UNIVERSITI PUTRA MALAYSIA

GENETIC CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE PATTERNS OF Burkholderia pseudomallei ISOLATES FROM ANIMALS AND ENVIRONMENT IN PENINSULAR MALAYSIA

ABUBAKAR SADIQ MUHAMMAD

FPV 2017 1

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By

ABUBAKAR SADIQ MUHAMMAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2017

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DEDICATION

This work is dedicated to my beloved parents late Alhaji Aisami Muhammad Idris and late Hajja Gambo Muhammad, may Allah (SWT) grant them His Mercy and Jannatul Firdaus.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

GENETIC CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE PATTERNS OF *Burkholderia pseudomallei* ISOLATES FROM ANIMALS AND ENVIRONMENT IN PENINSULAR MALAYSIA

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January 2017

Chairman : Latiffah Hassan, DVM, PhD
Faculty : Animal Medicine

Burkholderia pseudomallei is the aetiological agent of an emerging and potentially fatal human and animal disease melioidosis. Much of the knowledge about this organism in Malaysia and elsewhere revolved around human clinical isolates and much less is known about animal and environmental isolates. The present study was conducted with the aim generating more information on the phylogenetic variability of the animal and environmental *B. pseudomallei* isolates and its relatedness to antibiotic resistance pattern and genes. The specific objectives are to determine the molecular characteristics of *B. pseudomallei* isolates from animals and the environment (soil and water) in Peninsula Malaysia, compare the phylogeny of these isolates to those from elsewhere in the world, determine the antimicrobial resistance pattern among animal and environmental isolates, determine the occurrence of antimicrobial resistance genes and compare to the resistance pattern observed and determine the association between the sequence types to the physical and chemical characteristics of environments (soil and water) where the isolates originated.

A total of 113 Malaysian *B. pseudomallei* isolates from animals and farm environment (soil and water) were characterised by multilocus sequence typing (MLST). Eighteen alleles were recovered in this study, among which are novel allele 97 and 69 of gene locus *ace* and *lepA* respectively. The allelic combinations resolved the isolates into 12 distinct sequence types (STs) with five among which are novel STs; ST1130, ST1131, ST1338, ST1339 and ST1367. This study found no association between sources of isolates and ST whereby the STs recovered from animal cases co-cluster with those found in the environment and have also been previously reported in humans. The isolates were found to be highly clonal. Moreover, *B. pseudomallei* strains were recovered to have descended from a common ancestor clonal complex 48 (CC48) found regionally in Southeast Asia.

Disc diffusion or Kirby Bauer and E-test minimum inhibitory concentration (MIC) antibiotics susceptibility tests were conducted on 111 *B. pseudomallei* isolates. Twelve (12), common commercially prepared antimicrobial discs: ceftazidime, ceftriaxone, meropenem, ticarcillin, aztreonam, trimethoprim-sulfamethoxazole (cotrimoxazole), ciprofloxacin, imipenem, chloramphenicol, gentamicin, tetracycline and doxycycline were used in the disc diffusion method. All (100%) of the *B. pseudomallei* showed susceptibility to chloramphenicol, imipenem and doxycycline while all the isolates in this study were resistant to gentamicin and ticarcillin, and 99% were resistant to aztreonam. There was no significant association between the source of isolates (whether from animals or the environment) to the occurrence of resistance to any of the 12 antibiotics by disc diffusion method ($p > 0.05$), however there was a significant association between the occurrence of resistance to meropenem ($p<0.05$) and cotrimoxazole ($p<0.001$) to the STs of *B. pseudomallei* isolates. Five antibiotics namely meropenem, imipenem, ceftazidime, trimethoprim-sulfamethoxazole and amoxicillin-clavulanic acid, recommended in both acute and eradication phases of melioidosis treatment were tested using E-test MIC method. The majority of isolates were susceptible to all the antibiotics tested, however the existence of few resistant strains to meropenem, cotrimoxazole, ceftazidime and co-amoxiclav was observed among these isolates. A statistically significant association was found between resistance to meropenem and the animal isolates ($p<0.05$). The likelihood of resistance to meropenem was significantly higher among the novel sequence type (ST) 1130 isolates found in animal cases as compared to others.

