Institute of Health and Community Medicine

Antibiotic Susceptibility of *Burkholderia pseudomallei* Towards Co-trimoxazole and its Mechanism

Liana Lantong Anak Sumbu

Master of Science
2026

Antibiotic Susceptibility of *Burkholderia pseudomallei* Towards Co-trimoxazole and its Mechanism

Liana Lantong Anak Sumbu

A thesis submitted

In fulfillment of the requirements for the degree of Master of Science

(Emerging Infectious Diseases)

Institute of Health and Community Medicine
UNIVERSITI MALAYSIA SARAWAK
2026

DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

……

Signature

Name: Liana Lantong Anak Sumbu

Matric No.: 17020040

Institute of Health and Community Medicine

Universiti Malaysia Sarawak

Date: 26th February 2026

ACKNOWLEDGEMENT

All praise and worship I give to my Lord God for His guidance and graces that have eased my journey in completing this thesis. Thanks to Him for always delivering and securing me in all circumstances and never forsaking me, especially during this pandemic period. Also, special thanks to my lovely supervisor, Dr. Yuwana Podin. This work of graduate school would have overwhelmed me had it not for her thoughtfulness and unending guidance, support, and patience. May God repay you with abundance bless.

My sincere gratitude to my fellow Melioid Team, Diane, Sherene, Hidayah, and Lua for assisting me along the experiments and always lifting me up through time of need. Thanks to all of you that I had a great time learning, sharing knowledge, and memorable bittersweet memories drawn over the past five years. To my beloved friends, Team Samarahan, Stella and Adriana, thanks for always being my ears and standing by my side through everything.

I am grateful to all of the supportive members of IHCM for the help, insightful discussions and knowledge sharing during my study. Besides, I would like to thank Dr. Ooi Mong How, Dr. Wong, Dr. Tan, and Dr. Leslie for providing the clinical isolates and control strains used in this study. Thanks also to UNIMAS for granted the Zamalah scholarships that had greatly helped me financially.

Finally, I would like to extend my gratitude to my beloved family, dad; Sumbu Makam, mom; Mary Binit and sisters; Jacq, Rebecca and Felicia. Thank you for the endless love, prayers and unwavering support during these incredibly stressful and tumultuous years. This thesis is dedicated to all of you.

ABSTRACT

The etiologic agent, *Burkholderia pseudomallei* that causes the potentially fatal melioidosis, is known to be inherently resistant to a wide range of antimicrobial drugs. Co-trimoxazole, a combination of sulfamethoxazole and trimethoprim, is an important drug used in the eradication phase therapy of melioidosis. In recent years, there have been increasing reports of higher minimal inhibitory concentration (MIC) readings of co-trimoxazole for *B. pseudomallei* clinical isolates in Sarawak, Malaysian Borneo. Thus, this study is aimed to determine the prevalence and understand the mechanism of such phenomena in Sarawak. Antibiotic susceptibility was assessed using disk diffusion and E-test methods on *B. pseudomallei* clinical isolates collected from various hospitals in Sarawak. In addition, the characterisation of the isolates' susceptibility against the co-trimoxazole components, trimethoprim and sulfamethoxazole, was performed using the broth microdilution method. All analyses of the MIC breakpoints were done according to the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antibiotic Susceptibility Testing (EUCAST) guidelines. Overall, the Sarawak clinical *B. pseudomallei* isolates exhibited susceptibility of 96.3% (CLSI) and 97.6% (EUCAST) towards co-trimoxazole *in-vitro*. Broth microdilution demonstrated that the isolates were resistant when treated with the separate components of co-trimoxazole, sulfamethoxazole or trimethoprim alone. However, they were generally susceptible when treated with the combination of sulfamethoxazole and trimethoprim (co-trimoxazole). Thus, clinicians should be confident that the co-trimoxazole is effective for the treatment of melioidosis. Analysis of the MIC results showed that the CLSI guideline is a valid guide for antibiotic susceptibility testing and antimicrobial resistance surveillance works in Sarawak. Although the co-trimoxazole resistance determinant, the *bpeEF-oprC* genes, were not well characterised, this study has provided

some basis for future research pursuits. Furthermore, this study has established guidelines for the laboratory diagnosis, particularly for the betterment of antibiotic susceptibility testing and antimicrobial resistance surveillance of melioidosis cases in Sarawak.

Keywords: *Burkholderia pseudomallei*, co-trimoxazole, antibiotic susceptibility, Sarawak, melioidosis.

Kerentanan Antibiotik oleh* Burkholderia pseudomallei *terhadap Co-trimoxazole dan Mekanismenya

ABSTRAK

Ejen etiologi, Burkholderia pseudomallei *yang menyebabkan penyakit berpotensi maut melioidosis, adalah terkenal dengan ketahanan semulajadi terhadap pelbagai ubat antimikrob. Co-trimoxazole, iaitu gabungan sulfamethoxazole dan trimethoprim, merupakan ubat penting yang digunakan dalam terapi fasa pembasmian melioidosis. Sejak kebelakangan ini, terdapat peningkatan laporan bacaan kepekatan perencatan minimum (MIC) yang lebih tinggi bagi co-trimoxazole untuk merencat ketumbuhan isolat klinikal* B. pseudomallei *di Sarawak, Borneo Malaysia. Oleh itu, tujuan kajian ini adalah untuk menentukan kelaziman dan memahami mekanisme fenomena sedemikian di Sarawak. Kerentanan antibiotik dinilai menggunakan kaedah 'disk diffusion' dan 'E-test' bagi isolat klinikal* B. pseudomallei *yang dikumpulkan dari pelbagai hospital di Sarawak. Di samping itu, pencirian kerentanan isolat tersebut terhadap komponen co-trimoxazole, iaitu trimethoprim dan sulfamethoxazole pula dilakukan menggunakan kaedah 'broth microdilution'. Semua analisis titik putus MIC dibuat mengikut garis panduan dari Clinical and Laboratory Standards Institute (CLSI) dan European Committee on Antibiotic Susceptibility Testing (EUCAST). Secara keseluruhan, isolat klinikal* B. pseudomallei *Sarawak menunjukkan kerentanan sebanyak 96.3% (CLSI) dan 97.6% (EUCAST) terhadap co-trimoxazole secara* in-vitro*. 'Broth microdilution' menunjukkan bahawa pertumbuhan isolat klinikal* B. pseudomallei *Sarawak tidak rencat apabila didedahkan atau dirawat dengan komponen-komponen berasingan co-trimoxazole, iaitu sulfamethoxazole atau trimethoprim sahaja. Walau bagaimana pun, pertumbuhan isolat tersebut secara amnya dapat direncatkan apabila dirawat dengan gabungan sulfamethoxazole dan trimethoprim*

(co-trimoxazole). Oleh itu, pegawai perubatan seharusnya yakin bahawa co-trimoxazole adalah berkesan untuk rawatan melioidosis. Analisis keputusan MIC menunjukkan bahawa garis panduan CLSI adalah panduan yang sahih untuk ujian kerentanan antibiotik dan kerja-kerja pengawasan ketahanan antimicrob di Sarawak. Walaupun penentu ketahanan co-trimoxazole, yakni gen bpeEF-oprC *tidak dicirikan dengan baik, kajian ini telah menyediakan beberapa asas untuk usaha penyelidikan pada masa hadapan. Tambahan pula, kajian ini telah menetapkan garis panduan untuk diagnosis makmal, terutamanya untuk penambahbaikkan ujian kerentanan antibiotik dan pengawasan ketahanan antimikrob bagi kes-kes melioidosis di Sarawak.*

Kata kunci: Burkholderia pseudomallei*, co-trimoxazole, kerentanan antibiotik, Sarawak, melioidosis.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Absorbance
AMC	Amoxicillin
AMR	Antimicrobial resistance
AST	Antibiotic Susceptibility Testing
AUS	Abdominal Ultrasonography
AZ	Azithromycin
Bcc	*Burkholderia cepacia complex*
Bp	Base pair
CDs	Coding sequence(s)
CLSI	Clinical and Laboratory Standard Institute, USA.
CSF	Cerebrospinal fluid
CT	Computed topography
DC	Doxycycline
ddH_2O	Double-distilled water
dNTPs	Deoxynucleoside triphosphates
EUCAST	European Committee on Antimicrobial Susceptibility Testing, Europe.
GC	Guanine-cytosine
GEN	Gentamicin
GIs	Genomic island(s)
HGT	Horizontal gene transfer
IHCM	Institute of Health and Community Medicine
IZD	Inhibition Zone Diameter

IZ	Inhibition zone
LB	Luria-Bertani
LFI	Lateral flow immunoassays
Mbp	Mega base pair
MEM	Meropenem
MH	Mueller-Hinton agar (HiMedia, India)
MHb	Mueller-Hinton II broth (HiMedia, India)
$MgCI_2$	Magnesium chloride
MIC	Minimal Inhibitory Concentration
MRI	Magnetic resonance imaging
NZ	No inhibition zone detected
OD	Optical density
PCR	Polymerase Chain Reaction
QC	Quality control
rRNA	Ribosomal ribonucleic acid
SMX	Sulfamethoxazole
Spp.	Species
ST	Sequence Type
SXT	Co-trimoxazole (Sulfamethoxazole+Trimethoprim)
TMP	Trimethoprim
TSA	Tryptic Soy agar (HiMedia, India)
TTSS1	Type III Secretion System
TZ	Ceftazidime
UNIMAS	Universiti Malaysia Sarawak

CHAPTER 1

INTRODUCTION

Melioidosis is a severe and notable primary lead of fatal community-acquired sepsis and pneumonia in its area of endemicity (Currie & Kaestli, 2016). Historically, the disease is endemic in Northern Australia and some parts of Southeast Asia (Sahl et al., 2015) but has now been increasingly emerging in other parts of the globe, such as in Malawi, Northeastern Brazil, and Central America (Limmathurotsakul et al., 2016; Wiersinga et al., 2018; Sanchez-Villamil & Torres, 2018). A previous study by Limmathurotsakul et al. (2016) estimated that melioidosis would affect 165 000 individuals globally per year, and 54% (89 000) would be fatal. Meanwhile, in Malaysia, which is also endemic for melioidosis, 3703 people suffered from melioidosis annually, of which more than 2000 dead (Nathan et al., 2018). Furthermore, in Sarawak, Malaysian Borneo, 12.3 per 100 000 of the Central Sarawak population acquired melioidosis annually, of which 35% were fatal (Sia et al., 2021).

The etiologic agent of melioidosis is a Gram-negative environmental saprophyte called *Burkholderia pseudomallei* (White, 2003). It is characteristically resistant to a myriad of antimicrobial agents, including many β-lactams, macrolides and aminoglycosides (Rhodes & Schweizer, 2016). Nevertheless, interestingly in Sarawak, melioidosis infection is majorly caused by a rare aminoglycoside or specifically the gentamicin-susceptible *B. pseudomallei* of sequence type (ST)881 and ST997 (Podin et al., 2013). Based on previous phylogenetic studies of *B. pseudomallei*, the population of ST881 and ST997 has thus far, been confined to Sarawak (Podin et al., 2013; Arushothy et al., 2020). Besides, although there have been studies to understand the clinical epidemiology of melioidosis in Sarawak, there are still gaps of knowledge on the magnitude of the antibiotic susceptibility or

resistance in *Burkholderia pseudomallei* isolates, and the efficiency of antibiotics used for the treatment of melioidosis.

Basically, infection with the bacteria can occur through open wound inoculation (Wiersinga et al., 2012), inhalation or ingestion of the contaminated soil, water, or aerosolised dust particles that transport the bacteria (Chen et al., 2015). The infection has a diverse clinical spectrum that makes its diagnosis and treatment a challenge. For example, it can be manifested as bacteraemia without evident clinical focus, skin lesions, gastrointestinal tract ulcerations, and sometimes even mimic the symptoms of other diseases, such as pulmonary tuberculosis (Wiersinga et al., 2012; Hantrakun et al., 2019). Aside from that, the bacterium is known for being persistent in its infection. It can manifest as an asymptomatic latent infection for years and only progresses into acute melioidosis once the patient is immunocompromised (Ahmad et al., 2013). Hence, for these reasons, the treatment for melioidosis is biphasic and lengthy to minimise the risk of recurrence melioidosis.

For the first or acute phase treatment, ceftazidime or carbapenem drugs like meropenem and imipenem are administered intravenously for at least ten (10) days to prevent death from sepsis (Lipsitz et al., 2012). It is then followed by an eradicative-phase treatment with oral co-trimoxazole or doxycycline for at least twelve (12) weeks to prevent relapse (Dance, 2014). Despite that, lack of awareness and adherence to the lengthy treatment among the patients often leads to relapse or the acquisition of antibiotic resistance. Apart from that, although rare, the primary resistance of *B. pseudomallei* to prescribed antibiotics has been well-documented as a cause of treatment failure. For example, *B. pseudomallei*'s primary resistance to ceftazidime, which is the first-line of regimen for acute phase treatment, has been reported in Australia (4%) and Thailand (0.6%) (Jenney et al.,

2001; Wuthiekanun et al., 2011). Besides, there have been reports on the acquired resistance of *B. pseudomallei* towards co-trimoxazole in Thailand (16%) and Australia (2.5%) (Wuthiekanun et al., 2011; Sarovich et al., 2012). Similarly, there have also been increasing reports of higher minimal inhibitory concentrations (MIC) of co-trimoxazole that constituted for 16.7% to 37.5% resistance rate for melioidosis cases in Sarawak (Yong et al., 2016). Therefore, due to the unique sequence type of the *B. pseudomallei* from Sarawak, this incidence has sparked concerns among clinicians regarding the use and efficacy of the co-trimoxazole regimen for the eradicative-phase treatment of melioidosis in Sarawak.

Co-trimoxazole is a potent combination of trimethoprim and sulfamethoxazole that acts synergistically in inhibiting the folic acid synthesis (Kemnic & Coleman, 2021) of *B. pseudomallei*. Based on a previous study, such a resistance phenomenon is usually associated with the overexpression of the resistance-nodulation-division family efflux pumps, BpeEF-oprC (Podnecky et al., 2013). However, whether the previously characterised mechanism conforms to the co-trimoxazole resistance phenomenon in Sarawak warrants a study. Besides, the hypothesis of this study is that the nucleotide sequence of the *bpeEF-oprC* gene cluster for the rare Sarawak isolates may differ from the clinical *B. pseudomallei* isolates reported elsewhere. Furthermore, there is also a lack of standardised interpretation guidelines to aid in the accurate report of the co-trimoxazole susceptibility testing in Sarawak.

Therefore, to address the concern, the objectives of this study are:

1. To determine the co-trimoxazole susceptibility of Sarawak clinical *B. pseudomallei* isolates.
2. To determine a valid standardised interpretation guideline for the antibiotic susceptibility testing in Sarawak.

3. To elucidate the correlation between sulfamethoxazole and/or trimethoprim susceptibility in Sarawak clinical *B. pseudomallei* isolates.

4. To detect co-trimoxazole resistance determinant, the *bpeEF-oprC* genes in Sarawak clinical *B. pseudomallei* isolates.

The findings from this study should be able to provide more insights into the prevalence of co-trimoxazole susceptibility among the Sarawak clinical *B. pseudomallei* isolates. It is also hoped that the findings from this study are able to provide guidance on the laboratory diagnosis of melioidosis cases and to address clinicians' concerns on the efficacy of co-trimoxazole in the treatment of melioidosis.

CHAPTER 2

LITERATURE REVIEW

2.1 The etiologic agent: *Burkholderia pseudomallei*

The bacteria first emerged in 1911 as the etiologic agent of a glanders-like illness among the Burmese morphine addicts (White, 2003). It was discovered during the autopsy observation on those patients and differentiated from the causative agent of glanders by its swab culture. The autopsy swab grew as a new organism characterised as fast-growing, motile, Gram-negative bacilli (Cheng & Currie, 2005). It was then named *Bacillus pseudomallei* which derived from the Greek word 'pseudes', which means fake, due to its resemblance with the formerly characterised *Bacillus mallei*. However, it was known by various names during its early discovery and has been reassigned into different genera like *Malleomyces*, *Loefflerella*, and *Pseudomonas* (Schoch et al., 2020). Later, in 1992, it was incorporated into the genus *Burkholderia* (Yabuuchi et al., 1992 cited in Hardin, 2016) and henceforth, known as *Burkholderia pseudomallei*.

2.1.1 The Genus: *Burkholderia*

The bacterial genus *Burkholderia* belongs to the *Betaproteobacteria* subphylum in the family *Burkholderiaceae*. It was proposed by Yabuuchi et al. (1992) to cater to the seven (7) formerly characterised rRNA group II pseudomonads. The assignment was made according to the phenotypes, lipid compositions, DNA homology value, 16S rRNA sequences and the biochemical basis of the bacteria. At present (February 2022), there are 127 validly published species, and plenty of candidate species are still waiting for a formal description (Parte et al., 2020).

The members of the *Burkholderia* genus are remarkable for being well-adapted to diverse environmental and biological niches (Estrada-de los Santos et al., 2016). Most of them are plant pathogens and commensal such as the *Burkholderia gladioli* and *Burkholderia cepacia complex* (*Bcc*) species. They are metabolically versatile and harbour tremendous benefits for agricultural, pharmaceutical, and biotechnological use (Eberl & Vandamme, 2016; Estrada-de los Santos et al., 2016; Kunakom & Eustáquio, 2019). Despite that, due to their opportunistic characteristic of causing diseases in animals and humans, the use of the *Burkholderia* spp. in the aforementioned applications is controversial. The *Bcc* species, for example, may cause opportunistic infection in the immunosuppressed individuals and pseudo-infection by their ability to contaminate pharmaceutical products (Sahl et al., 2016; Depoorter et al., 2016). In addition to that, there are *B. mallei* which cause equine glanders and *B. pseudomallei*, which is responsible for the significant public health disease, melioidosis (Titball et al., 2017).

2.1.2 General Bacteriology of *Burkholderia pseudomallei*

Generally, the bacterium is a relatively small-sized non-sporulating aerobe measuring about 0.8×1.5 μm (Sprague & Nebauer, 2004; Barnewall et al., 2015). When cultured on standard nutrient agar, they typically exhibited differing colonial morphology, in which they appeared as round and mucoid colonies and became wrinkled metallic colonies on further incubation (Currie, 2010). These colony morphology variants were often associated with their response towards environmental stimuli and adaptation of the bacteria during human melioidosis (Chantratita et al., 2007). A further discussion on the colony morphology is in Section 4.1.

Aside from that, interestingly, the mature colonies of *B. pseudomallei* allow them to be distinguishable from their closely related species like *B. thailandensis,* by their musty and earthy odour (Inglis et al., 2015). The bacterium is also differentiable from the other avirulent *Burkholderia* spp. for their disability to assimilate L-arabinose as their sole carbon source (Moore et al., 2004).

Ubiquitous in the water surfaces and soil, the bacterium is constantly vulnerable to various adverse environments. Despite that, they are notoriously persistent in their surroundings, particularly the areas with high salinity, extremes in pH and temperature, nutrients depredation and even thrive in distilled water for years (Hantrakun et al., 2016; Chuah et al., 2017). Nevertheless, the bacterium is treatable from the contaminated water sources through ozonation, ultraviolet radiation and chlorination (Kumar et al., 2020).

2.1.3 Genomic Characteristics *B. pseudomallei*

Unlike the other prokaryotes that have only one chromosome, the genome of *B. pseudomallei* is composed of a bipartite enormous circular chromosome. For example, the reference strain *B. pseudomallei* K96243 consists of two chromosomes that are in total 7.27 mega base-pairs (Mbp) in size, each chromosome encoding for 3460 and 2395 coding sequences (CDs), respectively (Holden et al., 2004). According to Holden et al. (2004), the large chromosome is the carrier of housekeeping genes necessary for growth, cell metabolism and macromolecular biosynthesis, whereas the smaller chromosome carries auxiliary genes required for the virulence factors and bacterial adaptation to varying environments.

Over the years, sequences of *B. pseudomallei* have proven to be highly dynamic. For instance, in a study by Viberg et al. (2017), high genetic variability was observed in the *B.*

pseudomallei isolated from a chronic infection of cystic fibrosis. The sequences of the *B. pseudomallei* exhibited evolutionary patterns, such as the emergence of antibiotic resistance and mutation on its virulence genes like the Type III Secretion System (Viberg et al., 2017). These observations suggested rapid adaptations of the bacterium within its host, which resulted from frequent genomic recombination events.

Consequently, the frequent recombination events have caused the *B. pseudomallei* genome to have numerous DNA regions with unusual guanine-cytosine (GC) content that is either higher or lower than 68% if compared to the other bacterial genomes (Bugrysheva et al., 2015; Price et al., 2017). Additionally, the unusual GC content has also increased the difficulty of designing primer and genomic analysis or sequencing for the *B. pseudomallei* DNA.

Aside from that, frequent recombination events also led to the formation of diverse genomic islands (GI). According to Tuanyok et al. (2008), those GIs may harbour genes that encode for bacterial prophage, metabolism, pathogenicity, and hypothetical proteins with unknown functional roles. Those GIs can also be varied among the strains. Furthermore, it has been the prominent source of intraspecies genetic diversity, that in particular, contributed to different virulence among them.

2.1.4 Virulence factors of *B. pseudomallei*

B. pseudomallei is armed with innumerable virulence factors that facilitate its infectivity and survival in the host cells. For example, the adherence and invasion of the bacteria into its host cell can potentially be aided by the motility factor A (BimA) putative type V effector protein and several components of the Type III Secretion System (T3SS), such as the BsaZ (Duangurai et al., 2018).

Chronologically, the bacteria first modulate its uptake or engulfment into its host cell, at which point secretion of BsaZ T3SS effectors (Duangurai et al., 2018) rapidly disrupt the vacuolar membrane to facilitate the bacterium escapes into the host cell cytosol. Then, when it is finally inside the host cytosol, the bacterium activates the *BimA* protein to induce actin polymerisation to aid bacterial spread between adjacent cells (Stevens et al., 2005). It also concurrently promotes the bacterial proliferation and formation of multi-nucleated giant cells (Galyov et al., 2010).

Apart from its ability to invade the host cell, *B. pseudomallei* is remarkable in evading the host defenses. It is resistant to cationic peptides and complements lysosomal defensins (Gan, 2005 as cited in Lazar Adler et al., 2009). Besides, it can also synthesise numerous virulence factors such as catalase, peroxidase, protease, lecithinase, siderophore, hemolysin, and lipase, for its survival and maintenance (Jones et al., 1996).

Furthermore, *B. pseudomallei* can synthesise a glycocalyx polysaccharide capsule or biofilm (Galyov et al., 2017). This capsule is known as a significant virulence determinant, as it can enable the formation of microcolonies in a protective environment that would alter the organism's phenotype (Cheng & Currie, 2005). It usually leads to a significant reduction in antibiotic efficacy and may cause the latent infection of *B. pseudomallei*.

2.1.5 Select Agent Status

B. pseudomallei is classified as a Category B bioweapon agent by the United States Centres for Disease Control and Prevention (White, 2003). This classification was made due to the easy dissemination of the bacteria, their ability to cause moderate morbidity and fatality, and the lack of vaccines available that required enhanced disease surveillance (Limmathurotsakul et al., 2016). Therefore, all experiments involving the bacterium must be

performed in a Biosafety Level 2 (BSL 2) and following the Select Agent compliant standard procedure (Appendix 1).

2.2 The Disease: Melioidosis

Melioidosis is an emerging tropical bacterial infection caused by *B. pseudomallei* that occurs in human and animals. The infection is named based on the Greek "melis" (Stanton & Fletcher, 1921), which refers to the ability of the bacteria to manifest infection similar to glanders.

2.2.1 Epidemiology of Melioidosis

Melioidosis is endemic in Northern Australia and parts of Southeast Asia. It is, however, increasingly recognised across the 20th of northern and southern parallels that include Madagascar, Southern China, India, and Puerto Rico (Limmathurotsakul & Peacock, 2011). Imported incidences have also been reported in other temperate regions globally, such as in Central China (Yuan et al., 2019) and Europe (Le Tohic et al., 2019). The prevalence of melioidosis in the newly added regions is not known. The true incidence may also be underdiagnosed, especially with the diagnostic limitations of the infection. The predictive modelling study by Limmathurotsakul et al. (2016) estimated 165 000 cases of human melioidosis occur globally per year, of which 89 000 (54%) die. Based on the authors' prediction, the mortality burden of melioidosis is comparable to measles (96 000) and higher than that of leptospirosis (50 000) and dengue (9 100-12 500).

In Malaysia, the incidence of melioidosis usually involves the states that have active agriculture activities. For instance, in Pahang, the estimated human melioidosis case is 4.3 per 100 000 populations annually (Chua et al., 2010). Infection may occur in individuals of all ages. However, a previous study by Nathan et al. (2018) stated that the peak age-specific

melioidosis incidence in Malaysia is between 44 to 51 years, which is the age range during which most co-morbid conditions develop. The authors described type 2 diabetes mellitus as the most common co-morbid condition observed in 38 - 75% of the patients. Other co-morbid conditions described include chronic renal disease, tuberculosis, immune disorder, and solid tumors (Nathan et al., 2018). Kingsley et al. (2016) also added that the infection usually occurs in individuals with occupational exposure to the environment, such as farmers and blue-collar workers.

An individual could acquire the infection from contaminated soils and waters via percutaneous inoculation, indigestion, or inhalation of *B. pseudomallei* (Wiersinga et al., 2018). The peak incidence is often associated with intense rainfall and severe weather, such as during the wet season in Kelantan that occurred from November to February (Kaestli et al., 2016; Zueter et al., 2016). During the wet season, the bacteria can get churned up to the soil or water surface along with the flood, become aerosolized, and hence increasing the exposure potential. Additionally, although rare, Aziz et al. (2020) has confirmed a human-to-human melioidosis acquisition from a breastfeeding mother to her infant through comparative genomic analysis on their clinical isolates. Despite that, melioidosis is not contagious.

2.2.2 Epidemiology of Melioidosis in Sarawak, Malaysian Borneo

Sarawak is the largest state in Malaysia, situated on the northwest coast of Borneo Island. Melioidosis, which is hyper-endemic in Sarawak, has been notifiable since 2003 (Sarawak Health Department, unpublished data). The incidence of melioidosis has thus far clustered across the Central Sarawak regions, especially in Bintulu and Kapit (Podin et al., 2014). Factors that might contribute to the melioidosis prevalence are the rural lifestyle and

occupational hazards of the residents. Socio-economics in the said regions involve land-clearing activities for agricultural, industrial-scale logging, and infrastructure expansion projects. Besides, most residents resided in traditional longhouses, where gravity-fed water systems supply untreated water (reservoir of *B. pseudomallei*) from the streams.

Interestingly also, Podin et al. (2014) defined the predominant causal (86%) of melioidosis in Central Sarawak as gentamicin susceptible *B. pseudomallei* isolates (belongs to the multilocus sequence type (ST)881 or its single locus variant, ST 997), whereas *B. pseudomallei* has been intrinsically resistant to the aminoglycosides (Currie, 2015). According to the recent study by Sia et al. (2021), the average incidences of melioidosis in the said regions were about 12.3 per 100 000 populations annually. The commonest co-morbidities observed among the patients are diabetes mellitus and hypertension (Toh et al., 2021). Additionally, Mohan et al. (2017) estimated an average of 4.1 per 100 000 children under 15 years across Central Sarawak might acquire the infection yearly. The authors described a 24% fatality rate of pediatric melioidosis that primarily occurs among children with no known medical conditions but poor nutritional status.

