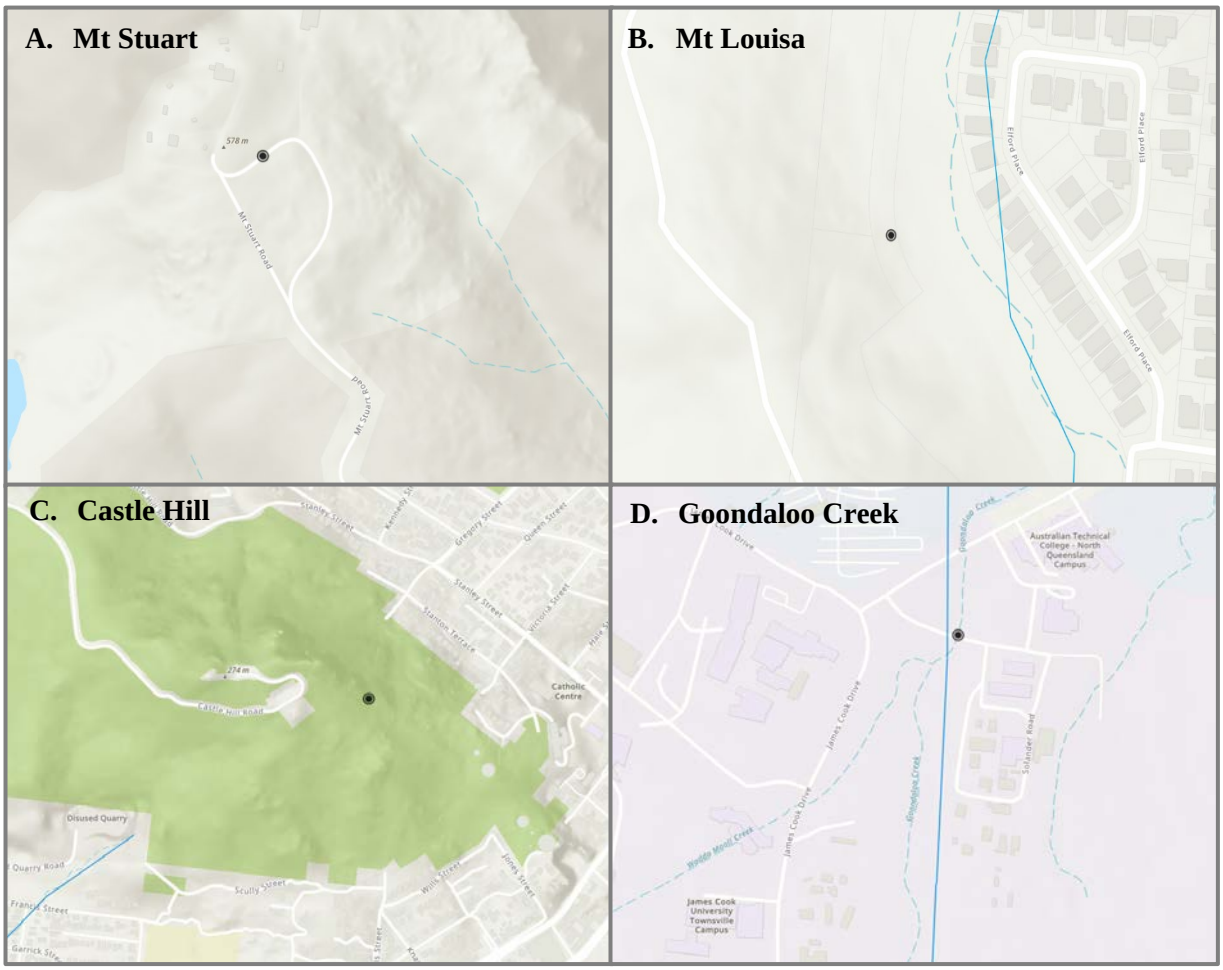

Figure A.1 – Initial sampling locations and their position relative to waterways to determine if collected water samples will represent a broad area. Major waterways are depicted as solid blue lines and minor waterways are shown as dotted blue lines. Initial sampling locations are shown as black circles. Maps created in ArcGIS Online.

Table A.4 - Direct plate count results from one-year longitudinal study of Goondaloo Creek.

Sample Date	**Total CFU/mL**	***Bps* CFU/mL**	**Sample Date**	**Total CFU/mL**	***Bps* CFU/mL**
26.1.22	822	14	21.10.22	1086	0
27.1.22	1593	154	26.10.22.1	141	0
2.2.22	29	0	26.10.22.2	148	0
3.2.22	273	0	26.10.22.3	393	0
4.2.22	343	24	2.11.22	132	0
9.2.22	20	0	22.11.22	422	3
16.2.22	2	0	23.11.22	992	1
23.2.22	343	0	28.11.22	556	22
2.3.22	112	0	30.11.22	431	0
9.3.22	2	0	1.12.22	149	8
16.3.22	7	0	7.12.22	51	0
22.4.22	1413	22	12.12.22	44	0
26.4.22	983	220	6.1.23	212	34
28.4.22	197	0	11.1.23	62	0
4.5.22	58	0	13.1.23	421	51
10.5.22	72	0	14.1.23	255	4
11.5.22	236	55	15.1.23	417	16
18.5.22	38	0	16.1.23	410	5
25.5.22	23	0	17.1.23	912	0
1.6.22	48	0	18.1.23	194	2
8.6.22	44	0	19.1.23	156	0
15.6.22	32	0	20.1.23	44	0
22.6.22	220	0	25.1.23	160	0
29.6.22	16	0	1.2.23	305	33
5.7.22	196	1	6.2.23	95	0
13.7.22	46	0	8.2.23	88	0
20.7.22	36	0	15.2.23	24	0
27.7.22	66	0	17.2.23	24	0
3.8.22	7	0	20.2.23	363	0
10.8.22	4	0	22.2.23	21	0
17.8.22	5	0			

Table A.5 - Daily rainfall (mm) data from January 2022 to February 2023 for Townsville Airport weather station (station number 032040, latitude: 19.25° S, longitude:146.77° E).

Day	Jan 2022	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 2023	Feb
1	0	0	0	16	0	0	0	0	0	0	0	27.8	9	31.4
2	0	0	0	0.2	0	0	0	0	0.2	0	0.2	0	12	13.4
3	22.4	0	0	0	0.2	0	0.4	0	10.4	0	0	0	0	2.6
4	0	92.4	0	0.2	0	0	12	0	0	0	0	0	0	25
5	0	5.2	0	0.2	0	0	36.4	0	0	0	0	0	0.4	64.8
6	0	0	0	0	0	0	1	0	0	0	0	0	50.2	81.2
7	16.8	0	0	0	0	24	0	0	0	0	0	0	0	2.2
8	0	7	0	0	3.8	0	0.2	0.4	0.6	0	0	0	0.2	11
9	0	0	0	0.2	7.8	0	0	0	0	0	0	0	0	0
10	0.2	0	0	0	12.4	0	0	0	0	0	0	0	0	0
11	2	0	0	0	102	0	0	0	0	0	0	0	0	0
12	0.2	0	0	0	19.6	0	0	0	0	0	0	0.8	0	0
13	0	0.2	0	0	5	0	0	0	0	0	0	0	30.8	0
14	0	2.8	36.4	0	0	0	0	0	0	0	0	0	32.4	0
15	0	0	0.2	0	0	0	0	0	0	0	0	0	160.2	0
16	0	5	0	0	0	0	0	0	0	0	5	0.8	14.2	25.2
17	0	0.2	0	0	0	0	0	0	0	0		5.6	14.4	8.2
18	0	6.2	0	0	0	0	0	0	0			8.2	70.6	1.2
19	5.6	0	0	0	0	0	0	0	0	0	0	2.4	0.8	30.4
20	0	0	0	0	0.6	0	0	0	0	0.2	0	0.2	0	2.6
21	0	0	0	0	1.6	0	0	0	0	20.4	0.2	0	0	2.2
22	0	0	0	95.6	0	0	0	0	0	8.4	87.2	0	0	0
23	0	0	0	4	0	0	0	0	0	0	43.6	0	0	2.4
24		0	0	0.2	0	0	0	0	0	0	0	0	0	3
25	0	0	0	15.6	0	0	0	0	0	0	0	25.2	0	0
26	84.8	0	2.4	153.2	0	0	0	0	0	1	0	1	0	0
27	172.2	0	0	8	0	0	0	0	0	7	79.6	0.6	6.8	0
28	18.6	1.2	0.4	0	0	0	0	0	0	4.6	62.2	0	3.6	0
29	14		0.2	0	0	0	0	0	10	0	0.2	0	5	
30	1.4		0	0	0	0	0	0	0	0	15	0	0	
31	0		0		0		0	0.6		0		0	0	

Table A.6 - Daily solar exposure (MJ m^{-2}) data from 20th January 2022 to 28th February 2023 for Townsville Airport weather station (station number 032040, latitude: 19.25° S, longitude:146.77° E).

