PHENOTYPIC AND MOLECULAR CHARACTERISATION OF CLINICAL CARBAPENEM-RESISTANT *Acinetobacter baumannii*

KONG BOON HONG

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2012

ABSTRACT

Acinetobacter baumannii is an opportunistic pathogen with increasing relevance in nosocomial infections. They cause a wide range of clinical complications, such as pneumonia, septicemia, urinary tract infection, wound infection, and meningitis, particularly in immunocompromised patients. Treatment of *A. baumannii* infections is often complicated by their resistance to multiple antimicrobial agents available. Carbapenem has remained as the effective antimicrobial agent for the treatment of *A. baumannii* infections. However, carbapenem-resistant *A. baumannii* is increasingly reported worldwide. In Malaysia, detailed information on the epidemiology and the mechanism of antimicrobial resistance of *A. baumannii* is still lacking. Hence, the objectives of this study were to investigate the prevalence of antimicrobial resistance, mechanisms of carbapenem resistance among the *A. baumannii* isolates and to provide sound scientific evidence of epidemiologic spread of *A. baumannii* in the hospital setting.

In 2006-2009, a total of 189 *A. baumannii* isolates were isolated from patients (n=171), environment (n=9) and hands of healthcare workers (HCWs) (n=9) in intensive care unit, University Malaya Medical Centre. One-hundred and eighty-five isolates (170 clinical; 7 environmental; 8 HCWs) were identified as *A. baumannii* by amplified ribosomal DNA restriction analysis (ARDRA). All the clinical, 7 environmental and 1 HCW *A. baumannii* isolates were multidrug-resistant to at least 3 groups of antimicrobial agents, with high resistance rates to the aminoglycoside, penicillin, cephalosporin, quinolone, carbapenem and foliate inhibitor. All the 2006 isolates appeared susceptible to cefoperazone/sulbactam. However, resistant isolates were detected in 2007 to 2009 isolates. Polymyxin B has remains effective against the *A. baumannii* isolates.

None of the 175 carbapenem-resistant isolates was metallo-β-lactamase (MBL)

producers based on phenotypic screening and PCR detection of the MBL genes. *bla*$_{OXA-51}$ gene which is intrinsic to *A. baumannii* was present in all the isolates and IS*Aba1*-*bla*$_{OXA-23}$ gene was detected in 174 isolates. Resistance to imipenem was mainly due to overexpression of the OXA-23 induced by the promoter sequences located in insertion element, IS*Aba1* upstream of the *bla*$_{OXA-23}$ gene.

Polymerase chain reaction detection of integrons showed class 1 integrons were predominant among the 185 isolates, with 17 isolates were also harbouring class 2 integrons. The integron gene cassettes contained the most number of resistance determinants to aminoglycosides (*aadB, aadA, aadDA1, aacC1* and *aacA4*). There was no correlation between the *bla*$_{OXA-23}$ gene and integrons, suggesting that integrons were unlikely involved in the mobility of *bla*$_{OXA-23}$ gene in the *A. baumannii* isolates.

Of the 175 carbapenem-resistant isolates, 164 (93.7%) isolates harboured 1-15 plasmids each, ranging from 1.6 kb to 125.1 kb. A total of 98 plasmid profiles were defined, with P49 (44.8 kb, 21.6 kb, 6.8 kb), P52 (44.8 kb, 6.8 kb) and P53 (44.8 kb, 16.1 kb, 6.8 kb) the predominant plasmid profiles, harbouring the common plasmids, 6.8 kb and 44.8 kb. Southern hybridisation analyses revealed that *bla*$_{OXA-23}$ gene was dispersed on diverse locations on the plasmids and chromosome among multiples isolates. However, the *bla*$_{OXA-23}$ gene was not transferable. To our knowledge, this is the first report of the plasmid- and chromosomal-mediated OXA-23-producing carbapenem-resistant *A. baumannii* in Malaysia.

PFGE and REP-PCR typing had successfully discriminated all the *A. baumannii* isolates. The non-multidrug-resistant had high genetic variability and were distinct from the multidrug-resistant isolates. However, the carbapenem-susceptible isolates could not be distinguished from the carbapenem-resistant isolates by both typing methods. The OXA-23-producing clinical, environmental and HCW isolates shared similar resistance phenotype and had closely related PFGE and REP-PCR profiles, indicating a possible

transmission route may occur between the environment, HCW and patients. OXA-23-producing *A. baumannii* isolates were observed in the ICU area throughout 2006-2009, indicating the endemicity of the isolates. In addition, an occurrence of new *A. baumannii* clone was observed in 2009 based on PFGE analysis.

In conclusion, the dissemination of the carbapenem-resistant *A. baumannii* within the ICU, UMMC from 2006-2009 was of OXA-23-producing isolates. PFGE and REP-PCR molecular typing were useful in discriminating the nosocomial related *A. baumannii* isolates. *A. baumannii* is able to obtain resistance genes, thus could confront the extensive exposure to antimicrobial agents and persisted in the ICU. Therefore, evaluation of effective antimicrobials and infection control measures are important to control the dissemination of carbapenem-resistant *A. baumannii* isolates within the hospital.

ABSTRAK

Acinetobacter baumannii ialah patogen oportunistik yang memainkan peranan penting dalam infeksi nosokomial. Ia boleh menyebabkan komplekasi klinikal yang luas seperti radang paru-paru, septicemia, jangkitan saluran kencing, jangkitan pada luka dan penyakit meningitis, terutamanya pada pesakit yang tidak memiliki imunokompetensi. Rawatan infeksi *A. baumannii* sering dikomplikasikan oleh keupayaan bakteria yang resisten terhadap pelbagai antibiotik yang ada dalam pasaran. Carbapenem adalah antara antibiotik yang masih kekal efektif dalam rawatan infeksi *A. baumannii*. Walau bagaimanapun, laporan tentang *A. baumannii* yang resisten terhadap carbapenem semakin banyak dilaporkan di dunia. Di Malaysia, maklumat berkenaan epidemilogi dan mekanisma rintangan antibiotik pada bakteria *A. baumannii* adalah tidak mencukupi. Oleh demikian, objektif kajian ini adalah untuk menyelidik kelaziman rintangan antibiotik, mekanisme rintangan terhadap carbapenem dalam bakteria *A. baumannii* dan mencari bukti saintifik bagi sebaran epidemilogi *A. baumannii* di hospital.

Sejumlah 189 *A. baumannii* strain telah dipencilkan daripada pesakit-pesakit (n=171), dari persekitaran (n=9) dan tangan pekerja penjaga kesihatan (HCWs) (n=9) dalam Unit Rawatan Rapi (ICU), Pusat Perubatan Universiti Malaya (PBUM). Seratus lapan puluh dan lima strain (170 klinikal; 7 persekitaran; 8 HCWs) telah dikenalpasti sebagai *A. baumannii* dengan menggunakan analisis restriksi DNA ribosom yang diamplifikasikan (ARDRA). Semua strain klinikal, 7 dari persekitaran dan 1 HCW *A. baumannii* strain adalah multi-rintangan terhadap tidak kurang daripada 3 jenis kumpulan agen antibiotik, dengan kadar resisten yang tinggi pada aminoglycoside, penicillin, cephalosporin, quinolone, carbapenem dan foliate inhibitor. Kesemua strain pada tahun 2006 adalah sensitif terhadap cefoperazone/sulbactam. Walau bagaimanapun, strain-strain yang resisten terhadap antibiotik ini telah ditemui di antara

tahun 2007 hingga 2009. Polymyxin B tetap kekal efektif terhadap kesemua *A. baumannii* strain.

Sejumlah 175 strain yang rintang terhadap carbapenem telah dikenalpasti tidak menghasilkan metallo-β-lactamase. Gen $bla_{\text{OXA-51}}$ yang intrinsik pada *A. baumannii* hadir dalam semua strain-strain yang diuji dan gen IS*Aba1*-$bla_{\text{OXA-23}}$ telah dikesan dalam 174 strain. Lebihan ekspresi OXA-23 yang didorong oleh jujukan promoter yang terletak dalam elemen penyisipan, IS*Aba1* di bahagian hulu gen $bla_{\text{OXA-23}}$ merupakan punca kepada rintangan terhadap imipenem.

Pengesanan integron-integron dengan tindakbalas rantaian polimerasi (PCR) menunjukkan integron kelas 1 adalah paling dominan dalam strain-strain dengan 17 strain-strain juga mengandungi integron kelas 2. Gen kaset integron membawa kebanyakan penentu resistensi terhadap aminoglycoside (*aadB, aadA, aadDA1, aacC1* and *aacA4*). Tidak ada korelasi di antara gen $bla_{\text{OXA-23}}$ dengan integron-integron, mencadangkan bahawa integron-integron adalah tidak terlibat dalam mobiliti gen $bla_{\text{OXA-23}}$ dalam strain-strain *A. baumannii*.

Daripada jumlah 175 strain yang rintang terhadap carbapenem, 164 (93.7%) strain membawa 1-15 plasmid dalam setiap strain, yang bersaiz 1.6 kb hingga 125.1 kb. Sejumlah 98 profil telah ditakrifkan dengan P49 (44.8 kb, 21.6 kb, 6.8 kb), P52 (44.8 kb, 6.8 kb) dan P53 (44.8 kb, 16.1 kb, 6.8 kb) merupakan profil-profil plasmid yang terdominan, membawa plasmid-plasmid biasa, 6.8 kb dan 44.8 kb. Analisis Southern hybridasi menunjukkan gen $bla_{\text{OXA-23}}$ tertabur di kepelbagaian lokasi pada plasmid dan kromosom antara pelbagai strain. Namun demikian, gen $bla_{\text{OXA-23}}$ tidak boleh dipindahkan. Untuk pengetahuan kami, ini merupakan laporan pertama di Malaysia berkenaan dengan *A. baumannii* yang resisten terhadap carbapenem dengan penghasilan OXA-23 yang dimediasikan oleh plasmid dan kromosom.

Semua strain-strain *A. baumannii* telah berjaya didiskriminasikan dengan menggunakan kaedah PFGE dan REP-PCR. Strain-strain yang bukan multi-rintangan mempunyai tahap perbezaan genetik yang tinggi dan berlainan daripada strain-strain yang multi-rintangan. Namun demikian, kedua-dua kaedah ini adalah tidak berupaya untuk membezakan strain-strain yang sensitif terhadap carbapenem daripada strain-strain yang rintang terhadap carbapenem. Strain-strain yang menghasilkan OXA-23 dipencilkan dari klinikal, persekitaran dan HCW berkongsi fenotip rintangan yang sama dan mempunyai profil-profil PFGE dan REP-PCR yang berkait rapat, mencadangkan kemungkinan kejadian transmisi di antara persekitaran, HCW dan pesakit-pesakit di ICU. Sepanjang tahun 2006 hingga 2009, strain-strain *A. baumannii* yang menghasilkan OXA-23 endemik di kawasan sekitar ICU. Tambahan pula, berdasarkan analisis PFGE klon baru *A. baumannii* telah diperhatikan pada tahun 2009.

Kesimpulannya, penyebaran strain-strain *A. baumannii* yang rintang terhadap carbapenem di ICU, PBUM sepanjang tahun 2006 hingga 2009 adalah strain-strain yang menghasilkan OXA-23. PFGE dan REP-PCR merupakan kaedah molecular yang berguna untuk mendiskriminasikan strain-strain *A. baumannii* yang berkaitan dengan nosokomial. *A. baumannii* memiliki keupayaan untuk memperoleh gen-gen rintangan, maka membolehkannya mengatasi pendedahan luas terhadap antimikrobial agen dan berkekalan dalam ICU. Oleh sebab itu, penilaian terhadap antimikrobial yang efektif dan langkah kawalan infeksi adalah penting untuk mengawal penyebaran strain-strain *A. baumannii* yang rintang terhadap carbapenem dalam hospital.

ACKNOWLEDGEMENT

I would like to express my deepest gratitude and thanks to my supervisor Professor Dr. Thong Kwai Lin and Associate Professor Dr. Yasmin Abu Hanifah for their patience guidance and advice throughout this project study.

Thanks to all my lab mates in the Laboratory of Biomedical Science and Molecular Microbiology for their generous help and support throughout my research.

I wish to express my deepest appreciation to University of Malaya for providing me scholarship and research grants which enabled me to complete my study without any financial worries.

My deepest gratitude goes to my family for their unflagging love and support throughout my life. This dissertation is simply impossible without them. My special thanks go to my sisters and brothers for their continuous support during all the years of my study.

Finally, I would like to thank all people who have helped and inspired me throughout the time of my study.

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ABBREVIATIONS

>	Greater than
≥	Same or greater than
~	Approximately
=	Equal to
^{o}C	Degree Celcius
%	Percent
3′-CS	3′ conserved segment
5′-CS	5′ conserved segment
ATCC	American Type Culture Collection
bp	basepair
CFU	Colony forming unit
D	Discriminatory Power
ddH_2O	Double distilled water
et al.	Et alii (and others)
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic
FDA	Food and Drug Administration
g	Gram
HCl	Hydrocloric acid
kb	Kilobase pair
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration

ml	Milliliter
mm	Millimeter
mM	Millimolar
NaCl	Natrium Chloride
NaOH	Natrium Hydroxide
No.	Number
n	Number of strains
OD	Optical density
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
psi	Pound per square inch
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
S/I/R	Sensitive/Intermediate/Resistant
spp.	Species
TBE	Tris-borate-EDTA
TE	Tris-EDTA
T_{Hyb}	Hybridisation temperature
Tm	Melting temperature
tRNA	Transporter ribonucleic acid
UV	Ultraviolet
V	Volt
µg	Microgram
µl	Microliter

μm	Micrometer
w/v	weight/volume
v/v	volume/volume

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Acinetobacter baumannii has emerged as an important nosocomial pathogen and constitutes a major problem in hospitals worldwide (Hanlon, 2005; Perez *et al*., 2007). *A. baumannii* causes a wide range of clinical complications such as bacteremia, meningitis, respiratory and urinary tract infections particularly in immunocompromised patients (Dijkshoorn *et al.,* 2007; Nemec *et al*., 2011). *A. baumannii* is widely distributed in nature and can be isolated from water, soil and even human skin. *A. baumannii* shares close relationship with *A. calcoaceticus*, genospecies 3 (*A. pittii*) and 13TU (*A. nosocomialis*) and is referred as *A. calcoaceticus-A. baumannii* complex. There are limitation in phenotypic tests to differentiate the *A. calcoaceticus-A. baumannii* complex strains (Gerner-Smidt *et al.,* 1991; Bernards *et al.,* 1995; Bernards *et al.,* 1996).

To date, a variety of molecular methods including DNA-based methods are available for genospeciating *Acinetobacter* spp., such as tRNA spacer fingerprinting (Ehrenstein *et al.* 1996), sequence analysis of 16S-23S rRNA gene spacer region (Nowak and Kur, 1995; Chang *et al*., 2005), *gyr*B genes (Yamamoto and Harayama, 1996) and *rpoB* (Gundi *et al*., 2009), PCR-RFLP (Jawad *et al.*, 1998; Krawczyk *et al*., 2002) and AFLP analysis (Jansen *et al*., 1997; Nemec *et al.*, 2001). Amplified ribosomal DNA restriction analysis (ARDRA) which has been validated with large numbers of strains with defined species is an ideal method for species identification of the genus *Acinetobacter* (Vanechoutte *et al*., 1995). The strain species in the *A. calcoaceticus-A. baumannii* complex can easily be identified by ARDRA and it is a feasible method to apply especially in hospital laboratories (Dijkshoorn *et al.,* 1998).

Treatment of *A. baumannii* infections is difficult and often complicated by their resistance to multiple antimicrobial agents that are currently available, including broad-

spectrum beta-lactams, aminoglycosides, tetracyclines and quinolones (Looveren *et al.,* 2004; Perez *et al*., 2007). The efficiency of carbapenems which remains as an alternative antimicrobial therapeutic agent for treatment of *A. baumannii* infections is being increasingly compromised by the emergence of carbapenem-hydrolysing β-lactamases; IMP, VIM, SIM, SPM and GIM-type class B metallo-β-lactamases and OXA-23, OXA-24, OXA-51 and OXA-58 type class D oxacillinases (Brown and Amyes, 2006; Peleg *et al*., 2008). The OXA enzymes are reported more prevalent in the *A. baumannii* worldwide compared to MBL enzymes (Poirel and Nordmann, 2006; Mugnier *et al*., 2010). Decreased susceptibility to carbapenem in *A. baumannii* is mainly associated to the presence of insertion sequence, IS*Aba1*. Promoter sequences in IS*Aba1* helps in genes regulation enhanced over production of the OXA genes enabling the organism to resist to carbapenem (Segal *et al*., 2005, Turton *et al*., 2006a).

Studies on the *A. baumannii* resistance mechanisms demonstrated the location of resistance genes on the mobile genetic elements, such as integrons and plasmids. Presence of the integrons often associated with the multi-resistance phenotypes of the strains. Integrons and plasmids possess specific recombination site, play an important role in acquisition and dissemination of the resistance determinants within the *Acinetobacter* spp.. With the ability to acquire antibiotic resistance genes and to survive on fomites or in the hospital environment for a prolonged period, endemic *A. baumannii* could persist in hospital (Musa *et al.,* 1990; Webster *et al.,* 2000; D'Agata *et al.,* 2000).

The persistence and spread of multidrug-resistant *A. baumannii* have reinforced the need for epidemiological studies describing the possible cross-infections among patients, sources and modes of transmission and the diversity of these strains (Dijkshoorn *et al*., 2008). Variety approaches have been developed for typing *Acinetobacter* spp. including DNA fragment-based, such as plasmid profiling, amplified fragment length polymorphism (AFLP), PCR fingerprinting (REP, ERIC, RAPD) and

pulsed-field gel electrophoresis (PFGE) or DNA sequence-based method, multilocus sequence typing (MLST) (Nemec *et al*., 2004; Nemec *et al*., 2001; Grundmann *et al*., 1997; Seifert *et al*., 2005; Bartual *et al*., 2005).

In Malaysia, detailed information on the antimicrobial resistance, resistance mechanisms and genetic relationship of *A. baumannii* is still lacking. In this study, the resistance phenotypes of the *A. baumannii* strains were determined using standard disk diffusion method. Strains that were resistant to carbapenem were used for further study on the carbapenem resistance mechanism, such as presence of the MBL and OXA carbapenemase genes, insertion elements, integrons and plasmids. Two genotyping methods, REP-PCR and PFGE, were applied to study the genetic relatedness of the strains. Documentation of the antibiogram patterns, resistance mechanisms and DNA fingerprinting data of *A. baumannii* is useful in determining the prevalence of these isolates within the hospital and its disease transmission in outbreaks. This could help to provide a better outbreak control and effectively manage the patients' infections in hospital settings.

1.2 Objectives

The overall aims of this study were to determine the genetic basis for the carbapenem resistance and genetic relatedness of the carbapenem-resistant *A. baumannii* strains isolated from patients, hands of healthcare workers (HCWs) and environment in the intensive care unit, University Malaya Medical Center on a 4-years period (2006 to 2009). Specifically, the objectives were:

a) To determine the antimicrobial resistance phenotypes of the *A. baumannii* strains.

b) To determine the carbapenem resistance genes and integrons by using polymerase chain reaction (PCR).

c) To determine the plasmid profiles of the strains.

d) To detect the localisation of the $bla_{\text{OXA-23}}$ gene on plasmid and/or chromosomal DNA.

e) To subtype the *A. baumannii* strains by using PCR-fingerprinting method (REP-PCR) and pulsed-field gel electrophoresis (PFGE).

CHAPTER 2: LITERATURE REVIEW

2.1 Genus *Acinetobacter*

The genus *Acinetobacter* was designated by Brisou and Prevot in 1954 (Brisou and Prevot). Species within the genus *Acinetobacter* could not be distinguished by phenotypic characterisation. Hence it was proposed that the genus contained only a single species, *A. calcoaceticus* (Peleg *et al*., 2008). In 1971, The Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria announced that the genus *Acinetobacter* should comprised only oxidase-negative strains and definition of the genus *Acinetobacter* was later accepted in the Bergey's Manual in 1984 (Bergogne-Berezin and Towner, 1996).

2.2 Taxonomy of genus *Acinetobacter*

The genus *Acinetobacter* was initially classified in the family *Neisseriaceae* due to a similar Gram-stained morphology with the *Neisseria* spp. (Bergogne-Berezin and Towner, 1996). In 1991, DNA-rRNA hybridisation has excluded the genus *Acinetobacter* from *Neisseriaceae* and was grouped in a new family *Moraxellaceae*, including *Moraxella*, *Psychrobacter* and allied organisms (Rossau *et al*., 1991).

To date, 40 species with 33 named and 7 unnamed genospecies have been described within the genus *Acinetobacter*. In 1986, using the DNA-DNA hybridisation tests, 12 genospecies of *Acinetobacter* (genospecies 1-12) was identified. Six of these genospecies were given the formal species names: *A. calcoaceticus* (genospecies 1), *A. baumannii* (genospecies 2), *A. haemolyticus* (genospecies 4), *A. junii* (genospecies 5), *A. johnsonii* (genospecies 7), and *A. lwoffii* (genospecies 8) (Bouvet and Grimont, 1986). The remaining genospecies which cannot be distinguished on the basis of phenotypic traits were not named (Bouvet and Grimont, 1986). *Acinetobacter*

genospecies 9 and *Acinetobacter lwoffii* were later found to belong to a single hybridisation group (Tjernberg and Ursing, 1989).

A new species of *Acinetobacter* was identified in 1988. This species was resistant to radiation, had different phenotypic and genotypic properties from other *Acinetobacter* species and was given the name *A. radioresistens*, formerly *Acinetobacter* genospecies 12 (Nishimura *et al*., 1988; Tjernberg and Ursing, 1989). Following a study by Tjernberg and Ursing, (1989), 3 additional *Acinetobacter* genospecies were described and numbered as *Acinetobacter* genospecies 13TU, 14TU and 15TU following the classification by Bouvet and Grimont, (1986). Concurrently, a study by Bouvet and JeanJean, (1989) found another 5 *Acinetobacter* genospecies, *Acinetobacter* genospecies 13BJ, *Acinetobacter* genospecies 14BJ, *Acinetobacter* genospecies 15 BJ, *Acinetobacter* genospecies 16 and *Acinetobacter* genospecies 17. *Acinetobacter* genospecies 13BJ was found to be similar to *Acinetobacter* genospecies 14TU (Tjernberg and Ursing, 1989).

A group of closely related *Acinetobacter* genospecies, comprising *A. calcoaceticus, A. baumannii, Acinetobacter* genospecies 3 and 13TU and two DNA hybridisation designated *Acinetobacter* genospecies "between 1 and 3" and "close to 13TU" were determined. These genospecies are closely related and could not be separated by phenotypic tests, thus were grouped as *Acinetobacter calcoaceticus-A. baumannii* complex (Gerner-Smidt *et al*., 1991 and 1993). A novel species which isolated from Venice lagoon was identified by Di Cello *et al*., (1997) and named as *A. venetianus* and was later validated by Vaneechouttee *et al*., (2009).

Within 3 years in 2001 to 2003, 14 novel species were identified, namely *A. schindleri, A. ursingii, A. parvus, A. berezinae, A. guillouiae, A. pittii, A. nosocomialis A. baylyi, A. bouvetii, A. gerneri, A. grimontii, A. tandoii, A. tjernbergiae* and *A. towneri* (Nemec *et al*., 2001, 2003; Carr *et al*., 2003). Later in 2007, 3 additional novel species, *A. marinus, A. seohaensis and A. septicus* were described (Yoon *et al*., 2007; Kilic *et*

al., 2007). However, Vaneechoutte *et al*., (2008) found that *A. grimontii* shared similar phenotypic and genotypic properties as *A. junii* and reclassified *A. grimontii* as later synonym of *A. junii.* Similarly, recent study indicated that *A. septicus* which proposed by Kilic *et al*., (2007) and *A. ursingii* species was represented one species (Nemec *et al*., 2008).

Another 10 novel species (*A. soli, A. antiviralis, A. beijerinckii, A. gyllenbergii, A. kyonggiensis, A. brisouii, A. rudis, A. oleivorans, A. oryzae* and *A. indicus*) were also identified by several research groups within 2008 to 2012 (Kim *et al*., 2008; Lee *et al*., 2009; Nemec *et al*., 2009; Lee and Lee, 2010; Anandham *et al*., 2010; Vaz-Moreira *et al*., 2011; Kang *et al*., 2011; Chaudhary *et al*., 2012, and Malhotra *et al*., 2012). Previously identified *Acinetobacter* genospecies 3, 10, 11 and 13TU have recently been proposed with formal species names, *A. pittii, A. berezinae, A. guillouiae* and *A. nosocomialis*, respectively (Nemec *et al*., 2010 and 2011).

2.3 Characterisation of genus *Acinetobacter*

Acinetobacter is short, plump, Gram negative rods with a DNA G+C content of 39% to 47%. They are non-fermenting, non-motile, strictly aerobic, oxidase-negative and can grow well on common complex media at incubation temperature between 33°C-37°C (Munoz-Price and Weinstein, 2008, Seifert and Dijkshoorn, 2008). The cell wall of *Acinetobacter* has the tendency to retain crystal violet causing difficulty in destain and often lead to misidentification as Gram-positive cocci. An overnight culture of *Acinetobacter* spp. form smooth, sometimes mucoid, pale yellow to greyish-white colonies on solid media with diameter size ranging 1.5 to 3.0 mm (Bergogne-Berezin and Towner, 1996; Peleg *et al*., 2008). Some *Acinetobacter* species (*Acinetobacter calcoaceticus-A. baumannii* complex) is able to grow on MacConkey agar and some (*A. haemolyticus*, *Acinetobacter* genospecies 6, 13BJ/14TU, 15BJ, 16, *A. venetianus, A.*

junii and *A. johnsonii*) has haemolytic activity on sheep blood agar (Vaneechoutte and De Baere, 2008). All *Acinetobacter* spp. is unable to reduce nitrate to nitrite, but could utilise various organic compounds and glucose for metabolism and energy production. However, no single metabolic test enables identification of *Acinetobacter* to the genus level. An unambiguous identification of *Acinetobacters* to the genus level is relied on transformation assay of Juni which based on the ability of a natural transformable tryptophan auxotroph, mutant *Acinetobacter* strain BD413 *trpE*27 which has been identified as *A. baylyi* (Vaneechouttee *et al*., 2006), to be transformed by crude DNA of any *Acinetobacter* species to a wild-type phenotype (Juni, 1972). Enrichment culture containing mineral media at relatively low pH with vigorous aeration and supplemented with acetate or other suitable carbon sources and nitrate as nitrogen source has proven useful to isolate *Acinetobacter* from environmental or clinical samples (Baumann, 1968). A selective medium, Leed *Acinetobacter* Medium (LAM) was formulated for isolation of *Acinetobacter calcoaceticus*-*A. baumannii* complex strains from the environment and clinical sources (Jawad *et al*., 1994).

2.4 Species identification

DNA-DNA hybridisation is recognised as the gold standard method for identification of *Acinetobacter* species (Bouvet and Grimont, 1986). Several different DNA-DNA hybridisation methods have been used for identification of *Acinetobacter* species, included S1 endonuclease method (Bouvet and Grimont, 1986), hydroxyapatite method with radioactive or non-radiactive DNA labelling (Tjernberg and Ursing, 1989; Carr *et al*., 2003), quantitative bacterial dot filter method (Nemec *et al*., 2001) and microplate method using photo-activable biotin labeled DNA (Nemec *et al*., 2003). However, these methods are not suitable for routine microbiology laboratories use as it is technically demanding, labour-intensive and time consuming (Dijkshoorn and

Nemec, 2008). The 28 phenotypic tests scheme proposed by Bouvet and Grimont (1986) has successfully discriminated 11 of the 12 genospecies initially described (Bouvet and Grimont, 1986; Bergogne-Beresin and Towner, 1996). Although this scheme has later been improved (Gerner-Smidt *et al*., 1991), cannot differentiate the genetically closely related genospecies 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3 (*A. pittii*) and 13 TU (*A. nosocomialis*) in the *A. calcoaceticus-A. baumannii* complex.

Several manual and semiautomated commercial phenotypic identification systems, such as API 20NE, VITEK 2/GNI card, ID 32 GN, Phoenix and MicroScan WalkAway are available for *Acinetobacter* species identification. However, these systems are problematic with poor accuracy of identification. The API 20NE and VITEK2 systems had failed and misidentified *A. ursingii* as other *Acinetobacter* genospecies (*A. lwofii, A. junii*, *A. johnsonii, A. baumannii* or *A. calcoaceticus*) (Dortet *et al*., 2006). In addition, the VITEK GNI card is unable to identify the strains within *A. calcoaceticus-A. baumannii* complex (Kuo *et al*., 2004; Lim *et al*., 2007). The ID 32 GN system was not able to differentiate *A. baumannii* from genospecies 13 (Shin *et al*., 2004). All of these commercial phenotypic identification systems are not reliable for species identification of the genus *Acinetobacter* particularly the member of the *A. calcoaceticus-A. baumannii* complex (Chang *et al*., 2005; Rodriguez-Banao *et al*., 2006).

To overcome the problems of phenotypic species identification, molecular identification methods have been developed and validated for identification of *Acinetobacter*, including ribotyping (Gerner-Smidt *et al*., 1992), tRNA spacer (tDNA) fingerprinting (Ehrenstein *et al*., 1996), sequence analysis of the 16S-23S rRNA gene spacer region (Nowak and Kur, 1995; Chang *et al*., 2005), *rpoB* gene and its flanking spacers (La Scola *et al*., 2006; Gundi *et al*., 2009) and *gyrB* genes (Yamamoto and Harayama, 1996), PCR-restriction fragment length polymorphism (RFLP) of 16S-23S

rRNA intergenic spacer sequences (Dolzani *et al*., 1995), *rec*A gene (Nowak and Kur, 1996; Jawad *et al*., 1998; Krawczyk *et al*., 2002) and 16S rDNA sequences (Vaneechoutte *et al*., 1995; Dijkshoorn *et al*., 1998) and amplified fragment length polymorphism (AFLP) analysis (Janssen *et al*., 1997; Nemec *et al.*, 2001).

In 1992, Gerner-Smidt, (1992) has introduced ribotyping method for the identification of the genetically related strains in *A. calcoaceticus-A. baumannii* complex by using 3 restriction enzymes *Eco*RI, *Cla*I and *Sal*I and hybridisation with a digoxigenin-11-dUTP-labeled probe derived from *Escherichia coli* rRNA (Gerner-Smidt, 1992). Ribotyping is highly discriminatory, the banding patterns generated are specific to the species level, reproducible and can be compared between laboratories. Thus, the method has been widely applied for typing and species identification of the *A. calcoaceticus-A. baumannii* complex strains (Chen *et al*., 2007; Huang *et al*., 2008).

Species identification by tDNA fingerprinting is a method that uses primers to amplify the spacer regions of tDNA clusters resulting in amplification profiles that are used for differentiation of strains at the species or genus level (Ehrenstein *et al*., 1996). Although this method is time-saving and reliable for the routine differentiation of most *Acinetobacter* spp. at the species level (17 out of 21 DNA-DNA hybridization groups), it is not able to differentiate strains between genospecies 1 (*A. calcoaceticus*) and 3 (*A. pittii*) and genospecies 2 (*A. baumannii*) and 13 TU (*A. nosocomialis*) (Ehrenstein *et al*., 1996).

Sequence analysis of the 16S-23S rRNA gene spacer region had high identification rate, at least 96% in species identification of the *A. calcoaceticus-A. baumannii* complex strains (Chang *et al*., 2005). The method involves an amplification and sequencing of the ITS region and a similarity comparison of the ITS sequence with those of the type and reference strains of *Acinetobacter* species. This method could also

be used to identify other named and unnamed *Acinetobacter* spp., but requires validation tests with more reference strains and clinical isolates.

Species identification using *gyrB* gene sequence analysis has also been proposed for identification of *Acinetobacter* isolates to the species level. However, it cannot delineate the genospecies 1 *(A. calcoaceticus),* 2 (*A. baumannii*), 3 (*A. pittii*) and 13TU (*A. nosocomialis*) in the *A. calcoaceticus-A. baumannii* complex, genospecies BJ15, BJ16 and BJ17 and genospecies 10 (*A. bereziniae*) and 11 (*A. guillouiae*) (Yamamoto *et al*., 1999). Sequence analysis of *rpoB* gene and its flanking spacer regions is a simple and accurate method which has been validated useful in the molecular identification of *Acinetobacter* (La Scola *et al*., 2006; Gundi *et al*., 2009). All of these sequence-based identification methods have contributed to a better identification of *Acinetobacter* at the species level, but are cost consuming and require a DNA sequencer which may not be accessible in most laboratories.

PCR-RFLP of conserved genes is a useful and easy-to-perform method for species identification of *Acinetobacter.* Amplification of 16S-23S rRNA intergenic spacer sequence followed by combined digestion using two restriction enzymes, *Alu*I and *Nde*II was a promising method for the identification of the genospecies belonging to the *A. calcoaceticus-A. baumannii* complex (Dolzani *et al*., 1995). The 16S-23S rRNA intergenic spacer-RFLP has later been successfully applied in other studies for *A. baumannii* identification (Kuo *et al*., 2004; Hernandez *et al*., 2011). Nowak and Kur, (1996) have proposed *rec*A-RFLP method in which 17 named and unnamed genospecies reference strains were successfully identified using two restriction enzymes, *Mbo*I and *Hinf*I. However, in another study, this application was failed to identify 32 well-characterized strains from six different genospecies (Jawad *et al*., 1998). Krawczyk *et al*., (2002) have later applied *rec*A-RFLP with an additional restriction enzyme, *Tsp*509I on 43 reference strains of 23 genospecies. The *Tsp*509I was

found to be most discriminative enzyme for species identification of *Acinetobacter* strains (Krawczyk *et al*., 2002) and *rec*A-RFLP might be an ideal method to identify large number of strains.

Amplified ribosomal 16S rDNA restriction analysis (ARDRA) is a robust and powerful method for identification of *Acinetobacter* species (Vaneechoutte *et al*., 1995). The ARDRA method has been validated using a large numbers of reference strains and seven restriction enzymes *Cfo*I, *Alu*I, *Mbo*I, *Rsa*I, *Msp*I, *Bfa*I and *Bsma*I. Database of ARDRA profiles of at least 21 named and unnamed genospecies reference strains are available for species identification of *Acinetobacters* (Dijkshoorn *et al*., 1998; http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html; Nemec *et al*., 2001 and 2003). Strains in *A. calcoaceticus-A. baumannii* complex can easily be identified using restriction profiling by five restriction enzymes, *Cfo*I, *Alu*I, *Mbo*I, *Rsa*I and *Msp*I (Dijkshoorn *et al*., 1998).

AFLP method for differentiation of genospecies in the genus *Acinetobacter* was introduced by Janssen *et al*., (1996). Initially, two restriction enzymes, *Hind*III and *Taq*I were used to digest the chromosomal DNA and primers T05 and 32P-labelled H01 with one or two adenosines as 3′extensions, respectively were used in selective amplification of the fragments (Janssen *et al*., 1996). The protocol was later been modified with digestion and ligation can be performed in a single step using *EcoR*I and *Mse*I as restriction enzymes and Cy5-labelled *Eco*RI-A primer (A=selective A base) and *Mse*I-C primer (C=selective C base) for selective amplification (Koeleman *et al*., 1998). The grouping level for species identification of the *Acinetobacters* was defined at 50% based on the analysis of AFLP profiles generated using 267 type and reference strains of the 31 described named and unnamed *Acinetobacter* genospecies (Dijkshoorn and Nemec, 2008). Using the AFLP method, 3 novel species, *A. ursingii, A. schindleri* and *A. parvus* were identified in 2001 and 2003 (Nemec *et al*., 2001 and 2003). AFLP analysis is

currently widely use for species identification of *Acinetobacters* (van den Broek *et al*., 2009; Donnarumma *et al*., 2010; Petersen *et al*., 2011).

Most recently, protein fingerprinting using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) is increasingly used for routine bacterial identification in clinical laboratories. MALDI-TOF MS is a promising and has an excellent ability in identification of *Acinetobacter* species. In a study by Seifert *et al*., (2007) MALDI-TOF MS was able to separate 552 well-characterized *Acinetobacter* strains into distinct clusters representing 15 different species. In another study by Nemec *et al*., (2010), a large set of *A. bereziniae* and *A. guillouiae* strains have also been successfully identified using the MALDI-TOF MS (Seifert *et al*., 2007; Nemec *et al*., 2010). Another approach, PCR detection of the $bla_{\text{OXA-51,}}$ the intrinsic carbapenemase gene in *A. baumannii* has been used as a rapid tool for identification of *A. baumannii* strains (Turton *et al*., 2006b). However, the $bla_{\text{OXA-51}}$ gene has recently been detected in non-*baumannii* strains, including *A. nosocomialis* and *Acinetobacter* genospecies "close to 13TU" (Lee *et al*., 2012). Therefore, the accuracy of identification using $bla_{\text{OXA-51}}$ detection is no longer reliable for differentiating *A. baumannii* from other *Acinetobacter* species.

2.5 Clinically important species of *Acinetobacter*

Among the 40 genospecies being described within the genus *Acinetobacter*, *A. calcoaceticus-A. baumannii* complex is the most associated with the clinical environment and nosocomial infections. *A. baumannii*, *A. pittii* (*Acinetobacter* genospecies 3) and *A. nosocomialis* (*Acinetobacter* genospecies 13TU) have been reported in many bloodstream infection related outbreaks (Wisplinghoff *et al*., 2000 and 2004; Montealegre *et al*., 2012; Lee *et al*., 2012). However, *A. lwoffii*, *A. johnsonii, A. ursingii, A. schindleri, A. haemolyticus* and *A. parvus* have been occasionally implicated

in hospital-acquired infections (Boo *et al*., 2009; Turton *et al*., 2010). *A. baumannii* is the most resistant to the antimicrobial agents, known as multi drug-resistant organism compared to other non-baumannii *Acinetobacter* isolates which are less resistant and easier to eradicate (Bergogne-Berezin, 2008).

2.6 Clinical significance of *A. baumannii*

A. baumannii is a successful opportunistic organism that has been associated with various diseases such as bacteraemia, septicaemia, wound infections, respiratory infections, meningitis, urinary tract infections and other miscellaneous infections (Peleg *et al*., 2008). *A. baumannii* mainly affects patients admitted to the intensive care units with severe underlying disease and a poor prognosis (Dijkshoorn *et al*., 2007). It is a common colonizer on human skin, but usually poses no threat to healthy individuals (Camp and Tatum, 2010).

Of the infections caused by *A. baumannii,* bacteraemia is the most significant infection with high morbidity and mortality often associated with MDR strains (Gkrania-Klotsas and Hershow, 2006; Lee *et al*., 2007). In a study carried out by Anunnatsiri and Tonsawan, (2011), almost half of the cases of *A. baumannii* bacteraemia in a tertiary care university hospital located in Northeast Thailand were due to MDR *A. baumannii* infections. Several factors have been identified as increasing risk factors of *A. baumannii* bacteraemia in patients admitted to an ICU, including colonization on the burn or open wounds, invasive procedures (central venous catheterization, mechanical ventilation and surgery) and frequent treatment with broad spectrum antimicrobials (Jung *et al.,* 2010). A high prevalence of *A. baumannii* bloodstream infections (6.11%) was recorded in the Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia, with majority of the cases reported were associated with nosocomial ventilator-associated pneumonia (48.3%), following by wound infection

(17.2%), intravascular catheter (6.9%) and urinary tract infections (1.7%) (Deris *et al*., 2009). *A. baumannii* also caused a significant bloodstream infections in the newborns hospitalised in the ICU. An outbreak of *A. baumannii* septicaemia was reported in the neonatal ICU in a Brazillian hospital, affecting 11 neonates, with 3 deaths. All the neonates had the predisposing risk factors for *A. baumannii* septicaemia such as low birth weight, long hospitalisation period, used of antibiotic for treatment and used of central or peripheral venous catheters (von Dolinger *et al*., 2005).

In the military population, *A. baumannii* is often causing deep wound or soft tissue infections in the injured soldiers (Camp and Tatum, 2010; Petersen *et al*., 2011). Several reports on the *A. baumannii* infections among military personnel with traumatic injuries during the conflicts in Iraq and Afghanistan have been reported (Davis *et al*., 2005; Zapor and Moran, 2005; Murray *et al*., 2008). Cases of osteomyelitis have been developed from deep wound infections due to *A. calcoaceticus-baumannii* complex among the soldiers in the 2003–2005 military operations in Iraq (Davis *et al*., 2005). MDR *A. calcoaceticus-baumannii* complex was also a common cause of infections among the burn patients in the US Army Institute of Surgical Research Burn Center (Albrecht *et al*., 2006). A high numbers of wounded soldiers and the transfer of these soldiers from one health care facility to another have assisted the spread and arising resistance of *A. baumannii* (Scott *et al*., 2007; Camp and Tatum, 2010).

A. baumannii isolates were frequently isolated from respiratory tracts of hospitalized patients particularly in the ICUs (Karageorgopoulos and Falagas, 2008). Most of the time, patients were simply colonised by *A. baumannii* rather than develop clinically significant infection of the respiratory tract (Fournier and Richet, 2006; Peleg *et al*., 2008). *A. baumannii* pneumonia was mostly associated with community- and hospital-acquired infections (Rodriguez-Bano *et al*., 2004; Peleg *et al*., 2008). Community-acquired pneumonia due to *A. baumannii* has been mostly reported in

Southeast Asia and tropical Australia (Anstey *et al*., 2002; Leung *et al*., 2006; Ong *et al*., 2009; Karageorgopoulos and Falagas, 2008). The risk factors of community-acquired *A. baumannii* pneumonia included diabetes mellitus, renal failure, excessive smoking and alcohol consumption and chronic obstructive pulmonary disease (Falagas *et al*., 2007a). Neonates with very low birth weight or premature admitted to the neonatal ICUs also posed very high risk to *A. baumannii* pneumonia (Touati *et al*., 2009).

A baumannii is an important cause of meningitis. *A. baumannii* meningitis mainly associated in patients undergone neurosurgical procedures or received intraventricular catheters (Wrobrewska *et al*., 2004; Krol *et al*., 2009; Yang *et al*., 2012). The mortality caused by *A. baumannii* meningitis ranged from 15% -71% (Kim *et al*., 2009). High mortality rate (71.4%) has been reported in patients with meningitis due to carbapenem-resistant *A. baumannii* (Metan *et al*., 2007). In a retrospective study, in which 51 patients were diagnosed with meningitis due to *A. baumannii*, 17 patients (33.3%) died due to the infection (Rodriguez-Guardado *et al*., 2008).

A. baumannii has rarely been reported as a causative agent for the development of nosocomial urinary tract infection. Typically, *A. baumannii* is associated with catheter-associated infection or colonization (Peleg *et al*., 2008). It is commonly found in patients with indwelling urinary catheters and prolonged hospital stays (Dijkshoorn *et al*., 2007; Rungruanghiranya *et al*., 2008). *A. baumannii* has been reported being responsible for 11% of urinary tract infections among 28 Spanish hospitals (Rodriguez-Bano *et al*., 2004). In a retrospective study performed in a teaching hospital Marseille, France, of the total *A. baumannii* infections occurred in 2002 to 2004, 25% were urinary tract infections (Fournier and Richet, 2006).

2.7 Treatment of *A. baumannii* infections and antimicrobial resistance

A. baumannii infections have usually been treated with carbapenems, sulbactam, aminoglycosides, fluoroquinolones, tetracyclines, glycylcyclines and polymyxins. However, the choice of appropriate antimicrobial therapy is limited due to build–up of resistance in *A. baumannii* to many of these agents. The most common mechanisms of antimicrobial resistance of *A. baumannii* including intrinsically or acquisition of resistance, production of β-lactamases, efflux pumps, decreased of permeability of the outer membrane, mutations in antibiotic targets and production of enzymes inactivating aminoglycosides (Karageorgopoulos and Falagas, 2008).

2.7.1 Carbapenems

Carbapenems are a class of β-lactam antibiotics with excellent antibacterial activity, are stable to most prevalent β-lactamases and have remained as the treatment of choice for serious infections caused by *A. baumannii* (Ehlers *et al*., 2012; Hawkey and Livermore, 2012). Although carbapenems may retain activity on *A. baumannii* isolates, carbapenem-resistant *A. baumannii* is increasingly reported (Higgins *et al*., 2010: He *et al*., 2011; Su *et al*., 2012).

Carbapenem resistance in *A. baumannii* is mediated by several different mechanisms, including acquired carbapenem-hydrolysing beta-lactamases (OXA-23-like, OXA-24-like, and OXA-58-like class D-oxacillanases, or IMP, VIM, SIM, GIM , SPM and NDM-type class B-metallo-β-lactamases), efflux pump mechanisms, modification of penicillin-binding-protein and alteration or loss of outer membrane proteins (Perez *et al*., 2007; Shahcheraghi *et al*., 2011; Mohamed and Raafat, 2011; Espinal *et al*., 2011; Hrabak *et al*., 2012). Over production of naturally occurring oxacillanase OXA-51-like has also contributed to carbapenem-resistance in *A.*

baumannii (Turton *et al*., 2006a). Presence of more than one of these resistance determinants could confer high-level resistance in the *A. baumannii* strains.

2.7.2 Sulbactam

Sulbactam is an active β-lactamase inhibitor, has intrinsic activity against many *Acinetobacter* strains (Rafailidis *et al*., 2007). Combination of sulbactam with β-lactam and sulbactam alone showed the highest activity against *A. baumannii* compared to clavulanate- and tazobactam-containing combinations (Higgins *et al*., 2004a). However, in sulbactam-containing combinations, the antimicrobial activity against isolates resistant to the β-lactam is determined by the intrinsic activity of sulbactam alone and does not result from β-lactamase inhibition (Brauers *et al*., 2005). The earliest report of ampicillin/sulbactam used for treatment was reported by Urban *et al*., (1993), in which 9 of 10 patients with imipenem-resistant *Acinetobacter* infections received ampicillin/sulbactam for 3 days and showed clinical improvement. Combination of sulbactam and ampicillin has also been used successfully for the treatment of MDR *A. baumannii* meningitis, ventilator-associated pneumonia, and catheter-related bacteraemia (Rodriguez-Guardado *et al*., 2008; Takahashi *et al*., 2009). A study from Korea demonstrated that treatment with cefoperazone/sulbactam has shown similar effectiveness to imipenem/cilastatin in patients with *A. baumannii* bacteraemia (Choi *et al*., 2006). Although the dosage of sulbactam for treatment of severe *A. baumannii* infections has been recommended at 6 g per day, use of high dose ampicillin/sulbactam (9 g intravenously every 8 hours) has been safely and effectively applied in the treatment of patients with MDR *A. baumannii* ventilator-associated pneumonia (Betrosian *et al*., 2008). Ampicillin/sulbactam-resistant *A. baumannii* has been reported in some countries, but the mechanism contributed to the resistance has yet been determined (Perez *et al*., 2007). Overexpression of the *adeB* gene of the efflux pump in

A. baumannii had shown a significant correlation with resistance to ampicillin/sulbactam (Chiu *et al*., 2010).

2.7.3 Aminoglycosides

Aminoglycosides are a class of antibiotics that could inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit (van Bambeke *et al*., 2010). The activity of aminoglycosides on MDR is lower compared with non-MDR *A. baumannii* isolates (Karageorgopoulos and Falagas, 2008). Two of the aminoglycoside agents, amikacin and tobramycin, have remained as therapeutic options for infection with MDR *A. baumannii* (Fishbain and Peleg, 2010). However, these agents are rarely used alone and are often used in combination with other classes of antimicrobial agents. Report on tobramycin used as monotherapy for *A. baumannii* infections showed similar risks of nephrotoxicity in patients compared with colistin (Gounden *et al*., 2009).

Resistance to aminoglycosides is mainly mediated by the production of aminoglycoside modifying enzymes (AMEs), including phosphotransferases, acetyltransferases, and nucleotidyltransferases (van Bambeke *et al*., 2010). These enzymes deactivating the aminoglycoside-modifying hydroxyl or amino groups and reducing their affinity for the target binding site (Esterly *et al*., 2011). The genes encoding for AMEs are mostly in association with class 1 integrons or can be located on plasmids or transposons (Gordon and Wareham, 2010). Another mechanism of resistance is the production of 16S rRNA methylase (*armA*, *rmtA*, *rmtB*, *rmtC*, and *rmtD*), which conferred high-level resistance to all clinically useful aminoglycosides, including gentamicin, tobramycin, and amikacin (Esterly *et al*., 2011). The *armA* is the most prevalence and has been described in *A. baumannii* from Korea, Japan, the United States and China (Lee *et al*., 2006; Doi and Arakawa, 2007; Adams-Haduch *et al*., 2008; Huang *et al*., 2012). Other mechanisms that involved in the resistance of

aminoglycosides include an alteration of membrane permeability, alteration of the target ribosomal protein, ineffective transportation of the antibiotic inside the bacteria (van Looveren *et al.*, 2004), and AdeABC and AbeM efflux pumps (Nemec *et al*., 2007).

2.7.4 Fluoroquinolones

Fluoroquinolones are antibiotics that work by inhibiting the activity of topoisomerases, including the DNA gyrase which responsible for supercoiling of the circular DNA and topoisomerase IV involved in the relaxation of the supercoiled circular DNA (van Bambeke *et al*., 2010). The effectiveness of the fluoroquinolones against *A. baumannii* has been decreased over the past decades (Higgins *et al*., 2010). The activity of the ciprofloxacin, gatifloxacin and levofloxacin against MDR or imipenem-resistant *Acinetobacter* isolates has been reported to be low (Gales *et al*., 2006; Scheetz *et al*., 2007; Valentine *et al*., 2008). Finafloxacin, a new fluoroquinolone agent, has a greater activity against ciprofloxacin-sensitive and -resistant *A. baumannii* and could be a promising new antimicrobial agent for treatment of *A. baumannii* infections at acidic body compartments (Higgins *et al*., 2010).

Fluoroquinolone resistance in *A. baumannii* is primarily due to chromosomal mutations in the quinolone-resistance-determining regions (QRDRs) of *gyrA* that encodes for DNA gyrase A subunit and *parC* that encodes for topoisomerase IV subunit (Doughari *et al*., 2011). In *A. baumannii,* the most frequent amino acid mutations that contributed to high-level of ciprofloxacin resistance occur at Gly 81 and Ser 83 in *gyrA*, and Ser 80 and Glu 84 in *parC* (Hamouda and Amyes, 2004; Valentine *et al*., 2008; Koo *et al*., 2010; Chiu *et al*., 2010). These mutations decrease the affinity of fluoroquinolone binding to the enzyme-DNA complex. Other mechanisms of fluoroquinolone resistance are membrane impermeabilisation and AdeABC and AbeM efflux systems (Vila *et al*., 2007).

2.7.5 Tetracyclines and glycylcyclines

Tetracyclines are antimicrobials that inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit (Karageorgopoulos and Falagas, 2008). The doxycycline and minocycline are the semi-synthetic tetracyclines which are available by intravenous infusion and minocycline is approved by the FDA for use in *Acinetobacter* infections (Fishbain and Peleg, 2010). A study by Akers *et al*., (2009) examined 133 *Acinetobacter baumannii-calcoaceticus* complex isolates of military origin; found that minocycline was the most active in vitro compared with tetracyclines and tigecycline. Both doxycycline and minocycline have been successfully used for treatment in patients with wound infections and ventilator-associated pneumonia caused by MDR *A. baumannii* (Wood *et al*., 2003; Bishburg and Bishburg, 2009).

Glycylcyclines are in a new class of antibiotics derived from tetracycline (Chopra, 2001). Tigecycline, a semi-synthetic derivative of minocycline, is the first antibiotic in the glycylcyclines. It has a similar mechanism of action as tetracyclines and shows bacteriostatic activity against *A. baumannii* (Maragakis and Perl, 2008; Fishbain and Peleg, 2010). The surveillance studies showed tigecycline is actively against *A baumannii* (Halstead *et al*., 2007; Reinert *et al*., 2007). A study by Bassetti *et al*., (2010) reported high eradication rates (69%) of *A. baumannii* isolates with tigecycline and tigecycline appeared to be safe and effective for treatment of serious hospital-acquired infections. Tigecycline in combination with other antimicrobials was effective for treatment of various infections caused by *A. baumannii*, including ventilator-associated pneumonia and primary or secondary bacteraemia (Karageorgopoulos *et al*., 2008; Principe *et al*., 2009).

Resistance to tetracyclines in *A. baumannii* is mediated by the expression of efflux pumps which are encoded by two efflux determinants, *tet*(A) and *tet*(B) (Huys *et al*., 2005; Akers *et al*., 2009; Mak *et al*., 2009). The *tetA* gene tends to confer resistance

to tetracycline and *tetB* confers resistance to both tetracycline and minocycline (Huys *et al*., 2005). Another mechanism that confers resistance to tetracyclines is the ribosomal protection system. *tetM* gene encodes ribosomal protection protein which helps to protect the ribosome from tetracycline, doxycycline, and minocycline (Perez *et al*., 2007). This *tetM* gene has rarely been described in clinical isolates of *A. baumannii* (Ribera *et al*., 2003). Compared with tetracyclines, neither efflux pumps nor the ribosomal protection protein is able to interfere with the action of tigecycline (Peleg *et al*., 2008). A study by Ruzin *et al*., (2007) has determined the role of the AdeABC efflux pump as a mechanism of resistance to tigecycline. The overexpression of the *adeB* gene codes for the transmembrane protein of the AdeABC efflux pump is associated with the increased of tigecycline MIC in *A. baumannii* strains (Peleg *et al*., 2007).

2.7.6 Polymyxins

Polymyxins are cationic polypeptides that interact with the lipopolysaccharide molecules of Gram-negative bacteria and increase the cell-envelope permeability to allow entry of polymyxins leading to a leakage of cytoplasmic contents (Falagas and Kasiakou, 2005). The clinical use of polymyxins has been abandoned in the 1960s and 1970s due to problems of toxicity (Munoz-Price and Weinstein, 2008; Karageorgopoulos *et al*., 2008). However, with the limited therapeutic options that are available, polymyxins have been considered for treatment of the MDR Gram-negative bacteria infections (Falagas *et al*., 2005). Colistin (polymyxin E) and polymyxin B are the two agents which currently available as a last resource drug for treatment of life-threatening MDR *A. baumannii* infections (Dijkshoorn *et al*., 2007). Colistin has been proved to be efficient and safe for the treatment of MDR *A. baumannii* infections, including ventilator-associated pneumonia, bloodstream, wound and urinary tract

infections, although nephrotoxicity remains a concern (Linden and Paterson, 2006; Gounden *et al*., 2009). Combined therapy with intrathecal and intravenous colistin is also an effective and safe option for treatment of nosocomial *A. baumannii* meningitis (Rodriguez-Guardado *et al*., 2008). Polymyxin B is effectively used for treatment in critically ill patients with MDR *A. baumnnnii* infections; with development of nephrotoxicity and neurotoxicity are concerns (Holloway *et al*., 2006).

Although polymyxin-resistant *A. baumannii* is still rare, increasing use of these agents may lead to the emergence of resistance. A study by Ko *et al*., (2007) has reported high rates of resistance to colistin and polymyxin B in *A baumannii* isolates from two South Korean hospitals. Polymyxin-resistant *A. baumannii* has also been reported in the United States, Brazillian, Greek and Spanish hospitals (Urban *et al*., 2001; Reis *et al*., 2001; Souli *et al*., 2006; Valencia *et al*., 2009). The resistance mechanism of polymyxins in *A. baumannii* has not been completely identified, but most probably is mediated by modifications in the lipopolysaccharide or changes of the outer membrane proteins (Esterly *et al*., 2011). Adam *et al*., (2009) has identified the mutations in the genes encoding the two component signalling proteins (PmrB and PmrA) leading to the colistin resistance in *A. baumannii*. In another study, mutations on the lipid A biosynthesis gene, *lpxA*, *lpxC*, or *lpxD*, causing loss of lipopolysaccharide production have resulted in increased MICs of colistin (Moffatt *et al*., 2010).