2.2.3 Clinical Manifestation of Melioidosis

According to Currie (2010), the clinical manifestation and severity of melioidosis depends on the patient's risk factors, infection mode, bacterial load, and strain virulence. The onset of acute melioidosis commonly occurs within three (3) weeks or an average of nine (9) days upon exposure (Wiersinga et al., 2018). Nevertheless, a latent infection may also occur, with the disease manifesting years after exposure (Dan, 2015). Melioidosis can affect all organs in our body and manifests a myriad of symptoms. It is infamous for mimicking

the symptoms of other community-acquired infections, tuberculosis, and malignancy. Hence, the reason that hampered clinical diagnosis of melioidosis.

Based on the review done by Nathan et al. (2018) on melioidosis in Malaysia, almost all cases reported (>90%) are of acute onset that manifests as an acute respiratory infection, acute bacteremia, and acute soft tissue infection with fever. The authors described the variable of melioidosis clinical spectrums include pneumonia (33 - 63%), septicemia (19 - 61%) that commonly occur in patients with diabetes mellitus, disseminated infection (16 - 37%) presenting as an abscess in multiple organs with or without bacteremia, and acute localized infection (10%) that may progress into septicemia or disseminate infection. The documented clinical presentations are consistent with most parts of the melioidosis endemic regions.

2.2.4 Diagnosis of melioidosis

The absence of pathognomonic presentation and unfamiliarity with melioidosis among attending clinicians often delay and misdiagnose the disease. Nevertheless, imaging modalities such as computed tomography (CT) scan, ultrasound, and magnetic resonance imaging (MRI) have demonstrated a significant role in providing crucial clues for the diagnosis and management of melioidosis. For instance, Mohan et al. (2020) suggested the utility of abdominal ultrasonography (AUS) in diagnosing pediatric melioidosis due to abdominal visceral involvement in most reported cases.

Definitive melioidosis diagnosis, however, still majorly relies on laboratory evaluations of clinical samples obtained from the suspected patient. The sample includes blood, urine, and sometimes cerebrospinal fluid (CSF), pus, and wound swabs (Gassiep et al., 2020). The culture of *B. pseudomallei* from the samples is the mainstay of melioidosis

diagnosis. It is routinely cultured on selective media such as blood agar, chocolate agar and MacConkey agar. Meanwhile, in Sarawak, selective media like modified Ashdown's agar (containing 50 µg/ml colistin) are used due to the prevalence of gentamicin-sensitive *B. pseudomallei* strain in Sarawak. Next, to differentiate the isolated *B. pseudomallei* from its closely related species, the bacterial culture is profiled through gram staining and the biochemical API 2ONE (bioMierieux, France) fast identification system (Choi et al*.,* 2020). Once culture-confirmed, the *B. pseudomallei* isolate is tested for its antibiotic susceptibility to ensure an appropriate antibiotic prescription for its therapy. The overall diagnosis process usually took approximately four (4) to seven (7) days.

Aside from that, some laboratory settings utilized more efficient diagnostic methods such as real-time Polymerase Chain Reaction (qPCR) and lateral flow immunoassay (LFI). The qPCR can rapidly detect the *B. pseudomallei* by targeting its Type III Secretion System (TTS1) (Novak et al*.,* 2006). Meanwhile, LFI utilizes a monoclonal antibody to target the *B. pseudomallei* capsular polysaccharide (Houghton et al*.,* 2014). These methods are faster and take approximately three (3) days to diagnose melioidosis and hence may significantly facilitate the therapy efficacy.

2.2.5 Treatment of Melioidosis

To date, standard melioidosis therapy has recommended an initial parenteral intensive phase followed by an oral eradication phase. The drug(s) dosage regimen prescribed for the biphasic treatment is dependent on the strain antibiotic susceptibility, patient risk factors, and disease severity (Zueter et al., 2016).

The parenteral intensive-phase usually involves a regimen of ceftazidime or carbapenem (meropenem and imipenem) that lasts for ten (10) to fourteen (14) days or longer

when clinically indicated (Lipsitz et al., 2012). In the case involving neurologic cutaneous, bone, and prostatic melioidosis, the combination of ceftazidime and co-trimoxazole is usually prescribed to achieve higher treatment efficacy (Currie, 2015). Additionally, Sia et al. (2018) described gentamicin regimen for the treatment of melioidosis caused by the gentamicin-susceptible *B. pseudomallei* in Sarawak. However, the administration of gentamicin in intensive-phase treatment is not standardised and still requires further pharmacokinetics (PK) study.

Additionally, the patient's blood culture is analysed weekly to detect the possible emergence of antibiotic resistance and ensure the efficacy of the antimicrobial therapy. Next, the subsequent oral eradication-phase treatment will start once the blood culture is negative and the patient has recovered from their symptoms. This extended eradication-phase treatment is crucial in minimising the recrudescence or relapse of melioidosis (Dance, 2014). The first-line drug for the therapy is the co-trimoxazole regimen for three (3) to six (6) months (Gassiep et al., 2020). However, Wen et al. (2008) reported that co-trimoxazole might cause serious adverse effects on pregnant women and children. Meanwhile, in Sarawak, the co-trimoxazole regimen is least preferred as clinicians are concerned with the reports of higher minimal concentration readings of co-trimoxazole against *B. pseudomallei* clinical isolate *in-vitro* (Yong et al., 2016). Hence, for those cases, the regimen of co-amoxiclav is recommended.

2.2.6 Vaccine Development

Vaccine development has long been anticipated and greatly needed in melioidosis-endemic areas. An effective vaccine shall protect both healthy and immunosuppressed individuals from the natural infection of melioidosis. There are several promising vaccine

candidates against melioidosis including live-attenuated strains, inactivated whole-cell and protein subunits that have been extensively studied (Titball et al., 2020).

Thus far, the live-attenuated vaccines have been exhibiting potent immune responses that can provide lasting immunity. It is evident in Khakhum et al. (2019) that the *B. pseudomallei* Δ*tonB* Δ*hcp1* (PBK001) mutant vaccine is capable of activating a robust immune response by humoral immunity and providing total protection for the mice model against aerosol infection. However, due in part to its potential for reverting to a virulent wild-type strain that can cause infection, it is unlikely that the live-attenuated vaccine can be licensed (Gassiep *et al.,* 2020).

Next, the inactivated whole-cell vaccine is cost-effective and capable of inducing immunity through multiple antigens (Titball et al., 2020). For instance, Petersen et al. (2014) proved the multivalent outer membrane vesicles (OMV) vaccine derived from *B. pseudomallei* 1026b to be safe and immunogenic in rhesus macaques. However, the vaccine is potentially reactogenic and needed in several doses. Besides, there is a concern for lesser protective antigens produced *in-vivo* compared to that shown in the *in-vitro* experiment.

Lastly, a safer option for vaccine candidates is the subunit vaccine. The vaccine utilizes protective antigens to induce the host's cellular and humoral immunity. Examples of extensively studied antigens include lipopolysaccharide (LPS), outer membrane proteins (OMP), and capsular polysaccharide (CPS). It was shown recently in Burtnick et al. (2018) that CPS-CRM97 and Hcp1 recombinant offered 70% sterilizing immunity with no culturable bacteria present in the organs of the mice models. However, despite these promising findings, there has yet been any performable clinical trial to check the validity

and effectiveness of the vaccine. Hence, until now, there is no licensed vaccine available for melioidosis (Titball et al., 2017).

2.3 *B. pseudomallei* and their clinically relevant antibiotics

2.3.1 Mode of action for the clinically relevant antibiotics

The antibiotics panel recommended for the standard melioidosis treatment include ceftazidime, carbapenems drugs, co-trimoxazole and co-amoxiclav (Lipsitz et al., 2012). These drugs can be grouped into different antimicrobial classes based on their mode of action or the cellular synthesis that they inhibit. Ceftazidime, carbapenems drugs and co-amoxiclav belonging to the β-lactams class are cell wall biosynthesis inhibitors. Meanwhile, the co-trimoxazole belongs to the sulfonamide class that targets the folate biosynthesis of the bacteria.

As a cell wall biosynthesis inhibitor, the target of β-lactams is an enzyme called penicillin-binding proteins. The β-lactams bind covalently and irreversibly to the enzyme and thus disrupt the synthesis of peptidoglycan monomers, transpeptidase and carboxypeptidase (Opal & Pop-Vicas, 2015). These compounds are the essential binding protein for the polymerisation, elongation, and cross-linking of the peptidoglycans (Zeng & Lin, 2013). The peptidoglycan is a mesh-like structure between the outer and inner (cytoplasmic) membrane. It maintains the cell wall and is essential for cellular division and the bacteria' adaptation to the diverse stresses they may encounter in the different niches (Zeng & Lin, 2013).

On the other hand, sulfonamide co-trimoxazole is a potent combination of sulfamethoxazole and trimethoprim (Kemnic & Coleman, 2021). The sulfamethoxazole and trimethoprim act in a bacteriostatic manner when used alone. Meanwhile, when combined,

the antibiotics work synergistically against two sequential steps of bacterial folate biosynthesis. First, during the synthesis of dihydrofolate, sulfamethoxazole acts as the competitor of p-aminobenzoic acid (PABA) to inhibit the action dihydropteroate synthase (Gorelova et al., 2019). This action is then immediately followed by the binding of trimethoprim to the dihydrofolate reductase and inhibits the production of tetrahydrofolic acid (Fernández-Villa et al., 2019). Tetrahydrofolate is an essential component in the synthesis of purines, which is needed for DNA and protein production (Gorelova et al., 2019). In short, these simultaneous and bactericidal actions of co-trimoxazole (sulfamethoxazole+trimethoprim) starved the bacteria of two bases, thymidine and uridine, which are vital for DNA replication and transcription. Hence, due in part to this, co-trimoxazole is chosen as the first-line regimen for the eradicative-phase treatment of melioidosis.

2.3.2 Intrinsic resistance

Burkholderia pseudomallei is inherently resistant to a multitude of antimicrobial classes, including B-lactams, aminoglycosides, and macrolides (Lipsitz et al., 2012). The primary resistance mechanism of *B. pseudomallei* to β-lactams, such as ceftazidime and penicillin, is due to the enzymatic inactivation of the antibiotics by the β-lactamase PenA (Sarovich et al., 2012). Meanwhile, the resistance mechanism for the aminoglycosides and macrolides is due to the upregulation of the BpeAB-oprB efflux pump (Sarovich et al., 2012).

2.3.3 Acquired resistance

Although rare, acquired resistance has been reported to occur during the acute and chronic phases of treatment. The incidence of acquired resistance is primarily associated

with the upregulation of the resistance-nodulation-division family (RND) efflux pump (Schweizer et al., 2012). For example, the AmrAB-oprA efflux pump is responsible for the acquired resistance of aminoglycosides. Besides, a study by Podnecky et al. (2017) also demonstrated that the acquired resistance to co-trimoxazole is due to the mutation on the transcriptional activator, *bpeT* gene of the BpeEF-oprC efflux pump.

2.4 Antibiotic Susceptibility Testing (AST)

Antimicrobial susceptibility testing (AST) is a procedure that defines the *in-vitro* antibacterial activity of antimicrobials within bacterial pathogens (Belkum et al., 2019). The result of the AST is crucial in predicting potentially emerging antimicrobial resistance in pathogens and aids in the timely inform of appropriate antimicrobial treatment. Therefore, it is commonly conducted as part of the diagnostic algorithm for bacterial infection.

Additionally, to ensure the reliability and validity of the antibiotic susceptibility profile, the AST results are interpreted in parallel with the standardised interpretive criteria. Those interpretative guidelines are regulated by authorities such as the United States Centre for Laboratory and Standard Institute (CLSI) and European Committee for Antibiotic Susceptibility Testing (EUCAST). Meanwhile, in Malaysia, including Sarawak, the guidelines from CLSI were adopted (Sia et al., 2021).

Generally, it takes at least 48 hours to identify the bacterial infection and assess its antimicrobial susceptibility. The AST methods usually yield either qualitative or quantitative results (Benkova et al., 2020). The phenotypic AST method known as disk diffusion test produces qualitative results that interpreted a bacterium as susceptible, intermediate or resistant to the tested antibiotic. Meanwhile, quantitative results infer the minimal inhibitory

concentration (MIC) of the tested antimicrobial, at which the visible bacterial growth is inhibited. This MIC result can be afforded through the E-test and microdilution method.

2.4.1 Kirby-Bauer Disk Diffusion method

Kirby-Bauer disk diffusion is the routine susceptibility method employed in most hospitals in the rural settings (Wuthiekanun et al., 2011), including Sarawak. According CLSI (2012), the fundamental of this method is based on the diffusion of predetermined antimicrobial concentration in a paper disk (see Figure 2.1) that is applied onto the surface of agar that had been inoculated with a standardised bacterial cell suspension. Later, as the antimicrobial diffuses into the agar, a zone in which the growth of the inoculated bacteria was inhibited is formed. The zone that is typically known as inhibition zone will then be utilised to interpret the bacterium as either "susceptible", "intermediate" or "resistance" against the tested antimicrobial (CLSI, 2017).

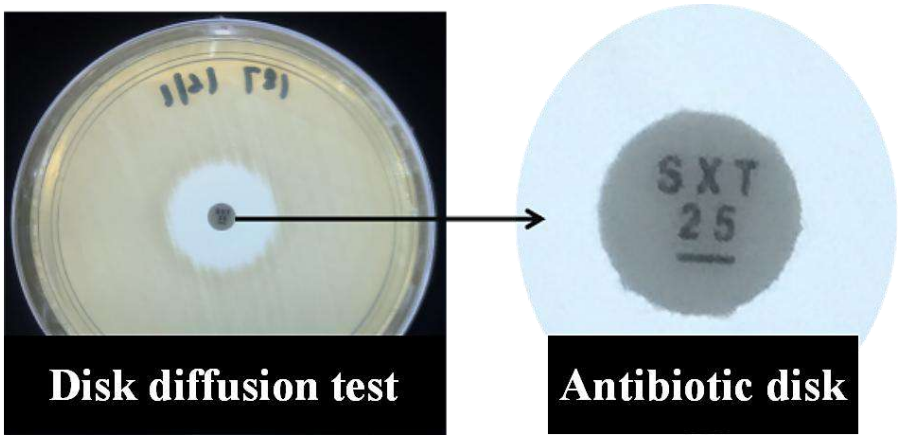


Figure 2.1: The 25 µg co-trimoxazole, commercialised antibiotic disk (OXOID, UK) that was used for the disk diffusion test in this study.

Basically, this method is favored due to its simple and cost-effective procedure that allowed the susceptibility assessment of multiple antimicrobial agents at one time (Reller et al., 2009). Besides, it produces qualitative results that are easy to be interpreted by the

clinician microbiologist (Khosravi et al., 2014). However, this disk diffusion test also tends to report a false resistant result. According to Wuthiekanun et al. (2005), 82% of the resistant isolates defined by disk diffusion is proved susceptible by the E-test method. Hence, in this study, the accuracy of disk diffusion results is analysed to ensure that it is still valid as the routine susceptibility testing method for melioidosis cases in Sarawak.

2.4.2 Epsilometer test (E-test) method

The Epsilometer test (E-test) or gradient diffusion is a quantitative approach to determining the antibiotic susceptibility of bacteria (Reller et al., 2009). Historically, this method was established to overcome several disadvantages of the disk diffusion and microdilution procedure (Sader & Pignatari, 1994). Therefore, the principle of the E-test is similar to that of disk diffusion, except that an E-test strip is used instead of the antibiotic disk. The E-test strip is technically innovated based on the microdilution principle. It is a non-porous plastic strip impregnated with the predetermined exponential and continuous gradient of antibiotic concentrations on the underside of the strip and printed with the MIC (μg/ml) scale on upper side of the strip, as shown in Figure 2.2.

This method is superior to the disk diffusion method as it provides quantitative MIC (μg/ml) results, which can be used in determining the dosing regimens for a personalised antibiotic therapy (Tenover, 2009). It is also typically employed to confirm the equivocal antibiotic susceptibility test results produced via disk diffusion method. In this study, the E-test was employed to confirm the co-trimoxazole susceptibility of isolates that were initially defined as intermediate and resistant by disk diffusion method. The difference between the susceptibility assessment using the E-test and disk diffusion method will be further discussed in Chapter 4.

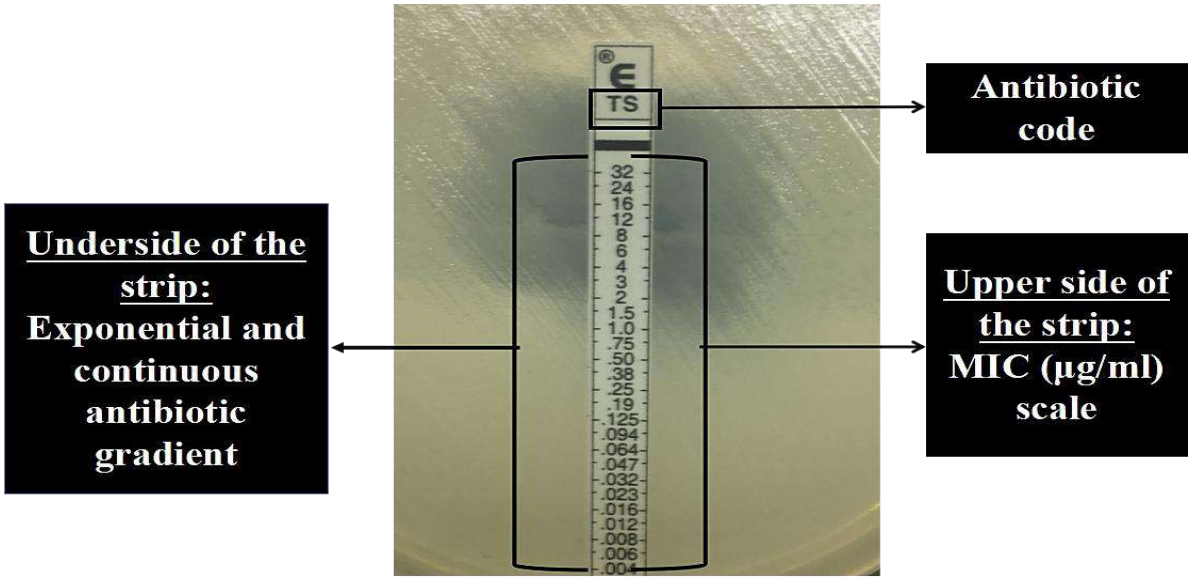


Figure 2.2: This is an example of co-trimoxazole (TS) E-test strip (bioMérieux, France) that was used for the E-test procedure in this study.

2.4.3 Microdilution method

Microdilution is considered the gold standard of the antibiotic susceptibility testing. It is also the only method recommended by the CLSI and EUCAST's committees for the testing of *B. pseudomallei* antibiotic susceptibility (Maloney et al., 2017). However, the procedure of microdilution required a tedious preparation for serial twofold dilution of antibiotic concentrations with the possibility of false resistant results due to the long incubation period (Waites et al., 2012). Therefore, the method is usually applied when there is need to confirm the results of antibiotic susceptibility by E-test or disk diffusion.

Apart from that, microdilution is employed for quantitative susceptibility assessment of a pathogen (ISO, 2019). The microdilution test produces extensive epidemiological MIC values for the tested pathogen. These epidemiological MIC values are an essential aid in determining the clinical breakpoints without causing any deleterious effect on the clinical performance of the antibiotic dosage (EUCAST, 2019). In this study, the microdilution

method was performed to assess the epidemiological MIC values of the Sarawak clinical *B. pseudomallei* isolates against the separate components of co-trimoxazole, the sulfamethoxazole and trimethoprim. A further discussion on the susceptibility assessment of the Sarawak isolates will be discussed in Section 4.2.

CHAPTER 3

METHODOLOGY

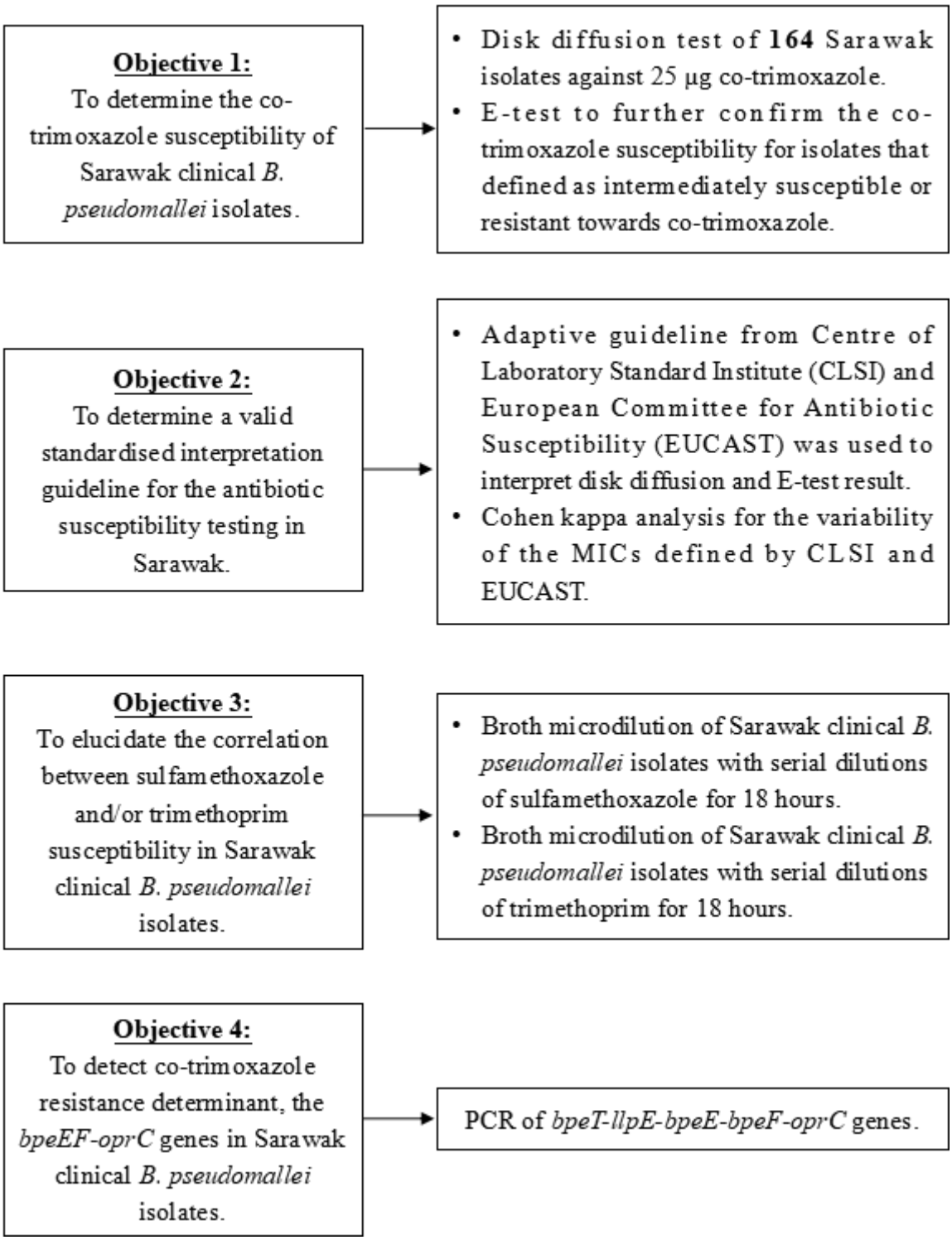


Figure 3.1: Flowchart above shows the overview of the works done in this study.

3.1 Materials Preparation

3.1.1 Growth Media for Bacterial Culture

There were three (3) types of growth media used for the bacterial culture throughout this study. The Ashdown's agar was modified with 50 µg/ml colistin as the selective agent for *B. pseudomallei* (Podin et al., 2014) and was used to check for the purity of the isolates. Meanwhile, tryptic soy agar (TSA) and broth (TSB) were used to grow the culture of *B. pseudomallei* from the 20% glycerol in Luria-Bertani (LB) stock and, for the sub-culture of the bacteria.

I. Ashdown's agar modified with 50 µg/ml colistin

Tryptone (HiMedia Laboratories, India)	: 6 g
Glycerol Biotechnology (Vivantis Technologies, Malaysia)	: 16 ml
Bacteriological agar powder (Scharlau Chemie s.a, Spain)	: 6 g
1% aqueous neutral red (Acros Organics, Belgium)	: 2 ml
1% crystal violet (Sigma-Aldrich, US)	: 2 ml
UHQ water (Veolia Water Technologies, UK)	: Top up to 400 ml
Colistin, 20 mg/L (Acros Organics, China)	: 8 mg

II. Tryptic soy agar (TSA)

Tryptic soy broth (OXOID, UK)	: 12 g
Bacteriological agar powder (Scharlau Chemie s.a, Spain)	: 4 g

UHQ water (Veolia Water Technologies, UK) : Top up to 400 ml

III. Tryptic soy broth (TSB)

Tryptic soy broth (OXOID, UK) : 12 g

UHQ water (Veolia Water Technologies, UK) : Top up to 400 ml

3.1.2 Solution and Media used in the Antibiotic Susceptibility Testing (AST)

The bacterial suspensions for all antibiotic susceptibility testing were prepared and diluted with 0.85% saline (NaCl) solution. For the media, Mueller Hinton agar (MHA) was used for Kirby-Bauer disk diffusion and E-test method, whereas the Mueller Hinton II broth (MHb) was used in broth microdilution. The selection of the media used for all AST methods were as specified in the standard protocols by CLSI (2017), CLSI (2018) and EUCAST (2018).