Day	Jan 2022	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 2023	Feb
1		29.1	26.7	23.6	20.4	17.9	10.1	15.8	18.6	25.4	25.6	25.8	16.5	18.2
2		28.5	27.3	12.8	15.9	17.5	10.9	19	7.8	23.6	28.3	29.6	21.2	28.5
3		25.6	27.2	23.1	19.4	17.2	10.7	17.7	21.9	24.7	28.9	25.7	26.8	25.5
4		9.7	27	22.8	19.8	17.8	6.1	16.8	23	23	19.3	27.3	29.6	25.6
5		24.7	26.8	23.4	20	17.6	8.7	17.7	22.8	22.8	27.5	30	18.9	19.1
6		27.9	25.9	23	19.1	13.5	17.8	18.6	21.5	24.3	17.1	30	14.6	26.5
7		17	26.4	22.5	12	11.7	17.8	15	20	25	28.4	30	27.7	23.6
8		28.6	25.5	22.3	9.4	17.3	17.8	12.1	16.1	26.4	29	29.2	25.6	22.7
9		28.9	25	14	9.8	17.6	17.8	20.1	21.6	24.6	28.5	29	28.9	24.5
10		28	25.4	20.4	7.2	17.6	17.9	20	23.4	26	24.1	27	29.4	27.3
11		27.5	21.5	21.3	6.3	17.5	17.9	16.9	23.4	26	28.8	29.5	27.2	28.7
12		28.3	22.2	22.1	14	17.2	17.9	18.4	23.6	26.2		29.7	25.9	28.7
13		24.3	10.4	21.9	19.1	17.5	18	19.2	23.8	26.6	27.9	28.8	13	28.4
14		23.7	19	21.8	19.2	17.5	18	20.7	32.6	27	26.1	29.9	0.7	18.9
15		15.9	16.1	21.8	19.1	16.1	17.9	20.7	23.6	26.8	27.4	30.1	14.6	16
16			24.8	21.8	19	15	15.3	20.7	22.8	27	29.5	28.3	15.2	17.7
17		18.9	23.3	15.4	18.9	17.2	18.1	20.8	24	24.5	28	26.4	9.8	12.3
18		26.4	21.5	21.3	18.8	17.2	18	20.9	23.7	27.1	27.7	20.5	14.1	8.4
19		28.2	24.4	21.1	14.7	17.1	18.3	20.9	23.2	26.5	28.6	19.9	27.7	26.1
20	28.6	25.5	24.7	20.6	12.1	17.3	15	21.1	23.9	15.5	24.6	24.2	29.8	18.3
21	24.6	27.6	23.9	11.3	15.5	16.6	14.5	20.3	23.8	11.6	27.7	28.3	29.6	20.6
22	24.8	27.3	22.7	9.1	15.8	17.1	17.5	19.3	23.4	28.1	26.7	23	29.5	9.5
23	17.7	23.3	24.6	16.9	16.3	17.3	18.6	20.9	24.8	26.9	24.8	28.9	29.4	17.2
24	26.6	26.6	24.5	12.1	18.4	16.8	18.6	21.5	24.7	26	29.7	20.4	29.6	19.4
25	7.1	27.6	23.2	13.4	16.9	14.6	18.5	21.6	22.2	27.1	28.6	10.9	28.4	25.7
26	9	27.4	20.5	12.9	17.8	15.4	17.3	15.4	24.8	18.1	19.8	17.8	29.4	23.8
27	15.3	27.4	23.7	18.7	17.9	16.4	18.1	16.6	23.8	23.9	23.6	13.8	25.7	17.2
28	16.5	24.4	23.3	19.8	18.2	15.9	18.9	14.9	21.5	26	22.6	27.6	21.4	25.3
29	22.8		24	20	18.1	15.2	18.9	16.1	21.2	28.6	26.9	29.8	28.5	
30	29.1		23.9	20.4	18	9.7	18.6	10.6	25.5	27.5	16	29.4	29.4	
31	28.7		23.7		16.9		18.6	18.6		25.4		27.9	23.8	

Table A.7 - Daily temperature (°C) data from 20th January 2022 to 28th February 2023 for Townsville Airport weather station (station number 032040, latitude: 19.25° S, longitude:146.77° E).

Day	Jan 2022	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 2023	Feb
1		32.6	34.9	32.9	29.6	27.8	25.8	25.9	27.7	28.2	33.2	30.3	29.3	30.7
2		32.7	36.3	30.2	28.4	26.9	24.5	25.1	24.8	29.6	33.2	28.9	31.4	33.9
3		33.5	36.2	31.7	29.5	27.5	21.5	25	27.8	30.2	31.6	30	30.8	33.7
4		29.7	37.2	32.5	29.1	29.8	15.1	26.1	26.1	29.9	31	29.5	32	31
5		32.6	36.6	31.9	29.3	28.1	15.9	27	25.9	29.5	30.5	31.1	30.8	31.8
6		30.5	35.1	31.6	29.8	28.9	22	27.6	27.1	30.2	30.4	31.1	30.8	32
7		30.4	35.9	32.1	28.3	27	23.5	28.1	26.4	30.6	30.9	32.6	31.2	33.1
8		30.5	36.1	32.7	26.7	26.2	23.7	24.4	27.5	30.3	30.8	31	30.9	32.9
9		30.9	35.4	31.6	26.4	23.3	23.8	24.4	29	30.4	31	31.6	31.5	34
10		31.7	35.4	33.2	26.4	22.3	23.6	24.5	29.9	31.2	31	32	32.4	31.9
11		32	34.2	33.5	23.3	22.3	20.3	26.1	28.8	30.4	31	32.1	31.5	31.7
12		31.2	34.1	32.8	27.2	23.5	24	26.6	27	30.4	31.1	32.6	32.3	32.3
13		32.1	29.4	32.3	30	23.9	23.4	26.6	28.2	29.7	31.4	32.7	28.9	32.4
14		33.3	32	32.4	28.9	25.2	21.7	28.4	27.7	29.7	32.1	33.3	25.4	31.8
15		31.8	31.1	31	29.8	26.7	22.6	26.3	27.5	30.9	34	34.6	29.6	31.5
16		32.2	32.3	30.9	29.9	27.6	25.1	27.9	29	31		33.5	29.3	32.2
17		31.9	32.9	30.2	29.8	26.8	25.3	26.2	30.4	31.1		32.4	28.5	30
18		32.3	31.5	31.3	29.8	26.2	24.9	24.7	30.6	31.4	32.4	31.7	28.5	27.7
19		31.7	32.3	31	27.9	25.1	25.5	25.9	30.5	31.5	33.5	31.3	31.5	31.3
20	34.2	32.4	31.9	31.5	28.8	26.7	25.4	26.6	30.2	31.8	32.9	30.9	30.6	29.6
21	34.5	32.2	31.8	28.5	27.4	26.8	25.9	25.8	30.9	27.4	34	31.9	30.5	30.8
22	33.8	32.9	31.1	25.9	27.1	27.2	26	27	31.2	32.5	35.7	30.8	30.6	27.7
23	30.7	32.8	30.9	30.1	27.9	26.4	24.9	25.8	32.2	31.2	31	31.3	30.9	29.1
24	31.8	34.6	31.2	28.8	28.4	26.9	24.9	27	31.3	31.8	31.4	31.9	30.9	31
25	31	33.8	31.5	28.5	27.5	26.2	24.9	23.7	31	32.5	31	28.8	31.3	30.9
26	28.6	35.7	31.3	27.1	27.8	26.4	26.4	24.3	30.3	33.9	31.2	30.2	31.9	31
27	29.1	35.4	32.7	30.3	27.9	27.6	27.1	26.4	30.4	34.3	30.6	30.2	31.8	30.6
28	30.5	34.4	32.7	30	28.3	24.8	23.3	27.2	31.6	33	29.8	31.4	30.6	31.8
29	30.9		35.3	30.6	27.6	25.9	24.9	28	27.4	35.6	31.7	30.4	31.4	
30	31.6		34.9	30.1	27.1	25.4	24.9	24.8	28.4	32.8	32	31.7	31.2	
31	31.6		34.5		28		26	28.3		32.5		31.2	31.5	

Table A.8 - Visual turbidity data (0-3) for collected water samples from Goondaloo Creek.

Sample Date	Visual Turbidity	Sample Date	Visual Turbidity
26.1.22	3	21.10.22	3
27.1.22	3	26.10.22	3
2.2.22	2	2.11.22	1
3.2.22	2	22.11.22	3
4.2.22	3	23.11.22	2
9.2.22	2	28.11.22	3
16.2.22	0	30.11.22	2
23.2.22	0	1.12.22	2
2.3.22	1	7.12.22	0
9.3.22	1	12.12.22	0
16.3.22	1	6.1.23	3
22.4.22	3	11.1.23	2
26.4.22	3	13.1.23	3
28.4.22	3	14.1.23	3
4.5.22	0	15.1.23	3
10.5.22	2	16.1.23	3
11.5.22	3	17.1.23	3
18.5.22	2	18.1.23	3
25.5.22	0	19.1.23	2
1.6.22	0	20.1.23	2
8.6.22	0	25.1.23	1
15.6.22	0	1.2.23	3
22.6.22	0	6.2.23	3
29.6.22	1	8.2.23	2
5.7.22	3	15.2.23	1
13.7.22	0	17.2.23	1
20.7.22	0	20.2.23	1
27.7.22	0	22.2.23	1
3.8.22	1		
10.8.22	1		
17.8.22	1		

Table A.9 - Flowing water data (yes/no) for collected water samples from Goondaloo Creek.

Sample Date	Flowing Data	Sample Date	Flowing Data
26.1.22	Yes	21.10.22	Yes
27.1.22	Yes	26.10.22	Yes
2.2.22	Yes	2.11.22	No
3.2.22	Yes	22.11.22	Yes
4.2.22	Yes	23.11.22	Yes
9.2.22	Yes	28.11.22	Yes
16.2.22	Yes	30.11.22	Yes
23.2.22	No	1.12.22	Yes
2.3.22	No	7.12.22	Yes
9.3.22	No	12.12.22	Yes
16.3.22	No	6.1.23	Yes
22.4.22	Yes	11.1.23	Yes
26.4.22	Yes	13.1.23	Yes
28.4.22	Yes	14.1.23	Yes
4.5.22	Yes	15.1.23	Yes
10.5.22	Yes	16.1.23	Yes
11.5.22	Yes	17.1.23	Yes
18.5.22	Yes	18.1.23	Yes
25.5.22	Yes	19.1.23	Yes
1.6.22	Yes	20.1.23	Yes
8.6.22	Yes	25.1.23	Yes
15.6.22	Yes	1.2.23	Yes
22.6.22	No	6.2.23	Yes
29.6.22	No	8.2.23	Yes
5.7.22	Yes	15.2.23	Yes
13.7.22	Yes	17.2.23	Yes
20.7.22	Yes	20.2.23	Yes
27.7.22	No	22.2.23	Yes
3.8.22	No		
10.8.22	No		
17.8.22	No		

Table A.10 - TTSS PCR results for selected colonies from direct plate count plates and broth plates created from Goondaloo Creek water samples.

