DEVELOPMENT OF A DNA-BASED METHOD FOR SIMULTANEOUS DETECTION OF *Acinetobacter baumannii*, ANTIMICROBIAL RESISTANCE GENES AND ITS GENOTYPES BY DNA FINGERPRINTING

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol/Abbreviation	Definition
®	Registered
™	Trademark
+	Plus or Positive
-	Minus or Negative
$\geq$	Greater than or equal to
$\leq$	Less than or equal to
$\pm$	Plus/Minus
=	Equal to
%	Percent
/	Per
'	Prime
π_j	Frequency n_j/n
σ^2	Variance
$\sum$	Sum
°C	Degree celsius
β	Beta
χ	Degree of precision
µg/ml	microgram per millilitre
µl	Microlitre
µm	Micrometer
µM	Micromolar
bp	Base pair
C	Concentration
CFU	Colony forming unit

Symbol/Abbreviation	Definition
CFU/ml	Colony forming unit per millilitre
Da	Dalton
g	Relative centrifugal force
g	Gram
kb	Kilobase
kDa	KiloDalton
L	Litre
M	Molar
mA	Milliampere
mBar	Millibar
mg/ml	milligram per millilitre
ml	Millilitre
mm	Millimeter
mM	Millimolar
N	Total number of isolates in the sample population
n	Sample size
n_j	Number of strains belonging to j^{th} type
ng	Nanogram
ng/μl	nanogram per microlitre
nm	Nanometer
p	Sensitivity or Specificity
pg	Picogram
rpm	Revolutions per minute
s	Total number of types described
T_a	Annealing temperature
T_e	Elevated temperature

Symbol/Abbreviation	Definition
T_m	Melting temperature
T_r	Room temperature
U	Enzyme unit
U/μl	Enzyme unit per microlitre
V	Volt or Volume
V/cm	Volt per centimeter
x	Time
A	Adenine/adenosine
ADC	*Acinetobacter*-derived cephalosporinase
AME	Aminoglycoside-modifying enzyme
AFLP	Amplified fragment length polymorphism
ARDRA	Amplified ribosomal DNA gene restriction analysis
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BCCM^{TM}/LMG	BCCM^{TM}/LMG Bacteria Collection
BHI	Brain heart infusion
BLAST	Basic Local Alignment Search Tool
C	Cytosine
Carbapenemase	Carbapenem-hydrolyzing β-lactamase
CI	Confidence interval
CHDL	Carbapenem-hydrolyzing class D β-lactamase
D	Simpson's index of diversity
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum β-lactamase

Symbol/Abbreviation	Definition
EU	European clone
G	Guanine/guanosine
GIM	German imipenemase
HCl	Hydrochloric acid
HGDI	Hunter-Gaston diversity index
HGM	Hospital Gua Musang
HM	Hospital Machang
Hospital USM	Hospital Universiti Sains Malaysia
HRPZII	Hospital Raja Perempuan Zainab II
HTA	Hospital Tengku Anis
IC	Internal control
ICL	International clonal lineage
ICU	Intensive care unit
Ig	Immunoglobulin
IMP	Active on imipenem
L-repeat VNTR	Large-repeat variable-number tandem-repeat
LAMP	Loop-mediated isothermal amplification
LB	Luria-Bertani
LMW	Low molecular weight
LoD	Limit of detection
MALDI-ToF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
$MgCl_2$	Magnesium chloride
MLST	Multilocus sequencing typing

Symbol/Abbreviation	Definition
MLVA	Multiple-locus variable-number tandem-repeat analysis
MRVP	Methyl-Red Vogas-Proskauer
MST	Minimum spanning tree
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
NDM	New Delhi metallo-β-lactamase
non-MDR	Non-multidrug-resistant
OMP	Outer membrane protein
PBP	Penicillin binding protein
PBS Tween-20	Phosphate buffer saline Tween-20
PCR	Polymerase chain reaction
PDR	Pandrug-resistant
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplified polymorphic DNA
rep-PCR	Repetitive sequence-based polymerase chain reaction
S-repeat VNTR	Small-repeat variable-number tandem-repeat
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	Seoul imipenemase
SPM	Sao Paulo metallo-β-lactamase
T	Typeability
T	Thymine/thymidine
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TBE	Tris-borate-EDTA
TE	Tris-EDTA

Symbol/Abbreviation	Definition
TR	Tandem repeat
UKMMC	Universiti Kebangsaan Malaysia Medical Centre
UPGMA	Unweighted pair group method with arithmetic averages
UV	Ultraviolet
VIM	Verona integron-encoded metallo-β-lactamase
VNTR	Variable-number tandem-repeat
W	Wallace coefficient
WGS	Whole-genome sequencing
WW	Worldwide
XDR	Extensive-drug-resistant

PEMBANGUNAN UJIAN DIAGNOSTIK BERASASKAN DNA BAGI PENGESANAN SERENTAK *Acinetobacter baumannii*, GEN-GEN KERINTANGAN ANTIBIOTIK DAN PENGENOTIPAN MELALUI KAEDAH PENJUJUKAN DNA

ABSTRAK

Spesies *Acinetobacter* telah muncul sebagai patogen penting yang berkait dengan penjagaan kesihatan di seluruh dunia. Berdasarkan liputan kajian terhadap *Acinetobacter*, *A. baumannii* merupakan patogen yang paling ketara implikasinya terhadap pelbagai jangkitan kronik dan mengakibatkan kadar morbiditi dan mortaliti yang tinggi dalam kalangan pesakit, terutamanya pneumonia yang terkait dengan ventilator dan jangkitan saluran darah. Kaedah rutin bagi pengenalpastian patogen pada masa kini tidak mampu mengenalpasti *Acinetobacter* sehingga ke peringkat spesies. Sehubungan itu, kajian ini bertujuan untuk membangunkan sebuah assai yang berkebolehan mengesan secara serentak genus *Acinetobacter*, *A. baumannii* serta empat gen yang mengekod *carbapenem-hydrolyzing class D β-lactamase* dan untuk mengkaji epidemiologi molekul bagi pencilan-pencilan klinikal yang mudah terdedah atau sebaliknya terhadap karbapenem. Bagi mencapai objektif kajian, sebuah assai PCR multipleks stabil suhu telah dibangunkan berdasarkan primer yang direka secara spesifik pada bahagian gen 16S rRNA, protein membran luaran bersaiz 33-36 kDa dan empat gen *carbapenem-hydrolyzing class D β-lactamase*. Satu kawalan dalaman telah dikombinasikan ke dalam assai ini supaya kebolehpercayaan dan kekuatan assai ini dapat disahkan. Pengesanan spesifik genus *Acinetobacter*, *A. calcoaceticus-A. baumannii* kompleks dan *A. baumannii* pada kultur bakteria tulen dan kultur darah *spike* telah dijalankan dengan menggunakan assai ini. Kajian ini mendapati bahawa had terendah pengesanan DNA yang ditulenkan adalah 100 pg, manakala DNA yang diperoleh secara penguraian termal sama ada daripada kultur bakteria atau spesimen *spike* dalam darah manusia adalah 10^6 CFU/ml. Assai ini juga mampu mengesan sekurang-kurangnya satu CFU sel bakteria daripada medium yang telah diperkaya. Assai ini mampu

