ISOLATION AND IDENTIFICATION OF *BURKHOLDERIA* SPECEIS IN SOILS AND VARIOUS WATER RESERVOIRS

By

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ABSTRACT

ISOLATION AND IDENTIFICATION OF *BURKHOLDERIA* SPECIES IN SOILS AND VARIOUS WATER RESERVOIRS

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The genus *Burkholderia* consists of diverse species which include both "friends" and "foes". They are gram-negative saprophytes living in the soil and water reservoirs in endemic regions such as the Southeast Asia and the tropical northern Australia. Some of the "friendly" *Burkholderia* species are widely used in the biotechnological and agricultural industries for bioremediation and biocontrol, respectively. However, several members of the genus, including *B. pseudomallei*, *B. mallei*, and *B. cepacia*, are known to cause fatal diseases in both humans and animals. Little is known about the environmental prevalence of *Burkholderia* species in Malaysia. Hence, this project was set out to detect and identify *Burkholderia* species in our environmental reservoirs, focusing on those around Kampar. Six soil samples and 12 water samples were collected from various locations, in which some involve direct human contact. Selective culture techniques using Galimand's, Ashdown's, and *Burkholderia cepacia* selective media were performed to ease the isolation and identification of *Burkholderia* species among environmental microorganisms. Results showed

that the use of Galimand's broth as a selective and enrichment medium gave better isolation yield with higher selectivity. The bacterial isolates were preliminarily identified based on their colony morphology, Gram staining appearance and biochemical features. Two PCR assays targeting the 16S rDNA sequences, one, universal and the other, *Burkholderia*-specific, were performed to identify the isolates to the species level. Four *Burkholderia* isolates were successfully isolated, and they were very likely of the *B. cepacia* complex. In addition, isolates belonging to a limited number of other genera (*Pseudomonas*, *Chromobacterium*, *Klebsiella*, *Cronobacter*, *Comamonas*, *Serratia* and *Escherichia*) were also isolated. Consequently, the health impacts of the *Burkholderia* isolates were briefly assessed. They were found to be clinically relevant and may pose threats to susceptible hosts in the event of physical contact. The potential usefulness of the two confirmed *B. cepacia* isolates (BCSA5 and BCSA14) in bioremediation for instance, remains to be investigated.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(AW YEE SHAN)

APPROVAL SHEET

This project report entitled **"ISOLATION AND IDENTIFICATION OF *BURKHOLDERIA* SPECIES IN SOILS AND VARIOUS WATER RESERVOIRS"** was prepared by AW YEE SHAN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

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I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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LIST OF ABBREVIATIONS

API	analytical profile index
ASH	Ashdown's agar
BCSA	*Burkholderia cepacia* selective agar
BLASTn	nucleotide-nucleotide Basic Local Alignment Search Tool
dH_2O	distilled water
DEBWorP	Detection of Environmental *Burkholderia pseudomallei* Working Party
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
D/W	deionized water
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
E-value	expect value
K_2HPO_4	dipotassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
LPSN	List of Prokaryotic Names with Standing in Nomenclature
MLST	multilocus sequence typing
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NTC	no-template control

OFPBL	oxidative-fermentative base, polymyxin b, bacitracin, lactose
PCR	polymerase chain reaction
PEG-DOC	polyethylene glycol-deoxycholate
rDNA	ribosomal deoxyribonucleic acid
sp. / spp.	species
TAE	Tris-acetate-EDTA
Tris-HCl	Tris hydrochloride
TSA	tryptic soy agar
TTC	triphenyltetrazolium chloride
℃	degree Celsius
x *g*	times gravity
bp	base pair
cm	centimeter
g	Gram
h	hour/hours
L	Liter
M	molar
min	minute/minutes
ml	milliliter
µl	Microliter
µM	Micromolar

rpm	revolution per minute
s	second/seconds
U	Unit
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Burkholderia is a bacterial genus of gram-negative, aerobic, bacilli created in 1992. It includes several species formerly classified under the genus *Pseudomonas.* According to the LPSN, there are at least 80 species under the genus *Burkholderia* (Euzeby, 1997). Some *Burkholderia* species are found to be useful for biotechnological applications while some may be harmful to human's health. With regard to the former, many *Burkholderia* species have great potential for agricultural and environmental uses such as plant growth stimulation, atmospheric nitrogen fixation, biological control and bioremediation (Estrada-De Los Santos, Bustillos-Cristales and Caballero-Mellado, 2001).

Several *Burkholderia* species are pathogenic to humans. *Burkholderia pseudomallei* is the etiological agent of melioidosis in humans and animals, which is endemic to Southeast Asia and Northern Australia (Cheng and Currie, 2005). The symptoms of meliodosis are very similar to other respiratory infections, causing it to be often misdiagnosed. Septicemia due to *B. pseudomallei* carries a high mortality rate in the absence of early aggressive therapy (Currie, Ward and Cheng, 2010). Another pathogenic species, *Burkholderia mallei,* causes glanders in horses and can be transmitted to humans and other animals. Due to the high mortality rates and the lack of reliable treatment and vaccine for both melioidosis and glanders, both

B. pseudomallei and *B. mallei* are recognized as bio-threatening agents. Another group of pathogenic species, the *Burkholderia cepacia* complex is made up of opportunistic pathogens which give rise to infections in patients with cystic fibrosis and chronic granulomatous disease.

A less virulent species, *B. thailandensis*, was described by Brett, DeShazer and Woods (1998). It displays very similar characteristics to those of *B. pseudomallei* in most conventional biochemical tests with the exception of assimilation of L-arabinose whereby only the former assimilates (Wuthiekanun et al., 1996). Interestingly, this organism is closely-related at the genetic level to pathogenic *Burkholderia* members such as *B. pseudomallei*, *B. mallei* and *B. cepacia* complex, but it exhibits difference in pathogenicity. *B. thailandensis* is apparently rarely pathogenic to humans and animal studies showed that its infection dose is far greater than that of *B. pseudomallei* (Deshazer, 2007). Although *B. thailandensis* is considered avirulent in mammals, rare cases of disease due to it have been documented (Glass et al., 2006).

Since *B. pseudomallei* and *B. mallei* are classified as Category B bioterrorism agents by the Centers for Disease Control and Prevention (CDC), works with these organisms are highly regulated and require special laboratory facilities. Due to these restrictions, the genetically similar but less virulent species, *B. thailandensis* is commonly used in the laboratory as a surrogate model for those pathogenic ones (Haraga et al., 2008). With the availability of this easily tractable experimental organism, acceleration in research on the pathogenesis of *Burkholderia* infection is promising.

Despite many investigations on the clinical importance and agronomical relevance of *Burkholderia* spp., information on the factors influencing their occurrence, abundance and diversity in the environment is still scarce (Stopnisek et al., 2013). Compared to endemically affected countries, there are not many studies on the occurrence of *Burkholderia* spp. in environmental reservoirs in Malaysia. Tracing the distribution of environmental *Burkholderia* species is essential for developing a risk map for burkholderial infection such as melioidosis, since this can provide the geographical setting for preventive measures as well as raising awareness among healthcare workers and residents in *Burkholderia*-positive areas. By means of environmental sampling in this project, the areas where people might be at risk or even before cases are recognized could be identified. Hence, the main intent of this project was to assess the prevalence of *Burkholderia* spp. in our environmental reservoirs, especially those pathogenic ones as well as the beneficial species.

In this project, isolation and identification of *Burkholderia* species in soil and water samples from the environment were carried out. The locations of sampling in this project were mainly those places where human might have a direct contact with contaminated water or soil, which might commit a risk of exposure to pathogenic *Burkholderia* species. These included rivers, lakes, stagnant water, waterfall, sea and different types of soil. The successfully isolated *Burkholderia* species could then be further studied on their pathogenesis and beneficial properties.

Due to the high degree of phenotypic and genotypic similarities among *Burkholderia* species, methods for their rapid detection and differentiation have been the popular topic of research. Culture-based methods that use selective and enrichment media, such as Ashdown's medium, *Burkholderia cepacia* selective medium and Galimand's broth, have been developed to ease burkholderial isolation. The phenotypic identification of Burkholderial isolates is usually carried out based on their colony morphology, gram staining appearances and biochemical features. However, some of the in-house biochemical tests are time-consuming and the use of commercial biochemical kits and automated identification systems are limited in accuracy, thus a molecular technique is also used as a supplement test to obtain a more accurate and reliable identification result. Several PCR assays for the identification of *Burkholderia* species have been developed (Lowe et al., 2013). Besides phenotypic identification, a universal 16S rDNA PCR assay and a genus-specific PCR assay targeting the *Burkholderia* 16S rDNA were also performed in this project to identify the isolates to their species level.

The aims and objectives of this project were:

I. To isolate *Burkholderia* species in water and soil samples collected from various locations.
II. To identify *Burkholderia* species among the bacterial isolates via phenotypic and genotypic identification methods.
III. To assess the presence of *Burkholderia* species in different types of environmental reservoirs.

CHAPTER 2

LITERATURE REVIEW

2.1 The genus *Burkholderia*

The genus *Burkholderia* consists of a wide variety of gram-negative, motile bacilli (1-5 μm in length and 0.5-1.0 μm in width), which are commonly found in the soil and water (Choh et al, 2013). Several *Burkholderia* species are shown to be useful for biotechnological and agricultural aspects such as biological control of plant diseases, stimulation of plant growth, improvement of nitrogen fixation and bioremediation (Stoyanova et al., 2007; Compant et al., 2008). Despite most *Burkholderia* species are non-pathogenic, a few including *B. pseudomallei, B. mallei*, and *B. cepacia* cause severe, life-threatening infections in both immunocompetent and immunocompromised individuals. Burkholderial infections are inherently hard to treat due to their properties of multiple antibiotic resistance, ability to form biofilms and establishment of intracellular and chronic infection in the hosts (Choh et al., 2013).

2.2 *Burkholderia* Species

2.2.1 Pathogenic *Burkholderia* spp.

The genus *Burkholderia* is well-known for its pathogenic members: *B. mallei*, *B. pseudomallei* and *B. cepacia* complex.

2.2.1.1 Human and Animal Pathogens

B. mallei is the causal agent of glanders, primarily infecting horses, mules, and donkeys. Unlike *B. pseudomallei*, *B. mallei* is a host-adapted pathogen which is unable to survive in nature outside its host. Human cases of glanders primarily occurred in veterinarians, horse and donkey caretakers, abattoir workers and laboratory workers. Human glanders can be acute, which can lead to failure in respiratory function and can be fatal if untreated.

Melioidosis is caused by *B. pseudomallei*. It is predominantly endemic in Southeast Asia, particularly Thailand, and northern Australia (Cheng and Currie, 2005). Inoculation through wounds, inhalation of contaminated dust and ingestion are the possible acquisition routes of this disease. Being associated with high mortality rates, melioidosis has received a lot of attention in many countries. The factor that makes this disease so problematic is the difficulty in its diagnosis. Its symptoms are very similar to those for other respiratory infections that it can often be misdiagnosed. Misidentification of *B. pseudomallei* as contaminant, *B. cepacia*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Chromobacterium* sp. often happens due to limited experience of laboratory workers or lack of validated diagnostic equipment. Atypical colony morphology of some *B. pseudomallei* strains and presence of species closely related to *Burkholderia* in the specimen can further increase the complexity of identification (Howard and Inglis, 2003). Detection using conventional media-based culturing methods and biochemical tests requires a few days to obtain results, thus leading to delayed initiation of treatment and subsequent death. Some laboratories rely on rapid biochemical test

kits and automated identificationsystems but the sensitivity of these approaches is unsatisfactory. Several cases of misidentification of *B. pseudomallei* by commercially available automated systems have been reported (Zong et al., 2012). *B. mallei* and *B. pseudomallei* are classified as category B bioterrorism agents by the CDC. So far, there is no available vaccine or effective therapy for glanders and melioidosis.

A group of closely-related *Burkholderia* species are classified as *B. cepacia* complex due to their high degree of similarity in their 16S rDNA and *recA* sequences. Although the members of this complex are generally non-pathogenic to healthy individuals, they can be pathogenic to those who are immunocompromised and cystic fibrosis patients (Coenye et al., 2001). Pulmonary infection in patient of cystic fibrosis patient due to *B. cepacia* complex is high in morbidity and mortality rate (Frangolias et al., 1999). Fulminating pneumonic infection with fever and respiratory failure, sometimes associated with septicaemia, is known as "*cepacia* syndrome" (Jones et al., 2004).

A *B. pseudomallei*-like species known as *B. thailandensis* is generally considered avirulent and its infection dose is far greater than that of *B. pseudomallei* (Deshazer, 2007). However, rare cases of infection due to this species have been documented. A case of pneumonia and septicemia due to *B. thailandensis* aspirated from drainage ditch water after an accident was reported by Glass et al. (2006).

2.2.1.2 Plant Pathogens

Several *Burkholderia* species infect a variety of plant types (Compant et al., 2008). The onion rot due to the infection of onion leaves and bulbs by *B. cepacia,* was first reported by Burkholder W. H. in 1950. *B. caryophylli* can induce bacterial wilt in various plant species (Furuya et al., 2000 cited in Compant et al, 2008, p. 609). *B. gladioli* was identified as the causal agent of bacterial soft rot in onions, grain rot and leaf-sheath browning in rice, flower rot in gladiolus and leaf and corm diseases in gladiolus and iris species (Ura et al, 2006 cited in Compant et al, 2008, p. 609). *B. plantarii* and *B. glumae* induce seedling and grain rot in rice and wilting symptoms in many plant species (Compant et al., 2008). More than 52 plant species can be infected by *B. andropogonis* (Compant et al., 2008).

2.2.2 Non-pathogenic and Beneficial *Burkholderia* spp.

Even though some members of *Burkholderia* are pathogenic, most of the plant-associated *Burkholderia* spp. are non-pathogenic and could even be beneficial to their hosts.

2.2.2.1 Stimulation of Plant Growth

As rhizobacteria, some *Burkholderia* spp. interact with their host plants in ways that induce plant growth (Bevivino et al., 2000). They do this by protecting plants from soil-borne pathogens and enhancing their resistance against abiotic stress. They can also stimulate plant growth directly by either facilitating the acquisition of nutrients such as nitrogen, phosphorus or iron, producting phytohormones such as auxin or cytokinin, or reducing plant ethylene levels by the activity of

1-aminocyclopropane-1-carboxylate deaminase and expression of quinolinate phosphoribosyl transferase (Glick, 2012).