Burkholderia pseudomallei was reported to exhibit high resistant to many antibiotics by employing several resistance mechanisms. Five components *B. pseudomallei* antibiotic resistance genes of resistance nodulation division (RND), namely MxFs_*BPSS1119*, *bpeA*, *bpeB*, *amrB* and *OprC*_RND and one *penA* β-lactamase, whose functions have been characterised were selected. The primer oligonucleotides for the genes *BBSS1119* (MxFs_*BPSS1119*), *bpeA* (*bpeA*_RND), *bpeB* (*bpeB*_RND), *amrB* (MxYs_*amrB*), *OprC*_RND and *penA* were used to amplify the respective gene fragments. The majority of isolates were susceptible to imipenem, ceftazidime and co-amoxiclav, however there were few resistant strains to meropenem, cotrimoxazole, ceftazidime and co-amoxiclav among the animal and environmental isolates. Polymerase chain reaction (PCR) amplification of *BPSS1119* RND, *bpeA*, *bpeB* and *amrB* gene fragment was obtained in all the 111 isolates of *B. pseudomallei* from animal or environment for all STs. However there was no amplification for *B. pseudomallei* RND efflux pump *OprC* and *penA* β-lactamase gene fragments. Although this study detected the four RND efflux pump genes *BPSS1119*, *bpeA*, *bpeB*, *amrB* it is still not clear whether these efflux pumps have been expressed or not. The inability to detect O*prC* and *penA* gene could be attributed to gene deletion or due to the occurrence of indels. We concluded that the efflux pump genes were widespread among animal and environmental *B. pseudomallei* regardless of isolate source and antibiotic resistance or susceptibility pattern.

Physicochemical properties or characteristics of the environment where organisms dwell have been shown to influence the distribution of organism. A total of 78 isolates (56 from soil and 22 from water) from livestock farms environment were molecularly characterised by multilocus sequence typing (MLST) and were analysed against the

environmental physicochemical properties from 33 livestock farms in four states of Pahang, Perak, Selangor and Negeri Sembilan in Peninsular Malaysia. Multinomial logistic regression performed found significant association between soil water content and ST84 (OR = 0.833, 95%CI 0.708 to 0.980; p=0.027) when compared to ST51. This shows unit increases in soil water content was associated with a 1.2 times increase in the odds of recovering ST51 compared with the odds of recovering ST84. Also statistically significant protective association was found between water pH and ST84 (OR=0.401 95%CI 0.195-0.828; p= 0.013) when compared to ST51. These findings suggest variation in the occurrence of various *B. pseudomallei* STs with the variations in the environmental (soil and water) physicochemical factors.

In conclusion, this study recovered two novel alleles *ace*97 and *lepA*, and five novel STs, ST1130, ST1131, ST1338, ST1339 and ST1367. *Burkholderia pseudomallei* is highly clonal and is likely to have originated from the CC48 found regionally in Southeast Asia. The existence of few resistant strains to drugs that are essential in the treatment of melioidosis among animal and environmental isolates poses a clinically significant threat to the management of infected animals and human patients. An association was demonstrated between *B. pseudomallei* STs and resistance to meropenem among the animal isolates. Polymerase chain reaction (PCR) detection of the efflux pump genes showed widespread prevalence of these genes among animal and environmental *B. pseudomallei* regardless of antibiogram, source or genotype. Physicochemical properties such as soil water content and water pH play a role in influencing the distribution of genotype of *B. pseudomallei* in endemic areas. This information is useful for planning control programs tailored towards environmental interventions to reduce contamination in non-endemic areas.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN GENETIK DAN CORAK RINTANGAN ANTIMIKROB *Burkholderia pseudomallei* TERASING DARI PERSEKITARAN LADANG DAN HAIWAN DI SEMANANJUNG MALAYSIA.