I. 0.08% saline (NaCl) solution

Sodium Chloride (NaCl) (R&M Chemicals, UK) : 8.5 g

UHQ water (Veolia Water Technologies, UK) : Top up to 1000 ml

II. Mueller Hinton agar (MHA)

Mueller-Hinton agar powder (HiMedia Laboratories, India) : 15.2 g

UHQ water (Veolia Water Technologies, UK) : Top up to 400 ml

III. Mueller Hinton II broth (MHb)

Mueller-Hinton broth powder (HiMedia Laboratories, India) : 8.4 g

UHQ water (Veolia Water Technologies, UK) : Top up to 400 ml

3.1.3 Agarose Gel and Buffer Solution for Gel Electrophoresis

For the analysis of PCR products via gel electrophoresis, 0.7% agarose gel and 1× Tris-Borate-EDTA (TBE) buffer solution was prepared as the following:

I. 0.7% Agarose gel

Agarose Powder (Biotechnology Grade, 1st BASE, Singapore) : 0.42 g

UHQ water (Veolia Water Technologies, UK) : 60 ml

II. 1× Tris-Borate-EDTA (TBE) buffer solution

10× Tris-Borate-EDTA (TBE) buffer, pH8.3, Ultra-Pure Grade (Vivantis Technologies, Malaysia) : 1 L

UHQ water (Veolia Water Technologies, UK) : 9 L

3.2 Bacterial Isolates Collection

Burkholderia pseudomallei isolate SWK-C 001 (also coded as MSHR 5078) was used as the prototype strain for antibiotic susceptibility testing by disk diffusion and Epsilometer test (E-test) methods in this study. Meanwhile, *Escherichia coli ATCC 25922* and *Escherichia coli ATCC 11775* were used as the quality control for the broth microdilution method. A total of 164 clinical *B. pseudomallei* isolates were obtained from the archival isolate collection at the Institute of Health and Community Medicine, Universiti Malaysia Sarawak (IHCM, UNIMAS). These isolates were isolated from melioidosis cases reported between 2011 until 2018 in six hospitals situated in Bintulu, Sibu, Kapit, Miri, and Kuching in Sarawak, Malaysian Borneo (see Table 3.1 for details). Additionally, all isolates had been characterised by its morphological appearance on modified Ashdown's selective agar with 50 µg/ml colistin (Acros Organics, China) (Podin et al., 2014), molecularly identified through TTS1 real-time PCR (Novak et al., 2006) and standard biochemical characterisation using API 20NE assay (bioMérieux, France). The isolates were kept at -80 ºC in 20% glycerol in Luria-Bertani (LB) stock for future use. All procedures involving *B. pseudomallei* were performed in the Select Agent approved Biosafety Level 2 (BSL 2) facility in IHCM following the Select Agent compliant protocols (refer to Appendix 1 for the pathogen safety data sheet of *B. pseudomallei*) prepared by the Office of Laboratory Security, Public Health Agency of Canada (2011).

Table 3.1: Number of Sarawak clinical *B. pseudomallei* isolates collected from the hospitals.

Hospital	Location	No. of isolates collected
Bintulu Hospital	Bintulu, Sarawak	98
Sibu Hospital	Sibu, Sarawak	10
Kapit Hospital	Kapit, Sarawak	45
Miri Hospital	Miri, Sarawak	7
Sarawak General Hospital	Kuching, Sarawak	2
Borneo Medical Centre	Kuching, Sarawak	2

3.3 Antibiotic Susceptibility Testing (AST)

There were three (3) procedures of antibiotic susceptibility testing outlined to analyse the minimal inhibitory concentration (MIC) of Sarawak clinical *B. pseudomallei* isolates against co-trimoxazole (SXT). All AST procedures and MIC definitions were performed based on the standardised international guidelines including:

a. Document M100, 27th Edition, Clinical and Laboratory Standard Institute, CLSI (2017),
b. Document M07-A11, 11th Edition, Clinical and Laboratory Standard Institute, CLSI (2018),
c. European Committee for Antimicrobial Susceptibility Testing (EUCAST) Guidelines Version 8.1 (2018).

The rationale for using the CLSI and EUCAST standard guidelines was to examine the MIC result variability based on the different standards and, to determine the suitable

guideline that will aid in a better understanding of the interpretations of the antibiotic susceptibility testing for the Sarawak clinical *B. pseudomallei* isolates.

Hence, following those guidelines, the co-trimoxazole susceptibility for the Sarawak clinical *B. pseudomallei* isolates was first determined using the Kirby-Bauer disk diffusion. Next, to confirm the consistency of the MICs cut-off, isolates were further analysed using the E-test method. Lastly, the standard microdilution was chosen for the epidemiological MIC cut-off values determination of the separate components of co-trimoxazole, sulfamethoxazole and trimethoprim were done in Mueller Hinton II broth (MHb) (HiMedia Laboratories, India). This experiment was conducted to elucidate the correlation between co-trimoxazole susceptibility in the Sarawak *B. pseudomallei* clinical isolates with their susceptibility against the respective components of co-trimoxazole. The selection of the bacterial isolates for each procedure is described in Table 3.2.

Table 3.2: Selection of isolates for each antibiotic susceptibility testing procedure.

Kirby-Bauer Disk diffusion	**Epsilometer test (E-test®)**	**Broth Microdilution**
All 164 Sarawak clinical *B. pseudomallei* isolates	Isolates defined as the following criteria during disk diffusion: i. **SXT resistant** based on **either** one or **both** CLSI (2017) and EUCAST (2018) standard ii. **SXT intermediate** based on **either** one or **both** CLSI (2017) and EUCAST (2018) standard	Isolates defined as the following criteria during E-test: i. MIC **2 µg/ml** ii. **SXT intermediate** MIC iii. demonstrate any possibly resistance phenotypic colonies iv. **SXT susceptible** isolates

3.3.1 Kirby-Bauer Disk Diffusion

The Kirby-Bauer disk diffusion test was done following the updated and modified protocols regulated by the CLSI (2017). Firstly, the *B. pseudomallei* clinical isolates were grown on Tryptic Soy agar (TSA) (OXOID, UK) for 24 hours at 37°C before setting up for MIC determination. On the following day, the cell suspension of those isolates was prepared by diluted a loopful of the overnight culture in 0.85% sterile saline solution (NaCl) (R&M Chemicals, Essex, UK) to the optical density ≈ 0.13 A at 600 nm (0.5 McFarland standard) using a spectrophotometer (BioPhotometer 6131, Eppendorf, Germany). The resulting bacterial cell suspension was then swabbed evenly in three directions using a wooden swab on Mueller Hinton agar (MH) (HiMedia Laboratories, India) plates to which the 25 µg co-trimoxazole disk (OXOID, UK) was applied. The inhibition zone diameter (IZD) was measured after 18 hours of incubation period at 37 ºC (Growth Chamber Model GC-1050, Protech Electronics, Malaysia). Interpretations of the IZD were defined following the CLSI and EUCAST guidelines, see Table 3.3. Additionally, the colonies (if any) that grew within the IZ(s) formed were sub-cultured onto modified Ashdown's agar (containing 50 µg/ml colistin) for purity check. The disk diffusion test was then repeated for those particular isolates to finalize their MIC. When the culture appeared to be pure, the isolated colonies were grown on TSA plates and stored in 20% glycerol in LB glycerol stock in -80 °C for further study.

Table 3.3: The summarised interpretive guidelines for the Inhibition Zone Diameter (IZD) extracted from Document M100, 27th Edition, CLSI (2017) and EUCAST (2018) guidelines Version 8.1.

CLSI's IZD interpretive for co-trimoxazole (mm)			**EUCAST's IZD interpretive for co-trimoxazole (mm)**		
S ≥	**I**	**R ≤**	**S ≥**	**I**	**R <**
16	11 - 15	10	14	11 - 14	11
• Disregard slight growth (20% or less) and measure a clearer margin as IZD. • For isolated colonies that grew within inhibition zone (IZ), measure the radius of the colonies closest to the disk until the outer zone edge and multiply by two (2) to get the IZD. • Measure only the clear IZ when there is two (2) IZs formed.			• Ignore slight growth or any isolated colonies if the zone edge can be seen. • Read as no zone if there is any growth up to the disk. • If there is formation of two (2) IZs or isolated colonies within the zone, check the purity of the culture and repeat the test. If the culture is pure, consider the colonies growth when taking the IZD.		

3.3.2 Epsilometer Test (E-test)

E-tests on the selected isolates were done following the manufacturer's instructions (bioMérieux, France). The isolates were initially grown on TSA (OXOID, UK) for 18 hours at 37°C. Afterwards, to prepare the cell suspension, a loopful of the overnight culture was diluted with 0.85% NaCl solution (R&M Chemicals, Essex, UK) to an optical density ≈ 0.13 A at 600 nm using a spectrophotometer (BioPhotometer 6131, Eppendorf, Germany). Then, the resultant bacterial cell suspension was streaked evenly in three directions onto a MH agar plate to prepare the bacterial lawn to which E-test® strips (bioMérieux, France) were applied. The plates were incubated in a growth chamber (Model GC-1050, Protech Electronics, Malaysia) for 18 hours at 37ºC, then the IZ was observed to define the MIC breakpoints for

the isolates. Isolates with MIC 2 µg/ml, intermediate MIC, or showing any possibly resistant phenotypic colonies were re-evaluated against other clinically relevant antibiotics to check for their multidrug-resistance profile. For instance, amoxicillin-clavulanate, ceftazidime, doxycycline, and meropenem that are routinely used in the treatment of melioidosis (Lipsitz et al., 2012). Additionally, the genotype of the isolates was determined through the MIC definitions for gentamicin and azithromycin. All the antibiotics MIC breakpoints were defined based on the standard set for *B. pseudomallei* (CLSI, 2017; EUCAST, 2018), except for azithromycin. The MIC interpretive standards of azithromycin for *non-Enterobacteriaceae* have not yet been established, thus their MIC was defined following the manufacturer's (bioMérieux, France) interpretive recommendations for aerobes, see Table 3.4 for details.

Table 3.4: E-test MIC's interpretation reference for the antibiotics tested in this study.

Antibiotic	**MIC interpretive according to both CLSI and EUCAST standards (µg/ml)**		
	S ≤	**I**	**R ≥**
Co-trimoxazole (SXT)	2	-	4
Amoxicillin-clavulanate (AMC)	8/4	16/8	32/16
Azithromycin (AZ)	2	4	8
Ceftazidime (TZ)	8	16	32
Doxycycline (DC)	4	8	16
Gentamicin (GEN)	4	8	16
Meropenem (MEM)	2	8	2

[Extracted from Document M100, 27th Edition, CLSI (2017) and EUCAST (2018) guidelines Version 8.1.]

3.3.3 Broth Microdilution

The standardised broth microdilution for the selected isolates was done according to CLSI in Document M07-A11, 11th Edition (CLSI, 2018) and Document M100, 27th Edition (CLSI, 2017). *Escherichia coli* ATCC 25922 is the recommended minimal quality control (QC) for broth microdilution involving *non-Enterobacteriaceae.* The expected MIC breakpoints of the *E. coli* ATCC 25922 against trimethoprim are within 0.5 µg/ml to 2 µg/ml, while the MIC breakpoints for sulfamethoxazole are within 8 µg/ml to 32 µg/ml. Initially, it was routinely tested against each of the antimicrobial agents but, due to some uncertain circumstances possibly contamination, the MIC breakpoints of the control strain were out of the expected limit. Therefore, the available *E. coli* ATCC 11775 in our archival strain collection was used as the QC.

The *E. coli* ATCC 11775 is commonly used as QC in food and water testing studies (ISO, 2014), and is rarely recommended or used in antibiotic susceptibility studies. Thus, the MIC breakpoints of the strain against most antibiotics including trimethoprim and sulfamethoxazole, have yet to be established. In this study, the MIC breakpoints of *E. coli* ATCC 11775 against co-trimoxazole were optimised by comparing the MIC values of the clinical *B. pseudomallei* SXT intermediate and SXT susceptible isolates with those of *E. coli* ATCC 25922. The MIC determination was performed in triplicate of Kirby-Bauer disk diffusion and E-test and, observation of the MIC values at 36 hours of prolonged incubation. Overall, the *E. coli* ATCC 11775 has shown consistent MIC breakpoints (see Table 3.5 for details) that fall within the expected limit as previously mentioned reference MIC breakpoints for *B. pseudomallei* in Table 3.3 and Table 3.4. Besides, the strain has proven to be a good susceptible control with no changes in its MIC breakpoints even with the prolonged incubation (see Table 3.6 and Figure 3.2 for details).

Table 3.5: MIC values of the tested isolates and *E. coli* ATCC 11775 against co-trimoxazole based on disk diffusion and E-test method.

Isolates	Description	Zone Diameter (mm)			MIC (µg/ml)		
		Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
SWK-C 106	SXT^{Int} in this study	12	16	14	2	3	2
SWK-C 118	SXT^{Int} in this study	12	16	12	3	2	2
SWK-C 145	SXT^{Sus} in this study	20	22	22	0.75	0.75	0.5
E. coli ATCC 25922	QC recommended by CLSI and EUCAST	NZ	NZ	NZ	NZ	NZ	NZ
***E. coli* ATCC 11775**	Alternative QC proposed in this study	32	32	33	0.125	0.125	0.125

Note: NZ = No inhibition zone detected.

Table 3.6: MIC values of the tested isolates and *E. coli* ATCC 11775 against co-trimoxazole with prolonged incubation.

Isolates	Zone Diameter (mm)			MIC (µg/ml)		
	18 hours	**24 hours**	**36 hours**	**18 hours**	**24 hours**	**36 hours**
SWK-C 106	16	11	NZ	3	4	NZ
SWK-C 118	16	16	9	2	4	NZ
SWK-C 145	22	16	13	0.75	1.5	3
E. coli ATCC 25922	NZ	NZ	NZ	NZ	NZ	NZ
***E. coli* ATCC 11775**	32	32	32	0.125	0.125	0.125

Note: NZ = No inhibition zone detected.

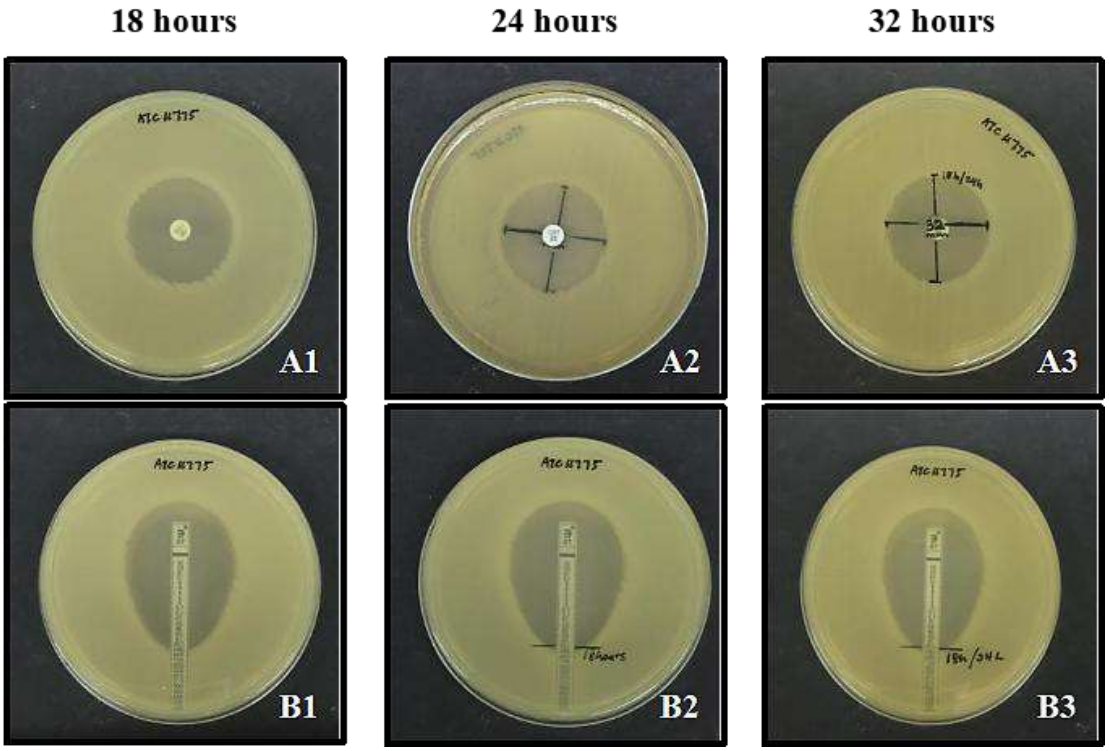


Figure 3.2: The inhibition zone of *E. coli* ATCC 11775 exhibited against co-trimoxazole with prolonged incubation based on disk diffusion (A1, A2 and A3) and E-test (B1, B2 and B3) method.

Next, the antimicrobial agents, trimethoprim and sulfamethoxazole (Santa Cruz Biotechnology, Texas, USA) were dissolved in the solvent as recommended by CLSI guidelines (see Table 3.7). The serial twofold dilution of the antimicrobial agents was performed using Mueller Hinton II broth (MHb) (HiMedia Laboratories, India) at pH 7.0. The dilutions ranging from 0.25 µg/ml to 128 µg/ml and 2 µg/ml to 1024 µg/ml were prepared for trimethoprim and sulfamethoxazole dilution, respectively (see Table 3.8 and Table 3.9). Subsequently, 180 µl of the resulting serial antimicrobial dilutions were inoculated into the respective wells of the previously labelled 96-well microtiter plate except for the wells at column 11 and 12, which were used as the growth and sterility controls for the isolates (see Figure 3.3).

Table 3.7: Solvents and Diluents for the Preparation of Stock Solutions of Trimethoprim and Sulfonamides.

Antimicrobial agent	Solvent	Diluent
Trimethoprim	0.05mol/L lactic or hydrochloric acid, 10% of final volume	Hot water
Sulfonamides (Sulfamethoxazole)	1/2 volume hot water and minimal amount of 2.5 mol/L NaOH to dissolve.	Water

[Extracted from Table 6A: Document M100, 27th Edition, CLSI (2017)]

Table 3.8: Modified dilution scheme for trimethoprim dilution following the guidelines set by CLSI (2017).

Antimicrobial solution (µg/ml)	Volume (ml)	Broth (ml)	Final concentration (µg/ml)
5 120	1	9	512
512	1	1	256
512	1	3	128
512	1	7	64
64	1	1	32
64	1	3	16
64	1	7	8
8	1	1	4
8	1	3	2
8	1	7	1
1	1	1	0.5
1	1	3	0.25
1	1	7	0.125

Table 3.9: Modified dilution scheme for sulfamethoxazole dilution following the guidelines set by CLSI (2017).

Antimicrobial solution (µg/ml)	Volume (ml)	Broth (ml)	Final concentration (µg/ml)
10 240	1	9	1 024
1 024	1	1	512
1 024	1	3	256
1 024	1	7	128
128	1	1	64
128	1	3	32
128	1	7	16
16	1	1	8
16	1	3	4
16	1	7	2

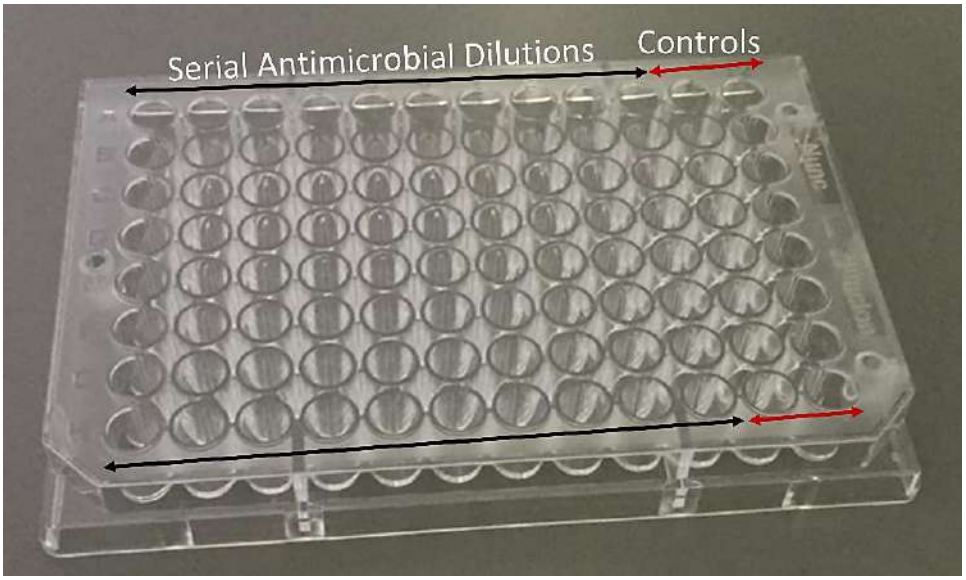


Figure 3.3: Layout of the 96 wells microtiter plate used in this study. The black arrow represents column 1 until 10, while the red arrow represents column 11 and 12.

Meanwhile, to prepare the bacterial cell suspension, the control organism and 14 selected clinical isolates were first cultured on modified Ashdown's agar with 50 µg/ml colistin (Acros Organics, China) for 16 to 24 hours at 37 ºC. Afterwards, those isolates were sub-cultured onto a TSA plate for a further 16 to 24 hours of incubation at 37 ºC (Growth Chamber Model GC-1050, Protech Electronics, Malaysia). Individual colonies were then sub-cultured into 5 mL of Tryptic Soy broth (TSB) (HiMedia Laboratories, India) for 4 hours in room temperature on an orbital shaker (Model 719, Protech Electronics, Malaysia) at 100 rpm aerobically. Once the incubation was done, the bacterial suspension was immediately prepared by inoculating the broth culture into 2 mL of MHb, and its turbidity was adjusted with MHb to an optical density ≈ 0.13 A at 600 nm (0.5 McFarland standard) using a spectrophotometer (BioPhotometer, Eppendorf, Germany). The resulting inoculated broth was then further diluted to give a final concentration of $5x10^5$ CFU/mL, following the bacterial cell suspension dilution factors suggested by CLSI (2017), see Figure 3.4.

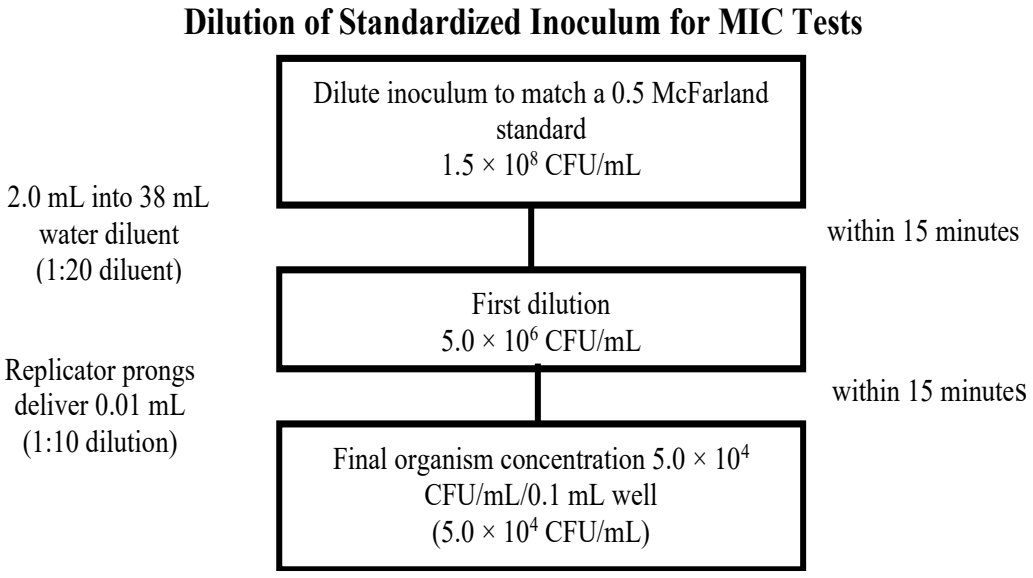


Figure 3.4: Dilution scheme for preparing a standardized inoculum for MIC tests (CLSI, 2017).

The layout of the microtiter plate allowed three (3) isolates to be tested per plate. The plate was then sealed and incubated at 37 ºC in ambient air (Growth Chamber Model GC-1050, Protech Electronics, Malaysia) for 16 to 24 hours before MIC determination using the BioTek Synergy H1 Microplate Reader (BioTek Instruments, USA). All the MICs were tested in a minimum of biological triplicate and the results were reported as the mode of replicates. The determinations for the MIC breakpoints or results were derived following Equation 3.1 and Equation 3.2.

$$\text{Final OD value} = \frac{(\text{OD value 1} + \text{OD value 2} + \text{OD value 3})}{3} \qquad \text{Equation 3.1}$$

$$\text{Bacterial kill \%} = \frac{(\text{Growth control OD value} - \text{Final OD value})}{\text{Growth control OD value}} \times 100\% \qquad \text{Equation 3.2}$$

The bacterial kill rate (%) represented the percentage of killed or inhibited bacterial population at the tested antibiotic concentration. The minimum inhibitory concentration (MIC) breakpoints of trimethoprim or sulfamethoxazole for the isolates were categorised as MIC_{50} and MIC_{80}. This was determined based on the lowest antibiotic concentration required to inhibit 50% (MIC_{50}) and 80% (MIC_{80}) of the bacterial population and, defined following the definitions provided by the CLSI (2017). Currently, since there are no established breakpoints of trimethoprim for *non-Enterobacteriaceae,* therefore the *Enterobacteriaceae* MIC breakpoints were used to define the susceptibility for trimethoprim, whereas *non-Enterobacteriaceae* MIC breakpoints were used to define the susceptibility for sulfamethoxazole, see Table 3.10 (CLSI, 2017).

Table 3.10: The MIC interpretive for trimethoprim and sulfamethoxazole (CLSI, 2017).

***Enterobacteriaceae* MIC interpretive for trimethoprim (µg/ml)**			***Non-Enterobacteriaceae* MIC interpretive for sulfamethoxazole (µg/ml)**		
S ≤	I	R ≥	S ≤	I	R ≥
8	-	16	256	-	512

3.3.4 Statistical Data Analysis

All MICs definitions from the three antibiotic susceptibility testing methods were analysed using Microsoft Excel 2010 (Microsoft, Redmond, Washington) and SPSS Statistics 23 (IBM, United States). The Cohen Kappa (κ) coefficient was used to analyse the concordance of the study parameters (CLSI and EUCAST's guidelines) used in this study. The measure of agreement according to the Cohen's Kappa value (κ) **i**nterpretation of the prevalence variability **(**Landis & Koch, 1977, as cited in Warrens, 2015)**:**

κ = 0.00 - 0.20, indicates slight agreement
κ = 0.21- 0.40, indicates fair agreement
κ = 0.41-0.60, indicates moderate agreement
κ = 0.61-0.80, indicates substantial agreement
κ = 0.81-1.00 indicates almost perfect agreement

3.4 Genomic Analysis

3.4.1 PCR of the *bpeEF-oprC* Genes Cluster

A series of polymerase chain reactions (PCR) were outlined to analyse and detect the co-trimoxazole resistance determinant, the *bpeEF-oprC* genes in Sarawak clinical *B. pseudomallei* isolates. All oligonucleotide primers used for the experiment were designed

using Primer3plus software (Untegrasser et al., 2012), see Table 3.11. The reference strain for the primer design was a Thailand isolate, *Burkholderia pseudomallei* K96243 (accession number: BX571996.1) (Holden et al., 2004). Meanwhile, the target gene(s) chosen were the bpeEF-oprC efflux pump genes cluster (*bpeT-llpE-bpeE-bpeF-oprC*), that were proposed by Biot et al. (2011) and Podnecky et al. (2013) to be responsible for co-trimoxazole resistance in clinical isolates.

Table 3.11: Oligonucleotide primers designed and used in this study.