Several *Burkholderia* spp. such as *B. cenocepacia*, *B. cepacia*, *B. ambifaria*, *B. pyrrocinia*, *B. vietnamiensis* and *B. phytofirmans* protect plants from soil-borne pathogens via the synthesis of antimicrobial compounds (Kang et al., 1998). The antagonistic effect of *Burkholderia* spp. toward other bacteria, protozoa and nematodes has been reported (Meyer et al., 2000). In the study of Vandamme et al. (2007), antifungal activities against phytopathogens and plant-growth-promoting properties were reported for *B. bryophila* and *B. megapolitana* isolated from moss. Their mechanisms of action include the ability to compete with other rhizobacteria, secretion of antibiotics and siderophores and induced systemic resistance (Van Loon et al., 1998; Compant et al., 2005). Besides that, the ability of *B. phytofirmans* strain PsJN to enhance plant's tolerance against abiotic stresses such as heat and cold and transplant stress of tissue culture plantlets were also reported (Theocharis et al., 2012; Poupin et al., 2013).

2.2.2.2 Bioremediation

In a recent study, Ajao et al. (2013) reported the use of a mixed culture of crude oil degrading *B. cepacia* and *Corynebacterium* sp for the bioremediation of refinery waste water. The wide substrate specificity of *Burkholderia* spp. makes them attractive bioremediation agents. Several non-pathogenic *Burkholderia* spp. have shown the ability to degrade many xenobiotic compounds as shown in Table 2.1.

Table 2.1: Pollutants known to be degraded by *Burkholderia* spp.

***Burkholderia* strain(s)**	**Pollutants degraded**
B. vietnamiensis strain G4 (ATCC 53617)	Benzene, *o*-cresol, *m*-cresol, *p*-cresol, phenol, toluene, Trichloroethylene, naphthalene, chloroform
Burkholderia kururiensis strain KP23 (JCM 10599)	Trichloroethylene
Burkholderia xenovorans strain LB400 (LMG 21463)	Biphenyl, polychlorinated biphenyls
Burkholderia phenoliruptrix strain AC1100 (LMG 22037)	2,4,5-trichlorophenoxyacetate, 2,3,4,6-tetrachlorophenol, pentachlorophenol
Burkholderia sp. strain JS150	Trichloroethylene, benzene, phenol, toluene, chlorobenzene, naphthalene
Burkholderia sp. strains CRE-7 and RP007	Polycyclic aromatic hydrocarbons
Burkholderia sp. strains CSV90, EML 1549, K712, RASC, TFD2 and TFD6	2,4-dichlorophenoxyacetate
Burkholderia sp. strain CBS3	4-chlorobenzoate, 2-nitrobenzoate, 3-nitrobenzoate, 4-nitrobenzoate, 3-nitrochlorobenzene, 2-nitrophenol, 3-nitrophenol, 2,4,6 trinitrotoluene
Burkholderia sp. strain 8	Benzoate, 4-flurobenzoate, 4-hydroxybenzoate
Burkholderia sp. strain KZ2	2-chlorobenzoate, 4-chlorobenzoate, 2,4-dichlorobenzoate
Burkholderia sp. strain NF100	Fenitrothion

(O'sullivan and Mahenthiralingam, 2005)

2.2.2.3 Production of Rhamnolipids

Rhamnolipids are a type of biosurfactants used for a wide range of industrial applications as well as bioremediation processes due to their excellent tensioactive properties, low toxicity and high biodegradability (Rahman and Gakpe, 2008). Dubeau et al. (2009) reported that orthologs of *rhl* gene clusters responsible for biosynthesis of rhamnolipids in *P. aeruginosa* have been found in *B. thailandensis*. This discovery has made it an ideal substitute for *P. aeruginosa* in the production of biodegradable biosurfactants, since the latter can be an opportunistic pathogen in humans.

2.3 Environmental *Burkholderia* Species and Their Natural Habitats

2.3.1 Previous Studies in Different Geographical Regions

As saprophytic bacteria, *Burkholderia* spp. inhabit a wide variety of environmental waters and soils. Their strong adaptation and survival properties can be attributed to their ability to utilize a wide range of substrates, form biofilms, and survive under low-nutrient conditions and in other organisms (Inglis and Sagripanti, 2006). In soil, *Burkholderia* spp. have been previously isolated from undisturbed lands such as forests and scrublands, disturbed lands such as agricultural lands, animal pens or paddocks, residential areas especially those inhabited by melioidosis and cystic fibrosis patients (Inglis et al., 2004; Kaestli et al., 2007; Stoyanova et al., 2007; Kaestli et al., 2009; Limmathurotsakul et al., 2013). These bacteria are also found in pooled surface waters like those in rice paddy fields, rivers, lakes or ponds, groundwater seeps, roads, gutters, water supplies and boreholes or domestic water tanks (Baker et al., 2011; Vongphayloth et al., 2012).

Among the *Burkholderia* species, the distribution of *B. pseudomallei* in the environment has received the greatest attention from researchers and public health workers since it is pathogenic to both humans and animals. Many studies had been carried out to examine its prevalence and the ecological factors that influence its presence. Rice paddy fields are most commonly associated with the presence of *B. pseudomallei* in endemic areas with high rates of melioidosis among rice farmers (Vuddhakul et al., 1999). This environment is favored by *B. pseudomallei* because of its low pH, high water content of muddy, moist and clay-rich soil and pooled surface water that ease the proliferation of *B. pseudomallei* (Palasatien et al.,

2008).

2.3.2 Isolation of *Burkholderia* spp. from the Environment

Based on the studies by Kaestli et al. (2007) and Limmathurotsakul et al. (2013), soil sampling for *Burkholderia* species is recommended at the depth of 30 cm. As *B. pseudomallei* cells will become nonculturable at low temperatures, soil samples must be stored at ambient temperature and away from heat source or direct sunlight.

The isolation from soil sample basically consists of two steps, which are the extraction of bacteria from the soil sample and then detection using culture, molecular technique or other methods. Distilled water, normal saline, detergent solution or enrichment medium (Inglis et al., 2004; Trung et al., 2011; Limmathurotsakul et al., 2012) have been used for bacterial extraction. The detachment from the soil matrix can be performed by manual shaking, vortexing or use of an orbital shaker (Warner et al., 2008; Kaestli et al., 2009).

One of the popular methodologies used in many endemic areas is the protocol introduced by Wuthiekanun et al. (1995). In the protocol, the soil sample is homogenized in distilled water to dissociate microorganisms from the soil matrix and then left for sedimentation overnight before culturing on the selective Ashdown's agar. However, Trung et al. (2011) reasoned that Wuthiekanun's protocol underestimates the true number of viable organisms because the bacterial cells are not efficiently dissociated from soil particles prior to culture. Thus, a

more efficient protocol was introduced by Trung et al. (2011) by substituting distilled water with a detergent solution of polyethylene glycol (PEG) and sodium deoxycholate (DOC). Besides that, an additional centrifugation step to separate bacteria from soil particles in order to obtain a supernatant for subsequent culture was also included and thereby replaced the overnight sedimentation step in the Wuthiekanun's method.

Since there is method variability among studies and lack of a simplified and standardized one, a standard operating procedure (SOP) for isolation of *B. pseudomallei* from soil was introduced by the Detection of Environmental *Burkholderia pseudomallei* Working Party (DEBWorP) in 2012 and is currently available online for investigators to use.

The isolation of *Burkholderia* species from water is usually carried out by filtration or centrifugation (Inglis et al., 2004; Levy et al., 2008). Vongphayloth et al. (2012) used the Moore's swab method, in which a gauze swab is used as a filter to trap and concentrate microorganisms in water to detect *B. pseudomallei* in surface water in Southern Laos.

2.4 Laboratory Identification of *Burkholderia* Species

2.4.1 Phenotypic Identification

2.4.1.1 Culture

In clinical laboratory, culture-based isolation method remains as the "gold standard" for diagnosis of burkholderial infections. For ease of detection, selective-differential media such as the Ashdown's agar (ASH) and *Burkholderia*

cepacia selective agar (BCSA) are commonly used. Ashdown medium containing crystal violet and gentamicin as selective agents is effective for isolating *B. pseudomallei*. *B. pseudomallei* often produces dry wrinkled colonies, while *B. thailandensis* colonies are smooth. Another selective medium, the *Burkholderia pseudomallei* selective agar, was developed by Howard and Inglis (2003), showing the following improvements in culture isolation: increased colony size, better recovery of certain strains of *B. pseudomallei* and better selection against B. *cepacia* and *P. aeruginosa*. However, Peacock et al. (2005) had compared these two media and concluded that the Ashdown's medium is more selective than the *Burkholderia pseudomallei* selective agar.

Three culture media, which include *Pseudomonas cepacia*, OFPBL, and BCSA media, were developed for the recovery of *B. cepacia*. Among these media, BCSA has been shown to be the most superior with regard to both sensitivity and specificity (Henry et al., 1999).

For environmental surveillance, selective-enrichment broths such as the Ashdown's broth and Galimand's broth are usually used. Ashdown's broth is a nutrient broth which contains crystal violet and colistin as the selective agents. On the other hand, Galimand's broth with colistin (TBSS-50) incorporates L-threonine as the sole carbon and nitrogen source. It is recommended as the primary enrichment medium with Ashdown's broth serves as an alternative (Limmathurotsakul et al, 2013).

Burkholderia spp. can grow over a wide range of temperature from 30 ℃ to 40 ℃ and some even at 42 ℃. The incubation temperature of 40 ℃ or 42 ℃ is recommended because they allow the growth of *B. pseudomallei* and *B. thailandensis*, but are inhibitory to other soil microbes (Limmathurotsakul et al., 2013). Incubation at 37 ℃ is more suitable to be used for the isolation of *B. cepacia* complex since some of its species cannot grow at 42 ℃.

2.4.1.2 Gram Staining

Preliminary identification of *Burkholderia* species is usually via observation of colony morphology on selective-differential media and microscopic examination (White, 2003). Most *Burkholderia* species are gram-negative bacilli or coccobacili. With the exception of *B. pseudomallei*, its cells show bipolar staining, giving them a "safety pin" appearance. This phenomenon can be attributed to the accumulation of polyhydroxybutyrate in *B. pseudomallei* cells (Inglis and Sagripanti, 2006).

2.4.1.3 Biochemical Tests

Nowadays, several biochemical tests kits such as the API kits and automated microbial identification systems are commercially available for identification of bacteria based on their biochemical profiles. However, the identification of *Burkholderia* species using these means is not that reliable due to poorly defined taxonomic status of these bacteria and that they harbor a vast intra-species genomic diversity as a result of high recombination frequency (Hodgson et al., 2009; Podin et al., 2013). Several cases of misidentification of *B. pseudomallei* as *B. cepacia*, *P. fluorescens*, *P. aeruginosa*, or *C. violaceum* have been reported

(Amornchai et al., 2007).

Simple operation and minimal requirement for equipment are the major advantages of biochemical testing. However, biochemical testing can be challenging in non-endemic areas due to the lack of test resources, experience and awareness. An accurate and rapid diagnosis is necessary especially for clinical aspects since many fatal cases of burkholderial infection happen due to the lack of prompt and appropriate treatment. Despite these difficulties, biochemical tests are still used to identify *Burkholderia* species along with other methods like monoclonal antibody agglutination test or molecular technique. Colony morphology on selective-differential agar, gram stain appearance, acid production from maltose, oxidation-fermentation reactions of glucose, heat-resistant alkaline phosphatase test and antibiotic susceptibility tests are common screening tests for the identification of pathogenic *B. pseudomallei* in clinical specimens (Hodgson et al., 2009).

Other than that, arginine and lysine tests can also be used to differentiate among the species within the genus *Burkholderia* and from members of the *Enterobacteriaceae*. Among nonfermentive gram-negative rods, *B. mallei*, *B. pseudomallei* and *B. thailandensis* showed positive results in the arginine dihydrolase test. A positive lysine decarboxylase reaction of *B. cepacia* complex can be used to distinguish the species within this complex from most other nonfermenting gram-negative rods. Furthermore, the L-arabinose assimilation test is essential to differentiate between *B. thailandensis* and *B. pseudomallei*, in which

they are indistinguishable in most biochemical tests.

2.4.2 Genotypic Identification

In order to have a more rapid detection method, many efforts have been made to develop molecular techniques for burkholderial identification in clinical and environmental samples. Genotypic approaches such as 16S rRNA and *recA* gene sequence analysis and multilocus sequence typing (MLST) have been demonstrated to be useful for *Burkholderia* species identification. Other than conventional PCR assays, probe-based real-time PCR and loop-mediated isothermal amplification assays that target the *Burkholderia* type III secretion system genes (Kaestli et al., 2007; Chantratita et al., 2008; Trung et al., 2011), and multiplex PCR assays (Ho et al., 2011) for burkholderial detection have also been used. Although many molecular assays have been developed, they are still not sensitive enough to replace the conventional culture-based methods.

2.4.3 Other Methods

Immunological techniques have also been developed and used for the detection of *Burkholderia* species. Serological tests such as indirect hemagglutination, immunofluorescent, enzyme-linked immunosorbent and immunoblot assays (Ashdown et al., 1989; Vadivelu et al., 1995; Puthucheary, Anuar and Tee, 2010) can yield results quicker than culture-based methods. Besides that, there are two commercially available monoclonal antibody-based latex agglutination test systems used to distinguish between *B. pseudomallei* and *B. thailandensis*; one is specific for the lipopolysaccharide and the other is specific for the 200-kDa

exopolysaccharide (Wuthiekanun et al., 2002). However, these methods may be low in sensitivity and specificity due to seroconversion for those previously exposed to the organism (White, 2003). Thus, it may be unreliable to be used in endemic areas (Cheng and Currie, 2005).

CHAPTER 3

METHODOLOGY

3.1 Experimental Design

The overall experimental design of this project is as shown in Figure 3.1.