mengesan *Acinetobacter* serta gen-gen resistannya dalam masa tiga jam dengan kadar spesifisiti dan sensitiviti yang tinggi (100%). Penilaian pecutan kestabilan reagen-reagen PCR multipleks stabil suhu menunjukkan bahawa campuran yang divakum-kering ini stabil pada suhu bilik selama 232 hari. Assai PCR multipleks stabil suhu ini turut membolehkan pengenalpastian bakteria secara serentak dan pengesanan gen resistan yang mungkin berguna dalam diagnosis segera bagi mengurangkan kadar morbiditi dan mortaliti pesakit yang mengalami jangkitan *Acinetobacter*. Pengkhususan terhadap pencilan-pencilan *Acinetobacter* yang diperoleh daripada 115 spesimen darah terkumpul selama 24 bulan turut dijalankan dengan menggunakan analisis pembatasan gen yang diamplifikasi daripada gen *ribosomal DNA* . *A. baumannii* (60.87%; 70 pencilan) didapati merupakan spesies predominan *Acinetobacter*, diikuti dengan *A. nosocomialis* (19.13%; 22 pencilan). Daripada sejumlah 115 pencilan *Acinetobacter*, 46.09% dan 5.22% daripada *A. baumannii* dan *Acinetobacter* spesies masing-masing adalah resistan karbapenem. *Pulsed-field gel electrophoresis* turut dijalankan untuk menentukan hubungan genetik antara pencilan-pencilan klinikal yang mudah terdedah dan yang tidak mudah terdedah terhadap karbapenem. Kesemua pencilan *A. baumannii* yang tidak mudah terdedah terhadap karbapenem didapati membawa gen β-laktamase secara konsisten, iaitu $bla_{\text{OXA-51-like}}$ (100%; semua 70 pencilan) dan $bla_{\text{OXA-23-like}}$ (34.29%; 24 pencilan). Dua kluster predominan merangkumi hampir kesemua pencilan *A. baumannii* yang tidak mudah terdedah terhadap karbapenem setiap tahun. Berdasarkan analisis *multiple-locus variable-number tandem-repeat analysis*, pencilan-pencilan *A. baumannii* dalam kajian ini kebanyakannya didapati berasal daripada klonal antarabangsa warisan II yang tersebar meluas serata dunia. Kajian ini seterusnya mendapati bahawa kemunculan ahli yang resistan karbapenem di kalangan genus *Acinetobacter* (selain daripada *A. baumannii*) menggambarkan kepentingan penggunaan agen antimikrobial secara berhemah serta pelaksanaan kaedah-kaedah kawalan jangkitan agar perkembangan fenotip dan penyebaran klonal resistan di penempatan hospital dapat dikurangkan.

DEVELOPMENT OF A DNA-BASED METHOD FOR SIMULTANEOUS DETECTION OF *Acinetobacter baumannii*, ANTIMICROBIAL RESISTANCE GENES AND ITS GENOTYPES BY DNA FINGERPRINTING

ABSTRACT

Acinetobacter species have emerged as important healthcare associated pathogens worldwide. Of the described *Acinetobacter*, *A. baumannii* constitutes as the most significant causative agent implicating various severe infections, especially ventilated associated pneumonia and bloodstream infections, associated high mortality and morbidity in patients. Current routine identification systems are unable to identify *Acinetobacter* to the species level. Hereof, this study aimed to develop a reliable tool for simultaneous detection of *Acinetobacter* genus, *A. baumannii* and four genes encoding the carbapenem-hydrolyzing class D β-lactamases, and to investigate the molecular epidemiology of carbapenem susceptible and non-susceptible *A. baumannii* clinical isolates in a hospital setting. A thermostabilized multiplex PCR assay was developed with primers designed on specific sequence regions of 16S rRNA gene, 33-36 kDa outer membrane protein and four carbapenem-hydrolyzing class D β-lactamase genes to achieve the goal of this study. An internal control was incorporated to validate reliability and robustness of the assay. Specific detection of *Acinetobacter* genus, *A. calcoaceticus-A. baumannii* complex and *A. baumannii* on pure bacterial cultures and spiked blood cultures was demonstrated using the developed assay. The assay yielded detection limits of 100 pg of purified DNA and 10^6 CFU/ml with DNA thermolysates prepared from either bacterial cultures or spiked human whole blood specimens. The assay was capable to detect at least one CFU of bacterial cells in a pre-enriched medium. The assay detected *Acinetobacter* with its resistance genes in three hours with high specificity and sensitivity (100%). Accelerated stability evaluation of thermostabilized multiplex PCR reagents demonstrated that vacuum-dried mixes were stable at room temperature for approximately 232 days. The developed thermostabilized multiplex

PCR assay enabled simultaneous bacterial identification and detection of its resistance gene which would be useful in rapid diagnosis to reduce morbidity and mortality of patients with *Acinetobacter* infections. Speciation on *Acinetobacter* isolates recovered from 115 blood specimens collected over 24-month period with amplified ribosomal DNA restriction analysis was performed. *A. baumannii* (60.87%; 70 isolates) was found to be the predominant *Acinetobacter* genomic species followed by *A. nosocomialis* (19.13%; 22 isolates). Of the total 115 *Acinetobacter* isolates, 46.09% and 5.22% of *A. baumannii* and *Acinetobacter* species, respectively, were carbapenems-resistant. Pulsed-field gel electrophoresis was performed to ascertain genetic relatedness of carbapenem susceptible and non-susceptible *A. baumannii* clinical isolates. All the carbapenem non-susceptible *A. baumannii* isolates were consistently found to harbour β-lactamase gene, *bla*$_{OXA-51-like}$ (100%; all 70 isolates) and *bla*$_{OXA-23-like}$ (34.29%; 24 isolates). Two predominant clusters contained of mostly carbapenems non-susceptible *A. baumannii* isolates were observed in each year of study. Based on the multiple-locus variable-number tandem-repeat analysis, the characterized *A. baumannii* isolates mostly belonged to the international clonal lineage II, of which is worldwide distributed. Finding of this study further demonstrated the emergence of carbapenem resistance in members within the *Acinetobacter* genus (other than *A. baumannii*), emphasizing the importance of wisely prescribed antimicrobial agents and stringent implementing infection control measures to reduce further the development of resistance phenotypes and clonal spreading in clinical settings.

CHAPTER ONE

INTRODUCTION

1.1   Introductory and microbiology of *Acinetobacter* genus

Acinetobacter, derived from a word of Greek, "akinetos", means non-motile, was proposed to distinguish non-motile bacteria from motile microorganisms within genus of *Achromobacter* (Brison and Prévot, 1954). However, the proposed genus designation was not widely accepted until year 1968 (Baumann *et al.*, 1968). Taxonomy of *Acinetobacter* genus underwent a great refinement since year 1980s parallel with the emergence of *Acinetobacter* as causative pathogens for healthcare associated infections in global hospital settings (Dijkshoorn *et al.*, 2007).

1.1.1   Taxonomy and microbiology of *Acinetobacter* species

In current taxonomic classifications, members of *Acinetobacter* genus are classified under family of Moraxellaceae within Gammaproteobacteria, a suborder of Proteobacteria (Peleg *et al.*, 2008). The *Acinetobacter* genus comprises Gram-negative, aerobic, non-fastidious, glucose non-fermentative, non-motile bacteria with guanine and cytosine content of 39% to 47% (Peleg *et al.*, 2008). Most *Acinetobacter* species are metabolic versatile which can grow easily on basic microbiological culture media (Visca *et al.*¸ 2011). In fact, a culture medium contains acetate and nitrate as source of carbon and nitrogen, respectively, is used to enrich *Acinetobacter* growth and recover them from composite microbial communities in specimens (Visca *et al.*¸ 2011). Furthermore, clinically relevant *Acinetobacter* genomic species demonstrate an optimal growth at 37°C, however, this growth temperature may not applicable to all environmental *Acinetobacter* species (Visca *et al.*¸ 2011).

In year 1986, 11 of 12 *Acinetobacter* isolations from various specimen types were unambiguously delineated with formal species names assigned for only a few genomic species (Bouvet and Grimont, 1986). Thereafter, valid species names were given to those

previously described *Acinetobacter* genomic species with provisional designations (Krizova *et al.*, 2015; Nemec *et al.*, 2011; Nemec *et al.*, 2010; Tjernberg and Ursing, 1989; Nishimura *et al.*, 1988; Bouvet and Grimont, 1986). Additional novel *Acinetobacter* genomic species were later isolated from various sources and identified, expanding number of members within the genus. Currently, *Acinetobacter* genus encompasses at least 38 genomic species, in which 30 and eight genomic species were assigned with valid species names and provisional designations, respectively (Table 1.1).

1.1.2   Natural reservoir of *Acinetobacter* species

As an overall, *Acinetobacter* species are ubiquitous microorganisms in nature, which can be easily recovered from various sources following bacterial enrichment cultivations (Peleg *et al.*, 2008). Indeed, common isolation of certain *Acinetobacter* genomic species, particularly *A. baumannii*, *A. nosocomialis* and *A. pittii*, from environments, animals and human commensals are controversial matters in existing literature (Towner, 2009). Occurrence and epidemiology of clinically important *Acinetobacter* in healthcare institutions have been documented. Due to the arguments, the existence of *Acinetobacter* outside hospitals with more focused on *A. baumannii* has progressively been elucidated in recent studies (Pailhoriès *et al.*, 2015a; Rafei *et al.*, 2015), providing a better perspective on their potential environmental and community reservoirs, albeit precise natural reservoirs are still obscure.