2.8 Mechanisms of carbapenem resistance in *A. baumannii*

A. baumannii infections are difficult to treat due to its tendency to acquire resistance to multiple antimicrobial agents that are available. Carbapenems are recognized as the last options for treatment in the life threatening infections caused by MDR *A. baumannii* (Fishbain and Peleg, 2010). In the past decade, increasing resistance of *A. baumannii* to carbapenems has been observed worldwide and raised a

global concern (Higgins *et al*., 2010). The carbapenem resistance in *A. baumannii* is mainly mediated by acquisition of metallo-β-lactamases and oxacillanases (Ehlers *et al*., 2012). Changes in outer membrane proteins, modifications of penicillin-binding proteins and efflux pumps could also mediate carbapenem resistance in *A. baumannii* (Mussi *et al*., 2005; Siroy *et al*., 2005)

2.8.1 Metallo-β-lactamases (MBLs)

MBLs are class B beta-lactamases capable of hydrolyzing carbapenems and other beta lactam antimicrobials with the exception of monobactams (aztreonam). These enzymes have a metal ion in the active site, usually zinc, to help in catalysis (Walsh *et al*., 2005). To date, 6 types of MBLs (IMP-, VIM-, SIM-, GIM-, SPM- and NDM-type) have been described in *A. baumannii* (Peleg *et al*., 2008; Shahcheraghi *et al*., 2011; Mohamed and Raafat, 2011; Espinal *et al*., 2011; Hrabak *et al*., 2012).

The IMP-1 was firstly described in a *Pseudomonas aeruginosa* strain in Japan in 1988 (Watanabe *et al*., 1991). This IMP-1 has later been identified in *A. baumannii* in Italy, Brazil, Japan and South Korea (Riccio *et al*., 2000; Tognim *et al*., 2006; Nishio *et al*., 2004; Sung *et al*., 2008). Recently, integron-borne $bla_{\text{IMP-1}}$ mediated imipenem resistance in *A. baumannii* isolates was reported in a Taiwanese hospital (Chiu *et al*., 2010). Several other IMP variants (IMP-2, IMP-4, IMP-5, IMP-6, IMP-11 and IMP-19) have also been described in *A. baumannii* in different geographic regions (Walsh *et al*., 2005; Yamamoto *et al.,* 2011). The IMP-2 was first described in *A. baumannii* in Italy with the amino sequence 85% identical to IMP-1, and was found in integron cassette In*42* ($bla_{\text{IMP-2}}$-*aacA4-aadA1*) (Riccio *et al*., 2000). The IMP-4 is identified from the imipenem-resistant *Acinetobacter* collected between 1994 and 1998 in Hong Kong. This enzyme shares amino acid sequence of 95.6% homology to IMP-1 and 89.3% homology to IMP-2 (Chu *et al*., 2001). The $bla_{\text{IMP-4}}$ gene was born in class 1 integron gene cassette

(*bla*$_{IMP-4}$-*qacG2-aacA4-catB3*) and on a plasmid (Houang *et al*., 2003). A new allelic variant of other *bla*$_{IMP}$ genes, named *bla*$_{IMP-5,}$ with a greater amino acid homology with IMP-1, IMP-3 and IMP-4 than with IMP-2 (93%, 92%, 91% and 87%, respectively) has been identified in an *A. baumannii* nosocomial isolate in Portugal (Da Silva *et al*., 2002). The *bla*$_{IMP-5}$ was reported in class 1 integron, In*76*, embedded in a TN*402*-like transposon (Domingues *et al*., 2011). IMP-6 described in *A. baumannii* in Brazil was first being described in a *Shigella flexneri* isolate. It had two amino acid changes from IMP-1 and display reduced activity against penicillin and piperacillin, and higher level of meropenem hydrolysis compared to imipenem (Walsh *et al*., 2005). Recently, *bla*$_{IMP-19}$ gene was found in *A. baumannii* isolates and located in a class 1 integron as a gene cassette array of *bla*$_{IMP-19}$-*aac6-31-bla*$_{OXA-21}$-*aadA1* (Yamamoto *et al*., 2011).

Verona/integron-encoded MBL or known as VIM was firstly described in Italy in 1997 from a *Pseudomonas aeruginosa* strain (Laurettii *et al*., 1999). To date, VIM enzymes have been rarely identified in *A. baumannii* (Lee *et al*., 2003; Yum *et al*., 2002; Yong *et al*., 2006; Tsakris *et al*., 2006). VIM-1 has been reported in Greek isolates and found in class 1 integron cassette of *bla*$_{VIM-1}$-*aacA7-dhfr*I-*aadA1* (Tsakris *et al*., 2006). The VIM-2 has only been identified in isolates from South Korea, and study by Yum *et al*., (2002) identified the VIM-2 gene in a novel integron In*105* (*bla*$_{VIM-2}$-*aacA7- aadA1*).

Seoul imipenemase (SIM-1) is a novel MBL identified in *A. baumannii* isolates in Korea (Lee *et al*., 2005). The SIM-1 enzyme is categorized in a new subclass B1 MBL, sharing 69% identity with IMP-12 and 64% identity with IMP-9. The *bla*$_{SIM-1}$ cassette was suggested for originate from *Pseudomonas alcaligenes* In*55044* superintegron (Lee *et al*., 2005).

The Sao Paulo MBL or known as SPM-1 was identified in a clinical *P. aeruginosa* isolate from Sao Paulo, Brazil in 1997 (Toleman *et al*., 2002). The sequence

of the SPM-1 is significant different from the IMP and VIM, with only 35.5% identities to that IMP-1 (Walsh *et al*., 2005). This SPM-1 enzyme has recently only been identified in *A. baumannii* (Shahcheraghi *et al*., 2011; Mohamed and Raafat, 2011).

German imipenemase (GIM-1) is identified in *P. aeruginosa* isolates from a medical site in Dusseldorf, Germany in 2002 (Castanheira *et al*., 2004). The GIM-1 contains amino acid sequence mostly identity to the IMP variants of IMP-6, IMP-1, and IMP-4 (43.5, 43.1, and 43.1%, respectively) and approximately 30% homology to VIM and 29% homology to SPM-1 (Walsh *et al*., 2005; Queenan and Bush, 2007). Recent study by Mohamed and Raafat, (2011) reported $bla_{GIM\text{-}1}$ in a single imipenem-resistant *A. baumannii* isolated from the Main University Hospital in Egypt.

Recently, a novel MBLs namely New Delhi metallo-β-lactamase (NDM) has been described in a Swedish patient who contracted a MDR *Klebsiella pneumoniae* urinary tract infection during his travel in India (Yong *et al*., 2009). NDM-1 shares very low identity with other MBLs, only 32.4% identity to VIM-1/VIM-2 and confers highly resistant to all carbapenems (Yong *et al*., 2009). NDM-1 is mostly found in *Klebsiella pneumoniae* and among a broad range of other Enterobacteriaceae with majority carrying the $bla_{NDM\text{-}1}$ on plasmids, a potential of spread between bacterial strains (Kumarasamy *et al*., 2010). The emergence and dissemination of NDM-1-producing *A. baumannii* isolates have been reported in several countries, including India, China, Japan, Czech Republic and Germany (Kumarasamy *et al*., 2010; Chen *et al*., 2011; Nakazawa *et al*., 2012; Pfeifer *et al*., 2011; Hrabak *et al*., 2012; Nemec and Krizova, 2012). Most recently, Kaase *et al*., (2011) described a new variant of NDM-1 in *A. baumannii* and named NDM-2. The sequence of NDM-2 had a substitution from C to G at position 82 from the start codon resulting in an amino acid substitution from proline to arginine at position 28 compared with NDM-1. A clonal dissemination of a NDM-2 producing *A. baumannii* has later been reported in an Israeli rehabilitation ward (Espinal

et al., 2011). The *bla*$_{NDM-2}$ has been suggested to be chromosomally encoded (Kaase *et al*., 2011; Espinal *et al*., 2011).

2.8.2 Carbapenem-hydrolysing oxacillanases (OXAs)

Carbapenem-hydrolysing oxacillanases (OXAs) are class D beta-lactamases uses a catalytically active serine residue for inactivation of the β-lactam antimicrobials, particularly carbapenems (Perez *et al*., 2007). To date, 4 clusters of OXA enzymes have been described in *A. baumannii*. The first described OXA-type enzyme in *A. baumannii* was ARI-1 (*Acinetobacter* Resistant to Imipenem), obtained from a clinical strain isolated in 1985 from Edinburgh, Scotland (Paton *et al*., 1993). The ARI-1 was encoded on a transferable plasmid and sequence analysis revealed that it belonged to the OXA–type enzyme, designated as OXA-23 (Scaife *et al*., 1995; Donald *et al*., 2000). Together with OXA-27 and OXA-49, the first gene cluster of OXA genes (*bla*$_{OXA-23}$) was defined in *A. baumannii* (Afzal-Shah *et al*., 2001; Brown and Amyes, 2006). The OXA-23-type enzymes have been described in carbapenem-resistant *A. baumannii* globally (Nordmann and Poirel, 2008; Peleg *et al*., 2008). Poirel *et al*., (2008) have suggested *A. radioresistens* is the reservoir of the *bla*$_{OXA-23}$ genes, and the findings of the *bla*$_{OXA-23}$ in environmental *A. baumannii* indicate possible niches of genes transfer (Girlich *et al*., 2010).

The second cluster of OXA enzymes which has been identified in *A. baumannii* comprising OXA-24, OXA-25, OXA-26, OXA-40 and OXA72, sharing less than 60% amino acid identity with OXA-23 (Brown and Amyes, 2006; Walther-Rasmussen and Hoiby, 2006). The OXA-24 and OXA-25 enzymes were first identified in carbapenem-resistant *A. baumannii* from Spain, whereas OXA-26 and OXA-40 were identified from Belgium and Portugal (Bou *et al*., 2000a; Afzal-Shah *et al*., 2001; Lopez-Otsoa *et al*., 2002; Heritier *et al*., 2003). The OXA-72 was first described in *Acinetobacter* strain

from Thailand in 2004 (GenBank accession no. AY739646), and has later been identified in several outbreaks of carbapenem-resistant *A. baumannii* in Taiwan, Brazil, France and Croatia (Lu *et al*., 2009; Werneck *et al*., 2010; Barnaud *et al*., 2010; Goic-Barisic *et al*., 2011). These OXA-24 type enzymes can be either chromosomal or plasmid-encoded (Walsh, 2010).

The third cluster consists of OXA-51 family enzymes (OXA-51, OXA-64 to -66, OXA-68 to -71, OXA-75 to -78, OXA-83, OXA-84, OXA-86 to -89, OXA-91, OXA-92, OXA-94 and OXA-95) which encoded by $bla_{\text{OXA-51}}$-like genes which is naturally occurring in *A. baumannii* (Queenan and Bush, 2007). OXA-51 was first described in two imipenem-resistant *A. baumannii* from Argentina (Brown and Amyes, 2005). This cluster of OXA β-lactamases shares less than 63% amino acid identity with OXA-23 and OXA-24 enzymes (Poirel and Nordmann, 2006). The $bla_{\text{OXA-51}}$-like genes are chromosomally encoded and its role in carbapenem resistance appears to be related to the presence of IS*Aba1* (Turton *et al*., 2006a and 2006b).

The fourth cluster of OXAs contains OXA-58, which has been identified in a carbapenem-resistant *A. baumannii* strain recovered in Toulouse, France (Poirel *et al*., 2005). The OXA-58 shares less than 50% amino-acid identity with other OXA enzyme. The OXA-97, which has been identified in *A. baumannii* from Tunisia, is the second member of the OXA-58 (Poirel *et al*., 2008). OXA-58 enzyme have been reported worldwide with sporadic outbreaks (Pournaras *et al*., 2006; Castanheira *et al*., 2008; Stoeva *et al*., 2009; Higgins *et al*., 2010). The $bla_{\text{OXA-58}}$-like genes have been identified as being plasmid-encoded and associated with insertion sequence (IS) elements which play a role in enhancing expression of OXA-58 (Poirel and Nordmann, 2006).

The IS elements have an important role for carbapenem resistance due to OXAs in *A. baumannii* (Turton *et al*., 2006a; Corvec *et al*., 2007). Several studies have demonstrated the role of IS*Aba1* in providing a strong promoter which helped in over

expression of intrinsic *bla*$_{OXA51}$-like and acquired *bla*$_{OXA-23}$ like genes in *A. baumannii* (Segal *et al*., 2005; Turton *et al*., 2006a; Corvec *et al*., 2007; Chen *et al*., 2009). The IS*Aba2*, IS*Aba3*, IS*18*, and IS*Aba825* have also been reported in providing strong hybrid promoters for *bla*$_{OXA-58}$-like gene in *A. baumannii* strains (Poirel *et al*., 2006; Gur *et al*., 2008; Ravasi *et al*., 2011). Whereas, over expression of the *bla*$_{OXA-23}$ like gene associated with IS*Aba4* has also been demonstrated (Corvec *et al*., 2007). Besides responsible in enhancing gene expression, IS*Aba1* is also responsible for the mobility of *bla*$_{OXA-23}$ like gene, with two copies of IS*Aba-1* elements bracketing the gene forming a putative composite transposon, Tn*2006,* or with only a copy located at one side of the gene forming one-ended transposon, named Tn*2008* (Corvec *et al*., 2007; Mugnier *et al*., 2009; Wang *et al*., 2011). In addition, a single copy of IS*Aba4* located at one side of *bla*$_{OXA-23}$ like gene formed a one-ended transposon, Tn*2007,* might responsible for the mobility of the gene (Corvec *et al*., 2007).

2.8.3 Changes in outer membrane proteins (OMPs), modifications of penicillin-binding proteins and efflux pumps

Contribution of porins or outer membrane proteins (OMPs) and penicillin binding proteins (PBPs) to antibiotic resistance in *A. baumannii* have been less well characterized. Carbapenem resistance due to loss of porins with reduced expression of 37-, 44- and 47- kDa OMPs has been reported in epidemic MDR *A. baumannii* in New York City (Quale *et al*., 2003). In Spain, reduced expression of 22- and 33- kDa OMPs in association with the production of OXA-24 have resulted in resistance to carbapenems in *A. baumannii* isolates (Bou *et al*., 2000a). The loss of a 29-kDa CarO protein in *A. baumannii* has played a role to mediate carbapenem resistance (Limansky *et al*., 2002, Mussi *et al*., 2005; Siroy *et al*., 2005; Lee *et al*., 2011). A study by Fernandez-Cuenca *et al*., (2003) has described reduced expression of PBP-2 together

with the production of oxacillanases were the most frequently observed mechanisms of resistance to carbapenems in *A. baumannii* from Spain.

The resistance-nodulation-division (RND)-type efflux pump AdeABC plays a role in acquiring antimicrobial resistance in *A. baumannii* has been well characterized (Magnet *et al*., 2001; Higgins *et al*., 2004b; Marchand *et al*., 2004). The AdeABC is regulated by a two-component system, the regulator (*adeR*) and sensor (*adeS*), and single point mutation in *adeR* or *adeS* gene will result in increase of AdeABC efflux pump expression (Marchand *et al*., 2004). However, its role in *A. baumannii* against carbapenems remains unclear (Bratu *et al*., 2008). Huang and colleagues (Huang *et al*., 2010) suggested that AdeABC efflux pump played a less important role in *A. baumannii* against carbapenems. They found no mutations occured in the *adeR* and *adeS* regulative genes of the carbapenem-resistant strains and suggested that co-existence of oxacillanase genes and efflux systems may play a role in the resistance to carbapenem (Huang *et al*., 2010). Presence of oxacillanase genes ($bla_{\text{OXA-23}}$, $bla_{\text{OXA-40}}$ and $bla_{\text{OXA-58}}$) together with over expression of the AdeABC efflux pump associated in high level of resistance to carbapenem in *A. baumannii* isolates which has been reported in Korea and France (Heritier *et al*., 2005; Lee *et al*., 2010).

2.9 Integrons

The emergence and rapid dissemination of antibiotic resistance genes among *A. baumannii* isolates is an increasing problem, globally. The resistance genes are usually acquired through mobile elements such as plasmids, transposons and integrons (Fournier *et al*., 2006). Integrons are genetic elements which capable to capture exogenous genes (mostly antibiotic resistance determinants) and rearrange open reading frames (ORFs) embedded in gene cassette, which are converted into functional genes upon correct expression (Cambray *et al*., 2010). Integrons possess three key elements

which are essential for capturing exogenous genes; a gene encoding for an integrase (*intI*), a primary recombination site (*attI*), and a promoter (Pc) for genes transcription (Hall and Collis, 1995).

To date, five classes of integrons have been described based on the sequence of the encoding integrase genes (40%-58% identity) (Mazel, 2006). All these classes are physically associated with mobile genetic elements, including insertion sequences, transposons and conjugative plasmids, which serve as genetics vehicles for transmission among bacterial of the same or different species (Mazel, 2006). Class 1 integrons are associated with active transposon, Tn*402*, which can be embedded in larger transposons, Tn*21* (Cambray *et al*., 2010). Class 2 integrons are embedded in transposon Tn*7*, which containing *tns* transposition region which is necessary for transposition (Patridge *et al*., 2009). Class 3 integrons were described in *Serratia marcescens* and are less prevalent compared to class 2 integrons. It is recognised to be located in a transposon inserted in as-yet-uncharacterized plasmids (Cambray *et al*., 2010). Both class 4 and class 5 integrons were identified through their involvement in the development of trimethoprim resistance in *Vibrio* species (Mazel, 2006). Class 4 integrons are located in a subset of SXT elements found in *Vibrio cholerae*, while class 5 integrons are located in a compound transposon carried on a plasmid of *Vibrio salmonicida* (Sorum *et al*., 1992; Hochhut *et al*., 2001).

Among the integrons, class 1 integrons are the most prevalent compared with class 2 which has rarely been defined in *A. baumannii* (Sirichot *et al*., 2009; Chen *et al*., 2009; Ramirez *et al*., 2012). Aminoglycosides resistance genes such as *aac* (acetyltransferases), *aad* (adenylyltransferase) and *aph* (phosphotransferases) are mostly associated with the class 1 integrons in *A. baumannii* (Nemec *et al*., 2004b). A total of 12 different combinations of aminoglycoside resistance genes in the gene cassettes have

been described among the aminoglycoside-resistant pan-European *A. baumannii* clones (Nemec *et al*., 2004b).

In *A. baumannii*, most of the acquired MBL genes such as bla_{IMP}, bla_{VIM} and bla_{SIM} have been found within class 1 integrons (Poirel and Nordmann, 2006). Unlike the MBLS, OXA-type carbapenemases in *A. baumannii* are not integrated into integrons as gene cassettes, but mostly are plasmid-encoded in association with transposons as genetic vehicle for their mobilisation (Poirel *et al*., 2010). Chromosomal- and plasmid-borne bla_{OXA-23} gene as part of the transposon structures, including Tn*2006*, Tn*2007*, and Tn*2008* has been reported in *A. baumannii* (Corvec *et al*., 2007; Mugnier *et al*., 2010)

Class 2 integrons are more prevalent in *A. baumannii* from South America, including Chile, Argentina, Uruguay and Brazil (Gonzalez *et al*., 1998; Ramirez *et al*., 2010a; Fonseca *et al*., 2011). Two class 2 integron variable regions, Tn*7*::In*2-8* and Tn*7*::In*2-7*, with array structures of *sat2-aadB-catB2-*(Δ*attC*)*-dfrA1-sat2-aadA1-orfX* and *dfrA1-sat2-aadA1-orfX-ybfA-ybfB-ybgA*, *dfrA1-sat2* and *sat2-aadA1-orfX-ybfA-ybfB-ybgA,* respectively have been identified in *A. baumannii* from Argentina and Uruguay. (Ramirez *et al*., 2005 and 2012).

2.10 Molecular typing of *A. baumannii*

Nosocomial outbreaks of *A. baumannii* infection have been increasingly reported worldwide. The ability of *A. baumannii* to gain antibiotic resistance genes and to survive on inanimate and dry surfaces has contributed to the persistence of endemic strains in hospital settings (Musa *et al.*1990; Webster *et al.* 2000; D'Agata *et al.* 2000). Therefore, identification and molecular typing are important to gain a better comprehension of the epidemiology and, in particular, the mode of spread of *A. baumannii*. A variety of molecular typing methods have been developed for

epidemiological studies of *A. baumannii*, including plasmid profiling, DNA fragment-based such as amplified fragment length polymorphism (AFLP), PCR fingerprinting (REP, ERIC, RAPD), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Nemec *et al*., 2001; Grundmann *et al*., 1997; Seifert *et al*., 2005; Bartual *et al*., 2005).

2.10.1 Plasmid profiling

Plasmids are extra chromosomal circular DNA elements that can present and replicate independently of the chromosomes in most clinical isolates (Tenover, 1985). Plasmid profiling which assesses the number and sizes of plasmids or restriction digestion profiles has been applied as molecular typing tool of many bacterial species (van Belkum *et al*., 2007). It has been applied for epidemiological typing of *Acinetobacter* as most of the species harbours indigenous plasmids (Pardesi *et al*., 2007; Peleg *et al*., 2008; Sevillano and Gallego, 2010). Plasmid profiling has also successfully been used for the epidemiological study of *A. baumannii* strains (Seifert *et al*., 1994; Patwardhan *et al*., 2008). However, plasmids can be easily lost, gained or transferred among strains, rendered it not a definitive typing method for epidemiological studies of *Acinetobacter* (van Belkum *et al*., 2007). It is recommended to be used with other robust molecular typing methods in order to obtain a better understanding of the epidemiology of *A. baumannii* infections. Plasmid analysis in conjunction with PFGE and MLST has been successfully determined the clonally dissemination of plasmid harbouring $bla_{\text{OXA-24}}$ carbapenem-resistant *A. baumannii* in Spain (Acosta *et al*., 2011). In addition, an occurrence of a novel epidemic *A. baumannii* clone harbouring plasmid-borne $bla_{\text{OXA-58}}$ gene has also been identified in the hospital in Naples, Italy (Giannouli *et al*., 2010). The combination of plasmid analysis with other molecular typing methods

is useful for global epidemiological studies of MDR *A. baumannii* in order to control the epidemic spread of MDR *A. baumannii* infections in hospital settings.

2.10.2 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a typing method based on amplification of selected restricted genomic DNA fragments, generated with one or two restriction enzymes, usually one cuts more frequently than the other (Vos *et al*., 1995; Janssen *et al*., 1996). Ligation of adaptors is then performed to the sticky ends of the restriction fragments, followed by selective amplification of sets of restriction fragments using primers designed to prime to the adaptor sequences, the remaining restriction site sequence, and an additional one or more selective nucleotides (Singh *et al*., 2006). The amplified fragments can either be separated by gel electrophoresis or using an automatic DNA sequencing instrument with automated data captures as one primer is usually labelled (van Belkum *et al*., 2007).

This AFLP method has been applied successfully to identify the clones within *A. baumannii* and epidemic strains in Netherlands and Italy (van den Broek *et al*., 2009; Donnarumma *et al*., 2010; Carretto *et al*., 2011). Although the AFLP DNA fingerprints are generally highly reproducible, it is not inter-centre reproducible when different electrophoresis platforms are used. In addition, it requires a dedicated software for cluster analysis as the profiles generated with labelled primers and automated sequencing instrument are highly complex (van Belkum *et al*., 2007).

2.10.3 PCR fingerprinting (REP, ERIC, RAPD)

PCR-based fingerprinting methods such as REP, ERIC and RAPD-PCR have been widely used in subtyping of *A. baumannii* isolates (Grundmann *et al*. 1997; Koelemen *et al*., 1998; Silbert *et al*., 2004). Repetitive extragenic palindromic PCR

(REP-PCR) uses consensus primers to amplify intervening sequences between highly conserved repetitve sequences to assess the genetic relatedness of *A. baumannii* strains (Snelling *et al*., 1996). REP-PCR has been described as a simple, rapid, and cost effective method for subtyping of nosocomial outbreak *A. baumannii* strains as it could provides reproducible and discriminative results (Bou *et al*., 2000b and 2000c; Martin-Lozano *et al*., 2002). A standardised and automated rep-PCR, known as DiversiLab system (bioMerieux) has been developed to allow a more efficient with higher discriminatory power and increased interlaboratory reproducibility for bacterial strain typing (Healy *et al*., 2005; Higgins *et al*., 2012). Recently, rep-PCR typing using the DiversiLab system has identified global dissemination of eight carbapenem-resistant *A. baumannii* clonal lineages (WW1-8) (Zander *et al*., 2012; Higgins *et al*., 2010).

The enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) is performed by using primers which complementary to the intergenic repetitive sequence that highly conserved in the genome DNA (Hulton *et al*., 1991). This method has been successfully applied for the genetic diversity study of $bla_{\text{OXA-143}}$- and $bla_{\text{OXA-23}}$-positive strains in Brazil (Antonio *et al*., 2011). Silbert *et al*., (2004) has indicated that ERIC-PCR is in comparable to PFGE for species evaluation and has suggested it can be used for initially typing of organisms, unless isolates are indistinguishable, a higher discriminatory method such as PFGE can be applied for confirmation.

Another approach that has been used for molecular subtyping of *A. baumannii* is randomly amplified polymorphic DNA PCR (RAPD-PCR) which involves random fragment amplifications of genomic DNA with single arbitrary primer (Wisplinghof *et al*., 2008; Chang *et al*., 2009). RAPD-PCR gave consistent results with PFGE and MLST in an investigation of the molecular epidemiology of *A. baumannii* isolates from Europe and the United States (Wisplinghoff *et al*., 2008). Chang *et al*., (2009), applied REP and RAPD-PCR to investigate a nosocomial outbreak of MDR *A. baumannii* in a

Taiwanese hospital, found all the isolates were belong to an epidemic strain that might have been transmitted among the health care workers and medical equipment.

PCR-based fingerprintings are useful as simple and rapid identification techniques for a quick estimate of epidemiological relatedness in a defined setting; however, their reproducibility, typeability and discriminatory power are lower than PFGE and MLST (Peleg *et al*., 2008).

2.10.4 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is introduced by Schwart and Cantor in 1984 for separation of larger DNA fragments (up to 2000 kb) (Schwartz and Cantor, 1984). In this method, bacteria cells are embedded in molten agarose which will be subjected to in situ detergent-enzyme lysis to release intact chromosomal DNA and is digested later with infrequently cutting restriction endonucleases (Olive and Bean, 1999). The digested DNA fragments will be separated on an agarose gel in a Contour-clamped Homogeneous Electric Fields (CHEF) apparatus in which the polarity of the current is changed at predetermined intervals (Dawkins, 1989). The restriction profiles obtained will be analysed using commercially available software.

PFGE is currently considered the gold standard method for epidemiological studies of *A. baumannii*. A standardised PFGE typing protocol for *A. baumannii* has been developed with sufficient interlaboratory reproducibility which enabled for set up an Internet-based database for monitoring of geographic spread of epidemic strains (Seifert *et al*., 2005). In a study by Mezzatesta *et al*., (2011), PFGE had same discriminatory power and reproducibility as MLST and showed concordant results with MLST in defining the carbapenem-resistant *A. baumannii* Italian clones and in correlating them with the two European clones I and II. Although PFGE can provide high level of reproducibility and discrimination for subtyping of *A. baumannii* (Corbella

et al., 2000; Seifert *et al*., 2005), it is labour-intensive, technically demanding and expensive, as it requires special pulsed-field apparatus.

2.10.5 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) is a highly discriminative typing method for bacteria based on the sequence comparison of internal fragments of seven housekeeping genes (Maiden *et al*., 1998). For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and each isolate is defined by the alleles at each of the housekeeping loci (the allelic profile or sequence type [ST]) (Bartual *et al*., 2005).

MLST has become a current standard for investigating the population structure of many bacterial species (Maiden, 2006) including *A. baumannii* (Bartual *et al*., 2005; Diancourt *et al*., 2010). Currently, two MLST schemes have been developed for study the genetic diversity of *A. baumannii* (Bartual *et al*., 2005; Diancourt *et al*., 2010). The first MLST scheme was described by Bartual *et al*., (2005) (http://pubmlst.org/abaumannii/), using seven housekeeping genes of citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), glucose dehydrogenase B (*gdhB*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*), glucose-6-phosphate isomerase (*gpi*) and RNA polymerase sigma factor rpoD (Sigma-70) (*rpoD*). The second scheme of MLST was developed by Diancourt *et al*., (2010) at the Pasteur Institute (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html), of which the seven targeted housekeeping genes are citrate synthase (*gltA*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*), protein elongation factor EF-G (*fusA*), CTP synthase (*pyrG*), 50S ribosomal protein L2 (*rplB*) and RNA polymerase subunit B (*rpoB*).

The *gyrB* and *gpi* loci have been reported to show discordant in relation to each other and to other five housekeeping genes in the phylogenetic tree analysis (Park *et al*., 2009a; Hamouda *et al*., 2010). It has been suggested the *gyrB* and *gpi* genes may be affected by horizontal gene transfer, and are thus not good candidates for phylogeny studies (Hamouda *et al*., 2010). In a study by Da Silva *et al*., (2010), both Bartual and Pasteur Institute schemes gave identical results that the spread of the carbapenem-resistant *A baumannii* isolates in Portugal between 1998 to 2009 was majority of European clone II (clonal complex, CC2) strains. Recently, many studies have reported the application of MLST for epidemiological studies of *A. baumannii* strains, for which the majority of the strains are associated with the spread of European clone II strains (Nemec *et al*., 2008; Fu *et al*., 2010; Runnegar *et al*., 2010; Grosso *et al*., 2011). The discriminatory power of the MLST for molecular typing of *A. baumannii* is comparable to both of PFGE and AFLP (Nemec *et al*., 2008; Villalon *et al*., 2010). With the readily available databases which can easily be accessed by internet, MLST is a portable method that may be suitable for global epidemiologic studies and allow the recognition of epidemic, multi resistant, and virulent *A. baumannii* clones and the monitoring of their national and international spread (Peleg *et al*., 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial isolates

A total of 189 *A. baumannii* isolates were collected from a local tertiary hospital, University of Malaya Medical Center (UMMC). One hundred and seventy-one *A. baumannii* isolates were obtained from tracheal secretions (n=86), tracheal aspirate (n=2), sputum (n=6), swab (n=22), catheter tips (n=20), blood (n=11), body fluids (n=15), nasal swabs (n=2), urine (n=3) and tissues (n=3) over a period of 2006 to May 2009 from patients admitted to Intensive Care Units (ICU), Universiti Malaya Medical Center (UMMC). Eighteen *A. baumannii* isolates from the ICU environment (beds, tables, buckets, washbasins, ventilators, mattress, washing sinks and mop) and hands of healthcare workers (HCWs) screened in April, August and September 2006 following the occurrence of an increasing incidence in March, June, July and September 2006 were included for analysis. All the isolates were initially identified as *A. baumannii* by the microbiology laboratory at UMMC. The purity of the isolates was carried out by sub-cultured on Brain Heart Infusion (BHI) agar. The isolates are listed in **APPENDIX I.**

3.1.2 Growth media, buffers and solutions/reagents

All the preparation of the growth media, buffers and solutions/reagents used in this study are listed in **APPENDIX II.**

3.2 Methods

3.2.1 Genospecies identification of the isolates by amplified ribosomal DNA restriction analysis (ARDRA)

3.2.1.1 Preparation of DNA template

A single bacterial colony on BHI agar plate was picked and resuspended in a 0.5 ml microfuge tube containing 50 µl of sterile deionized water. The cell suspensions were boiled for 5 minutes at 99ºC in a thermal cycler (Perkin Elmer) and were kept on ice immediately for 10 minutes. The cell debris was spun down at 7,500 x g for 2 minutes. Approximately 5 µl of the supernatant containing ~ 100 ng of bacterial genomic DNA was used for PCR amplification for each reaction.

3.2.1.2 Oligonucleotide primers for amplification 16S ribosomal DNA

The primers used for amplification of 16S ribosomal DNA are listed in Table 3.1.

Table 3.1: Primers sequences used for amplification of 1500 bp of 16S ribosomal DNA

Primers	Primer Sequence (5`- 3`)	Amplification target	Size	Reference
16S rDNA-F 16S rDNA-R	5` TGGCTCAGATTGAACGCTGGCGGC 3` 5` TACCTTGTTACGACTTCACCCCA 3`.	Ribosomal DNA	1500 bp	Vaneechoutte *et al*. 1995

F: forward primer, R: reverse primer

3.2.1.3 PCR reaction mixture and cycling condition

Four different sources of *Taq* DNA polymerases (GoTaq® DNA Polymerase, Promega, Madigan, USA; *i-Taq*™, iNtRON Biotechnology, Korea, *TaKaRa Ex Taq*™ Takara, Shiga, Japan and HotStarTaq, Qiagen, USA) were tested for PCR amplification of 1500 bp ribosomal DNA. The PCR reaction was carried out in a final volume of 25 µl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 200 nM (each) primer and 0.5 U of *Taq* DNA polymerase of each tested *Taq* polymerase, respectively. For the use of HotStarTaq DNA polymerase, 12.5 µl of HotStarTaq Master Mix

containing 2.5 U of HotStarTaq DNA polymerase, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs (QIAGEN, USA) was used. A DNA blank containing the same reaction mixture for each *Taq* polymerase used except the DNA template was included. The entire PCR reaction was performed in an Eppendorf thermal cycler at the conditions of an initial denaturation at 95ºC for 5 minutes, followed by 30 cycles at 95ºC for 40 seconds, 50ºC for 30 seconds, and 72ºC for 1 minutes and a final extension at 72ºC for 7 minutes. Annealing temperature at 60 ºC was used for HotStarTaq DNA polymerase.

3.2.1.4 Detection of PCR product by agarose gel electrophoresis

After PCR amplifications, the PCR products were analysed on an 1.5% (w/v) agarose gel submerged in 0.5 X TBE buffer at 90 V for approximately 25 minutes in a gel electrophoresis system (RunOne^{TM} Electophoresis Cell, USA). Five µl of the PCR products was then mixed with 2 µl of 6X gel loading dye and the mixture was loaded into the wells of the agarose gel. A 100 bp ladder was used as the molecular size marker. The gel was stained with ethidium bromide (0.5 µg/ml) for 5 minutes and destained in ddH_2O for 15 minutes. The gel was visualized under UV light and picture was captured by using Gel Doc^{TM} XR imaging system (Bio-Rad, USA).

3.2.1.5 Restriciton digestion of 16S rDNA PCR amplicon with restriction enzymes *AluI*, *Cfo*I, *Mbo*I , *Msp*I and *Rsa*I

Approximately 5 µl of the 16S rDNA PCR amplicon was digested with 2U restriction enzymes *Alu*I (AGCT), *Cfo*I (GCGC), *Mbo*I (GATC), *Msp*I (CCGG) and *Rsa*I (GTAC) in 20 µl total volume of commercially supplied restriction buffers, respectively and incubated at 37ºC for 4 hours. The digested fragments were

electrophoretically separated in an 1.5% (w/v) agarose gels as described in section 3.2.1.4.

3.2.1.6 Analysis of combined restriction pattern to identify the species level of *Acinetobacter*

Species identification was done by comparing the profiles consisting of the combination of restriction patterns generated with the different enzymes with reference to the scheme of Vaneechoutte *et al*., (1995) (http://allserv.rug.ac.be/_mvaneech/ARDRA/Acinetobacter.html). (**APPENDIX III**).

3.2.2 Antimicrobial susceptibility test (Disk diffusion method)

3.2.2.1 Growth of bacteria culture

A single bacterial colony was picked with inoculation loop from the BHI agar plate and inoculated into 5 ml of BHI broth in a 15 ml culture tube. The mixture was incubated overnight at 37ºC in a shaker water bath. *Escherichia coli* ATCC 25922 and were used as the control isolate to confirm the potency of the antimicrobial disks.

3.2.2.2 Standardisation of inoculum (CLSI, 2006)

The turbidity of the bacterial culture was standardised by using the method that described in the Clinical and Laboratory Standards Institute (CLSI, 2006). Three ml of sterile 0.85% (w/v) NaCl solution was aliquoted into 7 ml tubes. Bacterial culture in BHI broth was added into the 0.85% (w/v) NaCl by using pipette and mixed well. The turbidity of the culture was measured by using a turbidity meter. Three ml of fresh sterile 0.85% (w/v) NaCl solution was used as a blank for the purpose of calibration. The turbidity value of the culture was adjusted to 0.06-0.10 that was equivalent to 0.5

McFarland Standard. After standardized the turbidity of the culture, the tubes were kept on ice to inhibit the growth before spreading on Mueller Hinton agar was carried out.

3.2.2.3 Inoculation on Mueller Hinton agar

Spreading of the culture on Mueller Hinton agar was carried out within 15 minutes after the culture turbidity was adjusted. A sterile cotton swab was dipped into the culture and the excess liquid was removed by pressing the cotton swab on the wall of the tube. Then the swab was streaked over the entire surface of the agar for 3 times by rotating the plate approximately 60º after each application to ensure an even distribution of the inoculums on the agar. The plates were then allowed to air dry in the laminar flow for approximately 5 minutes before application of antimicrobial disks.

3.2.2.4 Application of antimicrobial disks

The antimicrobial disks used were amikacin (30 μg), ampicillin (10 μg), ampicillin/sulbactam (20 μg), amoxicillin/clavulanic (30 μg), cefuroxime (30 μg), ceftriaxone (30 μg), cefoperazone (30 μg), cefoperazone/sulbactam (105 μg), cefepime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), piperacillin/tazobactam (110 μg), polymyxin B (200 μg) and trimethoprim/sulfamethoxazole (25 μg). All the disks were purchased from Oxoid, Ltd, England.

The antibiotic disks were stored in the refrigerator (-20ºC). These antibiotic disks were allowed to warm to room temperature in order to reduce the amount of water condensation on the disks for at least 10 minutes before they were used. The disks were gently placed and pressed onto the surface of the agar for ensure complete contact of the disk with the agar by using a sterile forceps. Diffusion of the antibiotic drugs in the disks start once the disks contact with the agar. Therefore the disks were not moved

once it had contacted with the agar. The Muller Hinton agar plates were then inverted and incubated for 16 to 18 hours at 37ºC.

3.2.2.5 Interpretation of results

After 16-18 hours of incubation, the plates were examined and the diameter of inhibition zone was measured by using a divider and ruler. In cases of the presence of a clearer inner zone and more blurred outer zone, the diameter taken was that of the inner zone. The zone diameters recorded were then interpreted according to the CLSI guidelines (2006) (APPENDIX VI). The disk diffusion breakpoints for polymyxin B was evaluated based on the NCCLS documents (NCCLS, 1981). The organisms were subsequently reported as susceptible, intermediate or resistant to the antimicrobial agents. Analysis of resistance phenotypes were done by using BioNumeric Applied Math, Version 6.0. Interpretation of zone diameter for each antibiotic disk was showed in APPENDIX V.

3.2.3 Screening of MBLs in imipenem-resistant *A. baumannii* isolates

3.2.3.1 Combined disk test

Imipenem-EDTA double-disk synergy test was performed as described by Yong *et al*., (2002). Test isolates were adjusted to the McFarland 0.5 standard and inoculated onto plates of Mueller-Hinton agar as recommended by the CLSI, 2006. Two 10 µg imipenem disks were placed on the plate, and 4.03 µl of a 0.5M EDTA solution were added to one of them to obtain the desired concentration of 750 µg. The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 hours of incubation at 37°C. The inhibition zones with imipenem-EDTA disks were ≤14 mm for the MBL-negative isolates, while they were ≥17 mm for the MBL-positive isolates (Yong *et al*., 2002).

3.2.3.2 Imipenem-EDTA double-disk synergy test

Combined disk test was performed as described by Lee *et al*., (2001). Test isolates were adjusted to the McFarland 0.5 standard and inoculated onto plates of Mueller-Hinton agar as recommended by the CLSI, 2006. A 10 µg imipenem disk was placed on the plate and a blank filter paper disk was placed at a distance of 10 mm (edge to edge). To the blank disk, 10 µl of a 0.5 M EDTA solution was added. After 16 to 18 hours of incubation at 37°C, the presence of even a small synergistic inhibition zone was interpreted as MBL-positive isolates.

3.2.4 Screening of carbapenem resistance genes

3.2.4.1 Preparation of DNA template

DNA template preparation protocol was similar to section 3.2.1.1.

3.2.4.2 PCR detection of MBL resistance genes

Five specific primers pairs were used to detect five different MBL resistance genes which responsible for resistance to carbapenems antimicrobial agents.

Table 3.2: Primer sequences used for detection of MBL resistance genes

PCR	Primer name	Primer Sequence (5`- 3`)	Gene target	Product Size	Reference
Multiplex	GIM 1 F	TCG ACA CAC CTT GGT CTG AA	*bla*$_{GIM}$	477 bp	Ellington *et al*., 2007
	GIM 1 R	AAC TTC CAA CTT TGC CAT GC			
	SPM 1 F	AAA ATC TGG GTA CGC AAA CG	*bla*$_{SPM}$	271 bp	
	SPM 1 R	ACA TTA TCC GCT GGA ACA GG			
	SIM 1 F	TAC AAG GGA TTC GGC ATC G	*bla*$_{SIM}$	570 bp	
	SIM 1 R	TAA TGG CCT GTT CCC ATG TG			
	IMP F	GGA ATA GAG TGG CTT AAY TCT C	*bla*$_{IMP}$	188 bp	
	IMP R	CCA AAC YAC TAS GTT ATC T			
	VIM F	GAT GGT GTT TGG TCG CAT A	*bla*$_{VIM}$	390 bp	
	VIM R	CGA ATG CGC AGC ACC AG			

F: forward primer, R: reverse primer

A multiplex PCR to detect the presence of five MBL resistance genes, *bla*$_{GIM,}$ *bla*$_{SPM,}$ *bla*$_{SIM,}$ *bla*$_{IMP}$ and *bla*$_{VIM}$ was performed according to Ellington *et al*., (2007) with minor modifications. The PCR reaction was carried out in a final volume of 25 µl containing 1× PCR buffer, 1.5 mM $MgCl_{2,}$ 150 µM of each dNTP, 300 nM of each primer, 1.0 U of *Taq* DNA polymerase and 5 µl (~100 ng) of template DNA. A DNA blank containing the same reaction mixture except the DNA template was included. The entire PCR reaction was performed in the conditions of an initial denaturation at 94ºC for 5 minutes, followed by 30 cycles at 94ºC for 30 seconds, 52ºC for 40 seconds, and 72ºC for 50 seconds and a final extension at 72ºC for 5 minutes.

3.2.4.3 PCR detection of *bla*$_{OXA}$ genes encoding carbapenemases, presence of insertion sequence IS*Aba1* and upstream of IS*Aba1* of the *bla*$_{OXA-23}$ and *bla*$_{OXA-51}$ positive *A. baumannii*

Detection of four groups of OXA-carbapenemases (OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like) was carried out as described by Woodford *et al*., (2006) in a multiplex PCR assay using the primers as listed in the Table 3.3.

Table 3.3: Primer sequences used in PCR detection of OXA-carbapenemases, IS*Aba1* and upstream of IS*Aba1* of the *bla*$_{OXA-23}$ and *bla*$_{OXA-51}$ positive *A. baumannii* isolates

Primer name	Primer Sequence (5`- 3`)	Product Size	Reference
OXA-23likeF	GAT CGG ATT GGA GAA CCA GA	501 bp	Woodford *et al.*, 2006
OXA-23likeR	ATT TCT GAC CGC ATT TCC AT		
OXA-24likeF	GGT TAG TTG GCC CCC TTA AA	246 bp	
OXA-24likeR	AGT TGA GCG AAA AGG GGA TT		
OXA-51likeF	TAA TGC TTT GAT CGG CCT TG	353 bp	
OXA-51likeR	TGG ATT GCA CTT CAT CTT GG		
OXA-58likeF	CCC CTC TGC GCT GTA CAT AC	599 bp	
OXA-58likeR	AAG TAT TGG GGC TTG TGC TG		
ISF	CAC GAA TGC AGA AGT TG	549 bp	Segal *et al*., 2005
ISR	CGA CGA ATA CTA TGA CAC		
OXA-23F	GAT GTG TCA TAG TAT TCG TCG	-	

OXA-23R	TCA CAA CAA CTA AAA GCA CTG		Afzal-Shah *et al*., 2001
OXA-51-likeFr&c	CAA GGC CGA TCA AAG CAT TA	Sequencing of IS*Aba1* /bla_{OXA-51} like junction in IS*Aba1*F/OXA-51likeR PCR products	
IS*Aba1* Rr&c	GTG TCA TAG TAT TCG TCG		
OXA-51-like-front R	TTA GCA GTC ACT ATA TAA GG		Turton *et al*., 2006a
IS*Aba1* end F	CAT TGA GAT GTG TCA TAG		

F: forward primer, R: reverse primer

The PCR reaction was carried out in a final volume of 25 µl containing 1× PCR buffer, 1.2 mM $MgCl_2$, 120 µM of each dNTP, 500 nM of each primer, 1.5 U of *Taq* DNA polymerase and 5 µl (~100 ng) of template DNA. A negative control containing the same reaction mixture except the DNA template was included. The amplification condition was with an initial denaturation at 94°C for 5 minutes following by 30 cycles of 94°C for 25 seconds, 52°C for 40 seconds and 72°C for 50 seconds, and a final elongation at 72°C for 6 minutes.

The presence of IS*Aba1* in the *A. baumannii* isolates was carried out as described by Turton *et al*., (2006) with minor modifications. The primers sequence used are shown in Table 3.3. Primers pair of ISF/ISR was used to detect the presence of insertion sequence IS*Aba1* in the isolates. Combination primers of ISF and reverse primers of OXA-23R and OXA-51likeR were used to detect the presence of IS*Aba1* upstream of the bla_{OXA-23} and bla_{OXA-51} genes. The PCR reaction was carried out in a final volume of 25 µl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 300 nM of each primer, 1.0 U of *Taq* DNA polymerase and 5 µl (~100 ng) of template DNA. The PCR amplification condition used was an initial denaturation at 95 ºC for 5 minutes, followed by 35 cycles at 95ºC for 45 seconds, 56ºC for 45 seconds, and 72ºC for 3.5 minutes and a final extension at 72ºC for 5 minutes, except that an annealing temperature of 58ºC was used for the ISF/OXA-51likeR primers.

3.2.4.4 Detection of PCR products of the MBL genes, *bla*$_{OXA}$ genes encoding carbapenemases, ISF/OXA -23R and ISF/OXA-51likeR

Detection of the PCR products was described previously in section 3.2.1.4. Amplicons detected on agarose gel were purified and sent for sequencing.

3.2.5 PCR detection of class 1, 2 and 3 integrons

3.2.5.1 Bacterial isolates

A total of 175 carbapenem-resistant *A. baumannii* (167 clinical and 8 environmental) isolates were tested for the present of class 1, 2 and 3 integrons.

3.2.5.2 Preparation of DNA template

DNA template preparation protocol was similar to section 3.2.1.1.

3.2.5.3 PCR detection of *intI*1, *intI*2 and *intI*3 integrase genes

Detection of the 3 integrase genes was carried out in a multiplex PCR assay as described previously (Dillon *et al*., 2005). The primers used in the multiplex PCR and their sequences are shown in Table 3.4.

The PCR reaction was carried out in a final volume of 25 µl containing 1× PCR buffer, 1.4 mM $MgCl_2$, 150 µM of each dNTP, 500 nM of each primer, 1.0 U of *Taq* DNA polymerase and 5 µl (~100 ng) of template DNA. A negative control containing the same reaction mixture except the DNA template was included.

Amplicons obtained by agarose gel electrophoresis were purified and sent for sequencing.

Table 3.4: Primer sequences and amplification condition used for the detection of integron-encoded integrases

Primer name	Primer Sequence (5`- 3`)	Target	Position	Amplification condition	Product size	Reference
IntI1F	CAG TGG ACA TAA GCC TGT TC	*intI*1	2734–2751	Initial denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, and a final elongation at 72°C for 5 minutes.	160 bp	Koeleman *et al*., 2001
IntI1R	CCC GAG GCA TAG ACT GTA		2874–2891			
IntI2F	TTG CGA GTA TCC ATA ACC TG	*intI*2	11980–11999		287 bp	Koeleman *et al*., 2001
IntI2R	TTA CCT GCA CTG GAT TAA GC		12248–12267			
IntI3F	GCC TCC GGC AGC GAC TTT CAG	*intI*3	738–758		979 bp	Mazel *et al*., 2000
IntI3R	ACG GAT CTG CCA AAC CTG ACT		1697–1717			

F: forward primer, R: reverse primer

3.2.5.4 PCR amplification of integron-encoded gene cassettes within class 1 and class 2 integrons

Characterization of the integron variable regions which contain the integron-encoded gene cassettes was carried out using the primers listed in Table 3.5.

The primer pair 5'CS/3'CS was used to amplify the gene cassette in class 1 integrons in a final volume of 25 µl PCR reaction mixture containing 1× PCR buffer, 1.0 mM $MgCl_2$, 270 µM of each dNTP, 600 nM of each primer, 1.0 U of *Taq* DNA polymerase and 5 µl (~100 ng) of template DNA.

The primer pair hep74/hep51 was used to amplify the gene cassette in class 2 integrons in a final volume of 25 µl PCR reaction mixture containing 1× PCR buffer, 1.2 mM $MgCl_2$, 150 µM of each dNTP, 300 nM of each primer, 1.0 U of *Taq* DNA polymerase and 5 µl (~100 ng) of template DNA.

Table 3.5: Primer sequences and PCR conditions used to amplify the variable region within class 1 and class 2 integrons

Primer name	Primer Sequence (5`- 3`)	Target	Position	Amplification condition	Product size	Reference
5`CS	GGC ATC CAA GCA GCA AG	Class 1 integron 5' conserved segment	1190–1206	Initial denaturation at 94°C for 8 minutes, 30 cycles of 94°C for 45 seconds, 57°C for 30 seconds and 72°C for 7 minutes, and a final elongation at 72°C for 8 minutes	Variable	Levesque *et al.,* 1995
3`CS	AAG CAG ACT TGA CCT GA	Class 1 integron 3' conserved segment	1342–1326			
hep74	CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA	Array	1–30	Initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 4 minutes, and a final elongation at 72°C for 5 minutes	Variable	White *et al*., 2001
hep51	GAT GCC ATC GCA AGT ACG AG	Class 2	2205–2224			

F: forward primer, R: reverse primer

3.2.5.5 Restriciton digestion of class 1 and class 2 integrons gene cassettes with restriction enzymes *Alu*I.

Approximately 5 µl of the class 1 and class 2 integron gene cassette PCR amplicons were restricted with 2U restriction enzymes *Alu*I (AGCT) in 20 µl total volume of commercially supplied restriction buffers, separately and incubated at 37ºC for 4 hours. The fragments obtained were electrophoretically separated in an 1.5% (w/v) agarose gels as described in section 3.2.1.4. Class 1 and class 2 integron gene cassette profiles were determined and representative isolates of each determined profiles were selected and rerun for PCR. Amplicons obtained were purified and sent for sequencing.

3.2.6 Sequencing

3.2.6.1 Purification of PCR products

PCR-amplified products were purified using the MEGAquick-Spin™ PCR purification kit (iNtRON Biotechnology, INC, Korea). Briefly, 5 volume of BNL buffer was added to the PCR-amplified product in a microcentrifuge tube and mixed well. The mixture was then transferred into a MEGAquick-Spin™ column placed in a 2 ml collection tube and centrifuged at 7,500 x g for 1 minute at room temperature. The flow-through was discarded and the spin column was placed back to the same collection tube. Approximately 700 µl of washing buffer containing absolute ethanol was added to the column and centrifuged at 7,500 x g for 1 minute. The flow-through was discarded and the column was placed back to the same collection tube. The column was centrifuged for additional 1 minute at 7,500 x g to dry the membrane in the column. The column was then removed from the collection tube and placed in a sterile 1.5 ml microcentrifuge tube. To elute the bound DNA on the membrane, 22 µl of ddH_2O was added to the center of the column and incubated at room temperature for 1 minute. The column was centrifuged at 7,500 x g for 1 minute at room temperature. The column was discarded after centrifugation and the flow-through containing the eluted DNA was collected in the 1.5 ml microcentrifuge tube and stored at -20°C.

3.2.6.2 Sequencing

Purified PCR products were sent to a commercial laboratory for sequencing (1^{st} BASE, Pte. Ltd., Singapore). The BigDye® Terminator v3.1 cycle sequencing kit chemistry was used for the sequencing reaction. The results of the DNA sequence data were compared to data in the GenBank database by using the BLAST algorithm available at web site (http://www.ncbi.nih.gov)

3.2.7 Plasmid profiling

3.2.7.1 Plasmid extraction using conventional method, alkaline lysis

Plasmid extraction was carried out using alkaline lysis method as described by Birnboim and Dolly, with minor modifications. A single bacterial colony of *A. baumannii* isolates was grown in 5 ml of BHI broth. The cultures were incubated overnight in a shaker water bath at 37°C. The cells were then harvested by centrifugation at 7,500 x g for 2 minutes at 4°C. The cells pellet was resuspended in 100 µl of ice-cold Solution I. The mixture was then incubated at 0°C for 30 minutes. Two hundred microliter of Solution II was added to the mixture and mixed by gently inverting for 4-6 times before kept on ice for not more than 5 minutes. One-hundred and fifty microliter of ice-cold Solution III was added and mixed well by gently inverting for few times. The mixture was kept on ice for 20 minutes before centrifuged at 7,500 x g for 10 minutes at 4°C. After centrifugation, the clear supernatant was transferred to a clean 1.5 ml microcentrifuge tube. Approximately 100 µg/ml of RNase A was added to the supernatant and the mixture was incubated at 37°C for 30 min. Phenol-chloroform was added at half ratio to the volume of the mixture and mixed thoroughly. The mixture was then centrifuged at 7,500 x g for 10 minutes at 4°C. The upper layer aqueous phase was transferred to a new microcentrifuge tube by using sterile cut tips. One to ten ratios of the 3M sodium acetate and two volumes of cold absolute ethanol were added to the microcentrifuge tube and kept at -20°C for 1 hour. Then the mixture was centrifuged at 7,500 x g for 15 minutes at 4°C. The supernatant was discarded. The pellet was washed with cold 70% (v/v) ethanol and centrifuged at 7,500 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet was left for air dry in room temperature. The pellet was then dissolved in 50 µl of ddH_2O. The dissolved plasmid DNA was kept at -20°C or used immediately.

3.2.7.2 Detection of plasmid DNA by agarose gel electrophoresis

The extracted plasmid DNA was analysed on 0.7% (w/v) agarose gel subjected in 0.5 X TBE buffer at 90 V for approximately 3 hours in a gel electrophoresis system (Submarine Agarose Gel Unit, USA). Ten microliter of the plasmid extraction was mixed with 2 µl of 6X gel loading dye and the mixture was loaded into the wells of the agarose gel. *Escherichia coli* 39R and V517 were used as plasmid size markers. The gel was stained with ethidium bromide (0.5 µg/ml) for 5 minutes and destained in ddH_2O for 15 minutes. The gel was visualized under UV light and picture was captured by using the Gel Doc^{TM} XR imaging system (Bio-Rad, USA).

3.2.8 Polymerase chain reaction (PCR)-based methods

3.2.8.1 Preparation of template DNA

The template DNA was prepared as described by Sandvang *et al*., (1998). Cell culture was inoculated into 1 ml of BHI broth and incubated at 37ºC overnight. After incubation, cell was harvested by spinning at 7,500 x g for 3 minutes. The supernatant was discarded, and the pellet was resuspended and washed using 100 µl of PBS solution. The suspension was centrifuged at 7,500 x g for 3 minutes. The supernatant was discarded and 100 ul TE buffer was added to resuspend and wash the cell pellet. The suspension was centrifuged at 7,500 x g for 3 minutes. The supernatant was discarded. Then 50 µl of ddH_2O was added to resuspend the cell pellet to obtain homogenous solution. The cell suspension was boiled for 5 minutes at 99ºC and was immediately left on ice for 10 minutes. After snap cold, the tube was spin at 7,500 x g for 2 minutes. The supernatant containing the DNA was aliquoted into a clean sterile microcentrifuge tube. Five microliter (~100 ng) the DNA template was used for the REP-PCR.

3.2.8.2 Repetitive extragenic palindromic-PCR (REP-PCR)

Initially, 2 types of primers (Table 3.6) were tested in order to assess their usefulness in generating polymorphism and discriminatory power in subtyping the isolates.