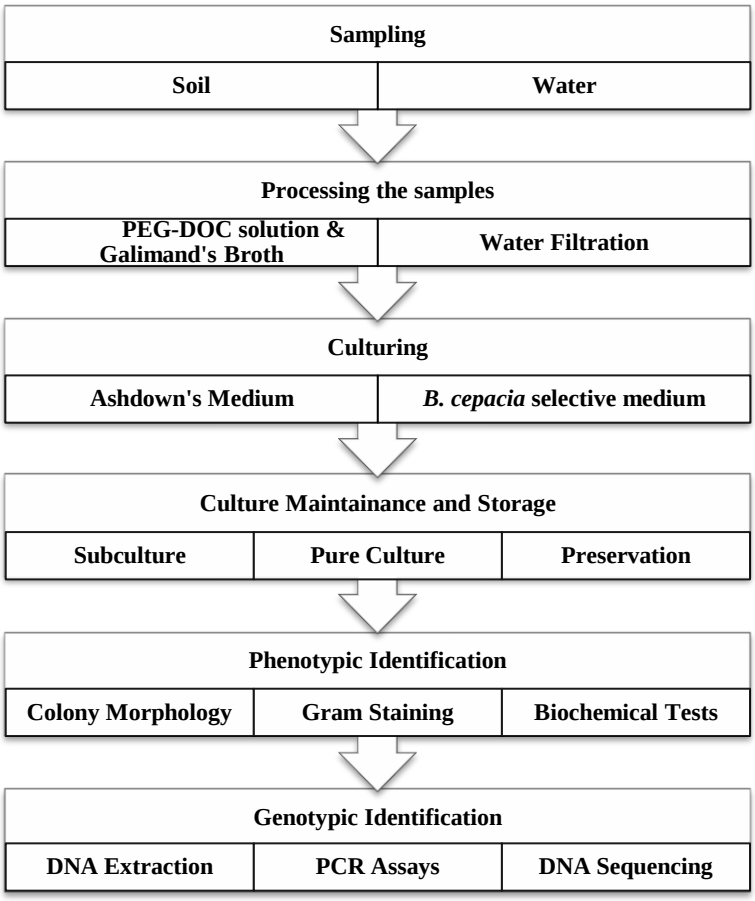


Figure 3.1: Overview of the experimental design of the project

3.2 Apparatus and Consumables

The apparatus and consumables used in this project are listed in Appendix A.

3.3 Preparation of Culture Media

All media were sterilized by autoclaving at 121℃ for 15 minutes unless otherwise stated.

3.3.1 Arginine Dihydrolase Agar

Arginine dihydrolayse agar was prepared by mixing 1 g of peptone, 5 g of NaCl, 0.3 g of K_2HPO_4, 10 g of L-arginine, 0.01 g of phenol red and 3 g of agar-agar with dH_2O to a final volume of 1 L. The pH of the mixture was adjusted to pH 6. The broth was distributed into 4 ml aliquots in test tubes and then autoclaved.

3.3.2 Ashdown's Agar

Ashdown's agar (ASH) was prepared by mixing 10 g of tryptic soy broth, 15 g of agar-agar powder, 5 ml of 0.1% w/v crystal violet, 40 ml of glycerol and 5 ml of 1% w/v neutral red with dH_2O to a final volume of 1 L. The mixture was stirred and boiled to completely dissolve the powder prior to autoclaving. After autoclaving, the agar was allowed to cool to 55℃ and then gentamicin was added to a final concentration of 4 mg/L.

3.3.3 *Burkholderia cepacia* Selective Agar

Burkholderia cepacia selective agar (BCSA) was prepared by mixing 7 g of sodium pyruvate, 5 g of peptone, 4.4 g of KH_2PO_4, 4 g of yeast extract, 1.5 g of

bile salts, 1.4 g of disodium hydrogen phosphate, 1 g of ammonium sulfate, 0.2 g of magnesium sulfate, 0.02 g of phenol red, 0.01 g of ferrous ammonium sulfate hexahydrate, 0.001 g of crystal violet and 12 g of bacteriological agar with dH_2O to a final volume of 1 L. After autoclaving, the agar was allowed to cool to 55 ℃ and then polymyxin B, gentamicin and penicillin were added to a final concentrations of 19.05 mg/L, 5 mg/L and 100 mg/L, respectively.

3.3.4 Galimand's Broth

Galimand's broth was prepared by mixing 0.451 g of KH_2PO_4, 1.73 g of K_2HPO_4, 0.123 g of magnesium sulfate heptahydrate, 0.0147 g of calcium chloride, 10 g of NaCl, 0.305 g of EDTA and 20 ml of solution A (Section 3.4.7) with dH_2O to a final volume of 1 L. The mixture was stirred and boiled to completely dissolve the powder. It was adjusted to pH 7.2 prior to autoclaving. After autoclaving, L-threonine and polymixin B were added to final concentrations of 0.05 M and 1 g/L, respectively.

3.3.5 Luria-Bertani Broth

Luria-Bertani (LB) broth was prepared by mixing 10 g of the broth powder with dH_2O to a final volume of 400 ml.

3.3.6 Lysine Decarboxylase Broth

Lysine decarboxylase broth was prepared by mixing 3 g of yeast extract, 5 g of peptone, 1 g of dextrose, 5 g of L-lysine and 0.02 g of phenol red with dH_2O to a final volume of 1 L. The pH of the mixture was adjusted to pH 6.8. The broth was distributed into 4 ml aliquots in test tubes and then autoclaved.

3.3.7 M9 Minimal Salt Agar with 0.2% w/v L-Arabinose

M9 minimal salt agar with 0.2% w/v L-arabinose was prepared by mixing 100 ml of M9 minimal salt solution and 300 ml of 2% w/v agar; both were autoclaved separately. A volume of 8 ml of 10% w/v L-arabinose solution was added to the agar before it was dispensed into plates.

3.3.8 MacConkey Agar

MacConkey agar was prepared by mixing 52 g of MacConkey powder with dH_2O to a final volume of 1 L. The mixture was stirred and boiled to completely dissolve the powder prior to autoclaving.

3.3.9 Motility Agar

Motility agar was prepared by mixing 3 g of beef extract, 10 g of tryptone, 5 g of NaCl, and 4 g of agar-agar with dH_2O to a final volume of 1 L. The mixture was stirred and boiled to completely dissolve the powder. A volume of 5 ml of 1% w/v TTC solution was then added. The agar was distributed into 5 ml

aliquots in test tubes and then autoclaved.

3.3.10 Tryptone Broth

Tryptone broth was prepared by mixing 10 g of beef extract and 5 g of NaCl with dH_2O to a final volume of 1 L. The broth was distributed into 4 ml aliquots in test tubes and then autoclaved.

3.3.11 Tryptic Soy Agar

Tryptic soy agar (TSA) was prepared by mixing 40 g of tryptic soy agar powder with dH_2O to a final volume of 1 L. The mixture was stirred and boiled to completely dissolve the powder prior to autoclaving.

3.4 Preparation of Reagents

All reagents were sterilized by autoclaving at 121℃ for 15 minutes or filter sterilized through 0.2 μm filter unit unless otherwise stated.

3.4.1 Crystal Violet, 0.1% w/v

Crystal violet solution (0.1% w/v) was prepared by dissolving 0.2 g of crystal violet powder in dH_2O to a final volume of 200 ml and then stirred overnight in a 250-ml amber reagent bottle. The solution was filter sterilized and then incubated at 37 ℃ for two weeks prior to use.

3.4.2 Glycerol Solution, 65% v/v

Glycerol solution (65% v/v) was prepared by mixing 162.5 g of glycerol, 20 ml of 1 M magnesium sulfate and 5 ml of 1 M Tris-HCl (pH 8) with dH_2O to a final volume of 200 ml.

3.4.3 L-Arabinose Solution, 10% w/v

L-arabinose solution (10% w/v) was prepared by dissolving 2.5 g of L-arabinose in dH_2O to a final volume of 200 ml. The solution was filter sterilized.

3.4.4 L-Threonine Solution

L-threonine solution was prepared by dissolving 5.95 g of L-threonine in dH_2O to a final volume of 100 ml. The solution was filter sterilized.

3.4.5 Neutral Red, 1% w/v

Neutral red solution (1% w/v) was prepared by dissolving 2 g of neutral red powder in dH_2O to a final volume of 200 ml. The solution was filter sterilized and stored at room temperature away from strong light.

3.4.6 PEG-DOC solution

PEG-DOC solution was prepared by dissolving 1 g of sodium deoxycholate and 25 g of polyethylene glycol 6000 in dH_2O to a final volume of 1 L. The

solution was filter sterilized.

3.4.7 Solution A

Solution A was prepared by dissolving 2.306 ml of 85% w/v phosphoric acid, 0.556 g of iron(II) sulfate heptahydrate, 0.297 g of zinc sulfate heptahydrate, 0.0218 g of copper(II) sulfate pentahydrate, 0.125 g of manganese(II) sulfate monohydrate, 0.03 g of cobalt(II) nitrate hexahydrate, 0.030 g of sodium molybdate and 0.062 g of boric acid in dH_2O to a final volume of 1 L and then autoclaved.

3.4.8 Tris-acetate-EDTA buffer

Tris-acetate-EDTA (TAE) buffer was prepared by dissolving 242 g of Tris base, 37.2 g of EDTA and 57.1 ml of glacial acetic acid in dH_2O to a final volume of 1 L. The buffer was diluted 50 times before use.

3.4.9 Triphenyltetrazolium Chloride Solution, 1% w/v

Triphenyltetrazolium chloride solution (TTC, 1% w/v) was prepared by dissolving 0.25 g of triphenyltetrazolium chloride in dH_2O to a final volume of 25ml. The solution was filter sterilized.

3.5 Soil and Water Sampling

Using a shovel and a soil sampling tube, approximately 30 g of soil was collected from a depth of 25-30 cm from each sampling site. A total of six sampling sites were selected for soil sampling. Within the UTAR Perak campus, soil samples were collected beside the ex-tin mining lake located behind the UTAR Library (designated S1 in this project), beside the river between Block E and Block G (S2), and from the C4 Land (S3). Besides that, soil samples were also collected from a cow farm in Taman Bandar Baru Selatan (S4) and beside and under the river located at the junction entering the Kampar New Town (S5 and S6, respectively). All soil samples were stored at room temperature and processed within 24 h.

Approximately 500 ml of water sample was collected from each sampling site into a sterile 500-ml bottle. A total of 12 sampling sites were selected for water sampling. They were categorized into eight sources which were ex-tin mining lake, river, aquarium, pond, stagnant water, sea, swimming pool and waterfall. Under the category of ex-tin mining lake, three water samples were collected from two ex-tin mining lakes within the UTAR Perak Campus, one located behind the UTAR Library (L1) and another near to Block A (L2), and a public recreational lake near the Rakan Muda Complex, Kampar Old Town, (L3). Two river samples were collected, in which one was from the river between Block E and Block G, UTAR Perak Campus (R1) and another from the river

located at the junction entering the Kampar New Town (R2). A water sample from aquarium source was collected from the aquarium in front of the UTAR Library (AQ). Pond water samples were collected from a pond located in Kampung Tersusun Batu Putih (P1) and another pond located near the cow farm (P2). Stagnant water was collected from puddles of rain water at the UTAR Block D car park after heavy rain (RAIN). Another three water samples were collected from the sea of Pulau Pangkor (SEA), a swimming pool (SW) and the Batu Berangkai Waterfall (WF). The volume of water sampled was approximately 500 ml each. All water samples were stored at 4℃ and processed within 24 h.

3.6 Primary Isolation of *Burkholderia* spp. from Environmental Samples

3.6.1 Soil Samples

In a sterile 250-ml bottle, 25 g of soil was mixed with 50 ml of PEG-DOC solution and the mixture was then shaken vigorously on an orbital shaker at 200 rpm for 2 h. After leaving the suspension to stand for 5 min, the upper layer of PEG-DOC solution was transferred into a 50-ml Falcon tube and then centrifuged at 1400 x *g* for 10 min. A volume of 1 ml of supernatant was inoculated into 9 ml of Galimand's broth. The broth was incubated at 42 ℃ for 4 days. Following incubation, the enriched culture was centrifuged at 2000 x *g* for 15 min and the resulting pellet resuspended in 500 µl of Galimand's broth. A volume of the 100 µl of the resulting suspension was spread onto ASH and

BCSA each in duplicates. The agar plates were incubated at 42 ℃ and 37 ℃, respectively, and inspected daily for up to 4 days.

3.6.2 Water Samples

A volume of 100 ml of water sample was filtered through 0.2 μm cellulose nitrate membrane. The membrane was then placed onto ASH and BCSA each in duplicates. ASH plates were incubated at 42 ℃ and BCSA plates were incubated at 37 ℃ for up to 1 week.

3.7 Subculture

The colonies in the primary isolation were subcultured onto ASH and BCSA, respectively. For water samples, colonies that grew on the filter membranes were picked and streaked onto ASH and BCSA. All agar plates were incubated as before. The colony morphology of the isolates was recorded. Each isolate on ASH and BCSA was also subcultured onto TSA and the morphology of its resulting colonies was recorded.

3.8 Gram Staining

Bacterial smear was prepared by emulsifying a colony in a drop of distilled water on a slide. Subsequently, the slide was heat-fixed. Then, the smear was flooded with crystal violet for 60 s. The slide was rinsed with tap water. Next, the smear was flooded with Gram's iodine for 60 s and rinsed as before. After that,

the slide was decolorized with alcohol for not more than 10 s. Following rinsing, the smear was stained with the counterstain safranin for 30 s. The slide was then rinsed and blotted dry. The stained smear was examined under oil immersion at 1000 x magnification.

3.9 Biochemical Tests

The following biochemical tests were performed on pure and fresh (18-24 h) cultures of isolates. Positive and negative controls were performed for each test.

3.9.1 Catalase Test

A small amount of bacterial colonies was placed on a slide. A drop of 3% w/v hydrogen peroxide was added onto the colonies. The formation of bubbles would indicate a positive result.

3.9.2 Oxidase Test

Six drops of each reagents A and B were applied to colonies on a TSA plate. For an oxidase-positive reaction, a change in color to blue would be observed within 20 min.

3.9.3 Motility Test

A well-isolated colony was picked and stabbed into the motility test medium. The tube was incubated at 37℃ until growth became evident. A positive reaction would be indicated by a red turbid area extending away from the line of inoculation. A negative reaction would be indicated by red growth along the stab line but no further.

3.9.4 L-Arabinose Assimilation Test

A loopful of isolate was streaked onto the M9 minimal salt agar with 0.2% w/v L-arabinose. The plate was incubated at 37℃ until bacterial growth became evident, which would indicate a positive result.

3.9.5 Lactose Fermentation Test

A loopful of isolate was streaked onto the MacConkey agar. The plate was incubated at 37℃. The growth of pink colonies would indicate a positive reaction while the growth of colorless colonies would indicate a negative reaction.

3.9.6 Indole Test

A well-isolated colony was inoculated into tryptone broth. The tube was incubated at 37℃ for 24 h. Five drops of Kovacs reagent were then added to the tube. The formation of a red ring on the surface of the medium would

indicate a positive result.

3.9.7 Arginine Dihydrolase Test

A well-isolated colony was stabbed into the arginine dihydrolase agar. The agar was covered with about 1 cm depth of mineral oil. The tube was incubated at 37℃ for 48 h. A color change from orange to yellow and then to light orange-pink would show a positive reaction. Failure to turn yellow or light orange-pink would indicate a negative reaction.