Species distribution of *Acinetobacter* on inanimate substances has been determined for water, soil, vegetable, meat, milk, fish and cheese samples (Rafei *et al.*, 2015; Choi *et al.*, 2012; Houang *et al.*, 2001; Berlau *et al.*, 1999b). An exploratory survey on swabs sampled from a range of environments together with soil samples in South Korea, found *Acinetobacter* isolations possessed 7.1% (29 isolates) of all 409 bacterial isolates, in which *A. baumannii*, *A. pittii* and *A. nosocomialis* only contained a total of seven isolates (Choi *et al.*, 2012). Another survey conducted in Hong Kong, *A. baumannii* represented 23% among *Acinetobacter* isolated from 66 soil samples (Houang *et al.*, 2001). Moreover, *A. baumannii*

Table 1.1   Classification of named and tentative genomic species within *Acinetobacter* genus

Species	Genomic species	Type or representative strain	Source of isolation	Reference
Acinetobacter baumannii	2	ATCC 19606	Human specimens and hospital environmental samples	Bouvet and Grimont, 1986
Acinetobacter baylyi		DSM 14961	Activated sludge	Carr *et al.*, 2003
Acinetobacter beijerinckii		NIPH838	Soil, water	Visca *et al.*, 2011
Acinetobacter bereziniae	10	ATCC 17924	Human specimens, hospital environmental samples, sewage	Nemec *et al.*, 2010; Bouvet and Grimont, 1986
Acinetobacter bouvetii		DSM 14964	Activated sludge	Carr *et al.*, 2003
Acinetobacter brisouii		DSM 18516	Peat layer	Anandham *et al.* 2010
Acinetobacter calcoaceticus	1	ATCC 23055	Soil	Bouvet and Grimont, 1986
Acinetobacter gerneri		DSM 14967	Activated sludge	Carr *et al.*, 2003
Acinetobacter grimontii		DSM 14968	Activated sludge	Carr *et al.*, 2003
Acinetobacter guillouiae	11	ATCC 11171	Human specimens, sewage, activated sludge, water, soil, raw milk, contact lens	Nemec *et al.*, 2010; Bouvet and Grimont, 1986
Acinetobacter gyllenbergii		NIPH2150	Human specimens	Visca *et al.*, 2011
Acinetobacter haemolyticus	4	ATCC 17906	Human specimens	Bouvet and Grimont, 1986
Acinetobacter Iwoffii	8/9	ATCC 15309	Human and animal specimens	Bouvet and Grimont, 1986
Acinetobacter johnsonii	7	ATCC 17909	Human and animal specimens, waste water	Bouvet and Grimont, 1986
Acinetobacter junii	5	ATCC 17908	Human specimens	Bouvet and Grimont, 1986
Acinetobacter kyonggiensis		KSL5401-037	Sewage treatment plant	Lee and Lee, 2010
Acinetobacter nosocomialis	13TU	ATCC 17903	Human specimens	Nemec et al., 2011; Tjernberg and Ursing, 1989
Acinetobacter parvus		NIPH384	Human specimens	Nemec *et al.*, 2003
Acinetobacter pittii	3	ATCC 19004	Human specimens and hospital environmental samples	Nemec *et al.*, 2011; Bouvet and Grimont, 1986
Acinetobacter radioresistens	12	IAM 13186	Cotton and soil	Nishimura *et al.*, 1988; Bouvet and Grimont, 1986

Table 1.1 continued

Species	Genomic species	Type or representative strain	Source of isolation	Reference
Acinetobacter rudis		LMG 26107	Raw milk and waste water	Vaz-Moreira *et al.*, 2011
Acinetobacter schindleri		NIPH1034	Human specimens	Nemec *et al.*, 2001
Acinetobacter septicus		AK001	Human specimens and catheter tip samples	Kilic *et al.*, 2008
Acinetobacter soli		KCTC 22184	Soil	Kim *et al.*, 2008
Acinetobacter tandoii		DSM 14670	Activated sludge	Carr *et al.*, 2003
Acinetobacter tjernbergiae		DSM 14971	Activated sludge	Carr *et al.*, 2003
Acinetobacter towneri		DSM 14962	Activated sludge	Carr *et al.*, 2003
Acinetobacter ursingii		NIPH137	Human specimens	Nemec *et al.*, 2001
Acinetobacter variabilis	15TU	NIPH2171	Human and animal specimens	Krizova *et al.*, 2015
Acinetobacter venetianus		ATCC 31012	Marine water	Visca *et al.*, 2011
	6	ATCC 17979	Human specimens	Bouvet and Grimont, 1986
	13BJ or 14TU	ATCC 17905	Human specimens	Visca *et al.*, 2011
	14BJ	CCUG 14816	Human specimens	Visca *et al.*, 2011
	15BJ	SEIP 23.78	Human specimens	Visca *et al.*, 2011
	16	ATCC 17988	Human specimens	Visca *et al.*, 2011
	17	SEIP Ac87.314	Human specimens, soil	Visca *et al.*, 2011
	Between 1 and 3	ATCC 17903	Human specimens	Bouvet and Grimont, 1986
	Close to 13TU	10090	Human specimens	Gerner-Smidt and Tjernberg, 1993

Abbreviations: 'BJ': Bouvet and Jeanjean; 'TU': Tjernberg and Ursing

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was also recovered from manure agricultural soil and pig slurry in United Kingdom (Byrne-Bailey *et al.*, 2009) and agriculture environments (fish and shrimp farms) in Southeast Asia (Huys *et al.*, 2007).

On the other hand, *Acinetobacter* carriage on human superficial body sites was found to be more frequent in hospitalized patients (75%) compared to healthy ambulatory volunteers (approximately 45%), with both populations were dominated by *A. Iwoffii* (47% to 61%) (Berlau *et al.*, 1999a; Seifert *et al.*, 1997). Moreover, *A. johnsonii*, *Acinetobacter* genomic species 15BJ, *A. radioresistens*, *A. pittii*, *A. nosocomialis* and *A. baumannii* were also isolated at lower percentages (21% to 0.5%). Controversial findings on the prevalence of *Acinetobacter* species distribution as human skin commensals were reported. Comparing to previous surveys, *A. pittii* (36%), *A. nosocomialis* (15%), *Acinetobacter* genomic species 15TU (6%) and *A. baumannii* (4%) were more frequently detected on skin of healthy Hong Kong residents (Chu *et al.*, 1999). Skin carriage of *A. calcoaceticus*, *A. baumannii*, *A. pittii* and *A. nosocomialis* was not detected on healthy United States soldiers (Griffith *et al.*, 2007). Furthermore, incidence of *Acinetobacter* in human faeces was determined with 224 specimens collected from non-hospitalized individuals, only 24 faecal specimens yielded three *Acinetobacter* genomic species of *A. johnsonii* (7.5%), *Acinetobacter* genomic species 11 (2.2%) and *A. baumannii* (0.9%) (Dijkshoorn *et al.*, 2005). Besides that, *A. baumannii* was also recovered from body lice sampled from elementary school children (Bouvresse *et al.*, 2011) and homeless people (La Scola and Raoult, 2004)..

Apart from human specimens and abiotic surfaces, *A. baumannii* is also regarded as an emerging opportunistic pathogen in veterinary medicine and its epidemic spread among animals in veterinary healthcare settings have been described (Pailhoriès *et al.*, 2015a; Endimiani *et al.*, 2011; Zordan *et al.*, 2011; Brachelente *et al.*, 2007). In a study conducted in Germany, *A. baumannii* isolates obtained from several veterinary clinics were resistance to multiple antimicrobial agents tested and most of them were genetically congruent with

epidemic clonal lineages (Zordan *et al.*, 2011). Among 141 animals sampled from veterinary clinics located at La Réunion Island, only 12 isolations yielded *A. baumannii* (8.5%) which exhibited susceptible or multidrug-resistant phenotype with diverse genotypic profiles (Pailhoriès *et al.*, 2015a). Outside hospital epidemiology of *A. baumannii* in Lebanon was investigated and yielded 30 *A. baumannii* of a total 110 *Acinetobacter* isolations from animals (Rafei *et al.*, 2015). All *A. baumannii* isolates were susceptible to most antimicrobial agents tested and exhibited novel genotypic profiles. These findings are therefore indicating that animals may constitute as a potential reservoir for *A. baumannii* transmission to environments, animals or humans (Pailhoriès *et al.*, 2015a; Rafei *et al.*, 2015). However, further systematic studies are needed to gain a better insight on interactions between humans and potential reservoirs in causing *Acinetobacter* infections.