Table 3.6: Primer sequences used for REP-PCR

Primer name	Primer Sequence (5`to 3`)	Amplification condition	Reference
REP1R	III ICG ICG ICA TCI GGC	Initial denaturation at 95°C for 3 minutes 30 cycles of 90°C for 30 seconds, 45°C for 1 minutes and 65°C for 8 minutes, and a final elongation at 65°C for 16 minutes.	Snelling *et al*., 1996
REP2	ICG ICT TAT CIG GCC TAC		

REP-PCR fingerprinting reactions were initially performed in three different PCR using primer REP1R, REP2 and REP1R+REP2. The reaction components for these 3 REP-PCR consisted of 1X PCR buffer, 1.6 mM $MgCl_2$, 220 µM each dNTPs, 1.2 µM PCR primer, 2.0 U *Taq*-DNA Polymerase in a final volume of 25 µl. The amplification condition is listed in Table 3.6. Primer that give the better discrimination of the isolates was selected to genotype all the *A. baumannii* isolates.

3.2.8.3 Detection of PCR products of REP-PCR

The amplified DNA bands of REP-PCR was analysed on an 1.5% (w/v) agarose gel submerged in 0.5 X TBE buffer at 90 V for approximately 3 hours in a gel electrophoresis system (Submarine Agarose Gel Unit, USA). Ten microliter of the PCR products was mixed with 2 µl of 6X gel loading dye and the mixture was loaded into the wells of the agarose gel. 100 bp marker (Promega) and 1 kb marker (Promega) were used as a molecular size marker. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) for 5 minutes and destained in ddH_2O for 15 minutes. The gel was visualized under UV light and picture was captured by using Gel Doc™ XR imaging system (Bio-Rad, USA).

3.2.9 Pulsed field Gel Electrophoresis (PFGE)

3.2.9.1 Preparation of PFGE plugs

PFGE analysis was carried out according to Seifert *et al*., (2005) with minor modifications. Briefly, a single colony was streaked on BHI agar and incubated at 37ºC for overnight. Cell suspension was prepared by transferred the cell culture into 2 ml of cell suspension buffer (CSB) by using a sterile cotton swab. The cell density was adjusted to 0.70-0.80 by using turbidity meter. One hundred microliter of the cell suspension was aliquoted into a sterile 1.5 ml microcentrifuge tube and 2 µl of

Proteinase K (20 mg/ml stock solution) was added. 120 µl of 1% Seakem Gold agarose (Cambrex Bio Science Rockland, Inc, USA) was mixed with cell mixture and gently dispensed into the wells of PFGE plug molds and allowed to solidify at room temperature for 20 min. The plugs were transferred into 50 ml falcon tubes contained 2ml of Cell Lysis Buffer (CLB) and 10 µl of Proteinase K (20 mg/ml). The plugs were then incubated at 54ºC for 2 hours in a shaker water bath. After lysis, the plugs were washed thoroughly in 10-15 ml of preheated (50ºC) sterile deionized water for twice and 5 times with preheated (50ºC) 1X TE buffer. After washing, the plugs were stored at 4ºC or used immediately.

3.2.9.2 Restriction digestion of DNA plugs

A slice of the plug (1 mm wide × 5 mm length) was cut and pre-restricted in 100 µl of pre-restriction buffer mixture (1X of commercially supplied restriction buffers and 0.1 mg/ml of BSA) at 37ºC for 15 minutes. After 15 minutes, the pre-restriction buffer was removed and replaced by added 100 µl of restriction enzyme mixture containing 1X of commercially supplied restriction buffers, 0.1 mg/ml of BSA and 10 U of *Apa*I restriction enzyme. Then the mixture was incubated at 37ºC for 4 hours.

3.2.9.3 PFGE DNA standard size marker

Salmonella serovar Braenderup H9812 was used as the DNA standard size marker (Hunter *et al*., 2005). The PFGE plug of *Salmonella* serovar Braenderup H9812 was prepared as described in section 3.2.8.1. DNA plug was restricted as described in section 3.2.8.2 with *Xba* I restriction enzyme was used.

3.2.9.4 Electrophoresis conditions

After restriction, the restricted plugs were loaded onto an 1% (w/v) agarose gel (Sigma Type 1, St. Louis, Mo). Restriction fragments obtained were separated in a CHEF DRIII system (Bio-Rad) in 0.5 X TBE buffer for 26 hours at 14°C following the conditions: initial switch time of 2.0 seconds, final switch time of 40 seconds, a constant voltage of 6V and angle of 120°.

3.2.9.5 Staining and documentation of PFGE agarose gel

After the electrophoresis, the gel was stained in ethidium bromide (0.5 μg/ml) for 10 minutes, and then destained in distilled water for 20 minutes, 2 times with water was changed for each interval times. The gel was visualized under UV light and picture was captured by using the Gel Doc^{TM} XR imaging system (Bio-Rad, USA).

3.2.10 Data analysis

The PFGE pulsotypes and REP-PCR, REP types were generated by using BioNumeric Version 6.0 (Applied Maths, Belgium) software. Cluster analysis of PFGE and REP-PCR were based on unweighted pair group arithmetic means methods (UPGMA). The similarity of the PFGE pulsotypes and REP types were calculated by the (Dice) coefficient, *F*. The discriminatory ability of the different techniques was determined by the Discriminatory Index, (*D*) (Hunter and Gaston, 1988). The *D* values represent the probability that two isolates would be distinguished by a particular subtyping method. If the test were capable of distinguishing all the isolates, then *D* = 1.0. If all isolates are indistinguishable, then *D* = 0. The Discriminatory Index (*D*) is given by the formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

Where *D* is the index of discriminatory power, *N* the number of unrelated isolates tested, *s* the number of different types, and *xj* the number of isolates belonging to the *j* type.

3.2.11 Southern hybridisation

Southern hybridisation was performed to detect the localisation of the $bla_{\text{OXA-23}}$ gene on plasmid DNA and/or chromosomal DNA.

3.2.11.1 Preparation of targeted gene probe

3.2.11.1.1 Genomic DNA

Genomic DNA was prepared using the Wizard® Genomic DNA Purification Kit (Promega, Madigan, USA). The protocol used was according to the manufacturer's instructions. Briefly, 1 ml of overnight culture was harvested in a 1.5 ml microcentrifuge tube by centrifugation at 7,500 x g for 2 minutes. The supernatant was discarded. Six hundred microliter of Nuclei Lysis Solution was added and mixed by gently pipeting. The mixture was incubated at 80°C for 5 minutes add left to cool to room temperature before adding the RNase Solution (3 µl). The mixture was then incubated at 37°C for 15-60 minutes and let to cool to room temperature. After that, 200 µl of Protein Precipitation Solution was added to the mixture and vortexed. The mixture was incubated on ice for 5 minutes. After incubation, the mixture was centrifuged at 7,500 x g for 3 minutes. The supernatant was transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol and mixed. The mixture was then centrifuged at 7,500 x g for 2 minutes. The supernatant was discarded and the DNA pellet was washed with 70% (v/v) of room temperature ethanol. The mixture was centrifuged at 7,500 x g for 2 minutes and the supernatant was discarded. The pellet was air-dried at room temperature. Approximately 100 µl of sterile

distilled water was added to dissolve the DNA pellet at 65°C for 1 hour or 4°C for overnight.

3.2.11.1.2 Labelling of *bla*$_{OXA-23}$ and 16S rDNA genes probe

PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was used in synthesizing labelled probe for hybridization. The protocol used was according to the manufacturer's instructions with minor modifications. Approximately 10 ng of genomic DNA was used as the template DNA for PCR. PCR mixture containing 1X buffer with $MgCl_2$, 100 µM of PCR DIG mix, 0.3 µM of primers and 1 U of Enzyme mix expand high fidelity was used. The primers used in amplification of *bla*$_{OXA-23}$ gene were similar to primers stated in Table 3.3 (Section 3.2.4.3). While the primers used to amplify 16S rDNA are 16F (5′ - AGT TTG ATC ATG GCT CAG-3′) and 16R (5′ -GGA CTA CCA GGG TAT CTA AT-3′) which would yield an expected amplicon size of 792 bp (Shukla *et al*., 2003 and Valera *et al*., 2004). A negative control containing the same reaction mixture except the DNA template was included. An unlabelled positive control with same reaction mixture except the non DIG-dUTP labelling dNTPs was also included. Labelled positive control which produced a labelled probe that recognizes human tissue plasminogen activator (tPA) sequences was included by using the DNA and primers as provided in the commercial kit. The amplification conditions for *bla*$_{OXA-23}$ gene was with an initial denaturation at 94°C for 5 minutes followed by 30 cycles of 94°C for 25 seconds, 52°C for 40 seconds and 72°C for 50 seconds, and a final elongation at 72°C for 6 minutes. While the amplification conditions for 16S rDNA was with an initial denaturation at 94°C for 5 minutes followed by 30 cycles of 94 °C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, and a final elongation at 72°C for 5 minutes. After PCR

amplification, the synthesized labelled probe was checked by running in the gel electrophoresis. The unlabelled probe was run together with the labelled probe.

3.2.11.2 Separating of DNA on an agarose gel

Sixteen selected *bla*$_{\text{OXA-23}}$-positive and 2 *bla*$_{\text{OXA-23}}$-negative *A. baumannii* isolates carrying different sizes of plasmid DNA were studied for the localisation of *bla*$_{\text{OXA-23}}$ gene on plasmid and/or chromosomal DNA. To detect the present of the *bla*$_{\text{OXA-23}}$ gene on plasmid DNA, alkaline lysis extracted plasmid DNA and S1 nuclease restricted PFGE plug DNA were used for hybridisation with *bla*$_{\text{OXA-23}}$ gene labelled probe. While to detect the chromosomal mediated *bla*$_{\text{OXA-23}}$ gene, I-*Ceu* I restricted PFGE plug DNA were used.

3.2.11.2.1 Plasmid DNA

Approximately 50 ng of plasmid DNA was loaded into a 0.7% (w/v) agarose gel and submerged in 0.5X TBE buffer at 90 V for approximately 3 hours in a gel electrophoresis system (Submarine Agarose Gel Unit, USA). *Escherichia coli* 39R and *E.coli* V517 were used as plasmid size markers. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) for 5 minutes and destained in ddH_2O for 15 minutes. The gel was visualized under UV light and picture was captured by using the Gel Doc^{TM} XR imaging system (Bio-Rad, USA) and kept for reference after hybridisation.

3.2.11.2.2 S1 nuclease restriction of PFGE plugs DNA

Restriction digestion of PFGE DNA plugs was similar to section 3.2.8.2 with the exception of 1U S1 nuclease enzyme was used and incubated at 37ºC for 45 minutes. *Salmonella* serovar Braenderup H9812 plug restricted with restriction enzyme *Xba* I

was used as the DNA standard size marker. The electrophoresis conditions were similar to section 3.2.8.4 with the exception of initial switch time of 5 seconds, final switch time of 20 seconds and run time for 20 hours. Unrestricted DNA plug was placed next to the restricted DNA plug as a control. After electrophoresis, the gel was stained in ethidium bromide (0.5 μg/ml) for 5 minutes, and then destained in distilled water for 20 minutes. The gel was visualized under UV light and picture was captured by using the Gel Doc™ XR imaging system (Bio-Rad, USA) and kept for reference after hybridisation.

3.2.11.2.3 I-*Ceu*I restriction of PFGE plugs DNA

Restriction digestion of PFGE DNA plugs was similar to section 3.2.8.2 with the exception of 1U of I-*Ceu*I restriction enzyme was used and incubated at 37ºC for 4 hours. *Salmonella* serovar Braenderup H9812 plug restricted with restriction enzyme *Xba* I was used as the DNA standard size marker. The electrophoresis conditions were described previously in section 3.2.8.4 with the exception of initial switch time of 5 seconds, final switch time of 60 seconds and run time for 20 hours. After electrophoresis, the gel was stained in ethidium bromide (0.5 μg/ml) for 5 minutes, and then destained in distilled water for 20 minutes. The gel was visualized under UV light and picture was captured by using the Gel Doc™ XR imaging system (Bio-Rad, USA) and kept for reference after hybridisation.

3.2.11.3 Transferring DNA from agarose gel to membrane

After viewing and capturing image, agarose gel was subjected to depurination step. Agarose gel was submerged in Depurination Solution (250 mM HCl) with shaking at room temperature for 10-20 minutes to depurinate the DNA prior to transfer. Then the agarose gel was rinsed with doubled distilled water before submerge in denaturation

solution (0.5 M NaOH; 1.5 M NaCl). To denature the DNA in the gel, agarose gel was submerged in denaturation solution for twice with each was shaking at room temperature for 15 minutes. Then the gel was rinsed with doubled distilled water and submerged in neutralization solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for twice, each was shaking for 15 minutes at room temperature. The agarose gel was equilibrating in 20X SSC for 10 minutes. A blot transfer was set up to transfer the DNA onto positively charged nylon membrane. First, a piece of Whatmann 3mm paper that has been soaked in 20X SSC was placed on the bridge that rests in a shallow reservoir of 20X SSC. The treated agarose gel was placed on the soaked Whatmann 3 mm paper and rolled over with a sterile glass rod to remove the air bubbles trapped between the gel and the paper. A piece of cut nylon membrane to the size of the gel was place on the top of the gel. One of the corners of the membrane was cut off to indicate the direction of the transferred DNA. Three sheets of dry Whatmann 3 mm papers were applied on the top of the membrane. A stack of paper towels, a glass plate and a 200-500g weight was added on the top. The completed blot was left to transfer for overnight in 20X SSC buffer. (Figure 3.1)

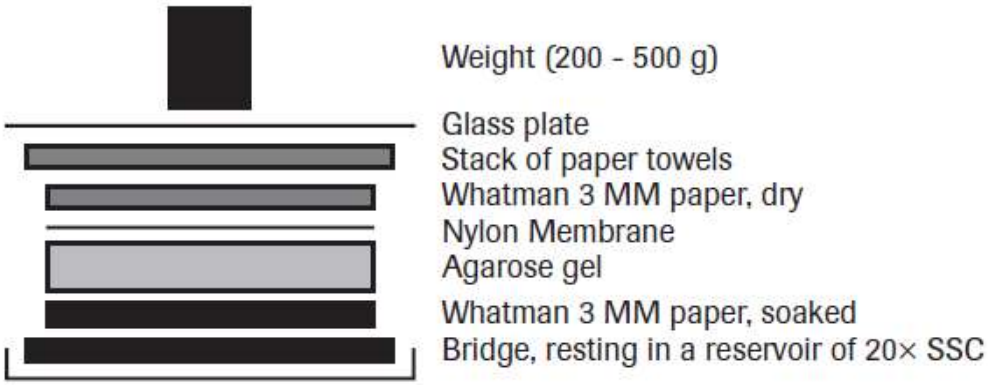


Figure 3.1: A complete blot transfer for transferring DNA in the gel onto positively charged nylon membrane (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany).

After an overnight transfer, the DNA on the membrane was fixed by UV for 1-3 minutes on a UV transilluminator. Then the membrane was rinsed with doubled distilled water and air dried. The blotted membrane was then can be used for hybridisation or kept between 2 sheets of dry Whatmann 3 mm paper in a sealed bag at 4°C for future hybridization experiment.

3.2.11.4 Prehybridisation and hybridisation

3.2.11.4.1 Prehybridisation

Prehybridisation was prepared by prewarmed 12 ml of DIG Easy Hybrid buffer (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) to the hybridisation temperature of 42°C. The hybridisation temperature was calculated according to the formula given by Roche Diagnostic:

$$Tm = 48.82 + 0.41\ (\%\ G+C) - 600/l$$

$$Thyb = Tm - (20°C - 25°C)$$

where, Tm = melting point of probe-target hybrid

(% G+C) = % of G and C residues in probe sequence

Thyb = optimal temperature for hybridisation of probe to target in DIG Easy Hyb, and

l = length of hybrid in base pairs

The blot membrane was placed in a hybridisation bag and prewarmed prehybridisation buffer was added. The bag was heat sealed and incubated in a shaking water bath at 42°C. The blot membrane was prehybridised for at least 30 minutes.

3.2.11.4.2 Hybridisation

Five milliliter of DIG Easy Hyb buffer was prewarmed to 42°C. 10 µl (~125 ng) of labelled probe was added into 50 µl of doubled distilled water and boiled for 5 minutes to denature the probe. The probe was chilled in an ice bath and added immediately into the prewarmed hybridisation and mixed by inverted for few times. The sealed bag was then cut off and prehybridisation buffer was discarded. Immediately, hybridisation buffer was added and the bag was heat sealed with no air bubbles. Hybridisation was carried out overnight at 42°C in a shaking water bath.

After an overnight hybridisation, the blot membrane was removed from the hybridisation bag and placed in a tray containing 200 ml of Low Stringency Buffer. The tray was shaked for 5 minutes at room temperature and the buffer was discarded. Two hundred milliliter of fresh Low Stringency Buffer was added and shaked for 5 minutes in room temperature. The Low Stringency Buffer was discarded and 200 ml of preheated (65°C) High Stringency Buffer was added. The blot membrane was washed twice with the High Stringency Buffer; each was shaked for 15 minutes at 65°C.

3.2.11.5 Detection of hybridised probe on blot membrane

DIG Wash and Block Buffer Set was purchased from Roche Diagnostic (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). The blotted membrane was transferred into a tray containing 100 ml of 1X washing buffer. The membrane was incubated for 2 minutes at room temperature with shaking. Washing buffer was discarded and 100 ml of 1X blocking solution was added. Membrane was incubated for 30 minutes with shaking at room temperature. Blocking solution was discarded and 30 ml of antibody solution containing 75 mU/ml of Anti-Digoxigenin-AP was added and incubated for 30 minutes. The antibody solution was discarded and the membrane was washed twice with 100 ml of 1X washing buffer, each was shaking for

15 minutes. After washing, the membrane was equilibrated in 30 ml of 1X detection buffer for 3 minutes. Chemiluminescent substrate, CSPD, was diluted in 1:100 in 1X detection buffer. The membrane was placed in hybridisation bag with the DNA side faced up. 1 ml of the diluted CSPD substrate was applied over the surface of the blotted membrane until the entire surface was soaked. The membrane was incubated at room temperature for 5 minutes. Excess liquid of the substrate was removed and the bag was heat sealed. The sealed membrane was incubated at 37°C for 10 minutes to enhance the luminescent reaction. Then the sealed membrane was exposed to the Lumi-Film X-ray film ((Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) for 30 minutes and the film was developed in a dark room.

3.2.11.6 Stripping membrane

After detection, the membrane was rinsed with distilled water for 1 minute. The membrane was washed with stripping buffer (0.2 M NaOH, 0.1% SDS) for twice, each was shake for 15 minutes at room temperature. The stripping buffer was discarded and the membrane was washed with 2X SSC buffer for 5 minutes. After washing, the membrane is ready for reprobing or can be stored at 4°C in 2X SSC buffer.

3.2.11.7 Reprobing membrane with *bla*$_{\text{OXA-23}}$ probe

The stripped membrane was reprobed with *bla*$_{\text{OXA-23}}$ probe to localize the *bla*$_{\text{OXA-23}}$ gene on plasmid and/or chromosomal DNA. The prehybridisation, hybridisation and detection of the probe were similar to section 3.2.11.4 and 3.2.11.5.

3.2.12 Transformation of plasmid borne *bla*$_{OXA-23}$ into competent *E. coli* 5-alpha

Commercial competent cells, NEB 5-alpha Competent *E. coli* (New England Biolabs, Inc, Ipswich, MA) was used as the recipient cells in the transformation of plasmid borne *bla*$_{OXA-23}$ from 2 representative donor *A. baumannii* isolates, AC/0606-22 and AC/0812-16. The procedure of the transformation was according to the manufacturer's instructions. The tube of NEB 5-alpha Competent *E. coli* cells were thawed on ice until the last ice crystals disappeared. Approximately 50 µl of the competent cells were transferred into a 15 ml falcon tube on ice. One microliter (~50 ng) of the plasmid DNA was added into the cell mixture and gently flicked for 4-5 times to mix the cells and DNA. The mixture was incubated on ice for 30 minutes. Then heat shock was carried out in a 42°C water bath for 30 seconds. After heat shocked, the mixture was incubated on ice for 5 minutes. 950 µl of room temperature SOC was added into the mixture and shake vigorously (180 rpm) at 37°C for 1 hour. Serial dilutions (10^{-1} to 10^{-4}) were carried out and 100 µl of the diluted transformant cells were plated onto TSA agar supplemented with 2 µg/ml of imipenem. The plates were inverted and incubated at 37ºC overnight. The same volume of untransformed competent cells was plated on TSA agar plates with and without selective antibiotics to serve as negative controls. Plasmid DNA of PGEX was used as positive control with 10 µl of the plasmid DNA (~1 µg) was used for transformation and the transformant cells was selected on the TSA agar containing 100 µg/ml of carbenicillin. Transformation efficiency (CFU/µg) was calculated according to the formula given by NEB manufacturer.

Transformation efficiency (TE) = Colonies/µg/Dilution

Colonies = the number of colonies counted on the plate

µg = the amount of DNA transformed expressed in µg

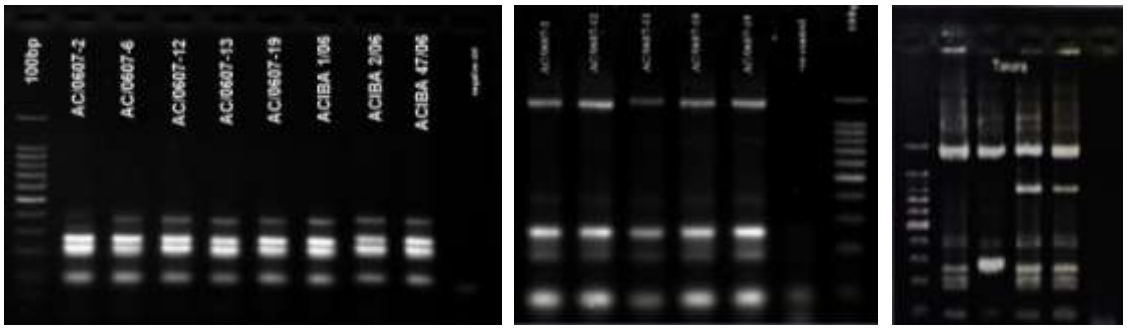
Dilution = the total dilution of the DNA before plating

CHAPTER 4: RESULTS

4.1 Genospecies identification of the isolates by amplified ribosomal DNA restriction analysis (ARDRA)

A total of 189 *A. baumannii* isolates were successfully confirmed by ARDRA method. Initially, four different types of *Taq* DNA polymerases (GoTaq® DNA polymerase (Promega, Madigan, USA), *i-Taq*™ (iNtRON Biotechnology, Korea), *TaKaRa Ex Taq*™ (Takara, Shiga, Japan) and HotStarTaq (Qiagen, USA)) were used to amplify the ribosomal DNA. However, the expected amplicon of approximately 1500 bp was only obtained with HotStarTaq DNA polymerase. GoTaq® DNA polymerase (Promega, Madigan, USA) failed to amplify the 1500 bp band with just ~100 bp to ~400 bp bands were observed on the agarose gel (Figure 4.1a). The other 2 sources of *Taq* DNA polymerases, *i-Taq*™ (iNtRON Biotechnology, Korea) and *TaKaRa Ex Taq*™ (Takara, Shiga, Japan) had generated unspecific DNA fragments (Figure 4.1b and 4.1c). HotStarTaq DNA polymerase was used for the subsequent PCR amplification of 16S rDNA for the rest of the isolates as it gave the expected size of 1500 bp amplicon (Figure 4.2).

Combination of the restriction patterns obtained from restriction of the 1500 bp amplicon with restriction enzymes *Alu*I, *Cfo*I, *Mbo*I, *Msp*I and *Rsa*I, gave identification to the species level of the isolates (Figure 4.3, Table 4.1). Among the 189 (clinical, n=171; environmental and hands of healthcare worker, n=18) isolates that were initially identified as *A. baumannii*, 185 (97.9%) isolates (170 clinical; 15 environmental) were confirmed as *A. baumannii,* 3 (1.6%) isolates (1 clinical; 1 environmental; 1 hands of healthcare worker) as genospecies 13TU (*A. nosocomialis*) and one environmental isolate as genospecies 15TU (**APPENDIX I**).



(a) (b) (c)

Figure 4.1: Representative gels of 16S rDNA gene amplified with different *Taq* DNA polymerases; (a) GoTaq® DNA polymerase (Promega, Madigan, USA); (b) *i-Taq*™ (iNtRON Biotechnology, Korea) and (c) *TaKaRa Ex Taq*™ (Takara, Shiga, Japan). These *Taq* DNA polymerases failed to amplify an expected 1500 bp amplicon for all the samples.

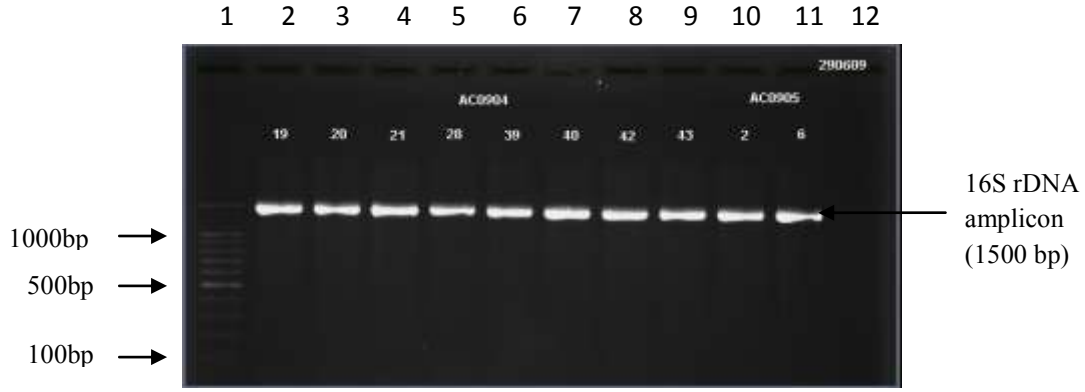


Figure 4.2: Representative gel of 1500 bp amplicon of the 16S rDNA gene amplified by HotStarTaq DNA polymerase. Lane 1: 100bp marker (Promega, USA); lane 2-lane 11: *A. baumannii* isolates and lane 12: DNA blank.

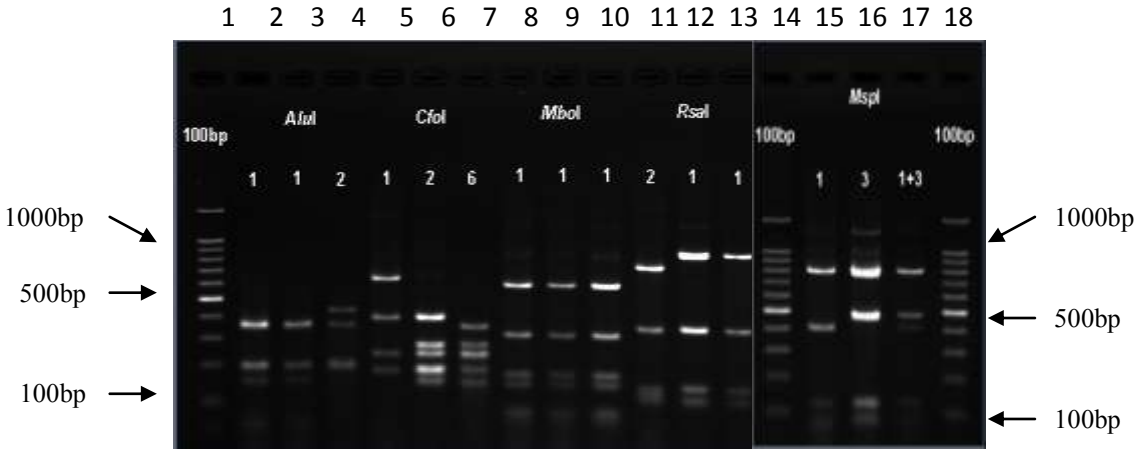


Figure 4.3: A composite of restriction patterns obtained after digestion with *Alu*I, *Cfo*I, *Mbo*I, *Msp*I and *Rsa*I for an amplified 1500bp of the 16S rDNA gene. Numbers on each lane refer to the ARDRA pattern of each restriction enzyme which interpretation was done based on scheme of Vaneechoutte *et al*., (1995). Lane 1, 14 and 18: 100bp DNA marker (Promega, USA); lane 2, 5, 8, 11 and 15: AC/0612-17 (*A. baumannii*); lane 3, 6, 9, 12 and 16: ACIBA 2006-66 (genospecies 13TU (*A. nosocomialis*)); lane 4, 7, 10, 13 and 17: ACIBA 2006-58 (*Acinetobacter* 15TU)

Table 4.1: Identification and differentiation of *Acinetobacter* based on ARDRA profiles

Genospecies	ARDRA patterns with restriction enzymes					Number of tested isolates
	***Alu*I**	***Cfo*I**	***Mbo*I**	***Rsa*I**	***Msp*I**	
Genospecies 1 (*A. calcoaceticus*)	2	2	1	1	3	0
Genospecies 2	1	1	1	2	1	81
(*A. baumannii*)	1	1	1	2	3	89
	1	1	1	2	1+3	15
Genospecies 3 (*A. pittii*)	1	2	3	1	3	0
Genospecies 13TU (*A. nosocomialis*)	1	2	1	1	1	1
	1	2	1	1	3	2
	1	2	1	1	1+3	0
Genospecies 15TU	2	6	1	1	3	1

The numerals indicate restriction pattern obtained after restriction digestion with restriction enzymes *Alu*I, *Cfo*I, *Mbo*I, *Msp*I and *Rsa*I, respectively. The interpretation was done based on scheme of Vaneechoutte *et al*., (1995).

4.2 Antimicrobial susceptibility profiles of *A. baumannii*

The resistance rates of *A. baumannii* isolates are summarized in Figure 4.4. Overall, all the 170 clinical isolates of *A. baumannii* showed 100% resistance to ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, ceftriaxone, cefoperazone, ceftazidime and cefepime. These isolates also exhibited high resistance rates to amikacin (78.8%), gentamicin (85.3%), ampicillin/sulbactam (81.8%), cefoperazone/sulbactam (34.7%), ciprofloxacin (99.4%), imipenem (96.5%), meropenem (98.2%) and trimethoprim/sulfamethoxazole (88.8%).

The resistance rates of the clinical isolates to amikacin decreased to 52.0% in 2007 from 70.5% in 2006 but increased again to 91.5% and 97.3% in 2008 and 2009, respectively. There was a decrease in resistance to gentamicin from 91.8% in 2006 to 78.7% in 2008 but slightly increased to 83.8% in 2009. Similarly, resistance rate to trimethoprim/sulfamethoxazole was dropped in 2007 (88.0%) and 2008 (76.6%) compared to 2006 (98.4%) but slightly increased in 2009 (89.2%). The clinical isolates were highly resistant to carbapenems (> 90%) and had high resistance rate towards ampicillin/sulbactam throughout the 4 year- period: 2006 (73.8%), 2007 (88.0%), 2008 (78.7%) and 2009 (94.6%). No cefoperazone/sulbactam-resistant isolate was observed in 2006. Resistance towards cefoperazone/sulbactam were first detected in 2007 (40.0%) and the resistance rates increased from 55.3% in 2008 to 62.2% in 2009.

A. baumannii isolates from the environment and hands of HCWs were 100% resistant to cefoperazone and had high resistance rates to ampicillin (83.3%) and cefuroxime (73.3%). These isolates had intermediate resistance rates, at varying levels, to the other antimicrobial agents except cefoperazone/sulbactam. Fortunately, all the clinical, environmental and hands of HCW isolates of *A. baumannii* were fully susceptible to polymyxin B.

Antimicrobial susceptibility tests had successfully subtyped the 185 *A. baumannii* isolates into 27 resistance phenotypes (R1-R27) (Figure 4.5). Two clusters (Cluster I and Cluster II) were generated. Cluster I consisted of all the multi-drug resistant (MDR) (resistant to at least 3 classes of antimicrobial agents) isolates and subdivided into 2 subclusters, Subcluster Ia and Subcluster Ib. Predominant resistance phenotype R19 (35.1%) which exhibiting resistance to all antimicrobial agents except polymyxin B and cefoperazone/sulbactam was located in Subcluster Ib. Second predominant resistance phenotype R01 in Subcluster Ia had 23.8% of the isolates being susceptible to polymyxin B. Seven environmental and a hands of HCW MDR isolates shared a similar resistance phenotype R19 with the clinical isolates in Subcluster Ia. Seven non-multidrug resistant (non-MDR) isolates from hands of HCWs were assigned to Cluster II with resistance phenotypes R23 to R27. The prevalence of MDR among the clinical and environmental isolates was 100% and 53.3%, respectively.

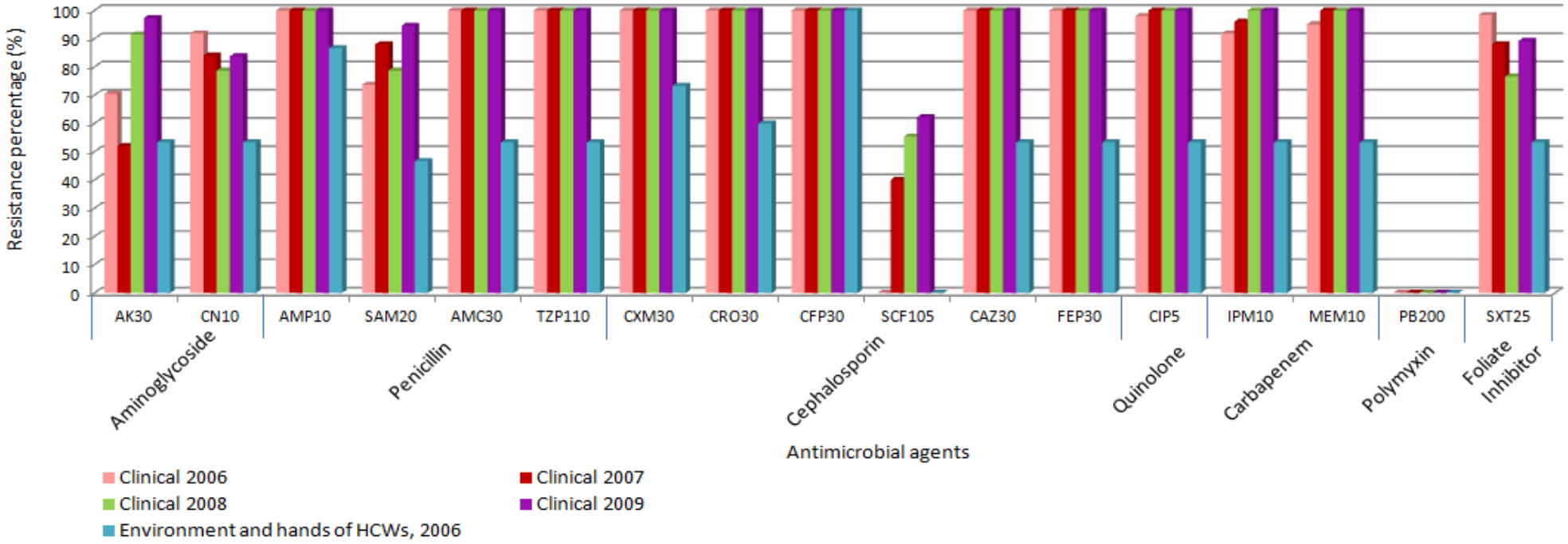
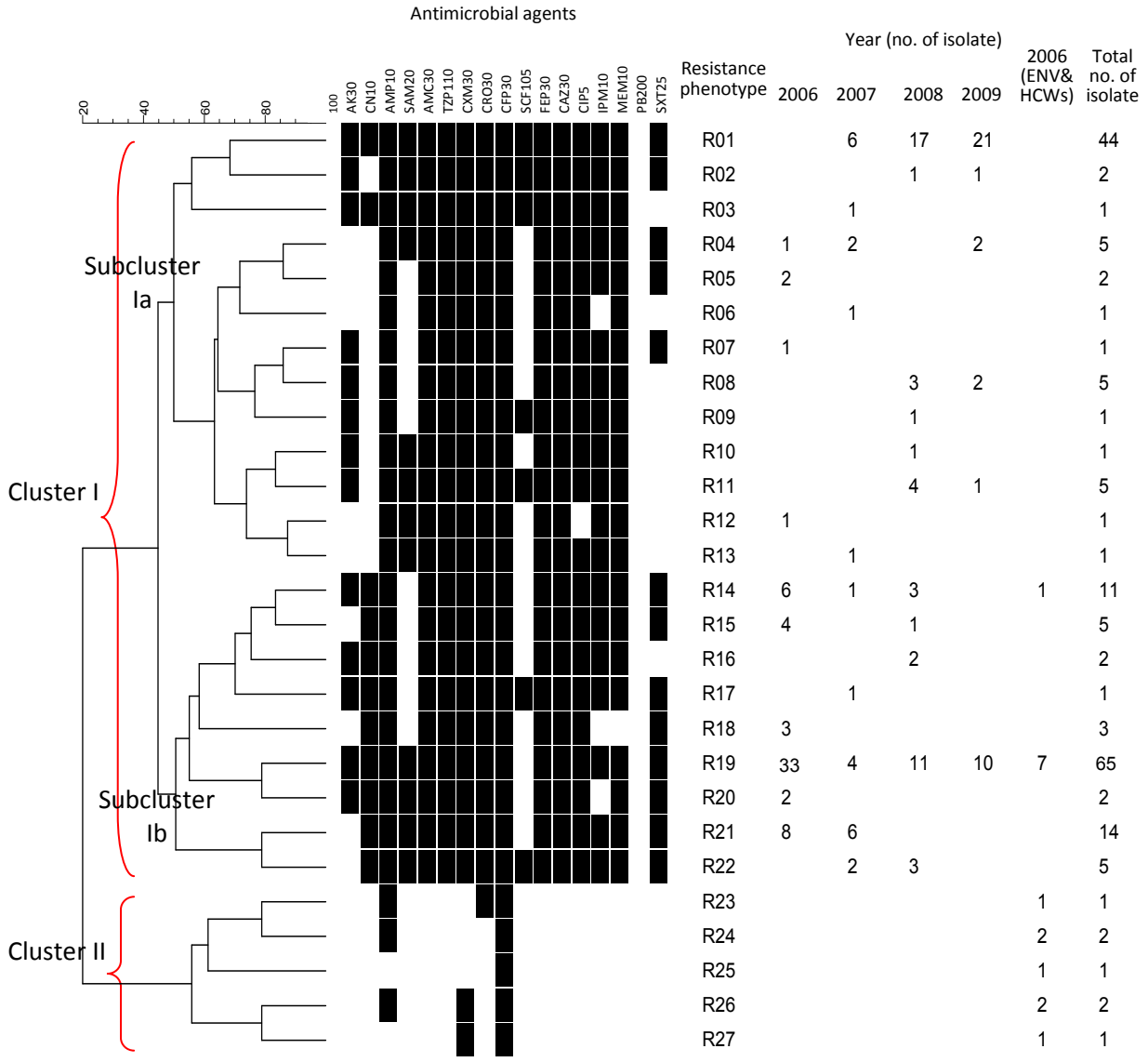


Figure 4.4: Resistance percentage of clinical, environmental and hands of HCWs *A. baumannnii* strains towards 17 tested antimicrobial agents. Abbreviations: AK30, Amikacin 30 µg; CN10, Gentamicin 10 µg; AMP10, Ampicillin 10 µg; SAM20, Ampicillin/sulbactam 20 µg; AMC30, Amoxicilin/clavulanic 30 µg; TZP110, Piperacillin/tazobactam 110 µg, CXM30, Cefuroxime 30 µg; CRO30, Ceftriaxone 30 µg; CFP30, Cefoperazone 30 µg; SCF105, Cefoperazone/sulbactam 105 µg; CAZ30, Ceftazidime 30 µg; FEP30, Cefepime 30 µg; CIP5, Ciprofloxacin 5 µg; IPM10, Imipenem 10 µg; MEM10, Meropenem 10 µg; PB200, Polymixin B 200 µg; SXT25, Trimethoprim/sulfamethoxazole 25 µg.



Resistance phenotype	Year (no. of isolate) 2006	2007	2008	2009	2006 (ENV& HCWs)	Total no. of isolate
R01		6	17	21		44
R02			1	1		2
R03		1				1
R04	1	2		2		5
R05	2					2
R06		1				1
R07	1					1
R08			3	2		5
R09			1			1
R10			1			1
R11			4	1		5
R12	1					1
R13		1				1
R14	6	1	3		1	11
R15	4		1			5
R16			2			2
R17		1				1
R18	3					3
R19	33	4	11	10	7	65
R20	2					2
R21	8	6				14
R22		2	3			5
R23					1	1
R24					2	2
R25					1	1
R26					2	2
R27					1	1

Figure 4.5: Twenty-seven representative resistance profiles of 185 *A. baumannii* isolates.

Abbreviations: AK30, Amikacin 30 µg; CN10, Gentamicin 10 µg; AMP10, Ampicillin 10 µg; SAM20, Ampicillin/sulbactam 20 µg; AMC30, Amoxicilin/clavulanic 30 µg; TZP110, Piperacillin/tazobactam 110 µg, CXM30, Cefuroxime 30 µg; CRO30, Ceftriaxone 30 µg; CFP30, Cefoperazone 30 µg; SCF105, Cefoperazone/sulbactam 105 µg; CAZ30, Ceftazidime 30 µg; FEP30, Cefepime 30 µg; CIP5, Ciprofloxacin 5 µg; IPM10, Imipenem 10 µg; MEM10, Meropenem 10 µg; PB200, Polymixin B 200 µg; SXT25, Trimethoprim/sulfamethoxazole 25 µg; ENV, environmental; HCWs, healthcare workers.

■ = Resistant

4.3 MBL activity in imipenem-resistant *A. baumannii* isolates

4.3.1 Combined disk test

Combined disk test was performed by measuring the diameters of the inhibition zone for imipenem +EDTA disks. The inhibition diameter of the imipenem-EDTA for all the resistant *A. baumannii* were ≤14 mm indicated that all the isolates were MBL-negative.

4.3.2 Imipenem-EDTA double-disk synergy test

According to Lee *et al*., 2003, presence of even a small synergistic inhibition zone between the imipenem and EDTA disks was interpreted as MBL-positive isolates. However, the synergistic inhibition zone was absent in all the imipenem-resistant *A. baumannii*. All the isolates were tested as MBL-negative.

4.4 Presence of carbapenem-resistance genes

4.4.1 MBL resistance genes

In this study, multiplex PCR assays were performed to detect the presence of 5 different MBL resistance genes: bla_{GIM}, bla_{SPM}, bla_{SIM}, bla_{IMP} and bla_{VIM} in 175 of carbapenem-resistant *A. baumannii* isolates. However, none of the isolates were positive for the MBL resistance genes in all the performed PCR assays.

4.4.2 Presence of OXA-carbapenemase genes

A multiplex PCR was carried out according to Woodford *et al*., 2006 to determine the presence of bla_{OXA} genes encoding carbapenemases ($bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$) in the *A. baumannii* isolates. All the 185 of *A. baumannii* isolates harboured $bla_{\text{OXA-51}}$ gene. Out of 175 carbapenem-resistant (imipenem and/or meropenem) isolates, 174 (99.0%) of the isolates harboured $bla_{\text{OXA-23}}$ gene. Both the $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$ genes were not detected in any of the *A. baumannii* isolates.

Genes encoding OXA-58-like enzyme was detected in one of the non-*baumannii* isolate (*A.* genospecies 13TU 0608-21) (Figure 4.6). Sequencing analysis of the $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$ PCR amplicons indicated complete identity to their respective sequences in the NCBI database (**APPENDIX VI** (a) and (b)).

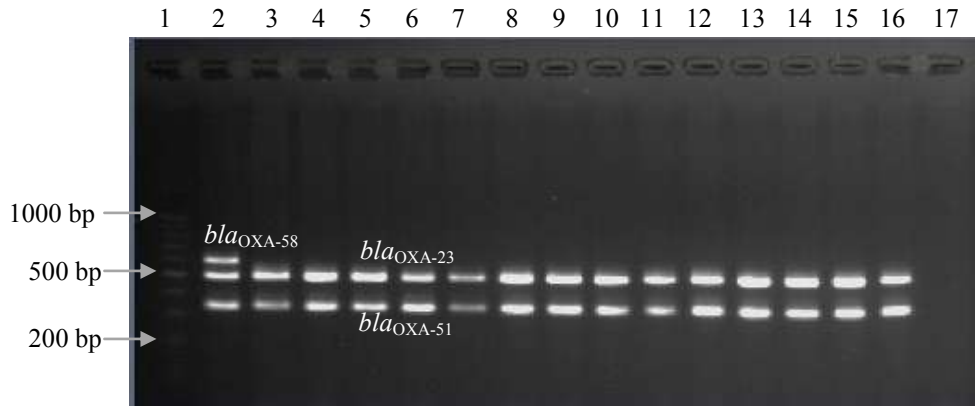


Figure 4.6: Representative gel picture of multiplex-PCR amplification of bla_{OXA} genes; $bla_{\text{OXA-23}}$ (501 bp), $bla_{\text{OXA-24}}$ (246 bp), $bla_{\text{OXA-51}}$ (353 bp) and $bla_{\text{OXA-58}}$ (599 bp). Lane 1: 100 bp DNA marker (Promega, USA), lane 2: *A.* genospecies 13TU, lane 3-16: *A. baumannii* isolates and lane 17: DNA blank.

4.4.3 Presence of insertion sequence IS*Aba1* and upstream of IS*Aba1* of the OXA-23 and OXA-51 positive *A. baumannii*

All the 175 carbapenem-resistant *A. baumannii* isolates were screened for the presence of insertion sequence IS*Aba1*. All the isolates were positive for a 549 bp band of IS*Aba1* insertion sequence (Figure 4.7). Sequencing analysis of the IS*Aba1* PCR amplicons from representative isolate revealed complete identity with IS*Aba1* sequence in the NCBI database (**APPENDIX VI** (c)).

PCR mapping followed by sequencing using ISF/OXA-23R primers revealed the presence of IS*Aba1* upstream to the $bla_{\text{OXA-23}}$. All the $bla_{\text{OXA-23}}$-positive *A. baumannii* gave an amplicon size of ~1.6 kb in the PCR using the primer pair of ISF/OXA-23R (Figure 4.8). Sequencing analysis of the PCR amplicon from representative isolate showed complete identity of the presence of insertion sequence IS*Aba1* upstream to the $bla_{\text{OXA-23}}$ gene in the NCBI database (**APPENDIX VI** (d)). There was no amplification for the primer pair of ISF/OXA-51likeR indicated IS*Aba1* was absent upstream of $bla_{\text{OXA-51}}$ gene. The presence of the bla_{OXA} genes and IS*Aba1* are summarized in Table 4.2.

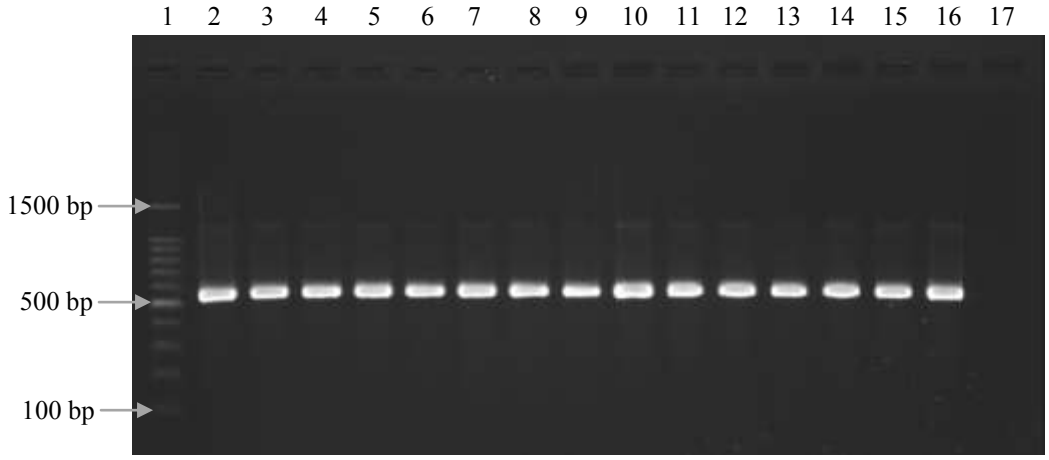


Figure 4.7: PCR amplification of I IS*Aba1*with primer pairs of ISF/ISR. Lane 1: 100 bp DNA marker (Promega, USA), lane 2-16: representative *A. baumannii* isolates and lane 17: DNA blank.

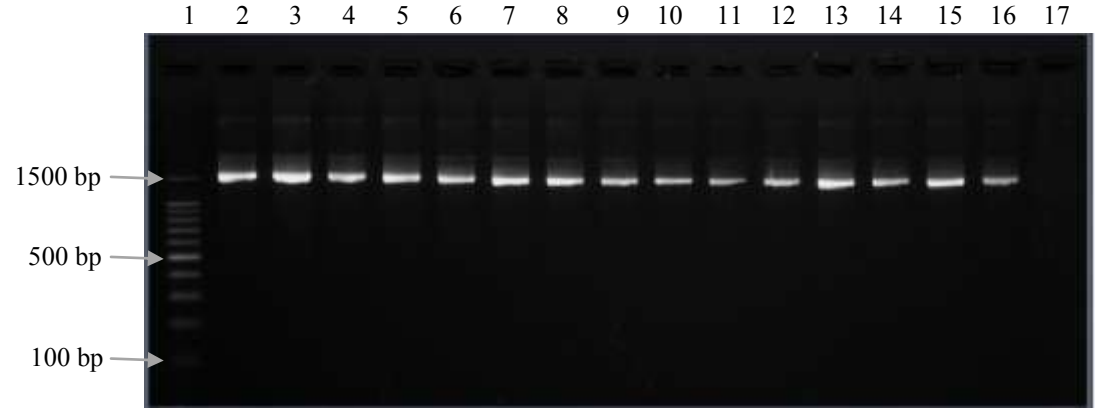


Figure 4.8: Representative gel picture of PCR amplification of IS*Aba1*upstream of $bla_{\text{OXA-23}}$ gene in *A. baumannii* isolates. Lane 1: 100 bp DNA marker (Promega, USA), lane 2-16: representative of *A. baumannii* isolates and lane 17: DNA blank.