Oleh

ABUBAKAR SADIQ MUHAMMAD

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Burkholderia pseudomallei adalah agen penyebab bagi penyakit melioidosis yang berpotensi menyebabkan kematian pada manusia dan haiwan. Pengetahuan mengenai organisma ini di Malaysia dan lain tempat berkisar mengenai pengasingan klinikal pada manusia dan sangat sedikit menumpu kepada haiwan dan persekitaran. Kajian ini telah dijalankan dengan tujuan mendapatkan maklumat lanjut mengenai kepelbagaian filogenetik *B. pseudomallei* pada haiwan dan persekitaran dan kaitannya dengan corak rintangan antibiotik dan gen. Objektif khusus adalah untuk menentukan ciri molekul pencilan *B. pseudomallei* daripada haiwan dan persekitaran (tanah dan air) di Semenanjung Malaysia, membandingkan filogeni pencilan dengan pencilan dari tempat lain di dunia, menentukan corak rintangan antimikrob di antara pencilan dari haiwan dan pencilan dari persekitaran, menentukan kehadiran gen ketahanan antimikrob dan membandingkan corak rintangan yang diperolehi, dan menentukan hubungkait di antara jenis urutan (ST) dengan ciri fizikal dan kimia persekitaran (tanah dan air) di mana pencilan berasal.

Sebanyak 113 pencilan *B. pseudomallei* dari haiwan dan persekitaran ladang di Malaysia telah dicirikan menggunakan *multilocus sequence typing* (MLST). Lapan belas alel telah dikenalpasti dalam kajian ini, iaitu alel novel 97 bagi gen *ace* dan 69 bagi gen *lep*A. Kombinasi alel ini telah menghasilkan 12 ST yang berbeza dengan lima di antaranya adalah ST novel; ST1130, ST1131, ST1338, ST1339 dan ST1367. Kajian ini mendapati tiada kaitan antara sumber pencilan diperolehi dan ST, dimana ST yang diperolehi daripada kes haiwan berkelompok dengan yang terdapat pada persekitaran dan juga telah dilaporkan pada manusia sebelum ini. Pencilan yang kami diperolehi adalah klonal. Selain itu, strain *B. pseudomallei* didapati berasal daripada kumpulan klonal kompleks 48 (CC48) yang biasa didapati di Asia Tenggara.

Teknik penyebaran cakera atau Kirby-Bauer dan E-test kepekatan perencatan minimum (MIC) antimikrob telah dijalankan ke atas 111 pencilan *B. pseudomallei*. Dua belas (12) cakera antimikrob yang komersial: ceftazidime, ceftriaxone, meropenem, ticarcillin, aztreonam, trimethoprim-sulfamethoxazole (cotrimoxazole), ciprofloxacin, imipenem, chloramphenicol, gentamicin, tetracycline dan doxycycline digunakan dalam kaedah penyebaran cakera. Semua (100%) *B. pseudomallei* rentan terhadap chloramphenicol, imipenem dan doxycycline manakala semua pencilan dalam kajian ini tahan terhadap gentamicin dan ticarcillin, manakala kira-kira 99% daripada pencilan menunjukkan kerintangan terhadap aztreonam. Tidak ada hubungan yang berkeertian antara sumber pencilan (sama ada dari haiwan atau persekitaran) kepada berlakunya ketahanan terhadap mana-mana 12 antibiotik dengan kaedah penyebaran cakera ($P>0.05$), bagaimanapun terdapat hubungan yang berkeertian antara berlakunya rintangan terhadap meropenem ($P <0.05$), cotrimoxazole ($P <0.001$) kepada ST pencilan *B. pseudomallei*. Lima antibiotik iaitu meropenem, imipenem, ceftazidime, cotrimoxazole dan amoxicillin-clavulanic acid yang disyorkan bagi rawatan fasa akut dan pembasmian melioidosis telah diuji menggunakan kaedah E-test MIC. Majoriti dari pencilan adalah rentan kepada semua antibiotik yang diuji, namun wujud beberapa strain yang tahan terhadap meropenem, cotrimoxazole, ceftazidime dan co-amoxiclav ditemui di kalangan pencilan ini. Secara statistik, terdapat hubungkait di antara rintangan terhadap meropenem dan pencilan dari haiwan ($P <0.05$). Kebarangkalian rintangan terhadap meropenem adalah jauh lebih tinggi di kalangan pencilan dari ST1130 dari kes haiwan berbanding dengan yang lain.