1.2   Clinical manifestation of *Acinetobacter* species

Acinetobacter are well recognized as significant nosocomial pathogens implicating various infections, such as pneumonia, secondary meningitis, skin and soft tissue infections, wound infections, urinary tract infections and bloodstream infections (Dijkshoorn *et al.*, 2007). Most of the infections are primarily attributed to *A. baumannii* and a lesser extent to *A. nosocomialis* and *A. pittii* (Visca *et al.*, 2011; Dijkshoorn *et al.*, 2007). While, other *Acinetobacter* genomic species of *A. Iwoffii*, *A. ursingii*, *A. johnsonii*, *A. parvus*, *A. radioresistens*, *A. haemolyticus*, *A. junii*, *A. beijerinckii*, *A. bereziniae*, *A. gyllenbergii*, *A. schindleri*, *Acinetobacter* genomic species 15TU and *Acinetobacter* genomic species 16 are regarded as emerging causative pathogens in human related nosocomial infections with majority restricted to bacteraemia, septicaemia and pneumonia, albeit low incidences were reported (Wisplinghoff *et al.*, 2012; Turton *et al.*, 2010; Boo *et al.*, 2009). Infections caused by non-*A. baumannii* are often benign clinical courses and least mortality on infected patients (Visca *et al.*, 2011).

1.2.1   Risk factor of *Acinetobacter* acquisition and infection

Several retrospective studies have been conducted to identify risk factors for acquisition and infection with *Acinetobacter*. The identified risk factors included previous antimicrobial therapy (Inchai *et al.*, 2015; Huang *et al.*, 2014; Zheng *et al.*¸ 2013; Huang *et al.*, 2012; Punpanich *et al.*¸ 2012), premature birth (Punpanich *et al.*¸ 2012; Al Jarousha *et al.*, 2009), intensive care unit admission (Huang *et al.*, 2014), undergone invasive procedures such as mechanical ventilation and total parenteral nutrition (Huang *et al.*, 2014; Zheng *et al.*, 2013; Huang *et al.*, 2012; Punpanich *et al.*¸ 2012), systemic and severe illnesses (Inchai *et al.*, 2015; Huang *et al.*, 2014; Zheng *et al.*¸ 2013; Huang *et al.*, 2012) as well as intubation of indwelling devices such as intravenous catheters and arterial line catheters (Huang *et al.*, 2014; Huang *et al.*, 2012).

1.2.2   Nosocomial infections

1.2.2.1   Ventilator associated pneumonia

Ventilator associated pneumonia is a common healthcare associated infection among patients admitted to critical care settings with mortality rates in ventilated patients ranged from 20% to 70% (Rosenthal *et al.*¸ 2011). A retrospective Vietnamese study reported substantial annual increments in *Acinetobacter* isolations from tracheal aspirate specimens of ventilated patients admitted to an intensive care unit over a 11-year duration (from year 2000 to year 2010), responsible for 46% of all pneumonia cases in year 2010 (Nhu *et al.*, 2014). Surveillance data retrieved from Asian Network revealed the prevalence of *Acinetobacter* in ten Asian countries, with the lowest percentage observed in Hong Kong (9.8%) to the highest percentage of 49.8% observed in Thailand (Chung *et al.*, 2011). The prevalence of *Acinetobacter* reported for ventilated associated pneumonia was 21.8% in Malaysia (Chung *et al.*, 2011). Recent data from The Surveillance Network database revealed 40.5% (12,915 respiratory specimens) of all significant 31,889 *A. baumannii* specimens from patients admitted to intensive care units throughout 217 United States hospitals (Zilberberg *et al.*, 2016). Patients with *A. baumannii* bacteremic pneumonia presented poor prognosis coupled

with high antimicrobials resistances and mortality rates compared to patients infected with *A. nosocomialis* (Lee *et al.*¸ 2013).

1.2.2.2   Bloodstream infection

Acinetobacter species ranked 10th among the most common etiologic pathogens responsible for nosocomial bloodstream infections in United States, accounted for 1.3% (273 of 20,978 cases) of monomicrobial cases in 49 hospitals over a 7.5-year period (Wisplinghoff *et al.*, 2004). Data retrieved from another surveillance network revealed 18.9% (7,431 of 39,320 cases) of *A. baumannii* cases were derived from patients with bloodstream infections during three different time periods throughout 217 United States hospitals (Zilberberg *et al.*, 2016). A prospective nationwide surveillance study, *Acinetobacter* species ranked 4th among the most common pathogens responsible for nosocomial bloodstream infections, accounted for 306 of 2447 monomicrobial cases (12.5%) in 16 Brazilian hospitals over a 2.8-year study period (Marra *et al.*, 2011). Besides that, the prevalence of *Acinetobacter* species responsible for bloodstream infections in South Africa, Malaysia and China was 16.0% (McKay and Bamford, 2015), 6.1% (Deris *et al.*, 2009) and 4.6% (Wu *et al.*, 2015a), respectively

Of *Acinetobacter* genomic species causing nosocomial bloodstream infections, *A. baumannii* accounted the most prevalent causative agent, followed by *A. nosocomialis*, *A. pittii* and other *Acinetobacter* genomic species (Wisplinghoff *et al.*, 2012; Chuang *et al.*, 2011). These pathogens are usually acquired by vulnerable patients undergoing treatment in intensive care units (Zilberberg *et al.*, 2016; Wisplinghoff *et al.*, 2012). Less favourable clinical outcomes have been observed in patients with *A. baumannii* infections who also suffered a considerable higher mortality than patients infected with other *Acinetobacter* genomic species (Wisplinghoff *et al.*, 2012). In fact, the attributable mortality of *A. baumannii* bacteraemia has been reported as high as approximately 59% in hospitalized population (Liu *et al.*¸ 2014; Chuang *et al.*, 2011).

1.2.2.3   Wound infection and traumatic battlefield

Acinetobacter baumannii is an ordinary microorganism recovered from burn wounds (Cen *et al.*, 2015; Keen III *et al.*, 2010) and traumatic wounds of repatriated casualties from Iraq (Operation Iraqi Freedom) and Afghanistan (Operation Enduring Freedom) combats (Sheppard *et al.*, 2010). Besides that, hospital acquired *A. baumannii* wound infections have also been reported in survivors from natural disasters (earthquake and tsunami), who underwent treatment in overloaded healthcare facilities where failures in implementation of standard hygiene measures were noted in these settings (Wang *et al.*, 2010; Ukay *et al.*, 2008). Of casualties suffered traumatic wounds infections, *A. baumannii* was likely acquired during their immediate care at field hospitals with environmental contamination or as a consequence of cross transmission between hospitalized soldiers and medical personnel in military hospitals (Scott *et al.*, 2007). Furthermore, this pathogen tended to develop greater resistance to a wide variety of antimicrobials during prolonged hospitalization and hence, leaving clinicians in a difficult situation to prescribe appropriate empirical antimicrobial therapy (Visca *et al.*, 2011; Keen III *et al.*, 2010).

1.2.2.4   Urinary tract infection

Nosocomial urinary tract infection is occasionally caused by *Acinetobacter* species (Djordjevic *et al.*, 2013; Visca *et al.*, 2011). It is often benign in association with urinary catheters and occurs in rehabilitation centres more frequently than critical care settings (Dijkshoorn *et al.*, 2007). Patients with male gender, previous antimicrobial therapy and transferation from other wards have been identified as risk factors relevant to *Acinetobacter* urinary tract infections (Djordjevic *et al.*, 2013).

1.2.3   Community acquired infections

Acinetobacter species occasionally cause community acquired infections such as urinary tract infection, soft tissue infection, wound infection, ocular infection, endocarditis, pneumonia and meningitis (Visca *et al.*, 2011; Falagas *et al.*, 2007b). A review article

concluded that most of the community acquired infections occurred in tropical or subtropical regions, in particular Asia and Australia (Falagas *et al.*¸ 2007b). Patients with community acquired *Acinetobacter* infections were typically had underlying conditions, such as chronic obstructive pulmonary disease, diabetes mellitus, renal disease, alcoholism and heavy smoking (Visca *et al.*¸ 2011; Falagas *et al.*¸ 2007b).