3.9.8 Lysine Decarboxylase Test

A loopful of isolate was inoculated into the lysine decarboxylase broth. The broth was covered with about 1 cm depth of mineral oil. The tube was incubated at 37℃ for 48 h. The interpretation for this test is similar to that for the arginine dihydrolase test (Section 3.9.7).

3.10 Genotypic Identification

3.10.1 DNA Extraction

A loopful of colonies from an isolate was suspended in 1 ml of autoclaved deionized water. DNA extraction was performed using the Geneaid DNA isolation Kit according to the manufacturer's instructions for gram-negative bacteria. The extracted DNA was assessed for both purity (A_{260}/A_{280}) and concentration using the nanospectrophotometer. Pure DNA should have

A_{260}/A_{280} ranging from 1.8 to 2.0. The extracted DNA was used as template in the PCR assays.

3.10.2 Universal 16S rDNA PCR Assay

Universal 16S rDNA PCR was carried out for the isolates in 25-µl reaction consisting of 5 µl of template DNA, 1x DreamTaq Green Buffer, 250 nM of each forward and reverse primers, 200 µM dNTPs, and 0.05 U DreamTaq DNA polymerase. The sequences for the forward and reverse primers were 5'-ACTCCTACGGGNGGCNGCA-3' and 5'-GTATTACCGCNNCTGCTGGCAC-3', respectively. PCR was performed in the thermocycler, beginning with 95˚C for 3 min for *Taq* polymerase activation, followed by 40 cycles of denaturation at 95˚C for 30 s, annealing at 60˚C for 60 s, and extension at 72˚C for 60 s. A no-template control (NTC) was included and *E. coli* DNA was used as the positive control in every PCR run. This PCR assay was designed and developed by K. Haldan (unpublished).

3.10.3 *Burkholderia*-specific 16S rDNA PCR Assay

Burkholderia-specific 16S rDNA PCR was carried out for the isolates in 25-µl reaction consisting of 5 µl of template DNA, 1x DreamTaq Green Buffer, 250 nM of each forward and reverse primers, 400 µM of dNTPs and 0.05 U DreamTaq DNA polymerase. The sequences for the forward and reverse primers were 5'-AGACACGGCCCAGACTCCTAC-3' and

5’-CAGTCACCAATGCAGT TCCCA-3’, respectively. PCR was performed in the thermocycler, beginning with 95˚C for 3 min for *Taq* polymerase activation, followed by 25 cycles of denaturation at 95˚C for 15 s, annealing at 62˚C for 30 s and extension at 72˚C for 30 s. A no-template control (NTC) was included in every PCR run. This PCR assay was designed and developed by Brett, Deshazer and Woods (1997).

3.10.4 Gel Analysis of PCR Amplicons

Universal 16S PCR amplicons were separated by electrophoresis on 2% w/v agarose gels; while those for the *Burkholderia*-specific 16S PCR were electrophoresed on 1.2% w/v agarose gels. A volume of 20 µl of each PCR reaction was analyzed on the gel along with 5 µl of GeneRuler 100-bp DNA ladder. Electrophoresis was performed at 80 V for 30 min. After that, the gel was stained with 0.5 g/ml ethidium bromide (EtBr) for 5-10 min and destained with dH_2O for 2 min. The EtBr-stained amplicons were visualized under an ultraviolet transilluminator.

3.10.5 Gel Purification of PCR Amplicons

Universal 16S PCR amplicons of the expected size of 199 bp and *Burkholderia*-specific 16S PCR amplicons of the expected size of 320 bp were excised from the gels and purified using the Invisorb Spin DNA Extraction Kit or Xprep Gel Purification Kit according to the manufacturers’ instructions. The

concentration of the purified PCR amplicons was assessed as before (Section 3.10.1) and it should be more than 10 ng/µl in order to be sent for DNA sequencing.

3.10.6 DNA Sequencing and Analyses

Purified PCR amplicons were sent to Medigene Sdn. Bhd. (Selangor, Malaysia) for sequencing. Analyses of the resulting DNA sequences were performed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website. The identity of each isolate was identified based on the sequence identity with the type strains and the expect value (E-value).

3.11 Long-term Storage of Isolates

Two loopfuls of colonies for each isolate were suspended in 0.5 ml of LB broth. The suspension was then mixed with 0.5 ml of 65% v/v glycerol solution and then stored at -80 ℃.

CHAPTER 4

RESULTS

4.1 Physical Appearance of Soil and Water Samples

Generally, the soil samples were visually and physically varied in their moisture, color and texture. In term of moisture, all soil samples were wet except for the one collected from S1. The soils collected from S5 and S6 were reddish in color, whereas those collected from other sites were brown in color. The soil from S4 was darker brown in color when compared to those from S1, S2 and S3. Apart from the soil from S2, all soil samples were sandy loam, in which the soil from S1 was the most sandy. The soil sample from S2 was sandy clay loam.

Visually, water samples were varied in their turbidity and the presence of sediments. Ranging in term of turbidity, the swimming pool water (SW) was the least turbid, followed by WF, AQ, R2, L1, L2, R1, L3, SEA, RAIN, P1 and P2. P2 appeared slightly brownish in color and it filtered with the slowest rate. Sediments such as soil and rock particles were observed in the water samples from RAIN, P1 and P2. Algae were present in the water samples from L1, L2 and R1. To minimize blockage during filtration, the algae were prior removed by filtration through a sterile 12-μm filter paper.

4.2 Primary Bacterial Isolation from Soil and Water Samples

The soil samples were firstly treated with PEG-DOC solution to separate the bacteria from the soil matrix and then inoculated into the Galimand's broth. All the inoculated broths became turbid after four days of incubation. The colonies observed on the spread plates (Figure 4.1) were subcultured on ASH and BCSA, respectively.

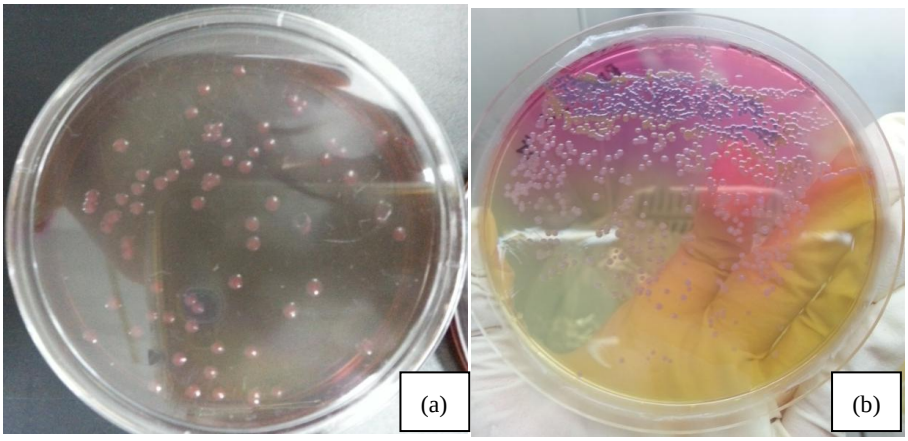


Figure 4.1: Spread plate cultures from Galimand's broth enrichments. (a) Soil sample S4; (b) Soil sample S3.

For water samples, they were filtered through a 0.2-μm membrane filter each, which was then placed onto a selective medium. After 48 hours of incubation, colonies were readily observed on the membrane filter (Figure 4.2a). Colonies of different morphology were selected and subcultured onto ASH and BCSA, respectively. Besides the presence of bacterial colonies, white and green molds were also observed for some samples after prolonged incubation (Figure 4.2b). No colony was observed on the membrane filters for the swimming pool sample.

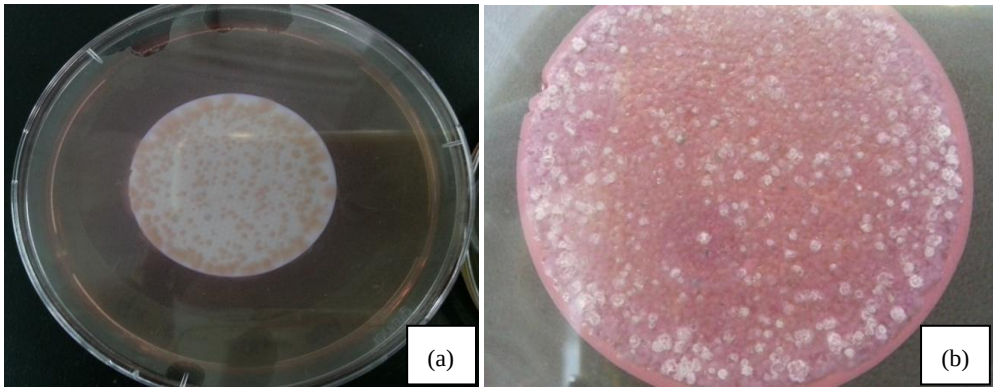


Figure 4.2: Appearance of cultures of water samples on the membrane filters. (a) The growth of colonies on the membrane filter placed on BCSA after 24 hours. (b) The presence of white molds on the membrane filter placed on ASH after 72 hours.

4.3 Culture Characteristics of Bacterial Isolates

4.3.1 Colony Morphology on Selective Media

A total of 21 and 14 isolates were obtained on ASH and BCSA, respectively (Tables 4.1 and 4.2, respectively). The colony morphology of the isolates was varied. The size, pigmentation, shape, elevation and edge for each type of colonies were observed and recorded in Tables 4.1 and 4.2. The colonies for most of the isolates were circular, convex and entire edged in appearances. None of the isolates yielded the typical wrinkled colonies of *B. pseudomallei* on ASH. The growth of colonies on BCSA turned the color of the medium to pink.

Unique colony morphology was shown by some of the isolates on the selective media used. The colonies for isolate ASH12 appeared black in color (Figure

4.3); those for ASH5 (Figure 4.3) and ASH8 were big and mucoid; those for BCSA14 was multicolor; and those for BCSA5 showed greenish yellow pigmentation. Except for ASH8, all isolates were subjected to identification via the 16S rDNA sequencing.

Table 4.1: Observation of colony morphology of bacterial isolates on ASH

Isolate	Sample Source	Observation on ASH				
		Color	**Size**	**Shape**	**Elevation**	**Edge &Texture**
ASH 1	L1	Red	Medium	Circular	Convex	Undulate
ASH 2	L2	Dark Purple	Big	Circular	Raised	Smooth
ASH 3	L2	Purple	Small	Circular	Convex	Entire
ASH 4	R1	Purple	Medium	Circular	Convex	Entire
ASH 5	R2	Purple	Big	Circular	Convex	Entire, Mucoid
ASH 6	R2	Purple	Medium	Irregular	Umbonate	Undulate, Dry
ASH 7	AQ	Dark Purple	Big	Irregular	Flat	Undulate
ASH 8	RAIN	Purple	Big	Circular	Convex	Entire, Mucoid
ASH 9	RAIN	Grayish	Small	Circular	Convex	Entire
ASH 10	RAIN	Purple	Medium	Circular	Convex	Entire
ASH 11	RAIN	Pink	Small	Circular	Convex	Entire
ASH 12	P1	Black	Medium	Irregular	Umbonate	Undulate, Rough
ASH 13	P1	Pink	Small	Circular	Convex	Entire
ASH 14	P2	Purple	Small	Circular	Convex	Entire
ASH 15	P2	Red	Small	Pinpoint		
ASH 16	SEA	Purple	Medium	Circular	Convex	Entire
ASH 17	SEA	Red	Medium	Circular	Flat	Entire
ASH 18	WF	Dark Purple	Small	Circular	Slightly Raised	Entire
ASH 19	WF	Dark Purple	Small	Circular	Slightly Raised	Entire
ASH 20	S1-4	Purple	Small	Circular	Convex	Entire
ASH 21	S5-6	Reddish Purple	Medium	Circular	Convex	Entire

Size: 1 mm or below is classified as small; 2-4 mm is classified as medium; 5 mm and above is classified as large.

Table 4.2: Observation of colony morphology of bacterial isolates on BCSA

Isolate	Sample Source	Observation on BCSA				
		Color	**Size**	**Shape**	**Elevation**	**Edge &Texture**
BCSA 1	L1	Grayish	Medium	Circular	Convex	Entire
BCSA 2	R1	Grayish	Medium	Circular	Convex	Entire
BCSA 3	R1	Grayish Purple	Medium	Circular	Convex	Concentric
BCSA 4	R2	Pink	Big	Circular	Convex	Entire
BCSA 5	R2	Greenish yellow	Medium	Circular	Convex	Entire
BCSA 6	AQ	Translucent Grey	Medium	Circular	Convex	Entire
BCSA 7	RAIN	Pink	Medium	Circular	Convex (48h Pulvinate)	Entire
BCSA 8	P2	Translucent Grey	Medium	Circular	Convex	Entire
BCSA 9	SEA	Translucent Grey	Medium	Circular	Slightly Convex	Undulate
BCSA 10	WF	Grayish	Small	Circular	Flat	Entire
BCSA 11	WF	Grayish	Medium	Circular	Convex	Entire
BCSA 12	WF	Pink	Medium	Circular	Convex	Entire
BCSA 13	WF	Pink	Small	Circular	Convex	Entire
BCSA 14	S1-4	Multicolor	Small	Circular	Convex	Entire

Size: 1 mm or below is classified as small; 2-4 mm is classified as medium; 5 mm and above is classified as large.

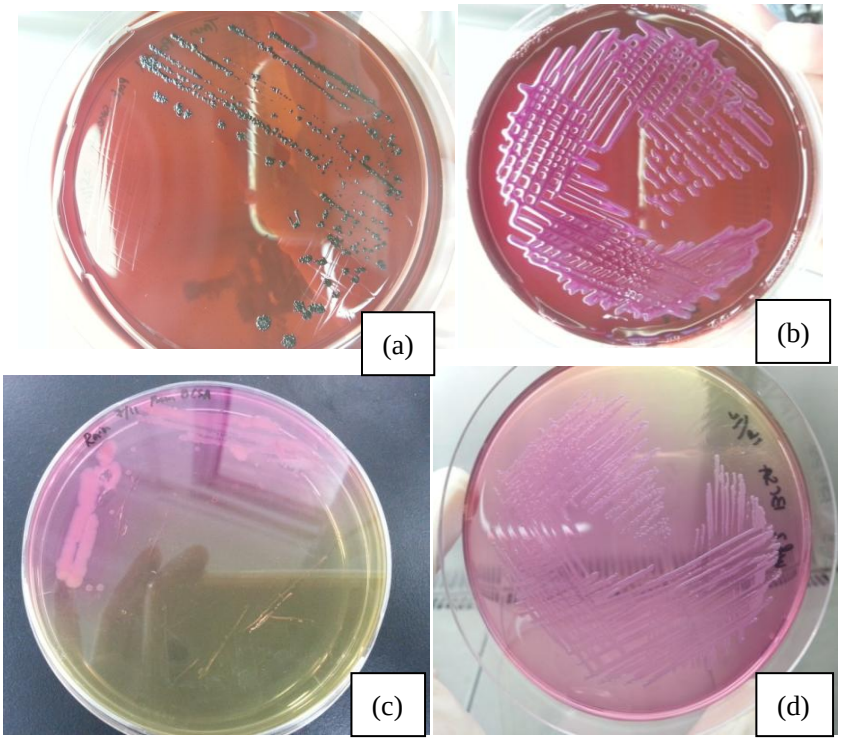


Figure 4.3: Varied colony morphology of bacterial isolates on ASH and BCSA. (a) ASH12; (b) ASH5; (c) BCSA7; (d) BCSA11.