Table 4.2: Summarized results of *bla*$_{OXA}$ genes and IS*Aba1* presence in the *A. baumannii* isolates

No	Isolate code	Resis-tance pheno-type	Oxacillinases genes(*bla*$_{OXA}$)		IS*Aba-1*	ISF/ OXA -23R	No	Isolate code	Resis-tance pheno-type	Oxacillinases genes (*bla*$_{OXA}$)		IS*Aba-1*	ISF/ OXA -23R	No	Isolate code	Resis-tance pheno-type	Oxacillinases genes (*bla*$_{OXA}$)		IS*Aba-1*	ISF/ OXA -23R
			23	51						23	51						23	51		
1	AC/0601-5	R19	+	+	+	+	**25**	AC/0607-2	R19	+	+	+	+	**49**	AC/0610-18	R19	+	+	+	+
2	AC/0601-8	R21	+	+	+	+	**26**	AC/0607-6	R19	+	+	+	+	**50**	AC/0611-5	R19	+	+	+	+
3	AC/0601-10	R19	+	+	+	+	**27**	AC/0607-12	R15	+	+	+	+	**51**	AC/0611-7	R19	+	+	+	+
4	AC/0602-19	R19	+	+	+	+	**28**	AC/0607-18	R15	+	+	+	+	**52**	AC/0611-10	R19	+	+	+	+
5	AC/0603-1	R21	+	+	+	+	**29**	AC/0607-19	R15	+	+	+	+	**53**	AC/0611-11	R21	+	+	+	+
6	AC/0603-2	R19	+	+	+	+	**30**	AC/0607-20	R19	+	+	+	+	**54**	AC/0611-15	R19	+	+	+	+
7	AC/0603-7	R19	+	+	+	+	**31**	AC/0607-22	R19	+	+	+	+	**55**	AC/0611-16	R19	+	+	+	+
8	AC/0603-9	R12	+	+	+	+	**32**	AC/0607-25	R20	-	+	+	-	**56**	AC/0611-18	R19	+	+	+	+
9	AC/0603-22	R04	+	+	+	+	**33**	AC/0607-28	R19	+	+	+	+	**57**	AC/0611-19	R21	+	+	+	+
10	AC/0603-25	R18	-	+	+	-	**34**	AC/0608-1	R21	+	+	+	+	**58**	AC/0612-7	R19	+	+	+	+
11	AC/0603-26	R05	+	+	+	+	**35**	AC/0608-5	R19	+	+	+	+	**59**	AC/0612-13	R19	+	+	+	+
12	AC/0604-6	R21	+	+	+	+	**36**	AC/0608-7	R19	+	+	+	+	**60**	AC/0612-16	R19	+	+	+	+
13	AC/0604-7	R18	-	+	+	-	**37**	AC/0608-17	R19	+	+	+	+	**61**	AC/0612-17	R19	+	+	+	+
14	AC/0604-11	R18	-	+	+	-	**38**	AC/0608-22	R19	+	+	+	+	**62**	ACIBA 2006- 1	R19	+	+	+	+
15	AC/0604-25	R15	+	+	+	+	**39**	AC/0609-1	R19	+	+	+	+	**63**	ACIBA 2006- 2	R19	+	+	+	+
16	AC/0605-3	R19	+	+	+	+	**40**	AC/0609-6	R19	+	+	+	+	**64**	ACIBA 2006- 36	R19	+	+	+	+
17	AC/0605-19	R07	+	+	+	+	**41**	AC/0609-8 tip	R19	+	+	+	+	**65**	ACIBA 2006- 43	R19	+	+	+	+
18	AC/0605-25	R21	+	+	+	+	**42**	AC/0609-10	R19	+	+	+	+	**66**	ACIBA 2006- 46	R19	+	+	+	+
19	AC/0606-11	R14	+	+	+	+	**43**	AC/0609-14	R19	+	+	+	+	**67**	ACIBA 2006- 47	R19	+	+	+	+
20	AC/0606-13	R05	+	+	+	+	**44**	AC/0609-25	R20	+	+	+	+	**68**	ACIBA 2006- 49	R26	-	+	-	-
21	AC/0606-16	R19	+	+	+	+	**45**	AC/0610-2	R19	+	+	+	+	**69**	ACIBA 2006- 50	R26	-	+	-	-
22	AC/0606-22	R19	+	+	+	+	**46**	AC/0610-8	R19	+	+	+	+	**70**	ACIBA 2006- 51	R27	-	+	-	-
23	AC/0606-23	R21	+	+	+	+	**47**	AC/0610-9	R19	+	+	+	+	**71**	ACIBA 2006- 52	R23	-	+	-	-
24	AC/0606-24	R14	+	+	+	+	**48**	AC/0610-12	R19	+	+	+	+	**72**	ACIBA 2006- 53	R25	-	+	-	-

No	Isolate code	Resis-tance pheno -type	Oxacillinases genes (bla_{OXA})		IS*Aba -1*	ISF/ OXA -23R	No	Isolate code	Resis-tance pheno -type	Oxacillinases genes (bla_{OXA})		IS*Aba -1*	ISF/ OXA - 23R	No	Isolate code	Resis-tance pheno-type	Oxacillinases genes (bla_{OXA})		IS*Aba -1*	ISF/ OXA -23R
			23	51						23	51						23	51		
73	ACIBA 2006- 56	R24	-	+	-	-	**97**	AC/0709-27	R14	+	+	+	+	**121**	AC/0806-18	R01	+	+	+	+
74	ACIBA 2006- 57	R24	-	+	-	-	**98**	AC/0710-3	R01	+	+	+	+	**122**	AC/0806-23	R11	+	+	+	+
75	ACIBA 2006- 63	R19	+	+	+	+	**99**	AC/0711-7	R06	+	+	+	+	**123**	AC/0806-24	R01	+	+	+	+
76	ACIBA 2006- 65	R19	+	+	+	+	**100**	AC/0712-3	R19	+	+	+	+	**124**	AC/0806-28	R22	+	+	+	+
77	AC/0701-11	R17	+	+	+	+	**101**	AC/0712-13	R03	+	+	+	+	**125**	AC/0807-20	R01	+	+	+	+
78	AC/0702-5	R01	+	+	+	+	**102**	AC/0801-4	R19	+	+	+	+	**126**	AC/0808-6	R01	+	+	+	+
79	AC/0702-17	R22	+	+	+	+	**103**	AC/0801-6	R19	+	+	+	+	**127**	AC/0808-14	R01	+	+	+	+
80	AC/0703-14	R21	+	+	+	+	**104**	AC/0801-11	R19	+	+	+	+	**128**	AC/0808-18	R01	+	+	+	+
81	AC/0703-21	R21	+	+	+	+	**105**	AC/0801-13	R19	+	+	+	+	**129**	AC/0808-20	R01	+	+	+	+
82	AC/0704-7	R19	+	+	+	+	**106**	AC/0802-1	R14	+	+	+	+	**130**	AC/0809-1	R01	+	+	+	+
83	AC/0705-3	R04	+	+	+	+	**107**	AC/0802-4	R15	+	+	+	+	**131**	AC/0809-9	R01	+	+	+	+
84	AC/0705-9	R21	+	+	+	+	**108**	AC/0802-14	R19	+	+	+	+	**132**	AC/0809-12	R09	+	+	+	+
85	AC/0705-15	R22	+	+	+	+	**109**	AC/0802-20	R19	+	+	+	+	**133**	AC/0809-29	R01	+	+	+	+
86	AC/0706-21	R21	+	+	+	+	**110**	AC/0803-15	R19	+	+	+	+	**134**	AC/0809-30	R01	+	+	+	+
87	AC/0707-8	R01	+	+	+	+	**111**	AC/0804-4	R19	+	+	+	+	**135**	AC/0810-8	R19	+	+	+	+
88	AC/0707-13	R01	+	+	+	+	**112**	AC/0804-19	R08	+	+	+	+	**136**	AC/0810-11	R01	+	+	+	+
89	AC/0707-26	R13	+	+	+	+	**113**	AC/0804-24	R01	+	+	+	+	**137**	AC/0810-12	R01	+	+	+	+
90	AC/0708-10	R01	+	+	+	+	**114**	AC/0804-31	R10	+	+	+	+	**138**	AC/0810-22	R22	+	+	+	+
91	AC/0708-16	R21	+	+	+	+	**115**	AC/0804-32	R11	+	+	+	+	**139**	AC/0810-26	R16	+	+	+	+
92	AC/0708-20	R19	+	+	+	+	**116**	AC/0805-4	R02	+	+	+	+	**140**	AC/0811-12	R01	+	+	+	+
93	AC/0709-5	R19	+	+	+	+	**117**	AC/0805-5	R14	+	+	+	+	**141**	AC/0811-13	R11	+	+	+	+
94	AC/0709-6	R21	+	+	+	+	**118**	AC/0805-20	R16	+	+	+	+	**142**	AC/0811-15	R19	+	+	+	+
95	AC/0709-7	R04	+	+	+	+	**119**	AC/0806-4	R14	+	+	+	+	**143**	AC/0811-25	R19	+	+	+	+
96	AC/0709-8	R01	+	+	+	+	**120**	AC/0806-10	R01	+	+	+	+	**144**	AC/0812-1	R01	+	+	+	+

No	Isolate code	Resis-tance pheno-type	Oxacillinases genes (bla_{OXA})		IS*Aba-1*	ISF/OXA-23R	No	Isolate code	Resis-tance pheno-type	Oxacillinases genes (bla_{OXA})		IS*Aba-1*	ISF/OXA-23R	No	Isolate code	Resis-tance pheno-type	Oxacillinases genes (bla_{OXA})		IS*Aba-1*	ISF/OXA-23R
			23	51						23	51						23	51		
145	AC/0812-8	R22	+	+	+	+	**159**	AC/0903-15	R19	+	+	+	+	**173**	AC/0904-40	R01	+	+	+	+
146	AC/0812-16	R11	+	+	+	+	**160**	AC/0903-19	R01	+	+	+	+	**174**	AC/0904-42	R04	+	+	+	+
147	AC/0812-29	R08	+	+	+	+	**161**	AC/0903-21	R02	+	+	+	+	**175**	AC/0904-43	R01	+	+	+	+
148	AC/0812-33	R08	+	+	+	+	**162**	AC/0903-28	R01	+	+	+	+	**176**	AC/0905-2	R01	+	+	+	+
149	AC/0901-5	R19	+	+	+	+	**163**	AC/0903-29	R01	+	+	+	+	**177**	AC/0905-6	R01	+	+	+	+
150	AC/0901-14	R19	+	+	+	+	**164**	AC/0903-31	R01	+	+	+	+	**178**	AC/0905-21	R01	+	+	+	+
151	AC/0901-36	R01	+	+	+	+	**165**	AC/0904-3	R01	+	+	+	+	**179**	AC/0905-22	R08	+	+	+	+
152	AC/0901-37	R19	+	+	+	+	**166**	AC/0904-7	R01	+	+	+	+	**180**	AC/0905-31	R01	+	+	+	+
153	AC/0902-5	R11	+	+	+	+	**167**	AC/0904-15	R01	+	+	+	+	**181**	AC/0905-42	R04	+	+	+	+
154	AC/0902-6	R01	+	+	+	+	**168**	AC/0904-19	R01	+	+	+	+	**182**	AC/0905-49	R19	+	+	+	+
155	AC/0902-13	R08	+	+	+	+	**169**	AC/0904-20	R01	+	+	+	+	**183**	AC/0905-53	R19	+	+	+	+
156	AC/0902-14	R19	+	+	+	+	**170**	AC/0904-21	R01	+	+	+	+	**184**	AC/0905-58	R19	+	+	+	+
157	AC/0902-15	R19	+	+	+	+	**171**	AC/0904-28	R01	+	+	+	+	**185**	AC/0905-60	R19	+	+	+	+
158	AC/0902-19	R01	+	+	+	+	**172**	AC/0904-39	R01	+	+	+	+							

+: gene present

- : gene absent

4.5 Integrons characterisation

4.5.1 Presence of integrase genes

Among 175 carbapenem-resistant *A. baumannii* isolates screened for the presence of class 1, 2 and 3 integron-encoded integrases (*intI*1, *intI*2 and *intI*3), 120 (68.6%) isolates were positive for *intI*1 and/or *intI*2 (Figure 4.9). Class 1 integron was predominant in the integron-positive isolates. One-hundred and nineteen (99.2%) of the integron-positive isolates were positive for *intI*1 gene whereas 18 (10.3%) isolates were positive for *intI*2 gene. Seventeen of the *intI*2-positive isolates were also harboured *intI*1 gene. Three carbapenem-susceptible clinical isolates were positive for *intI*1 gene. No *intI*1 and *intI*2 integrase genes were detected in the non-MDR hands of HCWs isolates. Class 3 integron-encoded *intI*3 integrase gene was not detected in any of the *A. baumannii* isolates. Sequencing analysis of the PCR amplified products from representative isolates indicated complete identity with *intI*1 and *intI2* sequences in the NCBI database **(APPENDIX VI** (e) and (f)).

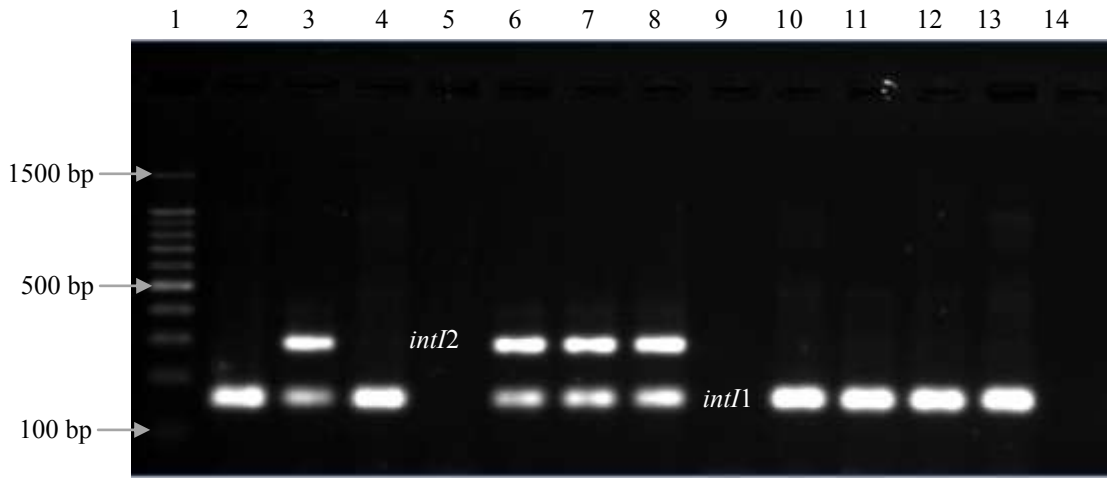


Figure 4.9: Multiplex-PCR amplification of class 1, 2 and 3 integron-encoded integrase genes, *intI*1 (160 bp), *intI*2 (287 bp) and *intI*3 (979bp). Lane 1: 100 bp DNA marker (Promega, USA), lane 2-13: representative *A. baumannii* isolates and lane 14: DNA blank.

4.5.2 Integron-encoded gene cassettes within class 1 and class 2 integrons

An amplicon size of approximately 2.5 kb was amplified in all the 119 *intI*1-positive carbapenem-resistant isolates and 3 of the carbapenem-susceptible isolates using 5′CS/3′CS primers pair (Figure 4.10). Restriction digestion with *Alu*I of the amplicon revealed 2 different profiles, IN1-a and IN1-b (Figure 4.11). Integron profile IN1-a was represented by 73 carbapenem-resistant isolates and 3 carbapenem-susceptible isolates, while IN1-b was represented by 46 carbapenem-resistant isolates.

The gene cassettes of class 2 integron amplified by hep54/hep71 primers pair gave an amplicon size of approximately 2.2 kb in all the 18 *intI*2-positive isolates (Figure 4.12). Based on the *Alu*I restricted patterns of the amplicons, a single profile for class 2 integron, IN2-a was determined (Figure 4.13). Seventeen of the IN2-a isolates were also harboured an IN1-a. (Table 4.3) Eleven and 6 of these IN1-a and IN2-a harbouring isolates were from 2006 and 2007, respectively (Table 4.4). A clinical isolate from 2006 (AC/0606-13) was harboured an IN2-a only. Integrons were mostly detected in the 2006 carbapenem-resistant isolates (96.6%), followed by isolates from 2007 (80.0%), 2008 (70.2%) and 2009 (8.0%).

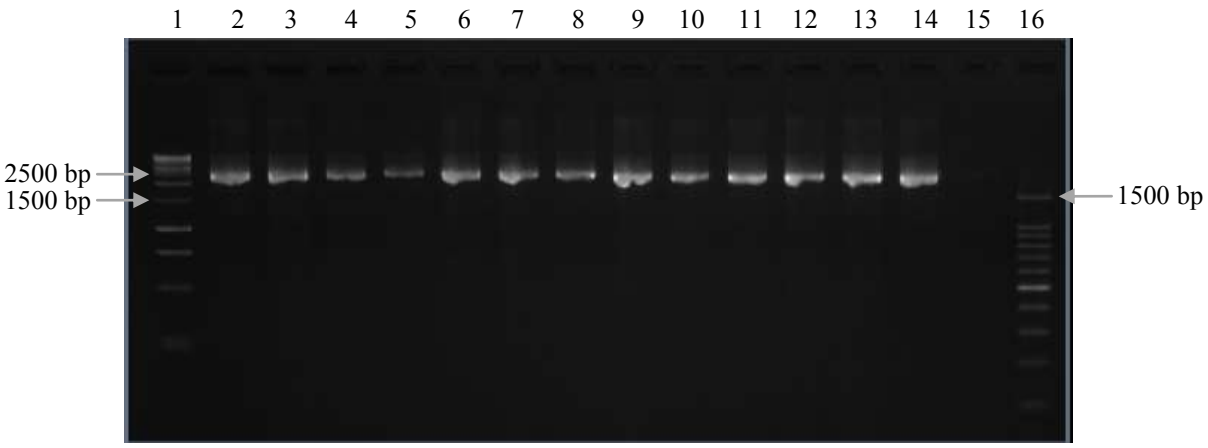


Figure 4.10: Amplification of class 1 integron gene cassettes using primers pair of 5`CS/3`CS. Lane 1: 1 kb DNA marker (Promega, USA), lane 2-14: representative *A. baumannii* isolates and lane 15: DNA blank, lane 16: 100 bp DNA marker (Promega, USA).

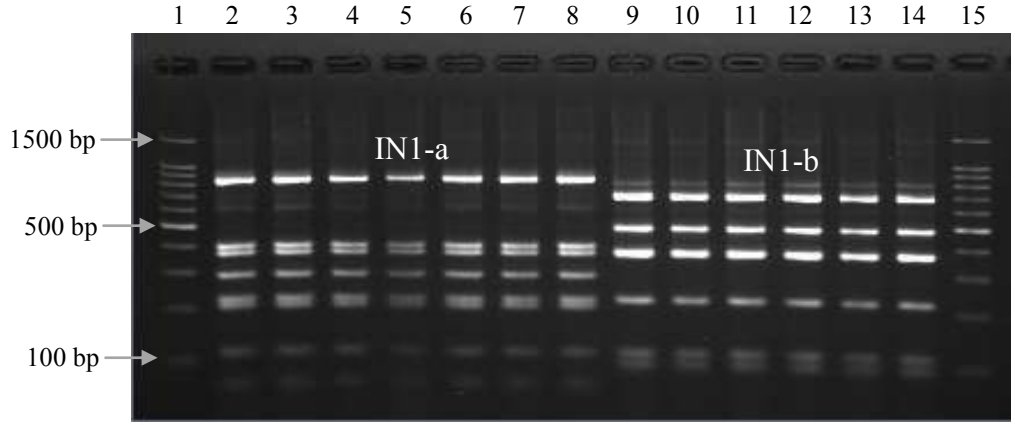


Figure 4.11: Restriction patterns obtained after digestion with *Alu*I for an amplified ~2.5 kb of the class 1 integron. Lane 1and 15: 100bp DNA marker (Promega, USA); lane 2-8: *A. baumannii* isolates with IN1-a integron profile; lane 9-14: *baumannii* isolates with IN1-b integron profile.

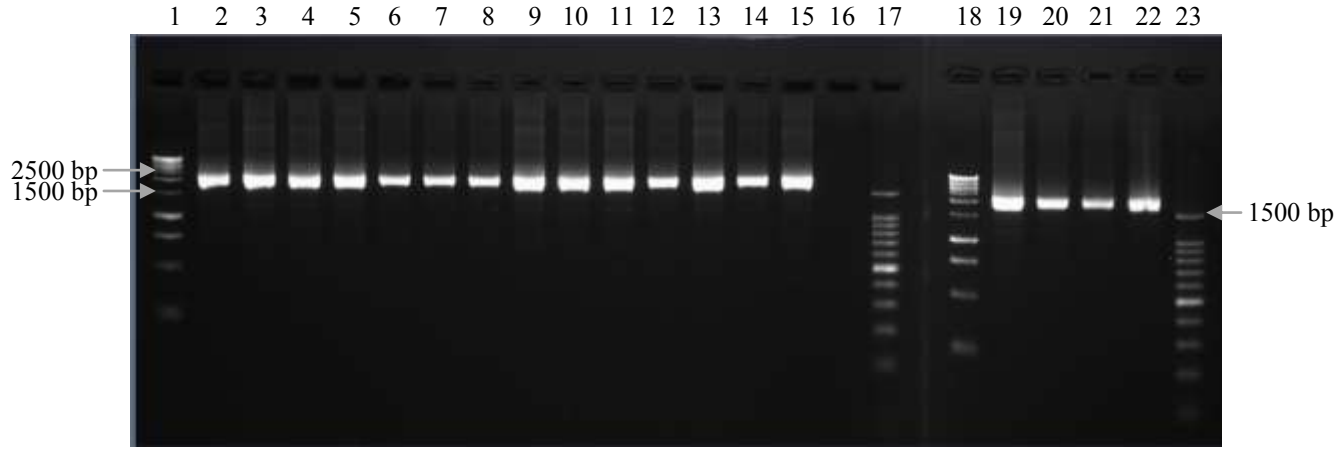


Figure 4.12: Amplification of class 2 integron gene cassettes using primers pair of hep54/hep71. Lane 1and 18: 1 kb DNA marker (Promega, USA), lane 2-14: representative *A. baumannii* isolates and lane 16: DNA blank, lane 17 and 23: 100 bp DNA marker (Promega, USA).

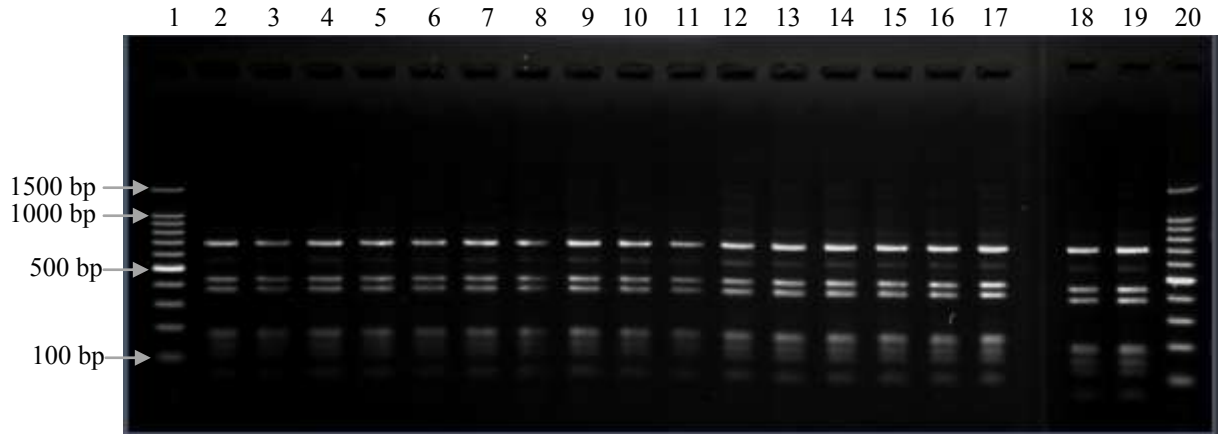


Figure 4.13: Restriction patterns obtained after digestion with *Alu*I for an amplified ~2.2 kb of the class 2 integron. Lane 1and 20: 100bp DNA marker (Promega, USA); lane 2-19: *A. baumannii* isolates with class 2 integron IN2-a profile.

Table 4.3: Summarized results of class 1 and class 2 integrons present in the *A. baumannii* isolates

No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2		No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2		No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2	
			Profile	Size (kb)	Profile	Size (kb)				Profile	Size (kb)	Profile	Size (kb)				Profile	Size (kb)	Profile	Size (kb)
1	AC/0601-5	R19	IN1-a	~2.5	IN2-a	~2.1	**28**	AC/0607-18	R15	IN1-a	~2.5	ND	-	**55**	AC/0611-16	R19	IN1-a	~2.5	ND	-
2	AC/0601-8	R21	IN1-a	~2.5	ND	-	**29**	AC/0607-19	R15	IN1-a	~2.5	ND	-	**56**	AC/0611-18	R19	IN1-b	~2.0	ND	-
3	AC/0601-10	R19	IN1-a	~2.5	ND	-	**30**	AC/0607-20	R19	IN1-a	~2.5	ND	-	**57**	AC/0611-19	R21	IN1-b	~2.0	ND	-
4	AC/0602-19	R19	IN1-a	~2.5	ND	-	**31**	AC/0607-22	R19	IN1-b	~2.0	ND	-	**58**	AC/0612-7	R19	IN1-a	~2.5	IN2-a	~2.1
5	AC/0603-1	R21	IN1-a	~2.5	ND	-	**32**	AC/0607-25	R20	IN1-b	~2.0	ND	-	**59**	AC/0612-13	R19	IN1-a	~2.5	ND	-
6	AC/0603-2	R19	IN1-b	~2.0	ND	-	**33**	AC/0607-28	R19	IN1-a	~2.5	IN2-a	~2.1	**60**	AC/0612-16	R19	IN1-a	~2.5	IN2-a	~2.1
7	AC/0603-7	R19	IN1-a	~2.5	ND	-	**34**	AC/0608-1	R21	IN1-a	~2.5	ND	-	**61**	AC/0612-17	R19	IN1-a	~2.5	ND	-
8	AC/0603-9	R12	ND	-	ND	-	**35**	AC/0608-5	R19	IN1-a	~2.5	IN2-a	~2.1	**62**	ACIBA 2006- 1	R19	IN1-a	~2.5	ND	-
9	AC/0603-22	R04	IN1-a	~2.5	ND	-	**36**	AC/0608-7	R19	IN1-a	~2.5	ND	-	**63**	ACIBA 2006- 2	R19	IN1-a	~2.5	ND	-
10	AC/0603-25	R18	IN1-a	~2.5	ND	-	**37**	AC/0608-17	R19	IN1-a	~2.5	ND	-	**64**	ACIBA 2006- 36	R19	IN1-a	~2.5	ND	-
11	AC/0603-26	R05	IN1-a	~2.5	ND	-	**38**	AC/0608-22	R19	IN1-b	~2.0	ND	-	**65**	ACIBA 2006- 43	R19	IN1-a	~2.5	ND	-
12	AC/0604-6	R21	IN1-a	~2.5	ND	-	**39**	AC/0609-1	R19	IN1-a	~2.5	ND	-	**66**	ACIBA 2006- 46	R19	IN1-b	~2.0	ND	-
13	AC/0604-7	R18	IN1-a	~2.5	ND	-	**40**	AC/0609-6	R19	IN1-a	~2.5	ND	-	**67**	ACIBA 2006- 47	R19	IN1-b	~2.0	ND	-
14	AC/0604-11	R18	IN1-a	~2.5	ND	-	**41**	AC/0609-8 tip	R19	IN1-b	~2.0	ND	-	**68**	ACIBA 2006- 49	R26	ND	-	ND	-
15	AC/0604-25	R15	IN1-a	~2.5	IN2-a	~2.1	**42**	AC/0609-10	R19	IN1-a	~2.5	ND	-	**69**	ACIBA 2006- 50	R26	ND	-	ND	-
16	AC/0605-3	R19	IN1-b	~2.0	ND	-	**43**	AC/0609-14	R19	IN1-a	~2.5	IN2-a	~2.1	**70**	ACIBA 2006- 51	R27	ND	-	ND	-
17	AC/0605-19	R07	IN1-a	~2.5	ND	-	**44**	AC/0609-25	R20	IN1-a	~2.5	IN2-a	~2.1	**71**	ACIBA 2006- 52	R23	ND	-	ND	-
18	AC/0605-25	R21	IN1-a	~2.5	ND	-	**45**	AC/0610-2	R19	IN1-b	~2.0	ND	-	**72**	ACIBA 2006- 53	R25	IN1-a	~2.5	ND	-
19	AC/0606-11	R14	IN1-a	~2.5	ND	-	**46**	AC/0610-8	R19	IN1-a	~2.5	IN2-a	~2.1	**73**	ACIBA 2006- 56	R24	ND	-	ND	-
20	AC/0606-13	R05	ND	-	IN2-a	~2.1	**47**	AC/0610-9	R19	IN1-a	~2.5	IN2-a	~2.1	**74**	ACIBA 2006- 57	R24	ND	-	ND	-
21	AC/0606-16	R19	IN1-b	~2.0	ND	-	**48**	AC/0610-12	R19	IN1-b	~2.0	ND	-	**75**	ACIBA 2006- 63	R19	ND	-	ND	-
22	AC/0606-22	R19	IN1-a	~2.5	ND	-	**49**	AC/0610-18	R19	IN1-b	~2.0	ND	-	**76**	ACIBA 2006- 65	R19	IN1-a	~2.5	ND	-
23	AC/0606-23	R21	IN1-a	~2.5	ND	-	**50**	AC/0611-5	R19	IN1-a	~2.5	ND	-	**77**	AC/0701-11	R17	IN1-a	~2.5	ND	-
24	AC/0606-24	R14	IN1-a	~2.5	ND	-	**51**	AC/0611-7	R19	IN1-b	~2.0	ND	-	**78**	AC/0702-5	R01	IN1-a	~2.5	ND	-
25	AC/0607-2	R19	IN1-a	~2.5	ND	-	**52**	AC/0611-10	R19	IN1-b	~2.0	ND	-	**79**	AC/0702-17	R22	IN1-a	~2.5	ND	-
26	AC/0607-6	R19	IN1-a	~2.5	ND	-	**53**	AC/0611-11	R21	IN1-a	~2.5	ND	-	**80**	AC/0703-14	R21	IN1-a	~2.5	ND	-
27	AC/0607-12	R15	IN1-a	~2.5	ND	-	**54**	AC/0611-15	R19	IN1-a	~2.5	IN2-a	~2.1	**81**	AC/0703-21	R21	IN1-a	~2.5	ND	-

No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2		No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2		No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2	
			Profile	Size (kb)	Profile	Size (kb)				Profile	Size (kb)	Profile	Size (kb)				Profile	Size (kb)	Profile	Size (kb)
82	AC/0704-7	R19	IN1-a	~2.5	ND	-	**108**	AC/0802-14	R19	IN1-a	~2.5	ND	-	**134**	AC/0809-30	R01	IN1-b	~2.0	ND	-
83	AC/0705-3	R04	ND	-	ND	-	**109**	AC/0802-20	R19	IN1-b	~2.0	ND	-	**135**	AC/0810-8	R19	IN1-b	~2.0	ND	-
84	AC/0705-9	R21	IN1-a	~2.5	ND	-	**110**	AC/0803-15	R19	IN1-b	~2.0	ND	-	**136**	AC/0810-11	R01	IN1-b	~2.0	ND	-
85	AC/0705-15	R22	ND	-	ND	-	**111**	AC/0804-4	R19	IN1-b	~2.0	ND	-	**137**	AC/0810-12	R01	IN1-b	~2.0	ND	-
86	AC/0706-21	R21	IN1-a	~2.5	ND	-	**112**	AC/0804-19	R08	ND	-	ND	-	**138**	AC/0810-22	R22	IN1-a	~2.5	ND	-
87	AC/0707-8	R01	IN1-a	~2.5	IN2-a	~2.1	**113**	AC/0804-24	R01	IN1-b	~2.0	ND	-	**139**	AC/0810-26	R16	ND	-	ND	-
88	AC/0707-13	R01	IN1-a	~2.5	IN2-a	~2.1	**114**	AC/0804-31	R10	ND	-	ND	-	**140**	AC/0811-12	R01	IN1-b	~2.0	ND	-
89	AC/0707-26	R13	ND	-	ND	-	**115**	AC/0804-32	R11	ND	-	ND	-	**141**	AC/0811-13	R11	ND	-	ND	-
90	AC/0708-10	R01	IN1-a	~2.5	ND	-	**116**	AC/0805-4	R02	ND	-	ND	-	**142**	AC/0811-15	R19	IN1-b	~2.0	ND	-
91	AC/0708-16	R21	IN1-a	~2.5	ND	-	**117**	AC/0805-5	R14	ND	-	ND	-	**143**	AC/0811-25	R19	IN1-b	~2.0	ND	-
92	AC/0708-20	R19	IN1-b	~2.0	ND	-	**118**	AC/0805-20	R16	ND	-	ND	-	**144**	AC/0812-1	R01	IN1-b	~2.0	ND	-
93	AC/0709-5	R19	IN1-a	~2.5	IN2-a	~2.1	**119**	AC/0806-4	R14	IN1-b	~2.0	ND	-	**145**	AC/0812-8	R22	IN1-a	~2.5	ND	-
94	AC/0709-6	R21	IN1-a	~2.5	ND	-	**120**	AC/0806-10	R01	IN1-b	~2.0	ND	-	**146**	AC/0812-16	R11	ND	-	ND	-
95	AC/0709-7	R04	ND	-	ND	-	**121**	AC/0806-18	R01	IN1-b	~2.0	ND	-	**147**	AC/0812-29	R08	ND	-	ND	-
96	AC/0709-8	R01	IN1-a	~2.5	IN2-a	~2.1	**122**	AC/0806-23	R11	ND	-	ND	-	**148**	AC/0812-33	R08	ND	-	ND	-
97	AC/0709-27	R14	IN1-a	~2.5	IN2-a	~2.1	**123**	AC/0806-24	R01	IN1-b	~2.0	ND	-	**149**	AC/0901-5	R19	IN1-b	~2.0	ND	-
98	AC/0710-3	R01	IN1-a	~2.5	IN2-a	~2.1	**124**	AC/0806-28	R22	IN1-a	~2.5	ND	-	**150**	AC/0901-14	R19	ND	-	ND	-
99	AC/0711-7	R06	ND	-	ND	-	**125**	AC/0807-20	R01	IN1-b	~2.0	ND	-	**151**	AC/0901-36	R01	ND	-	ND	-
100	AC/0712-3	R19	IN1-b	~2.0	ND	-	**126**	AC/0808-6	R01	IN1-b	~2.0	ND	-	**152**	AC/0901-37	R19	ND	-	ND	-
101	AC/0712-13	R03	IN1-a	~2.5	ND	-	**127**	AC/0808-14	R01	IN1-b	~2.0	ND	-	**153**	AC/0902-5	R11	ND	-	ND	-
102	AC/0801-4	R19	IN1-a	~2.5	ND	-	**128**	AC/0808-18	R01	IN1-b	~2.0	ND	-	**154**	AC/0902-6	R01	ND	-	ND	-
103	AC/0801-6	R19	IN1-b	~2.0	ND	-	**129**	AC/0808-20	R01	IN1-a	~2.5	ND	-	**155**	AC/0902-13	R08	ND	-	ND	-
104	AC/0801-11	R19	IN1-b	~2.0	ND	-	**130**	AC/0809-1	R01	IN1-b	~2.0	ND	-	**156**	AC/0902-14	R19	IN1-b	~2.0	ND	-
105	AC/0801-13	R19	IN1-a	~2.5	ND	-	**131**	AC/0809-9	R01	ND	-	ND	-	**157**	AC/0902-15	R19	ND	-	ND	-
106	AC/0802-1	R14	IN1-a	~2.5	ND	-	**132**	AC/0809-12	R09	ND	-	ND	-	**158**	AC/0902-19	R01	ND	-	ND	-
107	AC/0802-4	R15	IN1-b	~2.0	ND	-	**133**	AC/0809-29	R01	IN1-b	~2.0	ND	-	**159**	AC/0903-15	R19	ND	-	ND	-

No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2		No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2		No	Isolate code	Resis-tance pheno-type	Integron class 1		Integron class 2	
			Profile	Size (kb)	Profile	Size (kb)				Profile	Size (kb)	Profile	Size (kb)				Profile	Size (kb)	Profile	Size (kb)
160	AC/0903-19	R01	ND	-	ND	-	**169**	AC/0904-20	R01	ND	-	ND	-	**178**	AC/0905-21	R01	ND	-	ND	-
161	AC/0903-21	R02	ND	-	ND	-	**170**	AC/0904-21	R01	ND	-	ND	-	**179**	AC/0905-22	R08	ND	-	ND	-
162	AC/0903-28	R01	ND	-	ND	-	**171**	AC/0904-28	R01	ND	-	ND	-	**180**	AC/0905-31	R01	ND	-	ND	-
163	AC/0903-29	R01	ND	-	ND	-	**172**	AC/0904-39	R01	ND	-	ND	-	**181**	AC/0905-42	R04	ND	-	ND	-
164	AC/0903-31	R01	ND	-	ND	-	**173**	AC/0904-40	R01	ND	-	ND	-	**182**	AC/0905-49	R19	ND	-	ND	-
165	AC/0904-3	R01	ND	-	ND	-	**174**	AC/0904-42	R04	ND	-	ND	-	**183**	AC/0905-53	R19	ND	-	ND	-
166	AC/0904-7	R01	ND	-	ND	-	**175**	AC/0904-43	R01	ND	-	ND	-	**184**	AC/0905-58	R19	IN1-b	~2.0	ND	-
167	AC/0904-15	R01	ND	-	ND	-	**176**	AC/0905-2	R01	ND	-	ND	-	**185**	AC/0905-60	R19	ND	-	ND	-
168	AC/0904-19	R01	ND	-	ND	-	**177**	AC/0905-6	R01	ND	-	ND	-							

ND: Not detected
IN1-a: Integron class 1 profile a
IN1-b: Integron class 1 profile b
IN2-a: Integron class 2 profile a

4.5.3 DNA sequences of class 1 and class 2 integrons

The result of the DNA sequence data was compared to data in the GenBank database by using the BLAST algorithm (http://www.ncbi.nih.gov). From blast results of DNA sequences (Table 4.4), class 1 integron of profiles IN1-a and IN1-b consisted of 2 different types of gene cassettes, *aacC1-aadDA1-qacEΔdelta1-sul1* and *aacA4-catB8-aadA1*, respectively. The sequences of the class 2 integron amplicon displayed a gene cassette of *intI2 -sat2-aadB-catB2-dfrA1-aadA1* (**APPENDIX VI** (g), (h) and (i)).

The *aadB, aadA, aadDA1, aacC1* and *aacA4* genes encode enzyme aminoglycoside adenyltransferase enzymes and confer resistance to aminoglycosides. *dfrA1*gene encodes dihydrofolate reductase enzyme that confers resistance to trimethoprim. *sat2* gene encodes streptothricin acetyltransferase type 1 enzyme and confers resistance to streptothricin. *sul1* gene encodes dihydropteroate synthase type 1 enzyme and confers resistance to sulfonamide. Both the *catB2* and *catB8* genes encode the enzyme chloramphenicol acetyltransferase and confer resistance to chloramphenicol and the *qacEΔ1*gene encodes quaternary ammonium compound-resistance protein that confers resistance to quaternary ammonium compound.

Table 4.4: Summarized of class 1 and class 2 integrons gene cassettes in the carbapenem-resistant *A. baumannii* isolates isolated from 2006-2009

Integron	Profile	Gene cassette	Accessions	Year (no. of isolate)					Total
				Clinical				Env	
				2006 (n=58)	2007 (n=25)	2008 (n=47)	2009 (n=37)	2006 (n=8)	
Class 1	IN1-a	*aacC1-aadDA1-qacEdelta1-sul1*	EF033072	31	12	8	0	5	56
	IN1-b	*aacA4-catB8-aadA1*	AY557339	14	2	25	3	2	46
Class 2	IN2-a	*intI2 -sat2-aadB-catB2--dfrA1-aadA1*	DQ176450	1	0	0	0	0	1
Class 1 + class 2	IN1-a + IN2-a	*aacC1-aadDA1- qacEΔdelta1-sul1* and *intI2 -sat2-aadB-catB2-dfrA1-aadA1*	EF033072 DQ176450	11	6	0	0	0	17
		Total		57	20	33	3	7	120

4.6 Plasmid profiling of carbapenem-resistant *A. baumannii*

Among the 175 carbapenem-resistant isolates, 164 (93.7%) isolates harboured plasmids. 11 isolates from 2006 (n=4) and 2007 (n=7) lacked of visible plasmid bands. A total of 98 plasmid profiles with 48 different plasmids ranging from 1.6 kb to 125.1 kb were observed (Figure 4.14, Table 4.5). Profile P52 was the most predominant plasmid profile (n=20), followed by P49 (n=18), P53 (n=10), P1 (n=5), P54 (n=5) and P55 (n=3). Profiles P3, P4, P48, P57, P60, P61, P63, P64, P67, P77 and P94 each was represented by 2 isolates. The rest of the profiles were represented by one isolate each. The 6.8 kb plasmid was predominantly present in 142 (86.6%) plasmid harbouring isolates. Other common plasmids were 44.8 kb (n=85), 47.6 kb (n=48), 21.6 kb (n=48), 28.5 kb (n=34) and 2.4 kb (n=32).

Some isolates isolated from the same period displayed the same plasmid profiles. For example, 18 of 47 isolates isolated in 2008 were grouped into profile P49. Fourteen and six of 37 isolates isolated in 2009 possessed same plasmid profile of P52 and P53, respectively. However, it was also noted that isolates isolated from the environment had indistinguishable plasmid profile P52 with the 2009 human isolates. Isolates isolated in 2006 and 2007 exhibited very diverse plasmid profiles. Out of 54 plasmid harbouring isolates in 2006, 46 different profiles were observed. While 18 different plasmid profiles were detected from 19 plasmid harbouring isolates in 2007.

The carbapenem-susceptible isolates (AC/0603-25, AC/0604-7 and AC/0604-11) had similar plasmid profile, P11 with the isolate AC/0605-25. All the non-MDR resistant isolates did not harbour any plasmids.

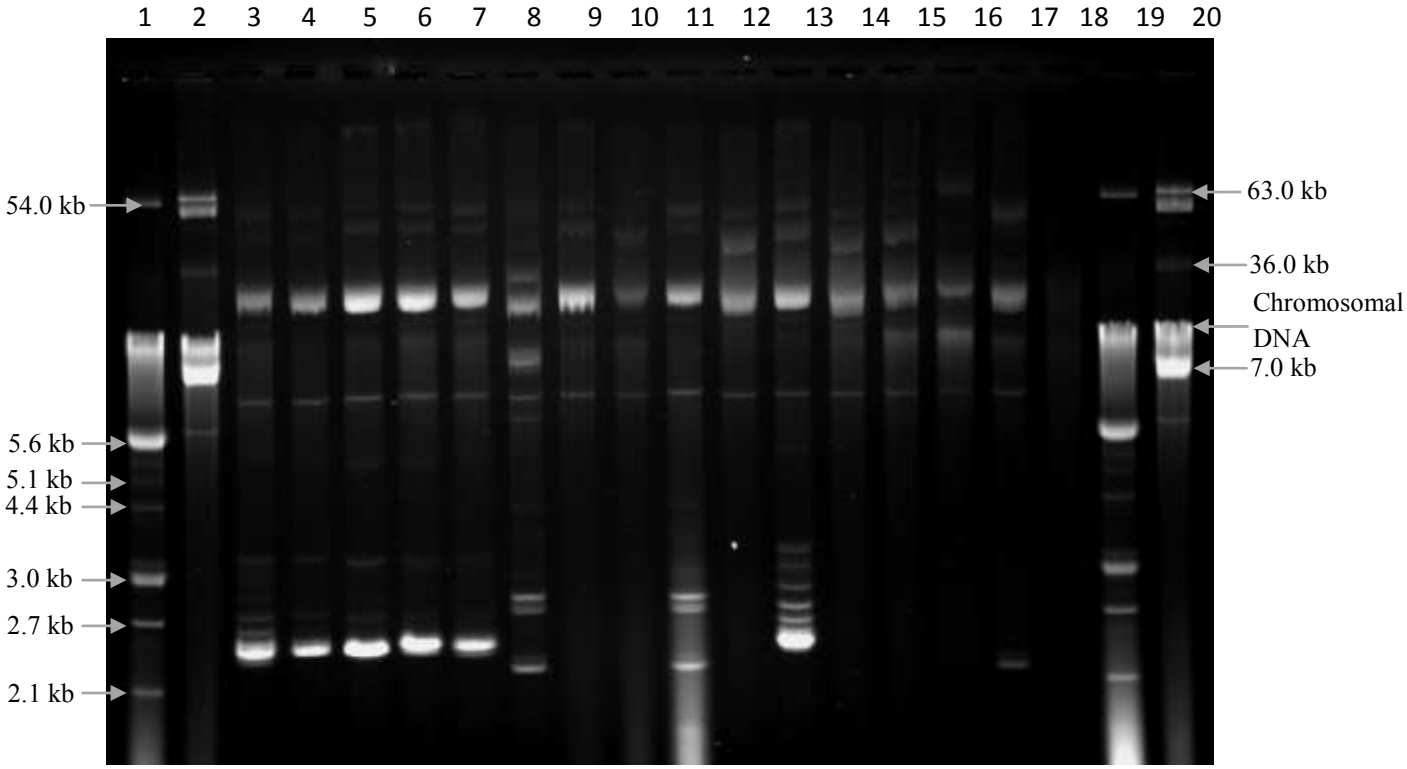


Figure 4.14: Representative plasmid DNA gel of *A. baumannii* isolates. Lane 1 and 19: *E. coli* V517; lane 2 and 20: *E. coli* 39R. lane 3: AC/0606-24; lane 4: AC/0607-6; lane 5: AC/0607-2; lane 6: AC/0607-20; lane 7: AC/0607-22; lane 8: AC/0607-28; lane 9: AC/0607-18; lane 10: AC/0611-18; lane 11: AC/0612-7; lane 12: AC/0608-7; lane 13: AC/0608-17; lane 14: AC/0608-22; lane 15: AC/0611-7; lane 16: AC/0703-21; lane 17: AC/0707-8 and lane 18: AC/0709-6.

Table 4.5: Plasmid profiles of the 164 plasmid harbouring of carbapenem-resistant *A.baumannii* isolates

Plasmid Profile	Size(s) of plasmid(s) (kb)	No. of plasmid	Year (no. of isolate)					Total
			Clinical				Env+hands of HCW	
			2006 (n=58)	2007 (n=25)	2008 (n=47)	2009 (n=37)	2006 (n=8)	
P01	32.4, 8.0	2		2	1	1	1	5
P02	44.8, 30.4, 8.0, 5.0, 4.3	5			1			1
P03	40.8, 8.0	2			1	1		2
P04	47.6, 28.5, 8.0, 4.6, 2.7, 2.4, 2.3	7	2					2
P05	28.5, 8.0, 4.8, 2.7, 2.4	5	1					1
P06	47.6, 28.5, 8.0, 4.8, 4.1, 3.1, 2.7, 2.5, 2.4, 2.3	10	1					1
P07	47.6, 28.5, 8.0, 4.8, 4.1, 3.1, 2.7, 2.5, 2.4	9	1					1
P08	47.6, 28.5, 8.0, 6.8, 5.9, 5.4, 4.8, 4.1, 3.8, 3.1, 2.7, 2.5, 2.4	13	1					1
P09	47.6, 36.9, 28.5, 8.0, 6.8, 2.3, 2.2	7	1					1
P10	47.6, 28.5, 8.0, 2.3, 2.2	5		1				1
P11	47.6, 28.5, 8.0	3	1					1
P12	49.8, 32.4, 5.0, 4.1, 3.3, 2.4, 2.3	7			1			1
P13	47.6, 9.3	2				1		1
P14	47.6, 40.8, 28.5, 16.1, 6.8, 3.8, 2.8, 2.6	8	1					1
P15	28.5, 6.8, 4.1, 3.8, 2.9, 2.8, 2.7	7		1				1
P16	47.6, 6.8, 4.3, 2.9, 2.8, 2.7	6	1					1
P17	47.6, 6.8, 2.8	3	1					1
P18	65.0, 47.6, 28.5, 25.6, 6.8, 5.4, 4.3, 3.8, 3.3, 2.8, 2.7, 2.2	12	1					1
P19	32.4, 21.6, 11.0, 9.3, 8.0, 6.8, 4.3, 3.1, 2.8, 2.7, 2.4, 2.3	12	1					1
P20	49.8, 44.8, 28.5, 6.8, 4.3, 3.1, 2.8, 2.7, 2.4, 2.3	10	1					1
P21	47.6, 28.5, 6.8, 3.1, 2.8, 2.5, 2.4, 2.3	8	1					1
P22	47.6, 28.5, 16.1, 6.8, 3.1, 2.9, 2.8, 2.7, 2.5, 2.4, 2.3	11	1					1
P23	47.6, 32.4, 16.1, 6.8, 3.1, 2.8, 2.7, 2.6, 2.5, 2.4, 2.3, 2.2	12	1					1

Plasmid Profile	Size(s) of plasmid(s) (kb)	No. of plasmid	Year (no. of isolate)					Total
			Clinical				Env+hands of HCW	
			2006 (n=58)	2007 (n=25)	2008 (n=47)	2009 (n=37)	2006 (n=8)	
P24	65.0, 47.6, 44.8, 36.9, 28.5, 9.3, 3.8, 3.1, 2.8, 2.7, 2.6, 2.4, 2.3	13			1			1
P25	44.8, 6.8, 3.3, 2.8, 2.4, 2.3	6	1					1
P26	49.8, 8.0, 6.8, 3.3, 2.8, 2.6, 2.5, 2.4, 2.3	9				1		1
P27	47.6, 6.8, 3.3, 2.8, 2.6, 2.5, 2.4	7	1					1
P28	47.6, 6.8, 5.4, 3.3, 2.8, 2.5, 2.4	7	1					1
P29	47.6, 44.8, 16.1, 6.8, 6.3, 5.4, 3.3, 2.8, 2.5, 2.4	10	1					1
P30	40.8, 8.0, 4.3, 3.3, 2.8, 2.7, 2.6	7	1					1
P31	8.0, 4.3, 3.3, 2.8, 2.7, 2.6, 2.3, 2.2	8		1				1
P32	32.4, 21.6, 8.0, 6.8, 4.3, 3.3, 2.8, 2.7, 2.6	9	1					1
P33	32.4, 21.6, 8.0, 6.8, 4.3, 3.3, 2.8, 2.7, 2.6, 2.2, 2.0	11		1				1
P34	49.8, 43.9, 6.8, 4.3, 2.8, 2.7, 2.6, 2.2, 2.1	9	1					1
P35	40.8, 8.0, 6.8, 2.7, 2.6, 2.0	6		1				1
P36	49.8, 44.8, 6.8, 5.0, 3.5, 2.9, 2.8, 2.7, 2.6, 2.5, 2.4, 2.3	12	1					1
P37	49.8, 44.8, 16.1, 6.8, 5.9, 5.0, 4.3, 3.5, 3.3, 2.9, 2.7, 2.6, 2.4, 2.3, 2.0	15			1			1
P38	49.8, 32.4, 28.5, 16.1, 11.0, 6.8, 5.9, 3.5, 2.9, 2.8, 2.7, 2.6, 2.5, 2.4,	14				1		1
P39	49.8, 44.8, 6.8, 4.3, 3.5, 3.3, 3.1, 2.9, 2.7, 2.6	10			1			1
P40	44.8, 6.8, 4.3, 3.8, 3.5, 3.3, 3.1, 2.9, 2.7, 2.6	10					1	1
P41	49.8, 44.8, 16.1, 6.8, 4.3, 3.8, 3.3, 3,1, 2.9, 2.7, 2.6	11	1					1
P42	49.8, 43.9, 28.5, 6.8, 5.7, 3.3, 3.1, 2.9, 2.7, 2.6, 2.5	11	1					1
P43	47.6, 36.9, 32.4, 16.1, 9.3, 8.0, 6.8, 6.0, 2.6, 2.3, 2.2	11	1					1
P44	47.6, 36.9, 32.4, 9.3, 8.0, 6.8, 6.0, 2.8, 2.6, 2.3, 2.2	11	1					1
P45	49.8, 47.6, 44.8, 16.1, 12.2, 9.3, 8.0, 6.8, 6.3, 6.0, 5.9	11	1					1
P46	65.0, 28.5, 4.6, 4.1, 3.3, 3.1, 2.8	7		1				1

Plasmid Profile	Size(s) of plasmid(s) (kb)	No. of plasmid	Year (no. of isolate)					Total
			Clinical				Env+hands of HCW	
			2006 (n=58)	2007 (n=25)	2008 (n=47)	2009 (n=37)	2006 (n=8)	
P47	47.6, 21.6, 11.0, 6.8, 4.6, 3.8, 3.3, 3.1, 2.9, 2.8	10			1			1
P48	44.8, 32.4, 21.6, 6.8	4			2			2
P49	44.8, 21.6, 6.8	3			18			18
P50	49.8, 44.8, 21.6, 6.8	4			1			1
P51	49.8, 44.8, 21.6, 11.0, 6.8	5			1			1
P52	44.8, 6.8	2	1			14	5	20
P53	44.8, 16.1, 6.8	3	3			6	1	10
P54	42.7, 16.1 6.8	3	4			1		5
P55	44.8, 42.7 16.1, 6.8	4	2	1				3
P56	49.8, 44.8, 25.6, 6.8	4	1					1
P57	44.8, 42.7, 21.6, 6.8	4			1	1		2
P58	44.8, 40.8, 21.6, 16.1, 6.8	5			1			1
P59	44.8, 42.7, 6.8	3	1					1
P60	42.7, 21.6, 6.8	3			2			2
P61	49.8, 42.7, 21.6, 6.8	4	2					2
P62	44.8, 40.8, 21.6, 8.0, 6.8	5			1			1
P63	44.8, 32.4, 21.6, 6.8, 5..0, 4.1	6			2			2
P64	42.7, 28.5, 6.8	3				2		2
P65	43.9, 40.8, 28.5, 6.8	4	1					1
P66	49.8, 43.9, 28.5, 6.8	4	1					1
P67	40.8, 28.5, 6.8	3	1			1		2
P68	47.6, 40.8, 28.5, 6.8	4		1				1
P69	28.5, 6.8	2	1					1

Plasmid Profile	Size(s) of plasmid(s) (kb)	No. of plasmid	Year (no. of isolate)					Total
			Clinical				Env+hands of HCW	
			2006 (n=58)	2007 (n=25)	2008 (n=47)	2009 (n=37)	2006 (n=8)	
P70	49.8, 47.6, 28.5, 6.8	4	1					1
P71	49.8, 28.5, 6.8	3		1				1
P72	47.6, 28.5, 6.8	3		1				1
P73	47.6, 28.5, 6.8, 2.2	4			1			1
P74	47.6, 44.8, 6.8	3	1					1
P75	47.6, 43.9, 28.5, 6.8	4		1				1
P76	65.0, 47.6, 44.8, 28.5, 11.0, 6.8, 2.2	7			1			1
P77	47.6, 44.8, 32.4, 6.8	4			1	1		2
P78	47.6, 44.8, 32.4, 25.6, 6.8	5				1		1
P79	47.6, 32.4, 25.6, 6.8	4				1		1
P80	47.6, 32.4, 6.8	3	1					1
P81	47.6, 32.4, 6.8, 2.2	4				1		1
P82	47.6, 42.7, 32.4, 6.8	4				1		1
P83	32.4, 6.8	2			1			1
P84	65.0, 47.6, 32.4, 25.6, 6.8, 3.1, 2.7,2.3	8			1			1
P85	47.6, 43.9, 28.5, 21.6, 6.8, 2.7, 2.5, 2.4	8	1					1
P86	47.6, 21.6, 6.8, 3.5, 2.5, 2.4, 2.3, 2.1	8			1			1
P87	47.6, 21.6, 6.8, 2.4	4		1				1
P88	47.6, 21.6, 6.8, 2.3, 2.2	5		1				1
P89	47.6, 21.6, 6.8	3		1				1
P90	65.0, 47.6, 44.8, 21.6, 6.8, 2.4	6		1				1
P91	125.1, 49.8, 21.6, 11.0, 6.8	5			1			1
P92	43.9, 28.5, 6.8, 3.1, 2.6, 2.5, 2.4, 2.3, 2.2, 1.9, 1.8, 1.7	12		1				1

Plasmid Profile	Size(s) of plasmid(s) (kb)	No. of plasmid	Year (no. of isolate)					Total
			Clinical				Env+hands of HCW	
			2006 (n=58)	2007 (n=25)	2008 (n=47)	2009 (n=37)	2006 (n=8)	
P93	42.7, 16.1, 6.8, 3.1, 2.8, 2.6, 2.4, 2.3, 2.2, 2.0, 1.8, 1.7	12			1			1
P94	44.8, 40.8, 21.6, 6.8, 2.4, 2.3, 2.0, 1.8, 1.7	9				2		2
P95	44.8, 21.6, 6.8, 2.4, 2.0, 1.8, 1.7, 1.6	8			1			1
P96	44.8, 21.6, 6.8, 5.0, 3.1, 2.8, 2.6, 2.5, 2.4, 2.3	10	1					1
P97	44.8, 21.6, 6.8, 2.6, 2.5, 2.4, 2.1, 1.9, 1.8	9			1			1
P98	2.2	1	1					1
Total			54	18	47	37	8	164

4.7 Genotyping of *A. baumannii* isolates by REP-PCR

Two different REP primers (REP1R and REP2) were tested to identify the primers that are useful for discrimination of *A. baumannii* isolates. The combination of primers REP1R and REP2 was tested useful and produced polymorphic REP profiles among the 185 isolates studied (Figure 4.15).

REP-PCR analysis of the 185 *A. baumannii* using REPIR and REP2 primers gave 62 REP types (F = 0.72 – 1.0). Each isolate contained 11 to 25 bands ranging in size from 150 bp to 3470 bp (Figure 4.16). Bands below 150 bp and above 3500 bp were not included in the analysis. Among the 62 REP types, REP033 was predominant (n=24), followed by REP006 (n=21), REP010 (n=10), REP011 (n=09), REP025 (n=7), REP012 (n=6), REP028 (n=6), REP043 (n=6), REP030 (n=5), REP001 (n=4), REP004 (n=4), REP008 (n=4), REP020 (n=4), REP021 (n=4), REP027 (n=4), REP031 (n=4), REP032 (n=4), REP026 (n=3), REP003 (n=2), REP005 (n=2), REP007 (n=2), REP018 (n=2), REP019 (n=2), REP022 (n=2), REP023 (n=2), REP034 (n=2), REP046 (n=2), REP047 (n=2), REP048 (n=2) and REP061 (n=2). The remaining of the 32 REP types was present in unique isolate for each REP type (Figure 4.17).

Based on 90.0% of similarity, the dendrogram of the 185 *A. baumannii* gave 6 clusters (A-F). Cluster B was predominant, consisted of 64 isolates subtyped into 13 REP types, followed by cluster C (n=47, 14 REP types), cluster D (n=30, 3 REP types), cluster E (n=13, 8 REP types), F (n=10, 7 REP types) and cluster A (n=5, 2 REP types). Sixteen of the *A. baumannii* isolates were not placed in any clusters (Figure 4.16, Table 4.6).

A majority of the isolates in cluster B (70%) were integron-bearing isolates. The remaining isolates were integron-negative isolates which were subtyped into 6 REP types with REP010 and REP012 being predominant. The 17 class 1 and class 2 integron-positive isolates were distributed in cluster A and cluster B. Four isolates

clustered in cluster A shared a similar REP type, REP001 and 12 isolates clustered in cluster B subtyped into 4 different REP types (REP003, n=2; REP004, n=4; REP007, n=2 and REP008, n=4), each REP type was different in 2 to 5 bands. Three MDR environmental isolates and a isolate from the hands of a HCW (ACIBA 2006-47) were clustered together with the 2006-2009 clinical isolates in the cluster B.

In cluster C, out of 47 isolates, 40 isolates had a similar integron profile IN1-b. Thirty-nine of these integron IN1-b-positive isolates were isolated from patients in 2006-2009 and one isolate was isolated from ICU environment in 2006. Three environmental isolates harbouring class 1 integron IN1-a profile were also grouped in this cluster. Sixteen out of 18 isolates harboured plasmid profile P49 were clustered in cluster C with all harboured integron profile IN1-b, 50% had resistance phenotype R01 and 50% had resistance phenotype R19.

Cluster D consists of 30 isolates with 94.1% of similarity. Most of the isolates were isolated in 2009 (n=28, 76%). These isolates were subtyped into 2 different REP types, REP032 (n=4) and REP033 (n=24) at 97.4% of similarity. There was only a single band difference between the isolates. These isolates do not harbour integrons. Twenty-one isolates carried resistance phenotype R01 and 6 isolates had resistance phenotype R19. However, there were 2 carbapenem-susceptible isolates clustered with these 2009 isolates.

Isolates in cluster E and cluster F were more diverse compared to the other clusters. In cluster E, out of 13 isolates, 8 different REP types were defined. While in cluster F, 10 isolates were subtyped into 7 different REP types. Most of isolates in cluster F (80%) were non-plasmid harboured carbapenem-resistant isolates.

All the 175 carbapenem-resistant *A. baumannii* isolates had closely genetic diversity as evidenced by at least 73% of the cut-off value of similarity. All the non-MDR hands of HCW isolates do not cluster with the MDR isolates and had unique REP

types. Only two isolates, ACIBA 2006-53 and ACIBA 2006-56 shared a similar REP type, REP061. REP-PCR had discriminatory index of D=0.96, indicated that it was useful for discriminating of *A. baumannii* isolates.