Target Gene	Primer	Primer Sequence (5'->3') [Expected base pair, bp]
bpeT	BpeT_F1	5' - TGC GCA AAC ATA TGA CGA AC -3'
	BpeT_R1	5'- CGA ATT CCA CTC ACG CTA CC -3'
		[768 bp]
	BpeT_F2	5' - GCG GCT CGA AAA GTA GTT GA - 3'
	BpeT_R2	5' - ACA ATT CAC GTC CCC TGA AC - 3'
		[684 bp]
llpe	llpE_F1	5' - GAT TGT TCA GGG GAC GTG A - 3'
	llpE_R1	5'- GAG CGA ATA ATC GAC CGA CA -3'
		[392 bp]
	llpE_F2	5'- CGG TGG TGC TTT ATT TCC AC -3'
	llpE_R2	5'- CGG GAA GTA CGC AAG ATA GC -3'
		[774 bp]
bpeE	BpeE_F1	5'- CGA CAA CCT GAG GGG TTT T -3'
	BpeE_R1	5'- GCC GAT GTA TTG CAG GTA GG -3'
		[730 bp]
	BpeE_F2	5'- TTA CGA CGA GAA GCA GAA CG -3'

Table 3.11 continued

	BpeE_R2	5'- TGA AAG GCT CTG TCT GAT TGG -3'
		[846 bp]
bpeF	BpeF_F1	5'- CCC AAT CAG ACA GAG CCT TT -3'
	BpeF_R1	5'- CGA ACT CGT CCT CGT TCT G -3'
		[769 bp]
	BpeF_F2	5'- ATT CGC GAG CAG AAC GTG -3'
	BpeF_R2	5'- GTC ATC GCG AAC TGC TTG TA -3'
		[797 bp]
	BpeF_F3	5'- CTA TTC GAT CAA CGC GCT CT -3'
	BpeF_R3	5'- CCG CGT ACT TCT GGT TCA G -3'
		[824 bp]
	BpeF_F4	5'- GTG AAC GGC TTC ACG AAC A -3'
	BpeF_R4	5'- TGA TCG GAA ACA CCC AGA AC -3'
		[796 bp]
	BpeF_F5	5'- GAC ATC CTG CAA CTG AAG ACG -3'
	BpeF_R5	5'- GCG TTC GTT GAT GTT GGT CT -3'
		[850 bp]
oprC	OprC_F1	5'- CGG ACG CTT GAG GAT AGA AA -3'
	OprC_R1	5'- CTC GCT GAA CGA GAA ATC C -3'
		[882 bp]
	OprC_F2	5'- CGC GGA TTT CTC GTT CAG -3'
	OprC_R2	5'- CGA CAT TCG CAT TTC GTC -3'
		[785 bp]

The PCR assays were performed in an overall thirteen (13) independent reactions using genomic DNA extracted with Chelex® 100 Resin (Bio-Rad Laboratories, USA) according to the manufacturer's protocols. The PCR amplification was performed using the Eppendorf Mastercycler® Nexus GX2 (Eppendorf, Germany). The PCR reaction of 15 µl consisted of 10× Taq Polymerase Buffer, dNTPs, autoclaved ddH2O, primers, Taq Polymerase and the DNA template were prepared as schemed in Table 3.12. Meanwhile, the cycling conditions were 95 °C for 5 minutes of initial denaturation, 45 cycles of 95 °C for 30 seconds, 52 °C to 55 °C for 30 seconds, and 72 °C for 1 minute, followed by a final extension at 72 °C for 5 minutes (Payne et al., 2005).

Table 3.12: List of reagents for the preparation of the PCR Master Mix (HotStarTaq Master Mix Kit, Qiagen, Germany).

PCR Reagents	Concentration	Volume per reaction (µl)
ddH_2O (autoclaved)	-	6.26
10× Taq Polymerase Buffer	1×	1.5
Magnesium chloride, $MgCl_2$	2 mM	1.8
ddNTPs/dNTPs	0.2 mM	0.3
Q solution	1.2 M	3.6
Foward Primer	0.4 µM	0.15
Reverse Primer	0.4 µM	0.15
Taq Polymerase	0.08 µ/µl	0.24
DNA template	-	1.0
TOTAL	-	**15.0**

3.4.2 Agarose Gel Electrophoresis

The procedure for the agarose gel electrophoresis was modified based on the manufacturer's recommended instructions (Invitrogen™, USA). All PCR products were electrophoresed on 0.7% agarose gel (Biotechnology Grade, 1st BASE, Singapore) in 1× Tris-Borate-EDTA (TBE) buffer at pH8.3 (Ultra-Pure Grade, Vivantis Technologies, Malaysia). The agarose gel was prepared with SYBR® Safe DNA gel stain at 1 in 10, 000 dilution (Invitrogen™, USA). A volume of 5 µL PCR products were mixed with 1 µL of 6× DNA Loading Dye (Thermo Scientific, US) and analysed on the gel along with 5 µL 100bp DNA ladder (Vivantis Technologies, Malaysia). The electrophoresis was run at constant voltage of 70 V using Bio-Rad 1000/500 Constant Voltage Power Supply (Bio-Rad Laboratories, USA). Once completed, the gel was viewed and documented using Gel Doc XR+ System, Model 1708195 (Bio-Rad Laboratories, USA) for the analysis of PCR products.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Co-trimoxazole (SXT) susceptibility of Sarawak clinical *B. pseudomallei* isolates

Apart from the previously described gentamicin susceptibility prevalence in Podin et al. (2013), currently, there is no other comprehensive antibiotic susceptibility profiling published for the Sarawak clinical *B. pseudomallei* isolates. *B. pseudomallei* is notorious for being resistant to a wide range of antibiotics, but it is generally susceptible to the antibiotic regimen used for melioidosis treatment. However, in recent years, there have been more reports of Sarawak clinical *B. pseudomallei* isolates having higher minimal inhibitory concentration (MIC) readings against co-trimoxazole (Yong et al., 2016). This incidence has sparked concerns about possible co-trimoxazole resistance among the Sarawak isolates. Particularly, because co-trimoxazole is the primary antibiotic regimen for the eradicative-phase treatment of melioidosis (Gassiep et al., 2020).

This study is aimed to determine the co-trimoxazole susceptibility profile for the Sarawak clinical *B. pseudomallei* isolates. The disk diffusion test (Section 4.1.1) and E-test (Section 4.1.2) was conducted to define the co-trimoxazole susceptibility for the Sarawak isolates. This two-step of antibiotic susceptibility testing was outlined due to the tendency of the co-trimoxazole false-resistant results reported by the disk diffusion method. Hence, the E-test method is necessitated to further validate the co-trimoxazole susceptibility.

Apart from that, a valid interpretation standardised guideline for the antibiotic susceptibility testing (AST) of the clinical *B. pseudomallei* isolates in Sarawak was also determined. The lack of standardised interpretation guidelines for the AST results may have

contributed to the false report of co-trimoxazole resistance among the Sarawak isolates. In this study, the MIC results obtained from the disk diffusion test and the E-test were interpreted using the guidelines from the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Susceptibility Testing (EUCAST). Then, the variability of the MIC results based on the different standards was analysed (Section 4.1.3) to determine the valid standardised interpretation guideline for the AST of *B. pseudomallei* in Sarawak.

4.1.1 Co-trimoxazole susceptibility testing by disk diffusion

A collection of 164 Sarawak clinical *B. pseudomallei* isolates were subjected to disk diffusion with 25 μg co-trimoxazole disk (OXOID, UK) to define their co-trimoxazole susceptibility. Based on the resultant inhibition zone observations, it is noted that 117 isolates exhibited only one (1) inhibition zone, whereas the remainder of 47 isolates exhibited two (2) inhibition zones. Figure 4.1 depicts the different types and characteristics of the inhibition zones formed by the isolates. Type A, the clear and single inhibition zone, is the most common type of inhibition zone. Meanwhile, Type B characterised two (2) inhibition zones and Type C referred to the inhibition zone formed with unclear edge and hazy growth of bacterial within the zone.

The phenotypic observation of the outermost zone in Type B inhibition zone indicates the hetero-resistance of the isolate against co-trimoxazole. Hetero-resistance refers to the incidence in which the subpopulation of cells in a bacterial isolate shows a substantial reduction of antibiotic susceptibility compared to its main population (Andersson et al., 2019). CLSI (2017) mentioned that the hetero-resistance phenotype is common for disk diffusion test involving co-trimoxazole. Moreover, it is an unstable phenotype and typically occurs when the co-trimoxazole disk does not completely inhibit the bacterial growth within

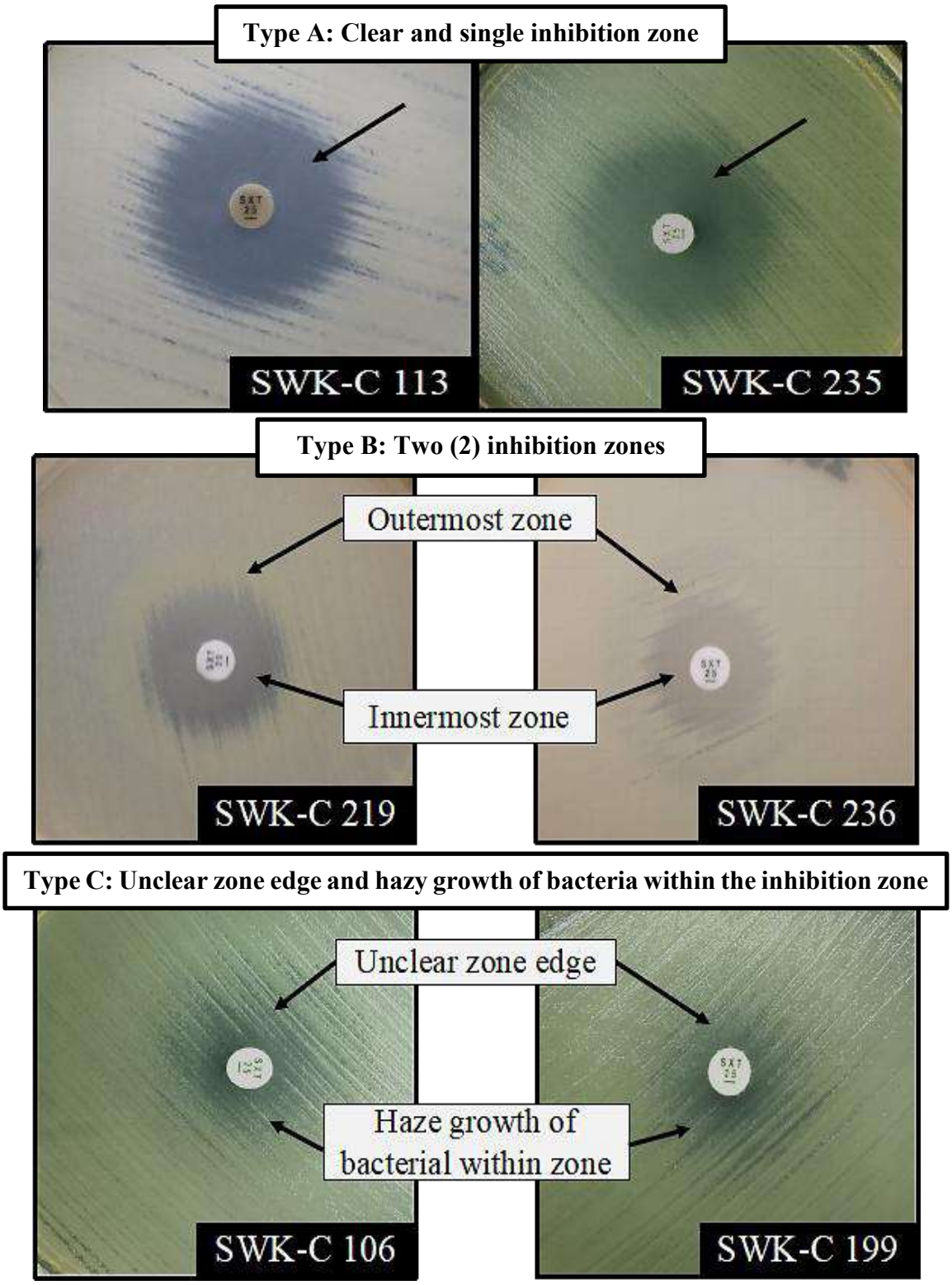


Figure 4.1: Type and characteristics of the inhibition zone(s) formed by Sarawak clinical *B. pseudomallei* isolates when tested with 25 µg co-trimoxazole disk.

the outermost zone (Andersson et al., 2019; CLSI, 2017). Therefore, in this circumstance, disk diffusion test was repeated before finalising their co-trimoxazole susceptibility report.

Aside from that, the Type B and Type C inhibition zones may have occurred as the antagonism effect to co-trimoxazole. The premade media, Mueller Hinton II agar (MHA) (HiMedia Laboratories, India) that was standardised for the disk diffusion test can possibly contained exogenous antagonistic compounds such as thymine and/or thymidine. Both thymine and thymidine are essential for the folate biosynthesis of *B. pseudomallei* and, are the competitive inhibitors of co-trimoxazole components, the sulfamethoxazole and trimethoprim, respectively (Fernández-Villa et al., 2019). Hence, the measurement of Type B and Type C's IZD would highly likely report a false-resistant result. Due in part to this, re-evaluation with disk diffusion test is necessitated to ensure a valid susceptibility report of the isolates.

Besides the phenotypic observations, the inhibition zone diameter of the isolates was measured and interpreted in parallel with the CLSI (2017) and EUCAST (2018) guidelines (see Table 3.3). Table 4.1 shows the disk diffusion results of 117 Sarawak clinical *B. pseudomallei* isolates that exhibited one (1) inhibition zone when tested against 25 µg co-trimoxazole (OXOID, UK). Meanwhile, the results for the remaining 47 isolates that exhibited two (2) inhibition zones were tabulated in Table 4.2. The measurement for the two (2) inhibition zones were categorised into MIC_{50} and MIC_{80}. The MIC_{50} was measured from the outermost zone margin at which 50% of the bacterial growth were inhibited. Meanwhile, the MIC_{80} measured at innermost zone margin where 80% of the bacterial growth were inhibited. However, the susceptibility interpretation was reported based on the MIC_{80}, as recommended in Table 3.3 (CLSI, 2017; EUCAST , 2018).

Table 4.1: Disk diffusion results of 117 Sarawak clinical *B. pseudomallei* isolates that exhibited one (1) inhibition zone when tested against 25 µg co-trimoxazole disk.

Isolates	Origin	IZD, mm (CLSI / EUCAST)	Remarks (Phenotype observation)
SWK-C 101	Bintulu	19 (S / S)	
SWK-C 103	Bintulu	10 (R / R)	**Type C inhibition zone**
SWK-C 105	Bintulu	23 (S / S)	
SWK-C 106	Bintulu	12 (I / R)	**Type C inhibition zone**
SWK-C 107	Bintulu	25 (S / S)	
SWK-C 108	Bintulu	11 (I / R)	**Type C inhibition zone**
SWK-C 109	Bintulu	18 (S / R)	**Type C inhibition zone**
SWK-C 111	Bintulu	18 (S / S)	
SWK-C 114	Bintulu	26 (S / S)	
SWK-C 115	Bintulu	21 (S / S)	
SWK-C 118	Bintulu	12 (I / R)	**Type C inhibition zone**
SWK-C 119	Bintulu	15 (I / R)	**Type C inhibition zone**
SWK-C 120	Bintulu	34 (S / S)	
SWK-C 123	Bintulu	14 (I / R)	**Type C inhibition zone**
SWK-C 143	Bintulu	24 (S / S)	
SWK-C 145	Bintulu	20 (S / S)	**Type C inhibition zone**
SWK-C 146 (a)	Bintulu	19 (S / S)	
SWK-C 146 (b)	Bintulu	21 (S / S)	
SWK-C 146 (c)	Bintulu	15 (I / R)	**Type C inhibition zone**
SWK-C 147	Bintulu	19 (S / S)	
SWK-C 148	Bintulu	19 (S / S)	
SWK-C 149 (a)	Bintulu	18 (S / S)	
SWK-C 149 (b)	Bintulu	16 (S / R)	**Type C inhibition zone**
SWK-C 150	Bintulu	15 (I / R)	**Type C inhibition zone**
SWK-C 151	Bintulu	20 (S / S)	
SWK-C 154 (b)	Bintulu	12 (I / I)	**Type C inhibition zone**
SWK-C 154 (c)	Bintulu	14 (I / R)	**Type C inhibition zone**
SWK-C 155 (a)	Bintulu	12 (I / R)	**Type C inhibition zone**

Table 4.1 continued

SWK-C 155 (b)	Bintulu	18 (S / R)	**Type C inhibition zone**
SWK-C 156	Bintulu	20 (S / S)	
SWK-C 158	Bintulu	12 (I / R)	**Type C inhibition zone**
SWK-C 159	Bintulu	17 (S / S)	
SWK-C 160	Bintulu	37 (S / S)	
SWK-C 161	Bintulu	37 (S / R)	**Type C inhibition zone**
SWK-C 162	Bintulu	25 (S / S)	
SWK-C 163 (a)	Bintulu	15 (I / R)	**Type C inhibition zone**
SWK-C 163 (b)	Bintulu	10 (R / R)	**Type C inhibition zone**
SWK-C 164	Bintulu	20 (S / S)	
SWK-C 167	Bintulu	21 (S / S)	
SWK-C 172	Bintulu	21 (S / S)	
SWK-C 175	Bintulu	21 (S / S)	
SWK-C 177	Bintulu	15 (I / R)	
SWK-C 180	Bintulu	25 (S / S)	
SWK-C 182	Bintulu	23 (S / S)	
SWK-C 184	Bintulu	24 (S / S)	
SWK-C 185	Bintulu	24 (S / S)	
MSHR 7905	Bintulu	22 (S / S)	
MSHR 7895	Bintulu	28 (S / S)	
MSHR 7903	Bintulu	21 (S / S)	
MSHR 7881	Bintulu	17 (S / S)	
MSHR 7882	Bintulu	20 (S / S)	
MSHR 7906	Bintulu	20 (S / S)	
MSHR 7887	Bintulu	22 (S / S)	
MSHR 7888	Bintulu	25 (S / S)	
SWK-C 192	Bintulu	20 (S / S)	
SWK-C 193	Bintulu	30 (S / S)	
SWK-C 194	Bintulu	15 (I / R)	**Type C inhibition zone**
SWK-C 195	Bintulu	21 (S / S)	
SWK-C 196	Bintulu	20 (S / S)	

Table 4.1 continued

SWK-C 197	Bintulu	28 (S / S)	
SWK-C 199	Bintulu	16 (S / R)	**Type C inhibition zone**
SWK-C 243	Bintulu	16 (S / S)	
SWK-C 267	Bintulu	12 (I / I)	
SWK-C 269	Bintulu	13 (I / I)	
SWK-C 270	Bintulu	20 (S / S)	
SWK-C 271	Bintulu	16 (S / S)	
SWK-C 290	Bintulu	15 (I / S)	
SWK-C 124	Kapit	19 (S / S)	
SWK-C 125	Kapit	24 (S / S)	
SWK-C 126	Kapit	22 (S / S)	
SWK-C 127	Kapit	19 (S / S)	
SWK-C 130	Kapit	22 (S / S)	
SWK-C 131	Kapit	19 (S / S)	**Type A inhibition zone**
SWK-C 132	Kapit	20 (S / S)	**Type A inhibition zone**
SWK-C 133	Kapit	21 (S / S)	**Type A inhibition zone**
SWK-C 134	Kapit	19 (S / S)	**Type A inhibition zone**
SWK-C 136	Kapit	21 (S / S)	**Type A inhibition zone**
SWK-C 138	Kapit	16 (S / S)	**Type A inhibition zone**
SWK-C 139	Kapit	15 (R / S)	**Type C inhibition zone**
SWK-C 140	Kapit	15 (I / R)	**Type C inhibition zone**
SWK-C 141	Kapit	24 (S / S)	
SWK-C 214	Kapit	22 (S / S)	
SWK-C 215	Kapit	24 (S / S)	
SWK-C 216	Kapit	22 (S / S)	
SWK-C 218	Kapit	19 (S / S)	
SWK-C 220	Kapit	20 (S / S)	
SWK-C 221	Kapit	20 (S / R)	**Type C inhibition zone**
SWK-C 222	Kapit	19 (S / S)	
SWK-C 223	Kapit	12 (I / I)	**Type C inhibition zone**
SWK-C 226	Kapit	16 (S / S)	

Table 4.1 continued

SWK-C 227	Kapit	26 (S / S)	
SWK-C 229	Kapit	30 (S / S)	
SWK-C 230	Kapit	26 (S / S)	
SWK-C 231	Kapit	19 (S / S)	
SWK-C 233	**Kapit**	**15 (I / R)**	**Type C inhibition zone**
SWK-C 234	Kapit	20 (S / S)	
SWK-C 235	Kapit	30 (S / S)	
SWK-C 236	Kapit	19 (S / S)	
SWK-C 237	Kapit	29 (S / S)	
SWK-C 238	Kapit	21 (S / S)	
SWK-C 241	Kapit	21 (S / S)	
SWK-C 242	Kapit	33 (S / S)	
SWK-C 089	Kapit	21 (S / S)	
SWK-C 097	Kuching	20 (S / S)	
SWK-C 187	Kuching	20 (S / S)	
MSHR 7891	Miri	21 (S / S)	
MSHR 7894	Miri	25 (S / S)	
SWK-C 063	Sibu	30 (S / S)	
SWK-C 064	Sibu	20.7 (S / S)	
MSHR 6392	Sibu	31 (S / S)	
MSHR 6802	Sibu	29 (S / S)	
MSHR 6401	Sibu	25 (S / S)	
MSHR 6404	Sibu	18 (S / S)	
MSHR 6816	Sibu	31 (S / S)	
SWK-C 084	Sibu	22 (S / S)	
SWK-C 085	Sibu	26 (S / S)	
SWK-C 087	Sibu	21 (S / S)	

Note: The **bold** details refer to the isolate and the criteria that was selected for E-test.

Table 4.2: Disk diffusion results of 47 Sarawak clinical *B. pseudomallei* isolates that exhibited Type B, two (2) inhibition zones when tested against 25 µg co-trimoxazole disk.

Isolates	Origin	Inhibition zone diameter		
		Diameter at MIC_{50} (mm)	Diameter at MIC_{80} (mm)	CLSI/EUCAST (MIC_{80})
SWK-C 102	Bintulu	35	21	S / S
SWK-C 104	Bintulu	32	22	S / S
SWK-C 112	Bintulu	23	**15**	**I / S**
SWK-C 113	Bintulu	22	**12**	**I / I**
SWK-C 116	Bintulu	35	21	S / S
SWK-C 117	Bintulu	28	20	S / S
SWK-C 152	Bintulu	30	16	S / S
SWK-C 153	Bintulu	33	24	S / S
SWK-C 154 (a)	Bintulu	24	**16**	**S / S**
SWK-C 157	Bintulu	34	20	S / S
SWK-C 165	Bintulu	29	20	S / S
SWK-C 168	Bintulu	28	18	S / S
SWK-C 169	Bintulu	29	20	S / S
SWK-C 170	Bintulu	25	19	S / S
SWK-C 173	Bintulu	27	17	S / S
SWK-C 176	Bintulu	30	18	S / S
MSHR 7883	Bintulu	29	24	S / S
MSHR 7884	Bintulu	27	22	S / S
MSHR 7896	Bintulu	35	20	S / S
MSHR 7904	Bintulu	32	17	S / S
MSHR 7885	Bintulu	35	26	S / S
MSHR 7897	Bintulu	32	**15**	**I / S**
MSHR 7886	Bintulu	29	18	S / S
SWK-C 200	Bintulu	30	20	S / S
SWK-C 201	Bintulu	24	17	S / S
SWK-C 204	Bintulu	27	20	S / S
SWK-C 207	Bintulu	31	21	S / S

Table 4.2 continued

SWK-C 209	Bintulu	25	20	S / S
SWK-C 210	Bintulu	32	25	S / S
SWK-C 212	Bintulu	33	22	S / S
SWK-C 262	Bintulu	26	16	S / S
SWK-C 129	Kapit	26	**15**	**I / S**
SWK-C 213	Kapit	33	22	S / S
SWK-C 219	Kapit	28	**9**	**R / R**
SWK-C 224	Kapit	28	22	S / S
SWK-C 225	Kapit	35	24	S / S
SWK-C 228	Kapit	25	23	S / S
SWK-C 232	Kapit	33	**12**	**I / I**
SWK-C 239	Kapit	33	**15**	**I / S**
SWK-C 240	Kapit	32	24	S / S
SWK-C 096	Kuching	28	**18**	**S / S**
SWK-C 100	Kuching	30	20	S / S
MSHR 7898 / M2	Miri	27	21	S / S
MSHR 7899 / M3	Miri	31	22	S / S
MSHR 7889 / M4	Miri	26	19	S / S
MSHR 7890 / M5	Miri	28	19	S / S
MSHR 7892 / M7	Miri	30	20	S / S

Note: The **bold** details refer to the isolate and the criteria that was selected for E-test.

Additionally, in both Table 4.1 and Table 4.2, there are an overall of 42 isolates that were highlighted. These are the isolates that were selected for further susceptibility testing with E-test. In general, these isolates exhibited either Type B or Type C inhibition zones that were interpreted as intermediate or resistant according to CLSI (2017) and/or EUCAST (2018) standards. There were also eight (8) susceptible isolates with Type A and Type B inhibition zones that were selected to understand the reproducibility of the susceptibility reported by the disk diffusion and E-test methods. The finalised co-trimoxazole

susceptibility of the said 42 Sarawak clinical *B. pseudomallei* isolates were further elaborated and discussed in Section 4.1.2.

On the other hand, analyses of the results shown in Table 4.1 revealed that the isolates that exhibited Type C inhibition zone (see Figure 4.1) are from Bintulu (21 isolates) and Kapit (5 isolates). The IZD obtained for isolates from Bintulu was within the range of 10 mm to 37 mm, while the IZD of Kapit isolates was ranging from 15 mm to 27 mm. Similarly, analyses on Table 4.2 also showed that four (4) isolates, each from Bintulu and Kapit that exhibited Type B inhibition zone were recorded with intermediate and resistant IZD. The Bintulu isolates had IZD (MIC_{80}) within range of 12 mm to 15 mm, while the Kapit isolates recorded IZD (MIC_{80}) ranged from 9 mm to 15 mm. In short, these analyses indicated the tendency of Bintulu and Kapit clinical *B. pseudomallei* isolates to complicate the co-trimoxazole susceptibility testing using the disk diffusion method. Thus, it is highly recommended that E-test method to be employed for all isolates that were observed with Type B or Type C inhibition zones (see Figure 4.1) and, isolates that were reported as intermediate or resistant during disk diffusion test.

Next, Table 4.3 shows the summary of the inhibition zone diameter distributions for Sarawak clinical *B. pseudomallei* isolates against the 25 µg co-trimoxazole (OXOID, UK). Overall, the inhibition zone's mode diameter for the Sarawak isolates (25/164) was 20 mm. There were 134/164 isolates with an inhibition zone diameter of more than 15 mm, the intermediate MIC cut-off. Meanwhile, the remaining 30/164 isolates exhibited inhibition zone diameters of less than 15 mm. These diameter distributions inferred that the majority of the Sarawak isolates are satisfactorily co-trimoxazole susceptible.

Table 4.3: Co-trimoxazole (25 µg disk) inhibition zone diameter distributions for the Sarawak clinical *B. pseudomallei* isolates.