Burkholderia pseudomallei dilaporkan menunjukkan ketahanan yang tinggi terhadap banyak antibiotik dengan menggunakan beberapa mekanisme rintangan. Lima komponen gen rintangan antibiotik *B. pseudomallei* dalam bahagian rintangan nodulation (RND) iaitu *MxFs_BPSS1119, bpeA, bpeB, amrB* dan *OprC_RND* dan satu *penA β-lactamase*, yang fungsinya telah dicirikan telah terpilih. Primer oligonukleotida untuk gen *BBSS1119 (MxFs_BPSS1119), bpeA (bpeA_RND), bpeB (bpeB_RND), armB (MxYs_amrB), OprC_RND* dan *penA* telah digunakan untuk Polymerase chain reaction (PCR) mengamplifikasikan fragmen gen masing-masing. Kebanyakan daripada pencilan adalah rentan kepada imipenem, ceftazidime dan co-amoxiclav, namun wujud beberapa strain yang tahan terhadap meropenem, cotrimoxazole, ceftazidime dan co-amoxiclav di kalangan pengasingan haiwan dan persekitaran. Pengamplifikasian fragmen gen *BPSS1119 RND, bpeA, bpeB* dan *amrB* telah dilihat dalam semua 111 pencilan *B. pseudomallei* dari haiwan atau persekitaran untuk semua ST. Walaubagaimanapun, tidak ada amplifikasi untuk *B. pseudomallei* RND pam efflux *OprC* dan fragmen gen *PenA β-lactamase*. Walaupun dalam kajian ini telah mengesan empat gen pam efflux RND *BPSS1119, bpeA, bpeB, amrB*, masih tidak jelas sama ada pam efflux telah ternyata atau tidak. Ketidakupayaan untuk mengesan gen *OprC* dan *penA* boleh dikaitkan dengan penghapusan gen atau kerana berlakunya indels. Kami membuat kesimpulan bahawa gen pam efflux adalah meluas di kalangan *B. pseudomallei* pada haiwan dan persekitaran tanpa mengira sumber pencilan dan rintangan terhadap antibiotik atau corak rentan antibiotik.

Sifat fizikokimia atau ciri persekitaran di mana organisma berada mempengaruhi taburan organisma. Sebanyak 78 pencilann (56 dari tanah dan 22 dari air) daripada persekitaran ladang ternakan telah dicirikan secara molekul menggunakan multilocus

sequence typing (MLST) dan sifat fizikokimia persekitaran telah dianalisis daripada 33 buah ladang ternakan di empat negeri iaitu Pahang, Perak, Selangor dan Negeri Sembilan di Semenanjung Malaysia. Logistik regresi multinomial mendapati terdapat hubungan yang berkeertian di antara kandungan air dalam tanah dan ST84 (OR = 0.833, 95% CI 0.708-0.980; P = 0.027) berbanding ST51. Ini menunjukkan bahawa dengan peningkatan kandungan air dalam tanah, ST51 adalah 1.2 kali lebih cenderung untuk hadir berbanding ST84. Secara statistik juga, terdapat hubungkait di antara pH air dan ST84 (OR = 0.401 95% CI 0.195-0.828; P = 0.013) berbanding ST51. Penemuan ini menunjukkan bahawa kejadian penyakit yang disebabkan oleh beberapa ST *B. pseudomallei* dipengaruhi oleh pelbagai faktor fizikokimia persekitaran (tanah dan air).