4.3.2 Colony Morphology on TSA

All isolates obtained on ASH and BCSA, respectively, were subcultured onto TSA. The morphology of the resulting colonies was observed and recorded as before (Appendix B). Most of the isolates yielded colonies that were translucent white or translucent yellow, medium in size, circular, convex and entire edged. Unique colony morphology was again shown by some of the isolates on TSA. Similar colony morphology to that on the selective media was observed for ASH12, BCSA14 and BCSA5 (Figure 4.4). In addition, the colonies for BCSA10 were wrinkled and dry (Figure 4.4d) and those for BCSA11 showed pink pigmentation (data not shown).

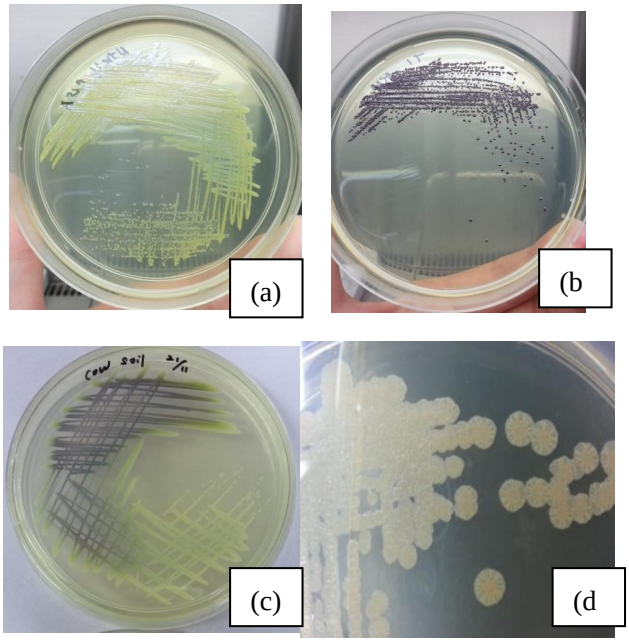


Figure 4.4: Varied colony morphology of bacterial isolates on TSA. (a) BCSA5; (b) ASH12; (c) BCSA14; (d) BCSA10.

4.4 Gram Staining of Bacterial Isolates

All isolates were confirmed to be gram-negative. A total of 24 out of 35 isolates were bacillus in shape and the remaining were coccobacillus in shape when viewed under the microscope (Figure 4.5).

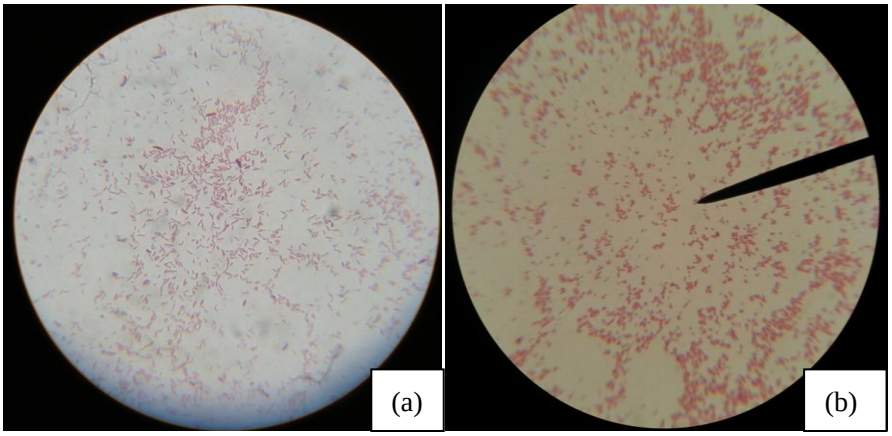


Figure 4.5: Gram-stained bacterial isolates observed under oil immersion at 1000x magnification. (a) Bacilli; (b) Coccobacilli.

4.5 Biochemical Tests on Bacterial Isolates

Apart from characterization based on colony morphology and gram staining appearance, biochemical testing on the bacterial isolates was also performed. Table 4.3 shows the biochemical profiles of the isolates. Most of the figures shown were from the tests performed on positive and negative controls.

Table 4.3: Biochemical profiles of the bacterial isolates

Isolate	Source	Catalase	Oxidase	Motility	Arabinose Assimilation	Lactose Fermentation	Indole Production	Arginine Dihydrolase	Lysine Decarboxylase
ASH 1	L1	+	+	+	-	-	-	+	+
ASH 2	L2	+	-	+	+	+	+	+	+
ASH 3	L2	+	+	+	-	-	-	+	-
ASH 4	R1	+	-	+	+	+	+	+	+
ASH 5	R2	+	-	-	+	+	+	-	+
ASH 6	R2	+	+	-	+	+	-	-	-
ASH 7	AQ	+	-	-	+	+	-	+	+
ASH 8	RAIN	+	-	+	+	+	-	+	-
ASH 9	RAIN	+	+	+	-	-	-	-	+
ASH 10	RAIN	+	+	+	-	-	-	-	+
ASH 11	RAIN	+	-	+	+	+	+	-	+
ASH 12	P1	+		+	-		-	+	-
ASH 13	P1	+	+	-	-	-	-	+	+
ASH 14	P2	+	-	-	-	-	-	-	-
ASH 15	P2	+	+	-	+	-	-	-	-
ASH 16	SEA	+	-	-	+	+	-	-	+
ASH 17	SEA	+	+	+	-	-	-	+	-
ASH 18	WF	+	-	+	+	+	+	-	-
ASH 19	WF	+	-	-	+	-	+	-	-
ASH 20	S1-4	+	+	-	+	-	-	-	+
ASH 21	S5-6	+	-	-	+	-	-	-	+

Table 4.3 (Continue……)

Isolate	Sample	Catalase	Oxidase	Motility	Arabinose Assimilation	Lactose Fermentation	Indole Production	Arginine Dihydrolase	Lysine Decarboxylation
BCSA 1	L1	+	-	+	+	-	+	+	+
BCSA 2	R1	+	-	-	+	+	+	-	+
BCSA 3	R1	+	+	+	-	-	-	+	-
BCSA 4	R2	+	-	-	+	+	-	-	+
BCSA 5	R2	+	-	-	+	-	-	-	+
BCSA 6	AQ	+	+	+	-	-	-	+	-
BCSA 7	RAIN	+	-	+	+	+	+	-	+
BCSA 8	P2	+	+	+	-	+	+	+	+
BCSA 9	SEA	+	+	+	-	-	-	+	-
BCSA 10	WF	+	+	+	-	-	-	+	-
BCSA 11	WF	+	-	+	-	+	-	-	-
BCSA 12	WF	+	-	+	-	-	-	-	-
BCSA 13	WF	+	-	+	+	-	-	-	-
BCSA 14	S1-4	+	+	-	+	-	-	-	+

"+" sign indicates positive reaction

"-" sign indicates negative reaction

4.5.1 Catalase Test

Upon the addition of the H_2O_2 reagent, formation of bubbles indicates positive result (Figure 4.6). All isolates were positive in this test (Table 4.3).

Figure 4.6: Screening for the presence of catalase. *E. coli* (left) and *Enterococcus faecalis* (right) were used as positive and negative controls, respectively.

4.5.2 Oxidase Test

Upon the addition of reagents A and B, the development of blue color in the bacterial colonies within two minutes indicates a positive reaction (Figure 4.7). Nine out of 21 isolates and six out of 14 isolates from ASH and BCSA, respectively, developed positive results in this test (Table 4.3). However, the test result for isolate ASH12 could not be interpreted due to the purple pigmentation of its colonies.

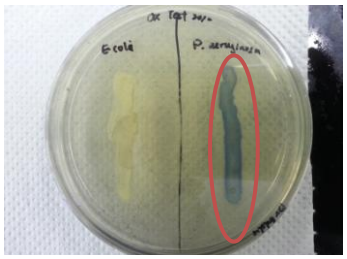
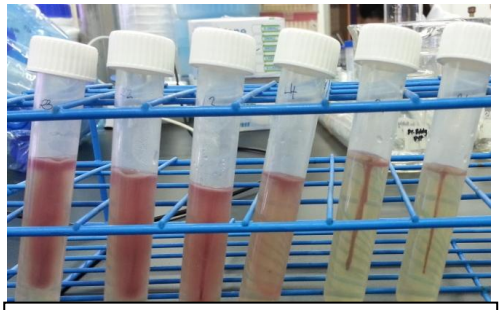


Figure 4.7: Screening for the presence of oxidase. *P. aeruginosa* (right) and *E. coli* (left) were used as positive and negative controls, respectively.

4.5.3 Motility Test

A diffuse growth from the line of inoculation or red turbid area indicates a positive reaction for motility (Figure 4.8). A negative test is indicated by red growth along the inoculation line but no further. Eleven out of 21 isolates and 10 out of 14 isolates from ASH and BCSA, respectively, showed diffuse growth away from the stabling line (Table 4.3).



(a) (b) (c) (d) (e) (f)

Figure 4.8: Screening for motility among the bacterial isolates. (a) ASH1; (b) ASH4; (c) BCSA1; (d) BCSA3; (e) ASH7; (f) BCSA4.

4.5.4 L-Arabinose Assimilation Test

To screen for the ability to utilize L-arabinose for growth, the bacterial isolates were streaked for growth on minimal salt agar supplemented with 0.2% w/v L-arabinose. Thirteen isolates and seven isolates from ASH and BCSA, respectively, grew on the test media, indicating that they were able to utilize L-arabinose (Table 4.3; Figure 4.9).

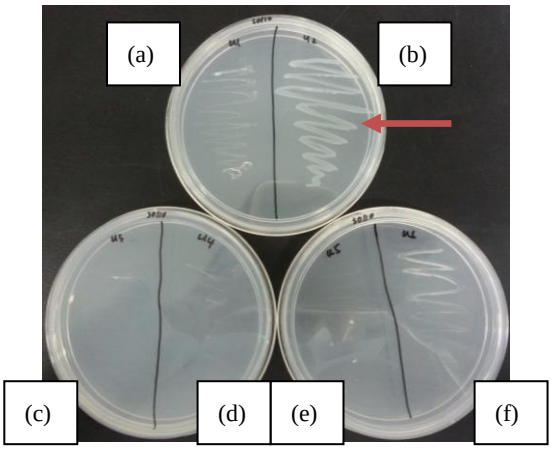


Figure 4.9: Screening for L-arabinose assimilation. (a) ASH4; (b) ASH2; (c) ASH3; (d) ASH1; (e) BCSA3; (f) BCSA2.

4.5.5 Lactose Fermentation Test

Since all the bacterial isolates were gram-negative, they were able to grow on the MacConkey agar. Nine isolates and five isolates from ASH and BCSA, respectively, were lactose fermenters (Table 4.3). Their colonies appeared pink in color or were surrounded by a pink halo each (Figure 4.10). Non-lactose fermenters yielded colorless colonies and the agar remained relatively translucent (Figure 4.10).

Figure 4.10: Screening for lactose fermentation on MacConkey agar. *E. coli* (left) and *P. aeruginosa* (right) were used as positive and negative controls, respectively.

4.5.6 Indole Test

Indole test was performed to screen for indole production. After the addition of the Kovac's reagent, the formation of red layer on the surface of the medium indicates a positive reaction (Figure 4.11). Six isolates and four isolates from ASH and BCSA, respectively, were indole-positive (Table 4.3).

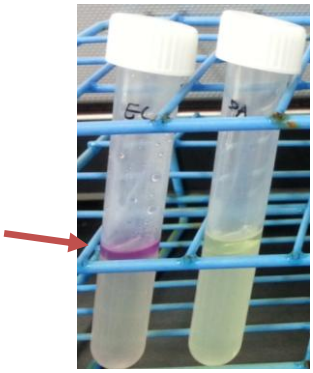


Figure 4.11: Screening for indole production. *E. coli* (left) and *P. aeruginosa* (right) were used as positive and negative controls, respectively.

4.5.7 Arginine Dihydrolase Test

For a positive arginine dihydrolase reaction, the color of the inoculated medium will change from orange to yellow then to pink color (Figure 4.12). Negative reaction is shown by no change in the color of the medium. In this test, nine isolates and six isolates from ASH and BCSA, respectively, yielded positive results, indicating that they were able to hydrolyze arginine in the medium (Table 4.3).

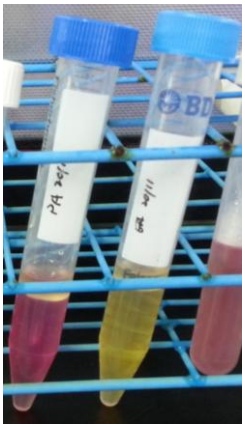


Figure 4.12: Screening for arginine dihydrolase activity. *P. aeruginosa* (left) and *E. aerogenes* (right) were used as positive and negative controls, respectively.

4.5.8 Lysine Decarboxylase Test

For a positive lysine decarboxylase reaction, the color of the inoculated medium will change from orange to yellow then to red color (Figure 4.13). Negative reaction is shown by no change in the color of the medium. Twelve isolates and 17 isolates from ASH and BCSA, respectively, gave positive results, indicating that they were able to hydrolyze arginine in the medium (Table 4.3).

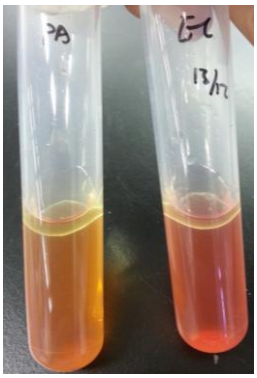


Figure 4.13: Screening for lysine decarboxylase activity. *P. aeruginosa* (left) and *E. coli* (right) were used as positive and negative controls, respectively.