IZD (mm)	≤ 10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	≥ 30
MIC_{80}, n = 117	2	1	7	1	2	10	6	2	5	11	**15**	**15**	7	2	6	6	4	0	2	2	11
MIC_{50}, n = 47	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	3	3	4	**6**	4	23
MIC_{80}, n = 47	1	0	2	0	0	4	3	3	4	3	**10**	4	6	1	4	1	1	0	0	0	0
Total, N = 164 (MIC_{80})	3	1	9	1	2	14	9	5	9	14	**25**	19	13	3	10	7	5	0	2	2	11

Note:

1. IZD is the abbreviation for the inhibition zone diameter (mm).
2. n = 117 refers to the inhibition zone diameter of 117 isolates that exhibited one (1) inhibition zone.
3. MIC_{50}, n = 47 refers to the outermost zone margin of the 47 isolates with two (2) inhibition zones.
4. MIC80, n = 47 refers to the innermost zone margin of the 47 isolates with two (2) inhibition zones.
5. The bold and underlined number is the mode for the inhibition zone diameter(mm).

In addition, Table 4.4 summaries the statistical analysis on the co-trimoxazole susceptibility of the Sarawak isolates. The co-trimoxazole susceptible frequencies suggested that the Sarawak clinical *B. pseudomallei* isolates are predominantly co-trimoxazole susceptible by disk diffusion test. This result is in contrast with the co-trimoxazole susceptibility rate (62.5%) by disk diffusion test previously reported by Yong et al. (2016).

In this experiment, the co-trimoxazole susceptibility of the Sarawak isolates was 81.7% (134/164) and 79.9% (131/164), as per CLSI and EUCAST standards, respectively. Besides, according to CLSI definitions, 15.9% (26/34) of the isolates were intermediately susceptible, and 2.4% (4/164) were co-trimoxazole resistant. In contrast, EUCAST criteria defined 3.7% (6/164) of the isolates as intermediately susceptible and 16.5% (27/164) as co-trimoxazole resistant. It is noteworthy that the difference in the intermediate and resistant frequencies was due to the guidelines used to interpret the inhibition zone of the isolates. The majority of those isolates have Type B and Type C phenotypic characteristics (see Figure 4.1). In this case, CLSI criteria defined a clearer margin by disregarding the slight bacterial growth of 20% within the zone (CLSI, 2017). Meanwhile, EUCAST (2018) suggested ignoring the minor bacterial growth if the zone edge can be seen and interpreting it as no zone if growth reaches the antibiotic disk.

Furthermore, Cohen's Kappa value (Ƙ) in Table 4.4 also reflected the difference in the said frequencies. Thus, the resulting value Ƙ = 0.458 (0.340 ~ 0.570) implied the moderate agreement or correlation between the CLSI and EUCAST guidelines used in this study. A summary and detailed discussion on the interpretation guidelines is presented in Section 4.1.3.

Table 4.4: Comparisons of CLSI and EUCAST susceptibility interpretations for the Sarawak clinical *B. pseudomallei* isolates against co-trimoxazole (25 µg disk).

MIC's Interpretive Standard	CLSI Interpretive Standard		EUCAST Interpretive Standard	
	Frequency, % (n/N)	**95% Confidence Interval**	**Frequency, % (n/N)**	**95% Confidence Interval**
Susceptible Isolates	81.7 % (134/164)	0.749 ~ 0.873	79.9% (131/164)	0.729 ~ 0.857
Intermediate Isolates	15.9% (26/164)	0.106 ~ 0.224	3.7% (6/164)	0.014 ~ 0.078
Resistant Isolates	2.4% (4/164)	0.007 ~ 0.061	16.5% (27/164)	0.111 ~ 0.230
Cohen's Kappa value, κ^a (95% Confidence Interval)	κ^a = 0.458 (0.340 ~ 0.570)			

Note: Cohen's Kappa value (κ) **i**nterpretation of the prevalence variability **(**Landis & Koch, 1977, as cited in Warrens, 2015)**:**

κ = 0.00 - 0.20, indicates slight agreement

κ = 0.21- 0.40, indicates fair agreement

κ = 0.41-0.60, indicates moderate agreement

κ = 0.61-0.80, indicates substantial agreement

κ = 0.81-1.00 indicates almost perfect agreement

4.1.2 Co-trimoxazole Susceptibility Testing by E-test

To further confirm the co-trimoxazole susceptibility by disk diffusion, the E-test was performed on 42 isolates previously defined as intermediately susceptible or resistant towards co-trimoxazole (refers to bolded isolates in Table 4.1 and Table 4.2). Figure 4.2 depicts the characteristics of the inhibition zone noted for the E-test. All isolates tested had a defined and clear inhibition zone, except for SWK-C 219, which exhibited two (2) inhibition zones. Thus, an E-test was repeated for SWK-C 219 to re-confirm its co-trimoxazole susceptibility.

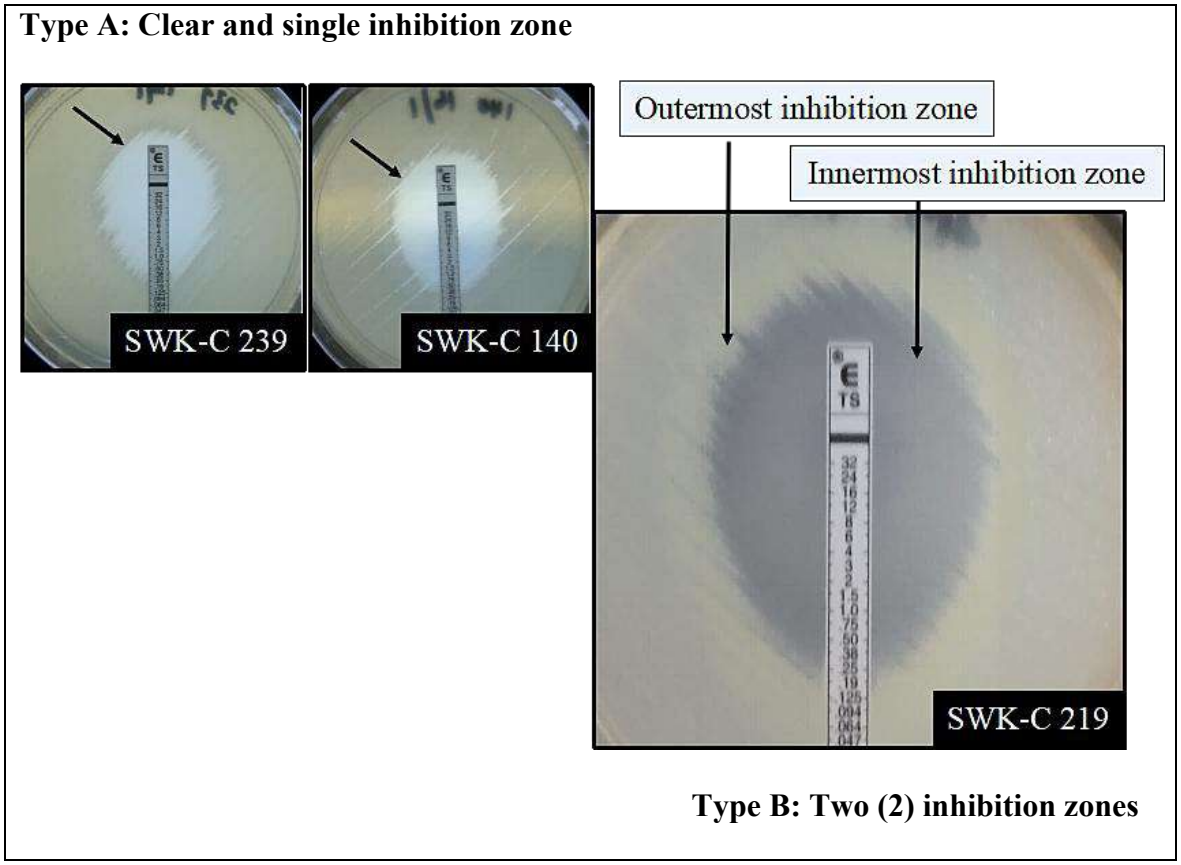


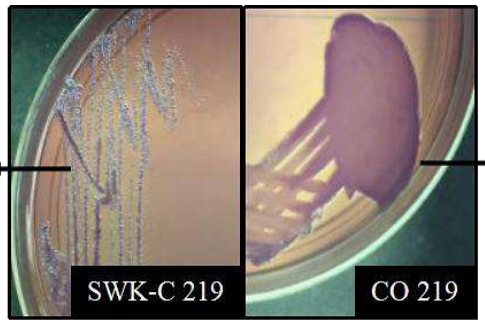
Figure 4.2: Type and characteristics of the inhibition zone(s) formed by Sarawak clinical *B. pseudomallei* isolates when tested with co-trimoxazole strip.

For the re-evaluation, the colonies in the outermost inhibition zone (coded as CO 219 in this study) were isolated and sub-cultured to ensure their purity. The morphological

comparison of the CO 219 and SWK-C 219 pure colony cultures was depicted in Figure 4.3. The pure colonies were a mix of dry and mucoid, whereas CO 219 appeared smooth and mucoid. Thus, the colonies were presumed to be of different strains. However, similar findings in Vipond et al. (2013) implied that these morphology variants are a common phenomenon. The authors suggested that the occurrence is unrelated to the genetic sequence diversity but happens due to epigenetic factors and variations of the gene expression and proteome.

Pure colonies of SWK-C 219:

- Mixture of dry and mucoid colonies.
- Co-trimoxazole MIC cut-off: 2 µg/ml



Colonies grown in the outermost inhibition zone, CO 219:
Smooth and mucoid colonies.
Co-trimoxazole MIC cut-off: 3 µg/ml

Figure 4.3: Morphology and characteristics of the pure colonies of SWK-C 219 and the colonies grown in the outermost inhibition zone of SWK-C 219 E-tested with co-trimoxazole.

Later, intriguingly, the E-test re-evaluation revealed that the MIC of CO 219 was 3 µg /ml, higher than that of SWK-C 219, which was 2 µg /ml. This observation concurred with the recent finding by Schnetterle et al. (2021), which documented the different antibiotic susceptibilities exhibited by morphotype variants isolated from a clinical *B. pseudomallei* isolate. In particular, the authors described that the co-trimoxazole resistance in the morphotype variant was due to the overexpression of the *bpeF* gene, the integral inner transporter of BpeEF-oprC efflux pumps. The overexpression of the *bpeF* was however, not due to any mutation on the BpeEF-oprC efflux pumps. Instead, it occurred due to transient

adaptive resistance in the clinical isolate that could be reversible in the absence of antibiotic pressure (Schnetterle et al., 2021).

In short, the findings in Schnetterle et al. (2021) suggest the likelihood that the presence of co-trimoxazole resistant morphotype variants within a clinical *B. pseudomallei* isolate may compromise the melioidosis eradicative-phase treatment with co-trimoxazole. Due in part to the lengthy treatment, transient adaptive resistance may develop with prolonged exposure to co-trimoxazole. Thus, it is purposed that routine ASTs are necessary during the treatment period to monitor possible adaptive resistance in the clinical *B. pseudomallei* isolates, especially for those that exhibit morphotype variants. Despite that, a future molecular study is warranted to understand the correlation of the morphotype variants observed in this study to the co-trimoxazole resistance phenomenon in Sarawak.

Aside from that, as shown in Table 4.5, the co-trimoxazole MIC profiles for Sarawak clinical *B. pseudomallei* isolates are all within the expected range. The results inferred that co-trimoxazole resistance in Sarawak isolates is not prevalent thus far. All tested isolates appeared to be co-trimoxazole susceptible, with MIC values ranging from 0.38 μg/ml to 2 μg/ml, except for SWK-C 118, which exhibited an intermediate MIC cut-off of 3 μg/ml. The predominant MIC cut-off was 0.75 μg/ml, suggesting that the isolates are substantially susceptible to co-trimoxazole. In general, these results indicated evidence of disk diffusion overcalled resistance for the co-trimoxazole, which is similar to the observations in Dance et al. (2014) and Saiprom et al. (2015). Both of the studies used the E-test method to re-evaluate and clarify the prevalence of co-trimoxazole resistance, which was initially tested with disk diffusion in Northern Thailand (Saiprom et al., 2015), Laos and Cambodia (Dance et al., 2014).

Table 4.5: Minimum inhibitory concentration results for Sarawak clinical *B. pseudomallei* isolates in this study.

Isolate(s)	Origin	Minimal Inhibitory Concentration ((µg/ml)	MIC Interpretation (CLSI and EUCAST)
SWK-C 103	Bintulu	1	S
SWK-C 106	Bintulu	2	S
SWK-C 108	Bintulu	0.75	S
SWK-C 109	Bintulu	1	S
SWK-C 112	Bintulu	0.5	S
SWK-C 113	Bintulu	0.75	S
SWK-C 118	Bintulu	**3**	I
SWK-C 119	Bintulu	1.5	S
SWK-C 123	Bintulu	1	S
SWK-C 145	Bintulu	0.38	S
SWK-C 146 (c)	Bintulu	1.5	S
SWK-C 149 (b)	Bintulu	2	S
SWK-C 150	Bintulu	1.5	S
SWK-C 154 (a)	Bintulu	2	S
SWK-C 154 (b)	Bintulu	2	S
SWK-C 154 (c)	Bintulu	2	S
SWK-C 155 (a)	Bintulu	1.5	S
SWK-C 155 (b)	Bintulu	1.5	S
SWK-C 158	Bintulu	1	S
SWK-C 161	Bintulu	0.5	S
SWK-C 163 (a)	Bintulu	1.5	S

Table 4.5 continued

SWK-C 163 (b)	Bintulu	2	S
MSHR 7897	Bintulu	2	S
SWK-C 194	Bintulu	0.38	S
SWK-C 199	Bintulu	0.5	S
SWK-C 129	Kapit	0.75	S
SWK-C 131	Kapit	1	S
SWK-C 132	Kapit	1	S
SWK-C 133	Kapit	0.75	S
SWK-C 134	Kapit	0.5	S
SWK-C 136	Kapit	1.5	S
SWK-C 138	Kapit	0.5	S
SWK-C 139	Kapit	0.75	S
SWK-C 140	Kapit	1	S
SWK-C 219	Kapit	2	S
SWK-C 221	Kapit	0.5	S
SWK-C 223	Kapit	0.75	S
SWK-C 226	Kapit	1.5	S
SWK-C 232	Kapit	1	S
SWK-C 233	Kapit	0.75	S
SWK-C 239	Kapit	0.75	S
SWK-C 096	Kuching	0.75	S

Note: Bold and underlined number refers to the intermediate MIC (µg/ml).

Based on the observations in both disk diffusion and the E-test, the main reason for the overcall resistance by disk diffusion was the difficulty of interpreting the MIC cut-offs while testing the isolate susceptibility to co-trimoxazole. As shown in Figure 4.4, the resultant inhibition zone (IZ) by disk diffusion test is difficult to assess visually. Specifically, when there is haze growth of bacteria within the IZ or unclear zone margin (depicted in Figure 4.4). Thus, depending on the reader's subjectivity (Karatuna et al., 2021), different interpretations could be made for the antibiotic susceptibility of the isolates tested. Besides, there was a lack of practical standardised methodology and interpretation criteria for the AST of *B. pseudomallei* using disk diffusion. In fact, for this study, the AST interpretation criteria were adaptive guidelines from CLSI (2017) and EUCAST (2018). In particular, the interpretive criteria for the *Enterobacteriaceae* and *non-Enterobacteriaceae* are applied for the interpretation of the co-trimoxazole susceptibility for *B. pseudomallei*.

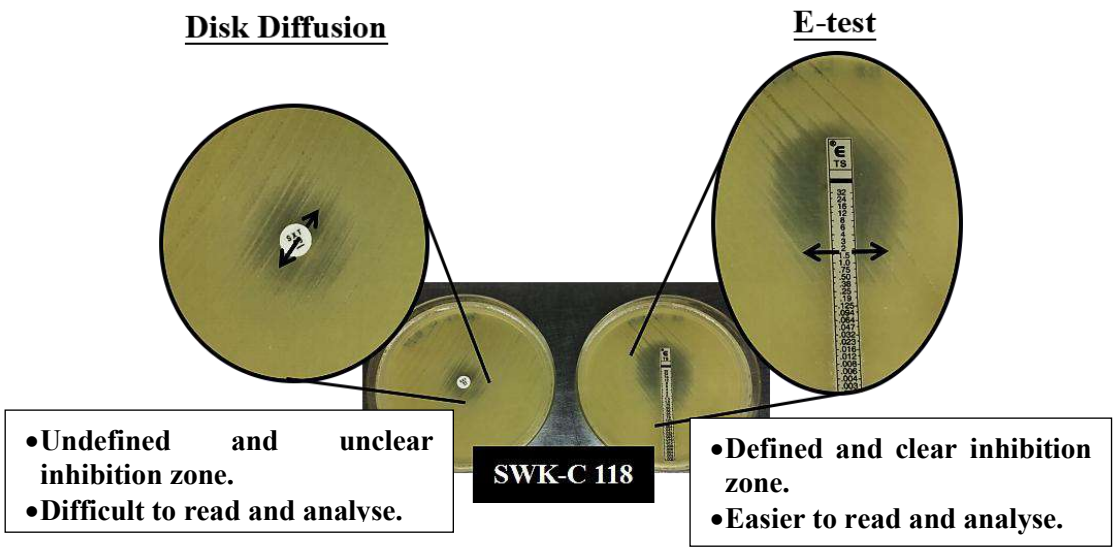


Figure 4.4: Comparison of phenotypic observations noted during disk diffusion and E-test.

Therefore, due to these reasons, E-test should be done for the isolates that had a hazy and poorly defined IZ. Furthermore, the defined resultant IZ by E-test (in Figure 4.4) is easy

to read and allows clinical microbiologists to directly determine the MIC of the tested antibiotic despite using adaptive guidelines. Likewise, the quantitative MIC results can also provide better reference in determining the appropriate drug dosage used for the treatment of melioidosis (Khosravi et al., 2014).

Subsequently, following the E-test results, a mathematical estimation for the true prevalence of co-trimoxazole susceptibility in the Sarawak clinical *B. pseudomallei* isolates was also derived. This was done by identifying the number of isolates whose susceptibility to co-trimoxazole had been re-tested and verified by the E-test. As shown in Table 4.6, out of 42 isolates that were E-tested, 97.6% (41/42) isolates were susceptible to co-trimoxazole. Only one (1) isolate, or 2.4% (1/41), was defined as intermediate. From this E-test result, the estimation was calculated by correcting the number of isolates that were initially overcalled as resistant and intermediate by the disk diffusion test. Therefore, in Table 4.7, the corrected CLSI and EUCAST-defined co-trimoxazole susceptibility prevalence were approximately 96.3% (158/164) and 97.6% (160/164), respectively. In addition, 3.7% (6/164) of the isolates were estimated to be intermediately susceptible according to CLSI definitions. Meanwhile, for EUCAST criteria, the intermediately susceptible and resistant isolates were approximately 1.2% (2/164).

Table 4.6: Co-trimoxazole susceptibility frequency of the 42 isolates by E-test.

Frequency (%)	**Disk Diffusion**		**E-Test (CLSI and EUCAST)**
	CLSI	EUCAST	
Susceptibility % (n/N)	40.5% (17/42)	28.6% (12/42)	97.6% (41/42)
Intermediate % (n/N)	50% (21/42)	9.5% (4/42)	2.4% (1/41)
Resistance % (n/N)	9.5% (4/42)	61.9% (26/42)	-

Table 4.7: Estimation for the true prevalence of co-trimoxazole susceptibility in the Sarawak clinical *B. pseudomallei* isolates.

Frequency (%)	CLSI's standard		EUCAST's standard	
	Disk Diffusion	**E-test**	**Disk Diffusion**	**E-test**
Susceptibility % (n/N)	81.7% (134/164)	96.3% (158/164)	79.9% (131/164)	97.6% (160/164)
Intermediate % (n/N)	15.9% (26/164)	3.7% (6/164)	3.7% (6/164)	1.2% (2/164)
Resistance % (n/N)	2.4% (4/164)	-	16.5% (27/164)	1.2% (2/164)

Based on the estimation result in Table 4.7, it is confirmed that the Sarawak clinical *B. pseudomallei* isolates are primarily co-trimoxazole susceptible. The statistics obtained closely mirror the co-trimoxazole susceptibility documented in Laos (99.2%), Australia (99.6%), Northeast Thailand (99.7%), Cambodia (100%), and Bangladesh (100%) (Dance et al., 2014; Saiprom et al., 2015; Dutta et al., 2017). In comparison, these figures are higher than the co-trimoxazole susceptibility reported in Northern Vietnam (89.1%) and Indonesia (86%) (Nhung et al., 2019; Tauran et al., 2018).

Meanwhile, in Malaysia, presently, there is still a lack of publication on the co-trimoxazole susceptibility rate of clinical *B. pseudomallei* isolates for the different localities. Thus, it is difficult to correlate the distribution of co-trimoxazole susceptibility for the isolates in Malaysia. However, Ahmad et al. (2013) previously reported that 90% of the Malaysian isolates are co-trimoxazole susceptible. The description was from the 170 clinical *B. pseudomallei* isolates reported in seven (7) states, including Sarawak (33/170). Therefore,

it is assumable that the current co-trimoxazole susceptibility profile in Sarawak isolates concurs with that of Ahmad et al. (2013).

Based on these comparisons, it can be inferred that co-trimoxazole exhibits a similar bactericidal effect against the *B. pseudomallei* isolates regardless of their geographical distribution and genetic variations. Hence, the clinicians should be confident that the co-trimoxazole regimen should be efficacious for the melioidosis treatment in Sarawak.

4.1.3 Multidrug susceptibility testing of 14 Sarawak isolates against clinically relevant antibiotics

Isolates that are susceptible at MIC 2 μg/ml, intermediate at MIC 3 μg/ml and, isolates that showed any antagonism or possibly resistant phenotype during E-test were chosen for further susceptibility studies by broth microdilution test. This selection was also made based on the previously discussed Type B and Type C isolates (refers to Section 4.1.2) that may have suggested the potentially antagonism activity against co-trimoxazole during disk diffusion test. Besides, to better understand such susceptibility phenomenon, isolates that exhibited a comparatively low MIC within the range of 0.38 μg/ml to 0.50 μg/ml during E-test were chosen. Overall, there were fourteen (14) isolates chosen. These isolates were E-tested against other clinically relevant antibiotics prior to the broth microdilution. This was done to understand the multidrug susceptibility of the isolates.

The panel of antibiotics tested includes meropenem (MEM), ceftazidime (TZ), doxycycline (DC), and amoxicillin-clavulanate (AMC) which are used in the treatment of melioidosis (Gassiep et al., 2020). Meanwhile, the E-test with gentamicin (GEN) and azithromycin (AZ) was to determine the genotype of the Sarawak isolates. The *B. pseudomallei* have been known to be resistant to aminoglycosides such as gentamicin

(Wiersinga et al., 2012), but the study conducted by Podin et al. (2013) showed that Sarawak clinical *B. pseudomallei* isolates are predominantly gentamicin-susceptible. Thus, it is important to differentiate the genotype of the isolates to provide more insights into the different antibiotic susceptibility profiles that they might exhibit.

The results showed (in Table 4.8) that 85.7% (12/14) of the Sarawak clinical *B. pseudomallei* isolates are gentamicin and azithromycin susceptible. Only two (2) isolates, SWK-C 145 and SWK-C 219 are gentamicin and azithromycin resistant. This result is similar to the findings by Podin et al. (2013), where approximately 86% of the Sarawak isolates are gentamicin-susceptible (Gen^S), while the rest are gentamicin-resistant (Gen^R). Furthermore, this result's analysis showed that both genotypes are majorly susceptible to co-trimoxazole (92.9%, 13/14), but their MIC values fell within distinct ranges. The Gen^S isolates exhibited higher MIC values ranging from 0.50 µg/ml to 2 µg/ml, while the MIC values of the Gen^R isolates ranged from 0.38 µg/ml to 0.50 µg/ml. However, the distinct ranges of MIC value for the two phenotypes of *B. pseudomallei* are not an alarming incidence. Instead, it is a suggested guidance for clinicians to determine the appropriate co-trimoxazole dosage needed for the melioidosis treatment (Dance et al., 2021).

Apart from that, there was no significant different in the susceptibility of both phenotypes toward the other tested antibiotics. All isolates are susceptible to meropenem, ceftazidime, doxycycline, and amoxicillin-clavulanate that was routinely recommended for the melioidosis treatment. This result also closely mirrors the antibiotic susceptibility profile for the clinical *B. pseudomallei* isolates reported in other countries. For instance, Crowe et al. (2014) reported 100% (N = 234) of meropenem and ceftazidime, 96.6% of doxycycline

Table 4.8: Antibiotic susceptibility profile of 14 isolates selected for microdilution.

SXT phenotype (Disk Diffusion)	Isolate(s)	SXT	GEN	MEM	TZ	DC	AZ	AMC
S	**SWK-C 145**	0.38 (S)	NZ (R)	1.5 (S)	1.5 (S)	1 (S)	NZ (R)	3 (S)
	SWK-C 133	0.5 (S)	1 (S)	1 (S)	2 (S)	0.75 (S)	3 (S)	4 (S)
	SWK-C 136	0.5 (S)	2 (S)	0.75 (S)	1.5 (S)	0.75 (S)	4 (S)	3 (S)
	SWK-C 161	0.5 (S)	2 (S)	2 (S)	2 (S)	0.75 (S)	4 (S)	3 (S)
R	**SWK-C 219**	0.5 (S)	96 (R)	2 (S)	3 (S)	1.5 (S)	NZ (R)	4 (S)
	SWK-C 109	1 (S)	1.5 (S)	1 (S)	1.5 (S)	0.75 (S)	6(S)	6 (S)
	SWK-C 118	3 (I)	6 (S)	1.5 (S)	2 (S)	1 (S)	6(S)	6 (S)
	SWK-C 106	2 (S)	1.5 (S)	1 (S)	2 (S)	0.75 (S)	4 (S)	4 (S)
	SWK-C 140	2 (S)	6 (S)	1.5 (S)	2 (S)	0.75 (S)	4 (S)	4 (S)
I	**SWK-C 149 (b)**	2 (S)	1.5 (S)	1.5 (S)	3 (S)	0.75 (S)	4 (S)	6 (S)
	SWK-C 154 (a)	2 (S)	1.5 (S)	1.5 (S)	2 (S)	0.75 (S)	3 (S)	4 (S)
	SWK-C 154 (b)	2 (S)	1.5 (S)	2 (S)	2 (S)	0.75 (S)	4 (S)	6 (S)
	SWK-C 154 (c)	2 (S)	4 (S)	2 (S)	1.5 (S)	0.75 (S)	4 (S)	4 (S)
	SWK-C 163 (b)	2 (S)	2 (S)	1.5 (S)	3 (S)	1 (S)	4 (S)	4 (S)
E-test Susceptibility frequency (%)		92.9%	85.7%	100%	100%	100%	85.7%	100%

Note: SXT = Co-trimoxazole; GEN = Gentamicin; MEM = Meropenem; TZ = Ceftazidime; DC = Doxycycline; AZ = Azithromycin; AMC = Amoxicillin-clavulanate; I = Intermediate; R = Resistance; S = Susceptible; NZ = No inhibition zone detected

and 99% of co-trimoxazole susceptibility rate in Northern Australia. In addition, a study by Nhung et al. (2019) also reported an almost similar antibiotic susceptibility profile for the *B. pseudomallei* in Northern Vietnam, where all 312 of tested isolates were susceptible to ceftazidime and amoxicillin-clavulanate, and 99.4% (310/312) were susceptible to doxycycline. Based on these comparisons, it is inferred that the antibiotic susceptibility profile of the Sarawak gentamicin-susceptible *B. pseudomallei* isolates is the same as that of the gentamicin-resistant *B. pseudomallei* isolates reported elsewhere.