1.2.4   Clinical impact of *Acinetobacter* infections

Clinical outcomes in patients with *Acinetobacter* infections have been assessed in numerous studies (Liu *et al.*, 2015; Chiang *et al.*, 2014; Liu *et al.*¸ 2014; Kuo *et al.*¸ 2013; Lee *et al.*, 2013b; Huang *et al.*, 2012a; Perez *et al.*¸ 2010). A poorer prognosis was observed in patients infected with carbapenem-resistant *A. baumannii* compared to other *Acinetobacter* genomic species (Liu *et al.*, 2015; Liu *et al.*¸ 2014; Lee *et al.*, 2013b; Huang *et al.*, 2012a). Furthermore, higher mortality rates were reported in hospitalized population with carbapenem-resistant *A. baumannii* bacteraemia or pneumonia (Liu *et al.*, 2015; Lee *et al.*, 2013b; Huang *et al.*, 2012a). However, true causes in attributing to mortality of patients with *A. baumannii* infections are difficult to assess as this pathogen mostly affects critically ill and immunosuppressed population (Visca *et al.*¸ 2011). In a review article, the attributable mortality rate of patients with *A. baumannii* infections was ranged from 8% to 32%, however, a conclusive rate could not be formulated due to the heterogeneity in study designs among the articles reviewed (Falagas and Rafailidis, 2007a).

1.3   Treatment of *Acinetobacter* infections

Antimicrobials susceptibility pattern of *Acinetobacter* guides clinicians to prescribe empirical therapy, however, multidrug-resistant isolates are increasingly encountered in clinical practices (Viale *et al.*, 2015; Peleg *et al.*¸ 2008). Current recommended therapeutic options differed between different susceptibility phenotypes of *Acinetobacter*. Aminoglycosides, third generation cephalosporins and a combination of β-lactam and β-lactamase inhibitors are prescribed for susceptible isolates, carbapenems, amikacin and

colistin are used for resistant isolates, while, colistin and tigecycline are used for treating multidrug-resistant isolates (Hatcher *et al.*, 2012).

1.3.1 Therapeutic options

1.3.1.1 Sulbactam

Sulbactam is a β-lactamase inhibitor with intrinsic bactericidal activity against *Acinetobacter* through its binding to penicillin-binding protein 2 (Michalopoulos and Falagas, 2010). This agent is commercially available in a combined formulation with ampicillin or cefoperazone. Clinical investigation on effectiveness of sulbactam-containing compound to treat mild to severe *A. baumannii* infections has been documented (Fishbain and Peleg, 2010). Majority of the previous studies reported that patients treated with a single sulbactam or ampicillin-sulbactam were cured or clinically improved, regardless of bacterial susceptibility profiles to carbapenems, aminoglycosides and polymyxin (Oliveira *et al.*, 2008; Lee *et al.*, 2007; Ko *et al.*, 2004). Moreover, a relatively lower pharmaceutical cost on sulbactam than imipenem was reported with sulbactam used to treat susceptible *A. baumannii* infections (Jellison *et al.*, 2001). Therefore, sulbactam-containing regimens were recommended as effective therapeutics to limit excessive use of carbapenems in clinical practices (Fishbain and Peleg, 2010; Michalopoulos and Falagas, 2010).

1.3.1.2 Polymyxins

Polymyxins (polymyxin E or colistin and polymyxin B) are polycationic lipopolypeptides that act on bacterial outer membrane resulting in the loss of membrane integrity (Neonakis *et al.*¸ 2011). In recent years, polymyxins have been administered in intensive care settings to treat multidrug-resistant *A. baumannii* infections (Neonakis *et al.*¸ 2011). Favourable or curative outcome has been documented on polymyxin therapy for hospital acquired infections caused by multidrug-resistant *A. baumannii* (Falagas *et al.*, 2010; Gounden *et al.*, 2009). However, the clinical outcome of patients with colistin administration is always a great concern, as treatment may implicate adverse effect of nephrotoxicity, neurotoxicity and

neuromuscular blockade (Michalopoulos and Falagas, 2010). Data from several studies revealed that accumulative doses of colistin methanesulfonate would increase the risk of nephrotoxicity in patients (Neonakis *et al.*¸ 2011; Hartzell *et al.*, 2009). Therefore, a lower dosage of polymyxin, with different antimicrobial formulations and careful monitoring on patients' renal function are required to reduce side effects (Michalopoulos and Falagas, 2010).

1.3.1.3   Tigecycline

Tigecycline, a 9-*t*-butylglycylamido semi-synthetic glycycline, represents a modified minocycline (Peleg *et al.*¸ 2008) that inhibits bacterial protein synthesis by binding to 30S ribosomal subunit (Fishbain and Peleg, 2010). Owing to lack of therapeutic options, tigecycline is primarily reserved for multidrug-resistant Gram-negative infections (Neonakis *et al.*¸ 2011). This antimicrobial agent has only been approved for treating intra-abdominal and skin infections by Food and Drug Administration (Michalopoulos and Falagas, 2010). Even though tigecycline demonstrates good *in vitro* bactericidal activity against multidrug-resistant *A. baumannii*, but its effectiveness in clinical use remains controversial (Gordon *et al.*, 2009; Anthony *et al.*, 2008; Gallagher and Rouse¸ 2008; Vasiley *et al.*, 2008). In addition, bacterial isolates can develop resistance to tigecycline during the treatment course (Viale *et al.*, 2015), thereby, tigecycline therapy is always recommended to be combined with other active antimicrobial agents (Michalopoulos and Falagas, 2010).

1.3.2   Antimicrobial combination therapy

Antimicrobial combination therapy is an alternative to improve bactericidal activity and prevent further development or emergence of resistance in *Acinetobacter* (Peleg *et al.*, 2008). Numerous *in vitro* assessments have been performed on multidrug-resistant *A. baumannii* against various antimicrobial combination regimens, including carbapenems, polymyxins, rifampicin, amikacin, tigecycline and cefeperazone-sulbactam (Kiratisin *et al.*, 2010; Principe *et al.*¸ 2009; Tripodi *et al.*, 2007; Timurkaynak *et al.*, 2006). Carbapenems are

always prescribed together with polymyxin, aminoglycoside or cefeperazone-sulbactam to exhibit a synergistic bactericidal effect (Kiratisin *et al.*, 2010; Principe *et al.*، 2009; Timurkaynak *et al.*, 2006). Besides that, other synergism combination regimens, carbapenem, tigecycline and sulbactam combine with colistin, rifampin or an aminoglycoside have also been demonstrated (Michalopoulos and Falagas, 2010).

Despite antimicrobial combination regimens demonstrated *in vitro* synergistic effects, evidences on their clinical effectiveness and safety are still scarce (Durante-Mangoni and Zarrilli, 2011). Better clinical outcomes were observed in patients treated with a combination regimen of colistin and carbapenem compared to patients treated with other regimens (Shields *et al.*، 2012; Falagas *et al.*, 2010). In a recent comparative study, 250 patients with bloodstream infections caused by extreme-drug-resistant *A. baumannii* were treated with different antimicrobial regimens (Batirel *et al.*, 2014). In this study, colistin monotherapy (36 patients) was compared with several antimicrobial combination regimens, such as colistin and meropenem (102 patients), colistin and sulbactam (69 patients), and colistin combined with other antimicrobials (43 patients) (Batirel *et al.*, 2014). Analyzed data showed that patients who received combination regimens demonstrated better outcomes in the 14 days survival, eradication rate and mortality than patients who were treated with colistin alone (Batirel *et al.*, 2014).

1.3.3   Future therapeutic alternatives

Bactericidal effects can also be achieved through non-antibiotic therapeutic approaches, such as phage therapy, iron chelation therapy, antimicrobial peptides, prophylactic vaccination and passive immunization, photodynamic therapy and nitric oxide-based therapy (García-Quintanilla *et al.*, 2013). All aforementioned approaches have been reviewed with limitations and problems needed to overcome for each approach were addressed (García-Quintanilla *et al.*, 2013). Among the non-antibiotic approaches, antimicrobial peptides are regarded as potential antibacterial candidates. In general, peptides comprise of 10 to 48

amino acid residues with non-conserved domains that possess biological activity (Conlon *et al.*, 2009). Most peptides share a cationic character and contain ⩾ 50% hydrophobic amino acids, allowing peptides to interact with microbial cell membranes (Conlon *et al.*, 2009; Pazgier *et al.*, 2006).