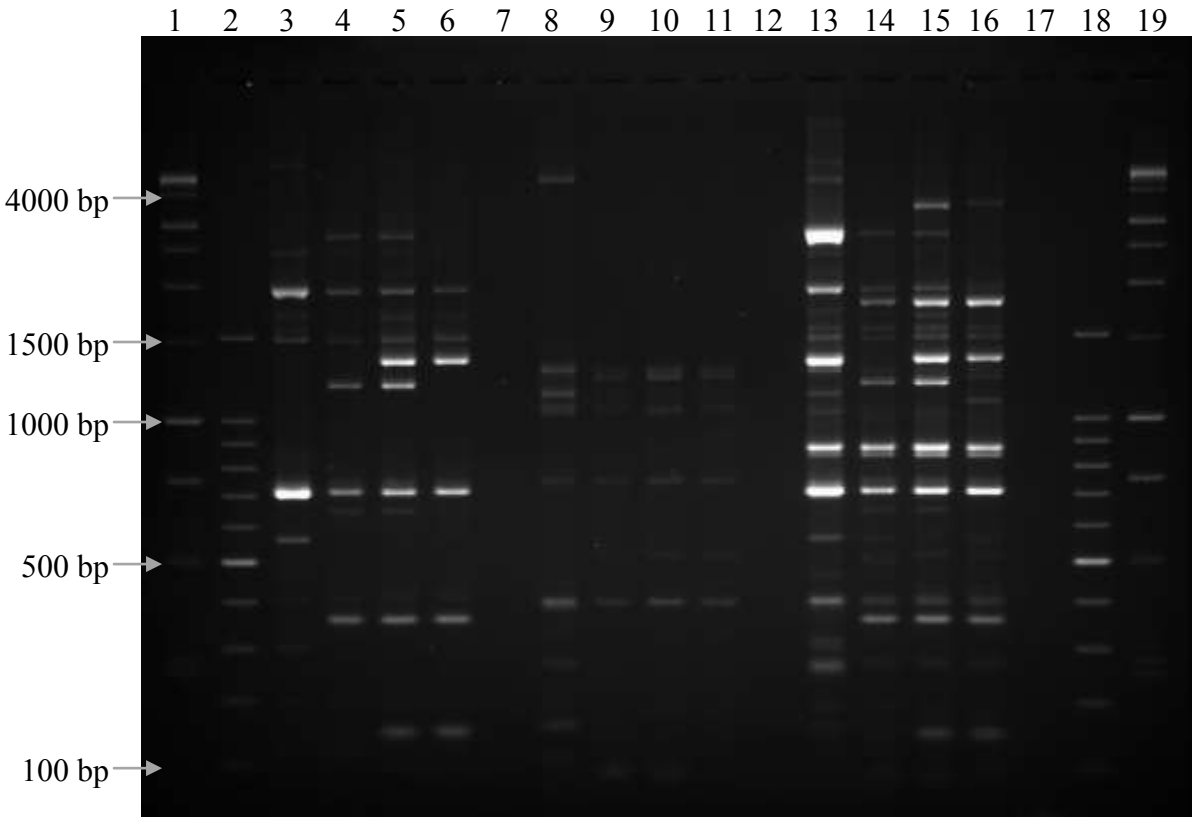


Figure 4.15: Optimisation of REP-PCR using different primers of REP1R, REP 2 and REP1R+REP2

Lane	Isolates	REP primer	Lane	Isolates	REP primer
1	1 kb marker (Promega)	/	11	AC/0603-2	REP2
2	100 bp marker (Promega)	/	12	Negative control	/
3	AC/0601-5	REP1R	13	AC/0601-5	REP1R+ REP2
4	AC/0601-8	REP1R	14	AC/0601-8	REP1R+ REP2
5	AC/0602-19	REP1R	15	AC/0602-19	REP1R+ REP2
6	AC/0603-2	REP1R	16	AC/0603-2	REP1R+ REP2
7	Negative control	/	17	Negative control	/
8	AC/0601-5	REP2	18	100 bp marker (Promega)	/
9	AC/0601-8	REP2	19	1 kb marker (Promega)	/
10	AC/0602-19	REP2			

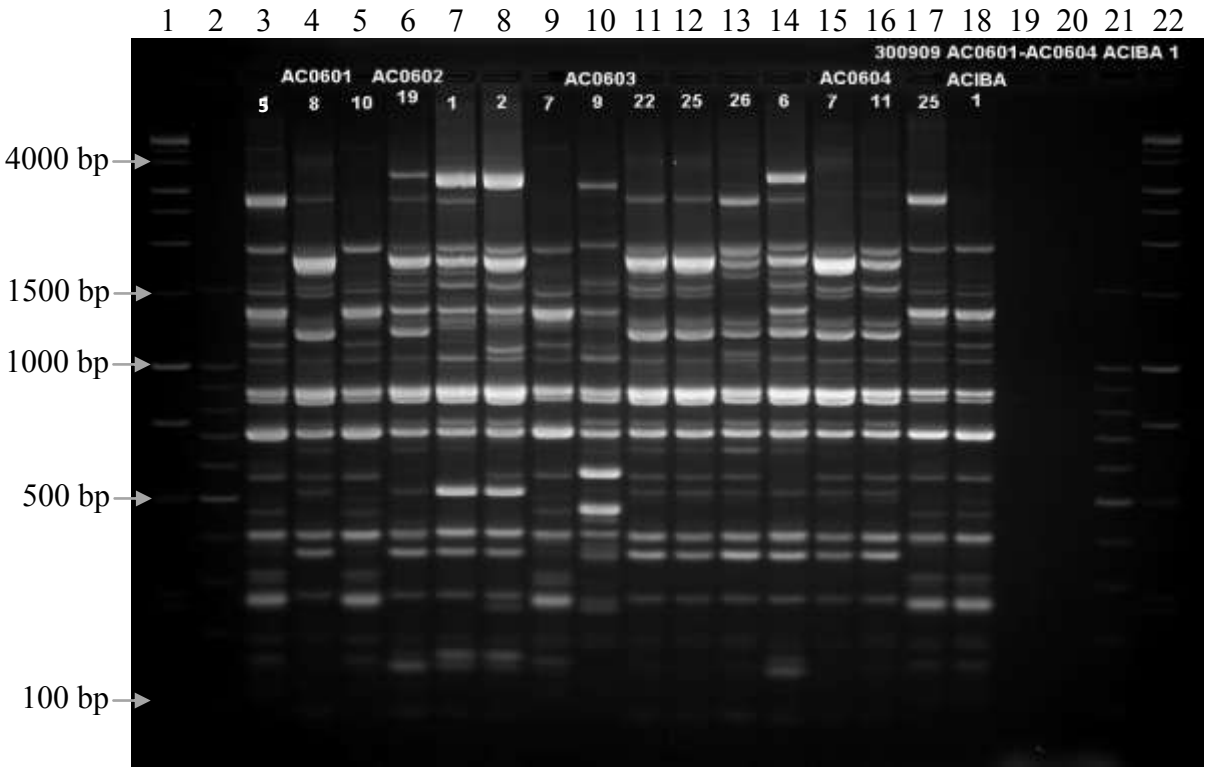
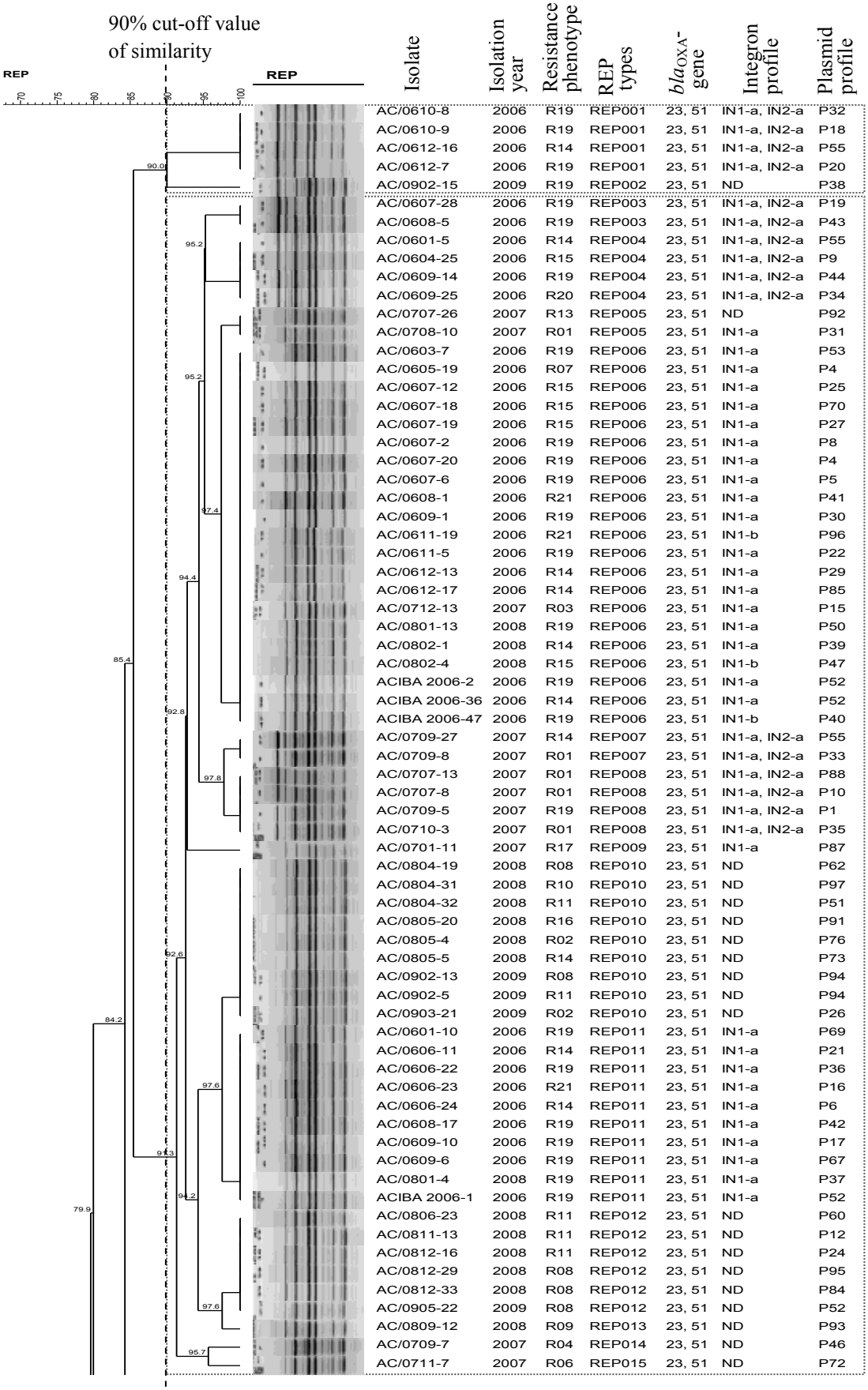
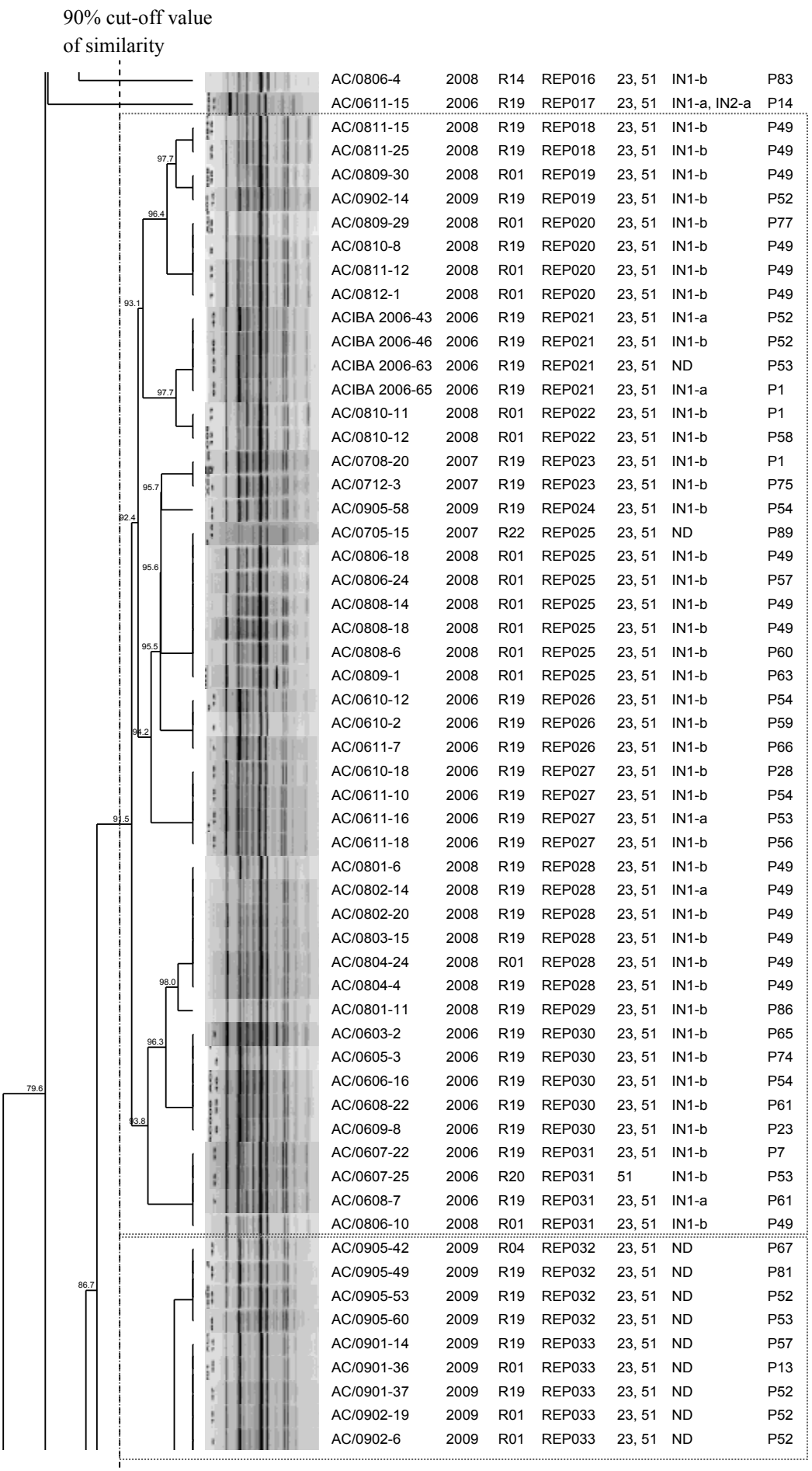


Figure 4.16: REP-PCR types of *A. baumannii* strains using REP1R and REP2 primers

Lane	Isolates	REP type	Lane	Isolates	REP type
1	1 kb marker (Promega)	/	12	AC/0603-25	REP043
2	100 bp marker (Promega)	/	13	AC/0603-26	REP041
3	AC/0601-5	REP004	14	AC/0604-6	REP048
4	AC/0601-8	REP043	15	AC/0604-7	REP034
5	AC/0601-10	REP011	16	AC/0604-11	REP034
6	AC/0602-19	REP048	17	AC/0604-25	REP004
7	AC/0603-1	REP049	18	ACIBA 2006-1	REP011
8	AC/0603-2	REP030	19	Negative control	/
9	AC/0603-7	REP006	20	/	/
10	AC/0603-9	REP053	21	100 bp marker (Promega)	/
11	AC/0603-22	REP043	22	1 kb marker (Promega)	/



Isolate	Isolation year	Resistance phenotype	REP types	bla_{OXA}-gene	Integron profile	Plasmid profile	
AC/0610-8	2006	R19	REP001	23, 51	IN1-a, IN2-a	P32	
AC/0610-9	2006	R19	REP001	23, 51	IN1-a, IN2-a	P18	
AC/0612-16	2006	R14	REP001	23, 51	IN1-a, IN2-a	P55	**Cluster A**
AC/0612-7	2006	R19	REP001	23, 51	IN1-a, IN2-a	P20	
AC/0902-15	2009	R19	REP002	23, 51	ND	P38	
AC/0607-28	2006	R19	REP003	23, 51	IN1-a, IN2-a	P19	
AC/0608-5	2006	R19	REP003	23, 51	IN1-a, IN2-a	P43	
AC/0601-5	2006	R14	REP004	23, 51	IN1-a, IN2-a	P55	
AC/0604-25	2006	R15	REP004	23, 51	IN1-a, IN2-a	P9	
AC/0609-14	2006	R19	REP004	23, 51	IN1-a, IN2-a	P44	
AC/0609-25	2006	R20	REP004	23, 51	IN1-a, IN2-a	P34	
AC/0707-26	2007	R13	REP005	23, 51	ND	P92	
AC/0708-10	2007	R01	REP005	23, 51	IN1-a	P31	
AC/0603-7	2006	R19	REP006	23, 51	IN1-a	P53	
AC/0605-19	2006	R07	REP006	23, 51	IN1-a	P4	
AC/0607-12	2006	R15	REP006	23, 51	IN1-a	P25	
AC/0607-18	2006	R15	REP006	23, 51	IN1-a	P70	
AC/0607-19	2006	R15	REP006	23, 51	IN1-a	P27	
AC/0607-2	2006	R19	REP006	23, 51	IN1-a	P8	
AC/0607-20	2006	R19	REP006	23, 51	IN1-a	P4	
AC/0607-6	2006	R19	REP006	23, 51	IN1-a	P5	
AC/0608-1	2006	R21	REP006	23, 51	IN1-a	P41	
AC/0609-1	2006	R19	REP006	23, 51	IN1-a	P30	
AC/0611-19	2006	R21	REP006	23, 51	IN1-b	P96	
AC/0611-5	2006	R19	REP006	23, 51	IN1-a	P22	
AC/0612-13	2006	R14	REP006	23, 51	IN1-a	P29	
AC/0612-17	2006	R14	REP006	23, 51	IN1-a	P85	
AC/0712-13	2007	R03	REP006	23, 51	IN1-a	P15	
AC/0801-13	2008	R19	REP006	23, 51	IN1-a	P50	
AC/0802-1	2008	R14	REP006	23, 51	IN1-a	P39	
AC/0802-4	2008	R15	REP006	23, 51	IN1-b	P47	
ACIBA 2006-2	2006	R19	REP006	23, 51	IN1-a	P52	
ACIBA 2006-36	2006	R14	REP006	23, 51	IN1-a	P52	
ACIBA 2006-47	2006	R19	REP006	23, 51	IN1-b	P40	
AC/0709-27	2007	R14	REP007	23, 51	IN1-a, IN2-a	P55	
AC/0709-8	2007	R01	REP007	23, 51	IN1-a, IN2-a	P33	
AC/0707-13	2007	R01	REP008	23, 51	IN1-a, IN2-a	P88	
AC/0707-8	2007	R01	REP008	23, 51	IN1-a, IN2-a	P10	**Cluster B**
AC/0709-5	2007	R19	REP008	23, 51	IN1-a, IN2-a	P1	
AC/0710-3	2007	R01	REP008	23, 51	IN1-a, IN2-a	P35	
AC/0701-11	2007	R17	REP009	23, 51	IN1-a	P87	
AC/0804-19	2008	R08	REP010	23, 51	ND	P62	
AC/0804-31	2008	R10	REP010	23, 51	ND	P97	
AC/0804-32	2008	R11	REP010	23, 51	ND	P51	
AC/0805-20	2008	R16	REP010	23, 51	ND	P91	
AC/0805-4	2008	R02	REP010	23, 51	ND	P76	
AC/0805-5	2008	R14	REP010	23, 51	ND	P73	
AC/0902-13	2009	R08	REP010	23, 51	ND	P94	
AC/0902-5	2009	R11	REP010	23, 51	ND	P94	
AC/0903-21	2009	R02	REP010	23, 51	ND	P26	
AC/0601-10	2006	R19	REP011	23, 51	IN1-a	P69	
AC/0606-11	2006	R14	REP011	23, 51	IN1-a	P21	
AC/0606-22	2006	R19	REP011	23, 51	IN1-a	P36	
AC/0606-23	2006	R21	REP011	23, 51	IN1-a	P16	
AC/0606-24	2006	R14	REP011	23, 51	IN1-a	P6	
AC/0608-17	2006	R19	REP011	23, 51	IN1-a	P42	
AC/0609-10	2006	R19	REP011	23, 51	IN1-a	P17	
AC/0609-6	2006	R19	REP011	23, 51	IN1-a	P67	
AC/0801-4	2008	R19	REP011	23, 51	IN1-a	P37	
ACIBA 2006-1	2006	R19	REP011	23, 51	IN1-a	P52	
AC/0806-23	2008	R11	REP012	23, 51	ND	P60	
AC/0811-13	2008	R11	REP012	23, 51	ND	P12	
AC/0812-16	2008	R11	REP012	23, 51	ND	P24	
AC/0812-29	2008	R08	REP012	23, 51	ND	P95	
AC/0812-33	2008	R08	REP012	23, 51	ND	P84	
AC/0905-22	2009	R08	REP012	23, 51	ND	P52	
AC/0809-12	2008	R09	REP013	23, 51	ND	P93	
AC/0709-7	2007	R04	REP014	23, 51	ND	P46	
AC/0711-7	2007	R06	REP015	23, 51	ND	P72	



AC/0806-4	2008	R14	REP016	23, 51	IN1-b	P83	
AC/0611-15	2006	R19	REP017	23, 51	IN1-a, IN2-a	P14	
AC/0811-15	2008	R19	REP018	23, 51	IN1-b	P49	
AC/0811-25	2008	R19	REP018	23, 51	IN1-b	P49	
AC/0809-30	2008	R01	REP019	23, 51	IN1-b	P49	
AC/0902-14	2009	R19	REP019	23, 51	IN1-b	P52	
AC/0809-29	2008	R01	REP020	23, 51	IN1-b	P77	
AC/0810-8	2008	R19	REP020	23, 51	IN1-b	P49	
AC/0811-12	2008	R01	REP020	23, 51	IN1-b	P49	
AC/0812-1	2008	R01	REP020	23, 51	IN1-b	P49	
ACIBA 2006-43	2006	R19	REP021	23, 51	IN1-a	P52	
ACIBA 2006-46	2006	R19	REP021	23, 51	IN1-b	P52	
ACIBA 2006-63	2006	R19	REP021	23, 51	ND	P53	
ACIBA 2006-65	2006	R19	REP021	23, 51	IN1-a	P1	
AC/0810-11	2008	R01	REP022	23, 51	IN1-b	P1	
AC/0810-12	2008	R01	REP022	23, 51	IN1-b	P58	
AC/0708-20	2007	R19	REP023	23, 51	IN1-b	P1	
AC/0712-3	2007	R19	REP023	23, 51	IN1-b	P75	
AC/0905-58	2009	R19	REP024	23, 51	IN1-b	P54	
AC/0705-15	2007	R22	REP025	23, 51	ND	P89	
AC/0806-18	2008	R01	REP025	23, 51	IN1-b	P49	
AC/0806-24	2008	R01	REP025	23, 51	IN1-b	P57	
AC/0808-14	2008	R01	REP025	23, 51	IN1-b	P49	
AC/0808-18	2008	R01	REP025	23, 51	IN1-b	P49	
AC/0808-6	2008	R01	REP025	23, 51	IN1-b	P60	
AC/0809-1	2008	R01	REP025	23, 51	IN1-b	P63	
AC/0610-12	2006	R19	REP026	23, 51	IN1-b	P54	Cluster C
AC/0610-2	2006	R19	REP026	23, 51	IN1-b	P59	
AC/0611-7	2006	R19	REP026	23, 51	IN1-b	P66	
AC/0610-18	2006	R19	REP027	23, 51	IN1-b	P28	
AC/0611-10	2006	R19	REP027	23, 51	IN1-b	P54	
AC/0611-16	2006	R19	REP027	23, 51	IN1-a	P53	
AC/0611-18	2006	R19	REP027	23, 51	IN1-b	P56	
AC/0801-6	2008	R19	REP028	23, 51	IN1-b	P49	
AC/0802-14	2008	R19	REP028	23, 51	IN1-a	P49	
AC/0802-20	2008	R19	REP028	23, 51	IN1-b	P49	
AC/0803-15	2008	R19	REP028	23, 51	IN1-b	P49	
AC/0804-24	2008	R01	REP028	23, 51	IN1-b	P49	
AC/0804-4	2008	R19	REP028	23, 51	IN1-b	P49	
AC/0801-11	2008	R19	REP029	23, 51	IN1-b	P86	
AC/0603-2	2006	R19	REP030	23, 51	IN1-b	P65	
AC/0605-3	2006	R19	REP030	23, 51	IN1-b	P74	
AC/0606-16	2006	R19	REP030	23, 51	IN1-b	P54	
AC/0608-22	2006	R19	REP030	23, 51	IN1-b	P61	
AC/0609-8	2006	R19	REP030	23, 51	IN1-b	P23	
AC/0607-22	2006	R19	REP031	23, 51	IN1-b	P7	
AC/0607-25	2006	R20	REP031	51	IN1-b	P53	
AC/0608-7	2006	R19	REP031	23, 51	IN1-a	P61	
AC/0806-10	2008	R01	REP031	23, 51	IN1-b	P49	
AC/0905-42	2009	R04	REP032	23, 51	ND	P67	
AC/0905-49	2009	R19	REP032	23, 51	ND	P81	
AC/0905-53	2009	R19	REP032	23, 51	ND	P52	
AC/0905-60	2009	R19	REP032	23, 51	ND	P53	
AC/0901-14	2009	R19	REP033	23, 51	ND	P57	Cluster D
AC/0901-36	2009	R01	REP033	23, 51	ND	P13	
AC/0901-37	2009	R19	REP033	23, 51	ND	P52	
AC/0902-19	2009	R01	REP033	23, 51	ND	P52	
AC/0902-6	2009	R01	REP033	23, 51	ND	P52	

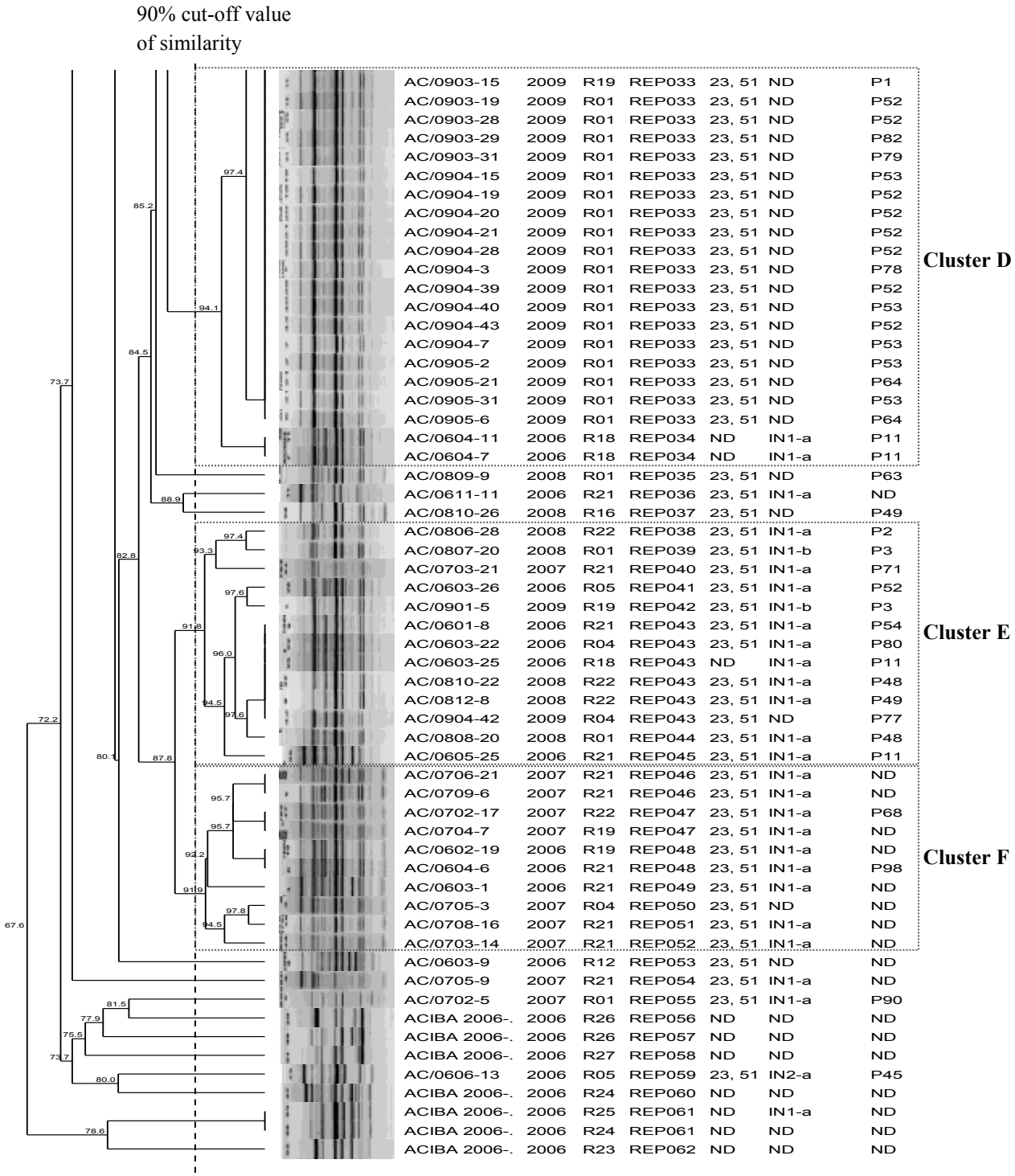


Figure 4.17: REP-PCR dendrogram cluster analysis of 185 *A. baumannii* generated using Bionumeric Version 6.0 (Applied Maths, Belgium) software and unweighted pair group arithmetic means methods (UPGMA).

Abbreviation: ND- not detected

Table 4.6: Distribution of *A. baumannii* isolates in the REP-PCR dendrogram cluster analysis based on 90% cut-off value of similarity

Cluster	Year (no. of isolate)					Total
	Clinical				Env + hands of HCW	
	2006	2007	2008	2009	2006	
A	4	0	0	1	0	5
B	28	12	16	4	4	64
C	15	3	23	2	4	47
D	2	0	0	28	0	30
E	5	1	5	2	0	13
F	3	7	0	0	0	10
Non-clustered	4	2	3	0	7	16
Total	61	25	47	37	15	185

4.8 Genotyping of *A. baumannii* isolates by PFGE

PFGE with *Apa*I subtyped all the 185 *A. baumannii* isolates into 98 pulsotypes comprising of 17 to 29 fragments ranging from approximately 25.9 kb to 680.1 kb (Figure 4.18). Pulsotype PFP068 represented the most number of isolates (n=14), followed by pulsotypes PFP089 (n=13), PFP001 (n=10), PFP009 (n=7), PFP072 (n=7), PFP085 (n=7), PFP004 (n=4), PFP008 (n=4), PFP011 (n=4), PFP026 (n=4), PFP088 (n=4), PFP032 (n=3), PFP067 (n=3), PFP071 (n=3), PFP072 (n=3), PFP022 (n=2), PFP023 (n=2), PFP025 (n=2), PFP035 (n=2), PFP043 (n=2), PFP045 (n=2), PFP046 (n=2), PFP060 (n=2), PFP063 (n=2), PFP075 (n=2), PFP078 (n=2) and PFP081 (n=2) (Figure 4.19).

Based on 70.0% of genetic similarity, 8 clusters, I-VIII (group of a clone with other clones or pulsotypes) with 17 clones (AC1-AC17) (isolates which shared ≥ 80% of similarity were defined as a similar clone) were observed. Clone AC15 was the most predominant, grouped 17 pulsotypes comprised of 45 isolates. This was followed by clone AC17 (7 pulsotypes, n=28), AC2 (10 pulsotypes, n=22) and AC1 (5 pulsotypes, n=17) (Table 4.7). Clone AC1 consisted of 2006 and 2007 isolates which harboured class 1 and class 2 integrons. Clone AC2 included a isolate from the hands of a HCW (ACIBA 2006-47), 3 MDR environmental isolates and 18 of the 2006 clinical isolates. The remaining 4 MDR environmental isolates were included in clone AC15 together with the clinical isolates isolated in 2006-2009. While 28 isolates isolated in 2009 were included in clone AC17 with all were lacked of integrons, 75% had similar resistance phenotype, R01 and 64% harboured plasmid profile of P52 or P53.

Isolates of clone AC2 were grouped in cluster I at 70% of similarity with a isolate with pulsotype of PFP018. All these isolates harboured integron profile IN1-a except the isolate isolated from the hands of a HCW. Cluster II consisted of clones AC3 and AC4 and 2 isolates with pulsotypes of PFP020 and PFP021, while cluster III consisted

of clone AC8 with a isolate isolated in 2009. Majority of the isolates in both clusters were isolated in 2008 (13/19, 68%) and do not harbour integrons except clone AC3 isolates.

Clusters IV, V. VI and VII were closely related with 60.9% of similarity, included 6 clones, AC9-AC14, contained of 24 isolates which mostly (22/24, 92%) harboured integron profile IN1-a. About 73% of non-plasmid harboured isolates were distributed in these clusters. Three carbapenem-susceptible isolates were grouped in cluster VI with 2 isolates was of clone AC13.

Cluster VIII grouped the predominant clone AC15 with the clone AC16 and a isolate isolated in 2007 at 74.7% of similarity. Thirty-six isolates of clone AC15 and isolates of the clone AC16 harboured integron profile IN1-b. Seventeen out of 18 (94%) isolates harboured plasmid profile P49 were of clone AC15 and clone AC16.

PFGE had differentiated the MDR from non-MDR isolates. Non-MDR isolates isolated from hands of HCW were of different clone and had unique pulsotypes of AC007, AC041, AC042 and AC095 to AC098. Wide genetic diversity was found among the 175 carbapenem-resistant *A. baumannii* isolates as evidenced by the *F*-values, which ranged from 0.40 to 1.00. PFGE had discriminatory index of D=0.98, indicated that PFGE was useful for discriminating of *A. baumannii* isolates.

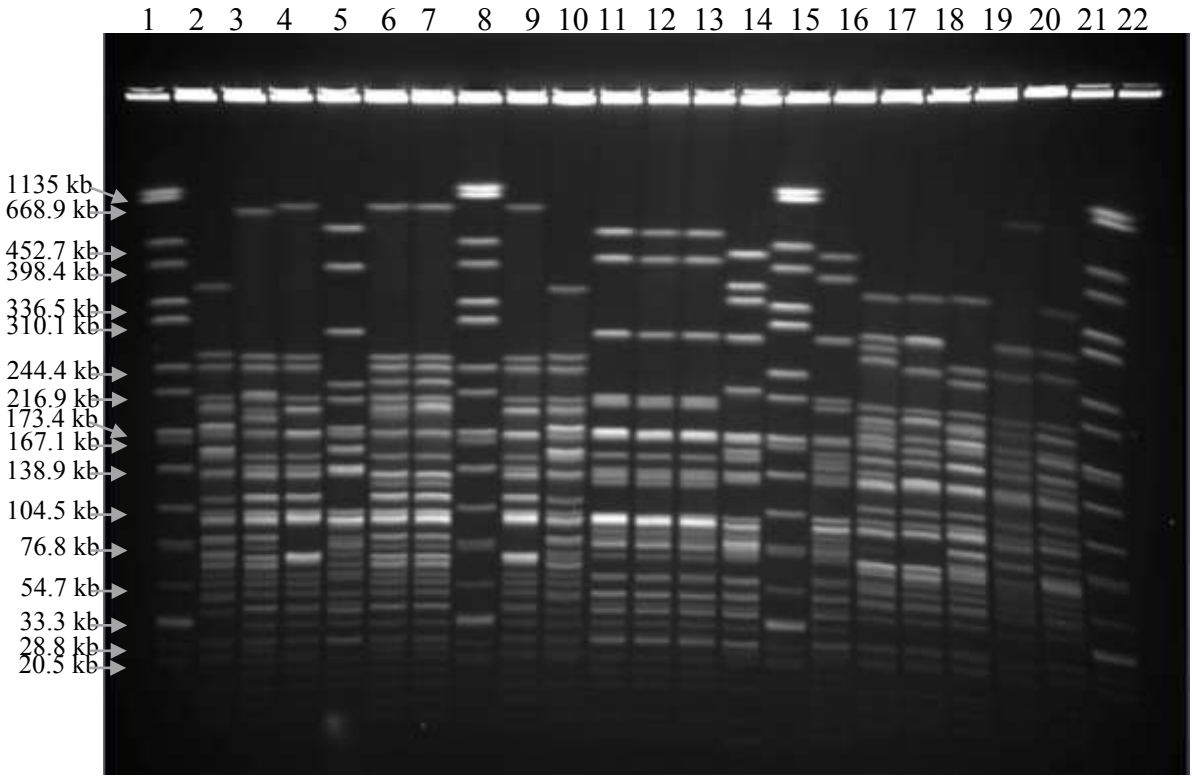
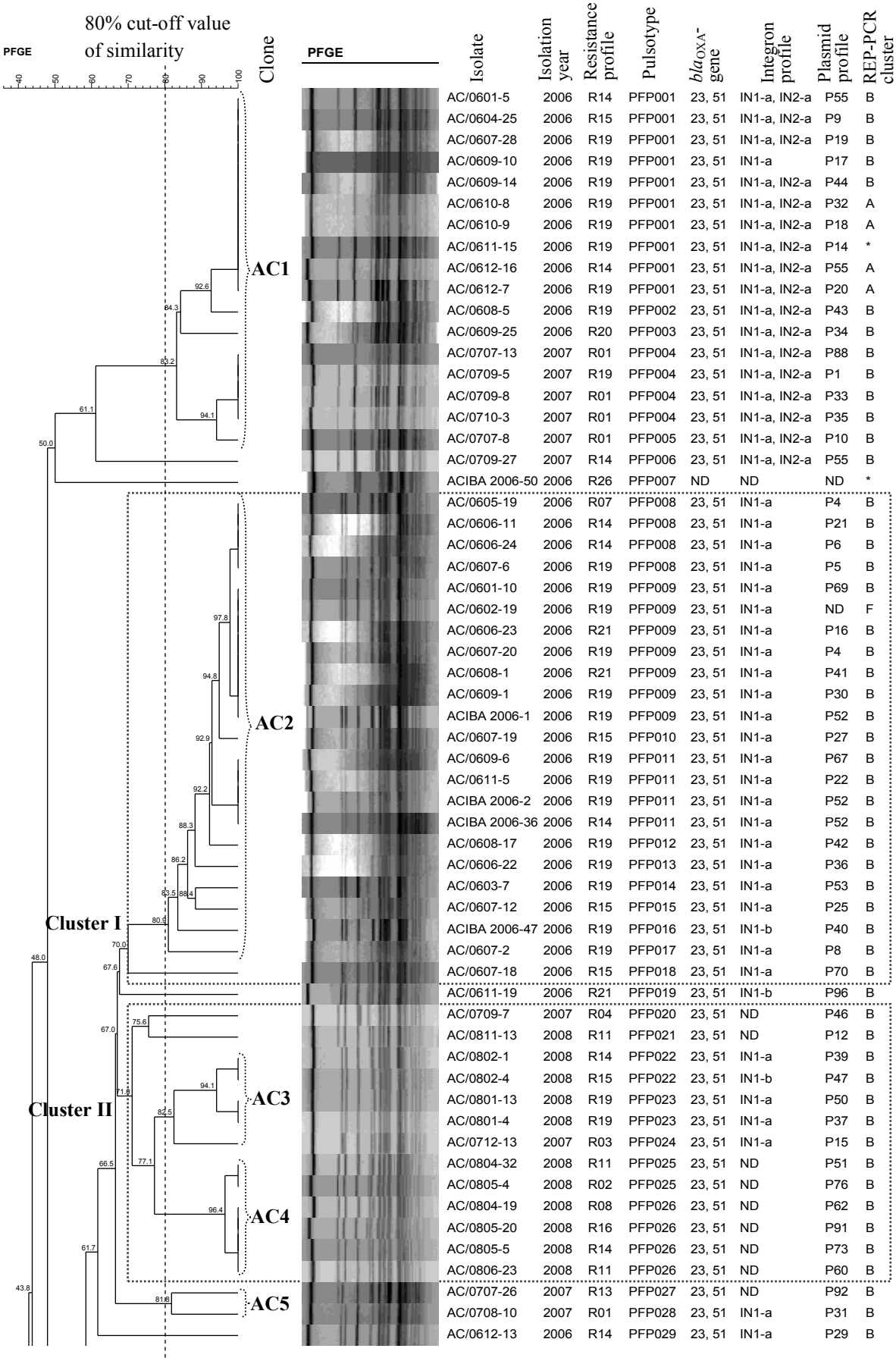
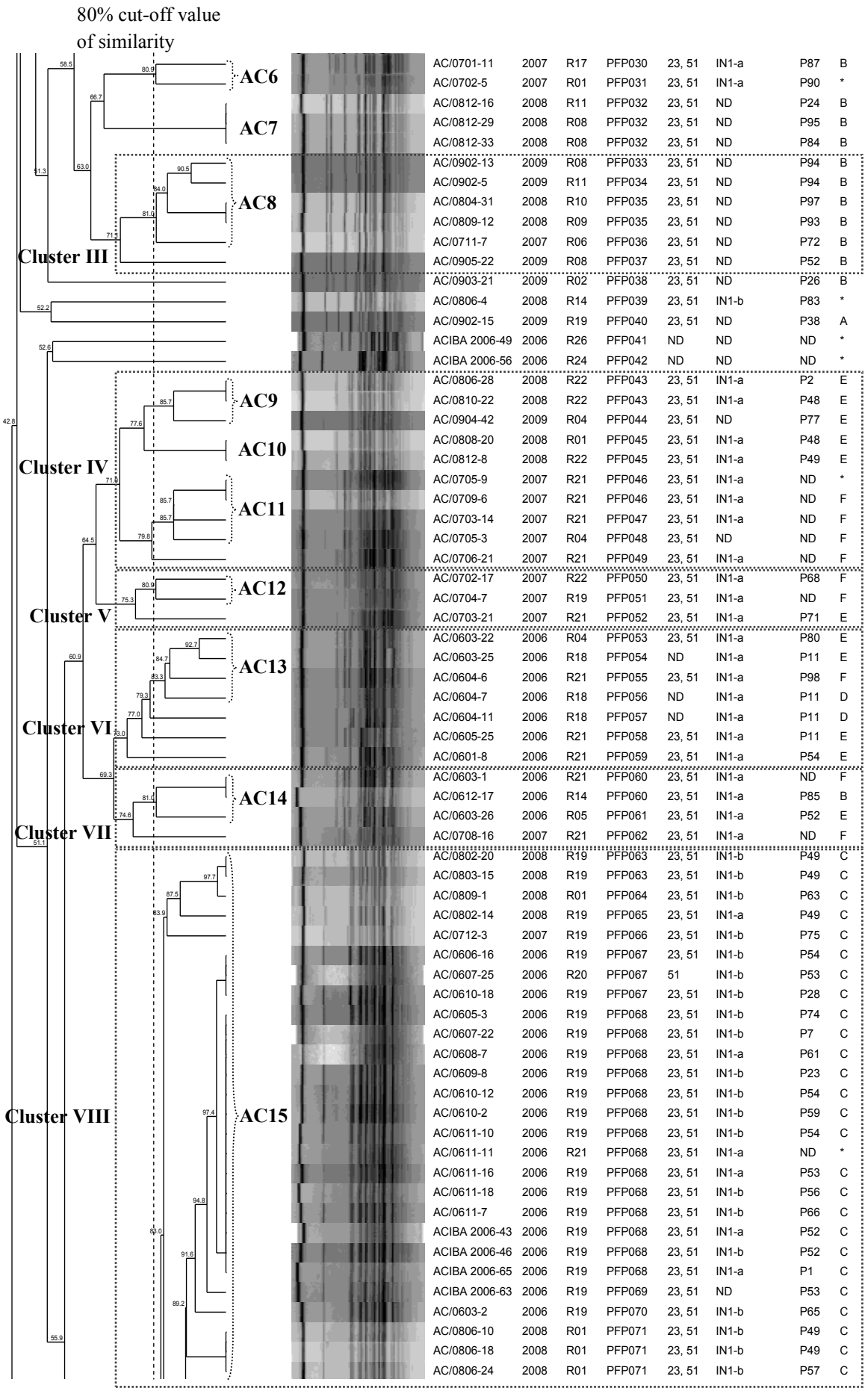


Figure 4.18: PFGE profiles of selected *Apa*I digested *A. baumannii* isolates

Lane	Isolates	PFPs	Lane	Isolates	PFPs
1	H9812	/	12	AC/0812-29	AC032
2	AC/0810-22	AC043	13	AC/0812-33	AC032
3	AC/0810-26	AC078	14	AC/0701-11	AC030
4	AC/0811-12	AC073	15	H9812	/
5	AC/0811-13	AC021	16	AC/0702-5	AC031
6	AC/0811-15	AC081	17	AC/0702-17	AC050
7	AC/0811-25	AC081	18	AC/0703-14	AC047
8	H9812	/	19	AC/0703-21	AC052
9	AC/0812-1	AC073	20	AC/0705-3	AC048
10	AC/0812-8	AC045	21	AC/0704-7	AC051
11	AC/0812-16	AC032	22	H9812	/



Isolate	Isolation year	Resistance profile	Pulsotype	bla_{OXA}-gene	Integron profile	Plasmid profile	REP-PCR cluster
AC/0601-5	2006	R14	PFP001	23, 51	IN1-a, IN2-a	P55	B
AC/0604-25	2006	R15	PFP001	23, 51	IN1-a, IN2-a	P9	B
AC/0607-28	2006	R19	PFP001	23, 51	IN1-a, IN2-a	P19	B
AC/0609-10	2006	R19	PFP001	23, 51	IN1-a	P17	B
AC/0609-14	2006	R19	PFP001	23, 51	IN1-a, IN2-a	P44	B
AC/0610-8	2006	R19	PFP001	23, 51	IN1-a, IN2-a	P32	A
AC/0610-9	2006	R19	PFP001	23, 51	IN1-a, IN2-a	P18	A
AC/0611-15	2006	R19	PFP001	23, 51	IN1-a, IN2-a	P14	*
AC/0612-16	2006	R14	PFP001	23, 51	IN1-a, IN2-a	P55	A
AC/0612-7	2006	R19	PFP001	23, 51	IN1-a, IN2-a	P20	A
AC/0608-5	2006	R19	PFP002	23, 51	IN1-a, IN2-a	P43	B
AC/0609-25	2006	R20	PFP003	23, 51	IN1-a, IN2-a	P34	B
AC/0707-13	2007	R01	PFP004	23, 51	IN1-a, IN2-a	P88	B
AC/0709-5	2007	R19	PFP004	23, 51	IN1-a, IN2-a	P1	B
AC/0709-8	2007	R01	PFP004	23, 51	IN1-a, IN2-a	P33	B
AC/0710-3	2007	R01	PFP004	23, 51	IN1-a, IN2-a	P35	B
AC/0707-8	2007	R01	PFP005	23, 51	IN1-a, IN2-a	P10	B
AC/0709-27	2007	R14	PFP006	23, 51	IN1-a, IN2-a	P55	B
ACIBA 2006-50	2006	R26	PFP007	ND	ND	ND	*
AC/0605-19	2006	R07	PFP008	23, 51	IN1-a	P4	B
AC/0606-11	2006	R14	PFP008	23, 51	IN1-a	P21	B
AC/0606-24	2006	R14	PFP008	23, 51	IN1-a	P6	B
AC/0607-6	2006	R19	PFP008	23, 51	IN1-a	P5	B
AC/0601-10	2006	R19	PFP009	23, 51	IN1-a	P69	B
AC/0602-19	2006	R19	PFP009	23, 51	IN1-a	ND	F
AC/0606-23	2006	R21	PFP009	23, 51	IN1-a	P16	B
AC/0607-20	2006	R19	PFP009	23, 51	IN1-a	P4	B
AC/0608-1	2006	R21	PFP009	23, 51	IN1-a	P41	B
AC/0609-1	2006	R19	PFP009	23, 51	IN1-a	P30	B
ACIBA 2006-1	2006	R19	PFP009	23, 51	IN1-a	P52	B
AC/0607-19	2006	R15	PFP010	23, 51	IN1-a	P27	B
AC/0609-6	2006	R19	PFP011	23, 51	IN1-a	P67	B
AC/0611-5	2006	R19	PFP011	23, 51	IN1-a	P22	B
ACIBA 2006-2	2006	R19	PFP011	23, 51	IN1-a	P52	B
ACIBA 2006-36	2006	R14	PFP011	23, 51	IN1-a	P52	B
AC/0608-17	2006	R19	PFP012	23, 51	IN1-a	P42	B
AC/0606-22	2006	R19	PFP013	23, 51	IN1-a	P36	B
AC/0603-7	2006	R19	PFP014	23, 51	IN1-a	P53	B
AC/0607-12	2006	R15	PFP015	23, 51	IN1-a	P25	B
ACIBA 2006-47	2006	R19	PFP016	23, 51	IN1-b	P40	B
AC/0607-2	2006	R19	PFP017	23, 51	IN1-a	P8	B
AC/0607-18	2006	R15	PFP018	23, 51	IN1-a	P70	B
AC/0611-19	2006	R21	PFP019	23, 51	IN1-b	P96	B
AC/0709-7	2007	R04	PFP020	23, 51	ND	P46	B
AC/0811-13	2008	R11	PFP021	23, 51	ND	P12	B
AC/0802-1	2008	R14	PFP022	23, 51	IN1-a	P39	B
AC/0802-4	2008	R15	PFP022	23, 51	IN1-b	P47	B
AC/0801-13	2008	R19	PFP023	23, 51	IN1-a	P50	B
AC/0801-4	2008	R19	PFP023	23, 51	IN1-a	P37	B
AC/0712-13	2007	R03	PFP024	23, 51	IN1-a	P15	B
AC/0804-32	2008	R11	PFP025	23, 51	ND	P51	B
AC/0805-4	2008	R02	PFP025	23, 51	ND	P76	B
AC/0804-19	2008	R08	PFP026	23, 51	ND	P62	B
AC/0805-20	2008	R16	PFP026	23, 51	ND	P91	B
AC/0805-5	2008	R14	PFP026	23, 51	ND	P73	B
AC/0806-23	2008	R11	PFP026	23, 51	ND	P60	B
AC/0707-26	2007	R13	PFP027	23, 51	ND	P92	B
AC/0708-10	2007	R01	PFP028	23, 51	IN1-a	P31	B
AC/0612-13	2006	R14	PFP029	23, 51	IN1-a	P29	B



AC/0701-11	2007	R17	PFP030	23, 51	IN1-a	P87	B
AC/0702-5	2007	R01	PFP031	23, 51	IN1-a	P90	*
AC/0812-16	2008	R11	PFP032	23, 51	ND	P24	B
AC/0812-29	2008	R08	PFP032	23, 51	ND	P95	B
AC/0812-33	2008	R08	PFP032	23, 51	ND	P84	B
AC/0902-13	2009	R08	PFP033	23, 51	ND	P94	B
AC/0902-5	2009	R11	PFP034	23, 51	ND	P94	B
AC/0804-31	2008	R10	PFP035	23, 51	ND	P97	B
AC/0809-12	2008	R09	PFP035	23, 51	ND	P93	B
AC/0711-7	2007	R06	PFP036	23, 51	ND	P72	B
AC/0905-22	2009	R08	PFP037	23, 51	ND	P52	B
AC/0903-21	2009	R02	PFP038	23, 51	ND	P26	B
AC/0806-4	2008	R14	PFP039	23, 51	IN1-b	P83	*
AC/0902-15	2009	R19	PFP040	23, 51	ND	P38	A
ACIBA 2006-49	2006	R26	PFP041	ND	ND	ND	*
ACIBA 2006-56	2006	R24	PFP042	ND	ND	ND	*
AC/0806-28	2008	R22	PFP043	23, 51	IN1-a	P2	E
AC/0810-22	2008	R22	PFP043	23, 51	IN1-a	P48	E
AC/0904-42	2009	R04	PFP044	23, 51	ND	P77	E
AC/0808-20	2008	R01	PFP045	23, 51	IN1-a	P48	E
AC/0812-8	2008	R22	PFP045	23, 51	IN1-a	P49	E
AC/0705-9	2007	R21	PFP046	23, 51	IN1-a	ND	*
AC/0709-6	2007	R21	PFP046	23, 51	IN1-a	ND	F
AC/0703-14	2007	R21	PFP047	23, 51	IN1-a	ND	F
AC/0705-3	2007	R04	PFP048	23, 51	ND	ND	F
AC/0706-21	2007	R21	PFP049	23, 51	IN1-a	ND	F
AC/0702-17	2007	R22	PFP050	23, 51	IN1-a	P68	F
AC/0704-7	2007	R19	PFP051	23, 51	IN1-a	ND	F
AC/0703-21	2007	R21	PFP052	23, 51	IN1-a	P71	E
AC/0603-22	2006	R04	PFP053	23, 51	IN1-a	P80	E
AC/0603-25	2006	R18	PFP054	ND	IN1-a	P11	E
AC/0604-6	2006	R21	PFP055	23, 51	IN1-a	P98	F
AC/0604-7	2006	R18	PFP056	ND	IN1-a	P11	D
AC/0604-11	2006	R18	PFP057	ND	IN1-a	P11	D
AC/0605-25	2006	R21	PFP058	23, 51	IN1-a	P11	E
AC/0601-8	2006	R21	PFP059	23, 51	IN1-a	P54	E
AC/0603-1	2006	R21	PFP060	23, 51	IN1-a	ND	F
AC/0612-17	2006	R14	PFP060	23, 51	IN1-a	P85	B
AC/0603-26	2006	R05	PFP061	23, 51	IN1-a	P52	E
AC/0708-16	2007	R21	PFP062	23, 51	IN1-a	ND	F
AC/0802-20	2008	R19	PFP063	23, 51	IN1-b	P49	C
AC/0803-15	2008	R19	PFP063	23, 51	IN1-b	P49	C
AC/0809-1	2008	R01	PFP064	23, 51	IN1-b	P63	C
AC/0802-14	2008	R19	PFP065	23, 51	IN1-a	P49	C
AC/0712-3	2007	R19	PFP066	23, 51	IN1-b	P75	C
AC/0606-16	2006	R19	PFP067	23, 51	IN1-b	P54	C
AC/0607-25	2006	R20	PFP067	51	IN1-b	P53	C
AC/0610-18	2006	R19	PFP067	23, 51	IN1-b	P28	C
AC/0605-3	2006	R19	PFP068	23, 51	IN1-b	P74	C
AC/0607-22	2006	R19	PFP068	23, 51	IN1-b	P7	C
AC/0608-7	2006	R19	PFP068	23, 51	IN1-a	P61	C
AC/0609-8	2006	R19	PFP068	23, 51	IN1-b	P23	C
AC/0610-12	2006	R19	PFP068	23, 51	IN1-b	P54	C
AC/0610-2	2006	R19	PFP068	23, 51	IN1-b	P59	C
AC/0611-10	2006	R19	PFP068	23, 51	IN1-b	P54	C
AC/0611-11	2006	R21	PFP068	23, 51	IN1-a	ND	*
AC/0611-16	2006	R19	PFP068	23, 51	IN1-a	P53	C
AC/0611-18	2006	R19	PFP068	23, 51	IN1-b	P56	C
AC/0611-7	2006	R19	PFP068	23, 51	IN1-b	P66	C
ACIBA 2006-43	2006	R19	PFP068	23, 51	IN1-a	P52	C
ACIBA 2006-46	2006	R19	PFP068	23, 51	IN1-b	P52	C
ACIBA 2006-65	2006	R19	PFP068	23, 51	IN1-a	P1	C
ACIBA 2006-63	2006	R19	PFP069	23, 51	ND	P53	C
AC/0603-2	2006	R19	PFP070	23, 51	IN1-b	P65	C
AC/0806-10	2008	R01	PFP071	23, 51	IN1-b	P49	C
AC/0806-18	2008	R01	PFP071	23, 51	IN1-b	P49	C
AC/0806-24	2008	R01	PFP071	23, 51	IN1-b	P57	C

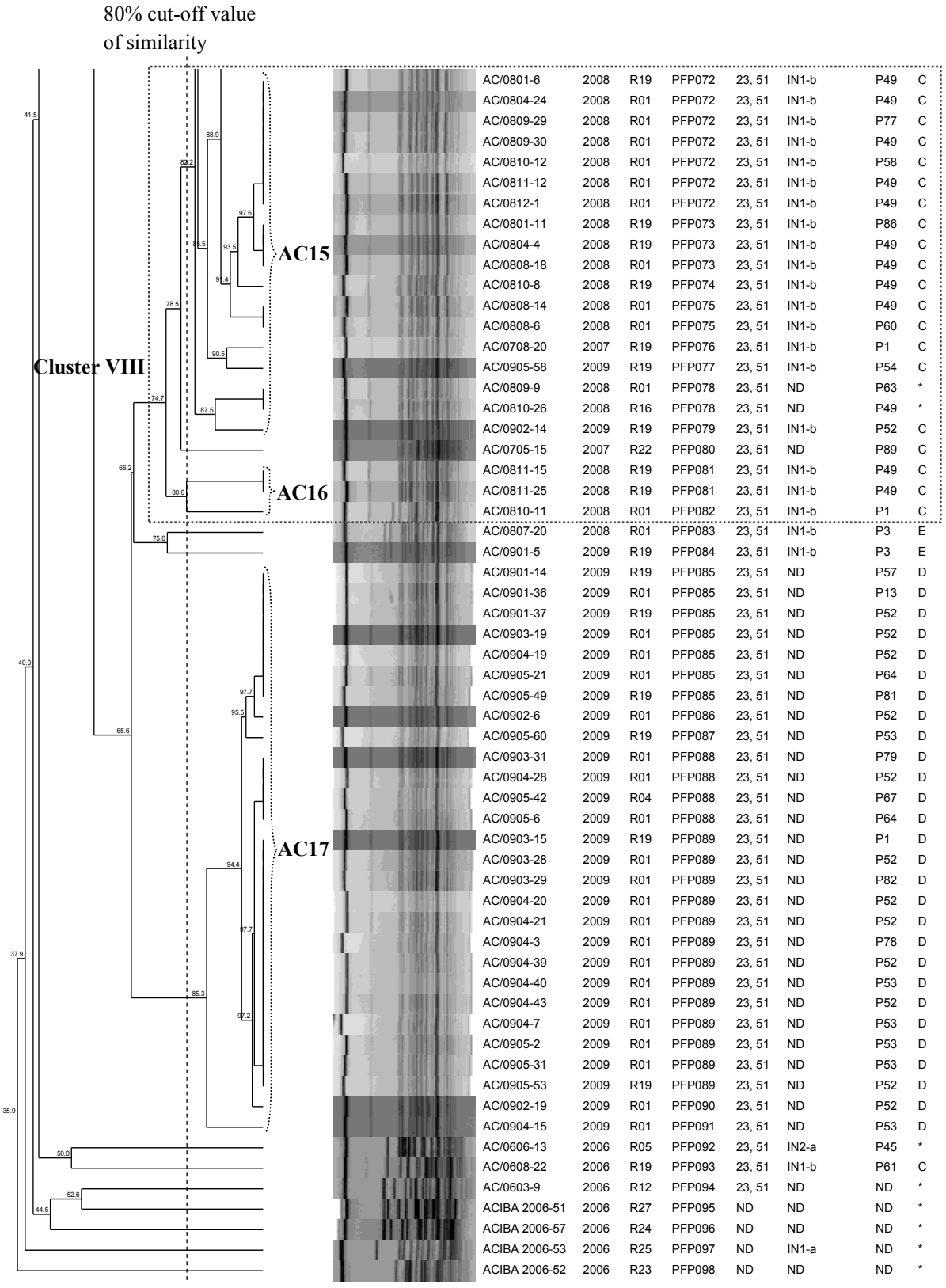


Figure 4.19: PFGE dendrogram cluster analysis generated using Bionumeric Version 6.0 (Applied Maths, Belgium) software and unweighted pair group arithmetic means methods (UPGMA) of *Apa*I digested *A. baumannii.*

Abbreviations: ND- not detected; *- non-clustered

Table 4.7: Distribution of *A. baumannii* isolates in the PFGE dendrogram cluster analysis

Cluster	Clone	PFP (N)	Year (no. of isolate)					Total (n=185)
			Clinical				Env + hands of HCW	
			2006 (n=61)	2007 (n=25)	2008 (n=47)	2009 (n=37)	2006 (n=8)	
I	AC2	10	18				4	22
	nc	1	1					1
II	AC3	3		1	4			5
	AC4	2			6			6
	nc	2		1	1			2
III	AC8	4		1	2	2		5
	nc	1				1		1
IV	AC9	2			2	1		3
	AC10	1			2			2
	AC11	3		4				4
	nc	1		1				1
V	AC12	2		2				2
	nc	1		1				1
VI	AC13	4	4					4
	nc	3	3					3
VII	AC14	2	3					3
	nc	1		1				1
VIII	AC15	17	15	2	22	2	4	45
	AC16	2			3			3
	nc	1		1				1
Non-clustered	AC1	5	12	5				17
	AC5	2		2				2
	AC6	2		2				2
	AC7	1			3			3
	AC17	7				28		28
	nc	18	5	1	2	3	7	18

Abbreviations: N- number of pulsotypes; nc- not clone

4.9 Comparison of REP-PCR and PFGE genotyping of *A. baumannii* isolates

REP-PCR and PFGE typing methods gave high value of discriminatory index. However, PFGE had higher D index (D=0.98) compared to REP-PCR (D=0.96). More diverse profiles were generated by PFGE (F_{value}= 0.35-1.00) compared to REP-PCR (F_{value}= 0.72-1.00). Of the 98 PFGE pulsotypes, 71 were unique pulsotypes. While in REP-PCR, there were 62 REP types were defined with 32 had unique REP types.