Sample Date	**PCR Result**	**Sample Date**	**PCR Result**	**Sample Date**	**PCR Result**	**Sample Date**	**PCR Result**
27.7.22-1	Positive	21.10.22-5	Negative	27.1.22-19	Positive	17.2.23-1	Negative
5.7.22-1	Positive	21.10.22-6	Negative	5.7.22-1	Positive	18.1.23-2	Negative
27.1.22-12	Positive	22.11.22-3	Negative	13.1.23-1	Positive	18.1.23-1	Positive
21.10.22	Negative	22.11.22-4	Negative	22.11.22-7	Negative	19.1.23-1	Negative
26.10.22-3	Negative	23.11.22-2	Negative	4.2.22-9	Positive	20.1.23-1	Negative
26.10.22-2	Negative	26.10.22-4	Negative	4.2.22-10	Positive	23.11.22-3	Negative
27.1.22-12	Positive	6.1.23-1	Positive	1.12.22-6	Negative	25.1.23-1	Negative
22.11.22-5	Negative	6.1.23-2	Positive	1.2.23-6	Negative	27.7.22-1	Positive
22.11.22-6	Negative	6.1.23-3	Positive	1.2.23-1	Positive	28.11.22-7	Negative
1.12.22-1	Positive	6.1.23-4	Positive	1.2.23-2	Positive	30.11.22-1	Negative
1.12.22-2	Negative	6.1.23-5	Positive	1.2.23-3	Positive	6.1.23-17	Negative
1.12.22-3	Positive	6.1.23-6	Positive	1.2.23-4	Positive	7.12.22-1	Negative
1.12.22-4	Negative	6.1.23-7	Positive	1.2.23-5	Positive	8.2.23-1	Negative
1.12.22-5	Positive	6.1.23-8	Positive	11.1.23	Negative	15.2.23-1	Negative
21.10.22-1	Negative	6.1.23-9	Positive	12.12.22	Negative	20.2.23-1	Negative
21.10.22-2	Negative	6.1.23-10	Positive	13.1.23-4	Negative	22.2.23-1	Negative
21.10.22-3	Negative	6.1.23-11	Positive	13.1.23-2	Positive	4.2.22-11	Positive
21.10.22-4	Negative	6.1.23-12	Positive	13.1.23-3	Positive	6.2.23-1	Negative
22.11.22-1	Negative	6.1.23-13	Positive	14.1.23-3	Negative	27.1.22-20	Positive
22.11.22-2	Positive	6.1.23-14	Positive	14.1.23-1	Positive	27.1.22-21	Positive
23.11.22-1	Positive	6.1.23-15	Positive	14.1.23-2	Positive	27.1.22-22	Positive
26.10.22-1	Negative	6.1.23-16	Positive	15.1.23-5	Negative	27.1.22-23	Positive
26.10.22-2	Negative	27.1.22-11	Positive	15.1.23-1	Positive	27.1.22-24	Positive
26.10.22-3	Negative	27.1.22-12	Positive	15.1.23-2	Positive		
28.11.22-1	Positive	27.1.22-13	Positive	15.1.23-3	Positive		
28.11.22-2	Positive	27.1.22-14	Positive	15.1.23-4	Positive		
28.11.22-3	Negative	27.1.22-15	Positive	16.1.23-3	Negative		
28.11.22-4	Positive	27.1.22-16	Positive	16.1.23-1	Positive		
28.11.22-5	Positive	27.1.22-17	Positive	16.1.23-2	Positive		
28.11.22-6	Positive	27.1.22-18	Positive	17.1.23-1	Negative		

B Appendix

Figure B.1 - Gel electrophoresis images from MLST PCR optimisation temperature gradient run for *gmhD* and *lepA*.

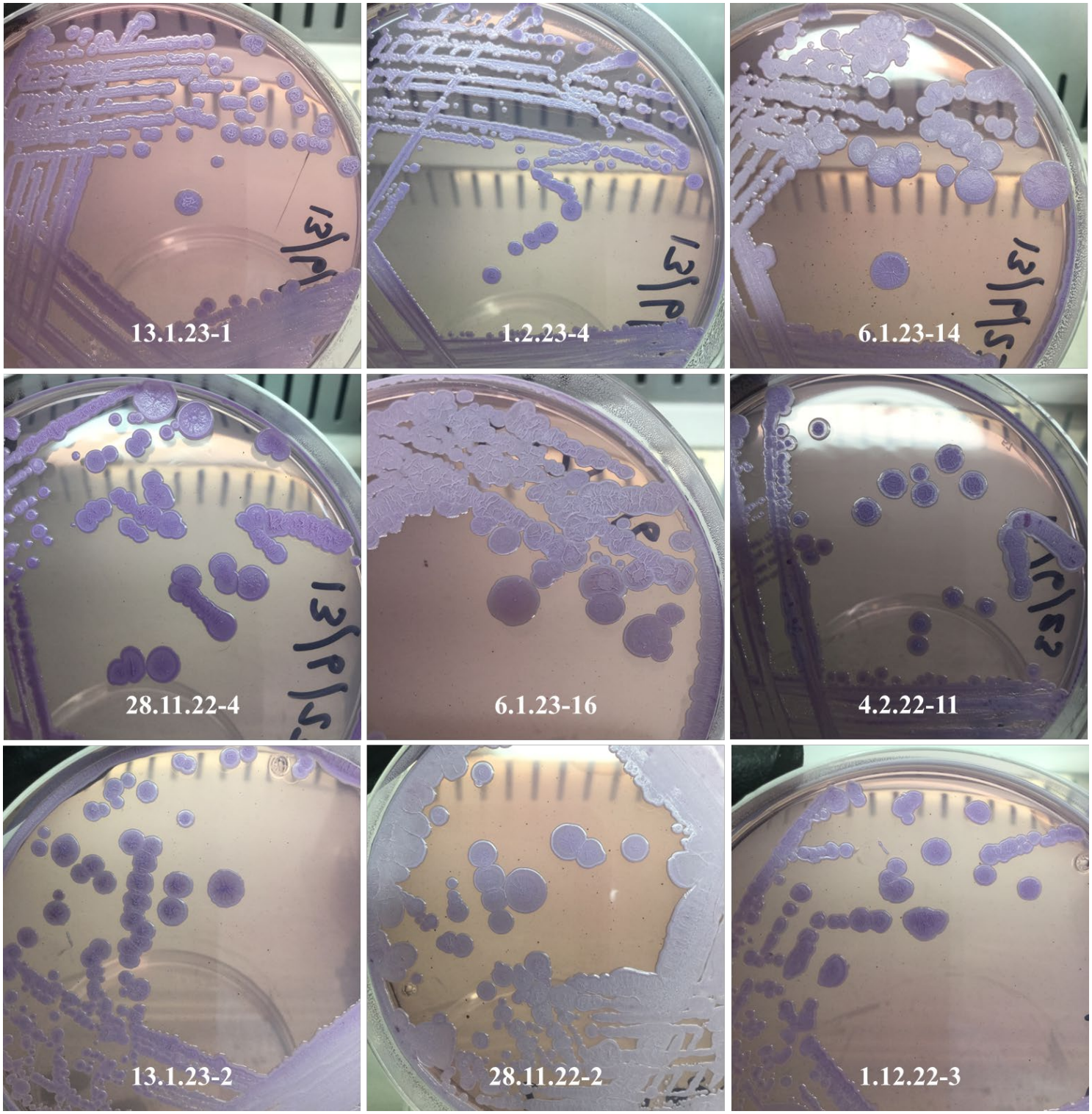

Figure B.2. - A selection of 48 hour cultured *B. pseudomallei* isolates used for DNA extraction.