4.6 Genotypic Identification of Bacterial Isolates

4.6.1 Universal 16S rDNA PCR Assay and DNA Sequencing

Due to limited resourses and time constraint, only 17 out of 35 isolates were subjected to genotypic identification. Since all these isolates were gram-negative, genomic DNA was extracted according to the protocol for gram-negative bacteria using the Geneaid DNA Isolation Kit. The extracted DNA samples were used as templates for PCR amplification of the 16S rDNA. Gel electrophoresis of the amplicons on 2% agarose gel following PCR showed bands of the expected size of 199 bp in great intensity and brightness (Figure 4.14).

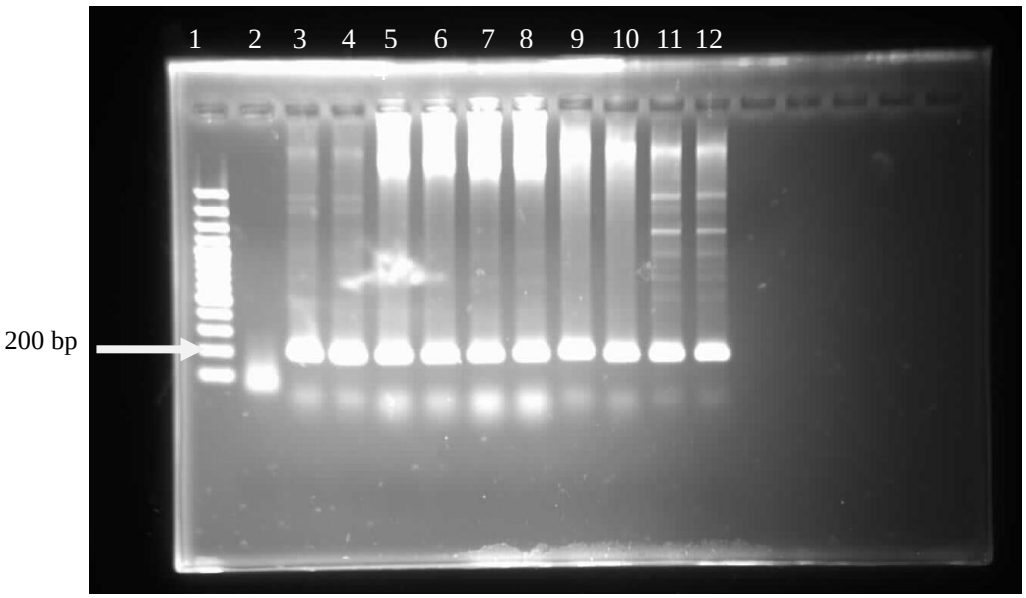


Figure 4.14: Gel analysis of 16S amplicons from the universal 16S rDNA assay.
Lane 1, GeneRuler 100-bp DNA ladder; lane 2, NTC; lanes 3 and 4, ASH5; lanes 5 and 6, BCSA14; lanes 7 and 8, BCSA5; lanes 9 and 10, BCSA11; lanes 11 and 12, *E. coli* (positive control).

The 16S amplicons were then excised from the gels and subjected to purification using commercial gel purification kits. Nine purified samples showed A_{260}/A_{280} values of 1.8-2.0 while the other three showed A_{260}/A_{280} values of slightly higher than 2.0 and five showed A_{260}/A_{280} values below 1.8. All samples yielded concentrations of more than 7 ng/µl.

Following DNA sequencing of the purified samples, the BLASTn result with the lowest E-value and the highest percentage of similarity for each isolate was selected (Table 4.4). Based on this, isolates ASH9, ASH10, BCSA5 and BCSA14 were identified to be those of *Burkholderia* spp.

Table 4.4: BLASTn analysis results of 16S rDNA amplicons for the bacterial isolates

Isolate	BLASTn identity	Score (bits)	Query cover (%)	E-value	Identity (%)	Accession
ASH1	*Comamonas terrigena* 16S rRNA gene	252	99	$7e^{-64}$	97	KC920989.1
ASH3	*Pseudomonas aeruginosa* 16S rRNA gene	196	98	$3e^{-47}$	92	DQ103761.1
ASH4	*Escherichia coli* 16S rRNA gene	183	93	$2e^{-43}$	97	KF500595.1
ASH5	*Escherichia coli* 16S rRNA gene	244	99	$1e^{-61}$	99	JF919882.1
	Enterobacter hormaechei 16S rRNA gene	244	99	$1e^{-61}$	99	GU419684.1
ASH8	*Cronobacter sakazakii* 16S rRNA gene	244	96	$1e^{-61}$	98	CP006731.1
	Cronobacter malonaticus 16S rRNA gene	244	96	$1e^{-61}$	98	KC818226.1
	Enterobacter cloacae 16S rRNA gene	244	96	$1e^{-61}$	98	KF498698.1
	Enterobacter asburiae 16S rRNA gene	244	96	$1e^{-61}$	98	KC434995.1
	Enterobacter amnigenus 16S rRNA gene	244	96	$1e^{-61}$	98	JN969309.1
ASH9	*Burkholderia sp*. 16S rRNA gene	268	99	$7e^{-69}$	98	Q912592.1
	Comamonas aquatica 16S rRNA gene	268	99	$7e^{-69}$	98	HQ893540.1
	Comamonas testosteroni 16S rRNA gene	268	99	$7e^{-69}$	98	EU072478.1
ASH10	*Burkholderia* sp. 16S rRNA gene	261	100	$1e^{-66}$	96	JQ912592.1
	Comamonas aquatica 16S rRNA gene	261	100	$1e^{-66}$	96	HQ893540.1

Table 4.4 (continued……)

Isolate	BLASTn identity	Score (bits)	Query cover (%)	E-value	Identity (%)	Accession
ASH12	*Chromobacterium violaceum* 16S rRNA gene	268	99	$7e^{-69}$	99	KF921009.1
	Iodobacter fluviatilis 16S rRNA gene	268	99	$7e^{-69}$	99	FJ753568.1
	Chromobacterium pseudoviolaceum 16S rRNA gene	268	99	$7e^{-69}$	99	AJ871128.2
	Chromobacterium subtsugae 16S rRNA gene	268	99	$7e^{-69}$	99	NR_042853.1
ASH17	*Pseudomonas aeruginosa* 16S rRNA gene	209	85	$5e^{-51}$	95	HM590705.1
BCSA2	*Klebsiella oxytoca* 16S rRNA gene	141	95	$9e^{-31}$	96	GU459206.1
BCSA3	*Pseudomonas aeruginosa* 16S rRNA gene	274	98	$2e^{-70}$	99	JX514422.1
BCSA5	*Burkholderia cepacia* 16S rRNA gene	255	99	$6e^{-65}$	98	KF974366.1
	Burkholderia stabilis 16S rRNA gene	255	99	$6e^{-65}$	98	KF836499.1
BCSA6	*Chromobacterium haemolyticum* 16S rRNA gene	239	99	$5e^{-60}$	99	JX500196.1
	Chromobacterium violaceum 16S rRNA gene	239	99	$5e^{-60}$	99	JQ312052.1
BCSA9	*Pseudomonas aeruginosa* 16S rRNA gene	117	79	$1e^{-23}$	100	KF164616.1
BCSA10	*Chromobacterium haemolyticum* 16S rRNA gene	206	99	$5e^{-50}$	97	JX500196.1
	Chromobacterium violaceum 16S rRNA gene	206	99	$5e^{-50}$	97	AB851804.1
BCSA11	*Serratia rubidaea* 16S rRNA gene	263	99	$3e^{-67}$	99	AB435617.1
BCSA14	*Burkholderia metallica* 16S rRNA gene	235	98	$7e^{-59}$	98	KF150376.1
	Burkholderia cepacia 16S rRNA gene	235	98	$7e^{-59}$	98	KF812860.1
	Burkholderia cenocepacia 16S rRNA gene	235	98	$7e^{-59}$	98	AM747720.1

4.6.2 *Burkholderia*-specific 16S rDNA PCR Assay and DNA Sequencing

The main advantage of performing this genus-specific PCR assay was that the presence of *Burkholderia* spp. could be determined directly from the gel analysis results. Once again, due to limited resources and time constraint, only nine isolates were selected for this genotypic identification, based on the percentage similarity of their phenotypic test results to those reported for *Burkholderia* spp. in the literature. Gel electrophoresis of the resulting 16S amplicons on 1.2% agarose gel showed bands of the expected size of 320 bp in great intensity and brightness (Figure 4.15). Besides that, four oxidase-negative isolates (ASH8, ASH12, ASH4 and BCSA2) were also tested. Unexpectedly, two of them were amplified (Figure 4.15). All 320-bp amplicons were purified, including those of the two oxidase-negative isolates. Nine purified samples showed A_{260}/A_{280} values of 1.8-2.0 while the other two showed A_{260}/A_{280} values below 1.8. All samples yielded concentrations of more than 7 ng/µl except for one that yielded concentration of less than 7 ng/µl. A total of seven gel-purified samples were sent for DNA sequencing, with the sample of low concentration (<7 ng/µl) and those previously sent for sequencing under universal 16S rDNA PCR excluded. The BLASTn results were analyzed as before. Only one (ASH10) out of the seven isolates was identified to be that of *Burkholderia* sp., while the other six were of the genera *Pseudomonas*, *Chromobacterium*, *Klebsiella* and *Escherichia*.

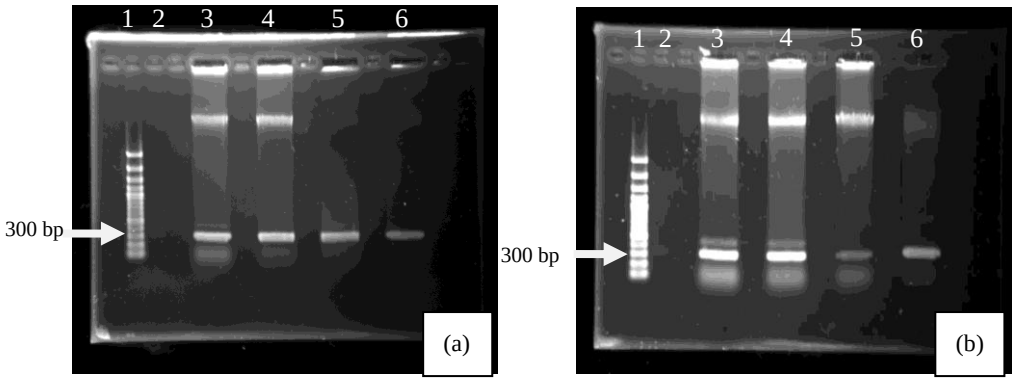


Figure 4.15: Gel analysis of 16S amplicons from the *Burkholderia*-specific 16S rDNA assay.

(a) Lane 1, GeneRuler 100-bp DNA ladder; lane 2, NTC; lane 3, ASH9; lane 4, ASH10; lane 5, ASH1; lane 6, ASH4. (b) Lane 1, GeneRuler 100-bp DNA ladder; lane 2, NTC; lane 3, BCSA10; lane 4, BCSA6; lane 5, BCSA3; lane 6, BCSA2.

To access the specificity of the primers used, the PCR assay was repeated with DNA extracts of non-burkholderial laboratory strains (*Bacillus cereus, Bacillus subtilis, E. aerogenes, E. coli, Klebsiella pneumoniae, P. aeruginosa Salmonella typhimurium* and *Staphylococcus aureus*). Unexpectedly, 320-bp bands were observed for five of these bacterial species, which are *E. aerogenes*, *E. coli*, *K. pneumoniae*, *S. typhimurium* and *P. aeruginosa* (Figure 4.16).

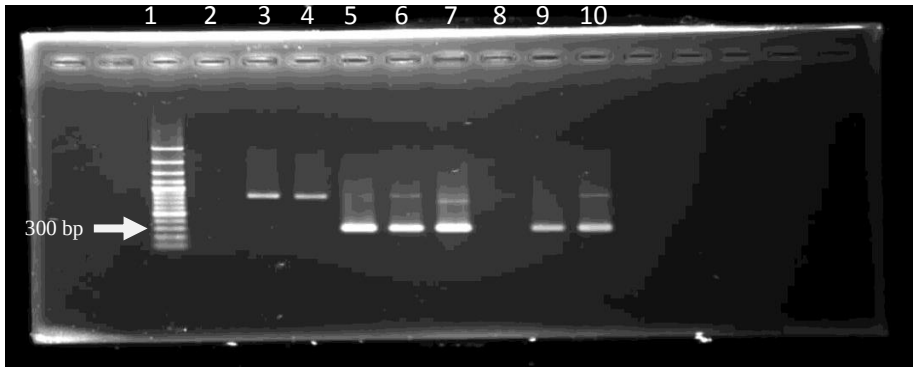


Figure 4.16: Gel analysis of 16S amplicons for the *Burkholderia*-specific 16S rDNA PCR assay with non-burkholderial species.

Lane 1, GeneRuler 100-bp DNA ladder; lane 2, NTC; lane 3, *B. cereus*; lane 4, *B. subtilis*; lane 5, *E. aerogenes*; lane 6, *E. coli*; lane 7, *K. pneumoniae*; lane 8, *S. aureus*; lane 9, *S. typhimurium*; lane 10, *P. aeruginosa*.

4.7 Final Identification of Bacterial Isolates

As shown in Table 4.4, each isolate corresponded to more than one bacterial species. Phenotypic test results for each isolate were compared against those reported in the literature; anomalous results and unreported characteristics were excluded (Tables 4.6, 4.7 and 4.8). Table 4.5 shows the results of the final identification. Four out of 17 isolates were identified to be those of the *Burkholderia* spp.; these include ASH9, ASH10, BCSA5 and BCSA14.