4.1.4 Interpretation guidelines for antibiotic susceptibility testing (AST)

One of the significant factors in determining the success rate of antibiotic therapy is the antibiotic susceptibility testing result. The definition of the disk diffusion or E-test results, such that "susceptible", "intermediate", and "resistance", can provide a crucial indicator for the suitable dosing regimen in the therapy. Still, due to a lack of standardised methodology for the result interpretation, most clinical microbiologists faced difficulty determining the endpoints of the antibiotic susceptibility testing (AST) result. Therefore, this study compared the adaptive guideline from CLSI (2017) that is presently used in Sarawak with the EUCAST (2018) guideline to determine the better guideline practices for the interpretation of the *B. pseudomallei* AST result.

Based on the disk diffusion findings in this study, the application of EUCAST criteria resulted in the reduction of co-trimoxazole susceptibility rate, but the difference with that of CLSI is not significant. Nevertheless, the discrepancies are apparent in determining the endpoints for the isolate that exhibited the hazy and unclear inhibition zone (Type B and Type C zone as in Figure 4.1). As a result (in Table 4.3), the EUCAST-defined resistance rate (16.5%, 27/164) is notably higher than the CLSI-defined resistance rate (2.4%, 4/164).

These findings are consistent with the observation by Cusack et al. (2019), who reported that the trend of reduced susceptibility and/or increased resistance was due to the EUCAST strict MIC cut-offs.

On the other hand, there is no significant difference between the susceptibility and resistance rates for the E-test results (see Table 4.6) interpreted using both guidelines. However, when comparing the overall MIC results obtained from both disk diffusion and E-test methods, it is apparent that the CLSI-defined MICs result were more consistent than that of EUCAST. In particular, there was a major discrepancy in the EUCAST-defined resistance rates by the disk diffusion (16.5%) and E-test method (1.2%). Meanwhile, for CLSI, the co-trimoxazole resistant rate was 2.4% by disk diffusion and 0% by E-test. These implied that interpreting the IZDs and/or MICs following the adaptive CLSI guideline can minimize the overcall resistance by disk diffusion test. Based on these comparisons, it is clear that the CLSI guideline is a valid option in determining the antibiotic susceptibility for the *B. pseudomallei* isolates.

Nevertheless, presently, EUCAST has established a standardised methodology specifically for the antibiotic susceptibility testing of *B. pseudomallei* (Karatuna et al., 2020). Besides, the criteria in determining the co-trimoxazole disk diffusion result that involves the aforementioned hazy and unclear inhibition zone are also emphasised. According to Karatuna et al. (2021), readers or clinical microbiologists should measure and record two (2) zone diameters at 80% of inhibited growth to define the co-trimoxazole susceptibility. The latest expected range for the co-trimoxazole zone diameter cut-offs are $S \geq 50$ mm and $R < 17$ mm, and the MIC cut-offs are $S \leq 0.001$ μg/ml and $R > 4$ μg/ml.

Aside from that, the definition of EUCAST criteria is revised in light of the new EUCAST guidelines. According to Dance et al. (2021), "intermediate" antibiotics are constantly avoided by clinicians. Due in part to its meaning, which indicates the level of antimicrobial agent activity associated with uncertain therapeutic effects (Kahlmeter et al., 2019). Therefore, Dance et al. (2021) decided to change the definition of "intermediate" to "susceptible, increased exposure". The authors inferred the new meaning as an interpretation for microorganisms when there is a high likelihood of therapeutic success due to an increased or adjusted dosing regimen. However, despite the change in the definition for EUCAST criteria, the standard treatment regimen recommended for melioidosis remain the same as previously described in Dance (2014). Therefore, adopting this recently established EUCAST guideline would not impact the current practice treatment of melioidosis. In conclusion, while the adaptive CLSI guidelines are sufficient for defining the antibiotic susceptibility of the Sarawak clinical *B. pseudomallei* isolates, it is suggested that the EUCAST recent standardised guideline should be gradually applied in future works on AST and antimicrobial resistance surveillance for the *B. pseudomallei* in Sarawak.

4.2 Sulfamethoxazole and trimethoprim susceptibility for the Sarawak clinical *B. pseudomallei* isolates by broth microdilution

The broth microdilution is an antimicrobial susceptibility test method that allowed the epidemiological MIC cut-off values determination for a pathogen (EUCAST, 2018). This method generated extensive MICs distribution that are important for the resistance surveillance and quantitative susceptibility assessment of the tested pathogen (ISO, 2019). According to EUCAST (2022), the epidemiological cut-off value is defined as the MIC that specifies the upper limit of the wild-type population of the pathogen and, the minimal

concentration at which clinical breakpoint can be determined without any deleterious effects on the clinical performance of the antibiotic susceptibility testing.

In this study, the standard broth microdilution was performed for Sarawak isolates against the separate components of co-trimoxazole, sulfamethoxazole or trimethoprim alone. The experiment was done with two (2) purposes. First, to assess the susceptibility of Sarawak clinical *B. pseudomallei* isolates against sulfamethoxazole and trimethoprim, respectively. Then, the generated sulfamethoxazole and trimethoprim MICs distribution were compared with the previously determined co-trimoxazole (sulfamethoxazole+trimethoprim) MICs by disk diffusion and E-test (in Section 4.1). The MICs comparison was done to understand the correlation between sulfamethoxazole and/or trimethoprim susceptibility with the co-trimoxazole susceptibility in Sarawak clinical *B. pseudomallei* isolates.

As previously mentioned in Section 4.1.2, fourteen (14) isolates (**2 gentamicin-resistant** isolates and **12 gentamicin-susceptible** isolates) that were either susceptible or intermediately susceptible to co-trimoxazole by E-test were subjected to the broth microdilution test. All MICs were assessed in a minimum of biological triplicates to avoid contamination and ensure consistent MIC results.

4.2.1 Sulfamethoxazole and trimethoprim susceptibility of Sarawak clinical *B. pseudomallei* isolates

The initial optical densities (ODs) of all isolates were kept constant at approximately 0.044 A. After 18 hours of treatment with serial concentrations of sulfamethoxazole (SMX), the OD for Sarawak clinical *B. pseudomallei* isolates were recorded. The final OD value was calculated following Equation 3.1 and, the epidemiological MIC values of the isolates were derived using Equation 3.2. The susceptibility was then defined based on the calculated 50%

(MIC_{50}) and 80% (MIC_{80}) of bacterial killed or inhibited via the broth microdilution test. All susceptibility interpretation and definition were according to the CLSI (2017) guideline.

$$\text{Final OD value} = \frac{(\text{OD value 1} + \text{OD value 2} + \text{OD value 3})}{3} \qquad \text{Equation 3.1}$$

$$\text{Bacterial kill \%} = \frac{(\text{Growth control OD value} - \text{Final OD value})}{\text{Growth control OD value}} \times 100\% \qquad \text{Equation 3.2}$$

The distributions of the final OD value for the isolates at the serial concentrations of SMX is tabulated in Table 4.9. Besides, the final ODs of the isolates that demonstrated MIC_{50} and MIC_{80} against SMX are also highlighted in the table. From the result's analyses, the sulfamethoxazole MICs at 50% of inhibited growth were all within the expected range. The MICs ranged from 4 µg/ml to 32 µg/ml, indicating that all isolates are susceptible at MIC_{50}. However, when analysed at 80% of inhibited bacterial growth, half (7/14) of the tested isolates exhibited resistant MICs. Four (4) of the isolates were inhibited at low-level resistance MIC_{80} of 512 µg/ml and 1024 µg/ml. The other three (3) isolates coded SWK-C 109, SWK-C 154(a) and, SWK-C 161 exhibited high-level resistance MIC_{80} of more than 1024 µg/ml. Meanwhile, the susceptible isolates (MIC_{80}) were inhibited at MIC cut-offs from 32 µg/ml to 256 µg/ml. The mode MICs were 32 µg/ml and 128 µg/ml for MIC_{50} and MIC_{80}, respectively (see Figure 4.5).

Table 4.9: Distributions of the optical density of Sarawak clinical *B. pseudomallei* isolates after 18 hours treated with serial concentrations of sulfamethoxazole.

	SMX	Optical Density (OD) of the SWK-C Isolate(s) after 18 hours														
	(µg/ml)	106	109	118	133	136	140	145	149b	154a	154b	154c	161	163b	219	QC*
	GC*	0.406	0.344	0.487	0.46	0.479	0.537	0.804	0.407	0.316	0.394	0.399	0.453	0.434	0.602	0.86
Susceptible	2	0.365	0.334	0.352	0.305	0.262	0.376	0.473	0.333	0.308	0.301	0.299	0.45	0.388	0.419	0.667
	4	0.311	0.267	0.261	0.229	0.166	0.328	0.37	0.274	0.243	0.229	0.21	0.328	0.319	0.335	0.535
	8	0.252	0.202	0.221	0.226	0.153	0.304	0.288	0.232	0.223	0.173	0.206	0.292	0.303	0.292	0.305
	16	0.217	0.176	0.197	0.197	0.133	0.257	0.257	0.184	0.18	0.14	0.175	0.273	0.246	0.223	0.208
	32	0.188	0.146	0.18	0.129	**0.077**	0.229	**0.183**	0.149	0.138	0.102	0.152	0.172	0.145	0.172	**0.166**
	64	0.137	0.125	0.141	0.096	0.067	0.204	0.13	0.129	0.108	0.085	0.11	0.12	0.114	0.128	0.121
	128	0.098	0.115	0.104	**0.084**	0.067	0.159	0.092	0.109	0.09	**0.077**	0.088	0.114	0.095	**0.092**	0.092
	256	0.083	0.104	**0.079**	0.082	0.065	0.11	0.083	**0.07**	0.084	0.089	0.081	0.112	0.089	0.073	0.094
Resistance	512	**0.078**	0.098	0.076	0.077	0.063	0.109	0.067	0.076	0.079	0.078	**0.071**	0.103	0.091	0.068	0.075
	1024	0.062	0.089	0.064	0.074	0.059	**0.107**	0.062	0.066	0.069	0.073	0.065	0.097	**0.087**	0.064	0.053

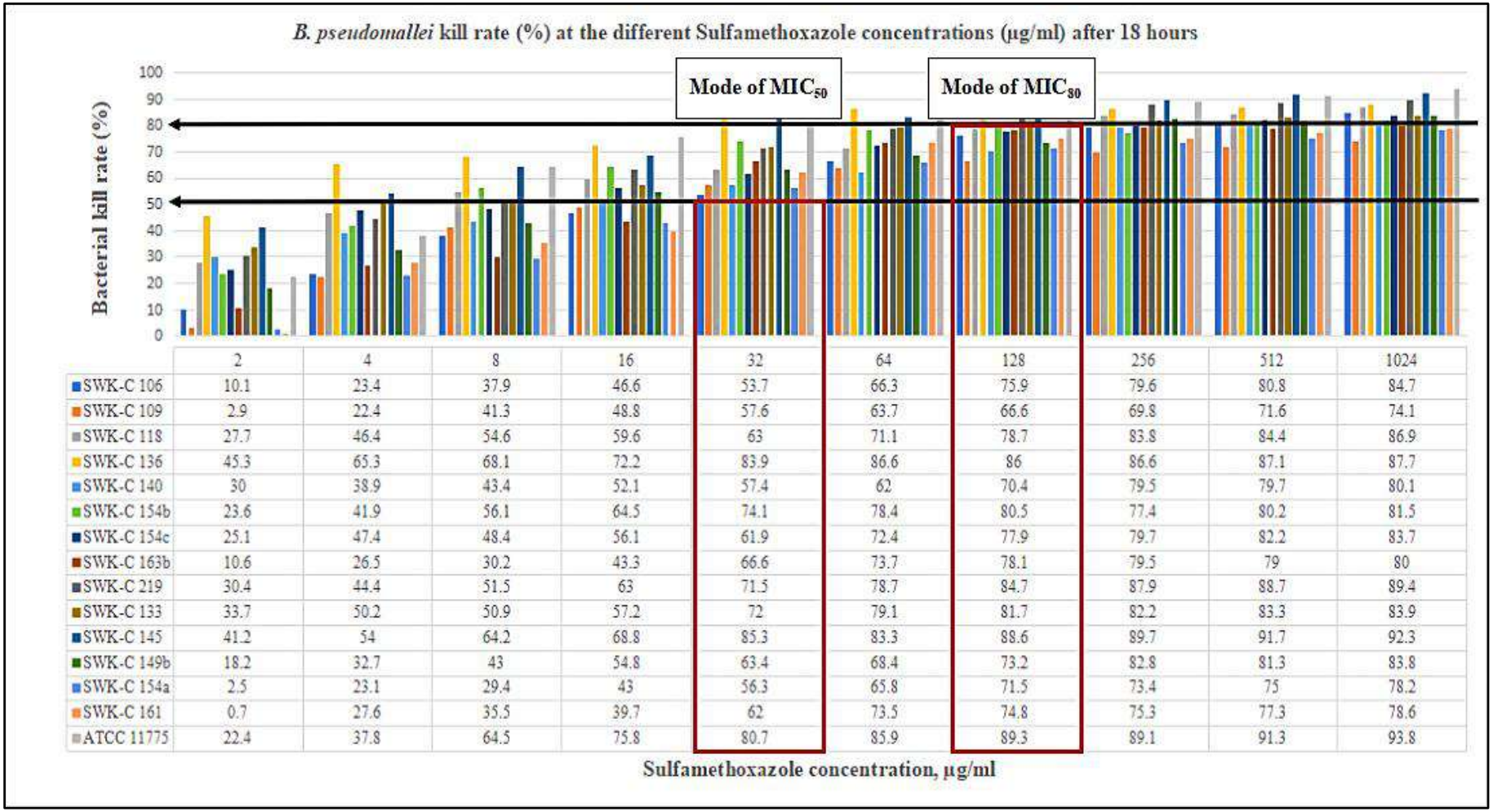
Note: a. **GC** = Growth control assay that contained **no** antibiotic.

b. **QC** = Quality control organism, *Escherichia coli* ATCC 11775.

c. **SMX** = Sulfamethoxazole

d. The underlined number is the optical density reading where **50% of bacterial growth is inhibited** by SMX (MIC_{50})**.**

e. Bold and underlined number is the optical density reading where **80% of bacterial growth is inhibited** by SMX (MIC_{80})**.**



	2	4	8	16	32	64	128	256	512	1024
SWK-C 106	10.1	23.4	37.9	46.6	53.7	66.3	75.9	79.6	80.8	84.7
SWK-C 109	2.9	22.4	41.3	48.8	57.6	63.7	66.6	69.8	71.6	74.1
SWK-C 118	27.7	46.4	54.6	59.6	63	71.1	78.7	83.8	84.4	86.9
SWK-C 136	45.3	65.3	68.1	72.2	83.9	86.6	86	86.6	87.1	87.7
SWK-C 140	30	38.9	43.4	52.1	57.4	62	70.4	79.5	79.7	80.1
SWK-C 154b	23.6	41.9	56.1	64.5	74.1	78.4	80.5	77.4	80.2	81.5
SWK-C 154c	25.1	47.4	48.4	56.1	61.9	72.4	77.9	79.7	82.2	83.7
SWK-C 163b	10.6	26.5	30.2	43.3	66.6	73.7	78.1	79.5	79	80
SWK-C 219	30.4	44.4	51.5	63	71.5	78.7	84.7	87.9	88.7	89.4
SWK-C 133	33.7	50.2	50.9	57.2	72	79.1	81.7	82.2	83.3	83.9
SWK-C 145	41.2	54	64.2	68.8	85.3	83.3	88.6	89.7	91.7	92.3
SWK-C 149b	18.2	32.7	43	54.8	63.4	68.4	73.2	82.8	81.3	83.8
SWK-C 154a	2.5	23.1	29.4	43	56.3	65.8	71.5	73.4	75	78.2
SWK-C 161	0.7	27.6	35.5	39.7	62	73.5	74.8	75.3	77.3	78.6
ATCC 11775	22.4	37.8	64.5	75.8	80.7	85.9	89.3	89.1	91.3	93.8

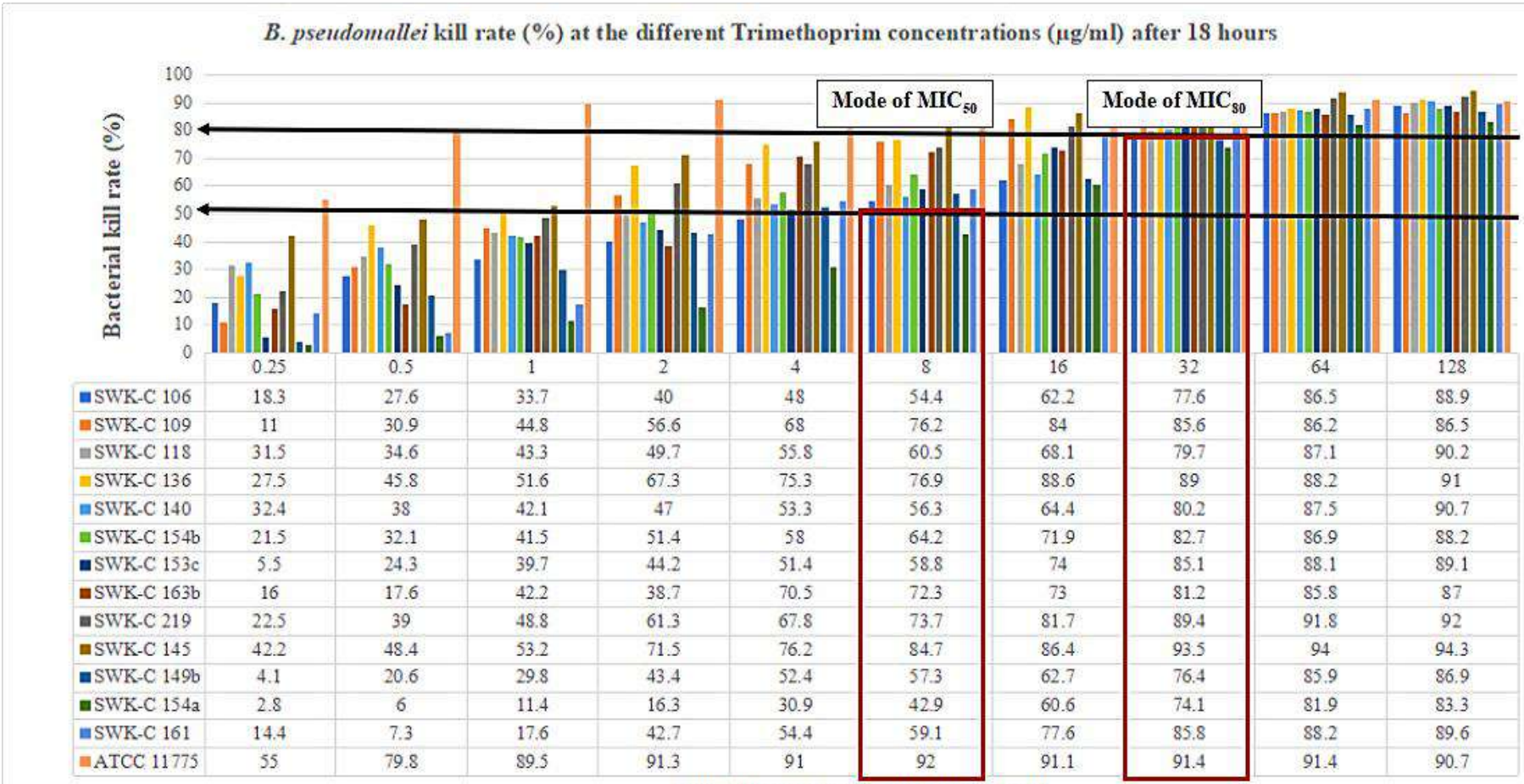
Figure 4.5: Sarawak clinical *B. pseudomallei* isolates kill rate (%) at the different sulfamethoxazole concentrations (µg/ml) after 18 hours.

Concurrently, the trimethoprim MICs were defined following a similar procedure to the sulfamethoxazole MIC definitions. Table 4.10 displays the distributions of the ODs for Sarawak clinical *B. pseudomallei* isolates after 18 hours treated with serial concentrations of trimethoprim. The analysis of the results indicated that the trimethoprim MIC_{50} was all within the expected range. All isolates were susceptible at the MICs ranging from 1 μg/ml to 8 μg/ml. However, the results for MIC_{80} revealed that trimethoprim resistance is prevalent among the tested isolates. Only one (1) isolate was susceptible to trimethoprim at 80% inhibited growth which is SWK-C 145 with the MIC of 8 μg/ml. Meanwhile, the remaining 13 resistant isolates had MICs ranging from 16 μg/ml to 128 μg/ml. The summarised MIC results illustrated in Figure 4.6 indicated that the mode MICs were 6 μg/ml and 32 μg/ml for MIC_{50} and MIC_{80}, respectively.

Table 4.10: Distributions of the optical density of Sarawak clinical B. pseudomallei isolates after 18 hours treated with serial concentrations of trimethoprim.

	TMP	Optical Density (OD) of the SWK-C Isolate(s) after 18 hours														
	(µg/ml)	106	109	118	133	136	140	145	149b	154a	154b	154c	161	163b	219	QC*
	GC*	0.46	0.362	0.511	0.469	0.498	0.655	0.804	0.389	0.282	0.405	0.403	0.45	0.393	0.574	0.813
Susceptible MIC	0.25	0.376	0.321	0.35	0.349	0.361	0.443	0.465	0.373	0.274	0.318	0.381	0.385	0.33	0.445	0.366
	0.5	0.333	0.25	0.334	0.28	0.27	0.406	0.415	0.309	0.265	0.275	0.305	0.417	0.324	0.35	**0.164**
	1	0.305	0.2	0.29	0.247	0.241	0.379	0.376	0.273	0.25	0.237	0.243	0.371	0.227	0.294	0.085
	2	0.276	0.157	0.257	0.19	0.163	0.347	0.229	0.22	0.236	0.197	0.225	0.258	0.241	0.222	0.071
	4	0.239	0.116	0.226	0.16	0.123	0.306	0.191	0.185	0.195	0.17	0.196	0.205	0.116	0.185	0.073
	8	0.21	0.086	0.202	0.143	0.115	0.286	**0.123**	0.166	0.161	0.145	0.166	0.184	0.109	0.151	0.065
Resistance MIC	16	0.174	**0.058**	0.163	**0.068**	**0.057**	0.233	0.109	0.145	0.111	0.114	0.105	0.101	0.106	**0.105**	0.072
	32	0.103	0.052	0.104	0.051	0.055	**0.13**	0.052	0.092	0.073	**0.07**	**0.06**	**0.064**	**0.074**	0.061	0.07
	64	**0.062**	0.05	**0.066**	0.048	0.059	0.082	0.048	**0.055**	**0.051**	0.053	0.048	0.053	0.056	0.047	0.07
	128	0.051	0.049	0.05	0.045	0.045	0.061	0.046	0.051	0.047	0.048	0.044	0.047	0.051	0.046	0.076

Note:
a. **GC** = Growth control assay that contained **no** antibiotic.
b. **QC** = Quality control organism, *Escherichia coli* ATCC 11775.
c. **TMP** = Trimethoprim
d. The **underlined** number is the optical density reading where **50% of bacterial growth is inhibited** by TMP (MIC_{50})**.**
e. **Bold** and **underlined** number is the optical density reading where **80% of bacterial growth is inhibited** by TMP (MIC_{80})**.**



	0.25	0.5	1	2	4	8	16	32	64	128
SWK-C 106	18.3	27.6	33.7	40	48	54.4	62.2	77.6	86.5	88.9
SWK-C 109	11	30.9	44.8	56.6	68	76.2	84	85.6	86.2	86.5
SWK-C 118	31.5	34.6	43.3	49.7	55.8	60.5	68.1	79.7	87.1	90.2
SWK-C 136	27.5	45.8	51.6	67.3	75.3	76.9	88.6	89	88.2	91
SWK-C 140	32.4	38	42.1	47	53.3	56.3	64.4	80.2	87.5	90.7
SWK-C 154b	21.5	32.1	41.5	51.4	58	64.2	71.9	82.7	86.9	88.2
SWK-C 153c	5.5	24.3	39.7	44.2	51.4	58.8	74	85.1	88.1	89.1
SWK-C 163b	16	17.6	42.2	38.7	70.5	72.3	73	81.2	85.8	87
SWK-C 219	22.5	39	48.8	61.3	67.8	73.7	81.7	89.4	91.8	92
SWK-C 145	42.2	48.4	53.2	71.5	76.2	84.7	86.4	93.5	94	94.3
SWK-C 149b	4.1	20.6	29.8	43.4	52.4	57.3	62.7	76.4	85.9	86.9
SWK-C 154a	2.8	6	11.4	16.3	30.9	42.9	60.6	74.1	81.9	83.3
SWK-C 161	14.4	7.3	17.6	42.7	54.4	59.1	77.6	85.8	88.2	89.6
ATCC 11775	55	79.8	89.5	91.3	91	92	91.1	91.4	91.4	90.7

Figure 4.6: Sarawak clinical *B. pseudomallei* isolates kill rate (%) at the different trimethoprim concentrations (µg/ml) after 18 hours.

4.2.2 Sulfamethoxazole and/or trimethoprim susceptibility in relation to co-trimoxazole susceptibility

The comparisons of the overall MIC results obtained from the broth microdilution testing, E-test, and disk diffusion is detailed in Table 4.11. Thus far, the occurrence of sulfamethoxazole resistance in co-trimoxazole susceptible clinical *B. pseudomallei* isolates is extremely rare, compared to trimethoprim resistance (Podnecky et al., 2017), which is common among susceptible and resistant isolates. However, in this study, the analysis revealed that a subset of co-trimoxazole susceptible isolates (7/14) were resistant to both sulfamethoxazole and trimethoprim. These isolates even exhibited sulfamethoxazole MIC cut-offs (512 μg/ml and ≥ 1024 μg/ml) higher than that shown by the co-trimoxazole intermediate isolate, SWK-C 118 (256 μg/ml). Besides, intriguingly also the isolates are all gentamicin-susceptible. To our knowledge, this is probably the first report of sulfamethoxazole resistance involving the wild-type gentamicin susceptible clinical *B. pseudomallei* isolate. However, since this is not the focus of this project, no further work is done to determine the true prevalence of the sulfamethoxazole resistance among our isolates. The possible mechanism confers to such phenomenon will be discussed in Section 4.3.

Based on the MICs result, it is apparent that the bacteriostatic activity of sulfamethoxazole (SMX) alone and trimethoprim (TMP) alone is insufficient in inhibiting the growth of the gentamicin susceptible Sarawak isolates. However, the E-test MICs (≤ 3 μg/ml) indicated that the synergistic activity of the combination of trimethoprim and sulfamethoxazole (co-trimoxazole) is potent and capable of inhibiting the growth of the B. pseudomallei isolates. Besides, the predominant MIC cut off for the co-trimoxazole against the Sarawak isolate for this analysis is at 2 μg/ml, suggesting a high likelihood of treatment success. The MIC cut-off is consistent to that reported in Dance et al. (2014) and Saiprom et

Table 4.11: Comparisons of the MICs and susceptibility interpretation for 14 Sarawak clinical *B. pseudomallei* isolates.