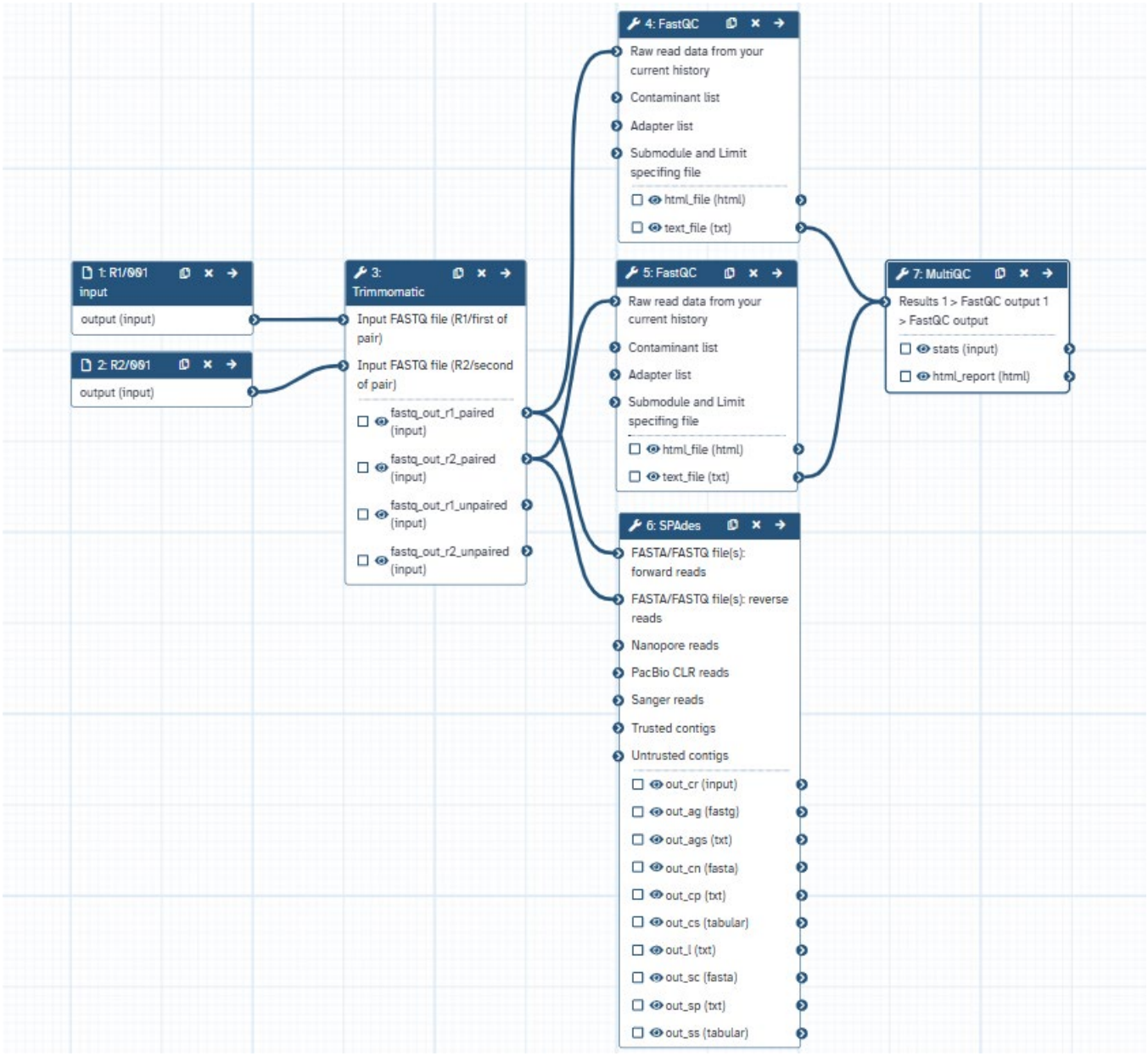

Figure B.3 - Image of Galaxy Australia workflow used to build whole genomes.

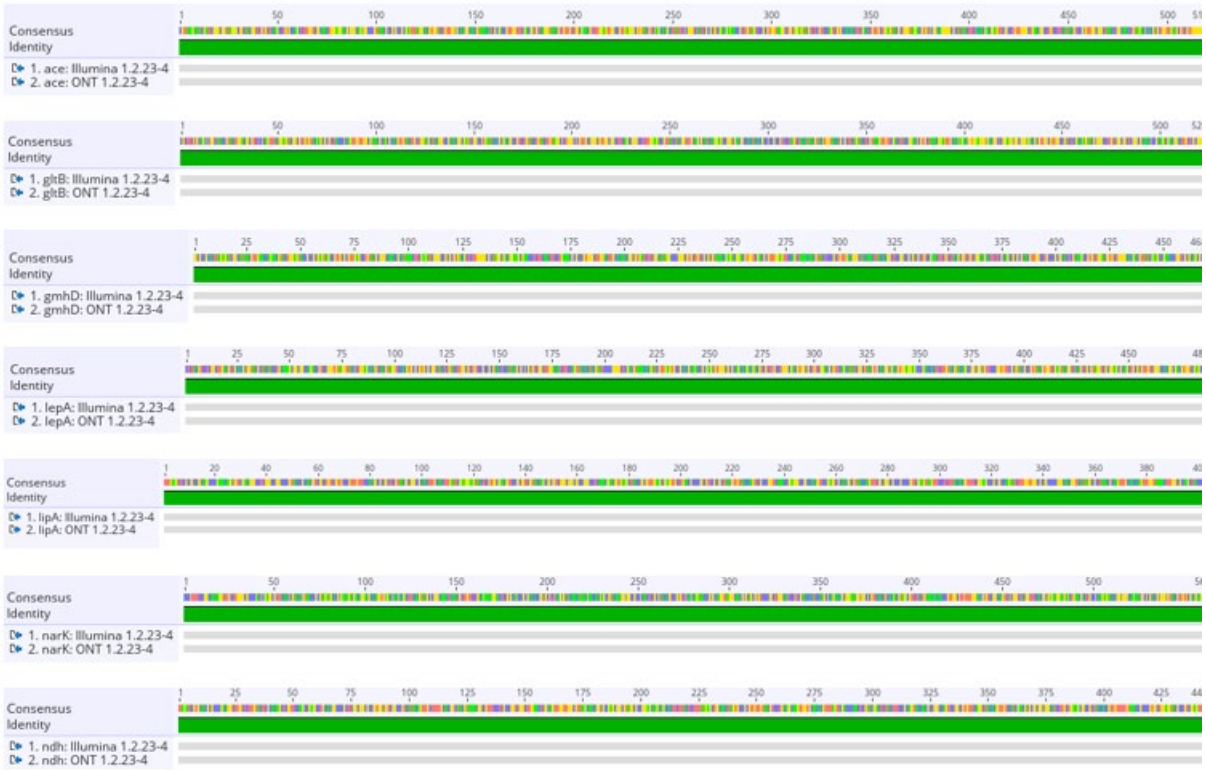

Figure B.4 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 1.2.23-4.

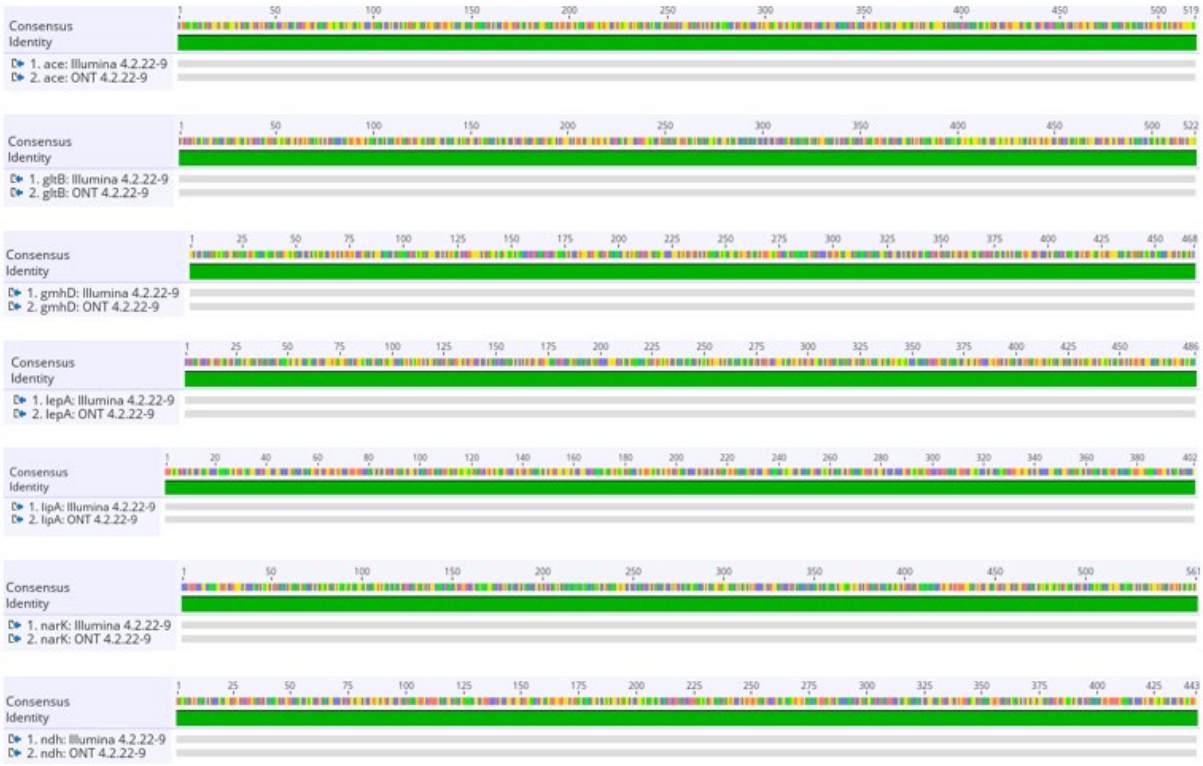

Figure B.5 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 4.2.22-9.