Kesimpulannya, kajian ini menemui dua novel alel *ace97* dan *lepA*, dan lima novel ST, ST1130, ST1131, ST1338, ST1339 dan ST1367. *Burkholderia pseudomallei* adalah klonal dan berasal dari CC48 di Asia Tenggara. Kewujudan beberapa strain yang tahan terhadap ubatt-ubatan yang penting dalam rawatan melioidosis di kalangan pencilan dari haiwan dan persekitaran menimbulkan ancaman klinikal kepada pengurusan haiwan atau peaskit yang terjangkit. Hubungkait telah ditunjukkan di antara ST *B. pseudomallei* dan kerintangan terhadap meropenem di kalangan pencilan haiwan. Pengesanan PCR gen pam efflux menunjukkan prevalen yang meluas bagi gen ini di kalangan *B. pseudomallei* dari haiwan dan persekitaran tanpa mengira antibiogram, sumber atau genotip. Sifat fizikokimia seperti kandungan air dalam tanah dan pH air memainkan peranan dalam mempengaruhi pengagihan genotip *B. pseudomallei* di kawasan endemik. Maklumat ini amat diperlukan dalam perancangan dan penilaian langkah kawalan melioidosis secara epidemiologi disesuaikan dengan terapi antibiotik dan intervensi persekitaran yang boleh mengubah faktor untuk mengurangkan pencemaran di kawasan bukan endemik.

ACKNOWLEDGEMENTS

I would like begin by expressing my profound gratitude to my creator Almighty Allah for giving me the opportunity, good health and wisdom to undergo this postgraduate study. I also wish to express my sincere appreciation to the chairman of my supervisory committee supervisors Assoc. Prof. Dr. Latiffah Hassan for her intellectual guidance, unreserved support, coaching, encouragement and construction of criticisms throughout the period of my study. I am most grateful for all that you did, enduring the rigors of meticulous reading, reviewing, corrections and observations for the success of this research. It is worthy of mention, those unrelenting persistence in questionings, insightful comments and useful advices she offered are pivotal in the achievements recorded in this work and above all for giving me the opportunity to work with her. I wish to equally express my sincere appreciation to other members of my supervisory committee, Assoc. Prof. Dr. Zunita Zakaria and Prof. Dr. Saleha A. Aziz for their relentless support, comments, guidance and insightful advice in the study design, data analysis and preparation of this thesis. Their support is invaluable in the accomplishment of the goals of this research and completion of this thesis.

I wish to extend my special appreciation to Dr. Maswati Mat Amin of Makmal Veterinar Kawasan Bukit Tengah, Pulau Pinang for providing us 10 *B. pseudomallei* animal isolates and Dr. Norlida Othman of Makmal Veterinar Kawasan Kubang Kerian, Kota Bharu, Kelantan for providing us two *B. pseudomallei* DNA extracts. I am thankful to Dr Hassan Ismail Musa for providing the environmental data and isolates used in this study. I wish to thank Prof. Mohammed, Omar Hussni of department of Population Medicine and Diagnostic Sciences, Cornell University USA for offering advice on data analysis. I equally appreciate the unreserved support and technical assistance rendered by Encik Mohammed Azri Roslan, Cik Krishnamma Kuppusami, Cik Rabiatul Adawyya and Puan Fauzia Nordin in the veterinary bacteriology laboratory, department of pathology and microbiology, faculty of animal medicine, UPM throughout the period of my the laboratory work. Also to Encik Mohammed Azizul Osman of the regional veterinary laboratory Bukit Tengah Penang for his technical assistance, Dr. Zarina Mohammed, Cpt (rtd) Mustafa and Encik Che Mat Bin Che Mud of the regional veterinary laboratory Kuban Krian, Kota Bharu, Kelantan for their technical assistance.

My special thanks go to my friends and colleagues Dr. Muhammad Modu Bukar, Dr. Adamu Kaikabo Ahmad Dawayo, Dr. Yakubu Ahmed Gaidam, Late Dr. Adulnasir Tijjani, Dr. Yusuf Abba, Dr. Konto Mohammed, Dr. Lawan Adamu for their support right from the beginning to completion of this thesis. My special thanks to Dr. Faez Firdaus Jesse Abdallah, Dr Saleh Mohammed Jajere, Dr Asimnamai Bitrus and Dr. Salina Ahmad for their support during the period of this study.