Isolate(s)	Disk Diffusion IZD (mm)	E-test MIC (µg/ml)	SMX, Broth Microdilution MIC (µg/ml)		TMP, Broth microdilution MIC (µg/ml)	
			MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}
SWK-C 219^{Gr}	9 (R)	2 (S)	8 (S)	128 (S)	2 (S)	16 (R)
SWK-C 163b^{Gs}	10 (R)	2 (S)	32 (S)	**1024 (R)**	4 (S)	**32 (R)**
SWK-C 106^{Gs}	12 (I)	2 (S)	32 (S)	**512 (R)**	8 (S)	**64 (R)**
SWK-C 118^{Gs}	12 (I)	3 (I)	8 (S)	256 (S)	4 (S)	64 (R)
SWK-C 154b^{Gs}	12 (I)	2 (S)	8 (S)	128 (S)	2 (S)	32 (R)
SWK-C 140^{Gs}	15 (I)	2 (S)	16 (S)	**1024 (R)**	4 (S)	**32 (R)**
SWK-C 154c^{Gs}	14 (I)	2 (S)	16 (S)	**512 (R)**	4 (S)	**32 (R)**
SWK-C 149b^{Gs}	16 (S)	2 (S)	16 (S)	256 (S)	4 (S)	64 (R)
SWK-C 154a^{Gs}	16 (S)	2 (S)	32 (S)	**ETR***	16 (R)	**64 (R)**
SWK-C 109^{Gs}	18 (S)	1 (S)	32 (S)	**ETR***	2 (S)	**16 (R)**
SWK-C 145^{Gr}	20 (S)	0.75 (S)	4 (S)	32 (S)	1 (S)	8 (S)
SWK-C 133^{Gs}	21 (S)	0.75 (S)	4 (S)	128 (S)	2 (S)	16 (R)
SWK-C 136^{Gs}	21 (S)	0.25 (S)	4 (S)	32 (S)	1 (S)	16 (R)
SWK-C 161^{Gs}	37 (S)	0.5 (S)	32 (S)	**ETR***	4 (S)	**32 (R)**

Note: IZD = Inhibition zone diameter; MIC = Minimal Inhibitory Concentration**;** **Gr** = Gentamicin resistant; **Gs** = Gentamicin susceptible
SMX = Sulfamethoxazole; TMP = Trimethoprim; **ETR** = Exceeded the tested concentration range;
Bold and underlined details referred to MIC cut-offs of the isolate that are resistant to **both** SMX and TMP.

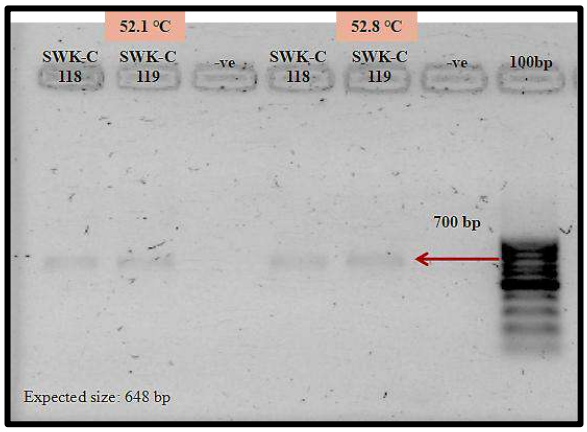
al. (2015), suggesting that the co-trimoxazole resistance is extremely rare. Therefore, despite the rare occurrence of sulfamethoxazole resistance among the isolates, the co-trimoxazole regimen are still relevant and useful for the treatment of melioidosis in Sarawak.

4.3 The *bpeEF-oprC* gene cluster PCR assays

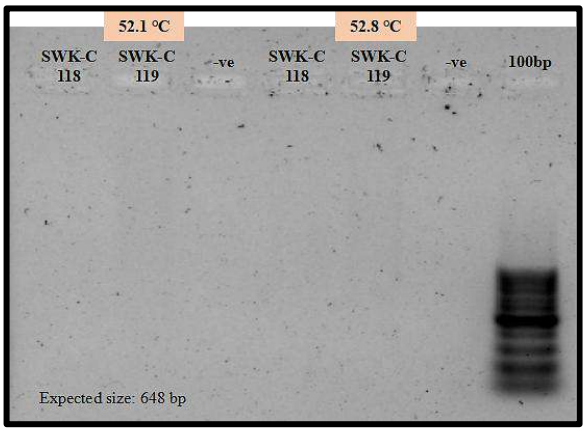
To detect co-trimoxazole resistance determinant, the *bpeEF-oprC* genes in Sarawak clinical *B. pseudomallei* isolates. Previous findings by Biot et al. (2011) and Podnecky et al. (2013) demonstrated the involvement of the BpeEF-oprC efflux pump in trimethoprim and sulfamethoxazole resistance. Following the findings, thirteen (13) primers targeting the *bpeEF-oprC* gene cluster were designed. The reference strain for the primer design was a Thai isolate, *Burkholderia pseudomallei* K96243 (accession number: BX571996.1) (Holden et al., 2004). Together with the strain genomic sequence, a few other Thai and Malaysian isolates that are supposedly closely related to Sarawak isolates were aligned to get the best fit primers for the study (as shown in Table 3.11). However, none of the primers managed to confirm the presence of the *bpeEF-oprC* gene cluster (*bpeT-llpE-bpeE-bpeF-oprC*) in the Sarawak isolates. The PCR products for the *bpeT* gene did have a faint band when electrophoresed, but the PCR retrial for the gene showed no band. All primers were troubleshot with gradient PCRs, but to no avail. Figure 4.7 depicts some of the results for the gradient PCR of the *bpeEF-oprC* gene cluster.

Nonetheless, these findings do not indicate the absence of the BpeEF-oprC efflux pump in the Sarawak clinical *B. pseudomallei* isolates. Instead, it is hypothesised that the nucleotide sequence of the *bpeEF-oprC* gene cluster for the Sarawak isolates differs from that of the clinical *B. pseudomallei* isolates reported elsewhere. The basis of this hypothesis corresponds to the previous findings in the Section 4.2.

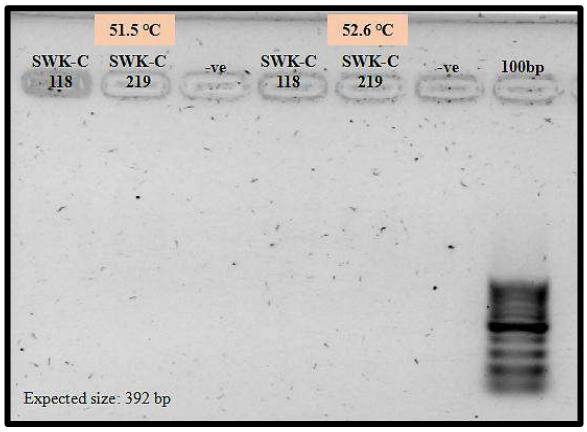
Target gene: *bpeT*



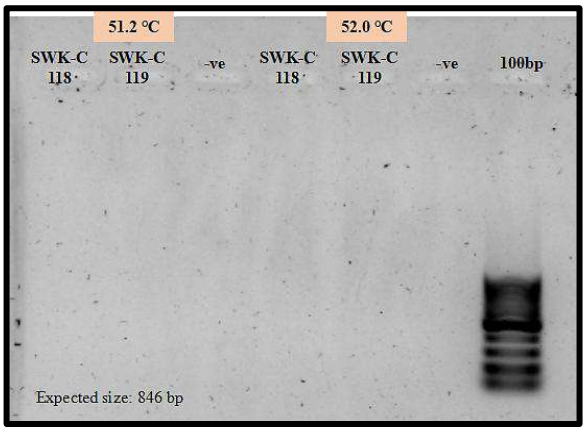
Target gene: *bpeT* (retrial)



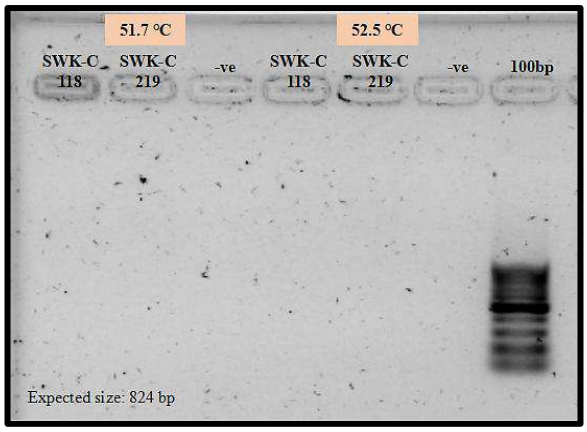
Target gene: *llpe*



Target gene: *bpeE*



Target gene: *bpeF*



Target gene: *oprC*

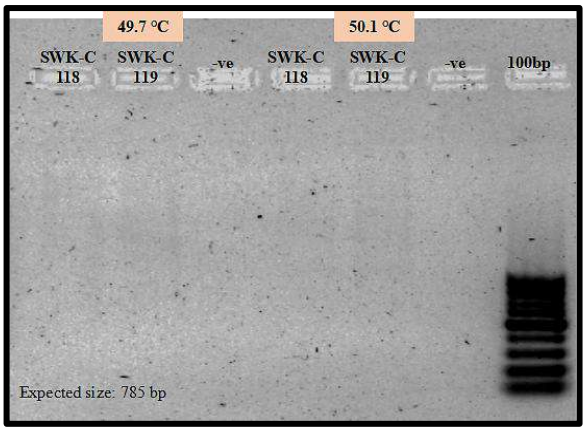


Figure 4.7: Results for some of the primers optimization for *bpeT-llpE-bpeE-bpeF-oprC* genes.

Based on Section 4.2, the broth microdilution test revealed a staggering 50% (7/14) of the Sarawak clinical *B. pseudomallei* isolates are resistance to sulfamethoxazole; a rare occurrence, especially when involving the wild-type co-trimoxazole susceptible *B. pseudomallei.* A previous study by Podnecky et al. (2017) demonstrated that the sulfamethoxazole resistance in the *B. pseudomallei* isolates is due to the overexpression of the BpeEF-oprC efflux pumps. The authors illustrated that the point mutations at the *bpeT* gene, the transcriptional activator of the BpeEF-oprC efflux pump reduced the susceptibility to trimethoprim (TMP), sulfamethoxazole (SMX), and co-trimoxazole (SXT) in the co-trimoxazole resistant mutant isolates. However, whether this resistance mechanism conforms to the resistance phenomenon exhibited by our isolates warrants a future study.

Aside from that, similar to report by Podin et al. (2013), the Sarawak clinical *B. pseudomallei* isolates (12/14) used for this experiment are unique and distinct from other *B. pseudomallei* isolates in being phenotypically gentamicin susceptible. They either belong to the multilocus sequence type (ST)881 or its single-locus variant ST997, both of which have an ancient and restricted dispersal to the Central Sarawak regions so far. Besides, they are also the first and sole wild-type of gentamicin susceptible *B. pseudomallei* isolates whose genome had novel point mutation within the highly conserved region of their *amrB* protein (*amrB*-1102G) that affects the expression of their resistance nodulation division family (RND) efflux pump, AmrAB-oprA. According to Podin et al. (2013), that is the first and only report of such nonsynonymous mutation involving wild-type the gentamicin-susceptible *B. pseudomallei* isolates so far. Therefore, although it remains to be proven, there could be a likelihood of a similar mutation occurring within the BpeEF-oprC efflux pumps of the Sarawak isolates used in this study.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Executive Summary

Burkholderia pseudomallei is 'hyper-endemic' in Sarawak, Malaysian Borneo. It is the causative agent of the extremely fatal infectious disease, melioidosis. Treatment of the disease is challenging due to its complexity and the adaptability of the bacteria to acquire resistance against antibiotics used in the treatment. One of the essential antibiotics for the treatment and management of the disease is co-trimoxazole. In recent years, there have been increased reports of *in-vitro* co-trimoxazole resistance among the Gentamicin-susceptible, ST188 and ST997 *B. pseudomallei* clinical isolates from Sarawak. This raised concerns among clinicians, particularly regarding the efficacy of the co-trimoxazole regimen for the eradicative-phase treatment of melioidosis. Hence, this study was conducted with the aim of understanding the magnitude of the co-trimoxazole resistance phenomenon among the Sarawak isolates.

Co-trimoxazole susceptibility of Sarawak clinical *B. pseudomallei* isolates was determined using two testing methods. Likewise, the MIC results were also defined using two guidelines: the Centre and Standard Laboratory Institute (CLSI) and the European Committee for Antibiotic Susceptibility Testing (EUCAST). Firstly, in **Section 4.1.1**, 164 isolates were subjected to disk diffusion testing. The result revealed that 81.7% (134/164) and 79.9% (131/164) of the Sarawak clinical *B. pseudomallei* isolates were susceptible to co-trimoxazole, as per CLSI and EUCAST standards. The results showed a high rate of co-trimoxazole susceptibility, but they did not reflect the true prevalence of co-trimoxazole susceptibility among the Sarawak isolates. Based on the phenotypic observations, most

isolates (42/164) that were intermediately susceptible and resistant to co-trimoxazole exhibited unclear zone edge and haze growth of bacterial within the zone. In this case, interpretation of the inhibition zone diameter will highly likely report a false-resistant result. Therefore, in **Section 4.1.2**, E-test was used to re-evaluate and confirm the co-trimoxazole susceptibility of those isolates. And, unsurprisingly, all isolates were co-trimoxazole susceptible by E-test. Subsequently, following the E-test results, the mathematical estimation for the true prevalence of co-trimoxazole susceptibility in the Sarawak clinical *B. pseudomallei* isolates was derived. The corrected CLSI and EUCAST-defined co-trimoxazole susceptibility prevalence was approximately 96.3% (158/164) and 97.6% (160/164), respectively. This result, therefore, confirmed the highly prevalent co-trimoxazole susceptibility in Sarawak clinical *B. pseudomallei* isolates. Besides, the co-trimoxazole susceptibility rate was comparable to that previously reported in studies involving Gentamicin-resistant *B. pseudomallei* in the other melioidosis endemic countries. This suggested that the antibacterial activity of co-trimoxazole against the *B. pseudomallei* isolate is similar regardless of their geographical distribution and genetic diversity. Hence, clinicians should be confident that the co-trimoxazole regimen is efficacious for the melioidosis treatment in Sarawak.

In **Section 4.1.3**, the variability of MIC results interpreted following the CLSI (2017) and EUCAST (2018) standards were compared. Based on the overall comparisons of the MIC results from both disk diffusion and the E-test, the CLSI-defined MICs were more consistent compared to that of EUCAST. In particular, EUCAST-defined MICs exhibited significant discrepancies for the resistance rates by disk diffusion (16.5%) and E-test method (1.2%). Meanwhile, the co-trimoxazole resistance rates by CLSI exhibited a minor difference of 2.4% by disk diffusion and 0% by E-test. These implied that the interpretation

of IZDs and MICs following the CLSI guideline reduced the risk of overcall resistance by disk diffusion test. Likewise, indicating that the CLSI standard is a valid guideline option to provide a reliable interpretation for the antibiotic susceptibility result.

In **Section 4.2**, the broth microdilution test was performed on 14 Sarawak isolates to assess their susceptibility toward sulfamethoxazole alone and trimethoprim alone. Interestingly, 50% (7/14) of the isolates were resistant to both sulfamethoxazole and trimethoprim. Despite that, the isolates were susceptible to the combination of sulfamethoxazole and trimethoprim (co-trimoxazole). This result inferred that resistance to sulfamethoxazole alone or trimethoprim alone does not affect the susceptibility of *B. pseudomallei* isolates toward co-trimoxazole. This is attributed to the fact that while the bacteriostatic activity of sulfamethoxazole alone and trimethoprim alone is insufficient in inhibiting *B. pseudomallei*, the synergistic activity of the combination of both sulfamethoxazole and trimethoprim (co-trimoxazole) is potent and capable of inhibiting the growth of the *B. pseudomalle*i isolates. Therefore, indicating that the co-trimoxazole should still be relevant and effective for the treatment regime of melioidosis in Sarawak.

Lastly, in **Section 4.3**, a series of independent PCR assays were performed to detect the co-trimoxazole resistance determinant *bpeEF-oprC* genes in the Sarawak isolates. However, none of the primers designed managed to confirm the presence of the *bpeEF-oprC* genes in the isolates. Although there was no detection of the *bpeEF-oprC* genes in the Sarawak isolates, the findings in **Section 4.2** suggested a hypothesis. In particular, the findings revealed a subset of Sarawak clinical *B. pseudomallei* isolates that were resistant to sulfamethoxazole. To our knowledge, this is a rare resistance occurrence, especially when involving wild-type *B. pseudomallei* isolates. Hence, it was hypothesised that the *bpeEF-*

oprC genes of the Sarawak isolate differ from that of the clinical *B. pseudomallei* isolates reported elsewhere.

5.2 Conclusion

In conclusion, the findings in this study unraveled the prevalence of co-trimoxazole susceptibility among the Sarawak clinical *B. pseudomallei* isolates. The susceptibility profile pooled from the Kirby-Bauer disk diffusion and E-test methods revealed that approximately 96.3% (158/164) to 97.6% (160/164) of the Sarawak isolates were co-trimoxazole susceptible based on CLSI and EUCAST standards, respectively. From the phenotypic observations for the disk diffusion test, the unclear inhibition zone edge and haze growth of bacteria within the inhibition zone may have contributed to the earlier reports of *in-vitro* co-trimoxazole resistance among the Sarawak isolates. However, this study demonstrated that such incidences are preventable by performing an E-test for isolates whose disk diffusion has been defined as intermediate and resistant. Aside from that, the broth microdilution test showed that the isolates from Sarawak were resistant to the separate components of co-trimoxazole but were generally susceptible to co-trimoxazole (sulfamethoxazole+trimethoprim). This result suggests that the Sarawak clinical *B. pseudomallei* isolates are manageable by the potent antibacterial activity of co-trimoxazole. Therefore, clinicians should be confident that the co-trimoxazole regimen is relevant and effective for the treatment of melioidosis in Sarawak. Next, analyses of MIC breakpoints in this study exhibited that the CLSI-defined MICs were more consistent than that defined by EUCAST. This result, therefore, indicated that the CLSI guideline is a valid option for antibiotic susceptibility testing and antimicrobial resistance surveillance in Sarawak. Aside from that, this study has revealed a rare circumstance of a staggering 50% (7/14) sulfamethoxazole-resistant Sarawak isolates by broth microdilution test. This finding has

raised the likelihood or hypothesis that the nucleotide sequence of co-trimoxazole resistance determinant *bpeEF-oprC* genes of the *B. pseudomallei* isolates from Sarawak is distinct from that reported elsewhere. Although the co-trimoxazole resistance determinant *bpeEF-oprC* genes were not well characterised in this study, the findings have laid some significant basis for future research pursuits.

5.3 Recommendations

Findings in this study had provided guidance and set path for future works, particularly on the laboratory diagnosis of melioidosis cases and the genomic analysis on the co-trimoxazole resistance determinant *bpeEF-oprC* genes for the Sarawak gentamicin susceptible *B. pseudomallei* isolates.

First, the melioidosis cases reported in Sarawak are predominantly clustered in rural regions (Sia et al., 2021), where the laboratory diagnosis of melioidosis is performed conventionally. For example, the antibiotic susceptibility of *B. pseudomallei* has been tested using the conventional disk diffusion method, as reported by Yong et al. (2016). Based on the findings in this study, I recommended the use of disk diffusion for the routine screening of antibiotic susceptibility but, isolates that are "intermediate" and "susceptible" should be re-tested using the E-test to confirm their antibiotic susceptibility.

Secondly, routine ASTs are necessary for the isolates that exhibited morphotype variants during the testing. Although the morphotype variants could have just been a response of the bacterium towards the environmental stress, as mentioned by Vipond et al. (2013), it can also be an indicator for transient adaptive resistance (Schnetterle et al., 2021) by the isolate. Therefore, routine ASTs are essential to monitor possible adaptive resistance in the clinical *B. pseudomallei* isolates while ensuring the efficacy of the treatment. This is

especially crucial due to prolonged use of co-trimoxazole for at least twelve (12) weeks in the eradication phase to prevent the relapse (Dance, 2014).

Thirdly, the adaptive CLSI guideline, which is currently used for the AST in Sarawak, is a valid option for the interpretation of antibiotic susceptibility testing results. Nevertheless, training and awareness among clinicians and microbiologists are important to address the concern of interpretative errors. Aside from that, the newly established EUCAST guideline by Karatuna et al. (2021) can also be gradually applied for the interpretation of AST and aid in the antimicrobial resistance surveillance for *B. pseudomallei* in Sarawak. However, in an adjunct to that, collaborative work between clinicians and microbiologists is needed to comprehensively study the impact of the new EUCAST guideline on the AST and treatment regime of melioidosis.

Lastly, as mentioned earlier, the co-trimoxazole resistance determinant *bpeEF-oprC* genes of the Sarawak isolates were hypothesised to be different from that of the clinical *B. pseudomallei* isolate reported elsewhere. Hence, to test this hypothesis, genomic analysis through whole-genome sequencing (WGS) or other genomic analysis method is recommended to elucidate the co-trimoxazole resistant determinant, *bpeEF-oprC* genes or if there are any other determinants exist among the Sarawak isolates in comparison with isolates found elsewhere. Through WGS, the entire nucleotide sequence (Hawroth et al., 2016) of the *B. pseudomallei* genome can be analysed for mutations at the specific genes of interest. In addition, other mechanisms that may contribute to the co-trimoxazole resistance will be able to be understood through genomic analysis.

REFERENCES

Ahmad, N., Hashim, R., & Mohd Noor, A. (2013). The in vitro antibiotic susceptibility of Malaysian isolates of *Burkholderia pseudomallei*. *International journal of microbiology*, 2013.

Andersson, D. I., Nicoloff, H., & Hjort, K. (2019). Mechanisms and clinical relevance of bacterial heteroresistance. *Nature Reviews Microbiology*, *17*(8), 479-496.

Aziz, A., Currie, B. J., Mayo, M., Sarovich, D. S., & Price, E. P. (2020). Comparative genomics confirms a rare melioidosis human-to-human transmission event and reveals incorrect phylogenomic reconstruction due to polyclonality. *Microbial genomics, 6*(2).

Barnewall, R. E., Benson, E. M., Brown, M. A., Fisher, D. A., Lindsay, A. S., Simmons, A. A., & Anderson, M. S. (2015). Characterization of a large animal aerosol exposure system for aerosolizing four strains of Burkholderia pseudomallei. *Journal of Aerosol Science*, *84*, 21-38.

Belkum, B. L.-G. (2019). Development roadmap for antimicrobial susceptibility testing systems. *Nature Reviews Microbiology*, 17, 51-62.

Benkova, M., Soukup, O., & Marek, J. (2020). Antimicrobial susceptibility testing: currently used methods and devices and the near future in clinical practice. *Journal of Applied Microbiology*, *129*(4), 806-822.

Biot, F. V., Valade, E., Garnotel, E., Chevalier, J., Villard, C., Thibault, F. M., ... & Pagès, J. M. (2011). Involvement of the efflux pumps in chloramphenicol selected strains of Burkholderia thailandensis: proteomic and mechanistic evidence. *PLoS One*, *6*(2), e16892.

Bugrysheva, J. V., Sue, D., Hakovirta, J., Loparev, V. N., Knipe, K., Sammons, S. A., ... & Weigel, L. M. (2015). Finished annotated genome sequence of *Burkholderia pseudomallei* strain Bp1651, a multidrug-resistant clinical isolate. *Genome announcements, 3*(6).

Chantratita, N., Wuthiekanun, V., Boonbumrung, K., Tiyawisutsri, R., Vesaratchavest, M., Limmathurotsakul, D., ... & Peacock, S. J. (2007). Biological relevance of colony morphology and phenotypic switching by Burkholderia pseudomallei. *Journal of bacteriology*, *189*(3), 807-817.

Chen, P. S., Chen, Y. S., Lin, H. H., Liu, P. J., Ni, W. F., Hsueh, P. T., ... & Chen, Y. L. (2015). Airborne transmission of melioidosis to humans from environmental aerosols contaminated with B. pseudomallei. *PLoS neglected tropical diseases*, *9*(6), e0003834.

Cheng, A. C., & Currie, B. J. (2005). Melioidosis: epidemiology, pathophysiology, and management. *Clinical microbiology reviews, 18*(2), 383-416.

Choi, J. Y., Hii, K. C., Bailey, E. S., Chuang, J. Y., Tang, W. Y., Wong, E. K. Y., ... & Gray, G. C. (2020). Burkholderia pseudomallei detection among hospitalized patients, Sarawak. *The American journal of tropical medicine and hygiene*, *102*(2), 388.

Chua, K. H., See, K. H., Thong, K. L., & Puthucheary, S. D. (2010). DNA fingerprinting of human isolates of Burkholderia pseudomallei from different geographical regions of Malaysia. *Tropical Biomedicine*, *27*(3), 517-524.

Chuah, C. J., Tan, E. K., Sermswan, R. W., & Ziegler, A. D. (2017). Hydrological connectivity and *Burkholderia pseudomalle*i prevalence in wetland environments: investigating rice-farming community's risk of exposure to melioidosis in North-East Thailand. *Environmental monitoring and assessment, 189*(6), 287.

CLSI. (2017). *Performance Standards for Antimicrobial Susceptibility Testing* 27th ed. CLSI supplement. M100. Wayne, PA: Clinical Laboratory Standards Institute.

CLSI. (2018). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically.* 11th ed. CLSI standard M07. Wayne, PA: Clinical and Laboratory Standards Institute.

Crowe, A., McMahon, N., Currie, B. J., & Baird, R. W. (2014). Current antimicrobial susceptibility of first-episode melioidosis Burkholderia pseudomallei isolates from the Northern Territory, Australia. *International journal of antimicrobial agents*, *44*(2), 160-162.

Currie, B. J. (2010). *Burkholderia pseudomallei* and *Burkholderia mallei*: melioidosis and glanders. *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases. 7th edn. Philadelphia: Churchill Livingstone Elsevier*, 2869-2885.

Currie, B. J. (2015). Melioidosis: evolving concepts in epidemiology, pathogenesis, and treatment. In *Seminars in respiratory and critical care medicine* (Vol. 36, No. 01, pp. 111-125). Thieme Medical Publishers.

Currie, B. J., & Kaestli, M. (2016). A global picture of melioidosis. *Nature*, *529*(7586), 290-291.

Cusack, T. P., Ashley, E. A., Ling, C. L., Rattanavong, S., Roberts, T., Turner, P., ... & Dance, D. A. B. (2019). Impact of CLSI and EUCAST breakpoint discrepancies on reporting of antimicrobial susceptibility and AMR surveillance. *Clinical Microbiology and Infection, 25*(7), 910-911.

Dan, M. (2015). Melioidosis in travelers: review of the literature. *Journal of travel medicine*, *22*(6), 410-414.