There was a general concordance in the clustering of *A. baumannii* isolates by PFGE and REP-PCR typing, although there were some exceptions (Figure 4.19). Isolates clustered by REP-PCR at ≥90% of similarity in cluster B were differentiated by PFGE into 8 different clones (AC1-AC8) which shared only 48% of similarity. Isolates in cluster E and F of REP-PCR (at 87.8% of similarity) were grouped into cluster IV to cluster VII by PFGE at 60.9% of similarity. However, the carbapenem-susceptible isolates, AC/0604-7 and AC/0604-11 which grouped separately from the AC/0603-25 by REP-PCR, were clustered into cluster VI by PFGE, shared 79% of similarity. PFGE showed the isolates which grouped by REP-PCR in cluster D with exception of the 2 carbapenem-susceptible isolates were a similar clone AC17 isolates. About 98% (46/47) of the isolates in cluster C of REP-PCR were determined as clone AC15 by PFGE.

These two typing methods had differentiated the non-MDR and MDR isolates. Both PFGE and REP-PCR had shown a genetic relationship between the environmental isolates and the isolates isolated from patients in 2006-2009.

4.10 Localisation of *bla*$_{OXA-23}$ on plasmid and/or chromosome by Southern hybridisation

4.10.1 *bla*$_{OXA-23}$ and 16Sr DNA gene probes

The DNA gene probes of *bla*$_{OXA-23}$ and 16S rDNA were successfully synthesized by PCR-labelling method using DIG Probe Synthesis Kit from Roche Diagnostics. The 550 bp band of tPA control probe was observed on the agarose gel and indicate that the labelling reaction was successful.

The labelled probe had a slightly greater molecular weight than the unlabeled probe. The labelled probe of *bla*$_{OXA-23}$ had an apparent band size of ~600 bp and the unlabelled probe yielded the actual expected band size of 501 bp (Figure 4.20 (a)). While the labelled probe of 16S rDNA gave an approximately 900 bp band compared to the unlabelled probe with the actual expected band size of 792 bp (Figure 4.20 (b)).

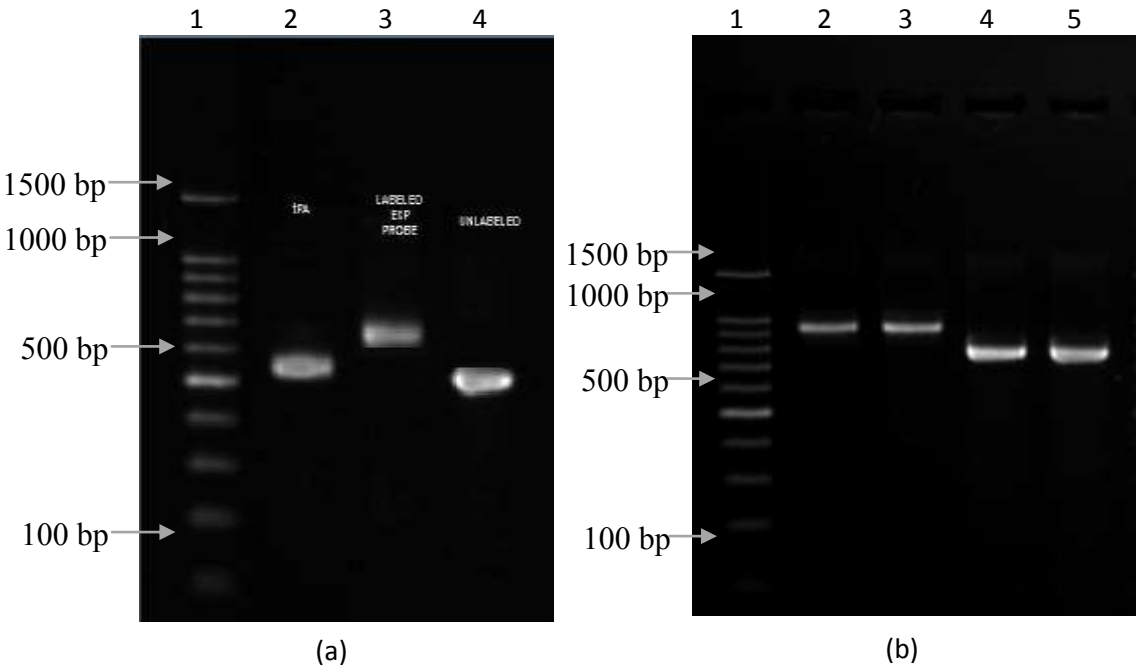


Figure 4.20: Evaluation of DIG-PCR labelled probe products on agarose gel. (a) DIG-PCR labelled probe of *bla*$_{OXA-23}$ gene. Lane 1: 100 bp DNA marker (Promega, USA); lane 2: tPA control probe; lane 3: labelled probe of *bla*$_{OXA-23}$ gene and lane 4: unlabelled probe of *bla*$_{OXA-23}$ gene (b) DIG-PCR labelled probe of 16S rDNA gene. Lane 1: 100 bp DNA marker (Promega, USA); lane 2 and 3: labelled probe of 16S rDNA gene; lane 4 and 5: unlabelled probe of 16S rDNA gene.

4.10.2 Localisation of *bla*$_{OXA-23}$ on plasmid DNA extracted by alkaline lysis

Uncut plasmids DNA of 16 selected *bla*$_{OXA-23}$-positive and 2 *bla*$_{OXA-23}$-negative *A. baumannii* isolates were separated by gel electrophoresis (Figure 4.21 (a)) and were successfully transferred onto positively charged nylon membrane. Under the high stringency conditions, *bla*$_{OXA-23}$ probe hybridised with the plasmid bands of the 2 selected isolates, AC/0606-22 and AC0812-16. No hybridisation was observed on the plasmid of other selected isolates. *bla*$_{OXA-23}$ probe hybridised with 2.3 kb plasmid band of AC/0606-22 and 4 plasmid bands (65.0 kb, 47.6 kb, 44.8 kb and 9.3 kb) of AC/0812-16 (Figure 4.21 (b)).

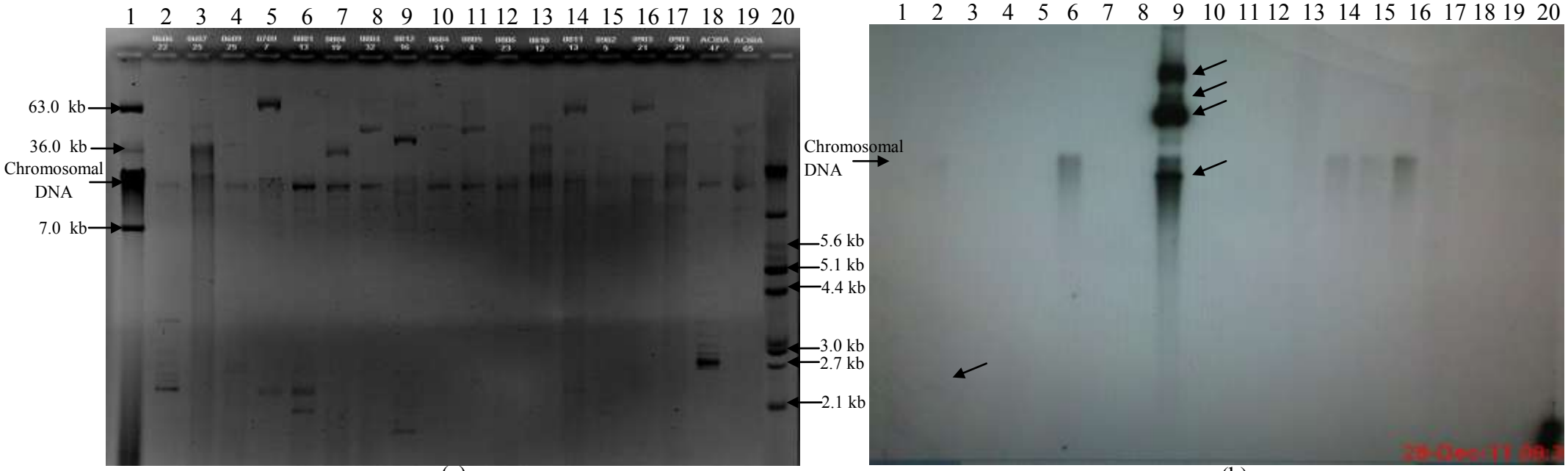


Figure 4.21: Localisation of *bla*$_{OXA-23}$ on plasmid DNA extracted by alkaline lysis. (a) Plasmid DNA gel. (b) Hybridisation with *bla*$_{OXA-23}$ probe. Arrows on the gel (b) indicated the co-hybridisation of *bla*$_{OXA-23}$ probe on the plasmid bands.

Lane	Isolates	Plasmid band co-hybridised with *bla*$_{OXA-23}$ probe (kb)	Lane	Isolates	Plasmid band co-hybridised with *bla*$_{OXA-23}$ probe (kb)
1	*E. coli* 39R	-	11	AC/0805-4	-
2	AC/0606-22	2.3	12	AC/0806-23	-
3	AC/0607-25	-	13	AC/0810-12	-
4	AC/0609-25	-	14	AC/0811-13	-
5	AC/0709-7	-	15	AC/0902-5	-
6	AC/0801-13	-	16	AC/0903-21	-
7	AC/0804-19	-	17	AC/0903-29	-
8	AC/0804-32	-	18	ACIBA 2006-47	-
9	AC/0812-16	65.0 ,58.6, 44.8, 9.3	19	ACIBA 2006-65	-
10	AC/0604-11	-	20	*E. coli* V517	-

4.10.3 Localisation of *bla*$_{OXA-23}$ on plasmid DNA by S1 nuclease digested PFGE plugs

The untreated PFGE plugs gave a linear band at approximately 1135.0 kb in the 18 selected *A. baumannii* isolates. Treated with S1 nuclease, the genomic DNA was digested and appeared at the bottom of the agarose gel after electrophoresis. While linearized plasmid DNA bands of >20.0 kb to 452.7 kb were observed (Figure 4.22 (a) and (d)).

After transferred onto positively charged nylon membrane, under the high stringency conditions, *bla*$_{OXA-23}$ probe hybridised with 1135.0 kb band of AC/0606-22, AC/0609-25, AC/0801-13, AC/0804-19, AC/0804-32, AC/0805-4, AC/0806-23, AC/0810-12, AC/0902-5 and ACIBA 2006-65; 452.7 kb, 398.4 kb, 102.0 kb, 44.8 kb and 18.0 kb bands of AC/0812-16; 102.0 kb band of AC/0801-13 and AC/0811-13 isolates (Figure 4.22 (c) and (f)).

Hybridisation with 16S rDNA probe, hybridisation signals were observed on 1135.0 kb band but not on the other bands. S1 nuclease with PFGE analysis showed that the *bla*$_{OXA-23}$ gene was carried on 452.7 kb, 398.4 kb, 102.0 kb, 45.0 kb and 18.0 kb plasmids (Figure 4.22 (b) and (e)).

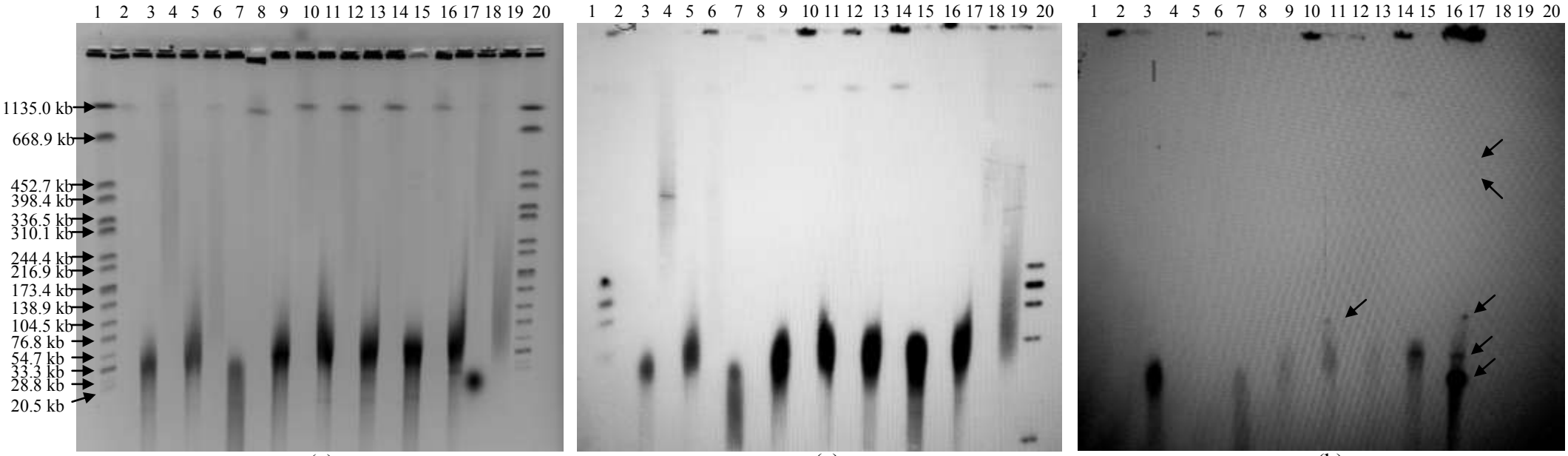
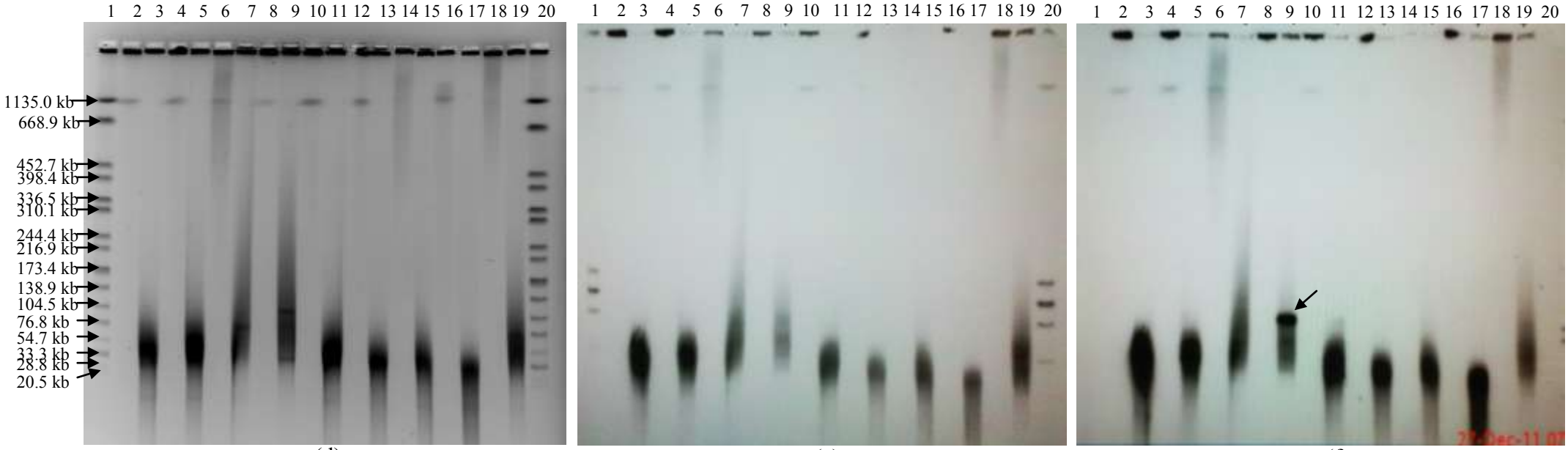


Figure 4.22: Plasmid identification by digestion with S1 nuclease. (a) PFGE gel (b). Hybridisation with 16S rDNA probe (c) Hybridisation with *bla*$_{\text{OXA-23}}$ probe. DNA plugs in lane 2, 4, 6, 8, 10, 12, 14, 16 and 18 were untreated with S1 nuclease. Arrows on the gel (c) indicated the co-hybridisation of *bla*$_{\text{OXA-23}}$ probe on the plasmid bands.

Lane	Isolates	Plasmid band co-hybridised with *bla*$_{\text{OXA-23}}$ probe (kb)	Lane	Isolates	Plasmid band co-hybridised with *bla*$_{\text{OXA-23}}$ probe (kb)
1	H9812	-	13	AC/0804-19	-
3	AC/0606-22	-	15	AC/0804-32	-
5	AC/0607-25	-	17	AC/0812-16	452.7, 398.4, 102.0, 44.8, 18.0
7	AC/0609-25	-	19	AC/0604-11	-
9	AC/0709-7	-	20	H9812	-
11	AC/0801-13	102.0			



(d) (e) (f)

Continued figure 4.22: Plasmid identification by digestion with S1 nuclease. (d) PFGE gel (e) Hybridisation with 16S rDNA probe (f) Hybridisation with *bla*$_{OXA-23}$ probe. DNA plugs in lane 2, 4, 6, 8, 10, 12, 14, 16 and 18 were untreated with S1 nuclease. Arrows on the gel (f) indicated the co-hybridisation of *bla*$_{OXA-23}$ probe on the plasmid bands.

Lane	Isolate	Plasmid band co-hybridised with *bla*$_{OXA-23}$ probe (kb)	Lane	Isolate	Plasmid band co-hybridised with *bla*$_{OXA-23}$ probe (kb)
1	H9812	-	13	AC/0903-21	-
3	AC/0805-4	-	15	AC/0903-29	-
5	AC/0806-23	-	17	ACIBA 2006-47	-
7	AC/0810-12	-	19	ACIBA 2006-65	-
9	AC/0811-13	102.0	20	H9812	-
11	AC/0902-5	-			

4.10.4 Localisation of *bla*$_{OXA-23}$ on chromosome by I-*Ceu*I digested PFGE plugs

I-*Ceu*I restricted DNA of the 18 *A. baumannii* isolates gave 4 to 7 bands which hybridised with 16S rDNA probe under the high stringency conditions (Figure 4.23 (a) (b)). Probed with *bla*$_{OXA-23}$ probe, hybridisation signal was detected on 1217.6 kb, 625.8 kb, 563.2 kb, 544.5 kb, 497.4 kb, 186.8 kb and 99.5 kb of chromosomal bands (Figure 4.23 (c). *bla*$_{OXA-23}$ gene was detected on the chromosome of 15 isolates. Twelve isolates showed a single copy of *bla*$_{OXA-23}$ gene, 6 isolates on 544.5 kb and 6 isolates on 217.6 kb chromosomal bands. Isolate AC/0609-25 and AC/0804-32 had 2 copies of *bla*$_{OXA-23}$ gene. AC/0609-25 carried *bla*$_{OXA-23}$ gene on 625.8 kb and 497.4 kb chromosomal bands. While AC/0804-32 carried *bla*$_{OXA-23}$ gene on 563.2 kb and 544.5 kb chromosomal bands. Isolate ACIBA 2006-47 had 3 copies of *bla*$_{OXA-23}$ gene carried on 1217.6 kb, 186.8 kb and 99.5 kb chromosomal bands.

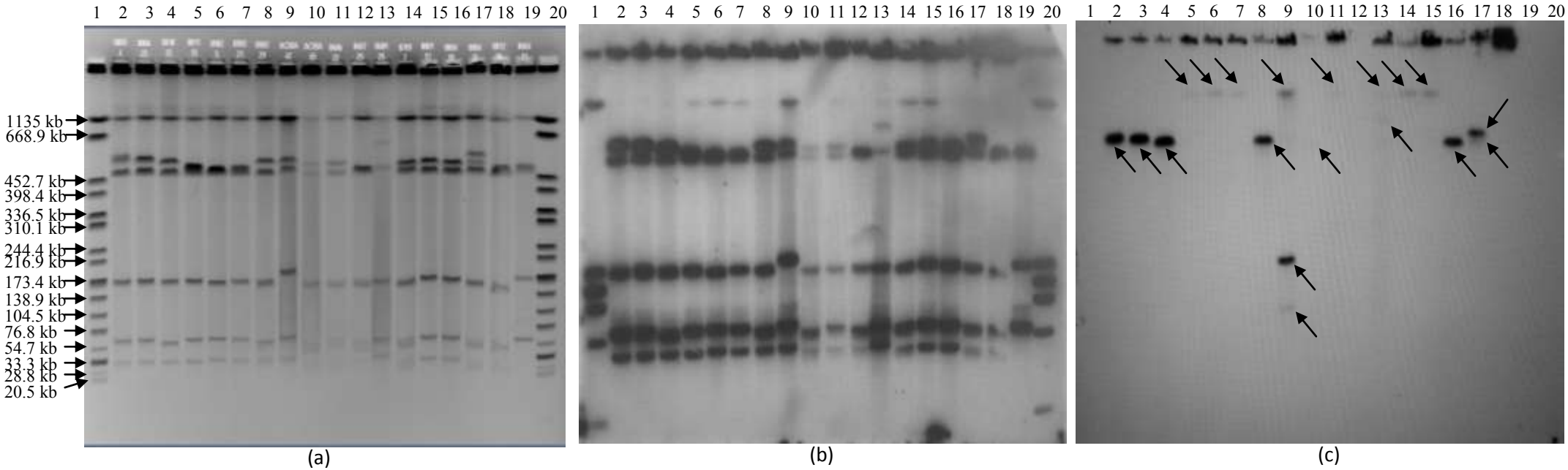


Figure 4.23: Localisation of *bla*$_{OXA-23}$ on chromosome by digestion with I-*Ceu*I. (a) PFGE gel (b) Hybridisation with 16S rDNA probe (c) Hybridisation with *bla*$_{OXA-23}$ probe. Arrow on the gel (c) indicated the co-hybridisation of *bla*$_{OXA-23}$ probe on the chromosomal bands.

Lane	Isolate	Chromosomal band co-hybridised with *bla*$_{OXA-23}$ probe (kb)	Lane	Isolate	Chromosomal band co-hybridised with *bla*$_{OXA-23}$ probe (kb)
1	H9812	-	11	AC/0606-22	1217.6
2	AC/0805-4	544.5	12	AC/0607-25	-
3	AC/0806-23	544.5	13	AC/0609-25	625.8, 497.4
4	AC/0810-12	544.5	14	AC/0709-7	1217.6
5	AC/0811-13	1217.6	15	AC/0801-13	1217.6
6	AC/0902-5	1217.6	16	AC/0804-19	544.5
7	AC/0903-21	1217.6	17	AC/0804-32	563.2, 544.5
8	AC/0903-29	544.5	18	AC/0812-16	-
9	ACIBA 2006-47	1217.6, 186.8, 99.5	19	AC/0604-11	-
10	ACIBA 2006-65	544.5	20	H9812	-

4.10.5 Summarized results of *bla*$_{OXA-23}$ gene location on plasmid and/or chromosome evaluated using alkaline lysis extracted plasmid, S1 nuclease and I-*Ceu*I methods

Neither plasmid nor chromosomal bands were hybridised with *bla*$_{OXA-23}$ probe in the *bla*$_{OXA-23}$-negative isolates indicated the hybridisation was specific. A single copy of *bla*$_{OXA-23}$ gene was detected on chromosome of 9 isolates. Three isolates (AC/0609-25, AC/0804-32 and ACIBA 2006-47) were carried more than one copy of *bla*$_{OXA-23}$ gene on chromosome. Two isolates had a similar location of *bla*$_{OXA-23}$ gene. AC/0801-3 and AC/0811-13 carried 2 copies of *bla*$_{OXA-23}$ gene, 1 on 102 kb plasmid according to the results of S1 nuclease method and 1 on chromosome with a hybridisation signal on 1217.6 kb band by I-*Ceu*I method. Isolate AC/0606-22 was also carried 2 copies of *bla*$_{OXA-23}$ gene, 1 on 2.3 kb plasmid using the alkaline lysis extracted plasmid and 1 on 1217.6 kb of chromosomal band using the I-*Ceu*I method. A isolate isolated in 2008 (AC/0812-16) had 8 copies of *bla*$_{OXA-23}$ gene on 9.3 kb, 18.0 kb, 44.8 kb, 65.0 kb, 102.0 kb, 151.2 kb, 398.4 kb and 452.7 kb plasmids which were detected by using two different methods, alkaline lysis extracted plasmid and S1 nuclease (Table 4.8).

Table 4.8: Summarized results of *bla*$_{OXA-23}$ gene location on plasmid and/or chromosome

Isolates	Southern hybridisation with *bla*$_{OXA-23}$		
	Plasmid (kb)		Chromosomal (kb)
	Alkaline lysis	S1 nuclease with PFGE	I-*Ceu*I with PFGE
AC/0606-22	2.3	-	1217.6
AC/0607-25	-	-	-
AC/0609-25	-	-	625.8, 497.4
AC/0709-7	-	-	1217.6
AC/0801-13	-	102.0	1217.6
AC/0804-19	-	-	544.5
AC/0804-32	-	-	563.2, 544.5
AC/0812-16	151.2, 65.0, 44.8, 9.3	452.7, 398.4, 102.0, 44.8, 18.0	-
AC/0604-11	-	-	-
AC/0805-4	-	-	544.5
AC/0806-23	-	-	544.5
AC/0810-12	-	-	544.5
AC/0811-13	-	102.0	1217.6
AC/0902-5	-	-	1217.6
AC/0903-21	-	-	1217.6
AC/0903-29	-	-	544.5
ACIBA 2006-47	-	-	1217.6, 186.8, 99.5
ACIBA 2006-65	-	-	544.5

4.11 Transformation of plasmid borne *bla*$_{\text{OXA-23}}$ into competent *E. coli* 5-alpha

PGEX plasmid DNA was successfully transformed into *E. coli* 5-alpha competent cells indicating that the transformation method used in this study was successful. However, the plasmids borne *bla*$_{\text{OXA-23}}$ gene for AC/0606-22 and AC/0812-16 could not be transferred into the *E. coli* 5-alpha competent cells, although the experiment was repeated twice.

CHAPTER 5: DISCUSSIONS

5.1 Species identification of *A. baumannii*

Application of phenotypic method to identify the species of the genus *Acinetobacter* require specific media and is time consuming as it needs few days of incubation. Besides, this method is also insufficient and inaccurate to differentiate the *A. calcoaceticus-A. baumannii* complex which comprises *A. calcoaceticus*, *A. baumannii*, *A. pittii*, and *A. nosocomialis* (Gerner-Smidt *et al*., 1991; Bergogne-Berezin and Towner, 1996).

In this study, ARDRA had successfully differentiated the *A. baumannii* from the non-*baumannii* isolates. In order to obtain the 1500 bp of 16S rDNA, different sources of *Taq* DNA polymerases have effects on the gene amplification. Initially GoTaq® DNA Polymerase (Promega, Madigan, USA) was used to perform PCR for 16S rDNA with the primers according to Vaneechoutte *et al*., (1995). The expected 1500 bp product did not amplified, although optimisation of the PCR reagent concentrations was done. Few smaller fragments were obtained, and DNA sequences analyses showed that the amplified products were partial sequence of the 16S rDNA gene. Thus, we suspected that the quality and source of *Taq* polymerase could be the main problem. Therefore, different brands of *Taq* polymerase were utilised in attempt to obtain the single 1500 bp band, but unspecific bands were still present. That led us to explore the efficacy of the different brands of *Taq* polymerase such as the *i-Taq*™ (iNtRON Biotechnology, Korea) and *TaKaRa Ex Taq*™ (Takara, Shiga). When the HotStarTaq, Qiagen, USA was tested, the single 1500 bp band was successfully amplified. In order to perform ARDRA, it is necessary to obtain the 1500 bp band without any unspecific bands; hence, source of *Taq* is important in the PCR amplification.

Identification of *Acinetobacter* genospecies using ARDRA method has been successfully and widely applied in many studies (Houang *et al*., 2001; Anstey *et al*.,

2002; Lim *et al*., 2007; D'Arezzo *et al*., 2011). ARDRA is found to be more definitive and rapidly in differentiate *A. baumannii* from *Acinetobacter* genospecies 13TU compared to ID 32 GN system (Shin *et al*., 2004). ARDRA has also successfully discriminated the *A. baumannii* from *Acinetobacter* genospecies 10 and 13TU which has been previously identified using the commercial phenotypic systems, Phoenix (Becton Dickinson, Sparks, MD, USA) and Vitek 2 (BioMerieux, Marcy-L'Etoile, France) (D'Arezzo *et al*., 2009). Study by Lee *et al*., (2007) has determined 224 *Acinetobacter* isolates into seven genospecies: *A. baumannii*, *A. junii, A. johnsonii*, *Acinetobacter* genomic species 13TU, 3, 10, and 14BJ. However, Lee *et al*., (2007) has also reported 8 unclassified genospecies isolates were differentiated by ARDRA. There is limitation in ARDRA as some species could have few restriction profiles and particular restriction profiles may be shared among different species (Seifert *et al*., 2008). Recently, Chang *et al*., (2005) have reported the use of ITS sequencing for identification of the *A. calcoaceticus-A. baumannii* complex. However this method is only feasible if a DNA sequencing facility is cheaply and readily available. In the absence of such a capital intensive facility, the ARDRA method, which has been validated by various laboratories, is the best alternative, since PCR machines are easily accessible in most clinical microbiology laboratories (Chang *et al*., 2005). Even though detection of $bla_{\text{OXA-51}}$ gene which intrinsically harboured by *A. baumannii* can be used to identify *A. baumannii* isolates (Turton *et al*., 2006b), ARDRA is able to give more information to identify other species of *Acinetobacter* caused infections in the hospitals. Therefore, in this study, ARDRA was applied and proved to be sufficient and useful for identification of *A. baumannii* isolates.

5.2 Antimicrobial resistance phenotypes of *A. baumannii*

In this study, all the clinical *A. baumannii* isolates exhibited high resistance to the antimicrobial agents tested except for polymyxin B. A previous study from a local hospital reported that *Acinetobacter* spp. isolated in 1996-1998 were 100% resistant to amoxicillin clavulanate, ampicillin, cefoperazone and cefuroxime and highly resistant to ceftazidime, gentamicin, ceftriaxone and ciprofloxacin (Misbah *et al*., 2004). However, the present study showed that there was an emergence of imipenem-resistant and amikacin-resistant isolates which were absent in the previous report (Misbah *et al*., 2004). Similar result was reported in United States that absence of the imipenem-resistant *A. baumannii* isolates in 2004 was found to increase dramatically in 2005 (52%) to 2007 (96%) (Qi *et al*., 2008). Based on the reports on the national surveillance on antibiotics resistance (NSAR) for 2003-2010 from the Institute of Medical Research, Ministry of Health, Malaysia (http://www.imr.gov.my/report/nsar_b.htm), resistance rates of *Acinetobacter* spp. to amikacin (2003, 8.8%; 2004, 18.3%; 2005, 19.3%; 2007, 29.2%; 2008, 40.6%; 2009, 39.3%; 2010, 48.2%) and imipenem (2003, 29.3% 2004, 35.0%; 2005, 40.3%; 2006, 44.5%; 2007, 46.7%; 2008, 42.3%; 2009, 48.1%; 2010, 56.5%) were increasing but lower when compared to this study. However, direct comparison of resistance rates is difficult as the national data is for *Acinetobacter* spp. and not for *A. baumannii*.

As observed in Singapore hospitals, *A. baumannii* isolates obtained in 2006-2007 were also highly resistant to carbapenem (Tan *et al*., 2008). Hsu *et al*., (2010) has also reported high resistance (50%) of carbapenem in *Acinetobacter* spp. blood isolates recovered from four Singaporean public hospitals. A similar increase in the resistance rate of *A. baumannii* isolates to imipenem and amikacin from 1996 to 2006 was reported in Greece (Falagas *et al*., 2007b). However, *A. baumannii* isolates from a tertiary care hospital in Georgia remained highly susceptible to the aminoglycosides and

carbapenems (Dauner *et al*., 2008). Lim *et al*., (2007) has also reported highly susceptible of imipenem *A. baumannii* isolates (67.5%) isolated from a University hospital in Korea. Increase in the *A. baumannii* resistance rates toward aminoglycosides, trimethoprim/sulfamethoxazole and carbapenems in ICU, UMMC may be caused by the selective pressure for resistance build-up in the isolates as a consequence of the routine use of same antimicrobial agents for treatment. Due to the emergence of resistance and high antibiotic tolerance by selective pressure in hospitals, the first therapeutic options for treatment, carbapenem might be compromised.

The addition of sulbactam, tazobactam and clavulanic acid as β-lactamase inhibitor was able to increase the susceptibility of *A. baumannii* isolates to penicillins and cephalosporins (Williams, 1999), since all the clinical *A. baumannii* isolates from ICU, UMMC were fully resistant to β-lactam antibiotics. Unfortunately, all the isolates were 100% resistant to amoxicillin clavulanic and piperacillin/tazobactam and had high resistance rate to ampicillin/sulbactam (84.1%) as compared to the study by Levin *et al*., (2003) and Yu *et al*., (2004) who reported 67.0% and 43.5%, respectively of the carbapenem-resistant isolates being susceptible to ampicillin/sulbactam. Study by Chun *et al*., (2006) has reported cefoperazone/sulbactam has greater inhibition effect than the β-lactam alone and more than 50% of the tested *A. baumannii* isolates have been inhibited by this antimicrobial agent. In the present study, the combination of cefoperazone and sulbactam appears to be effective against *A. baumannii* isolates in 2006 as all were cefoperazone-resistant but cefoperazone/sulbactam-susceptible. However, this combination of antimicrobial agents had lost its effectiveness as cefoperazone/sulbactam-resistant isolates were detected in 2007-2009. Similar result was reported by Zhou *et al*., (2007), 30.6% and 67.6% of the imipenem-resistant *A. baumannii* isolated from a Chinese hospital was resistant to cefoperazone/sulbactam and ampicillin/sulbactam, respectively. Intermediate resistance rate to

cefoperazone/sulbactam (33.3%) was also found in the *A. baumannii* isolates in India (Shareek *et al*., 2012).

Ballow and Schentag (1992) had reported the recovery of penicillin susceptibility and reduction of ceftazidime resistance by replacement use of piperacillin plus an aminoglycoside in cephalosporins resistant *Enterobacter cloacae* isolates. In our study, the substitution of cefoperazone/sulbactam in the therapeutic treatment might have resulted in decrease resistance to aminoglycosides and trimethoprim/sulfamethoxazole. However, increased use of cefoperazone/sulbactam might have resulted in increase resistance as observed in 2007. Thus, there is a need for proper scheduled antibiotic class changes in selection of appropriate drugs for treatment.

Polymyxins have been used as the therapeutic options for the treatment of MDR *A. baumannii* infections (Choi *et al*., 2006; Zavascki *et al*., 2007). However, there were reports of resistance to polymyxins in *A. baumannii* in USA (Urban *et al*., 2001) and Korea (Ko *et al*., 2007). In 2007, an emergence of polymyxin B-resistant *A. baumannii* has been repored in Korea by Park *et al*., (2009b). Gales *et al*., (2006) has also identified 2.8% of polymyxin B-resistant *A. baumannii* (2.8%) in the European arm of the SENTRY antimicrobial surveillance programme, 2001–2004. Fortunately, the *A. baumannii* isolates in our hospital remain sensitive to polymyxin B.

5.3 Phenotypic and genotypic of carbapenem resistance in *A. baumannii*

MBL-producing *A. baumannii* is increasingly reported in Asia, Europe and South America (Poirel and Nordmann, 2006). Among the regions of Asia continent, Southern Asia has higher prevalence of MBL-producing *A. baumannii* compared to Eastern and South-eastern. As reported in Pakistan, 96.6% and 84.0% of MBL-producing *A. baumannii* isolates were determined in military and tertiary care hospitals, respectively (Irfan *et al*., 2006; Kaleem *et al*., 2010). MBL-producing *A. baumannii* was also found

in 49.0% of the carbapenem-resistant *A. baumannii* isolates isolated in a teaching hospital located at northwest, Iran (Peymani *et al*., 2011). Recent studies by Karthika *et al*., (2009) and Kumar *et al*., (2011) have also reported high percentage, 70.9% and 21.0%, respectively, of MBL-producing *A. baumannii* isolates in India. In contrast to this, only 1.1% of the 178 *A. baumannii* isolates from 12 Korean hospitals in 2007 were MBL-producing isolates (Kim *et al*., 2010). Low prevalence of MBL-producing *A. baumannii* has also been reported by Koh *et al*., (2007) in Singapore (3.5%) and Chu *et al*., (2001) in Hong Kong (5.7%). However, in the present study, combined disks and IMP-EDTA double disks tests showed disagreement with the above research findings where there is no MBL activity was detected among all the carbapenem-resistant *A. baumannii* isolates. Similar finding was reported by Castanheira *et al*., 2008, no MBL-producer was detected among all the carbapenem-resistant *A. baumannii* isolates collected from a hospital in Texas, USA.

Conventional phenotypic tests based on EDTA inhibition of β-lactamase activity may not be reliable in detection of MBL-producing isolates as negative DDST with $bla_{\text{VIM-2}}$ and bla_{IMP} PCR-positive *A. baumannii* isolates have been reported in Spain (Canduela *et al*., 2006). Similar findings were also reported by researchers (Ikonomidis *et al*., 2008; Loli *et al*., 2008) in Greece, MBL phenotype-negative *A. baumannii* isolates from hospitals in Greece harboured a $bla_{\text{VIM-1}}$ gene. Thus, they suggested that PCR detection of MBL genes is necessary in order to identify MBL-producing isolates although the phenotypic MBL tests were performed (Ikonomidis *et al*., 2008). Therefore, in this study, regardless the negative MBL-phenotype, PCR detection of MBL genes was performed on all the isolates. Our results showed the MBL genotype is in agreement with the phenotype of the isolates as no MBL genes are present.

However, in a previous study conducted by Wong *et al*., (2009b), MBL-producing *A. calcoaceticus* isolates have been isolated in the Universiti Malaya Medical Centre

and harboured a $bla_{\text{IMP-4}}$ gene. The $bla_{\text{IMP-4}}$ gene was first detected from *A. baumannii* isolated in 1994 in a Hong Kong hospital (Chu *et al*., 2001) and recovered in a *A. baumannii* isolate isolated in 1998 (Houang *et al*., 2003). This gene was then discovered in 1996 and 2001 Singaporean *A. baumannii* isolates (Koh *et al*., 2007). In 2003, researchers in Australia have found $bla_{\text{IMP-4}}$ gene in the *Enterobacteriaceae* from Sydney and Melbourne and suggested the gene could be imported from Southeast Asia (Peleg *et al*., 2006; Espedido *et al*., 2008; Walsh *et al*., 2010). Besides $bla_{\text{IMP-4}}$, several IMP-variant genes have also been described in *A. baumannii*: $bla_{\text{IMP-1}}$ in Italy, Brazil, Japan and South Korea (Riccio *et al*., 2000; Tognim *et al*., 2006; Nishio *et al*., 2004; Sung *et al*., 2008); $bla_{\text{IMP-2}}$ in Italy and Japan (Riccio *et al*., 2000; Shibata *et al*.,2003); $bla_{\text{IMP-5}}$ in Portugal (Da Silva *et al*., 2002; Domingues *et al*., 2011) and $bla_{\text{IMP-6}}$ in Brazil (Gales *et al*., 2003). The VIM enzymes are rarely found in *A. baumannii:* VIM-1 in Greece (Tsakris *et al*., 2006 and 2008), VIM-2 in South Korea (Yum *et al*., 2002) and VIM-1/VIM-2 in Poland (Wroblewska *et al*., 2007). SPM-1 has only been detected in Iranian *A. baumannii* isolates in a recent study by Shahcheraghi *et al*., (2011). SIM-1 is reported in *A. baumannii* from Korea (Lee *et al*., 2005 and Yong *et al*., 2006) and GIM enzymes are reported in *A. baumannii* isolate in Egypt (Mohamed and Raafat, 2011). GIM enzymes are reported in non-*baumannii* isolates (Lee *et al*., 2010).

Absence of the MBL genes indicated possible presence of the carbapenem-hydrolysing oxacillanase genes in the carbapenem-resistant *A. baumannii* isolates. Therefore, PCR amplification of the OXA genes ($bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$) was performed and results showed $bla_{\text{OXA-23}}$ (n=174, 99.4%) and $bla_{\text{OXA-51}}$ (n=175, 100%) genes were prevalent amongst the isolates. The $bla_{\text{OXA-51}}$ gene is known to be intrinsic to *A. baumannii* (Turton *et al*., 2006b) and was present in all the studied isolates. This is in agreement to the identity of the isolates as confirmed by ARDRA; hence, the detection of this intrinsic gene is proved to be reliable to identify the *A.*

baumannii at species level (Turton *et al*., 2006b). A isolate isolated in 2006 (AC/0607-25) has imipenem-susceptible and meropenem-resistant profile, harboured *bla*$_{\text{OXA-51}}$ as the sole carbapenemase gene. However, *bla*$_{\text{OXA-51}}$ gene is known to express at low level and has weak carbapenem-hydrolysing activity. Presence of the insertion element, IS*Aba1* upstream of the chromosomal gene encoding OXA-51 enzyme could increase the expression of this gene and result in carbapenem resistance (Turton *et al*., 2006a). Although all of our isolates carried the IS*Aba1* element, they did not locate upstream of the *bla*$_{\text{OXA-51}}$ gene. In contrast, widespread of *A. baumannii* with IS*Aba1* upstream of the *bla*$_{\text{OXA-51}}$ was reported in Taiwan (Hu *et al*., 2007; Chen *et al*., 2009). In a previously published data, up-regulation of efflux pump has conferred resistant to meropenem in the *A. baumannii* isolates isolated from the same hospital (Wong *et al*., 2009a). Thus, similar resistance mechanism may be responsible for meropenem resistance in this AC/0607-25 isolate.

The *bla*$_{\text{OXA-23}}$ gene has been repeatedly reported in many countries in the outbreaks of carbapenem-resistant *A. baumannii*, such as in Europe (Naas *et al*., 2005; Kohlenberg *et al*., 2009; Boo *et al*., 2009; Mendes *et al*., 2009; D'Arezzo *et al*., 2011; Liakopoulas *et al*., 2012), Latin America (Villegas *et al*., 2007; Merkier *et al*., 2008; Carvalho *et al*., 2009; Martins *et al*., 2009), Africa (Andriamanantena *et al*., 2010) and Asia (Jeon *et al*., 2005; Lee *et al*., 2007; Wang *et al*., 2007; Zhou *et al*., 2007; Koh *et al*., 2007; Zong *et al*., 2008; Feizabadi *et al*., 2008; Niumsup *et al*., 2009). In the present study, 99.4% of the carbapenem-resistant *A. baumannii* isolates were confirmed as OXA-23 producers. Insertion element, IS*Aba1* was upstream of the *bla*$_{\text{OXA-23}}$ gene which likely provided a promoter for the gene expression in the isolates. Association of the IS*AbaI* and *bla*$_{\text{OXA-23}}$ has been commonly found in *A. baumannii* as reported in United States, Ireland, Hong Kong, China, Brazil and Norway (Adam Haduch *et al*., 2008; Boo *et al*., 2009; Chu *et al*., 2009; He *et al*., 2011; Carvalho *et al*., 2011; Karah *et*

al., 2011). Besides IS*AbaI*, IS*Aba4* has also been reported related to the $bla_{\text{OXA-23}}$ gene in *A. baumannii* in France and Belgium (Corvec *et al*., 2007; Bogaerts *et al*., 2008).

The $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$ genes are seldom found in *A. baumannii* isolates (references). Neither $bla_{\text{OXA-24}}$ nor $bla_{\text{OXA-58}}$ was found in the *A. baumannii* isolates in this study. In contrast, OXA-24-positive *A. baumannii* isolates have been isolated in the outbreaks in Spain (Bou *et al*., 2000a and Acosta *et al*., 2011) and OXA-58-positive *A. baumannii* isolates were isolated in France (Heritier *et al*., 2005), Italy (Bertini *et al*., 2006) and Greece (Poirel *et al*., 2006 and Pournaras *et al*., 2006).

These findings showed that IS*Aba1*-$bla_{\text{OXA-23}}$ gene was widespread in the carbapenem-resistant *A. baumannii* in the ICU, UMMC. Carbapenem resistance particularly imipenem could be a result of over expression of the $bla_{\text{OXA-23}}$ gene by a promoter located in upstream insertion element IS*Aba1*.

In this study, the carbapenem-susceptible isolates do not harbour $bla_{\text{OXA-23}}$ gene. This is in contrast to the reports from Ireland and Brazil where $bla_{\text{OXA-23}}$ gene was present in the carbapenem-susceptible *A. baumannii* isolates (Boo and Crowley, 2009; Carvalho *et al*., 2011). Although this gene is not detectable in our isolates, laboratory screening of such gene in *A. baumannii* with regardless the carbapenem susceptibility profile is important as it may be silently spread in a hospital environment and poses a possible threat of undetected reservoirs of carbapenemase genes.

5.4 Integrons

Mobile genetic elements such as integrons and plasmids are important in dissemination of antimicrobial resistance among bacterial population. In the present study, integron-typing of the carbapenem-resistant *A. baumannii* isolates revealed class 1 integrons were widely distributed (68.6%) as compared with class 2 integrons which was only found in 10.3% of the carbapenem-resistant *A. baumannii* isolates. These

findings were concordant with other studies that class 1 integrons were the most common integrons found in *A. baumannii* isolates (Gallego and Towner, 2001; Nemec *et al*., 2004b; Abbott *et al.*, 2005; Zarrilli *et al.*, 2007; Sirichot *et al*., 2009; Lin *et al*., 2010) and the rare occurrence of class 2 integrons in *Acinetobacter* spp. (Gonzalez *et al.*, 1998; Seward and Towner 1999; Koeleman *et al.*, 2001; Ramirez *et al*., 2010a). However, class 2 integrons associated with Tn*7* transposon have been reported prevalent in the Chilean and Argentinean *A. baumannii* isolates (Ramirez *et al*., 2005 and Ramirez *et al*., 2010b). Unlike other oxacillanases, OXA-type carbapenemases are not integrated into integrons as gene cassettes (Walther-Ramussen and Hoiby, 2006; Poirel *et al*., 2010). Similar finding was observed in the studied isolates, $bla_{\text{OXA-23}}$ gene was not embedded in the integrons, and hence, suggesting integrons are unlikely mobile vehicle for the $bla_{\text{OXA-23}}$ gene.

Analysis of the DNA sequences of the integron cassette arrays showed that gene-encoding aminoglycoside resistance, *aadB, aadA, aadDA1, aacC1* and *aacA4* were most frequent. This data is in agreement to the aminoglycoside susceptibility profiles of the isolates. Although there were differences in the number and contents of the integron cassette arrays with the 2003-2004 UMMC isolates in another study by Wong *et al*., (2009b), both studies showed that integrons were responsible for the carriage of aminoglycoside resistance genes in *A. baumannii*. Similar finding was also reported in other studies in Spain, Greece, Taiwan and United States (Ribera *et al*., 2004; Kraniotaki *et al*., 2006; Chen *et al*., 2009, Golanbar, *et al*., 2011) the common genes found on *Acinetobacter* integrons are those encoding for aminoglycosides resistance. Aminoglycosides are used for the treatment of infections caused by aminoglycoside-susceptible *A. baumannii* isolates (Maragakis *et al*., 2008). A proper management in the usage of this drug is important to prevent the build-up of selective pressure which could

maintain the persistence of the resistance genes not only in *A. baumannii* but also in other nosocomial pathogens.

We noted that the prevalence of integrons in the isolates has changed over time. A majority of the isolates isolated in 2006 and 2007 harboured integron profile IN1-a, contained gene cassettes of *aacC1-aadDA1-qacEdelta1-sul1*. However, in 2008, isolates with integron profile IN1-b with *aacA4-catB8-aadA* cassette have emerged or became predominant and the three integron bearing 2009 isolates were also harboured the similar cassette array. The presence of class 1 integrons with consideration into cassette array has been suggested as useful marker for identification of epidemic *A. baumannii* isolates (Turton *et al*., 2005; Valenzuela *et al*., 2007). In China, this class 1 integron cassette array, *aacA4-catB8-aadA* has been reported in *A. baumannii* from Nanjing and Shenzhen (Gu *et al*., 2007 and Xu *et al*., 2008). Similar gene cassette has also been reported in *A. baumannii* from Taiwan by Lee *et al*., (2009) and Lin *et al*., (2010). Therefore, these findings postulate that *aacA4-catB8-aadA* is prevalent in the *A. baumannii* in Asia and *A. baumannii* isolates harbouring this cassette may be the epidemic isolates. In addition, the presence of integrons did not correlate with the number of antimicrobial resistance. All the OXA-23-producing isolates were multidrug-resistant to at least 13 antimicrobial agents. However, 55 (31%) isolates did not possess any integron. Isolates harboured gene cassette of *aacC1-aadDA1-qacEdelta1-sul1* and *aacA4-catB8-aadA* were persisted in the ICU's environment throughout 2006 to 2009.

5.5 Plasmid profiles of *A. baumannii*

A majority of *Acinetobacter* species harbours indigenous plasmids (Gerner-Smidt, 1989; Seifert *et al.*, 1994a; Pardesi *et al*., 2007). Analysis of plasmid DNA profiles has been applied for molecular subtyping of *A. baumannii* since 1990 (Garcia *et al*., 1996). In this study, 164 (93.7%) of the 175 carbapenem-resistant *A. baumannii* isolates were

successfully subtyped into 98 different plasmid profiles, ranging from 1.6 kb to 125.1 kb. Six common plasmids, 2.4 kb, 6.8 kb, 21.6 kb, 28.5 kb, 44.8 kb and 47.6 kb were recognized among the plasmid profiles. Similar results were reported by Patwardhan *et al*., (2008), who found multiple plasmid profiles in 26 of *A. baumannii* isolates isolated from clinical samples in India with sizes ranging from 4.0 kb to 50.0 kb. Study by Gallego, (2010) reported the European clone I and clone II *A. baumannii* isolates isolated from a Spanish hospital harboured plasmids of different sizes ranging from 2.5 kb to 125.0 kb, with the 2.5 kb, 8.0 kb and 32.0 kb were the most common in the isolates. Compared to our study, only 11.0%, 17.1% and 12.2% of the isolates were carried the 2.5 kb, 8.0 kb and 32.0 kb, respectively. Three predominant plasmid profiles, P49, P52 and P53 shared the two common plasmids, 6.8 kb and 44.8 kb. These common plasmids were isolated in the 2006, 2008 and 2009 isolates indicating the common plasmids were stable in the isolates and increase of multiple antibiotic resistance in the *A. baumannii* population at these sites may mediated by transfer of the common plasmids. However, no plasmid was detected in 11 carbapenem-resistant *A. baumannii* isolates which were also MDR. Shear damage or co-precipitation of the plasmid DNA with chromosomal DNA during the extraction process may be the reason of undetectable plasmids in the MDR isolates (Hansen and Olsen, 1978). In addition, instability of the plasmids may also the possible cause for the loss of plasmids in the isolates. The absence of the plasmids in MDR isolates may indicate presence of other mobile elements such as integrons and transposons. Of the 11 non-plasmid harboured isolates, 9 isolates had class 1 integrons and 2 isolates do not carry any integron.