I am particularly grateful to my late parents Alhaji Aisami Muhammad Idris and Hajja Gambo Muhammad of blessed memory, may Allah (SWT) cleanse you from all your sins and may He grant you His Mercy and Jannatul Firdaus. For your love, care, guidance, admonishments and prayers have surely had influence in my sojourn through

life. I wish to extend my special hearty gratitude to my lovely wife Fatimah Kolo Abubakar for her unreserved love, care, support, encouragement, patience, tolerance, dedication and unalloyed confidence in me. She stood firmly behind me taking care of the children and giving me confidence and encouraging me to carry on with the struggle in the field of research. To my children Aisha, Fatima (Mamma), Ahmad (Amir), Fatimah Zahra and Muhammad (Walid) are indeed my source of joy, motivation, perseverance and strength throughout the research journey. May Allah guide and protect you all.

I certify that a Thesis Examination Committee has met on 13 January 2017 to conduct the final examination of Abubakar Sadiq Muhammad on his thesis entitled "Genetic Characterization and Antimicrobial Resistance Patterns of *Burkholderia pseudomallei* Isolates from Animals and Environment in Peninsular Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by graduate student

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ADH	Arginine dihydrolase
AHL	N-acyl-homoserine lactone
AMC	Amoxicillin-Clavulanic acid (co-amoxiclav)
ASA	Ashdown's selective agar
ASM	Ashdown's selective medium
ATM	Aztreonam
BLS-2	Biosafety level two
BLS-3	Biosafety level three
BPSA	*Burkholderia pseudomallei* selective agar
BTFC	*Burkholderia thailandensis*-like flagellum and chemotaxis
CAZ	Ceftazidime
CC	Clonal complex
CEC	Cation exchange capacity
CFU	Colony forming unit
CHL	Chloramphenicol
CI	Confidence interval
CIP	Ciprofloxacin
CLDC	Cationic lipid-DNA complex
CLSI	Clinical Laboratory Standards Institute
COD	Chemical oxygen demand
CRO	Ceftriaxone
CSIs	Conserved sequence indels
DLV	Double locus variant
DNA	Deoxyribonucleic acid
DOX	Doxycycline
DVS	Department of Animal Services

EC	Exchangeable calcium
EI	Extractive iron
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended spectrum β-lactamase
FAO	Food and agricultural organization
g	Gram
GEN	Gentamicin
GIT	Gastrointestinal tract
HAT	Haemagglutination test
IgG	Immunoglobulin G
IHA	Indirect haemagglutination assay
IPM	Imipenem
Kg	Kilogram
LDC	Lysine decarboxylase
LPS	Lipopolysaccharide
MDR	Multidrug resistant
MEGA 6	Molecular evolutionary genetics analysis six
MEM	Meropenem
meq	Milliequivalent
mg	Milligram
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
mL	Millilitre
MLST	Multilocus sequence typing
mm	Millimetre
MRSA	Methicillin-resistant *Staphylococcus aureus*
ng	Nanogram

NCBI	National center for biotechnology information
NIT	Nitrate to nitrite reduction test
NJ	Neighbour joining
OMP	Outer membrane protein
ONPG	Ortho-nitrophenyl-gamma-d-galactopyranosidase
OR	Odds ratio
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
qPCR	Quantitative real-time PCR
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RND	Resistance nodulation division
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SE	Standard error
SEA	Southeast Asia
SLV	Single locus variant
ST	Sequence type
STM	Signature-tagged mutagenesis
SXT	Trimethoprim-Sulfamethoxazole (cotrimoxazole)
TET	Tetracycline
TIC	Ticarcillin
TLV	Triple locus variant
TSA	Trypticase Soy Agar
TTSS	Type III secretion system
VNTR	Variable number tandem repeats

VRE	Vancomycin-resistant enterococci
YLF	*Yersinia*-like fimbrial gene
µg	Microgram
µL	Microliter

CHAPTER 1

INTRODUCTION

Melioidosis is an emerging, potentially fatal human and animal disease caused by the saprophytic soil and water dwelling bacteria *Burkholderia pseudomallei*. This organism, a member of the genus *Burkholderia*, is a β-proteobacteria, oxidase positive, motile, aerobic, non-spore-forming, Gram-negative rods ranging from 1 to 5μm in length and 0.5 to 1.0μm in width (Stoyanova *et al.*, 2007; Currie, 2010). They are ubiquitous, therefore readily recovered from water and wet soil such as in paddy fields within endemic areas (Coenye and Vandamme, 2003; Limmathurotsakul *et al.*, 2014). Melioidosis is endemic in Southeast Asia and northern Australia, regions located approximately between tropical latitudes 20°N and 20°S (Cheng and Currie, 2005). It has also been reported in other tropical regions, while endemic regions now expand to include the majority of the Indian subcontinent, southern China, Hong Kong and Taiwan (Currie *et al.*, 2008). Over the past two decades, melioidosis is emerging as it is being increasingly recognised more frequently both within established endemic areas and elsewhere in the world (Dance, 2000b).