Dance, D. (2014). Treatment and prophylaxis of melioidosis. *International journal of antimicrobial agents, 43*(4), 310-318.

Dance, D. A. B., Davong, V., Soeng, S., Phetsouvanh, R., Newton, P. N., & Turner, P. (2014). Trimethoprim/sulfamethoxazole resistance in *Burkholderia pseudomallei*. *International journal of antimicrobial agents*, *44*(4), 368-369.

Dance, D. A., Wuthiekanun, V., Baird, R. W., Norton, R., Limmathurotsakul, D., & Currie, B. J. (2021). Interpreting *Burkholderia pseudomallei* disc diffusion susceptibility test results by the EUCAST method. *Clinical Microbiology and Infection*.

Depoorter, E., Bull, M. J., Peeters, C., Coenye, T., Vandamme, P., & Mahenthiralingam, E. (2016). *Burkholderia*: an update on taxonomy and biotechnological potential as antibiotic producers. *Applied microbiology and biotechnology, 100*(12), 5215-5229.

Duangurai, T., Indrawattana, N., & Pumirat, P. (2018). Burkholderia pseudomallei adaptation for survival in stressful conditions. *BioMed research international*, *2018*.

Dutta, S., Haq, S., Hasan, M. R., & Haq, J. A. (2017). Antimicrobial susceptibility pattern of clinical isolates of *Burkholderia pseudomallei* in Bangladesh. *BMC research notes, 10*(1), 1-5.

Eberl, L., & Vandamme, P. (2016). Members of the genus *Burkholderia*: good and bad guys. *F1000Research, 5*.

Estrada-De Los Santos, P., Rojas-Rojas, F. U., Tapia-García, E. Y., Vásquez-Murrieta, M. S., & Hirsch, A. M. (2016). To split or not to split: an opinion on dividing the genus Burkholderia. *Annals of Microbiology, 66*(3), 1303-1314.

European Committee on Antimicrobial Susceptibility Testing. (2018). *Breakpoint tables for interpretation of MICs and zone diameters*. Version 8.1, valid from 2018-05-15. [online] Available:

https://www.eucast.org/ast_of_bacteria/previous_versions_of_documents/ [Accessed Nov. 12, 2018]

Fernández-Villa, D., Aguilar, M. R., & Rojo, L. (2019). Folic acid antagonists: antimicrobial and immunomodulating mechanisms and applications. *International journal of molecular sciences*, *20*(20), 4996.

Galyov, E. E., Brett, P. J., & DeShazer, D. (2010). Molecular insights into Burkholderia pseudomallei and Burkholderia mallei pathogenesis. *Annual review of microbiology*, *64*, 495-517.

Gassiep, I., Armstrong, M., & Norton, R. (2020). Human melioidosis. *Clinical microbiology reviews*, *33*(2), e00006-19.

Gorelova, V., Bastien, O., De Clerck, O., Lespinats, S., Rébeillé, F., & Van Der Straeten, D. (2019). Evolution of folate biosynthesis and metabolism across algae and land plant lineages. *Scientific reports*, *9*(1), 1-16.

Hantrakun, V., Rongkard, P., Oyuchua, M., Amornchai, P., Lim, C., Wuthiekanun, V., ... & Limmathurotsakul, D. (2016). Soil nutrient depletion is associated with the presence of *Burkholderia pseudomallei*. *Applied and environmental microbiology, 82*(24), 7086-7092.

Hantrakun, V., Kongyu, S., Klaytong, P., Rongsumlee, S., Day, N. P., Peacock, S. J., ... & Limmathurotsakul, D. (2019, December). Clinical epidemiology of 7126 melioidosis patients in Thailand and the implications for a national notifiable diseases surveillance system. In *Open forum infectious diseases* (Vol. 6, No. 12, p. ofz498). US: Oxford University Press.

Hardin, J. W. (2016). Chapter 137 - *Burkholderia pseudomallei* (MELIOIDOSIS) ATTACK. In *Ciottone's Disaster Medicine* (Ciottone, G. R., 2nd Ed), pp. 740-742.

Elsevier.

Haworth, A., Savage, H., & Lench, N. (2016). Diagnostic Genomics and Clinical Bioinformatics. *Medical and Health Genomics*, 37-50.

Holden, M. T., Titball, R. W., Peacock, S. J., Cerdeño-Tárraga, A. M., Atkins, T., Crossman, L. C., ... & Parkhill, J. (2004). Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proceedings of the National Academy of Sciences, 101*(39), 14240-14245.

Houghton, R. L., Reed, D. E., Hubbard, M. A., Dillon, M. J., Chen, H., Currie, B. J., ... & AuCoin, D. P. (2014). Development of a prototype lateral flow immunoassay (LFI) for the rapid diagnosis of melioidosis. *PLoS neglected tropical diseases*, *8*(3), e2727.

Inglis, T. J., Hahne, D. R., Merritt, A. J., & Clarke, M. W. (2015). Volatile-sulfur-compound profile distinguishes *Burkholderia pseudomallei* from *Burkholderia thailandensis*. *Journal of clinical microbiology, 53*(3), 1009-1011.

International Organization for Standardization. (2014). Microbiology of food, animal feed and water—preparation, production, storage, and performance testing of culture media. ISO 11133: 2014.

International Organization for Standardization. (2019). Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices—Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases: ISO 20776-1.

Jenney, Lum, G., Fisher, D. A., & Currie, B. J. (2001). Antibiotic susceptibility of *Burkholderia pseudomallei* from tropical northern Australia and implications for therapy of melioidosis. *International Journal of Antimicrobial Agents, 17*(2), 109–

113.

Jones, A. L., Beveridge, T. J., & Woods, D. E. (1996). Intracellular survival of Burkholderia pseudomallei. *Infection and immunity*, *64*(3), 782-790.

Kaestli, M., Grist, E. P., Ward, L., Hill, A., Mayo, M., & Currie, B. J. (2016). The association of melioidosis with climatic factors in Darwin, Australia: A 23-year time-series analysis. *Journal of Infection, 72*(6), 687-697.

Kahlmeter, G., Giske, C. G., Kirn, T. J., & Sharp, S. E. (2019). Point-counterpoint: differences between the European Committee on Antimicrobial Susceptibility Testing and Clinical and Laboratory Standards Institute recommendations for reporting antimicrobial susceptibility results. *Journal of clinical microbiology, 57*(9), e01129-19.

Karatuna, O., Dance, D. A. B., Matuschek, E., Åhman, J., Turner, P., Hopkins, J., ... & Kahlmeter, G. (2021). *Burkholderia pseudomallei* multi-centre study to establish EUCAST MIC and zone diameter distributions and epidemiological cut-off values. *Clinical Microbiology and Infection*, *27*(5), 736-741.

Kemnic, T. R., & Coleman, M. (2021). Trimethoprim Sulfamethoxazole.[Updated 2020 Dec 11]. *StatPearls [Internet]; StatPearls Publishing: Treasure Island, FL, USA.*

Khosravi, Y., Vellasamy, K. M., Mariappan, V., Ng, S. L., & Vadivelu, J. (2014). Antimicrobial susceptibility and genetic characterisation of Burkholderia pseudomallei isolated from Malaysian patients. *The Scientific World Journal*, *2014*.

Kingsley, P. V., Leader, M., Nagodawithana, N. S., Tipre, M., & Sathiakumar, N. (2016). Melioidosis in Malaysia: a review of case reports. *PLoS neglected tropical diseases, 10*(12), e0005182.

Kumar, S., Gupta, A. K., Maurya, A., & Singh, M. K. (2020). Chemical treatment for

removal of waterborne pathogens. In *Waterborne Pathogens* (pp. 205-218). Butterworth-Heinemann.

Kunakom, S., & Eustáquio, A. S. (2019). *Burkholderia* as a source of natural products. *Journal of natural products, 82*(7), 2018-2037.

Lazar Adler, N. R., Govan, B., Cullinane, M., Harper, M., Adler, B., & Boyce, J. D. (2009). The molecular and cellular basis of pathogenesis in melioidosis: how does Burkholderia pseudomallei cause disease?. *FEMS microbiology reviews*, *33*(6), 1079-1099.

Le Tohic, S., Montana, M., Koch, L., Curti, C., & Vanelle, P. (2019). A review of melioidosis cases imported into Europe. *European Journal of Clinical Microbiology & Infectious Diseases, 3*8(8), 1395-1408.

Limmathurotsakul, D., & Peacock, S. J. (2011). Melioidosis: a clinical overview. *British medical bulletin, 99*(1), 125-139.

Limmathurotsakul, D., Golding, N., Dance, D. A., Messina, J. P., Pigott, D. M., Moyes, C. L., ... & Hay, S. I. (2016). Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nature microbiology, 1*(1), 1-5.

Limmathurotsakul, W. A. (2014). Melioidosis Caused by Burkholderia pseudomallei in Drinking Water, Thailand, 2012. *Emerging Infectious Disease*, 20(2): 265-268.

Lipsitz, R., Garges, S., Aurigemma, R., Baccam, P., Blaney, D. D., Cheng, A. C., ... & Smith, T. L. (2012). Workshop on treatment of and postexposure prophylaxis for Burkholderia pseudomallei and B. mallei infection, 2010. *Emerging infectious diseases*, *18*(12), e2.

Maloney, S., Engler, C., & Norton, R. (2017). Epidemiological cut-off value of clinical isolates of Burkholderia pseudomallei from northern Queensland to meropenem,

ceftazidime, trimethoprim/sulfamethoxazole and doxycycline by the microbroth dilution method. *Journal of global antimicrobial resistance*, *10*, 291-294.

Mohan, A., Podin, Y., Tai, N., Chieng, C. H., Rigas, V., Machunter, B., ... & Ooi, M. H. (2017). Pediatric melioidosis in Sarawak, Malaysia: epidemiological, clinical and microbiological characteristics. *PLoS neglected tropical diseases*, *11*(6), e0005650.

Mohan, A., Manan, K., Tan, L. S., Tan, Y. C., Chin, S. T., Ahmad, R., ... & Ooi, M. H. (2020). Detection of spleen abscesses facilitates diagnosis of melioidosis in Malaysian children. *International Journal of Infectious Diseases*, *98*, 59-66.

Moore, R. A., Reckseidler-Zenteno, S., Kim, H., Nierman, W., Yu, Y., Tuanyok, A., ... & Woods, D. E. (2004). Contribution of gene loss to the pathogenic evolution of Burkholderia pseudomallei and Burkholderia mallei. *Infection and immunity, 72*(7), 4172-4187.

Nathan, S., Chieng, S., Kingsley, P. V., Mohan, A., Podin, Y., Ooi, M. H., ... & How, S. H. (2018). Melioidosis in Malaysia: incidence, clinical challenges, and advances in understanding pathogenesis. *Tropical medicine and infectious disease*, *3*(1), 25.

Nhung, P. H., Van, V. H., Anh, N. Q., & Phuong, D. M. (2019). Antimicrobial susceptibility of *Burkholderia pseudomallei* isolates in Northern Vietnam. J*ournal of global antimicrobial resistance, 18*, 34-36.

Novak, R. T., Glass, M. B., Gee, J. E., Gal, D., Mayo, M. J., Currie, B. J., & Wilkins, P. P. (2006). Development and evaluation of a real-time PCR assay targeting the type III secretion system of Burkholderia pseudomallei. *Journal of clinical microbiology*, *44*(1), 85-90.

Office of Laboratory Security, Public Health Agency of Canada (2011). Retrieved from https://www.canada.ca/en/public-health/services/laboratory-biosafety-

biosecurity/pathogen-safety-data-sheets-risk-assessment/burkholderia-pseudomonas-pseudomallei-material-safety-data-sheets-msds.html

Opal, S. M., & Pop-Vicas, A. (2015). 18 - Molecular mechanisms of antibiotic resistance in bacteria. In *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases* (pp. 235-251). WB Saunders.

Parte, A. C., Carbasse, J. S., Meier-Kolthoff, J. P., Reimer, L. C., & Göker, M. (2020). List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *International journal of systematic and evolutionary microbiology, 70*(11), 5607.

Payne, G. W., Vandamme, P., Morgan, S. H., LiPuma, J. J., Coenye, T., Weightman, A. J., ... & Mahenthiralingam, E. (2005). Development of a recA gene-based identification approach for the entire Burkholderia genus. *Applied and environmental microbiology*, *71*(7), 3917-3927.

Price, E. P., Currie, B. J., & Sarovich, D. S. (2017). Genomic insights into the melioidosis pathogen, *Burkholderia pseudomallei*. *Current Tropical Medicine Reports, 4*(3), 95-102.

Podin, Y., Kaestli, M., McMahon, N., Hennessy, J., Ngian, H. U., Wong, J. S., ... & Currie, B. J. (2013). Reliability of automated biochemical identification of Burkholderia pseudomallei is regionally dependent. *Journal of clinical microbiology*, *51*(9), 3076-3078.

Podin, Y., Sarovich, D. S., Price, E. P., Kaestli, M., Mayo, M., Hii, K., ... & Currie, B. J. (2014). *Burkholderia pseudomallei* isolates from Sarawak, Malaysian Borneo, are predominantly susceptible to aminoglycosides and macrolides. *Antimicrobial agents and chemotherapy*, *58*(1), 162-166.

Podnecky, N. L., Wuthiekanun, V., Peacock, S. J., & Schweizer, H. P. (2013). The BpeEF-OprC efflux pump is responsible for widespread trimethoprim resistance in clinical and environmental Burkholderia pseudomallei isolates. *Antimicrobial agents and chemotherapy*, *57*(9), 4381-4386.

Podnecky, N. L., Rhodes, K. A., Mima, T., Drew, H. R., Chirakul, S., Wuthiekanun V., ... & Schweizer, H. P. (2017). Mechanisms of resistance to folate pathway inhibitors in *Burkholderia pseudomallei*: deviation from the norm. *MBio, 8*(5), e01357-17.

Reller, L. B., Weinstein, M., Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical infectious diseases*, *49*(11), 1749-1755.

Sader, H. S., & Pignatari, A. C. C. (1994). E test: a novel technique for antimicrobial susceptibility testing. *Sao Paulo Medical Journal*, *112*, 635-638.

Sahl, J. W., Vazquez, A. J., Hall, C. M., Busch, J. D., Tuanyok, A., Mayo, M., ... & Wagner, D. M. (2016). The effects of signal erosion and core genome reduction on the identification of diagnostic markers. *MBio, 7*(5).

Sahl, J. W., Allender, C. J., Colman, R. E., Califf, K. J., Schupp, J. M., Currie, B. J., ... & Tuanyok, A. (2015). Genomic characterization of Burkholderia pseudomallei isolates selected for medical countermeasures testing: comparative genomics associated with differential virulence. *PLoS One*, *10*(3), e0121052.

Saiprom, N., Amornchai, P., Wuthiekanun, V., Day, N. P., Limmathurotsakul, D., Peacock, S. J., & Chantratita, N. (2015). Trimethoprim/sulfamethoxazole resistance in clinical isolates of *Burkholderia pseudomallei* from Thailand. *International journal of antimicrobial agents, 45*(5), 557.

Sanchez-Villamil, J. I., & Torres, A. G. (2018). Melioidosis in Mexico, Central America,

and the Caribbean. *Tropical medicine and infectious disease*, *3*(1), 24.

Sarovich, D. S., Price, E. P., Von Schulze, A. T., Cook, J. M., Mayo, M., Watson, L. M., ... & Wagner, D. M. (2012). Characterization of ceftazidime resistance mechanisms in clinical isolates of Burkholderia pseudomallei from Australia. *PloS one*, *7*(2), e30789.

Schnetterle, M., Gorgé, O., Nolent, F., Boughammoura, A., Sarilar, V., Vigier, C., ... & Neulat-Ripoll, F. (2021). Genomic and RT-qPCR analysis of trimethoprim-sulfamethoxazole and meropenem resistance in *Burkholderia pseudomallei* clinical isolates. *PLoS neglected tropical diseases*, *15*(2), e0008913.

Schoch, C. L., Ciufo, S., Domrachev, M., Hotton, C. L., Kannan, S., Khovanskaya, R., ... & Karsch-Mizrachi, I. (2020). NCBI Taxonomy: a comprehensive update on curation, resources and tools. *Database, 2020*.

Schweizer, H. P. (2012). Mechanisms of antibiotic resistance in Burkholderia pseudomallei: implications for treatment of melioidosis. *Future microbiology*, *7*(12), 1389-1399.

Sia, T., Podin, Y., Chuah, T. B., & Wong, J. S. (2018). Melioidosis: An unusual cause of infective endocarditis: A case report. *European Heart Journal-Case Reports*.

Sia, T. L., Mohan, A., Ooi, M. H., Chien, S. L., Tan, L. S., Goh, C., ... & Podin, Y. (2021). Epidemiological and clinical characteristics of melioidosis caused by gentamicin-susceptible Burkholderia pseudomallei in Sarawak, Malaysia. In *Open Forum Infectious Diseases* (Vol. 8, No. 10, p. ofab460). US: Oxford University Press.

Sprague, L. D., & Neubauer, H. (2004). Melioidosis in animals: a review on epizootiology, diagnosis and clinical presentation. *Journal of Veterinary Medicine, Series B, 51*(7), 305-320.

Stanton, A. T., & Fletcher, W. (1921). Melioidosis, a New Disease of the

Tropics. *Melioidosis, a New Disease of the Tropics*.

Stephen, J., Ronald, J., Yvette, S., José, H., Ivonne, D., Robert, L., ... & Carol, A. (2005). Manual of antimicrobial susceptibility testing. *Washington: American Society for Microbiology*, 39-40.

Stevens, M. P., Stevens, J. M., Jeng, R.L., Taylor, L. A., Wood, M. W., Hawes, P., Monaghan, P., Welch, M. D., & Galyov, E. E. (2005). Identification of a bacterial factor required for actin-based motility of Burkholderia pseudomallei. *Molecular Microbiology, 56*(1), 40–53.

Tauran, P. M., Wahyunie, S., Saad, F., Dahesihdewi, A., Graciella, M., Muhammad, M., ... & Iskandriati, D. (2018). Emergence of melioidosis in Indonesia and today's challenges. *Tropical medicine and infectious disease, 3*(1), 32.

Tenover, F. C. (2009). Antimicrobial susceptibility testing methods for bacterial pathogens. *Antimicrobial Drug Resistance: Clinical and Epidemiological Aspects*, 1151-1159.

Titball, R. W., Burtnick, M. N., Bancroft, G. J., & Brett, P. (2017). *Burkholderia pseudomallei* and *Burkholderia mallei* vaccines: are we close to clinical trials?. *Vaccine, 35*(44), 5981-5989.

Toh, V., Tee, S. P., & Lee, S. H. (2021). Clinical characteristics and predictors of mortality in patients with melioidosis: the Kapit experience. *Tropical Medicine & International Health*, *26*(6), 664-671.

Tuanyok, A., Leadem, B. R., Auerbach, R. K., Beckstrom-Sternberg, S. M., Beckstrom-Sternberg, J. S., Mayo, M., ... & Keim, P. (2008). Genomic islands from five strains of *Burkholderia pseudomallei*. *BMC genomics, 9*(1), 1-14.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen,

S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic acids research*, *40*(15), e115-e115.

Viberg, L. T., Sarovich, D. S., Kidd, T. J., Geake, J. B., Bell, S. C., Currie, B. J., & Price, E. P. (2017). Within-host evolution of Burkholderia pseudomallei during chronic infection of seven Australasian cystic fibrosis patients. *MBio, 8*(2), e00356-17.

Vipond, J., Kane, J., Hatch, G., McCorrison, J., Nierman, W. C., & Losada, L. (2013). Sequence determination of Burkholderia pseudomallei strain NCTC 13392 colony morphology variants. *Genome announcements*, *1*(6), e00925-13.

Waites, K. B., Duffy, L. B., Bébéar, C. M., Matlow, A., Talkington, D. F., Kenny, G. E., ... & Brown, S. D. (2012). Standardized methods and quality control limits for agar and broth microdilution susceptibility testing of Mycoplasma pneumoniae, Mycoplasma hominis, and Ureaplasma urealyticum. *Journal of clinical microbiology*, *50*(11), 3542-3547.

Warrens, M. J. (2015). Five ways to look at Cohen's kappa. *Journal of Psychology & Psychotherapy*, 5(4), 1.

Wen, S. W., Yang, T., Krewski, D., Yang, Q., Nimrod, C., Garner, P., ... & Walker, M. C. (2008). Patterns of pregnancy exposure to prescription FDA C, D and X drugs in a Canadian population. *Journal of Perinatology*, *28*(5), 324-329.

White, N. J. (2003). Melioidosis. *The Lancet*, *361*(9370), 1715-1722.

Wiersinga, W. J., Virk, H. S., Torres, A. G., Currie, B. J., Peacock, S. J., Dance, D. A., & Limmathurotsakul, D. (2018). Melioidosis. *Nature reviews Disease primers, 4*(1), 1-22.

Wiersinga, W. J., Currie, B. J., & Peacock, S. J. (2012). Melioidosis. *New England Journal of Medicine*, *367*(11), 1035-1044.

Wuthiekanun, V., Desakorn, V., Wongsuvan, G., Amornchai, P., Cheng, A. C., Maharjan, B., ... & Peacock, S. J. (2005). Rapid immunofluorescence microscopy for diagnosis of melioidosis. *Clinical and Vaccine Immunology*, *12*(4), 555-556.

Wuthiekanun, V., Amornchai, P., Saiprom, N., Chantratita, N., Chierakul, W., Koh, G. C., ... & Peacock, S. J. (2011). Survey of antimicrobial resistance in clinical Burkholderia pseudomallei isolates over two decades in Northeast Thailand. *Antimicrobial agents and chemotherapy*, *55*(11), 5388-5391.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., ... & Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. Nov. *Microbiology and immunology, 36*(12), 1251-1275.

Yong, K. Y., Tang, A. S. O., Teh, Y. C., Fam, T. L., & Chua, H. H. (2016). Prevalence of Antibiotic Resistance In *Burkholderia Pseudomallei* Cases Presented to Miri General Hospital. *Sarawak Health Journal, 3,* 26.

Yuan, Y., Yao, Z., Xiao, E., Zhang, J., Wang, B., Ma, B., ... & Fan, E. (2019). The first imported case of melioidosis in a patient in Central China. *Emerging microbes & infections, 8*(1), 1223-1228.

Zeng, X., & Lin, J. (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Frontiers in microbiology*, *4*, 128.

APPENDICES

Appendix 1: Pathogen Safety Data Sheet – *Burkholderia pseudomallei*

SECTION I – INFECTIOUS AGENT

NAME: *Burkholderia (Pseudomonas) pseudomallei*

SYNONYM OR CROSS REFERENCE: Melioidosis, Whitmore disease; (formerly Pseudomonas)

CHARACTERISTICS: Gram negative rod, motile, aerobic, young cultures exhibit bipolar staining (safety pin appearance), wrinkled colonies on agar media

SECTION II – HEALTH HAZARDS

PATHOGENICITY: Melioidosis - an endemic glanders-like disease; clinical symptoms vary from inapparent infection to chronic infection to rapidly fatal septicemia; may simulate typhoid fever or more commonly tuberculosis, with symptoms such as empyema, chronic abscesses and osteomyelitis

EPIDEMIOLOGY: Worldwide distribution, however, found primarily in tropical or subtropical regions, especially in Southeast Asia and northern Australia; individuals who have had intimate contact with soil and surface water

HOST RANGE: Humans and various animals (see reservoir)

INFECTIOUS DOSE: Unknown

MODE OF TRANSMISSION: Acquired by ingestion, inhalation or contact of abraded, wounded or burned skin with contaminated water or soil

INCUBATION PERIOD: 2 days (several months or years may elapse between presumed exposure and clinical disease)

COMMUNICABILITY: Person to person transmission is extremely rare; human carriers are not known

SECTION III – DISSEMINATION

RESERVOIR: Environmental organism found in certain waters and soils; animals include sheep, goats, horses, swine, monkey and rodents

ZOONOSIS: Yes - direct or indirect contact of mucous membranes with lesion discharge of infected animals

VECTORS: None

SECTION IV – VIABILITY

DRUG SUSCEPTIBILITY: TMP-SMX is most effective; susceptible to ceftazidime, imipenem, doxycycline, ciprofloxacinsulphas, chloramphenicol, tetracycline

DRUG RESISTANCE: Resistant to penicillin G, ampicillin, carbenicillum, aminoglycosides, cephalosporins; resistance to TMP-SMX has been reported

SUSCEPTIBILITY TO DISINFECTANTS: Susceptible to many disinfectants - 1% sodium hypochlorite, 70% ethanol, glutaraldehyde, formaldehyde

PHYSICAL ACTIVATION: Susceptible to moist heat (121°C for at least 15 min) and dry heat (160-170°C for at least 1 hour)

SURVIVAL OUTSIDE HOST: Survives for years in soil and water

SECTION V – MEDICAL

SURVEILLANCE: Monitor for symptoms; confirm by rise in antibody titre and isolation of organism

FIRST AID/TREATMENT: Antibiotic therapy; multiple drugs for septicemic cases; pulmonary resection may be considered for chronic cases

IMMUNISATION: None

PROPHYLAXIS: None

SECTION VI - LABORATORY HAZARDS

LABORATORY-ACQUIRED INFECTIONS: 20 cases of infection, with 7 deaths, reported up to 1976; one case associated with massive aerosol and skin contact exposure; an additional infection resulted from an aerosol created during open-flask sonication of a culture presumed to be *P. cepacia*; 3 laboratory workers were reported to have subclinical infections in 1992.

SOURCES/SPECIMENS: Sputa, blood, wound exudates, tissues

PRIMARY HAZARDS: Direct contact with cultures and infectious materials from humans, animals or the environment; ingestion; autoinoculation; exposure to infectious aerosols and droplets

SPECIAL HAZARDS: May be present in soil and water samples from endemic areas

SECTION VII - RECOMMENDED PRECAUTIONS

CONTAINMENT REQUIREMENTS: Biosafety level 3 practices and containment for activities utilizing infectious body fluids and tissues; also for activities with a high potential for aerosol or droplet production or the production of large quantities of infectious materials;

Agriculture Canada may impose additional requirements or restrictions on the use of this agent

CONTAINMENT REQUIREMENTS: Biosafety level 3 practices and containment for activities utilizing infectious body fluids and tissues; also for activities with a high potential for aerosol or droplet production or the production of large quantities of infectious materials; Agriculture Canada may impose additional requirements or restrictions on the use of this agent

OTHER PRECAUTIONS: Gloves should be worn when handling, and during necropsy of infected animals

SECTION VIII - HANDLING INFORMATION

SPILLS: Allow aerosols to settle; wear protective clothing; gently cover spill with paper towels and apply 1% sodium hypochlorite, starting at perimeter and working towards the centre; allow sufficient contact time (30 min) and clean area

DISPOSAL: Decontaminate before disposal; steam sterilization, incineration, chemical disinfection

STORAGE: In sealed containers that are appropriately labelled

SECTION IX - MISCELLANEOUS INFORMATION

Date prepared: November 1999 (Updated 18th February 2011)

Adapted from: Office of Laboratory Security, Public Health Agency of Canada (https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment/burkholderia-pseudomonas-pseudomallei-material-safety-data-sheets-msds.html)