Although plasmid analysis has been successfully used as an epidemiological tool for subtyping of *A. baumannii* (Wang *et al*., 2007; Sevillano and Gallego, 2010), it is not a definitive typing method as plasmids can be easily lost, gained or transferred among isolates (van Belkum *et al*., 2007). Therefore, additional molecular typing

methods are recommended for epidemiological studies of *A. baumannii* (van Belkum *et al*., 2007; Snelling *et al*., 1996).

5.7 Location and transferability of *bla*$_{\text{OXA-23}}$ gene

OXA-type carbapenemase genes are known to be plasmid or chromosomal-encoded in *A. baumannii*. Deposition of multiple copies of OXA-type carbapenemase on plasmid and chromosomal has been reported in many studies (Brown and Amyes, 2006). In Spain, Gallego, (2010) has reported plasmid-borne *bla*$_{\text{OXA-40}}$ gene with 32 kb plasmid was predominant in 1999-2005 isolates and 34 kb and 40 kb plasmids in 2008. Similarly, plasmid encoding of *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{OXA-58}}$ has recently been reported in Taiwan (Chen *et al*., 2010), Turkey (Ozen *et al*., 2009) and Bolivia (Sevillano *et al*., 2012)

In this study, in 1 of the 16 OXA-23-producing isolates examined, *bla*$_{\text{OXA-23}}$ was plasmid encoded; 3 isolates had both chromosomal and plasmid-encoded and others were chromosomally encoded. Study conducted by Mendes *et al*., (2009) has reported 32 kb and 44 kb plasmid- and 487 kb chromosomal fragments-borne *bla*$_{\text{OXA-23}}$ in *A. baumannii* isolates in Italy. The *bla*$_{\text{OXA-23}}$ gene has also been found in the 44.8 kb plasmid and 497 kb chromosomal fragments of the studied isolates. The 44.8 kb plasmid was one of the common plasmids observed in these isolates. However, it does not responsible for the dissemination of the *bla*$_{\text{OXA-23}}$ gene as no hybridisation was observed on the 44.8 kb plasmid of the other tested isolates.

A diverse location of the *bla*$_{\text{OXA-23}}$ gene on the chromosome and plasmids among multiples isolates was observed in this study. Transposition of the *bla*$_{\text{OXA-23}}$ gene in *A. baumannii* isolates was associated with the transposon elements (Corvec *et al*., 2007; Mugnier *et al*., 2010; Wang *et al*., 2011). Presence of the transposons will further enhance the potential spread of the *bla*$_{\text{OXA-23}}$ gene among the species. However, study

on transposons is not included in this study. Further study should be carried out to determine whether the Tn*2006*, Tn*2007* and Tn*2008* are responsible in the *bla*$_{\text{OXA-23}}$ gene transposition in the plasmids and chromosome of the isolates.

However, due to its plasmidic location, the dissemination of this gene among the isolates in the hospital settings should be monitored. Le Hello *et al*., (2008) have successfully transferred plasmid harbouring *bla*$_{\text{OXA-23}}$ gene into rifampicin-resistant *A. baumannii* CIP 7020 but at a low frequency. In this present study, the *bla*$_{\text{OXA-23}}$ gene could not able to be transferred into the *E. coli* 5-alpha recipient cells. Although the attempts to transfer the *bla*$_{\text{OXA-23}}$ gene were failed, natural interspecies and intraspecies plasmidic transfer could have occurred naturally in the hospital environment.

5.6 Genetic diversity of *A. baumannii* isolates

In this study, the genetic relatedness of the *A. baumannii* isolates was investigated by PCR fingerprinting (REP-PCR) and PFGE. REP-PCR and PFGE typing had successfully discriminated all the 185 *A. baumannii* isolates into 62 REP types and 98 pulsotypes, respectively. PFGE gave a high discriminative index (D=0.98) in subtyping the isolates. This method has been recognized in other studies as the most discriminative methods for molecular subtyping of *A. baumannii* isolates (Silbert *et al*., 2004). However, REP-PCR technique is also useful and has comparable discrimination with PFGE, showing discriminative index of D=0.96 and is in agreement to the previously study by Bou *et al*., (2000), Saeed *et al*., (2006) and Kohlenberg *et al*., (2009). REP-PCR has also been successfully differentiated the *A. baumannii* lineage isolates from the *Acinetobacter* spp. isolates isolated from the similar hospital (Misbah *et al*., 2004).

Comparison of the REP-PCR and PFGE cluster analyses showed there was a correlation in the isolates grouping. Both typing methods had distinctly discriminated

the non-MDR from the MDR isolates. The non-MDR isolates had wide genetic variability among one another. PFGE showed the carbapenem-susceptible isolates shared a close genetic similarity compared to REP-PCR. However, these typing methods do not distinguish the carbapenem-susceptible isolates from the carbapenem-resistant isolates as they shared close genetic relatedness, at more than 70% of similarity. Isolates which gathered in same cluster in REP-PCR were determined as similar clone by PFGE.

REP-PCR and PFGE clustering of the *A. baumannii* isolates also showed an association with the presence of mobile genetic elements, integrons and plasmids. Isolates harboured similar integron profiles were clustered into similar clusters by REP-PCR or similar clone by PFGE. In addition, isolates harboured predominant plasmid profiles P49, P52 and P53 were grouped in cluster C and cluster D in REP-PCR cluster analysis, while determined as clone AC15, AC16 and AC17 by PFGE.

The OXA-23-producing *A. baumannii* isolates recovered from patients during the periods of increased incidence of infections reported in 2006 was mainly of clone AC2 isolates. Three environmental isolates and a isolate from the hands of a HCW screened on April and August 2006, following the high incidence of *A. baumannii* infections was of similar clone, AC2. This suggesting possible transmission route may have occurred between the patients, healthcare workers and the fomites, although the direction was unknown.

Clone AC15 were believed to be endemic in the ICU ward of UMMC throughout the study period. An indication of this endemic clone of OXA-23 producing *A. baumannii* isolates was the observed persistence of environmental isolates and multiple isolates of similar clone isolated from the patients admitted in 2006 to 2009. Isolates of this clone AC15 shared closely related resistance phenotypes, integron and plasmid profiles. The selective pressure from intense antibiotic usage could have prolonged its

survival in the environment. Close genetic relatedness between the environmental, hands of healthcare worker and the clinical isolates also indicated the presence of a reservoir of *A. baumannii* contamination in the ICU, UMMC. Therefore, careful hand hygiene and environmental cleaning would contribute to the successful control of MDR *A. baumannii* outbreak in ICUs.

An occurrence of new *A. baumannii* clone, AC17 was observed in the ICU ward. These AC17 isolates were first observed and had emerged in 2009. Based on PFGE analysis, this clone of isolates shared <66% of similarity with the endemic *A. baumannii* clone AC15. However, these isolates shared very similar resistance characteristics with the environmental isolate which also did not harbour integrons and carried similar plasmid profiles, P52. In addition, these isolates were also resistant to the similar antimicrobial agents, except few of the AC17 isolates were resistant to cefoperazone/sulbactam. Therefore, these clone AC17 isolates may have derived from the endemic clone AC15 isolate which was first isolated in 2006 and has persisted in the hospital settings.

In general, the two different molecular typing methods applied in this study have successfully discriminated the *A. baumannii* isolates. However, PFGE remains as the gold standard for subtyping of *A. baumannii* isolates due to its stability, high discriminatory and high reproducibility (Corbella *et al*., 2000; Seifert *et al*., 2005). Using a standardized PFGE protocols, interlaboratory comparisons of the *A. baumannii* isolates is available. However, this method is labour-intensive which requires at least 3 days generating the results. In addition, PFGE is cost-consuming and requires special pulsed-field apparatus that is expensive and mostly not available in the hospital laboratories. PCR based fingerprinting, REP-PCR, could be useful as an alternative subtyping tool for *A. baumannii* isolates. REP-PCR is easier and could generate results more rapidly than PFGE. It is important for identifying the source of infection and the

spread of the organism during an outbreak in a hospital setting (Snelling *et al*., 1996). However, REP-PCR is not suitable for interlaboratory and epidemiological studies as its instability and low reproducibility level.

CHAPTER 6: CONCLUSION

A. baumannii is an important nosocomial pathogen causing infections to the immunocompromised patients in hospitals. Since the beginning of 1990s, the prevalence of multidrug-resistant (MDR) *A. baumannii* has been increased and widely reported in many countries.

Species level identification of the *Acinetobacter* spp. is important as most of the outbreak infections in the hospitals involved *A. baumannii*. ARDRA is useful and capable to differentiate *A. baumannii* from other *A. calcoaceticus-A. baumannii* complex isolates, and results was supported by the presence of the intrinsic $bla_{\text{OXA-51}}$ gene in *A. baumannii*.

Antibiotic susceptibility test showed the 178 isolates including 7 environmental and a isolate from hands of HCW was MDR to at least 10 antimicrobial agents tested. All the isolates were highly resistant to the carbapenem, the drugs of choice used in the treatment of *A. baumannii* infections. An emergence of cefaperazone/sulbactam-resistant isolates was observed in 2007. Polymyxin B has remained as the most effective antimicrobial agent against the *A. baumannii* isolates.

Carbapenem resistance in the *A. baumannii* isolates was mediated by the presence of the $bla_{\text{OXA-23}}$ gene with an insertion element, IS*Aba1* upstream of the gene. Majority of the isolates harboured class 1 integrons with some also carried class 2 integrons. Integron cassette arrays contained mostly the gene encoding resistance to aminoglycoside which supported the resistance profile of the isolates. However, IS*Aba1*-$bla_{\text{OXA-23}}$ gene were not integrated in the integron cassette arrays.

Plasmid profiling showed majority of the *A. baumannii* isolates harboured plasmids sizes ranging from 1.6 kb to 125.1 kb. Common plasmids 6.8 kb and 44.8 kb were present stably in the isolates as observed in 2006, 2008 and 2009 isolates. The $bla_{\text{OXA-23}}$ gene was plasmid- and chromosomal-mediated, found on diverse locations on

the plasmid and chromosomal fragments. Transferability of the plasmid-borne $bla_{\text{OXA-23}}$ was unsuccessful; however, possible transmission of the resistance genes among the species could have occurred in nature environment.

Integrons typing, plasmid profiling, REP-PCR and PFGE typing were proven reliable epidemiological markers for *Acinetobacter*. Genotyping of the isolates by REP-PCR and PFGE showed a correlation with the presence of integrons and plasmids in the isolates. A reservoir of *A. baumannii* contamination in the ICU, UMMC were determined. PFGE has confirmed the presence of an endemic clone AC15 and newly emerged clone AC17 in the ICU, UMMC. REP-PCR could be an alternative tool for molecular subtyping of *A. baumannii* isolates as it gave high discriminatory power and is comparable to PFGE.

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APPENDIX I

List of *A. baumannii* isolates collected from ICU, UMMC from year 2006-May 2009

Isolates collected from patients admitted to ICU, UMMC

No	Isolate code	Patient'sWard	Date of specimen sent	Specimen	Organisms	Month	Year	ARDRA confirmation
1	AC/0601-5	ICU	8-Jan	T/sec	*A. baumannii*	January	2006	*A. baumannii*
2	AC/0601-8	ICU	12-Jan	T/sec	*A. baumannii*	January	2006	*A. baumannii*
3	AC/0601-10	ICU	17-Jan	T/sec	*A. baumannii*	January	2006	*A. baumannii*
4	AC/0602-19	ICU	22-Feb	Sputum	*A. baumannii*	February	2006	*A. baumannii*
5	AC/0603-1	ICU	2-Mar	Sputum	*A. baumannii*	March	2006	*A. baumannii*
6	AC/0603-2	ICU	2-Mar	T/asp	*A. baumannii*	March	2006	*A. baumannii*
7	AC/0603-7	ICU	8-Mar	Tripple lumen tip	*A. baumannii*	March	2006	*A. baumannii*
8	AC/0603-9	ICU	13-Mar	T/sec	*A. baumannii*	March	2006	*A. baumannii*
9	AC/0603-22	ICU	25-Mar	CVL tip	*A. baumannii*	March	2006	*A. baumannii*
10	AC/0603-25	12U→ICU	28-Mar	T/sec	*A. baumannii*	March	2006	*A. baumannii*
11	AC/0603-26	ICU	29-Mar	T/sec	*A. baumannii*	March	2006	*A. baumannii*
12	AC/0604-6	ICU	5-Apr	Abdomen wound swab	*A. baumannii*	April	2006	*A. baumannii*
13	AC/0604-7	ICU	4-Apr	Peritoneal swab	*A. baumannii*	April	2006	*A. baumannii*
14	AC/0604-11	ICU	8-Apr	T/sec	*A. baumannii*	April	2006	*A. baumannii*
15	AC/0604-25	7U→ICU	25-Apr	T/sec	*A. baumannii*	April	2006	*A. baumannii*
16	AC/0605-3	ICU	9-May	T/sec	*A. baumannii*	May	2006	*A. baumannii*
17	AC/0605-19	ICU	24-May	Wound swab	*A. baumannii*	May	2006	*A. baumannii*
18	AC/0605-25	8D→ICU	28-May	T/sec	*A. baumannii*	May	2006	*A. baumannii*
19	AC 0606-11	ICU	15-Jun	T/sec	*A. baumannii*	June	2006	*A. baumannii*
20	AC 0606-13	ICU	15-Jun	T/sec	*A. baumannii*	June	2006	*A. baumannii*
21	AC 0606-16	ICU	22-Jun	BAL	*A. baumannii*	June	2006	*A. baumannii*
22	AC 0606-22	ICU	26-Jun	T/sec	*A. baumannii*	June	2006	*A. baumannii*

23	AC 0606-23	ICU	27-Jun	Abdomen swab	*A. baumannii*	June	2006	*A. baumannii*
24	AC 0606-24	ICU	29-Jun	Blood	*A. baumannii*	June	2006	*A. baumannii*
25	AC 0607-2	ICU	5-Jul	Right Lung Empyema fluid	*A. baumannii*	July	2006	*A. baumannii*
26	AC 0607-6	ICU	6-Jul	Pus swab from back carbuncle	*A. baumannii*	July	2006	*A. baumannii*
27	AC 0607-12	ICU	8-Jul	CVL site pus swab	*A. baumannii*	July	2006	*A. baumannii*
28	AC/0607-18	ICU	18-Jul	T/sec	*A. baumannii*	July	2006	*A. baumannii*
29	AC 0607-19	ICU	19-Jul	Blood	*A. baumannii*	July	2006	*A. baumannii*
30	AC 0607-20	ICU	19-Jul	Tripple lumen tip	*A. baumannii*	July	2006	*A. baumannii*
31	AC 0607-22	ICU	20-Jul	T/sec	*A. baumannii*	July	2006	*A. baumannii*
32	AC 0607-25	ICU	25-Jul	T/sec	*A. baumannii*	July	2006	*A. baumannii*
33	AC 0607-28	ICU	26-Jul	T/sec	*A. baumannii*	July	2006	*A. baumannii*
34	AC 0608-1	ICU	1-Aug	Wound	*A. baumannii*	August	2006	*A. baumannii*
35	AC 0608-5	ICU	5-Aug	BAL	*A. baumannii*	August	2006	*A. baumannii*
36	AC 0608-7	4U→ICU	9-Aug	Tripple lumen tip	*A. baumannii*	August	2006	*A. baumannii*
37	AC 0608-17	ICU	26-Aug	Tissue	*A. baumannii*	August	2006	*A. baumannii*
38	AC 0608-22	ICU	29-Aug	BAL	*A. baumannii*	August	2006	*A. baumannii*
39	AC 0609-1	ICU	1-Sep	Urine	*A. baumannii*	September	2006	*A. baumannii*
40	AC 0609-6	ICU	11-Sep	T/sec	*A. baumannii*	September	2006	*A. baumannii*
41	AC 0609-8	ICU	11-Sep	CVL tip	*A. baumannii*	September	2006	*A. baumannii*
42	AC 0609-10	ICU	13-Sep	Peritoneal fluid	*A. baumannii*	September	2006	*A. baumannii*
43	AC 0609-14	ICU	14-Sep	T/sec	*A. baumannii*	September	2006	*A. baumannii*
44	AC 0609-25	ICU	26-Sep	Catheter/tube site	*A. baumannii*	September	2006	*A. baumannii*
45	AC 0610-2	ICU	5-Oct	Double lumen tip	*A. baumannii*	October	2006	*A. baumannii*
46	AC 0610-8	ICU	16-Oct	Blood swab	*A. baumannii*	October	2006	*A. baumannii*
47	AC 0610-9	ICU	20-Oct	T/sec	*A. baumannii*	October	2006	*A. baumannii*
48	AC 0610-12	ICU	23-Oct	Double lumen tip	*A. baumannii*	October	2006	*A. baumannii*
49	AC 0610-18	ICU	30-Oct	T/sec	*A. baumannii*	October	2006	*A. baumannii*
50	AC 0611-5	ICU	5-Nov	Nasal	*A. baumannii*	November	2006	*A. baumannii*

51	AC 0611-7	ICU	3-Nov	Blood	*A. baumannii*	November	2006	*A. baumannii*
52	AC 0611-10	ICU	8-Nov	Right knee pus swab	*A. baumannii*	November	2006	*A. baumannii*
53	AC 0611-11	ICU	12-Nov	T/sec	*A. baumannii*	November	2006	*A. baumannii*
54	AC/0611-15	ICU	15-Nov	T/sec	*A. baumannii*	November	2006	*A. baumannii*
55	AC/0611-16	ICU	15-Nov	Tracheal aspirate	*A. baumannii*	November	2006	*A. baumannii*
56	AC 0611-18	ICU	22-Nov	T/sec	*A. baumannii*	November	2006	*A. baumannii*
57	AC 0611-19	ICU	21-Nov	Tripple lumen tip	*A. baumannii*	November	2006	*A. baumannii*
58	AC/0612-7	ICU	12-Dec	Double lumen tip	*A. baumannii*	December	2006	*A. baumannii*
59	AC/0612-13	ICU	26-Dec	T/sec	*A. baumannii*	December	2006	*A. baumannii*
60	AC/0612-16	ICU	29-Dec	Peritoneal fluid	*A. baumannii*	December	2006	*A. baumannii*
61	AC/0612-17	ICU	31-Dec	Peritoneal fluid	*A. baumannii*	December	2006	*A. baumannii*
62	AC/0701-11	ICU	22-Jan	Blood	*A. baumannii*	January	2007	*A. baumannii*
63	AC/0702-5	ICU	12-Feb	T/sec	*A. baumannii*	February	2007	*A. baumannii*
64	AC/0702-17	ICU	27-Feb	Tripple lumen tip	*A. baumannii*	February	2007	*A. baumannii*
65	AC/0703-14	ICU	16-Mar	T/sec	*A. baumannii*	March	2007	*A. baumannii*
66	AC/0703-21	ICU	19-Mar	T/sec	*A. baumannii*	March	2007	*A. baumannii*
67	AC/0704-7	ICU	9-Apr	T/sec	*A. baumannii*	April	2007	*A. baumannii*
68	AC/0705-3	ICU	2-May	T/sec	*A. baumannii*	May	2007	*A. baumannii*
69	AC/0705-9	ICU	18-May	T/sec	*A. baumannii*	May	2007	*A. baumannii*
70	AC/0705-15	ICU	27-May	T/sec	*A. baumannii*	May	2007	*A. baumannii*
71	AC/0706-21	ICU	29-Jun	T/sec	*A. baumannii*	June	2007	*A. baumannii*
72	AC/0707-8	ICU	5-Jul	Peritoneal fluid	*A. baumannii*	July	2007	*A. baumannii*
73	AC/0707-13	ICU	13-Jul	Wound	*A. baumannii*	July	2007	*A. baumannii*
74	AC/0707-26	ICU	29-Jul	T/sec	*A. baumannii*	July	2007	*A. baumannii*
75	AC/0708-10	ICU	8-Aug	Swab	*A. baumannii*	August	2007	*A. baumannii*
76	AC/0708-16	ICU	23-Aug	Tripple lumen tip	*A. baumannii*	August	2007	*A. baumannii*
77	AC/0708-20	ICU	31-Aug	Tripple lumen tip	*A. baumannii*	August	2007	*A. baumannii*
78	AC/0709-5	ICU	6-Sep	T/sec	*A. baumannii*	September	2007	*A. baumannii*
79	AC/0709-6	ICU	7-Sep	T/sec	*A. baumannii*	September	2007	*A. baumannii*

80	AC/0709-7	ICU	7-Sep	T/sec	*A. baumannii*	September	2007	*A. baumannii*
81	AC/0709-8	ICU	8-Sep	T/sec	*A. baumannii*	September	2007	*A. baumannii*
82	AC/0709-27	ICU	23-Sep	T/sec	*A. baumannii*	September	2007	*A. baumannii*
83	AC/0710-3	ICU	5-Oct	T/sec	*A. baumannii*	October	2007	*A. baumannii*
84	AC/0711-7	ICU	5-Nov	T/sec	*A. baumannii*	November	2007	*A. baumannii*
85	AC/0712-13	ICU	28-Dec	Swab	*A. baumannii*	December	2007	*A. baumannii*
86	AC/0712-3	ICU	13-Dec	T/sec	*A. baumannii*	December	2007	*A. baumannii*
87	AC/0801-4	ICU	3-Jan	BAL	*A. baumannii*	January	2008	*A. baumannii*
88	AC/0801-6	ICU	12-Jan	T/sec	*A. baumannii*	January	2008	*A. baumannii*
89	AC/0801-11	ICU	21-Jan	T/sec	*A. baumannii*	January	2008	*A. baumannii*
90	AC/0801-13	ICU	31-Jan	Peritoneal Fluid	*A. baumannii*	January	2008	*A. baumannii*
91	AC/0802-1	ICU	1-Feb	Wound	*A. baumannii*	February	2008	*A. baumannii*
92	AC/0802-4	ICU	1-Feb	T/sec	*A. baumannii*	February	2008	*A. baumannii*
93	AC/0802-14	ICU	15-Feb	Peritoneal Fluid	*A. baumannii*	February	2008	*A. baumannii*
94	AC/0802-20	ICU	28-Feb	Sputum	*A. baumannii*	February	2008	*A. baumannii*
95	AC/0803-15	ICU	24-Mar	Swab	*A. baumannii*	March	2008	*A. baumannii*
96	AC/0804-4	ICU	4-Apr	T/sec	*A. baumannii*	April	2008	*A. baumannii*
97	AC/0804-19	ICU	17-Apr	T/sec	*A. baumannii*	April	2008	*A. baumannii*
98	AC/0804-24	ICU	19-Apr	Blood	*A. baumannii*	April	2008	*A. baumannii*
99	AC/0804-31	ICU	28-Apr	CVL tip	*A. baumannii*	April	2008	*A. baumannii*
100	AC/0804-32	ICU	28-Apr	Tissue	*A. baumannii*	April	2008	*A. baumannii*
101	AC/0805-4	ICU	6-May	Wound	*A. baumannii*	May	2008	*A. baumannii*
102	AC/0805-5	ICU	8-May	Tripple Lumen tip	*A. baumannii*	May	2008	*A. baumannii*
103	AC/0805-20	ICU	24-May	Swab	*A. baumannii*	May	2008	*A. baumannii*
104	AC/0806-4	ICU	3-Jun	BAL	*A. baumannii*	June	2008	*A. baumannii*
105	AC/0806-10	ICU	9-Jun	Wound	*A. baumannii*	June	2008	*A. baumannii*
106	AC/0806-14	ICU	17-Jun	T/sec	*A. baumannii*	June	2008	*Acinetobacter* genospecies 13TU
107	AC/0806-18	ICU	20-Jun	T/sec	*A. baumannii*	June	2008	*A. baumannii*

108	AC/0806-23	ICU	20-Jun	Swab	*A. baumannii*	June	2008	*A. baumannii*
109	AC/0806-24	ICU	23-Jun	Nasal	*A. baumannii*	June	2008	*A. baumannii*
110	AC/0806-28	ICU	26-Jun	Sputum	*A. baumannii*	June	2008	*A. baumannii*
111	AC/0807-20	ICU	23-Jul	T/sec	*A. baumannii*	July	2008	*A. baumannii*
112	AC/0808-6	ICU	6-Aug	T/sec	*A. baumannii*	August	2008	*A. baumannii*
113	AC/0808-14	ICU	13-Aug	Tripple Lumen tip	*A. baumannii*	August	2008	*A. baumannii*
114	AC/0808-18	ICU	14-Aug	Tripple Lumen tip	*A. baumannii*	August	2008	*A. baumannii*
115	AC/0808-20	ICU	16-Aug	Tripple Lumen tip	*A. baumannii*	August	2008	*A. baumannii*
116	AC/0809-1	ICU	31-Aug	T/sec	*A. baumannii*	August	2008	*A. baumannii*
117	AC/0809-9	ICU	5-Sep	T/sec	*A. baumannii*	September	2008	*A. baumannii*
118	AC/0809-12	ICU	10-Sep	Peritoneal Fluid	*A. baumannii*	September	2008	*A. baumannii*
119	AC/0809-29	ICU	30-Sep	T/sec	*A. baumannii*	September	2008	*A. baumannii*
120	AC/0809-30	ICU	30-Sep	T/sec	*A. baumannii*	September	2008	*A. baumannii*
121	AC/0810-8	ICU	8-Oct	T/sec	*A. baumannii*	October	2008	*A. baumannii*
122	AC/0810-11	ICU	15-Oct	Urine	*A. baumannii*	October	2008	*A. baumannii*
123	AC/0810-12	ICU	15-Oct	T/sec	*A. baumannii*	October	2008	*A. baumannii*
124	AC/0810-22	ICU	22-Oct	T/sec	*A. baumannii*	October	2008	*A. baumannii*
125	AC/0810-26	ICU	27-Oct	T/sec	*A. baumannii*	October	2008	*A. baumannii*
126	AC/0811-12	ICU	11-Nov	BAL	*A. baumannii*	November	2008	*A. baumannii*
127	AC/0811-13	ICU	10-Nov	T/sec	*A. baumannii*	November	2008	*A. baumannii*
128	AC/0811-15	ICU	14-Nov	T/sec	*A. baumannii*	November	2008	*A. baumannii*
129	AC/0811-25	ICU	25-Nov	Blood	*A. baumannii*	November	2008	*A. baumannii*
130	AC/0812-1	ICU	2-Dec	T/sec	*A. baumannii*	December	2008	*A. baumannii*
131	AC/0812-8	ICU	7-Dec	T/sec	*A. baumannii*	December	2008	*A. baumannii*
132	AC/0812-16	ICU	16-Dec	T/sec	*A. baumannii*	December	2008	*A. baumannii*
133	AC/0812-29	ICU	23-Dec	Blood	*A. baumannii*	December	2008	*A. baumannii*
134	AC/0812-33	ICU	31-Dec	Wound	*A. baumannii*	December	2008	*A. baumannii*
135	AC/0901-5	ICU	5-Jan	T/sec	*A. baumannii*	January	2009	*A. baumannii*
136	AC/0901-14	ICU	13-Jan	Sputum	*A. baumannii*	January	2009	*A. baumannii*

137	AC/0901-36	ICU	28-Jan	T/sec	*A. baumannii*	January	2009	*A. baumannii*
138	AC/0901-37	ICU	29-Jan	T/sec	*A. baumannii*	January	2009	*A. baumannii*
139	AC/0902-5	ICU	14-Feb	Tripple Lumen tip	*A. baumannii*	February	2009	*A. baumannii*
140	AC/0902-6	ICU	14-Feb	T/sec	*A. baumannii*	February	2009	*A. baumannii*
141	AC/0902-13	ICU	23-Feb	T/sec	*A. baumannii*	February	2009	*A. baumannii*
142	AC/0902-14	ICU	28-Feb	Sputum	*A. baumannii*	February	2009	*A. baumannii*
143	AC/0902-15	ICU	23-Feb	Blood	*A. baumannii*	February	2009	*A. baumannii*
144	AC/0902-19	ICU	26-Feb	Urine	*A. baumannii*	February	2009	*A. baumannii*
145	AC/0903-15	ICU	11-Mar	Wound	*A. baumannii*	March	2009	*A. baumannii*
146	AC/0903-19	ICU	10-Mar	T/sec	*A. baumannii*	March	2009	*A. baumannii*
147	AC/0903-21	ICU	13-Mar	T/sec	*A. baumannii*	March	2009	*A. baumannii*
148	AC/0903-28	ICU	19-Mar	Peritoneal Fluid	*A. baumannii*	March	2009	*A. baumannii*
149	AC/0903-29	ICU	21-Mar	T/sec	*A. baumannii*	March	2009	*A. baumannii*
150	AC/0903-31	ICU	23-Mar	T/sec	*A. baumannii*	March	2009	*A. baumannii*
151	AC/0904-3	ICU	2-Apr	T/sec	*A. baumannii*	April	2009	*A. baumannii*
152	AC/0904-7	ICU	30-Mar	Blood	*A. baumannii*	March	2009	*A. baumannii*
153	AC/0904-15	ICU	10-Apr	T/sec	*A. baumannii*	April	2009	*A. baumannii*
154	AC/0904-19	ICU	13-Apr	Pus	*A. baumannii*	April	2009	*A. baumannii*
155	AC/0904-20	ICU	13-Apr	T/sec	*A. baumannii*	April	2009	*A. baumannii*
156	AC/0904-21	ICU	13-Apr	T/sec	*A. baumannii*	April	2009	*A. baumannii*
157	AC/0904-28	ICU	16-Apr	CVL tip	*A. baumannii*	April	2009	*A. baumannii*
158	AC/0904-39	ICU	23-Apr	T/sec	*A. baumannii*	April	2009	*A. baumannii*
159	AC/0904-40	ICU	24-Apr	Swab	*A. baumannii*	April	2009	*A. baumannii*
160	AC/0904-42	ICU	27-Apr	Blood	*A. baumannii*	April	2009	*A. baumannii*
161	AC/0904-43	ICU	28-Apr	Wound	*A. baumannii*	April	2009	*A. baumannii*
162	AC/0905-2	ICU	4-May	T/sec	*A. baumannii*	May	2009	*A. baumannii*
163	AC/0905-6	ICU	6-May	T/sec	*A. baumannii*	May	2009	*A. baumannii*
164	AC/0905-21	ICU	14-May	Tissue	*A. baumannii*	May	2009	*A. baumannii*
165	AC/0905-22	ICU	12-May	T/sec	*A. baumannii*	May	2009	*A. baumannii*

166	AC/0905-31	ICU	18-May	T/sec	*A. baumannii*	May	2009	*A. baumannii*
167	AC/0905-42	ICU	21-May	T/sec	*A. baumannii*	May	2009	*A. baumannii*
168	AC/0905-49	ICU	26-May	T/sec	*A. baumannii*	May	2009	*A. baumannii*
169	AC/0905-53	ICU	27-May	T/sec	*A. baumannii*	May	2009	*A. baumannii*
170	AC/0905-58	ICU	30-May	Swab	*A. baumannii*	May	2009	*A. baumannii*
171	AC/0905-60	ICU	30-May	T/sec	*A. baumannii*	May	2009	*A. baumannii*

Abbreviations: ICU, Intensive care unit; T/sec, tracheal secretion; T/asp, tracheal aspirate; CVL, central venous line; BAL, bronchoalveolar lavage;

APPENDIX I (continued…)

Isolates collected from environment and hands of healthcare workers screening during the high incidence in ICU

No	Isolate code	Date of specimen sent	Isolation site	Organisms	Month	Year	ARDRA confirmation
1	ACIBA 2006 1	12-Apr	ICU 2 overhead table R12 (No patient)	*A. baumannii*	April	2006	*A. baumannii*
2	ACIBA 2006 2	12-Apr	ICU 7 bed R3 (No patient)	*A. baumannii*	April	2006	*A. baumannii*
3	ACIBA 2006 36	11-Aug	Bucket	*A. baumannii*	August	2006	*A. baumannii*
4	ACIBA 2006 43	11-Aug	Clean wash basin	*A. baumannii*	August	2006	*A. baumannii*
5	ACIBA 2006 46	15-Aug	Clean ventilator	*A. baumannii*	August	2006	*A. baumannii*
6	ACIBA 2006 47	15-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*A. baumannii*
7	ACIBA 2006 49	15-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*A. baumannii*
8	ACIBA 2006 50	15-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*A. baumannii*
9	ACIBA 2006 51	15-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*A. baumannii*
10	ACIBA 2006 52	15-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*A. baumannii*
11	ACIBA 2006 53	15-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*A. baumannii*
12	ACIBA 2006 56	16-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*A. baumannii*
13	ACIBA 2006 57	16-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*A. baumannii*
14	ACIBA 2006 58	16-Aug	Healthcare worker's fingerprint	*A. baumannii*	August	2006	*Acinetobacter* genospecies 15TU
15	ACIBA 2006 63	18-Aug	Pillow Sari RM 6	*A. baumannii*	August	2006	*A. baumannii*
16	ACIBA 2006 65	18-Aug	Mattress RM 17	*A. baumannii*	August	2006	*A. baumannii*
17	ACIBA 2006 66	18-Aug	Sink B18	*A. baumannii*	August	2006	*Acinetobacter* genospecies 13TU
18	ACIBA 2006 73	26-Sep	Mop ICU	*A. baumannii*	September	2006	*Acinetobacter* genospecies 13TU

APPENDIX II

Growth media, buffers, solutions/reagents and other chemicals

All the growth media, buffers, solutions/reagents and other chemicals were sterilized by autoclaving at 121 °C and 15 psi for 15 minutes, unless otherwise stated.

1. Brain Heart Infusion (BHI) agar

BHI agar powder (Oxoid Ltd., England)	14.1 g
Distilled water	300 ml

2. Brain Heart Infusion (BHI) broth

BHI agar powder (Oxoid Ltd., England)	11.1 g
Distilled water	300 ml

3. Mueller Hinton (MH) agar

MH agar powder (Oxoid Ltd., England)	11.4 g
Distilled water	300 ml

4. SOB medium

Tryptone (Oxoid Ltd., England)	2 g
Yeast extract (Oxoid Ltd., England)	0.5 g
NaCl (BDH Lab Supplies, England)	0.058 g
KCl (BDH Lab Supplies, England)	0.019 g
$MgCl_2$ (Oxoid Ltd., England)	0.203 g
$MgSO_4$ (Oxoid Ltd., England)	0.247 g
Distilled water	100 ml

5. Glucose solution (100 mM)

Glucose powder (BDH Lab Supplies, England)	0.9 g
Distilled water	50 ml

The glucose powder was stirred until fully dissolved in the distilled water and then filter sterilized through a 0.2 μM filter membrane (Millipore, Cork, Ireland)

6. SOC medium

SOB medium	80 ml
Glucose solution (100mM)	20 ml

Do not autoclave.

7. Tryptone Soya Agar (TSA)

TSA powder (Oxoid Ltd., England)	12 g
Distilled water	300 ml

8. 50% glycerol

Ultra pure glycerol (Invitrogen, USA)	50 ml
Distilled water	50 ml

9. 0.85 % NaCl (saline)

NaCl (BDH Lab Supplies, England)	0.85 g
Distilled water	100 ml

10. 10X Tris-EDTA buffer (TBE, pH 8.3)

Trizma base (Sigma-Aldrich, USA)	121.2 g
Orthoboric/Boric acid (BDH Lab Supplies, England)	61.8 g
EDTA (Invitrogen, USA)	0.745 g
Deionised water	1000 ml

At first, the above ingredients were dissolved in 500 ml of deionised water by stirring on the heated magnetic stirrer plate. Then buffer was left to cool to room temperature and pH was adjusted to 8.3 and top up to 1000 ml with deionised water.

11. 0.5X TBE

10X TBE buffer (pH 8.3)	50 ml
Deionised water top up to	1000 ml

Sterilized by autoclaving is unnecessary.

12. 1 M Tris, pH 8.0

Trizma-base (Sigma-Aldrich, USA)	60.57 g
Deionised water	250 ml

The trizma-base powder was dissolved in 250 ml of deionised water by stirring on the heated magnetic stirrer plate. The buffer was left to cool to room temperature and pH was adjusted to 8.0. Then the buffer was top up to 500 ml.

13. 0.5 M EDTA, pH 8.0

EDTA (Invitrogen, USA)	93.06 g
Deionised water	250 ml

Few pellets of sodium hydroxide (BDH Lab Supplies, England) were added slowly to dissolve the EDTA on the heated magnetic stirrer plate. The buffer was left to

cool to room temperature and pH was adjusted to 8.0. Then the buffer was top up to 500 ml.

14. Tris-EDTA (TE) buffer (10 mM Tris ; 1 mM EDTA; pH 8.0)

1 M Tris, pH 8.0	10 ml
0.5 M EDTA, pH 8.0	2 ml
Top up with deionised water to	1000 ml

15. Cell Suspension Buffer (100 mM Tris; 100 mM EDTA; pH 8.0)

1 M Tris, pH 8.0	30 ml
0.5 M EDTA, pH 8.0	60 ml
Top up with deionised water to	300 ml

16. 10 % Sarcosyl (N-Lauryl-Sarcosine, Sodium Salt)

Sodium N-Lauryl-Sarcosinate solution (Sigma-Aldrich, USA)	10 ml
Top up with deionised water to	100 ml

17. Cell Lysis Buffer (50 mM Tris; 50 mM EDTA, pH 8.0; 1 % Sarcosine)

1 M Tris, pH 8.0	25 ml
0.5 M EDTA, pH 8.0	50 ml
10 % Sarcosyl	50 ml
Top up with deionised water to	500 ml

18. Depurination solution (250mM HCl)

Concentrated HCl (37.5%) (BDH Lab Supplies, England)	21 ml
Top up with sterilized distilled water to	1000 ml

Do not autoclave.

19. Denaturation solution (0.5 M NaOH; 1.5 M NaCl)

NaCl (BDH Lab Supplies, England)	87.66 g
Distilled water	800 ml

Autoclaved at 121 °C and 15 psi for 15 minutes.

NaOH (BDH Lab Supplies, England)	20 g

NaOH pellets were added and dissolved in the autoclaved NaCl solution and top up by sterilized distilled water to 1000 ml.

20. Neutralization solution (0.5 M Tris; 1.5 M NaCl)

Tris-HCL (Promega, Madison, USA)	60.57 g
NaCl (BDH Lab Supplies, England)	87.66 g
Distilled water	700 ml

The Tris-HCl and NaCl powders were dissolved in 700 ml of distilled water by stirring on the heated magnetic stirrer plate. pH was adjusted to 7.5 and finally top up to 1000 ml.

21. 10% Sodium dodecyl sulphate (SDS)

SDS powder (Sigma-Aldrich, USA)	10 g
Distilled water	100 ml

The SDS powder was slowly dissolved in 100 ml of distilled water in 65 °C water bath. Do not autoclave.

22. 1 M Sodium hydroxide solution (NaOH)

NaOH (BDH Lab Supplies, England)	40 g
Sterilized distilled water	100 ml

NaOH pellets were dissolved in 100 ml sterilized distilled water without autoclave.

23. 2X SSC (Saline-sodium citrate)

20X SSC solution (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany)	10 ml
Top up with sterilized distilled water to	100 ml

Do not autoclave.

24. Low Stringency Buffer (LSB) (2X SSC; 0.1 % SDS)

20X SSC solution (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany)	20 ml
10% SDS solution	2 ml
Top up with sterilized distilled water to	200 ml

Do not autoclave.

25. High Stringency Buffer (HSB) (0.5X SSC; 0.1 % SDS)

20X SSC solution (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany)	5 ml
10% SDS solution	2 ml
Top up with sterilized distilled water to	200 ml

Do not autoclave.

26. DIG Wash and Block Buffer set

DIG Wash and Block Buffer set was purchased from Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany and contained 5 bottles of 1 L buffers:

a) 10X Washing Buffer (2 bottles)

b) 10X Blocking Solution

c) 10X Detection Buffer

d) 10X Maleic Acid Solution

27. 1X Washing Buffer

10X Washing Buffer	20 ml
Top up with sterilized distilled water to	200 ml

Clean and sterilized glassware was used. Do not autoclave the buffer.

28. 1X Maleic Acid

10X Maleic Acid solution	50 ml
Top up with sterilized distilled water to	500 ml

Clean and sterilized glassware was used. Do not autoclave the solution.

29. 1X Blocking Solution

10X Blocking Solution	13 ml
Diluted with 1X Maleic Acid to	130 ml

Clean and sterilized glassware was used. Do not autoclave the solution.

30. 1X Detection Buffer

10X Detection Buffer	3.1 ml
Top up with sterilized distilled water to	31 ml

Clean and sterilized glassware was used. Do not autoclave the buffer.

31. Antibody Solution

Anti-digoxigenin-AP, Fab fragments, (750 U/ml)	3 µl
(Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany)	
1X Blocking Solution	29.997 ml

The Anti-Digoxigenin-AP was centrifuged at 7, 500 x g for 10 minutes before adding into 1X blocking solution.

Clean and sterilized glassware was used. Do not autoclave the solution.

32. Chemiluminescent substrate, CSPD solution

CSPD (11.6 mg/ml)	10 µl
1X Detection Buffer	990 µl

The CSPD substrate can be reused up to 3 times and was stored in dark at 4 °C.

Do not autoclave.

33. Stripping solution (0.2 M NaOH; 0.1% SDS)

10% SDS solution	1 ml
1 M NaOH solution	20 ml
Top up with sterilized distilled water to	100 ml

Do not autoclave.

34. Developer and replenisher (Kodak, Carestream Health, Inc, Rochester, NY, USA)

Distilled water	365.025 ml
RP X-OMAT Developer and Replenisher, Part A	125 ml
RP X-OMAT Developer and Replenisher, Part B	5.1 ml
RP X-OMAT Developer and Replenisher, Part C	4.875 ml

Do not autoclave.

35. Fixer and replenisher (Kodak, Carestream Health, Inc, Rochester, NY, USA)

Distilled water	363.5 ml
RP X-OMAT LO Fixer and Replenisher, Part A	125 ml
RP X-OMAT LO Fixer and Replenisher, Part B	11.5 ml

Do not autoclave.

36. Ethidium bromide (EtBr)

Ethidium Bromide (Sigma-Aldrich, USA)	30 µl
Distilled water	300 ml

Do not autoclave. This solution was stored in a dark bottle at room temperature, and diluted to 0.5 µg/ml with distilled water before used.

37. Proteinase K (20 mg/ml)

Proteinase K (Promega, Madison, USA)	100 mg
Sterilized distilled water	5 ml

After added the sterilized distilled water, the Proteinase K powder was left to dissolve at room temperature before aliquoted into few sterile1.5 ml tubes and kept in -20 ºC fridge.

38. 1% SeaKem Gold (SKG) Agarose

Seakem Gold Agarose base (Cambrex Bio Science Rockland, Inc., USA)	0.1 g
1X TE buffer	10 ml

0.1 g of Seakem Gold Agarose powder was dissolved in 10 ml of 1X TE buffer by slowly boiled in the microwave. The temperature of the agarose was adjusted to 50 °C-54°C in water bath. Do not autoclave.

39. 0.7% Agarose gel for PCR

Agarose powder (LE Analytical Grade) (Promega, Madison, USA)	0.7 g
0.5X TBE buffer	100 ml

1% Agarose gel for PCR

Agarose powder (LE Analytical Grade) (Promega, Madison, USA)	0.35 g
0.5X TBE buffer	35 ml

1.5% Agarose gel for PCR

Agarose powder (LE Analytical Grade) (Promega, Madison, USA)	0.525 g
0.5X TBE buffer	35 ml

The agarose mixture was heated slowly until the agarose particles completely dissolved. Then it was kept in water bath equilibrated to 55-60 ºC before pour for casting the gel. Do not autoclave.

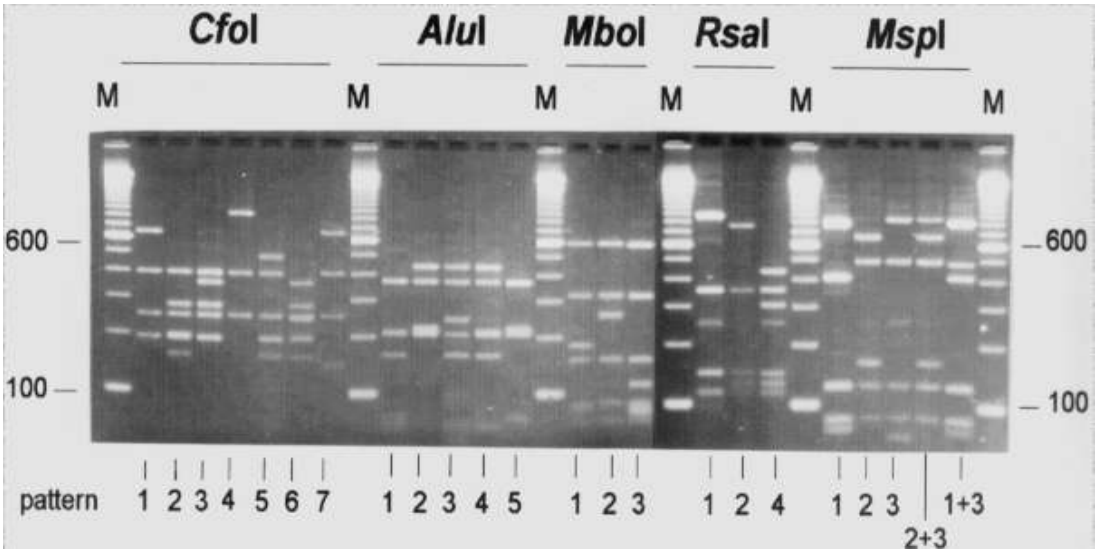
40. Agarose gel (1%) for PFGE

Agarose Type I (Sigma-Aldrich, USA)	1.5 g
0.5X TBE buffer	150 ml

The agarose mixture was heated slowly until the agarose particles completely dissolved. Then it was kept in water bath equilibrated to 55-60 ºC before pour for casting the gel. Do not autoclave.

APPENDIX III

ARDRA profiles of *A. baumannii* - Scheme of Vaneechoutte *et al.*, (1995)



Restriction patterns obtained after restriction digestion with *Cfo*I, *Alu*I, *Mbo*I, *Rsa*I and *Msp*I for amplified 16S rDNA of different *Acinetobacter* species. (http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html accessed on 20 May 2008)

ARDRA profiles of 202 *Acinetobacter* strains previously identified to genomic species by the use of DNA-DNA hybridization (http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html accessed on 20 May 2008)

Genomic species		Pattern with enzyme					Strains	Reference strain
		CfoI	AluI	MboI	RsaI	MspI	(N)	
1 (*A. calcoaceticus*)		2	2	1	1	3	7	ATCC 23055^T
		3	2	1	1	3	1	RUH 583
2 (*A. baumannii*)		1	1	1	2	3	27	ATCC 17904
		1	1	1	2	1	33	ATCC 19606^T
		1	1	1	2	1+3	4	LMD 82.54
3		2	1	3	1	3	24	ATCC 19004
		2+3	1	3	1	3	2	RUH 1163
4 (*A. haemolyticus*)	u	1	4	1	2	2	7	ATCC 17906^T
5 (*A. junii*)	l	1	2	1	2	3	6	ATCC 17908^T
6		1	1	2	2	2	2	ATCC 17979
7 (*A. johnsonii*)	u	1	4	1	2	2	8	ATCC 17909^T
	n	1	4	1	2	2+3	1	RUH 2859
8/9 (*A. lwoffii*)		3	3	2	1	2	19	NCTC 5866^T
10	D	4	2	1	2	3	3	ATCC 17924
11	D	4	2	1	2	3	7	ATCC 11171

12 (*A. radioresistens*)		7	3	2	2	1	3	SEIP 12.81
		7	3	2	2	3	3	IAM 13186^{T}
		7	3	2	2	1+3	2	RUH 2862
13 BJ/14 TU	u	1	4	1	2	2	3	SEIP 5.84
		1	4	1	2	3	6	114^{C}, RUH 2866
	n	1	4	1	2	2+3	6	ATCC 17905
14 BJ		5	5	1	2	3	5	CCUG 14816
15 BJ		1	2	1	2	2	2	79^{d}, RUH 1729
16		1	2	1	4	2	3	ATCC 17988
		1	2	1	2+4	2	1	LUH 1734
17	l	1	2	1	2	3	2	SEIP 2/87
13 TU		2	1	1	1	1	2	ATCC 17903
		2	1	1	1	3	9	100 ^{d}
		2	1	1	1	1+3	2	RUH 2624
15 TU		6	2	1	1	3	2	151a^{C}, RUH 1090

Abbreviations: T, type strain; ATCC, American Type Culture Collection, Rockville, Md, USA; LUH, RUH, collection Leiden University Hospital, Leiden, The Netherlands; LMD, LMD Culture Collection, University of Technology, Delft, The Netherlands; NCTC, National Collection of Type Cultures, London, United Kingdom; SEIP, Service des Entérobactéries de l'Institut Pasteur, Paris, France; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden.

(N), Number of strains tested. Markings (u, l, n,D) denote profiles occurring in different genomic species. Most of these species can be further differentiated by restriction digestion with *Bfa*I and *Bsma*I

^{c} Strain and designation used by *Tjernberg* and *Ursing*, 1989.

^{d} Strain and designation used by *Bouvet* and *Jeanjean*, 1989.