Several antimicrobial peptides have been demonstrated as having promising synergistic effects on multidrug-resistant *A. baumannii*. Human β-defensin 2, 3 and 4 (Routsias *et al.*, 2010; Supp *et al.*, 2009; Maisetta *et al.*, 2006) as well as alyteserin-1c (Conlon *et al.*, 2010; Conlon *et al.*, 2009) have been reported as potent peptide candidates with rapid bactericidal actions against multidrug-resistant *A. baumannii*. Good bactericidal effects have been demonstrated with melittin and mastoparan tested against colistin susceptible and resistant *A. baumannii* clinical isolates (Vila-Farres *et al.*, 2012). Importantly, no bacterial regrowth was observed in mastoparan tested against colistin susceptible and resistant isolates, contrary findings were observed when those isolates tested with colistin. Therefore, mastoparan has been proposed as an alternative therapy for colistin-resistant *Acinetobacter* infections (Vila-Farres *et al.*, 2012).

1.4   Mechanism of antimicrobial resistance in *Acinetobacter* species

Members of *Acinetobacter* genus are increasingly resistance to numerous antibacterial agents via intrinsic and acquired resistance mechanisms (Espinal *et al.*, 2011b). Of all described *Acinetobacter* genomic species, *A. baumannii* possesses an outstanding ability to rapidly accumulate and express various genetic determinants conferring resistance against a broad spectrum of antimicrobials, which are usually prescribed for empirical therapy (Poirel *et al.*, 2011). The foremost mechanisms contributed to antimicrobial resistance, including acquisition of resistance determinants through horizontal genes transfer, modification of endogenous genes and up regulation of efflux systems (Roca *et al.*, 2012).

Wide dissemination of resistance genes among inter- and intra- genetic relatedness bacterial species is facilitated by mobile elements, such as integrative and conjugative elements, plasmids, transposons, insertion sequence elements, gene cassettes and integrons (Stokes and Gillings, 2011). Genomic analysis on distribution of mobile elements, in particular integrons and insertion sequence elements, revealed abundance of these elements in different *Acinetobacter* genomic species at various percentages (Touchon *et al.*, 2014). Mobile elements play important roles in horizontal acquiring foreign genetic determinants via conjugation, transduction or transformation process and then, integrating the acquired determinants into bacterial genome (Stokes and Gillings, 2011), thereby, contributing to high genetic plasticity leading to genomic diversification among species within *Acinetobacter* genus (Touchon *et al.*, 2014).

1.4.1   Genetic basis of resistance to aminoglycosides, quinolones and polymyxins

Resistance to aminoglycosides is achieved through modification of hydroxyl or amino group by aminoglycoside-modifying enzymes (AMEs). Three AMEs are responsible for aminoglycosides resistance, including aminoglycoside acetyltransferases, aminoglycoside nucleotidyltransferases and aminoglycoside phosphotransferases (Cho *et al.*, 2009; Nemec *et al.*¸ 2004b). These enzymes are usually associated with class 1 integron and resistance islands (Poirel *et al.*, 2011), therefore, they can horizontally transfer and disseminate among *A. baumannii* isolates (Nemec *et al.*¸ 2004b). The 16S rRNA methylation, mediated by *armA* gene, is another mechanism contributes to the high level of resistance against all formulated aminoglycosides (Cho *et al.*, 2009). Genetic analysis on *armA* gene structure revealed that the gene is flanked by an insertion sequence IS*CR1*, embedded within a transposon Tn*1528* (Tada *et al.*, 2015). Moreover, the presence of *armA* gene is often co-detected with a bla_{OXA-23} or bla_{NDM-1} gene (Tada *et al.*, 2015; Adams-Haduch *et al.*, 2008).

Resistance to quinolones and fluoroquinolones is due to modification of gyrase or topoisomerase IV through point mutation in *gyrA* and *parC* gene located within quinolone-

resistance-determining-region (Lopes *et al.*, 2013; Karah *et al.*, 2011; Zhao *et al.*, 2011), resulting in a low affinity for antimicrobials to bind to the enzyme-DNA complex (Poirel *et al.*, 2011). The common *gyrA* and *parC* mutations, Ser83 to Leu83 substitution in GyrA and Ser80 to Leu80 substitution in ParC, have been identified in quinolones- and fluoroquinolones-resistant isolates (Jiang *et al.*, 2014; Lopes *et al.*, 2013; Karah *et al.*, 2011; Lin *et al.*, 2010b; Hujer *et al.*, 2006). Moreover, amino acid substitutions are often associated with ciprofloxacin resistance in *Acinetobacter* clinical isolates (Lin *et al.*, 2010b; Adams-Haduch *et al.*, 2008; Hujer *et al.*, 2006).

Resistance to polymyxins is mediated by loss of bacterial lipopolysaccharide (Moffatt *et al.*, 2011) and PmrAB mutations (Adam *et al.*, 2009). The PmrAB is a two-component signal transduction system that encoded by *pmrA* and *pmrB* genes, governing expression of *pmrC* gene encodes a phosphoethanolamine transferase enzyme (Poirel *et al.*, 2011). Mutations in *pmrA* and *pmrB* genes cause overexpression of *pmrC* gene with addition phosphoethanolamine moieties to lipid A, resulting in a decrease of negative charge at bacterial outer membrane and eventually impair the affinity of lipopolysaccharide against polymyxins (Beceiro *et al.*, 2011). Further, mutations in *IpxA* and *IpxC* genes could implicate lipid A biosynthesis cause a complete loss of lipopolysaccharide production and a high level of colistin resistance in *A. baumannii* (Moffatt *et al.*, 2011).

1.4.2   Genetic basis of resistance to beta-lactams

Resistance to β-lactams is typically ascribed to enzymatic and non-enzymatic mechanisms. Enzymatic mechanisms involve the production of β-lactam hydrolyzing enzymes, also known as β-lactamases, capable to hydrolyze and inactivate β-lactamic antimicrobials. The β-lactamases are chromosomal or plasmid encoded by *bla* gene. While, non-enzymatic mechanisms are referred to resistance modes without the production of enzymes to degrade and inactivate antibacterial agents.

1.4.2.1 Non-enzymatic mechanisms for resistance to beta-lactams

1.4.2.1.1 Alteration of outer membrane proteins

The outer membrane of Gram-negative bacteria contains proteins that mediate transmembrane passage of amino acids, ions and small molecules needed for cell growth and function. Hence, alteration of the membrane permeability can implicate in uptake of amino acids and other molecules into the bacterial cells. Correlation between *A. baumannii* proteomic profile and carbapenem susceptibility has been established, reduced in certain protein expression may diminish carbapenem diffusion across bacterial outer membrane and thereby, decreased susceptibility to carbapenem (Dupont *et al.*, 2005).

A heat-modifiable protein, 29 kDa outer membrane protein, also recognized as CarO protein, which was absence from imipenem-resistant *A. baumannii* with no carbapenemase activity was detected in the isolates under studied (Dupont *et al.*, 2005; Limansky *et al.*, 2002). Apparent loss of CarO protein in carbapenem-resistant *A. baumannii* was resulted from *carO* gene disruption by distinct insertion elements (Mussi *et al.*, 2005). Further studies were conducted to decipher structural characteristic and function of CarO protein in *A. baumannii* (Zahn *et al.*, 2015; Catel-Ferreira *et al.*, 2011; Siroy *et al.*, 2005; Limansky *et al.*, 2002). Findings revealed the alteration in CarO protein expression could implicate ornithine uptake (Siroy *et al.*, 2005) and reduce susceptibility to imipenem but not meropenem (Catel-Ferreira *et al.*, 2011).

Analysis of outer membrane protein profiles among carbapenems-resistant *A. baumannii* isolates revealed a reduced expression on 43 kDa protein, also known as OprD homologue, with 49% homology to *Pseudomonas aeruginosa* OprD (Dupont *et al.*, 2005). Findings from a study suggested that OprD homologue may not be involved in carbapenem resistance (Catel-Ferreira *et al.*, 2012). Rather, the regulation of OprD homologue can assist bacterium to adapt and survive in magnesium and iron depleted environments (Catel-Ferreira *et al.*, 2012). Besides, another outer membrane protein, namely 33-36 kDa protein, was also absent

from bacterial outer membrane or showed decreased expression in association with carbapenem resistance in *A. baumannii* (del Mar Tomás *et al.*, 2005; Clark, 1996).