Melioidosis have been reported in animals by several authors in Malaysia and elsewhere (O'Brien *et al.*, 2003; Ouadah *et al.*, 2007; Naama *et al.*, 2012). Overall a serological prevalence of 5.7% has been reported among livestock in Malaysia (Musa *et al.*, 2012). The species-specific seroprevalence rates of melioidosis among livestock in Malaysia were reported as follows: cattle 7.6%, buffaloes 48.2%, goats 2.6%, sheep 13.6% and pigs 3.6% (Musa *et al.*, 2012). In Malaysia most studies on melioidosis agent have mainly focused on those that affect humans and there is a dearth of information about animal and environmental (soil and water) isolates of *B. pseudomallei*. Among humans, infections due to *B. pseudomallei* occur in most communities in endemic areas without obvious clustering in time or place. It is still not clear whether the epidemiology of melioidosis in animals and humans are directly linked. There is no suggestive evidence from previous studies that linked specific bacterial strains or sequence type to a particular host or to the likelihood of enhanced ability to cause invasive melioidosis. In addition, there is dearth of information on phylogenetic variability and its relatedness to antibiotic resistance pattern, resistance genes and environmental properties in animal and environmental *B. pseudomallei* isolates in Peninsular Malaysia.

Genotyping of environmental (soil and water) and animal clinical isolates is essential to link outbreaks of infection by this saprophyte to a common contaminated source (Currie *et al.*, 2001). Nucleotide sequence-based methods for bacterial typing (multilocus sequence typing; MLST), that indexes the dissimilarity in the sequences of the seven housekeeping genes, allow rapid and global comparisons between results from different laboratories (Cooper and Feil, 2004). Multilocus sequence typing (MLST) of *Burkholderia pseudomallei* has been described (Godoy *et al.*, 2003; McCombie *et al.*, 2006). Previous studies have suggested the biogeographical

clustering of *B. pseudomallei* strains and sequence type (STs) (Vesaratchavest *et al.*, 2006; Currie *et al.*, 2007; Pearson *et al.*, 2009; Dale *et al.*, 2011; McRobb *et al.*, 2014). However, in Malaysia, works has not been done to determine the diversity of STs among the local (Malaysian) *B. pseudomallei* isolates from animal melioidosis cases and from the environments. Therefore, it remains to be determined if certain *B. pseudomallei* STs have an enhanced potential to cause melioidosis in animals and if biogeographical clustering exist for animal melioidosis cases.

Infections with *B. pseudomallei* can be treated with antibiotics but it is biphasic and usually a lengthy treatment regimen consisting of a short-term parenteral acute phase and a long-term oral eradication phase treatments (Wuthiekanun and Peacock, 2006; Estes *et al.*, 2010). *Burkholderia pseudomallei* resistance to cotrimoxazole, ceftriaxone, amoxicillin/clavulanate and doxycycline has been reported (Jenney *et al.*, 2001). Resistance of clinical isolates of *B. pseudomallei* to parenteral amoxicillin-clavulanic acid and ceftazidime has been demonstrated among melioidosis patients (Wuthiekanun *et al.*, 2011). Various methods for determining antimicrobial susceptibility of bacteria have been described, these include agar or broth dilution methods (CLSI, 2014), disc diffusion method (Coyle, 2005) and minimum inhibitory concentration (MIC) testing using E-test strips (Jorgensen and Ferraro, 1998). Disc diffusion test and E-test has been compared to assess the agreement of the two tests to the susceptibility and resistance patterns of *B. pseudomallei* to cotrimoxazole. Where significantly poor agreement was observed suggesting the superiority of E-test, as disc diffusion method was found to overestimate resistance to cotrimoxazole (Piliouras *et al.*, 2002). A previous study by Thomas *et al.* (1981b) in Australia on antibiotic resistance pattern in animal and environmental *B. pseudomallei* did not cover their phylogenetic relatedness. While some works have been performed on human clinical isolates in Malaysia with regards to antibiotic resistance pattern (Ahmad *et al.*, 2012; Ahmad *et al.*, 2013; Khosravi *et al.*, 2014), none have reported the resistance pattern for animal or environmental isolates.