APPENDIX IV

Antimicrobial agents' breakpoints (CLSI, 2006)

Agent	Abbreviation	S	I	R
Amikacin	AK30	>=17	15-16	<=14
Ampicillin	AMP10	>=17	14-16	<=13
Ampicillin/Sulbactam	SAM20	>=15	12-14	<=11
Amoxicillin/ clavulanic	AMC30	>=18	14-17	<=13
Cefuroxime	CXM30	>=18	15-17	<=14
Ceftriaxone	CRO30	>=21	14-20	<=13
Cefoperazone	CFP30	>=21	16-20	<=15
Cefoperazone/Sulbactam	SCF105	>=21	16-20	<=15
Cefepim	FEP30	>=18	15-17	<=14
Ceftazidime	CAZ30	>=18	15-17	<=14
Ciprofloxacin	CIP5	>=21	16-20	<=15
Gentamicin	CN10	>=15	13-14	<=12
Imipenem	IPM10	>=16	14-15	<=13
Meropenem	MEM10	>=16	14-15	<=13
Piperacillin/ tazobactam	TZP10	>=21	18-20	<=17
Polymyxins*	PB200	>=12	10-11	<=9
Sulphamethoxazole/ Trimethoprim	SXT25	>=16	11-15	<=10

* Breakpoint of polymyxin B according to NCCLS, 1981

APPENDIX V

Inhibition zone diameter/interpretation of S/I/R of 185 *A. baumannii*

No	Strain	Antimicrobial agents																
		Aminoglycoside		Penicillin				Cephalosporin						Quinolone	Carbapenem		Colistin	Foliate Inhibitor
		Amikacin	Gentamicin	Ampicillin	Ampicillin/ sulbactam	Amoxicillin clavulanic acid	Piperacillin/ Tazobactam	Cefuroxime	Ceftriaxone	Cefoperaone	Cefoperazone/ sulbactam	Cefepime	Ceftazidime	Ciprofloxacin	Imipenem	Meropenem	Polymyxin B	Trimethoprim/ sulfamethoxazole
		AK30	CN10	AMP 10	SAM 20	AMC 30	TZP 110	CXM 30	CRO 30	CFP 30	SCF 105	FEP3 0	CAZ3 0	CIP5	IPM 10	MEM 10	PB 200	SXT 25
1	AC/0601-5	0/R	0/R	0/R	12/I	0/R	9/R	0/R	0/R	0/R	21/S	10/R	0/R	8/R	13/R	9/R	12/S	0/R
2	AC/0601-8	19/S	8/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	16/I	9/R	0/R	0/R	9/R	0/R	12/S	0/R
3	AC/0601-10	9/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	19/I	9/R	0/R	0/R	10/R	0/R	12/S	0/R
4	AC/0602-19	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	17/I	0/R	0/R	0/R	0/R	0/R	14/S	0/R
5	AC/0603-1	16/I	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	17/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
6	AC/0603-2	0/R	0/R	0/R	9/R	0/R	8/R	0/R	0/R	0/R	18/I	0/R	0/R	0/R	8/R	0/R	13/S	0/R
7	AC/0603-7	9/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	18/I	0/R	0/R	0/R	0/R	9/R	12/S	0/R
8	AC/0603-9	19/S	19/S	0/R	10/R	0/R	0/R	11/R	12/R	0/R	19/I	8/R	9/R	21/S	8/R	0/R	13/S	21/S

9	AC/0603-22	17/S	19/S	0/R	11/R	0/R	8/R	0/R	0/R	0/R	20/I	9/R	0/R	0/R	11/R	8/R	12/S	0/R
10	AC/0603-25	18/S	8/R	0/R	20/S	10/R	15/R	0/R	0/R	0/R	25/S	9/R	0/R	0/R	25/S	20/S	12/S	0/R
11	AC/0603-26	20/S	15/S	0/R	14/I	0/R	9/R	0/R	0/R	0/R	21/S	10/R	0/R	0/R	10/R	0/R	12/S	0/R
12	AC/0604-6	21/S	9/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	18/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
13	AC/0604-7	19/S	0/R	0/R	21/S	10/R	14/R	0/R	0/R	0/R	28/S	10/R	0/R	0/R	25/S	20/S	13/S	0/R
14	AC/0604-11	20/S	9/R	0/R	22/S	11/R	15/R	0/R	0/R	0/R	26/S	11/R	0/R	0/R	27/S	22/S	13/S	0/R
15	AC/0604-25	20/S	0/R	0/R	12/I	0/R	8/R	0/R	0/R	0/R	21/S	11/R	0/R	8/R	13/R	10/R	13/S	0/R
16	AC/0605-3	0/R	0/R	0/R	0/R	0/R	9/R	0/R	0/R	0/R	17/I	8/R	0/R	0/R	0/R	0/R	12/S	0/R
17	AC/0605-19	13/R	15/S	0/R	17/S	0/R	10/R	0/R	0/R	0/R	30/S	13/R	0/R	12/R	11/R	10/R	15/S	10/R
18	AC/0605-25	21/S	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	17/I	0/R	0/R	0/R	10/R	0/R	13/S	0/R
19	AC/0606-11	13/R	0/R	0/R	12/I	0/R	0/R	0/R	0/R	0/R	21/S	0/R	0/R	0/R	10/R	0/R	13/S	0/R
20	AC/0606-13	23/S	23/S	0/R	12/I	0/R	0/R	0/R	0/R	0/R	24/S	0/R	0/R	0/R	0/R	0/R	12/S	0/R
21	AC/0606-16	8/R	0/R	0/R	7/R	0/R	0/R	0/R	0/R	0/R	18/I	0/R	0/R	0/R	8/R	0/R	12/S	0/R
22	AC/0606-22	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	19/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
23	AC/0606-23	19/S	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	18/I	0/R	0/R	0/R	0/R	0/R	12/S	0/R
24	AC/0606-24	11/R	0/R	0/R	12/I	0/R	0/R	0/R	0/R	0/R	23/S	0/R	0/R	0/R	10/R	0/R	14/S	9/R
25	AC/0607-2	13/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	23/S	0/R	0/R	0/R	10/R	0/R	14/S	0/R
26	AC/0607-6	10/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	21/S	0/R	0/R	0/R	10/R	0/R	13/S	0/R
27	AC/0607-12	19/S	0/R	0/R	13/I	0/R	12/R	0/R	0/R	0/R	20/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
28	AC/0607-18	19/S	0/R	0/R	14/I	0/R	8/R	0/R	0/R	0/R	23/S	8/R	0/R	0/R	10/R	0/R	13/S	0/R
29	AC/0607-19	14/R	11/R	0/R	13/I	0/R	0/R	0/R	0/R	0/R	24/S	0/R	0/R	0/R	10/R	0/R	14/S	10/R
30	AC/0607-20	11/R	0/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	20/I	0/R	0/R	0/R	9/R	8/R	12/S	0/R
31	AC/0607-22	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	19/I	0/R	0/R	0/R	9/R	0/R	14/S	0/R

32	AC/0607-25	14/R	11/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	22/S	0/R	0/R	0/R	15/I	0/R	14/S	0/R
33	AC/0607-28	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	17/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
34	AC/0608-1	19/S	9/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	15/I	0/R	0/R	0/R	0/R	0/R	14/S	0/R
35	AC/0608-5	0/R	0/R	0/R	10/R	0/R	8/R	0/R	0/R	0/R	17/I	11/R	0/R	0/R	10/R	9/R	13/S	0/R
36	AC/0608-7	11/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	18/I	0/R	0/R	0/R	0/R	0/R	14/S	0/R
37	AC/0608-17	11/R	0/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	20/I	0/R	0/R	0/R	10/R	0/R	12/S	0/R
38	AC/0608-22	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	16/I	0/R	0/R	0/R	0/R	0/R	12/S	0/R
39	AC/0609-1	11/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	19/I	0/R	0/R	0/R	0/R	0/R	13/S	9/R
40	AC/0609-6	13/R	9/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	22/S	9/R	0/R	0/R	12/R	9/R	12/S	0/R
41	AC/0609-8	13/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	23/S	0/R	0/R	0/R	10/R	0/R	13/S	0/R
42	AC/0609-10	14/R	9/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	19/I	8/R	0/R	0/R	12/R	9/R	13/S	0/R
43	AC/0609-14	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	17/I	0/R	0/R	0/R	0/R	0/R	12/S	0/R
44	AC/0609-25	0/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	22/S	0/R	0/R	0/R	14/I	0/R	14/S	0/R
45	AC/0610-2	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	19/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
46	AC/0610-8	0/R	0/R	0/R	11/R	0/R	7/R	0/R	0/R	0/R	17/I	11/R	0/R	0/R	10/R	7/R	13/S	0/R
47	AC/0610-9	0/R	0/R	0/R	9/R	0/R	7/R	0/R	0/R	0/R	17/I	11/R	0/R	0/R	10/R	7/R	13/S	0/R
48	AC/0610-12	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	20/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
49	AC/0610-18	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	19/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
50	AC/0611-5	14/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	23/S	0/R	0/R	0/R	0/R	0/R	14/S	0/R
51	AC/0611-7	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	19/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
52	AC/0611-10	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	16/I	9/R	0/R	10/R	0/R	0/R	13/S	0/R
53	AC/0611-11	21/S	10/R	0/R	9/R	0/R	12/R	0/R	0/R	0/R	20/I	0/R	0/R	13/R	0/R	0/R	13/S	0/R
54	AC/0611-15	9/R	0/R	0/R	11/R	0/R	R	0/R	0/R	0/R	16/I	8/R	0/R	9/R	11/R	8/R	13/S	0/R

55	AC/0611-16	0/R	0/R	0/R	9/R	0/R	9/R	0/R	0/R	0/R	21/S	11/R	0/R	R	10/R	R	13/S	0/R
56	AC/0611-18	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	20/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
57	AC/0611-19	19/S	0/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	19/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
58	AC/0612-7	0/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	20/I	11/R	0/R	0/R	11/R	0/R	14/S	0/R
59	AC/0612-13	10/R	0/R	0/R	13/I	0/R	8/R	0/R	0/R	0/R	22/S	9/R	0/R	0/R	12/R	10/R	13/S	0/R
60	AC/0612-16	0/R	0/R	0/R	12/I	0/R	0/R	0/R	0/R	0/R	19/I	12/R	0/R	0/R	12/R	10/R	13/S	0/R
61	AC/0612-17	10/R	0/R	0/R	12/I	0/R	0/R	0/R	0/R	0/R	23/S	9/R	0/R	0/R	9/R	9/R	14/S	0/R
62	ACIBA 2006- 1	9/R	0/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	18/I	8/R	0/R	0/R	10/R	9/R	12/S	0/R
63	ACIBA 2006- 2	10/R	0/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	19/I	R	0/R	0/R	9/R	9/R	13/S	0/R
64	ACIBA 2006- 36	11/R	0/R	0/R	12/I	0/R	0/R	0/R	0/R	0/R	19/I	9/R	0/R	0/R	8/R	8/R	13/S	7/R
65	ACIBA 2006- 43	0/R	0/R	0/R	10/R	0/R	9/R	0/R	0/R	0/R	18/I	10/R	0/R	0/R	9/R	9/R	13/S	0/R
66	ACIBA 2006- 46	0/R	0/R	0/R	10/R	0/R	10/R	0/R	0/R	0/R	20/I	10/R	9/R	0/R	9/R	9/R	13/S	0/R
67	ACIBA 2006- 47	11/R	0/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	20/I	8/R	0/R	0/R	0/R	8/R	13/S	10/R
68	ACIBA 2006- 49	21/S	20/S	10/R	25/S	15/I	22/S	14/R	17/I	12/R	30/S	21/S	20/S	23/S	26/S	25/S	14/S	23/S
69	ACIBA 2006- 50	21/S	20/S	9/R	21/S	15/I	22/S	12/R	16/I	9/R	31/S	22/S	20/S	24/S	26/S	23/S	14/S	25/S
70	ACIBA 2006- 51	22/S	20/S	14/I	26/S	17/I	23/S	14/R	16/I	11/R	32/S	19/S	20/S	22/S	26/S	22/S	13/S	21/S
71	ACIBA 2006- 52	19/S	18/S	13/R	23/S	18/S	22/S	16/I	12/R	13/R	28/S	21/S	19/S	20/I	24/S	22/S	12/S	19/S
72	ACIBA 2006- 53	22/S	23/S	24/S	28/S	28/S	28/S	22/S	21/S	15/R	29/S	25/S	22/S	27/S	30/S	26/S	13/S	29/S
73	ACIBA 2006- 56	21/S	22/S	12/R	27/S	17/I	24/S	17/I	17/I	15/R	31/S	22/S	21/S	24/S	23/S	22/S	13/S	25/S
74	ACIBA 2006- 57	21/S	19/S	10/R	25/S	16/I	19/I	17/I	14/I	12/R	29/S	18/S	18/S	23/S	20/S	19/S	13/S	21/S
75	ACIBA 2006- 63	8/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	17/I	8/R	0/R	0/R	0/R	0/R	14/S	0/R
76	ACIBA 2006- 65	0/R	0/R	0/R	9/R	0/R	9/R	0/R	0/R	0/R	21/S	10/R	0/R	0/R	0/R	0/R	14/S	0/R
77	AC/0701-11	8/R	0/R	0/R	12/I	0/R	0/R	0/R	0/R	0/R	15/R	8/R	0/R	0/R	8/R	8/R	12/S	0/R

78	AC/0702-5	10/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	13/R	0/R	0/R	0/R	0/R	0/R	12/S	8/R
79	AC/0702-17	23/S	9/R	0/R	10/R	0/R	9/R	0/R	0/R	0/R	15/R	11/R	8/R	11/R	8/R	0/R	14/S	0/R
80	AC/0703-14	18/S	0/R	0/R	10/R	0/R	9/R	0/R	0/R	0/R	19/I	10/R	0/R	0/R	12/R	11/R	13/S	0/R
81	AC/0703-21	17/S	0/R	0/R	9/R	0/R	8/R	0/R	0/R	0/R	17/I	8/R	0/R	0/R	8/R	8/R	12/S	0/R
82	AC/0704-7	10/R	8/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	16/I	8/R	0/R	8/R	8/R	0/R	13/S	0/R
83	AC/0705-3	19/S	14/I	0/R	9/R	0/R	8/R	0/R	0/R	0/R	17/I	8/R	0/R	0/R	9/R	8/R	13/S	0/R
84	AC/0705-9	19/S	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	16/I	8/R	0/R	0/R	8/R	8/R	12/S	0/R
85	AC/0705-15	17/S	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	8/R	0/R	0/R	0/R	0/R	13/S	0/R
86	AC/0706-21	17/S	7/R	0/R	9/R	0/R	8/R	0/R	0/R	0/R	18/I	9/R	0/R	0/R	10/R	10/R	12/S	0/R
87	AC/0707-8	0/R	8/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	13/R	9/R	0/R	0/R	8/R	7/R	13/S	0/R
88	AC/0707-13	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	12/R	0/R	0/R	0/R	8/R	7/R	13/S	0/R
89	AC/0707-26	20/S	15/S	0/R	10/R	0/R	0/R	0/R	0/R	0/R	16/I	8/R	0/R	0/R	10/R	9/R	14/S	17/S
90	AC/0708-10	11/R	7/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	13/R	0/R	0/R	0/R	7/R	0/R	13/S	0/R
91	AC/0708-16	19/S	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	16/I	0/R	0/R	0/R	10/R	8/R	14/S	0/R
92	AC/0708-20	0/R	0/R	0/R	0/R	0/R	8/R	0/R	0/R	0/R	17/I	9/R	8/R	0/R	0/R	8/R	13/S	0/R
93	AC/0709-5	11/R	0/R	0/R	0/R	0/R	8/R	0/R	0/R	0/R	17/I	12/R	0/R	0/R	0/R	0/R	13/S	0/R
94	AC/0709-6	20/S	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	17/I	8/R	0/R	0/R	9/R	0/R	13/S	0/R
95	AC/0709-7	21/S	16/S	0/R	9/R	0/R	0/R	0/R	0/R	0/R	18/I	9/R	0/R	0/R	12/R	10/R	14/S	0/R
96	AC/0709-8	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	15/R	9/R	0/R	0/R	0/R	0/R	13/S	0/R
97	AC/0709-27	0/R	0/R	0/R	12/I	0/R	9/R	0/R	0/R	0/R	19/I	11/R	0/R	0/R	10/R	9/R	13/S	0/R
98	AC/0710-3	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	14/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
99	AC/0711-7	21/S	23/S	0/R	13/I	0/R	10/R	0/R	0/R	0/R	20/I	12/R	0/R	0/R	14/I	12R	18/S	21/S
100	AC/0712-3	0/R	0/R	0/R	8/R	0/R	9/R	0/R	0/R	0/R	17/I	9/R	9/R	0/R	8/R	0/R	13/S	0/R

101	AC/0712-13	13/R	8/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	9/R	8/R	0/R	13/S	12/I
102	AC/0801-4	7/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	16/I	10/R	8/R	0/R	0/R	0/R	13/S	0/R
103	AC/0801-6	0/R	0/R	0/R	10/R	0/R	8/R	0/R	0/R	0/R	17/I	9/R	0/R	0/R	8/R	8/R	13/S	0/R
104	AC/0801-11	0/R	0/R	0/R	10/R	0/R	9/R	0/R	0/R	0/R	18/I	10/R	8/R	0/R	8/R	8/R	13/S	0/R
105	AC/0801-13	7/R	0/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	20/I	9/R	0/R	0/R	8/R	0/R	14/S	0/R
106	AC/0802-1	10/R	0/R	0/R	12/I	0/R	0/R	0/R	0/R	0/R	18/I	9/R	0/R	0/R	9/R	9/R	13/S	0/R
107	AC/0802-4	17/S	0/R	0/R	13/I	0/R	9/R	0/R	0/R	0/R	19/I	9/R	0/R	0/R	12/R	10/R	12/S	0/R
108	AC/0802-14	0/R	0/R	0/R	9/R	0/R	8/R	0/R	0/R	0/R	18/I	9/R	8/R	0/R	8/R	8/R	13/S	0/R
109	AC/0802-20	0/R	0/R	0/R	10/R	0/R	8/R	0/R	0/R	0/R	17/I	10/R	8/R	0/R	9/R	9/R	13/S	0/R
110	AC/0803-15	0/R	0/R	0/R	10/R	0/R	9/R	0/R	0/R	0/R	18/I	10/R	8/R	0/R	8/R	8/R	14/S	0/R
111	AC/0804-4	0/R	0/R	0/R	10/R	0/R	8/R	0/R	0/R	0/R	18/I	9/R	8/R	0/R	8/R	8/R	13/S	0/R
112	AC/0804-19	8/R	14/I	0/R	13/I	0/R	0/R	0/R	0/R	0/R	19/I	9/R	0/R	0/R	11/R	9/R	13/S	17/S
113	AC/0804-24	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	15/R	8/R	8/R	0/R	0/R	0/R	14/S	0/R
114	AC/0804-31	9/R	15/S	0/R	11/R	0/R	0/R	0/R	0/R	0/R	20/I	9/R	0/R	0/R	10/R	8/R	13/S	19/S
115	AC/0804-32	0/R	13/I	0/R	9/R	0/R	0/R	0/R	0/R	0/R	15/R	7/R	0/R	0/R	8/R	0/R	12/S	16/S
116	AC/0805-4	8/R	13/I	0/R	9/R	0/R	0/R	0/R	0/R	0/R	15/R	9/R	0/R	0/R	8/R	0/R	13/S	0/R
117	AC/0805-5	0/R	12/R	0/R	13/I	0/R	8/R	0/R	0/R	0/R	18/I	10/R	0/R	0/R	10/R	10/R	13/S	10/R
118	AC/0805-20	0/R	9/R	0/R	12/I	0/R	7/R	0/R	0/R	0/R	19/I	10/R	0/R	0/R	10/R	9/R	12/S	14/I
119	AC/0806-4	0/R	0/R	0/R	12/I	0/R	9/R	0/R	0/R	0/R	18/I	10/R	9/R	0/R	8/R	9/R	12/S	0/R
120	AC/0806-10	0/R	0/R	0/R	11/R	0/R	8/R	0/R	0/R	0/R	13/R	9/R	0/R	0/R	8/R	8/R	13/S	0/R
121	AC/0806-18	0/R	0/R	0/R	9/R	0/R	8/R	0/R	0/R	0/R	13/R	10/R	0/R	0/R	8/R	8/R	13/S	0/R
122	AC/0806-23	0/R	13/I	0/R	10/R	0/R	0/R	0/R	0/R	0/R	13/R	8/R	0/R	0/R	8/R	7/R	13/S	15/I
123	AC/0806-24	0/R	0/R	0/R	10/R	0/R	8/R	0/R	0/R	0/R	13/R	10/R	8/R	0/R	9/R	9/R	13/S	0/R

124	AC/0806-28	17/S	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	13/R	9/R	0/R	0/R	8/R	8/R	12/S	0/R
125	AC/0807-20	0/R	0/R	0/R	11/R	0/R	0/R	0/R	0/R	0/R	14/R	12/R	9/R	0/R	9/R	9/R	13/S	0/R
126	AC/0808-6	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	11/R	9/R	0/R	0/R	0/R	0/R	13/S	0/R
127	AC/0808-14	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	10/R	8/R	0/R	0/R	0/R	0/R	13/S	0/R
128	AC/0808-18	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	7/R	7/R	0/R	0/R	0/R	13/S	0/R
129	AC/0808-20	0/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	12/R	9/R	0/R	0/R	8/R	8/R	13/S	0/R
130	AC/0809-1	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	10/R	8/R	8/R	0/R	0/R	0/R	13/S	0/R
131	AC/0809-9	8/R	0/R	0/R	10/R	0/R	0/R	0/R	0/R	0/R	14/R	10/R	0/R	0/R	7/R	7/R	13/S	0/R
132	AC/0809-12	11/R	16/S	0/R	12/I	0/R	7/R	0/R	0/R	0/R	15/R	9/R	0/R	0/R	12/R	12/R	13/S	16/S
133	AC/0809-29	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	14/R	0/R	8/R	0/R	0/R	0/R	13/S	0/R
134	AC/0809-30	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	8/R	8/R	0/R	0/R	0/R	13/S	0/R
135	AC/0810-8	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	16/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
136	AC/0810-11	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
137	AC/0810-12	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	12/S	0/R
138	AC/0810-22	18/S	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	14/R	0/R	0/R	0/R	8/R	0/R	13/S	0/R
139	AC/0810-26	0/R	0/R	0/R	14/I	0/R	9/R	0/R	0/R	0/R	19/I	9/R	8/R	0/R	8/R	9/R	13/S	13/I
140	AC/0811-12	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	8/R	8/R	0/R	0/R	0/R	13/S	0/R
141	AC/0811-13	8/R	15/S	0/R	0/R	0/R	0/R	0/R	0/R	0/R	12/R	0/R	0/R	0/R	8/R	0/R	13/S	22/S
142	AC/0811-15	0/R	0/R	0/R	10/R	0/R	8/R	0/R	0/R	0/R	17/I	9/R	8/R	0/R	9/R	8/R	14/S	0/R
143	AC/0811-25	0/R	0/R	0/R	9/R	0/R	8/R	0/R	0/R	0/R	17/I	9/R	8/R	0/R	9/R	8/R	13/S	0/R
144	AC/0812-1	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	8/R	8/R	0/R	0/R	0/R	13/S	0/R
145	AC/0812-8	19/S	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	14/R	8/R	0/R	0/R	8/R	0/R	13/S	0/R
146	AC/0812-16	11/R	19/S	0/R	0/R	0/R	0/R	0/R	0/R	0/R	14/R	0/R	0/R	0/R	0/R	0/R	13/S	24/S

147	AC/0812-29	11/R	16/S	0/R	14/I	0/R	0/R	0/R	0/R	0/R	20/I	9/R	0/R	0/R	11/R	9/R	13/S	23/S
148	AC/0812-33	12/R	17/S	0/R	13/I	0/R	8/R	0/R	0/R	0/R	21/S	9/R	0/R	0/R	11/R	9/R	14/S	23/S
149	AC/0901-5	0/R	0/R	0/R	10/R	0/R	9/R	0/R	0/R	0/R	20/I	10/R	10/R	0/R	0/R	0/R	13/S	0/R
150	AC/0901-14	0/R	0/R	0/R	8/R	0/R	8/R	0/R	0/R	0/R	16/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
151	AC/0901-36	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
152	AC/0901-37	0/R	0/R	0/R	8/R	0/R	8/R	0/R	0/R	0/R	18/I	8/R	0/R	0/R	0/R	0/R	12/S	0/R
153	AC/0902-5	9/R	15/S	0/R	9/R	0/R	0/R	0/R	0/R	0/R	15/R	10/R	0/R	0/R	8/R	8/R	13/S	23/S
154	AC/0902-6	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
155	AC/0902-13	12/R	16/S	0/R	14/I	0/R	8/R	0/R	0/R	0/R	20/I	10/R	0/R	0/R	11/R	10/R	13/S	16/S
156	AC/0902-14	0/R	0/R	0/R	10/R	0/R	9/R	0/R	0/R	0/R	19/I	11/R	8/R	0/R	9/R	9/R	13/S	0/R
157	AC/0902-15	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	17/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
158	AC/0902-19	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
159	AC/0903-15	0/R	0/R	0/R	9/R	0/R	8/R	0/R	0/R	0/R	16/I	0/R	0/R	0/R	8/R	0/R	13/S	0/R
160	AC/0903-19	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	13/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
161	AC/0903-21	9/R	14/I	0/R	8/R	0/R	0/R	0/R	0/R	0/R	11/R	8/R	0/R	0/R	0/R	0/R	12/S	16/S
162	AC/0903-28	0/R	0/R	0/R	9/R	0/R	7/R	0/R	0/R	0/R	14/R	8/R	0/R	0/R	0/R	0/R	13/S	0/R
163	AC/0903-29	0/R	0/R	0/R	9/R	0/R	8/R	0/R	0/R	0/R	15/R	8/R	0/R	0/R	0/R	0/R	13/S	0/R
164	AC/0903-31	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	13/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
165	AC/0904-3	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
166	AC/0904-7	0/R	0/R	0/R	9/R	0/R	7/R	0/R	0/R	0/R	15/R	7/R	0/R	0/R	0/R	0/R	13/S	0/R
167	AC/0904-15	0/R	0/R	0/R	7/R	0/R	0/R	0/R	0/R	0/R	14/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
168	AC/0904-19	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	15/R	8/R	0/R	0/R	0/R	0/R	13/S	0/R
169	AC/0904-28	0/R	0/R	0/R	7/R	0/R	0/R	0/R	0/R	0/R	13/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R

170	AC/0904-39	0/R	0/R	0/R	7/R	0/R	0/R	0/R	0/R	0/R	13/R	0/R	0/R	0/R	0/R	0/R	12/S	0/R
171	AC/0904-40	0/R	0/R	0/R	9/R	0/R	7/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	12/S	0/R
172	AC/0904-42	17/S	14/I	0/R	10/R	0/R	8/R	0/R	0/R	0/R	16/I	9/R	0/R	0/R	10/R	9/R	12/S	0/R
173	AC/0904-43	0/R	8/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
174	AC/0904-20	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	14/R	9/R	0/R	0/R	0/R	0/R	13/S	0/R
175	AC/0904-21	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	14/R	7/R	0/R	0/R	0/R	0/R	13/S	0/R
176	AC/0905-2	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	14/R	0/R	0/R	0/R	0/R	0/R	12/S	0/R
177	AC/0905-6	0/R	0/R	0/R	7/R	0/R	0/R	0/R	0/R	0/R	14/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
178	AC/0905-21	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	14/R	0/R	0/R	0/R	0/R	0/R	12/S	0/R
179	AC/0905-22	10/R	15/S	0/R	13/I	0/R	0/R	0/R	0/R	0/R	20/I	10/R	0/R	0/R	12/R	9/R	13/S	20/S
180	AC/0905-31	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	15/R	0/R	0/R	0/R	0/R	0/R	13/S	0/R
181	AC/0905-42	19/S	15/S	0/R	10/R	0/R	9/R	0/R	0/R	0/R	18I/	10/R	0/R	0/R	10/R	9/R	13/S	0/R
182	AC/0905-49	0/R	0/R	0/R	0/R	0/R	8/R	0/R	0/R	0/R	17/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
183	AC/0905-53	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	16/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
184	AC/0905-58	0/R	0/R	0/R	9/R	0/R	0/R	0/R	0/R	0/R	17/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R
185	AC/0905-60	0/R	0/R	0/R	8/R	0/R	0/R	0/R	0/R	0/R	16/I	0/R	0/R	0/R	0/R	0/R	13/S	0/R

APPENDIX VI

DNA Sequencing Results

(a) *bla*$_{\text{OXA-23}}$ gene

Selected isolate for sequencing: AC/0608-5

```
>gb|HM053483.1|  Acinetobacter baumannii isolate HKU 2H9 carbapenemase OXA-
23 (OXA-23) gene, complete cds

Length=822
Score = 830 bits (449), Expect = 0.0
Identities = 454/456 (99%), Gaps = 2/456 (0%)
Strand=Plus/Plus

Query  13   ATGG-AGGGCGAG-AAAGGTCATTTACCGCTTGGGAAAAAGACATGACACTAGGAGAAGC  70
            |||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  306  ATGGAAGGGCGAGAAAAGGTCATTTACCGCTTGGGAAAAAGACATGACACTAGGAGAAGC  365

Query  71   CATGAAGCTTTCTGCAGTCCCAGTCTATCAGGAACTTGCGCGACGTATCGGTCTTGATCT  130
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  366  CATGAAGCTTTCTGCAGTCCCAGTCTATCAGGAACTTGCGCGACGTATCGGTCTTGATCT  425

Query  131  CATGCAAAAAGAAGTAAAACGTATTGGTTTCGGTAATGCTGAAATTGGACAGCAGGTTGA  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  426  CATGCAAAAAGAAGTAAAACGTATTGGTTTCGGTAATGCTGAAATTGGACAGCAGGTTGA  485

Query  191  TAATTTCTGGTTGGTAGGACCATTAAAGGTTACGCCTATTCAAGAGGTAGAGTTTGTTTC  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  486  TAATTTCTGGTTGGTAGGACCATTAAAGGTTACGCCTATTCAAGAGGTAGAGTTTGTTTC  545

Query  251  CCAATTAGCACATACACAGCTTCCATTTAGTGAAAAAGTGCAGGCTAATGTAAAAAATAT  310
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  546  CCAATTAGCACATACACAGCTTCCATTTAGTGAAAAAGTGCAGGCTAATGTAAAAAATAT  605

Query  311  GCTTCTTTTAGAAGAGAGTAATGGCTACAAAATTTTTGGAAAGACTGGTTGGGCAATGGA  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  606  GCTTCTTTTAGAAGAGAGTAATGGCTACAAAATTTTTGGAAAGACTGGTTGGGCAATGGA  665

Query  371  TATAAAACCACAAGTGGGCTGGTTGACCGGCTGGGTTGAGCAGCCAGATGGAAAAATTGT  430
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  666  TATAAAACCACAAGTGGGCTGGTTGACCGGCTGGGTTGAGCAGCCAGATGGAAAAATTGT  725

Query  431  CGCTTTTGCATTAAATATGGAAATGCGGTCAGAAAT  466
            |||||||||||||||||||||||||||||||||||
Sbjct  726  CGCTTTTGCATTAAATATGGAAATGCGGTCAGAAAT  761
```

(b) *bla*$_{\text{OXA-51}}$ gene

Selected isolate for sequencing: AC/0608-5

```
>emb|AJ309734.2|  Acinetobacter baumannii oxa-51 gene for beta-lactamase

Length=825
Score = 540 bits (292), Expect = 3e-150
Identities = 309/317 (97%), Gaps = 2/317 (1%)
Strand=Plus/Plus

Query  7    CACCACAG-AGTATTTAA-TGGGATGGGGAAAAAAGGCTATTCCCAGAATGGGAAAAGAA  64
            |||||||| ||||||||| ||||| ||| |||||||||||||||||||||||||||||| |
Sbjct  291  CACCACAGAAGTATTTAAGTGGGACGGGCAAAAAAGGCTATTCCCAGAATGGGAAAAGGA  350

Query  65   CATGACCCTAGGCGATGCTATGAAAGCTTCCGCTATTCCGGTTTATCAAGATTTAGCTCG  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  351  CATGACCCTAGGCGATGCTATGAAAGCTTCCGCTATTCCGGTTTATCAAGATTTAGCTCG  410

Query  125  TCGTATTGGACTTGAGCTCATGTCTAAGGAAGTGAAGCGTGTTGGTTATGGCAATGCAGA  184
            ||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  411  TCGTATTGGACTTGAACTCATGTCTAAGGAAGTGAAGCGTGTTGGTTATGGCAATGCAGA  470

Query  185  TATCGGTACCCAAGTCGATAATTTTTGGCTGGTGGGTCCTCTAAAAATTACTCCTCAGCA  244
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  471  TATCGGTACCCAAGTCGATAATTTTTGGCTGGTGGGTCCTTTAAAAATTACTCCTCAGCA  530

Query  245  AGAGGCACAGTTTGCTTACAAGCTAGCTAATAAAACGCTTCCATTTAGCCAAAAAGTCCA  304
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  531  AGAGGCACAGTTTGCTTACAAGCTAGCTAATAAAACGCTTCCATTTAGCCCAAAAGTCCA  590

Query  305  AGATGAAGTGCAATCCA  321
            ||||||||||||||||
Sbjct  591  AGATGAAGTGCAATCCA  607
```

(c) IS*Aba1*

Selected isolate for sequencing: AC/0601-10

```
>gb|JN696403.1|     Acinetobacter baumannii insertion sequence ISAba1
transposase genes, complete cds; truncated DEAD helicase pseudogene and
blaOXA-23 gene, complete sequence; beta-lactamase OXA-23 gene, complete cds;
and insertion sequence ISAba1 transposase genes, complete cds

Length=4805
Sort alignments for this subject sequence by:
E value  Score  Percent identity
Query start position  Subject start position

Score = 900 bits (487), Expect = 0.0
Identities = 489/490 (99%), Gaps = 0/490 (0%)
Strand=Plus/Plus

Query  14    CACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAA  73
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  616   CACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAA  675

Query  74    ATGGCTATAAAGCGTTGAATCAAAGCAATGCGCTCTTTCGTATCTGAATTTCCACGTTTA  133
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  676   ATGGCTATAAAGCGTTGAATCAAAGCAATGCGCTCTTTCGTATCTGAATTTCCACGTTTA  735

Query  134   TTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGG  193
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  736   TTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGG  795

Query  194   ATATTAATATTTCGTTTTCCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGG  253
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  796   ATATTAATATTTCGTTTTCCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGG  855

Query  254   TCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTGACCT  313
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  856   TCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTGACCT  915

Query  314   GCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAGCACTTGATGGGCAAGGCT  373
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  916   GCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAGCACTTGATGGGCAAGGCT  975

Query  374   TTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAAATAATCACAAGCATGATGAGCGCA  433
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  976   TTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAAATAATCACAAGCATGATGAGCGCA  1035

Query  434   AAGCACTTTAAATGTGACTTGTTCCATTTTAGATATTTGTTTAAGATAAGATATAACTCA  493
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  1036  AAGCACTTTAAATGTGACTTGTTCCATTTTAGAGATTTGTTTAAGATAAGATATAACTCA  1095

Query  494   TTGAGATGTG  503
             ||||||||||
Sbjct  1096  TTGAGATGTG  1105


Score = 900 bits (487), Expect = 0.0
Identities = 489/490 (99%), Gaps = 0/490 (0%)
Strand=Plus/Minus

Query  14    CACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAA  73
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4190  CACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTTTACCAAAA  4131

Query  74    ATGGCTATAAAGCGTTGAATCAAAGCAATGCGCTCTTTCGTATCTGAATTTCCACGTTTA  133
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4130  ATGGCTATAAAGCGTTGAATCAAAGCAATGCGCTCTTTCGTATCTGAATTTCCACGTTTA  4071

Query  134   TTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGG  193
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  4070  TTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGCGAGCATCAGG  4011

Query  194   ATATTAATATTTCGTTTTCCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGG  253
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4010  ATATTAATATTTCGTTTTCCCCATTTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGG  3951

Query  254   TCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTGACCT  313
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3950  TCGAATGAAAACATATTGAAAATCAACTGAGAAATTTGACGATAATCAAAATACTGACCT  3891

Query  314   GCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAGCACTTGATGGGCAAGGCT  373
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3890  GCAAAGAAGCGCTGCATACGTCGATAAAATGATTGTGGTAAGCACTTGATGGGCAAGGCT  3831

Query  374   TTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAAATAATCACAAGCATGATGAGCGCA  433
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3830  TTAGATGCAGAAGAAAGATTACATGTTTGCTTTAAAATAATCACAAGCATGATGAGCGCA  3771

Query  434   AAGCACTTTAAATGTGACTTGTTCCATTTTAGATATTTGTTTAAGATAAGATATAACTCA  493
              |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  3770  AAGCACTTTAAATGTGACTTGTTCCATTTTAGAGATTTGTTTAAGATAAGATATAACTCA  3711

Query  494   TTGAGATGTG  503
              ||||||||||
Sbjct  3710  TTGAGATGTG  3701
```

(d) Upstream of IS*Aba1* to the *bla*$_{\text{OXA-23}}$ gene

Selected isolate for sequencing: AC/0608-5

```
>gb|GQ861439.1|     Acinetobacter baumannii transposon Tn2006 insertion
sequence ISAba1, complete sequence, beta-lactamase OXA-23 (blaOXA-23) gene,
complete cds, and insertion sequence ISAba1, complete sequence

Length=5292
Sort alignments for this subject sequence by:
E value  Score  Percent identity
Query start position  Subject start position

Score = 1890 bits (1023), Expect = 0.0
Identities = 1028/1030 (99%), Gaps = 1/1030 (0%)
Strand=Plus/Minus

Query  5     ACTAG-CAAATTAACAGGTTTCATTATTAGTCAGATTATAAAAGGCCCATTTATCTCAAA  63
             |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2337  ACTAGAAAAATTAACAGGTTTCATTATTAGTCAGATTATAAAAGGCCCATTTATCTCAAA  2278

Query  64    TGGGCTTTTTATAAAATTAGAGTTTCTGTCAAGCTCTTAAATAATATTCAGCTGTTTTAA  123
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2277  TGGGCTTTTTATAAAATTAGAGTTTCTGTCAAGCTCTTAAATAATATTCAGCTGTTTTAA  2218

Query  124   TGATTTCATCAATAATTCATTACGTATAGATGCCGGCATTTCTGACCGCATTTCCATATT  183
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2217  TGATTTCATCAATAATTCATTACGTATAGATGCCGGCATTTCTGACCGCATTTCCATATT  2158

Query  184   TAATGCAAAAGCGACAATTTTTCCATCTGGCTGCTCAACCCAGCCGGTCAACCAGCCCAC  243
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2157  TAATGCAAAAGCGACAATTTTTCCATCTGGCTGCTCAACCCAGCCGGTCAACCAGCCCAC  2098

Query  244   TTGTGGTTTTATATCCATTGCCCAACCAGTCTTTCCAAAAATTTTGTAGCCATTACTCTC  303
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2097  TTGTGGTTTTATATCCATTGCCCAACCAGTCTTTCCAAAAATTTTGTAGCCATTACTCTC  2038

Query  304   TTCTAAAAGAAGCATATTTTTTACATTAGCCTGCACTTTTTCACTAAATGGAAGCTGTGT  363
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2037  TTCTAAAAGAAGCATATTTTTTACATTAGCCTGCACTTTTTCACTAAATGGAAGCTGTGT  1978

Query  364   ATGTGCTAATTGGGAAACAAACTCTACCTCTTGAATAGGCGTAACCTTTAATGGTCCTAC  423
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1977  ATGTGCTAATTGGGAAACAAACTCTACCTCTTGAATAGGCGTAACCTTTAATGGTCCTAC  1918

Query  424   CAACCAGAAATTATCAACCTGCTGTCCAATTTCAGCATTACCGAAACCAATACGTTTTAC  483
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1917  CAACCAGAAATTATCAACCTGCTGTCCAATTTCAGCATTACCGAAACCAATACGTTTTAC  1858

Query  484   TTCTTTTTGCATGAGATCAAGACCGATACGTCGCGCAAGTTCCTGATAGACTGGGACTGC  543
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1857  TTCTTTTTGCATGAGATCAAGACCGATACGTCGCGCAAGTTCCTGATAGACTGGGACTGC  1798

Query  544   AGAAAGCTTCATGGCTTCTCCTAGTGTCATGTCTTTTTCCCAAGCGGTAAATGACCTTTT  603
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1797  AGAAAGCTTCATGGCTTCTCCTAGTGTCATGTCTTTTTCCCAAGCGGTAAATGACCTTTT  1738

Query  604   CTCGCCCTTCCATTTAAATATTTCATTAATATCCGTTTTCTGGTTCTCCAATCCGATCAG  663
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  CTCGCCCTTCCATTTAAATATTTCATTAATATCCGTTTTCTGGTTCTCCAATCCGATCAG  1678

Query  664   GGCATTCAACATTTTAAATGTAGAGGCTGGCACATATTCTGTATTTGCGCGGCTTAGAGC  723
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  GGCATTCAACATTTTAAATGTAGAGGCTGGCACATATTCTGTATTTGCGCGGCTTAGAGC  1618

Query  724   ATTACCATATAGATTAAttttttttATCTGTTTGAATAACCAGCACACCTGAGGTGttttt  783
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  ATTACCATATAGATTAATTTTTTTTATCTGTTTGAATAACCAGCACACCTGAGGTGTTTTT  1558
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Query  784   ttCATCAAAGTATTGATGAATCACCTGATTATGTCCTTGAACAATCTGACTCGGGGTTTC  843
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1557  TTCATCAAAGTATTGATGAATCACCTGATTATGTCCTTGAACAATCTGACTCGGGGTTTC  1498

Query  844   ATTTATTAAATTATGCTGAACCGTACAACCAGAAAGAAAAAGAGAAGCAACCACATAGCA  903
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1497  ATTTATTAAATTATGCTGAACCGTACAACCAGAAAGAAAAAGAGAAGCAACCACATAGCA  1438

Query  904   AGTAAAATATTTATTCATTTTAAACACCAGATCAATAGAAAATAAAAAAGAGCTCTGTAC  963
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1437  AGTAAAATATTTATTCATTTTAAACACCAGATCAATAGAAAATAAAAAAGAGCTCTGTAC  1378

Query  964   ACGACAAAAATAGATAACTCATTGAAATAATGTCATAATAATTGTTTTCTAACGACGAAT  1023
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1377  ACGACAAAAATAGATAACTCATTGAAATAATGTCATAATAATTGTTTTCTAACGACGAAT  1318

Query  1024  ACTATGACAC  1033
             ||||||||||
Sbjct  1317  ACTATGACAC  1308


Score = 145 bits (78), Expect = 8e-31
Identities = 78/78 (100%), Gaps = 0/78 (0%)
Strand=Plus/Plus

Query  956   CTCTGTACACGACAAAAATAGATAACTCATTGAAATAATGTCATAATAATTGTTTTCTAA  1015
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3831  CTCTGTACACGACAAAAATAGATAACTCATTGAAATAATGTCATAATAATTGTTTTCTAA  3890

Query  1016  CGACGAATACTATGACAC  1033
             ||||||||||||||||||
Sbjct  3891  CGACGAATACTATGACAC  3908
```

(e) Integrase class 1 (*IntI*1)

Selected isolate for sequencing: AC/0606-13

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>gb|EF033072.1|  Acinetobacter baumannii isolate 17368 class 1 integron 3-N-
aminoglycoside    acetyltransferase    (aacC1),    hypothetical    proteins,
aminoglycoside adenyltransferase (aadDA1), and quaternary ammonium compound-
resistance protein(qacEdelta1) genes, complete cds, and dihydropteroate
synthase type 1 (sul1) gene, partial cds

Length=3168
Score = 872 bits (472), Expect = 0.0
Identities = 491/500 (98%), Gaps = 1/500 (0%)
Strand=Plus/Plus

Query  15   TTTGATCTTATGGATCAGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCA  74
            |||||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4    TTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCA  63

Query  75   GCCCCCCCTAAAACAAAGTTAGGTGGCTCAAGTATGGGCATCATTCGCACATGTAGGCTC  134
            |  | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   G-TCGCCCTAAAACAAAGTTAGGTGGCTCAAGTATGGGCATCATTCGCACATGTAGGCTC  122

Query  135  GGCCCTGACCAAGTCAAATCCATGAGGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGA  194
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  GGCCCTGACCAAGTCAAATCCATGAGGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGA  182

Query  195  GACGTAGCCACCTACTCCCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGCTCCGT  254
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  GACGTAGCCACCTACTCCCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGCTCCGT  242

Query  255  AGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAGCGGTTGTTGGCGCTCTCGCG  314
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  AGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAGCGGTTGTTGGCGCTCTCGCG  302

Query  315  GCTTACGTTCTGCCAAAGTTTGAGCAGGCGCGTAGTGAGATCTATATCTATGATCTCGCA  374
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  GCTTACGTTCTGCCAAAGTTTGAGCAGGCGCGTAGTGAGATCTATATCTATGATCTCGCA  362

Query  375  GTCTCCGGCGAGCACCGGAGGCAAGGCATTGCCACCGCGCTCATCAATCTCCTCAAGCAT  434
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  GTCTCCGGCGAGCACCGGAGGCAAGGCATTGCCACCGCGCTCATCAATCTCCTCAAGCAT  422

Query  435  GAGGCCAACGCGCTTGGTGCTTATGTGATCTACGTGCAAGCAGATTACGGTGACTATCCC  494
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  423  GAGGCCAACGCGCTTGGTGCTTATGTGATCTACGTGCAAGCAGATTACGGTGACGATCCC  482

Query  495  TTAGTGGTTCTCTATACAAA  514
               ||||| ||||||||||||
Sbjct  483  GCAGTGGCTCTCTATACAAA  502
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(f) Integrase class 2 (*IntI*2)

Selected isolate for sequencing: AC/0606-13

>gb|DQ176450.1| Acinetobacter baumannii isolate AB28 transposon Tn7 tyrosine recombinase (intI2) pseudogene, complete sequence, and streptothricin acetyltransferase (sat2), aminoglycoside adenyltransferase (aadB), chloramphenicol acetyltransferase (catB2), dihydrofolate reductase type I (dfrA1), streptothricin acetyltransferase (sat2), streptomycin 3''-adenyltransferase (aadA1), hypothetical protein (ybeA), hypothetical protein (ybfA), hypothetical protein (ybfB),hypothetical protein (ybgA), transposition site target selection protein E (tnsE), transposition site target selection protein D (tnsD), transposition regulator protein (tnsC), transposase B (tnsB), and transposase A (tnsA) genes, complete cds

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Length=14850
Score = 379 bits (205), Expect = 4e-102
Identities = 228/238 (96%), Gaps = 6/238 (3%)
Strand=Plus/Plus

Query  8    CAGAAGATGAACAAGAGAGGAAAGCCG-CTAGGCTTGCTTGCAGGGATATAATCAATATC  66
            ||| |||| ||| |||| ||  ||||| ||||||||||||||||||||||||||||||||
Sbjct  216  CAG-AGAT-AAC-AGAG-GG-TAGCCGTCTAGGCTTGCTTGCAGGGATATAATCAATATC  270

Query  67   GCCCAACGGCTGTTGTAAAAACCTGTTGTACAAAAAAGCTAGGGCATTTAAAGCGATTTT  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  271  GCCCAACGGCTGTTGTAAAAACCTGTTGTACAAAAAAGCTAGGGCATTTAAAGCGATTTT  330

Query  127  CTGCGTGTTTATGGCTACATGTCTGCTGTTTGCTAAGCTGGATAAAAACAGCCTGACCTC  186
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  331  CTGCGTGTTTATGGCTACATGTCTGCTGTTTGCTAAGCTGGATAAAAACAGCCTGACCTC  390

Query  187  TTCACTGCCCATGGTCTGAGGATTACGTTTTTTGTGAAACACAATAAAACCCTTAATC  244
            |||||||||||||||||||||| |||||||||||||||| |||||||| |||||||
Sbjct  391  TTCACTGCCCATGGTCTGAGGATGACGTTTTTTGTGAAACAGAATAAAACGCTTAATC  448
```

(g) Integron class 1 (profile IN1-a)

Selected isolate for sequencing: AC/0601-10

>gb|EF033072.1| Acinetobacter baumannii isolate 17368 class 1 integron 3-N-aminoglycoside acetyltransferase (aacC1), hypothetical proteins, aminoglycoside adenyltransferase (aadDA1), and quaternary ammonium compound-resistance protein (qacEdelta1) genes, complete cds, and dihydropteroate synthase type 1 (sul1) gene, partial cds

Length=3168
Score = 2032 bits (1100), Expect = 0.0
Identities = 1173/1203 (98%), Gaps = 25/1203 (2%)
Strand=Plus/Minus

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Query  7     AATTATTGTGCTTAGTGCATCTAACGCTTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGC  66
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2473  AATTA-TGTGCTTAGTGCATCTAACGCTTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGC  2415

Query  67    TTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGT  126
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2414  TTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGT  2355

Query  127   GGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGAT  186
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2354  GGACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGAT  2295

Query  187   AAGCCTGTCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCC  246
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2294  AAGCCTGTCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCC  2235

Query  247   AGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCAAATGCGGG  306
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2234  AGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCAAATGCGGG  2175

Query  307   ACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCG  366
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2174  ACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCG  2115

Query  367   TTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCT  426
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2114  TTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCT  2055

Query  427   CCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCA  486
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2054  CCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCA  1995

Query  487   GATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATT  546
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1994  GATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATT  1935

Query  547   CTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAA  606
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1934  CTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAA  1875

Query  607   CAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCA  666
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1874  CAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCA  1815

Query  667   AAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCA  726
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1814  AAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCA  1755

Query  727   GCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTA  786
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1754  GCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTA  1695

Query  787   CGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAG  846
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1694 CGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAG 1635

Query  847  TCGATACTTCGGCGATCACCGCTTCCCTCATGATGTTTAACGCCCAGCTTCAGACGCGCG 906
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1634 TCGATACTTCGGCGATCACCGCTTCCCTCATGATGTTTAACGCCCAGCTTCAGACGCGCG 1575

Query  907  GAGGCCGTAAAGGCCGTAGCGTCGGCTGGAAGCATTGGGTTAGAGCATGAGCGATTTACT 966
            |||||||||| |||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  1574 GAGGCCGTAA-GGCCGTAGCGTCGGCTGGAAGCATTGG-TTAGAGCATGAGCGATTTACT 1517

Query  967  GTATTCATGGGCTTTCGAAAAATTGTATCTGCACAGGTTGCTAGTACGACGTACACA-TA 1025
            |||||||||| |||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1516 GTATTCATGG-CTTTCGAAAAATTGTATCTGCACAGGTTGCTAGTACGACGTACACAATA 1458

Query  1026 G-CCCGAGCAGTATGAGCGCCGCCCAGCCCTGAGGAGAACTTGGCGCTT-ACCCGCGTAG 1083
            | ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1457 GGCCCGAGCAGTATGAGCGCCGCCCAGCCCTGAGGAGAACTTGGCGCTTTACCCGCGTAG 1398

Query  1084 -TGACGATTCTC-ACCATCA-TAGCGTCAAG-C-GCA-G-AATATGGCGATGTTTTCCCG 1136
             ||||||| ||| |||| || ||||| | || | ||| | |||||||||||||| | ||
Sbjct  1397 GTGACGAT-CTCCACCACCAGTAGCGCCCAGGCCGCAAGGAATATGGCGATGTTT-CGCG 1340

Query  1137 -C-TTA-CCA-GTGCGCCTTCTGGAACCT-GTCTAGAAGAATTCACCAACGTCACTACCG 1191
             | ||| ||| |||||| ||||| | ||| |||||||||| ||||||| ||||||||||
Sbjct  1339 GCCTTAACCAAGTGCGC-TTCTG-AGCCTTGTCTAGAAGA-TTCACCA-CGTCACTACCG 1284

Query  1192 TCA  1194
            |||
Sbjct  1283 TCA  1281
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(h) Integron class 1 (profile IN1-b)

Selected isolate for sequencing: AC/0810-12

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>gb|AY557339.1|  Acinetobacter baumannii isolate 1-43 class I integron Intw1
aminoglycoside      6'-N-acetyltransferase      (aacA4),      chloramphenicol
acetyltransferase (catB8), and aminoglycoside 3'-adenyltransferase (aadA1)
genes, complete cds

Length=2496
Score = 2056 bits (1113), Expect = 0.0
Identities = 1155/1172 (99%), Gaps = 16/1172 (1%)
Strand=Plus/Minus

Query  9     ATGTGCTTAGTGCATCTAACGCTTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGAA  68
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2481  ATGTGCTTAGTGCATCTAACGCTTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGAA  2422

Query  69    CGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGAACA  128
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2421  CGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGAACA  2362

Query  129   AATTCTTCCAACTGATCTGCGCGGGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCC  188
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2361  AATTCTTCCAACTGATCTGCGCGGGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCC  2302

Query  189   TGTCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCG  248
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2301  TGTCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCG  2242

Query  249   GCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCAAATGCGGGACAAC  308
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2241  GCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTACCAAATGCGGGACAAC  2182

Query  309   GTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAG  368
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2181  GTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTAAG  2122

Query  369   GTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCC  428
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2121  GTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAAAGAGTTCCTCCGCC  2062

Query  429   GCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCA  488
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2061  GCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCA  2002

Query  489   ATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCA  548
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2001  ATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCA  1942

Query  549   AATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATG  608
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1941  AATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATG  1882

Query  609   GTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGG  668
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1881  GTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGG  1822

Query  669   TCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAA  728
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1821  TCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAA  1762

Query  729   TCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCC  788
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1761  TCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCCGTACAAATGTACGGCC  1702

Query  789   AGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGAT  848
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1701  AGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGAT  1642
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Query  849   ACTTCGGCGATCACCGCTTCCCTCATGATGTTTAACGCCAGAATTAAGCCGTGCCGCGAA  908
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1641  ACTTCGGCGATCACCGCTTCCCTCATGATGTTTAACGCCAGAATTAAGCCGTGCCGCGAA  1582

Query  909   GCGGCGTCGGCTTGAATGAATTGTTAGACGGCAAACTCGCGCCAATACTTATGCAGACCA  968
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1581  GCGGCGTCGGCTTGAATGAATTGTTAGACGGCAAACTCGCGCCAATACTTATGCAGACCA  1522

Query  969   AAAATATTTGACGAGCACAGCAGAGGCATTGCTGTCTTAATTTTATCTAGTGGCCAGGTT  1028
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1521  AAAATATTTGACGAGCACAGCAGAGGCATTGCTGTCTTAATTTTATCTAGTGGCCAG-TT  1463

Query  1029  CCACCACTCCATCTCCATGAGCA-TGAGATT-C-TCATCGGAAAAGCGCTTCT-A-TT-G  1082
             |||||||||||||||||||||| ||||||| | |||||||| |||||||||| | || |
Sbjct  1462  CCACCACTCCATCTCCATGAGCAATGAGATTTCCTCATCGGAGAAGCGCTTCTTAATTTG  1403

Query  1083  CTT-GCGG-ATT-CCCCCGATGATG-CATAAG-CTCTACATCTTTTTGTCAC-AACGAAG  1136
             ||| |||| ||| |||||||||||| |||||| |||||||||||||| ||||| ||||| |
Sbjct  1402  CTTTGCGGGATTTCCCCCGATGATGGCATAAGGCTCTACATCTTTT-GTCACCAACGA-G  1345

Query  1137  CGACTACCTATCACG-CACCGTCTCCAATTTT  1167
             ||||||||||||||| ||||||||||||||||
Sbjct  1344  CGACTACCTATCACGGCACCGTCTCCAATTTT  1313
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(i) Integron class 2 (profile IN2-a)

Selected isolate for sequencing: AC/0606-13

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>gb|DQ176450.1|  Acinetobacter baumannii isolate AB28 transposon Tn7
tyrosine recombinase (intI2) pseudogene, complete sequence, and
streptothricin acetyltransferase (sat2), aminoglycoside adenyltransferase
(aadB), chloramphenicol acetyltransferase (catB2), dihydrofolate reductase
type I (dfrA1), streptothricin acetyltransferase(sat2), streptomycin 3''-
adenyltransferase (aadA1), hypothetical protein (ybeA), hypothetical protein
(ybfA), hypothetical protein(ybfB), hypothetical protein (ybgA),
transposition site target selection protein E tnsE), transposition site
target selection protein D (tnsD), transposition regulator protein (tnsC),
transposase B (tnsB), and transposase A (tnsA) genes, complete cds

Length=14850

Sort alignments for this subject sequence by:
E value  Score  Percent identity
Query start position  Subject start position

Score = 1871 bits (1013), Expect = 0.0
Identities = 1018/1020 (99%), Gaps = 2/1020 (0%)
Strand=Plus/Minus

Query  5     CTTC-AC-GATCGCGCATAGTCTTCCCCAGCTCTCTAACGCTTGAGTTAAGCCGCGCCGC  62
             |||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4992  CTTCAACAGATCGCGCATAGTCTTCCCCAGCTCTCTAACGCTTGAGTTAAGCCGCGCCGC  4933

Query  63    GAAGCGGCGTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTC  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4932  GAAGCGGCGTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATCTC  4873

Query  123   GCCTTTCACGTAGTGAACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTC  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4872  GCCTTTCACGTAGTGAACAAATTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTC  4813

Query  183   TTGTCCAAGATAAGCCTGCCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCG  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4812  TTGTCCAAGATAAGCCTGCCTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCG  4753

Query  243   CTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTA  302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4752  CTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTA  4693

Query  303   CCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGA  362
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4692  CCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGA  4633

Query  363   GTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATC  422
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4632  GTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATC  4573

Query  423   AAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAG  482
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4572  AAAGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAG  4513

Query  483   CAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATT  542
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4512  CAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATT  4453

Query  543   GCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTC  602
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4452  GCGCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTC  4393

Query  603   GTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGC  662
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4392  GTCGTGCACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAAGC  4333
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Query  663   CGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGT  722
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4332  CGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCAAGCCTTACGGT  4273

Query  723   CACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCC  782
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4272  CACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGAGCC  4213

Query  783   GTACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTC  842
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4212  GTACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTC  4153

Query  843   TGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATGATGTTTAACTCCTGAAT  902
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4152  TGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATGATGTTTAACTCCTGAAT  4093

Query  903   TAAGCCGCGCCGCGAAGCGGTGTCGGCTTGAATGAATTGTTAGGCGTCATCCTGTGCTCC  962
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4092  TAAGCCGCGCCGCGAAGCGGTGTCGGCTTGAATGAATTGTTAGGCGTCATCCTGTGCTCC  4033

Query  963   CGAGAACCAGTACCAGTACATCGCTGTTTCGTTCGAGACTTGAGGTCTAGTTTTATACGT  1022
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4032  CGAGAACCAGTACCAGTACATCGCTGTTTCGTTCGAGACTTGAGGTCTAGTTTTATACGT  3973


Score = 244 bits (132), Expect = 7e-61
Identities = 132/132 (100%), Gaps = 0/132 (0%)
Strand=Plus/Minus

Query  891   TAACTCCTGAATTAAGCCGCGCCGCGAAGCGGTGTCGGCTTGAATGAATTGTTAGGCGTC  950
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1435  TAACTCCTGAATTAAGCCGCGCCGCGAAGCGGTGTCGGCTTGAATGAATTGTTAGGCGTC  1376

Query  951   ATCCTGTGCTCCCGAGAACCAGTACCAGTACATCGCTGTTTCGTTCGAGACTTGAGGTCT  1010
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1375  ATCCTGTGCTCCCGAGAACCAGTACCAGTACATCGCTGTTTCGTTCGAGACTTGAGGTCT  1316

Query  1011  AGTTTTATACGT  1022
             ||||||||||||
Sbjct  1315  AGTTTTATACGT  1304


Score = 87.9 bits (47), Expect = 1e-13
Identities = 53/56 (95%), Gaps = 0/56 (0%)
Strand=Plus/Minus

Query  37    CTAACGCTTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGAACGAATTGTTAG  92
             ||||| | ||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  2027  CTAACACCTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGGACGAATTGTTAG  1972
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APPENDIX VII

Presentations and Publications

Presentations

1. Boon Hong Kong, Yasmin Abu Hanifah, Mohd Yasim Yusof and Kwai Lin Thong. (2010). Prevalence of carbapenem-hydrolyzing oxacillanases producing Acinetobacter baumannii in a tertiary hospital, Malaysia. 1st AMDI-International Biohealth Science Conference (IBSC). 29th Nov -1st December 2010. Universiti Sains Malaysia. (International)
2. Boon Hong Kong and Kwai Lin Thong. (2010). Antimicrobial Susceptibility and Genetic Diversity of Nosocomial *Acinetobacter baumannii*. Innovation and Creativity Expo University Malaya 2010, University of Malaya, Kuala Lumpur, Malaysia. *Meeting Abstract:* pg 87. (Silver medal award)
3. Boon Hong Kong, Yasmin Abu Hanifah, Mohd Yasim Yusof and Kwai Lin Thong. (2009). Antimicrobial Susceptibility and Molecular Characterization of Nosocomial *Acinetobacter baumannii* using Pulsed-Field Gel Electrophoresis. 4th National Medical Microbiology Seminar and Workshop 2009 - Advanced Techniques on Multiple Drug Resistance (MDR) Epidemiology and Genetics, University Putra Malaysia, Selangor, Malaysia, *Meeting Abstract:* pg 24. (Poster- Best presentation)
4. Boon Hong Kong, Yasmin Abu Hanifah, Mohd Yasim Yusof and Kwai Lin Thong (2009). Antimicrobial Susceptibility and Molecular Characterization of Nosocomial *Acinetobacter baumannii* using Pulsed-Field Gel Electrophoresis. 18th Scientific Meeting of Malaysian Society for Molecular Biology and Biotechnology Symposium, Kuala Lumpur, Malaysia, *Meeting Abstract:* pg 53.
5. Boon Hong Kong, Yasmin Abu Hanifah, Mohd Yasim Yusof and Kwai Lin Thong (2009). Resistant Phenotypes and Genetic Diversity of Nosocomial *Acinetobacter* spp. Using Pulsed Field Gel Electrophoresis. 7th International Symposium on Antimicrobial Agents and Resistance, Bangkok Thailand. *Meeting Abstract:* pg 244.

Publications

1. Kong, B. H., Hanifah, Y. A., Yusof, M. Y. and Thong, K. L. (2011). Antimicrobial Susceptibility Profiling and Genomic Diversity of Multidrug-Resistant *Acinetobacter baumannii* Isolates from a Teaching Hospital,Malaysia. *Japanese Journal of Infectious Diseases* **64**:337-340. (*ISI-Cited Publication*)

2. Kong, B. H., Hanifah, Y. A., Yusof, M. Y. and Thong, K. L. (2011). Application of Amplified Ribosomal DNA Restriction Analysis in Identification of *Acinetobacter baumannii* from a Tertiary Teaching Hospital, Malaysia. *Tropical Biomedicine* **28**:563-568 (*ISI-Cited Publication*)

3. Kong, B. H., Hanifah, Y. A., Yusof, M. Y. and Thong, K. L. Characterisation of carbapenem-resistant *Acinetobacter baumannii* producing OXA-23 in a University Hospital, Malaysia. (Manuscript in submission).