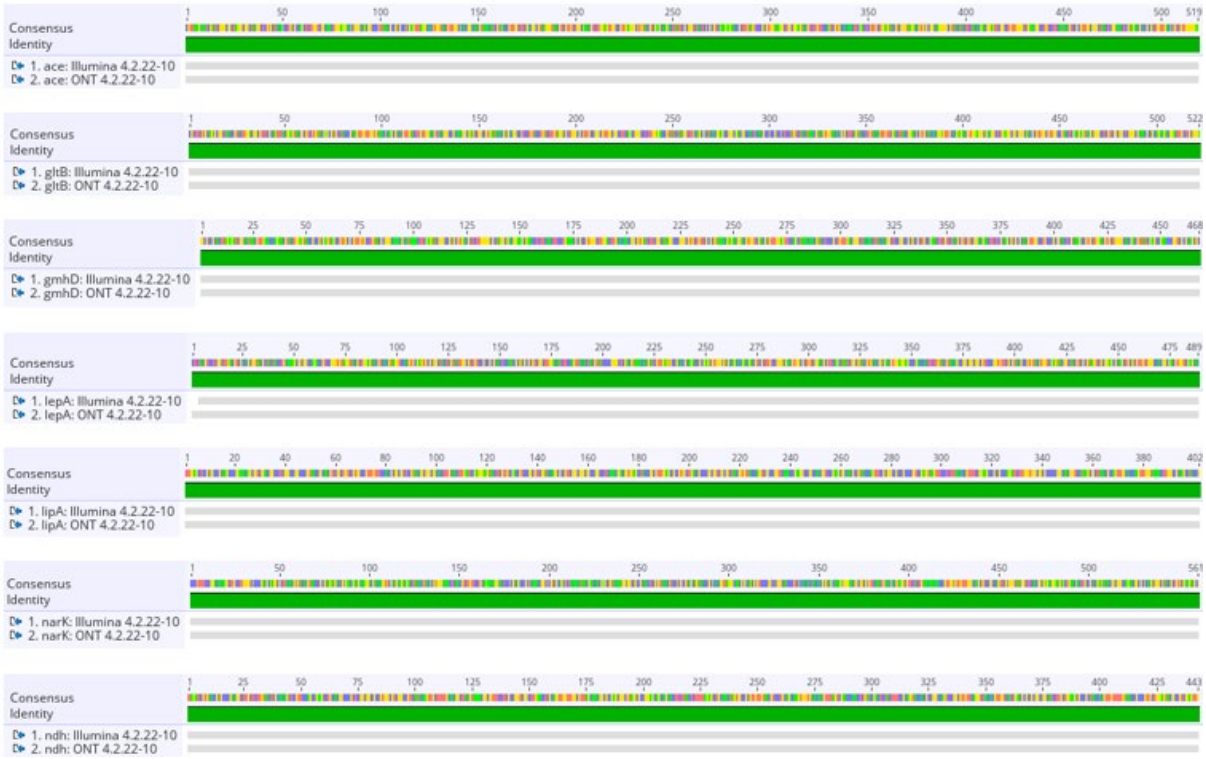

Figure B.6 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 4.2.22-10. Contains novel *lepA* allele.

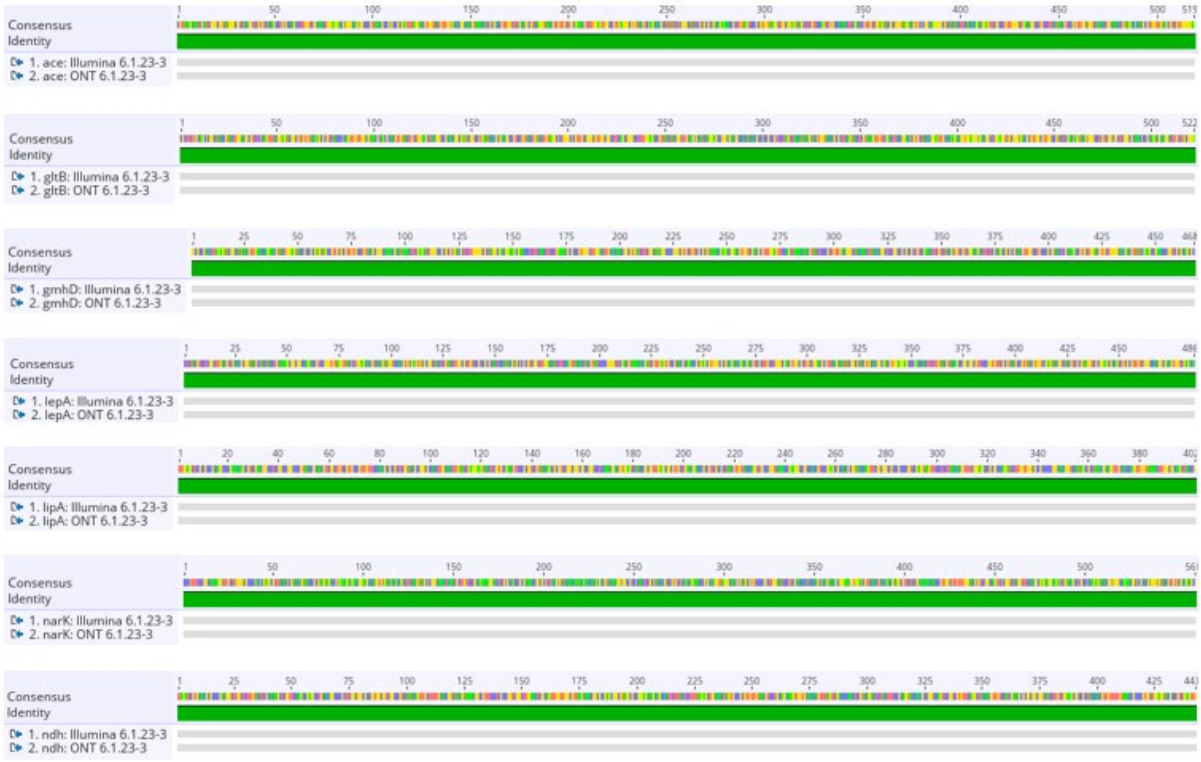

Figure B.7 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 6.1.23-3.

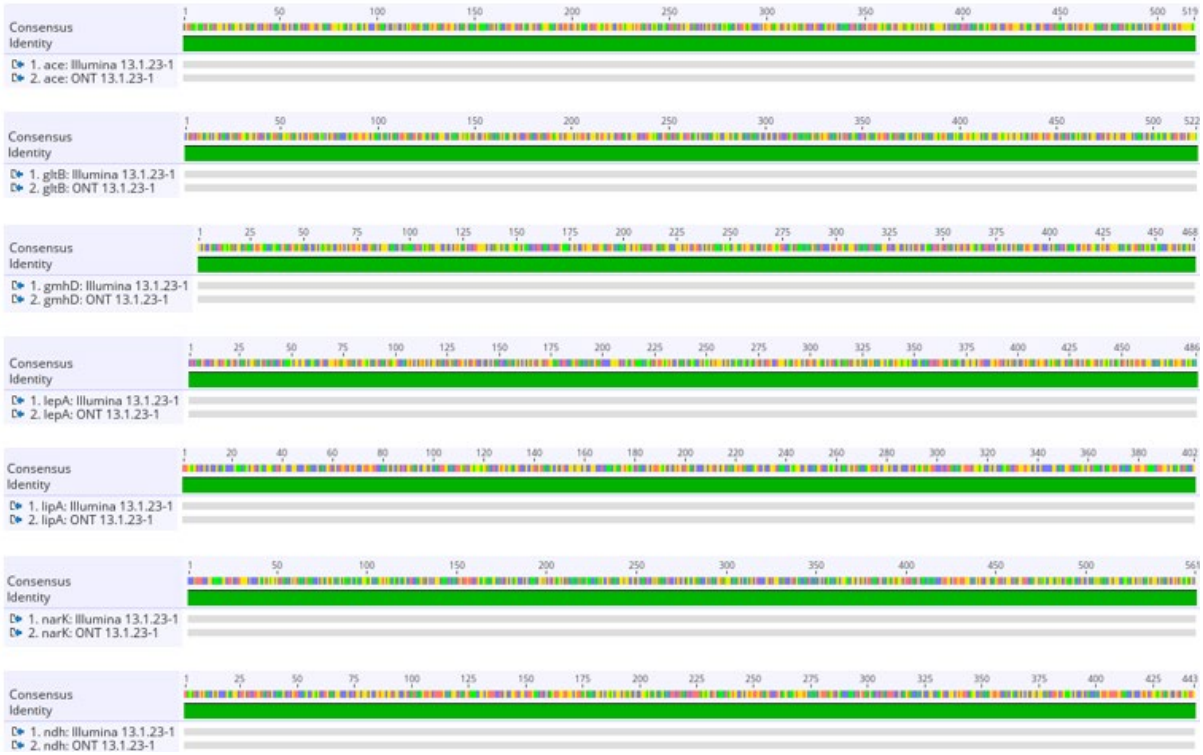

Figure B.8 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 13.1.23-1.

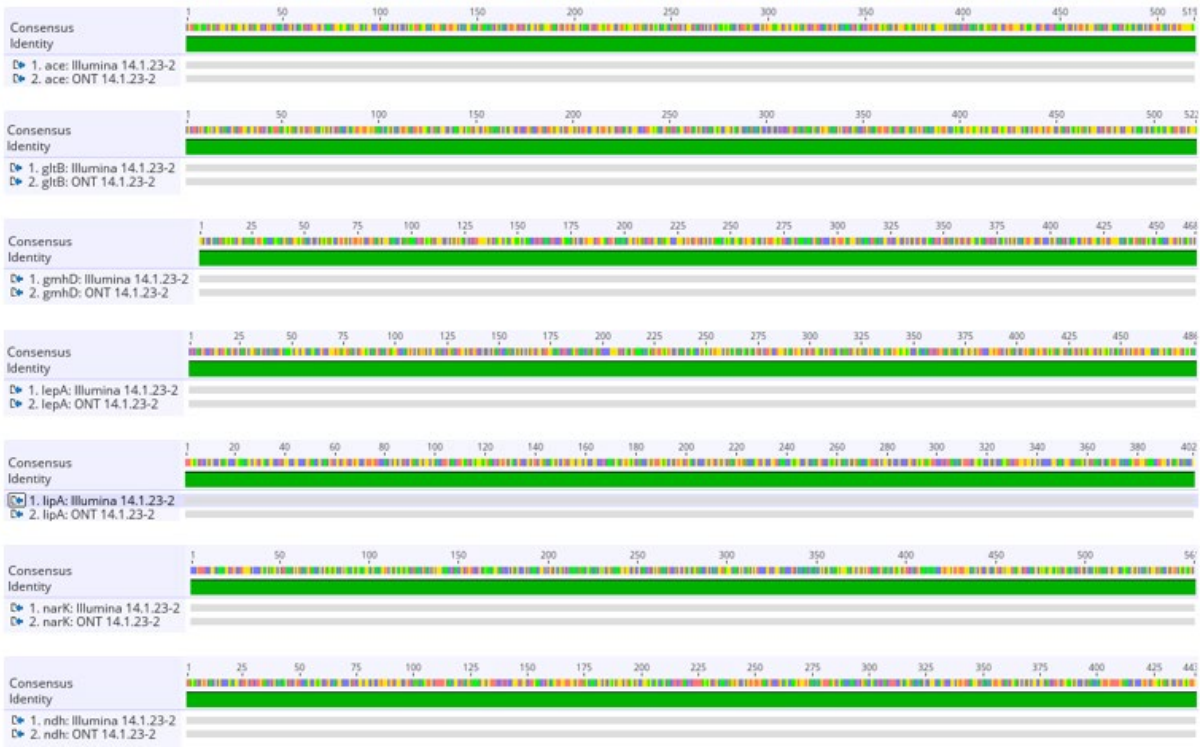

Figure B.9 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 14.1.23-2.