Table 4.5: The most possible identities for the bacterial isolates obtained in this study based on both phenotypic and genotypic results

Isolate	Bacterial Species
ASH1	*Comamonas terrigena*
ASH3	*Pseudomonas aeruginosa*
ASH4	*Escherichia coli*
ASH5	*Escherichia coli*
ASH8	*Cronobacter sakazakii*
ASH9	*Burkholderia* sp.
ASH10	*Burkholderia* sp.
ASH12	*Chromobacterium violaceum*
ASH17	*Pseudomonas aeruginosa*
BCSA2	*Klebsiella oxytoca*
BCSA3	*Pseudomonas aeruginosa*
BCSA5	*Burkholderia cepacia*
BCSA 6	*Chromobacterium haemolyticum*
BCSA9	*Pseudomonas aeruginosa*
BCSA10	*Chromobacterium haemolyticum*
BCSA11	*Serratia rubidaea*
BCSA14	*Burkholderia metallica* or *Burkholderia cepacia*

Table 4.6: Examples of final identification of bacterial isolates based on phenotypic and genotypic results

A) Isolates ASH9 and ASH10

Phenotypic feature / Species	Growth at 42 ℃	Pigment production	Gram staining	Cell morphology	Catalase	Oxidase	Motility	Arabinose Assimlation	Lactose Fermentation	Indole Production	Arginine Dihydrolase	Lysine Decarboxylase	Similarity (%)
B. pseudomallei	+	-	Gram negative	Bacillus	+	+	+	-	-	-	+	-	83.3
B. thailandensis	+	-	Gram negative	Bacillus	+	+	+	+	-	-	+	-	75
***B. cepacia* complex**	V	V	Gram negative	Bacillus	+	+	+	+	-	-	-	+	91.7
ASH9	+	-	Gram negative	Bacillus	+	+	+	-	-	-	-	+	
ASH10	+	-	Gram negative	Bacillus	+	+	+	-	-	-	-	+	

V, 10 to 90% are positive

Table 4.6 (continued……)

B) Isolate BCSA5

Phenotypic feature / Species	Pigment production	Gram staining	Cell morphology	Catalase	Oxidase	Motility	Arabinose Assimilation	Lactose Fermentaion	Indole Production	Arginine Dihydrolase	Lysine Decarboxylase	Similarity (%)
B. cepacia	V (Yellow)	Gram negative	Bacillus	+	+	+	+	-	-	-	+	81.8
B. stabilis	-	Gram negative	Bacillus	+	+	+	V	-	-	-	+	72.7
BCSA5	+ (Yellow)	Gram negative	Bacillus	+	-	-	+	-	-	-	+	

V, 10 to 90% are positive

Table 4.6 (continued……)

C) Isolate BCSA14

Phenotypic feature / Species	Pigment production	Gram staining	Cell morphology	Catalase	Oxidase	Motility	Arabinose Assimilation	Lactose Fermentation	Indole Producation	Arginine Dihydrolase	Lysine Decarboxylase	Similarity (%)
B. metallica	V (Yellow)	Gram negative	Bacillus	+	+	+	+	-	-	-	+	90.9
B. cepacia	V (Yellow)	Gram negative	Bacillus	+	+	+	+	-	-	-	+	90.9
B. cenocepacia	V (Brown)	Gram negative	Bacillus	+	+	+	+	-	-	-	+	81.8
BCSA14	+ (Yellow and violet)	Gram negative	Bacillus	+	+	-	+	-	-	-	+	

V, 10 to 90% are positive

CHAPTER 5

DISCUSSION

5.1 Environmental Sampling

5.1.1 Collection of Soil and Water Samples

Burkholderia spp. can be found in a wide range of ecological habitats such as soil, water and rhizosphere but their prevalence and distribution in the environment are not clear (Stoyanova et al., 2007). In this project, soil and water samples were collected from 18 sites that are potential reservoirs for human exposure to *Burkholderia* spp., for example, rain puddles on the roadside (Baker et al., 2011).

Collection of soil samples from the depth of approximately 25-cm was due to the fact that the top layer of soil was exposed to sunlight. *Burkholderia* spp. are susceptible to UV radiation (Mustafa 2010). They prefer moist environments and the higher residual water content is found at greater depths (Inglis and Sagripanti, 2006). Inglis and Sagripanti (2006) also explained that a rising water table after rainfall is the reason for the reappearance of *Burkholderia* spp. in the superficial layers of soil prior to human exposure.

Back in the laboratory, soil samples were stored at room temperature before further processing was carried out. Storing at room temperature was important to maintain the soil samples under an environment that resembled the field conditions to avoid drastic change in their mineral contents and the bacterial populations that were in their present (Limmathurotsakul et al., 2013). Meanwhile, water samples were kept at 4℃ to slow down the growth of bacteria and potentially antagonistic organisms that might hinder the recovery of the target organisms (Baker et al., 2011). All water samples were processed within 24 h.

5.1.2 Physical Appearance of Soil and Water Samples

In this project, soil samples collected were different in their moisture, color and texture. Inglis and Sagripanti (2006) reported that waterlogged and high clay content soil is much better at supporting *Burkholderia* persistence. A similar finding was also reported by Kaestli et al. (2007) in which they reasoned that it might be due to the clay's properties that can withhold nutrients and water better, interactions of clay particles with the bacteria and iron-containing compounds present in the clay might further support the growth of *B. pseudomallei*. However, the soil sampling sites in this study were around Kampar which is an ex-tin mining land. This kind of land consists mostly of sand and little clay content, which may only be occupied by a limited numbers of microorganisms, as compared to undisturbed land, due to nutrient deficiency

and extreme environmental condition. This might result in low presence of *Burkholderia* spp in this area.

In this project, the successful isolation of *Burkholderia* spp. from S1, S2, S3 and S4 shows the presence of this organism in various environmental soils. However, due to the lack of significant data, the correlation between the occurrence of *Burkholderia* spp. and soil texture and other environmental factors could not be determined in this study.

5.2 Culture Characteristics of Bacterial Isolates

5.2.1 Primary Isolation

Although the culture method is the "gold standard" for detection of *Burkholderia* spp., isolating these organisms from the environment is difficult due to the limited selectivity of the media available. Many non-*Burkholderia* species were able to grow on ASH and BCSA in this study. Besides that, the growth of fungi over the membrane filters for water samples might mask the bacterial colonies, leading to difficulty in their isolation. However, this problem was minimized by prior enrichment of the soil sample in Galimand's broth. There was only one type of bacterial isolate obtained from each enriched soil sample. This shows that the Galimand's broth, with the addition of 1 g/L polymixin B sulfate, was more selective for *Burkholderia* species than ASH and BCSA. Due to the presence of small numbers of bacteria in enriched soil

samples, the Trung's method was modified by concentrating the enrichment cultures through centrifugation and then spreading of culture instead of streaking them to ensure that most of the bacterial cells in the enrichment cultures were plated out.

Bacteria were isolated from all samples except the water sample from swimming pool. This was very likely due to the presence of chlorine for disinfection in the swimming pool that used for. The cidal effect of chlorine against pathogenic *B. pseudomallei* is of particular interest since this chemical is widely used as biodefense disinfectant and treatment of drinking water supplies. The susceptibility of *B. pseudomallei* to chlorine was studied by Inglis and Sagripanti (2006), in which they showed that the cells were able to tolerate 1 ppm chlorine, which is the commonly used chlorine concentration for treating water supplies. In contrast, swimming pools are usually adequately chlorinated and therefore they might not harbor *Burkholderia* spp.

5.2.2 Colony Morphology

Various types of colonies were observed on ASH and BCSA in this study. The color of colonies of *B. cepacia* complex vary, in which they may be pigmented or non-pigmented. In this study, most of the isolates on ASH were purple, smooth, circular and convex. One of the isolates, ASH12, produced black colonies on ASH and purple colonies on TSA, and it was later identified to be

that of *C. violaceum*. Although the typical wrinkled colony morphology of *B. pseudomallei* was not observed on ASH, the possibility of their presence could not be concluded. Atypical colony morphology of *B. pseudomallei* had been reported by Chantratita et al. (2007) to be due to starvation, iron limitation and growth at 42 ℃.

BCSA medium was initially yellowish orange in color when there was bacterial growth, but the medium then turned pink. This was due to the response of phenol red present in the medium to the metabolitc activity of *B. cepacia* complex. As the result of pyruvate metabolism due to the growth of *B. cepacia* complex, the alkaline end products caused a raise in the pH of the medium, thus changing its color from orange to pink in the growth area.

However, pigmentation is not a universal characteristic of *Burkholderia* spp.. Some *B. cepacia* species can produce phenazine pigments that are made up of a variety of colors. A single species can synthesize one type of pigment, different types of pigments or no pigment at all (Garrity et al., 2005). In this study, isolate BCSA14 yielded colonies of varied colors on BSCA and TSA, and BCSA5 yielded bright yellow colonies on TSA. They were later identified as a *Burkholderia* spp. via genotyping methods.

5.3 Gram Staining of Bacterial Isolates

In this study, gram staining revealed that all the isolates were gram-negative bacilli or coccobacilli. The results of gram staining show that the concentrations of crystal violet used in ASH (5 mg/L) and BCSA (1 mg/L) were sufficient to inhibit the growth of gram-positive bacteria. *Burkholderia* species are expected to be gram-negative bacilli.

5.4 Biochemical Tests on Bacterial Isolates

5.4.1 Catalase Test

The catalase test is used to assess the ability of microbes to decompose hydrogen peroxide to water and oxygen. In a positive reaction, immediate formation of bubbles after the addition of hydrogen peroxide will be observed. All isolates in this study were catalase positive. *Burkholderia* species are expected to be positive in this test.

5.4.2 Oxidase Test

The oxidase test is used to determine the presence of cytochrome oxidase in bacteria. Tetra-methyl-*p*-phenylenediamine dihydrochloride reagent is often used as an artificial electron donor for cytochrome *c* in this test. When the reagent is oxidized by cytochrome *c*, it changes from colorless to a dark blue or purple compound, indophenol blue (Shields and Cathcart, 2010).

In total, 44% of the isolates were oxidase positive. Majority of *Burkholderia* species are expected to be positive in the oxidase test but some species of *B. cepacia* complex such as *B. contaminans*, *B. lata* and *B. pyrrocinia* are oxidase negative (Garrity, Bell and Liburn, 2005).

5.4.3 Motility Test

In this study, 60% of the isolates were positive for motility. Motility test can be performed easily in the laboratory to check for bacterial motility. The motility test medium used in the study was supplemented with the colored dye tetrazolium which made it much easier to interpret the result. The colorless tetrazolium turned red when reduced as a result of bacterial metabolism.

Motility can be observed in all *Burkholderia* species due to the presence of one or several polar flagella. Unlike *P. aeruginosa*, twitching motility on the surface of solid media is not usually shown by *Burkholderia* species (Garrity, Bell and Liburn, 2005). However, similar gene clusters necessary for biosynthesis of rhamnolipids and swarming motility of *P. aeruginosa* had been found in *B. thailandensis* (Dubeau et al., 2009). The reason for hidden swarming motility among *Burkholderia* species might be due to the repression of rhamnolipid synthesis by the sole nitrogen and carbon sources provided in the medium (Kohler et al., 2000).

5.4.4 L-Arabinose Assimilation Test

In this study, isolates were tested for arabinose assimilation via growth on minimal salt agar containing 0.2% L-arabinose (Wuthiekanun et al. 1996). A total of 57% of the isolates were able to assimilate L-arabinose. L-arabinose assimilation test is a simple preliminary test which can discriminate between the highly virulent *B. pseudomallei* and the generally avirulent *B. thailandensis* (Wuthiekanun et al., 1996). The arabinose assimilation property had been shown to be probably one of the virulence determinants of *Burkholderia* species (Moore et al., 2004).

5.4.5 Lactose Fermentation Test

The lactose fermentation test was carried out by growing the isolates on the MacConkey agar. From the results, 60% of the isolates were non-lactose fermenting. The growth of all isolates on MacConkey agar further supported the gram stain results. This test was used to differentiate the lactose fermenters, such as the species from the genera *Escherichia*, *Enterobacter* and *Klebsiella*, and non-lactose fermenters such as *Burkholderia* spp. and *Pseudomonas* spp. Fermentation of lactose caused the pH of the media to dropped. As the pH drop, the pH indicator, neutral red, turned red and was absorbed by the bacteria. Thus, they yielded bright pink to red colonies on the agar. Bacteria with strong lactose fermentation produced adequate acid to cause precipitation of the bile salts, resulting in a pink halo in the medium surrounding the individual

colonies. Weakly lactose-fermenting bacteria growing on MacConkey agar would still yielded pink to red colonies but they would not be surrounded by a pink halo in the surrounding medium. Non-lactose fermenting bacteria yielded colorless colonies on the agar.

5.4.6 Indole Test

The indole test is simple and easy to be performed in the laboratory. The results show that 71% of the isolates were indole-negative. *Burkholderia* spp. are negative in the indole test and this can differentiate them from indole-positive bacteria such as *E.coli*, *Klebsiella oxytoca*, *Vibrio* spp. and *Providenica* spp. Indole test assesses the ability of the organism to synthesize tryptophanase that catalyses the conversion of tryptophan to indole. Detection of indole is based on the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) which is present in the Kovac's reagent under acidic condition to produce the red dye rosindole (MacWilliams 2009).

5.4.7 Arginine Dihydrolase Test

The arginine dihydrolase test aids the differentiation among *Burkholderia* species. *B. pseudomallei* and *B. thailandensis* are expected to be positive, whereas most species of the *B. cepacia* complex are negative in this test. In this study, 43% of the isolates were found to be positive for arginine dihydrolase.

In the arginine dihydrolase test, the inoculated bacteria fermented the dextrose in the medium, resulting in a drop in pH, which was detected by the pH indicator, phenol red, and showed a color change to yellow. Under acidic condition, arginine dihydrolase was stimulated and then the bacteria decarboxylated the arginine present in the medium to putrescine. The production of putrescine raised the pH and phenol red turned pink color under alkaline condition. A color change from initially pink to yellow and then back to pink indicated a positive reaction. A thin layer of oil was added to each inoculated tube to prevent alkalinization at the surface of the medium in the presence of air, which could cause inaccurate result.

5.4.8 Lysine Decarboxylase Test

Similar interpretation for the arginine dihydrolase test applies to the lysine decarboxylase test. The bacteria that possessed lysine decarboxylase stimulate the decarboxylation of lysine under acidic condition and produce cadaverine as the end-product. In this study, 83% of the isolates were able to produce lysine decarboxylase. Some *Burkholderia* spp. are expected to be negative in this test and these include *B. dolosa* and *B. ubonensis*, members of the *B. cepacia* complex, *B. pseudomallei* and *B. thailandensis*; other species of the *B. cepacia* complex are expected to be positive in this test.

5.5 Genotypic Identification of Bacterial Isolates

5.5.1 Universal 16S rDNA PCR Assay and DNA Sequencing

Gel analyses showed that the 16S amplicons of 17 selected isolates yielded bands of the expected size of 199 bp. Some smearing and non-specific bands were also observed on the gels. Non-specific amplification could be due to the low purity of the DNA extracts, in which medium residues could have interfered with primer annealing, resulting in non-specific amplification (Weighardt et al., 1993). Nonetheless, non-specific bands would not be a problem as the 199-bp amplicons could be selectively excised out for purification and sequencing

Using the BLASTn program available on the NCBI website, four (24%) isolates were identified to be those of *Burkholderia* species, whereas other isolates were those of the genera *Chromobacterium*, *Comomonas*, *Cronobacter, Serratia, Escherichia* and *Pseudomonas*, which are ubiquitous gram-negative rods that are found naturally in soil and water.