The antibiotic treatment of *B. pseudomallei* infection is difficult, because these bacteria demonstrate a high level of intrinsic resistance to most common available antibiotics. These organisms have been found to demonstrate intrinsic resistance to antibiotics such as quinolones, polymyxins and beta-lactam (β-lactam) agents, including monobactams and carbapenems (Waters and Ratjen, 2006). *Burkholderia pseudomallei* have been described to exhibit natural resistance to gentamicin and other aminoglycosides (Schweizer and Peacock, 2008). Multiple drug efflux pumps of the resistance-nodulation-cell division (RND) superfamily, production of hydrolytic enzymes, β-lactamases, decreased cellular permeability through outer membrane protein (Omp) and deletion of antibiotic target were the four main antibiotic resistance mechanisms of *B. pseudomallei* so far established (Schweizer, 2012a). It is believed that all resistance mechanisms of *B. pseudomallei* to antibiotics are due to chromosomally encoded RND and β-lactamases (Livemore *et al.*, 1987; Godfrey *et al.*, 1991). The efflux pumps have been described to be the most dominant multidrug resistance mechanism in *B. pseudomallei* affecting various classes of clinically relevant antibiotics (Schweizer, 2012a). Previously, Kumar *et al.* (2008), reported detection of varying proportion of RND efflux pump genes amongst

human clinical isolates of *B. pseudomallei* in Australia. Currently there are no reports of detection of these antibiotic resistance genes among animal and environmental isolates from Malaysia or elsewhere.

The saprophytic organism *B. pseudomallei* is ubiquitous in endemic areas and can be readily isolated from the environmental niches such as water, moist soil and rice paddies (Brook *et al.*, 1997). However the occurrence of the organism is known to be affected by physicochemical factors in the environment (Ngamsang *et al.*, 2015). The occurrence of *B. pseudomallei* in the soil was reported to be influenced by soil moisture, texture, pH, soil organic matter, mineral content and salinity (Kaestli *et al.*, 2009; Draper *et al.*, 2010). Previous studies have demonstrated the differences in distribution of sequence type (ST) between sampling areas (Wuthiekanun *et al.*, 2009; McRobb *et al.*, 2014). The effects of environmental physicochemical factors on the occurrence and distribution of *B. pseudomallei* STs have not been elucidated.

The main objective of this study was to generate epidemiological information on phylogenetic variability and its relatedness to antibiotic resistance pattern and genes among animal and environmental (soil and water) *B. pseudomallei* isolates in Peninsular Malaysia.

The hypotheses of this study are:

1. Similar sequence types of *B. pseudomallei* can be recovered from environmental (soil and water) and animal sources in Peninsular Malaysia.
2. The *B. pseudomallei* sequence types obtained in this study are consistent with those obtained elsewhere.
3. The antimicrobial resistance patterns of *B. pseudomallei* isolates from animals and environment are similar.
4. There is no difference in the frequency of antimicrobial resistance genes between isolates from animals and the environment
5. There is no association between the molecular sequence types to the physical and chemical properties of the environment.

The objectives of this study are:

1. To determine the genotypes of *B. pseudomallei* isolates from animals and the environment (soil and water) in Peninsular Malaysia.
2. To compare the phylogeny of local *B. pseudomallei* isolates to those from elsewhere in the world.
3. To determine the antimicrobial resistance pattern among clinical and environmental isolates.
4. To determine the occurrence of antimicrobial resistance genes and compare to the resistance pattern observed.
5. To determine the association between the sequence types to the physical and chemical characteristics of environments (soil and water) where the isolates originated.

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