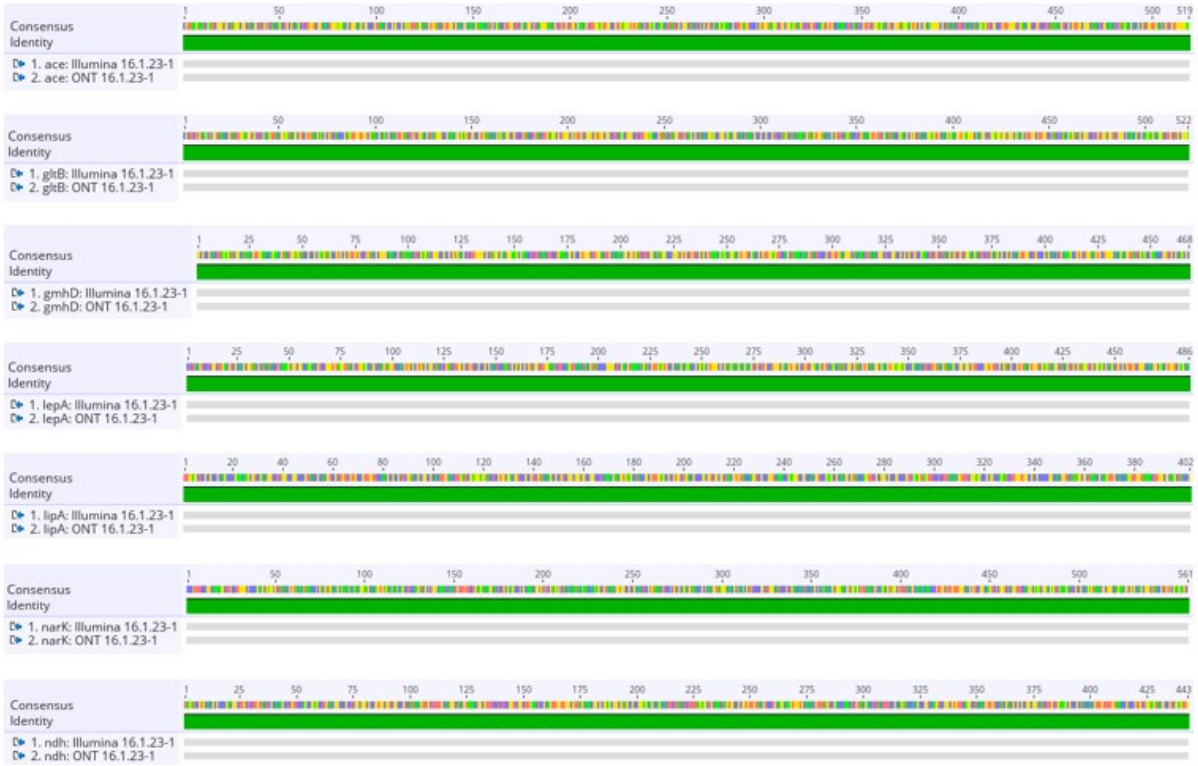

Figure B.10 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 16.1.23-1.

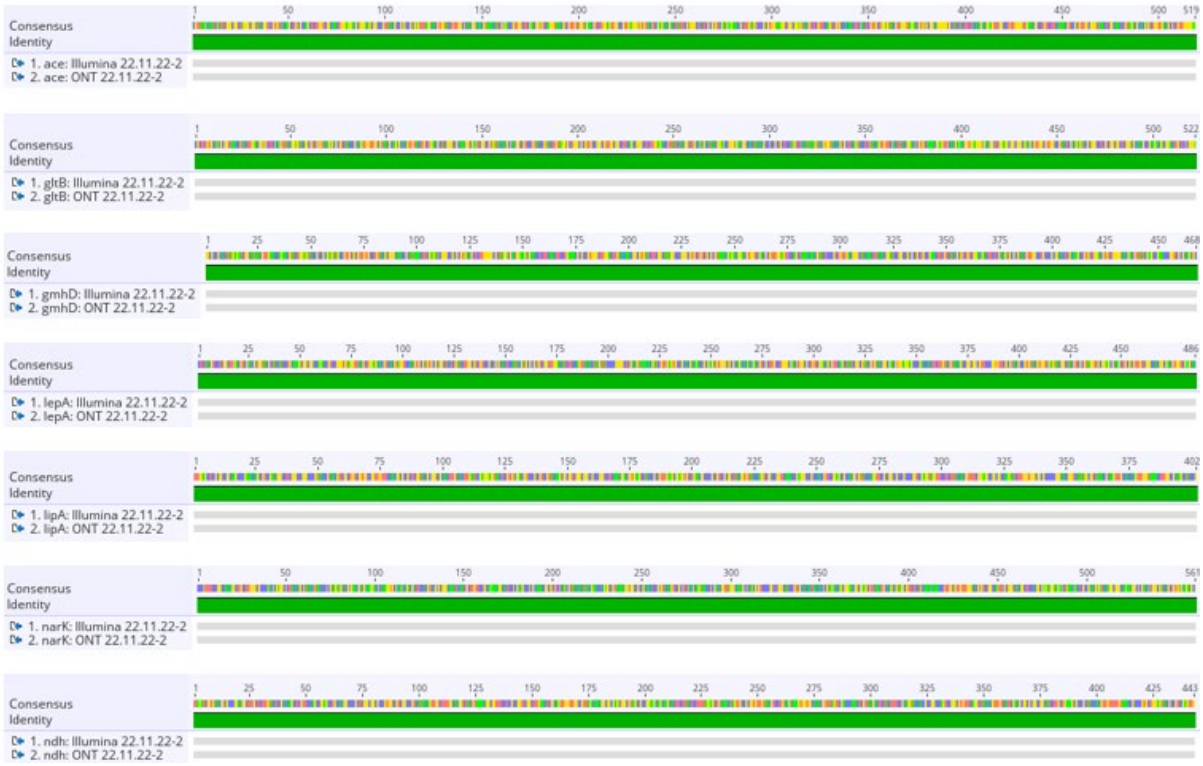

Figure B.11 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 22.11.22-2.

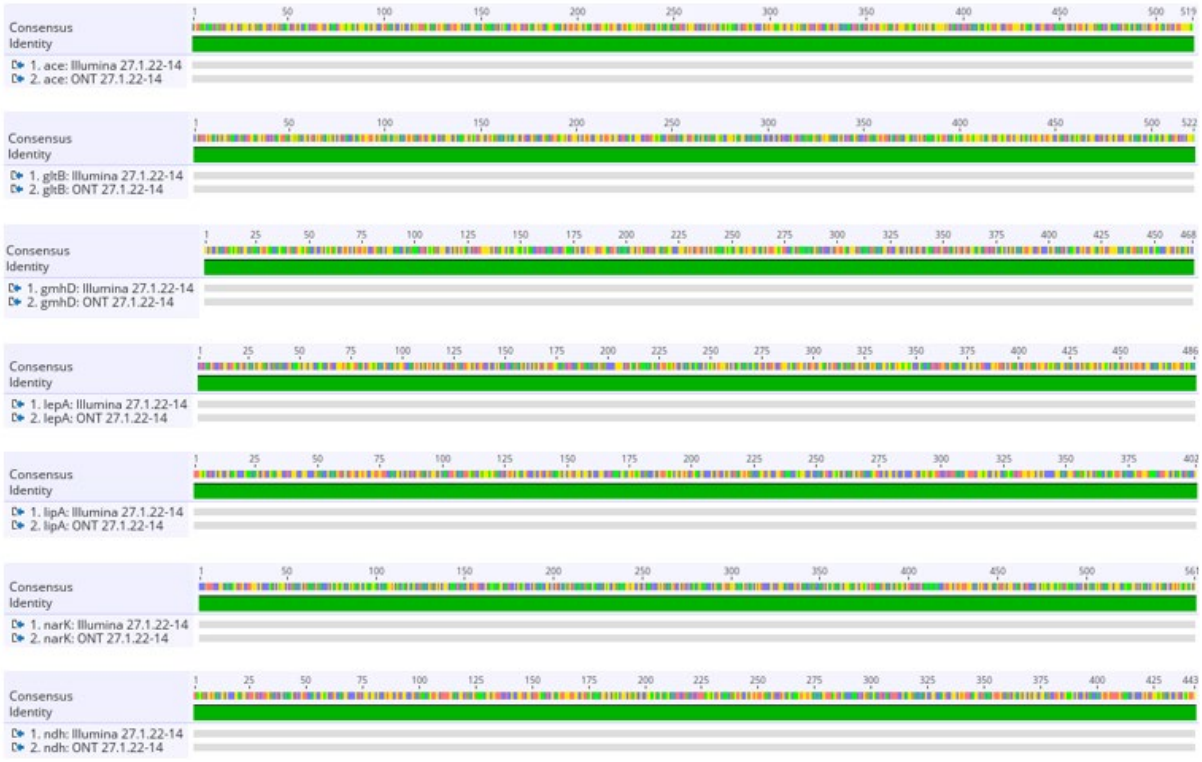

Figure B.12 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 27.1.22-14.