Due to the unsatisfying sequencing results for their *Burkhoderia*-specific 16S amplicons, seven isolates were subjected to sequencing of their universal 16S amplicons. However, the sequencing results were also unsatisfactory. These failures might be attributed to the low concentrations and purities of the samples analyzed. The former could be due to degradation of template DNA or

a result of prolonged storage and frequent freeze-thrawing, thus contributing to low amount of amplicons obtained after PCR. In addition, DNA degradation might also occur during transport to the sequencing facility. Nevertheless, the final identification of these isolates was done based on sequenicng results for both the *Burkholderia*-specific and universal 16S amplicons.

5.5.2 *Burkholderia*-specific 16S rDNA PCR Assay and DNA Sequencing

The *Burkholderia*-specific 16S rDNA PCR assay amplifying a 320-bp portion (nucleotide 325-645) of the 16S rDNA of *Burkholderia* species described by Brett et al. (1997) was used in this study. Nine isolates (ASH3, ASH17, ASH9, ASH10, ASH1, BCSA9, BCSA10, BCSA6 and BCSA3) were selected based on the highest similarity in biochemical profiles with those for *Burkholderia* species for this PCR assay. Besides that, an additional four oxidase-negative isolates (ASH8, ASH12, ASH4 and BCSA2) were randomly selected to be used as negative controls since *Burkholderia* species are mostly oxidase-positive. Unexpectedly, 320-bp amplicons were amplified from all these for isolates. For further identification, all amplicons with the expected size of 320-bp were purified from the gels and sent for DNA sequencing.

From the BLASTn result, the amplicon sequence for isolate ASH10 shows high similarity to *Burkholderia* 16S rDNA sequence, with maximum identity of 99%. However, most isolates yielded poor DNA sequencing results that were

difficult for bioinformatics analysis. This might be attributed to the low concentrations and purities of the samples analyzed. In addition, DNA degradation might also occur during transport to the sequencing facility. Hence, the isolates were reanalyzed under universal 16S rDNA PCR assay and the results from both sequencing tests were compared for final identification of the isolates.

The specificity of the *Burkholderia*-specific 16S rDNA PCR assay was also assessed and the results show that it has low specificity. There were five false positives for the whole panel of eight negative controls tested. However, it amplified all the gram-negative controls but not the gram-positive ones. This indicates that this genus-specific PCR assay is not reliable to be used for identification of *Burkholderia* spp.

5.6 Final Identification of Bacterial Isolates

In this study, four *Burkholderia* species were successfully isolated and identified. The phenotypic features of these isolates are not totally identical to those reported, which might be due to the variations among different strains. Individually, both the genotypic and phenotypic identification methods yielded a list of possible identities for each of the bacterial isolates screened. Furthermore, relying on the findings of the latter alone was not sufficient to preliminarily identify *Burkholderia* spp. among the isolates. Thus, it is

important to take into account the findings of both approaches for reliable identification. A few non-burkholderial species such as *P. aeruginosa*, *C. violaceum, C. haemolyticum* and *E .coli* were also isolated. They are ubiquitous gram-negative rods found in the environment.

5.7 Assessment of Health Impacts of Isolated *Burkholderia* spp. on Humans

In this study, the presence of *Burkholderia* spp. and other opportunistic pathogens such as *P. aeruginosa, K. oxytoca, C. violaceum* and *C. haemolyticum* was reported in the environmental reservoirs screened. Among the four *Burkholderia* spp. isolated, two were identified to be *B. cepacia* and *B. metallica*, respectively; all belong to the *B. cepacia* complex.

The members of the *B. cepacia* complex are not obligate pathogens, although they can become pathogenic to humans under some predisposing conditions. They can affect those suffering from cystic fibrosis, chronic granulomatous disease, cancer and acquired immunodeficiency syndrome (Sousa, Ramos and Leitao, 2011). The species of *B. cepacia* complex can sporadically cause infections in immunocompetent individuals; these include chronic suppurative otitis media, pharyngeal infections and pediatric neck infections (Sousa, Ramos and Leitao, 2011; Karanth et al., 2012).

Opportunistic infections due to *Burkholderia* spp. are likely to occur via environment-to-human transmission. Direct contact and inhalation of contaminated dusts or aerosols are the possible modes of acquisition of *Burkholderia* infection. Soil-associated *Burkholderia* spp. can be aerosolized by agitation and by the wind. *B. cepacia* in inhaled droplets can colonize the lungs, leading to host inflammatory response that slowly deteriorates the lung infections and inflammation that lead to a slow deterioration of lung function (Tomich et al., 2002). This is aided by its ability to invade and survive within respiratory epithelial cells of infected individuals (Martin and Mohr, 2000) and resist intracellular killing by phagocytic cells (Saini et al., 1999). However, the risk of infection is very low for immunocompetent individuals. The risk increases with frequent and continuous exposure, especially in those involved in agricultural activities (farmers in the UTAR C4 Land and the cow farm in this study). Thus, it is recommended to minimize exposure by avoiding walking bare-footed on *Burkholderia*-positive sites and covering all open wounds with waterproof dressings.

Due to the presence of *Burkholderia* spp. in puddles of rain water on the roadside, their health impacts on pedestrians, cyclists and motorcyclists were also evaluated. Indirect exposure due to the aerosols generated from the splashing of water is possible, and therefore it is very important for cyclists and pedestrians to wear covered shoes and cover all open wounds. It is also

advisable not to step on the puddles.

5.8 Future Works

5.8.1 Improvement of PCR Assays

Since non-specific amplification was observed upon gel analysis, dimethyl sulfoxide and formamide can be added into PCR reactions to increase their stringency (Cheah, 2010). Due to the unsatisfactory results obtained, the *Burkholderia*-specific PCR assay should be redesigned or replaced with another genus-specific PCR assay, for example, that developed by Payne et al. (2005). With a reliable *Burkholderia*-specific PCR, the isolates can be selectively outsourced for DNA sequencing.

5.8.2 Direct Detection of *Burkholderia* spp. in the Environment Using Molecular Method

Several factors such as the overgrowth of environmental fungi and other bacterial species and the presence of viable but nonculturable *Burkholderia* cells could hinder their detection by culture-based methods. In this case, the use of molecular methods to directly detect *Burkholderia* spp. in environmental reservoirs would be more appropriate. Soil DNA extraction can be performed using commercially available kits, for example the SoilMaster DNA Extraction Kit or the Fast DNA Spin Kit for soil, or using the Trung's method developed in-house (Trung et al., 2011). For direct burkholderial detection in water

samples, the microorganisms trapped on the membrane filter can be eluted and then subjected to DNA extraction, followed by a PCR assay downstream. A multiplex PCR assay should be considered for simultaneous detection of multiple *Burkholderia* spp.

5.8.3 Study on the Environmental Factors Influencing the Existence of *Burkholderia* spp.

Environmental factors such as temperature, moisture and pH should be looked into to assess whether they play important roles in influencing the existence of *Burkholderia* spp. A better understanding on the occurrence and distribution of *Burkholderia* spp. in different environmental reservoirs could then be obtained.

5.8.4 Further Characterization of Isolated *Burkholderia* spp.

Further characterization can be performed to identify the *Burkholderia* isolates, ASH4 and ASH5, to the species level through an improved molecular assay and a commercial biochemical test kit. For the successfully speciated *Burkholderia* isolates (BCSA5 and BCSA14), further investigation into their potential usefulness can be carried out. For instance, their potentials in bioremediation can be investigated. (Juhasz, Britz and Stanley, 1997; Widada, Nojiri and Omori, 2002).

CHAPTER 6

CONCLUSIONS

To detect and identify *Burkholderia* spp. in various environmental reservoirs, six soil samples and 12 water samples were collected and processed. Selective culture techniques using the Ashdown's medium, Galimand's medium and *Burkholderia cepacia* selective medium were used to detect and isolate these organisms and determine its presence in our environment. Pure isolates recovered from the samples were further characterized based on their phenotypic and genotypic features. Besides differentiation based on colony morphology and gram staining appearance, eight biochemical assays were performed to preliminarily identify the isolates. The isolates were categorized based on their biochemical characteristics. The isolates that are biochemically similar to *Burkholderia* spp. were proceeded to genotypic identification. The use of PCR as a method of identification was shown to be valuable, as biochemical characteristics were inconsistent and not all strains gave the typical pattern of characteristics. Especially for the identification of *Burkholderia cepacia* complex, it was impossible to distinguish them based on phenotypic characteristics. Results of DNA sequencing and bioinformatics analyses revealed that four environmental isolates corresponded to *Burkholderia* species. By comparison to the phenotypic characteristics of the

isolates, the most probable species isolated were of the *Burkholderia cepacia* complex. They were isolated from puddles of rain water, river at Kampar New Town, C4 Land, cow farm, soil near to the lake behind the UTAR library and river in UTAR Perak Campus. With the use of selective and enrichment media, isolates belonging to a limited number of other genera (*Pseudomonas, Chromobacterium*, *Klebsiella*, *Cronobacter*, *Comamonas*, *Serratia* and *Escherichia*) were also recovered on these media. Consequently, the health impacts of *Burkholderia* isolates on human around the sampling sites were briefly evaluated based on their clinical significance. The species of *B. cepacia* complex detected in some of the sampling sites are opportunistic pathogens that infect immunocompromised people. Although healthy individuals are not thought to be at risk, rare cases of infection could happen. Hence, people should be advised to avoid unprotected exposure to the environment and cover wounds as necessary.

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APPENDIX A

APPARATUS AND CONSUMABLES

The lists of apparatus and consumables used in this project are as follow:

Table A1: List of apparatus and their respective manufacturers.

Apparatus	Manufacturer
BSL-2 cabinet	Telstar
Centrifuge machines	Hettich Zentrifugen, Sigma, Beckman Coulter
Electrophoresis system	Major Science
Gel imaging system	Syngene Bio Imaging
Laminar air flow cabinet	Telstar
Light microscope	Leica CME
Microcentrifuge machines	Labo Gene
Nanophotometer	Thermoscientific
Orbital shaker	N-Biotek
PCR thermocycler	Kyratec

Table A2: List of consumables and their respective manufacturers.

Consumable	Manufacturer
Agar-agar powder	R & M Chemicals
Agarose powder	1st Base
Crystal violet	Bio Basic
Deoxyribonucleoside triphosphate (dNTP) mix	Fermentas
DNA ladder	Fermentas
DNA extraction kit	Geneaid
Ethidium bromide (EtBr)	Bio Basic
Ethylenediaminetetraacetic acid (EDTA) disodium salt	EMD Chemicals
Filter membrane (0.2-µm)	Whatman
Gel purification kit	Invitek, Xprep
Gentamicin	BioBasic
Glycerol	QReC
Hydrochloric acid	Merck
Hydrogen peroxide	HmbG Chemicals
Kovac's reagent	Merck
L-Arabinose	Merck
L-Arginine	Sigma
L-Lysine	Bio Basic
L-Threonine	Bio Basic
Luria-Bertani (LB) broth powder	Pronadisa
M9 medium broth	Amresco
MacConkey agar powder	Oxoid
Neutral red	Amresco
Penicillin	BioBasic
Peptone	BD
Phenol red	R & M Chemicals
Polymyxin B sulfate	Amresco
Primers	1st Base, Integrated DNA Technologies
Reagents A & B	Fluka
Sodium chloride (NaCl)	Merck
Sodium hydroxide (NaOH)	Merck
Syringe	Terumo
Syringe filters (0.2 µm)	Pall Life Science
Taq polymerase and PCR buffer	Fermentas
Triphenyltetrazolium chloride	Nacalaitesque
Tris base	MP Biomedicals
Tryptic soy broth and agar powder	Merck
Tryptone	Pronadisa

APPENDIX B

Table B: Observation of colony morphology of bacterial isolates on TSA.

Isolate	Sample Source	Observation on TSA				
		Color	Size	Shape	Elevation	Edge
ASH 1	L1	Translucent Yellow	Medium	Circular	Convex	Entire
ASH 2	L2	Translucent White	Medium	Circular	Convex	Entire
ASH 3	L2	Translucent Yellow	Medium	Oval	Flat	Entire
ASH 4	R1	Translucent Yellow	Medium	Circular	Flat	Entire
ASH 5	R2	Translucent White	Medium	Circular	Flat	Entire
ASH 6	R2	Translucent White	Medium	Circular	Convex	Entire
ASH 7	AQ	Translucent Yellow	Medium	Circular	Convex	Entire
ASH 8	RAIN	Translucent Yellow	Small	Circular	Convex	Entire
ASH 9	RAIN	Translucent Yellow	Medium	Circular	Slightly raised	Entire
ASH 10	RAIN	Translucent Yellow	Medium	Circular	Convex	Entire
ASH 11	RAIN	Translucent White	Medium	Circular	Convex	Entire
ASH 12	P1	Dark Purple	Small	Circular	Raised	Entire
ASH 13	P1	Translucent White	Medium	Circular	Convex	Entire
ASH 14	P2	White	Medium	Circular	Convex	Entire
ASH 15	P2	White	Medium	Circular	Convex	Entire
ASH 16	SEA	Translucent White	Medium	Circular	Convex	Entire
ASH 17	SEA	Translucent Yellow	Small	Circular	Convex	Entire
ASH 18	WF	Translucent Yellow	Medium	Circular	Convex	Entire
ASH 19	WF	Translucent Yellow	Medium	Circular	Convex	Entire
ASH 20	S1-4	Creamy White	Small	Pinpoint		
ASH 21	S5-6	Translucent White	Medium	Circular	Convex	Entire
BCSA 1	L1	White	Medium	Circular	Convex	Entire
BCSA 2	R1	White	Medium	Circular	Convex	Entire
BCSA 3	R1	Translucent Yellow	Big	Oval	Flat	Entire
BCSA 4	R2	Translucent White	Medium	Circular	Convex	Entire
BCSA 5	R2	Greenish Yellow	Small	Circular	Convex	Entire
BCSA 6	AQ	White	Small	Circular	Convex	Entire
BCSA 7	RAIN	Translucent Yellow	Medium	Circular	Convex	Entire
BCSA 8	P2	Translucent Yellow	Medium	Circular	Raised	Undulate
BCSA 9	SEA	Translucent Yellow	Small	Circular	Convex	Entire
BCSA 10	WF	Translucent Yellow	Medium	Circular	Flat	Entire, Dry Wrinkled
BCSA 11	WF	White, Slightly Pink	Medium	Circular	Convex	Entire
BCSA 12	WF	Translucent Yellow	Medium	Circular	Convex	Entire
BCSA 13	WF	Translucent Yellow	Small	Circular	Convex	Entire
BCSA 14	S1-4	Multicolor	Small	Circular	Convex	Entire