1.4.2.1.2   Alteration of penicillin binding proteins

Role of penicillin binding proteins (PBPs) in resistance mechanism is rarely investigated. As an overall, PBPs only confer marginal levels of carbapenem resistance but association with the production of β-lactamases, overexpression of efflux pumps or alteration of expression level or structure in outer membrane proteins could contribute a higher level of phenotypic carbapenems resistance (Yun *et al.*, 2011).

1.4.2.2   Enzymatic mechanisms

Ambler classification scheme has been applied to classify β-lactamases according to their molecular structures into four molecular classes, represented by Ambler class A extended-spectrum β-lactamases, Ambler class B metallo-β-lactamases, Ambler class C cephalosporinases and Ambler class D oxacillinases (Queenan and Bush, 2007).

1.4.2.2.1   Ambler class A extended-spectrum beta-lactamases

Ambler class A extended-spectrum β-lactamases is always regarded to have minor effects on bacterial resistance phenotypes (Roca *et al.*, 2012; Peleg *et al.*, 2008). Extended-spectrum β-lactamases (ESBLs) of KPC-, CARB-, TEM-, SHV-, CTX-M-, GES-, SCO-, PER- and VEB-type have thus far detected in *Acinetobacter*, compromising efficacy of penicillins, cephalosporins and oxyimino-β-lactams, but their hydrolysis capacities can be inhibited by clavulanic acid (Roca *et al.*, 2012; Espinal *et al.*, 2011b). Among the described ESBLs, PER- and VEB-type, β-lactamases are more frequently detected in *Acinetobacter* (Roca *et al.*, 2012; Espinal *et al.*, 2011b; Kolayli *et al.*, 2005) and nosocomial outbreaks caused by isolates harbouring both enzymes have been reported (Jeong *et al.*¸ 2005; Poirel *et al.*, 2003). Genetic context analysis revealed an insertion sequence IS*26* which is often preceded *bla*$_{VEB-}$

₁ gene, forming part of class 1 integron (Poirel *et al.*, 2003). Genes encoding PER-type variants (*bla*PER-1, *bla*PER-2 and *bla*PER-7) are associated with insertion sequence elements, IS*Pa12*, IS*Pa13* and IS*CR1*, as promoter sequence leading to genes expression (Bonnin *et al.*, 2011b; Pasterán *et al.*, 2006; Poirel *et al.*, 2005a). The *bla*PER-1 gene is flanked by upstream IS*Pa12* and downstream IS*Pa13* forming a composite transposon Tn*1213* (Poirel *et al.*, 2005a), *bla*PER-2 gene is only flanked by a single copy of IS*Pa12* (Pasterán *et al.*, 2006) while IS*CR1* present at upstream immediate from *bla*PER-7 gene (Bonnin *et al.*, 2011). Comparing to *bla*PER-1, expression of *bla*PER-7 gene could result in a greater resistance to cephalosporins and monobactams (Poirel *et al.*, 2011).

1.4.2.2.2   Ambler class B metallo-beta-lactamases

Ambler class B metallo-β-lactamases is capable of hydrolyzing all β-lactams except monobactam aztreonam (Tang *et al.*, 2014). Metallo-β-lactamases (MBLs) are zinc-dependent metalloproteins, which can be inhibited by ethylenediaminetetraacetic acid but do not physically interact with β-lactams and β-lactamase inhibitors, such as tazobactam, sulbactam and clavulanic acid (Bush and Jacoby, 2010). Metallo-β-lactamases have been grouped into six families with most designations derived from the sites of their first positive isolations, including active on imipenem (IMP), Verona integron-encoded metallo-β-lactamase (VIM), Seoul imipenemase (SIM), Sao Paulo metallo-β-lactamase (SPM), German imipenemase (GIM) and New Delhi metallo-β-lactamase (NDM) (Yong *et al.*, 2009; Queenan and Bush, 2007).

Allelic variants belonging to IMP, VIM, SIM and NDM families have thus far been described in *Acinetobacter* (Tang *et al.*, 2014). Despite MBLs are less frequently detected in *Acinetobacter* than Ambler class D oxacillinases, these enzymes demonstrate 100 to 1000 folds of hydrolytic activities toward carbapenems (Poirel and Nordmann, 2006a). Furthermore, *bla* gene encoded MBLs are usually located in class 1 integron that contains an

array of resistance gene cassettes (Yamamota *et al.*¸ 2013; Poirel and Nordmann, 2006a). Insertion of genes encoding MBLs onto resistance gene cassettes located in integrons are embedded within transferable plasmids, act as important vehicles to disseminate resistance determinants among *Acinetobacter* species and across different genera (Poirel and Nordmann, 2006a). The IMP enzyme was first described in *Pseudomonas aeruginosa* isolated in Japan (Watanabe *et al.*, 1991). Thereafter, IMP-type enzymes have widely disseminated among Gram-negative bacteria including *Acinetobacter*. The 37 IMP-type variants that have been identified with common detection in *Acinetobacter* included IMP-1, IMP-2, IMP-4, IMP-5, IMP-6, IMP-8, IMP-11 and IMP-19 (Zarrilli *et al.*, 2013; Espinal *et al.*, 2011b).

Verona integron-encoded metallo-β-lactamases exhibit less than 40% amino acid homology to IMP-type enzymes, but share similar substrates profile with a higher affinity towards carbapenems (Docquier *et al.*, 2003). The first VIM-type variant, VIM-1, was detected in *Pseudomonas aeruginosa* isolated from Verona, Italy (Lauretti *et al.*, 1999). Five VIM-type variants, VIM-1, VIM-2, VIM-3, VIM-4 and VIM-11, have sporadically been detected in *Acinetobacter* isolations from China (Zhao *et al.*, 2015), Egypt (Fouad *et al.*¸ 2013), India (Chaudhary and Payasi, 2013; Amudhan *et al.*, 2012), Saudi Arabia (Al-Sultan *et al.*, 2015) and Taiwan (Ku *et al.*, 2015; Lin *et al.*, 2010a). For SIM family, SIM-1 enzyme was initially described in *A. baumannii* derived from a tertiary hospital in Seoul, Korea (Lee *et al.*, 2005). This β-lactamase exhibits 64% to 69% amino acid homology to IMP-type variants and hydrolyzes penicillins, narrow-spectrum cephalosporins, extended-spectrum cephalosporins and carbapenems (Lee *et al.*, 2005).

An allelic variant belonging to NDM family, NDM-1 enzyme, was initially detected in *Klebsiella pneumoniae* and *Escherichia coli* isolates recovered from a Swedish who returned from India (Yong *et al.*, 2009). As similar to other MBLs, NDM-type enzymes confer resistance to all β-lactams except aztreonam (Roca *et al.*¸ 2012). Genes encoding NDM-type

variants can be located in bacterial chromosome or plasmids with approximate sizes ranged from 30 kb to 50 kb (Chen *et al.*, 2011; Espinal *et al.*, 2011a). Genetic context surrounding *bla*_{NDM-type} showed to be different from other MBLs, often in association with insertion sequence IS*Aba125* and IS*Aba11* on a transposon Tn*125* (Yang *et al.*, 2012; Espinal *et al.*, 2011a). In most NDM-type positive isolates, *bla*_{NDM} gene is flanked by two copies of insertion sequence IS*Aba125*, alongside *ble* (bleomycin resistance) and *trpF* (*N*-(5'-phosphoribosyl) anthranilate isomerase) genes at downstream immediate from that gene (Espinal *et al.*, 2011a).

1.4.2.2.3   Ambler class C cephalosporinases

Ambler class C cephalosporinases, also known as AmpC-type β-lactamases, encompasses non-inducible chromosomal enzymes are now recognized as *Acinetobacter*-derived cephalosporinases (ADCs) (Tang *et al.*, 2014; Hujer *et al.*, 2005). Fifty six ADC-type variants have been identified in *Acinetobacter*, with more than half of those allelic variants are found in *A. baumannii* (Zhao and Hu, 2012; Hujer *et al.*, 2005). The ADC-type enzymes confer resistance to all penicillins, extended-spectrum cephalosporins and a combination of β-lactam and β-lactamase inhibitor (Drawz *et al.*, 2010). These enzymes constitute basal expression levels that possess no effects on β-lactams (Héritier *et al.*, 2006). Nonetheless, *bla*_{ADC-type} gene could be overexpressed with acquisition of an upstream insertion sequence IS*Aba1* or IS*Aba125*, resulting in bacterial resistance against extended-spectrum cephalosporins but does not compromise efficacy of cefepime and carbapenems (Rezaee *et al.*, 2013; Héritier *et al.*, 2006).