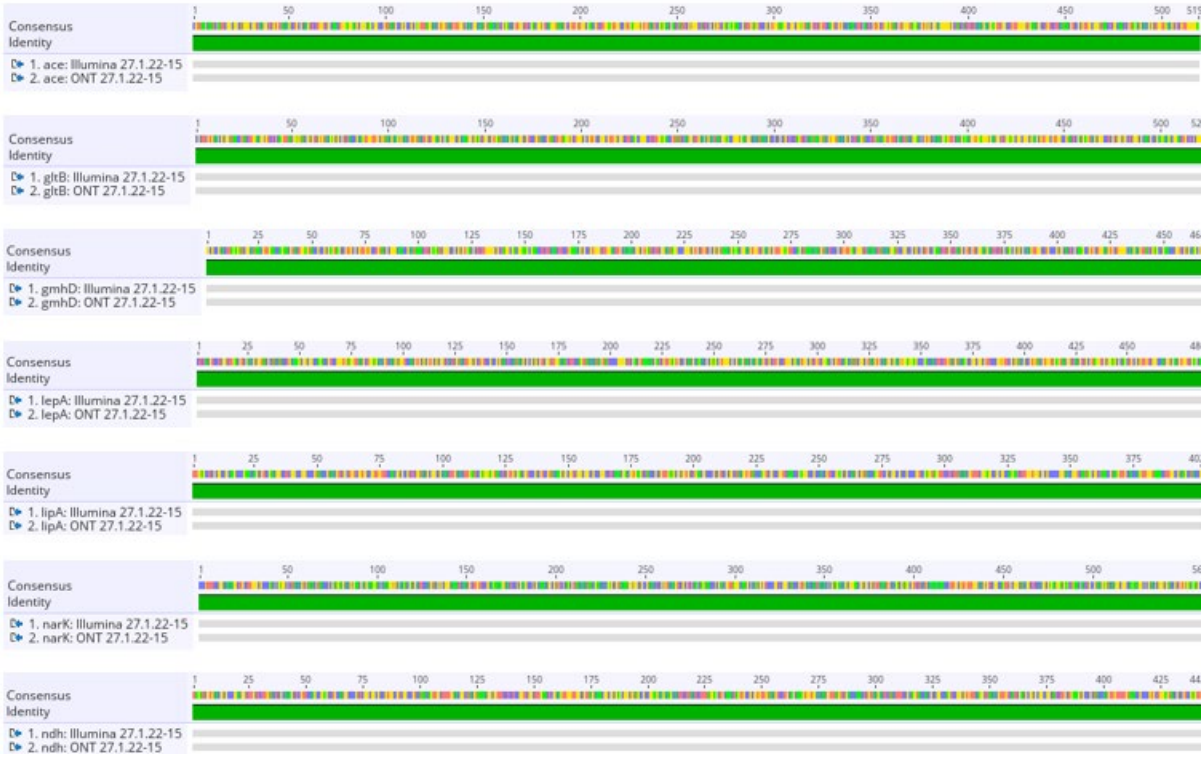

Figure B.13 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 27.1.22-15.

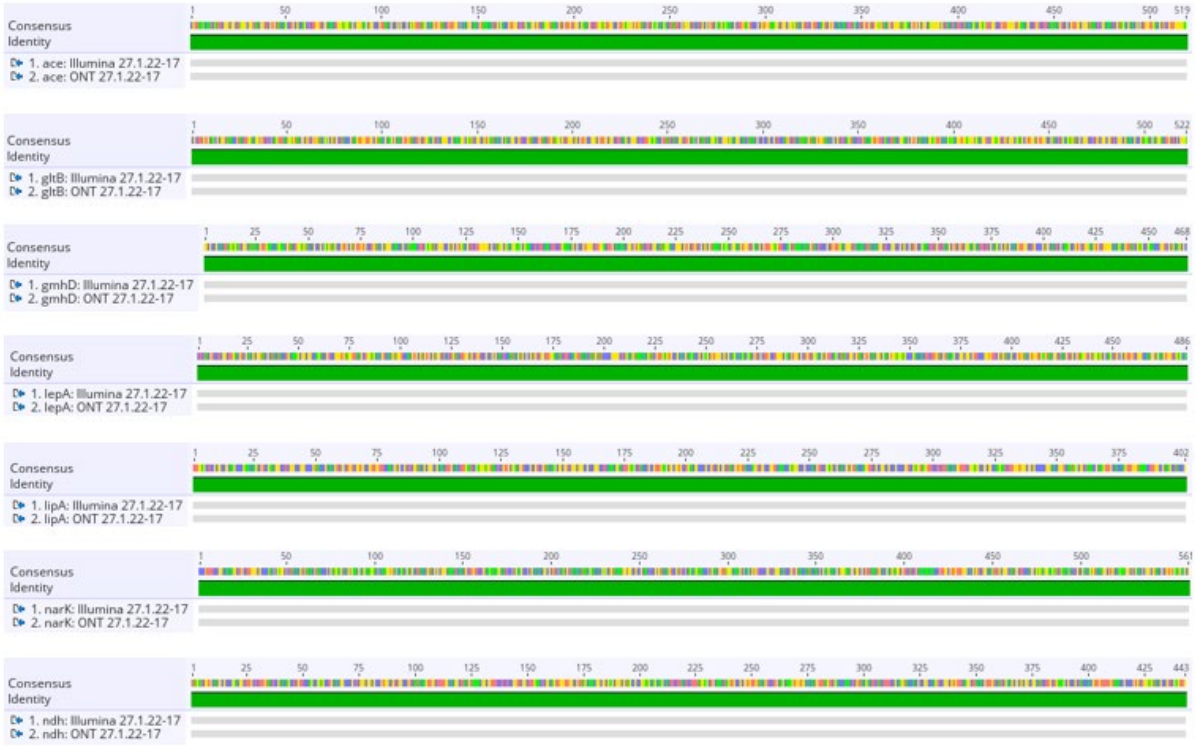

Figure B.14 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 27.1.22-17. Contains novel *lipA* allele, now classified as *lipA* 189.

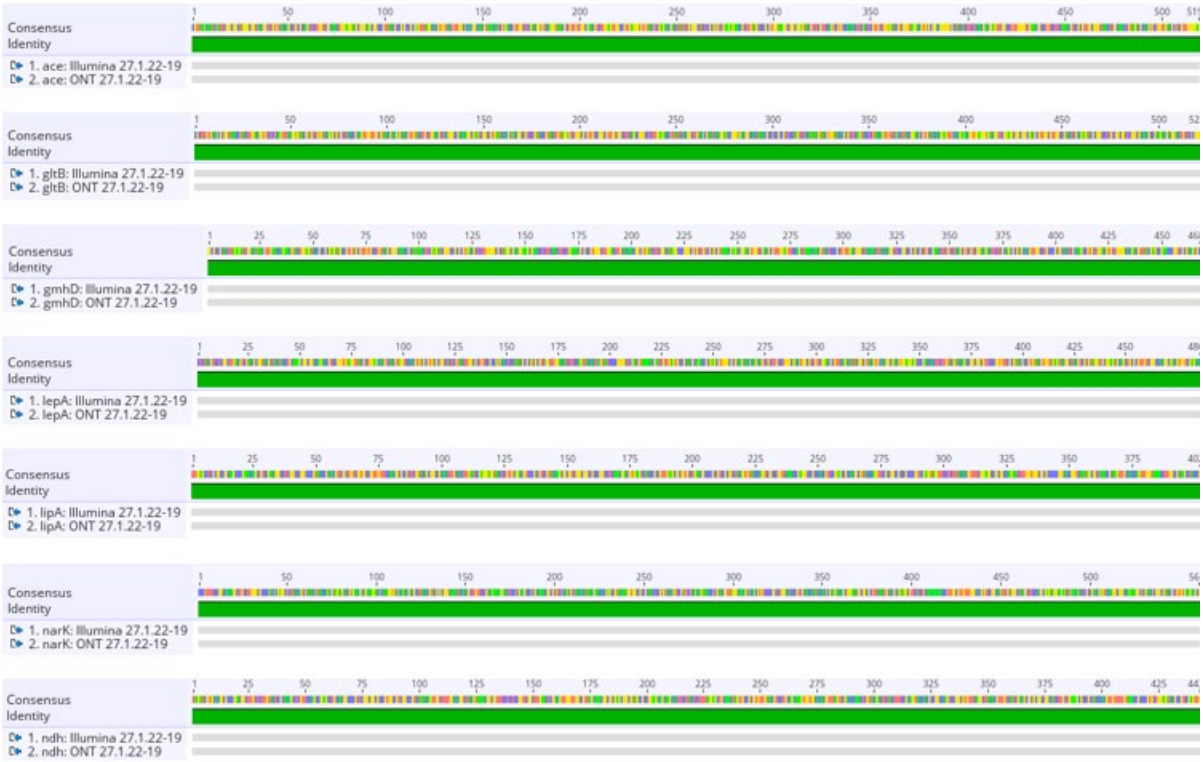

Figure B.15 - MAFFT pairwise alignment of Illumina and ONT MLST alleles sequenced from isolate 27.1.22-19.

Table B.1 - Cost analysis table for targeted ONT sequencing workflow.

ONT Workflow Step	Consumables/Reagents	Number of Preparations per Item	Expense Total ($) (50 Preparations)	Expense per Preparation ($) per Item
DNA Extraction	Roche High Pure PCR Template Preparation Kit	100.00	465.00	4.65
	Lysozyme	20000.00	117.00	0.01
	Isopropanol	5000.00	89.27	0.02
	Ethyl Alcohol	500.00	82.00	0.16
PCR	GoTaq Colourless Mastermix (Promega M7132)	69.00	402.00	5.83
	Primers	4000.00	65.34	0.02
	Nuclease Free Water	10869.57	162.00	0.01
Gel Electrophoresis	DNA Ladder	321.43	273.00	0.85
	Loading Dye	5000.00	50.40	0.01
	Gelred	214.29	145.00	0.68
	50x TAE	214.29	226.00	1.05
	Agarose Powder	68.57	134.00	1.95
PCR Clean-Up	Wizard SV Gel and PCR Clean Up System	250.00	597.00	2.39
Sequencing Preparation	Native Barcoding Kit	144.00	1080.00	7.50
	NEBNEXT Ultra II End Repair/dA-Tailing Module	96.00	411.82	4.29
	NEB Blunt/TA Ligase Mastermix	50.00	345.61	6.91
	NEBNext Quick Ligation Module	480.00	555.56	1.16
	Bovine Serum Albumin	4800.00	241.00	0.05
Sequencing	Flow Cell	72.00	1090.00	15.14
Sequencing Clean Up	Flow Cell Wash Kit	144.00	154.00	1.07
Extra	Eppendorf 1mL Tubes	50.00	56.55	1.13
	10µL Tips	50.00	158.00	3.16
	20µL Tips	50.00	79.00	1.58
	200µL Tips	50.00	86.90	1.74
	1000µL Tips	50.00	93.50	1.87
	PCR Tubes and Lids	50.00	756.00	15.12
	Qubit Tubes	500.00	468.29	0.94
	Qubit Assay Kit	500.00	162.00	0.32
TOTAL:			**$8546.24**	**$79.61**

Table B.2 - Cost analysis table for Illumina (AGRF) WGS workflow.

(AGRF) Illumina Workflow Step	Consumables/Reagents/ Service	Number of Preparations per Item	Expense Total ($) (50 Preparations)	Expense per Preparation ($) per Item
DNA Extraction	Roche High Pure PCR Template Preparation Kit	100.00	465.00	4.65
	Lysozyme	20000.00	117.00	0.01
	Isopropanol	5000.00	89.27	0.02
	Ethyl Alcohol	500.00	82.00	0.16
Gel Electrophoresis	DNA Ladder	2250.00	273.00	0.12
	Loading Dye	5000.00	50.40	0.01
	Gelred	1500.00	145.00	0.10
	50x TAE	1500.00	226.00	0.15
	Agarose Powder	900.00	134.00	0.15
Sequencing	Illumina Service (16+ Runs)	50.00	6250.00	125.00
Extra	Qubit Tubes	500.00	468.29	0.94
	Qubit Assay Kit	500.00	162.00	0.32
	Eppendorf 1mL Tubes	50.00	56.55	1.13
	10µL Tips	120.00	79.00	0.66
	20µL Tips	120.00	79.00	0.66
	200µL Tips	120.00	86.90	0.72
	1000µL Tips	120.00	93.50	0.78
	Packaging and Shipping	1.00	20.00	20.00
TOTAL:			**$8876.91**	**$155.58**

C Appendix

Table C.1 - Goondaloo Creek sequenced isolates and their corresponding ONT ST and PubMLST ID number.

Isolate ID	PubMLST ID	ST	Isolate ID	PubMLST ID	ST
27.1.22-11	6745	283	6.1.23-1	6765	283
27.1.22-12	6746	283	6.1.23-3	6766	2074
27.1.22-13	6747	1966	6.1.23-4	6767	2072
27.1.22-14	6784	2077	6.1.23-5	6768	2071
27.1.22-15	6748	2070	6.1.23-14	6769	2072
27.1.22-16	6749	283	6.1.23-16	6786	283
27.1.22-17	6750	2080	13.1.23-1	6770	2075
27.1.22-18	6751	1966	13.1.23-2	6771	2073
27.1.22-19	6752	2071	13.1.23-3	6772	2072
27.1.22-21	6789	1966	14.1.23-1	6773	2072
27.1.22-22	6785	1966	14.1.23-2	6787	2078
4.2.22-9	6753	2072	15.1.23-1	NA*	Unknown
4.2.22-10	NA*	Unknown	15.1.23-2	6774	283
4.2.22-11	6754	283	15.1.23-3	6775	283
5.7.22-1	6755	276	15.1.23-4	6776	283
27.7.22-1	NA*	Unknown	16.1.23-1	6788	2079
22.11.22-2	6756	2073	16.1.23-2	6777	1664
23.11.22-1	6757	283	18.1.23-1	6778	283
28.11.22-1	6758	1969	1.2.23-1	6779	276
28.11.22-2	6759	283	1.2.23-2	6780	283
28.11.22-4	6760	2080	1.2.23-3	6781	276
28.11.22-5	6761	283	1.2.23-4	6782	2076
1.12.22-1	6762	624	1.2.23-5	6783	283
1.12.22-3	6763	1966	TSV1 (ST276 control)	2270	276
1.12.22-5	6764	2070	TSV2 (ST814 control)	2271	814

*Not applicable (NA) as these isolates have not been submitted due to incomplete profile.

Table C.2 – DNA sequences for *lipA* and *lepA* alleles determined from ONT sequencing of Goondaloo Creek *B. pseudomallei* isolates.

Alleles	**PubMLST Allele Number**	**Sequence Length (bp)**	**DNA Sequence**
lipA	189	402	AAGTGCACGCGCCGCTGCCCGTTCTGCGACGTCGGC CACGGCCGGCCCGATCCGCTCGACGCAGACGAGCCG AAGAACCTCGCGCGCACGATCGCGGCGCTCAAGCTC AAGTACGTGGTGATCACGAGCGTCGACCGCGACGAT CTGCGCGACGGCGGCGCCGGCCACTTCGTCAAGTGC ATCCGCGAAGTGCGCGAGCAGTCGCCCGCGACGCGC ATCGAGATCCTGACGCCGGACTTCCGTGGCCGCCTC GACCGTGCGCTCGCGATCCTGAACGCGGCGCCGCCC GACGTGATGAACCACAATCTCGAAACGGTGCCGCGC CTGTACAAGGAGGCGCGCCCCGGCTCGGACTATGCG CATTCGCTGAAGCTCCTGAAGGATTTCAAGGCGCTG CATCCG
lepA	NA*	489	CACATCGACCACGGCAAGTCGACGCTCGCGGATCGC ATCATCCAGCTTTGCGGCGGCCTGTCCGACCGGGAG ATGGAATCGCAGGTGCTCGACTCGATGGACCTCGAG CGTGAGCGCGGCATCACGATCAAGGCGCAGACCGCC GCGCTCACCTATCGCGCGCGCGACGGCAAGGTCTAC AACCTGAATCTCATCGATACCCCGGGGCACGTCGAT TTCTCGTACGAAGTGAGCCGCTCGCTGTCCGCGTGCG AGGGCGCGCTGCTCGTCGTCGACGCAAGCCAGGGCG TCGAGGCGCAGACGGTCGCGAACTGCTATACGGCGA TCGAGCTCGGCGTCGAGGTGGTGCCCGTCCTCAACA AGATCGATCTGCCGGCGGCGGCGAACCCGGAGAACG CGATCGCCGAGATCGAGGACGTGATCGGCATCGACG CGATGGACGCGGTGCGCTGCAGCGCGAAGACGGGCC TCGGCGTCGAGGACGTGCTC

*Not accepted by PubMLST due to three base pair insertion.

Table C.3 – Number and percentage totals of sequenced Goondaloo Creek *B. pseudomallei* isolates for each ST.

ST	**Number of Total Sequenced Isolates**	**Percentage of Total Sequenced Isolates (%)**
276	3	6.25
283	15	31.25
624	1	2.083
1664	1	2.083
1966	5	10.417
1969	1	2.083
2070	2	4.167
2071	2	4.167
2072	5	10.417
2073	2	4.167
2074	1	2.083
2075	1	2.083
2076	1	2.083
2077	1	2.083
2078	1	2.083
2079	1	2.083
2080	2	4.167
Unknown	3	6.25

```
#!/bin/bash
#PBS -j oe
#PBS -m ae
#PBS -N AlignmentCkseq
#PBS -M kaitlin.burns@jcu.edu.au
#PBS -l walltime=24:00:00
#PBS -l select=1:ncpus=1:mem=2gb

cd $PBS_O_WORKDIR
shopt -s expand_aliases
source /etc/profile.d/modules.sh
echo "Job identifier is $PBS_JOBID"
echo "Working directory is $PBS_O_WORKDIR"


module load mafft/7.505
mafft --auto Creek_Sequences_Final.fas > Creek_Sequences_Align.fas

```

Figure C.1 - Shell script code used for aligning sequences using MAFFT.

```
#!/bin/bash
#PBS -j oe
#PBS -m ae
#PBS -N TreeCreek
#PBS -M kaitlin.burns@jcu.edu.au
#PBS -l walltime=24:00:00
#PBS -l select=1:ncpus=1:mem=16gb

cd $PBS_O_WORKDIR
shopt -s expand_aliases
source /etc/profile.d/modules.sh
echo "Job identifier is $PBS_JOBID"
echo "Working directory is $PBS_O_WORKDIR"


module load iqtree/2.2.2.2
iqtree -s Creek_Sequences_Align.fas -m MFP -b 1000 --prefix Creek_iqtree
```

Figure C.2 - Shell script code used for creating phylogenetic trees with IQ-TREE.