1.4.2.2.4   Ambler class D oxacillinases

Ambler class D oxacillinases involves carbapenem-hydrolyzing activity (hydrolyzes imipenem and meropenem but not extended-spectrum cephalosporins and aztreonam), hence, β-lactamases belonging to this class are also termed as carbapenem-hydrolyzing class D β-

lactamases (Poirel and Nordmann, 2006a). Five phylogenetic subgroups of carbapenem-hydrolyzing class D β-lactamases (CHDLs) have been identified, represented by OXA-51, OXA-23, OXA-24, OXA-58 and OXA-143 (Roca *et al.*, 2012). Each subgroup comprises allelic variants with different amino acid substitutions in respective sequences. All variants are chromosomal or plasmid encoded by *bla* gene ($bla_{OXA-51/23/24/58/143-like}$ gene), which are often associated with mobile elements, such as insertion sequence elements and transposons.

Naturally occurring oxacillinases, represented by OXA-51 subgroup, intrinsic enzymes in *A. baumannii* allowing weak hydrolysis of β-lactamic substrates, mainly to penicillins (benzylpenicillin, ampicillin, ticarcillin and piperacillin) and carbapenems (imipenem and meropenem) but do not active against expanded-spectrum cephalosporins (Durante-Mangoni and Zarrilli, 2011; Poirel and Nordmann, 2006a). More than 68 variants belonging to OXA-51 subgroup have currently been described in *A. baumannii* isolations from diverse geographical regions (Zhao and Hu, 2012). Presence of an upstream insertion sequence IS*Aba1* or IS*Aba9* at $bla_{OXA-51-like}$ gene often acts as a strong transcriptional promoter to enhance gene expression (Figueiredo *et al.*, 2009; Turton *et al.*, 2006a).

OXA-23 subgroup constitutes OXA-23, OXA-27, OXA-49 and OXA-73 variants (Roca *et al.*, 2012; Afzal-Shah *et al.*, 2001). It shares 56% amino acid identity with OXA-51 subgroup (Poirel and Nordmann, 2006a). The OXA-23 enzyme was first detected in an *A. baumannii* clinical strain isolated from Scotland in year 1985, initially named as ARI-1 (Paton *et al.*, 1993). Presence of $bla_{OXA-23-like}$ gene in bacterial chromosome or plasmid seems to be exclusive in *Acinetobacter* genus, with an exception when that gene was detected in *Proteus mirabilis* isolated from France (Bonnet *et al.*, 2002). Interestingly, *A. radioresistens* has been identified as the progenitor for bla_{OXA-23} gene, whose chromosomal encoded bla_{OXA-23} gene mobilized into *A. baumannii* through a transposition event (Poirel *et al.*, 2008). An insertion sequence IS*Aba1* or IS*Aba4* preceding $bla_{OXA-23-like}$ gene could increase gene expression level (Wang *et al.*, 2014; Manageiro *et al.*, 2012). Two genetic structures of

*bla*_{OXA-23-like} gene are identified in multidrug-resistant *A. baumannii*, one copy of upstream insertion sequence IS*Aba1* preceding *bla*_{OXA-23-like} gene and *bla*_{OXA-23-like} gene is flanked by two copies of IS*Aba1* in opposite orientations, both structures are embedded on transposon Tn*2006*, Tn*2007* or Tn*2008* (Wang *et al.*, 2014; Manageiro *et al.*, 2012; Wang *et al.*, 2011).

OXA-24 subgroup comprises of OXA-24, OXA-25, OXA-26 and OXA-72 variants (Wang *et al.*, 2007; Afzal-Shah *et al.*, 2001). Of this subgroup, chromosomal encoded *bla*_{OXA-24} gene was first described in carbapenem-resistant *A. baumannii* derived from Spain (Bou *et al.*, 2000). Oxacillinases classified under this subgroup share 63% and 60% amino acid homology with OXA-51 and OXA-23 subgroup, respectively (Poirel and Nordmann, 2006a). Genes encoding allelic variants within OXA-24 subgroup can be located in bacterial chromosome or plasmid (Roca *et al.*, 2012) in association with XerC/XerD-like recombinant sites, a different genetic context mapped for *bla*_{OXA-23-like} and *bla*_{OXA-58-like} genes (Merino *et al.*, 2010).

OXA-58 subgroup comprises of OXA-58, OXA-96 and OXA-97 variants. It shares 59% amino acid identity with OXA-51 subgroup and less than 50% amino acid identity with both OXA-23 and OXA-24 subgroups (Poirel and Nordmann, 2006a). The OXA-58 enzyme was first detected in a carbapenem-resistant *A. baumannii* isolated from Toulouse, France (Poirel *et al.*, 2005b). The *bla*_{OXA-58-like} gene is usually plasmid-borne and associated with insertion sequence elements of IS*Aba1*, IS*Aba2* or IS*Aba3* (Villalón *et al.*, 2015; Migliavacca *et al.*, 2013; Lee *et al.*, 2012). Similar to genetic structure surrounding *bla*_{OXA-23-like} gene, *bla*_{OXA-58-like} gene is also flanked by two copies of insertion sequence elements, with insertion sequence IS*Aba3* is usually detected at 3' end, forming a composite transposon (Giannouli *et al.*, 2009; Poirel and Nordmann, 2006b).

In OXA-143 subgroup, OXA-143 and OXA-231, are plasmid encoded β-lactamases sharing 88%, 63% and 52% amino acid identity with OXA-24, OXA-23 and OXA-58 subgroup,

respectively (Higgins *et al.*, 2009). The OXA-143 enzyme was first detected in a carbapenem-resistant *A. baumannii* isolated from Brazil in year 2004 (Higgins *et al.*, 2009). In contrast to other CHDLs, *bla*$_{OXA-143}$ gene appears being associated with neither insertion sequence element nor integron, but it is flanked by two copies of replicase genes (Higgins *et al.*, 2009).

1.5 Epidemiology of *Acinetobacter baumannii* harboured carbapenem-hydrolyzing class D beta-lactamases genes of *bla*$_{OXA-23/24/58/143-like}$

Three distinct *A. baumannii* clonal lineages, European clone (EU) I, II and III, that were responsible for outbreaks in European hospitals have successfully been delineated (Nemec *et al.*, 2004a; van Dessel *et al.*, 2004). Sequence-based typing schemes were subsequently developed to characterize *A. baumannii* epidemic clones, represented by group or clonal complex 1, 2 and 3 corresponding to EU I, II and III (Wisplinghoff *et al.*, 2008; Turton *et al.*, 2007; Bartual *et al.*, 2005). Epidemic EU clones have been reported to be disseminated in healthcare institutions worldwide and are now recognized as international clone (ICL) I, II and III (Antunes *et al.*, 2014; Visca *et al.*, 2011). Additional five distinct clonal lineages, named as worldwide clonal lineage 4, 5, 6, 7 and 8, have been reported as global epidemic spread (Higgins *et al.*, 2010a). Indeed, at least nine major clonal complexes including the three international clonal lineages have been showed to be distributed worldwide, as revealed in a population structure analysis (Zarrilli *et al.*, 2013).

Hospital outbreaks are mostly caused by carbapenem-resistant *A. baumannii* strains harboured *bla* gene encoding Ambler class D oxacillinases (Kulah *et al.*, 2010; Kohlenberg *et al.*, 2009; Ozen *et al.*, 2009). The *bla*$_{OXA-23-like}$ gene is frequently detected in carbapenem-resistant isolates and currently, this corresponding gene has been disseminated worldwide and spread to different genomic species within the *Acinetobacter* genus (Roca *et al.*, 2012). Epidemic *A. baumannii* strains harbouring *bla*$_{OXA-23-like}$ gene are generally assigned to ICL I and ICL II clones (Peymani *et al.*, 2012; He *et al.*, 2011; Irfan *et al.*, 2011; Runnegar *et al.*,